AN ABSTRACT OF THE DISSERTATION OF

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Title: Harnessing the Potential of Gut Microbiome in the Context of Type 2 Diabetes

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Gut microbiota plays an essential role in health and diseases, a fact already established. With the explosive increase in obesity and its main complication, Type 2 Diabetes Mellitus (T2D), there is an immediate need for novel methods of diagnosis, prevention and treatment of these conditions. In the last one and half decades, the gut microbiota has been a focal point of study in obesity and T2D. Several studies have proposed using gut microbiota to predict, diagnose, prevent, and treat obesity and T2D. However, there is no consistency between studies except for few microbes. The U.S Food and Drug Administration (FDA) has not approved any microbe or microbial products for the treatment of T2D, though many are in the stages of clinical trials. Diet rich in processed fat and sugar, also known as the western diet (WD), is the main culprit of T2D. WD changes the metabolic phenotypes leading to obesity, insulin resistance, glucose intolerance and T2D. WD also changes gut microbiota composition, as evidenced by the difference between obese and lean individuals. So, there seems obvious crosstalk between diet, microbiota and metabolic complications. In this dissertation, using WD induced T2D mouse model, we established the interactions between WD, microbiota and host. We identified and validated gut microbes that mediate beneficial effects in systemic glucose homeostasis. We also determined the effects of the western diet that are dependent on microbiota and identified a gut microbe associated with WD, promoting insulin resistance via induction of a metallopeptidase in adipose tissue.

In the second chapter, we did a comprehensive review of current literature involving human subjects to identify the potential role of different bacterial taxa affecting glucose intolerance, insulin resistance and T2D. This systemic review showed a negative association of T2D with

genera of *Bifidobacterium, Bacteroides, Faecalibacterium, Akkermansia* and *Roseburia* and positive association with the genera of *Ruminococcus, Fusobacterium and Blautia.* We also discussed several molecular mechanisms of microbiota effects in the onset and progression of T2D.

In the third chapter, we used animal models, systems biology, & *invitro* systems to infer, validate and identify the potential probiotic microbiota. We also showed that these microbes improved glucose metabolism by promoting healthy hepatic mitochondria, hepatic beta-oxidation, and lipid composition. We used the western diet-induced mouse model of T2D as the *in-vivo* animal model. Using the data-driven approach called Transkingdom Network analysis, developed in Shulzhenko and Morgun labs, we modeled the host-microbiome interactions under WD. This network analysis inferred us about the microbes that can potentially contribute to the altered host metabolism due to WD. We identified two species of *Lactobacill*us as the beneficial microbes that improve glucose metabolism in mice. Similarly, one species of *Rombutsia* was identified and tested as a microbe that worsens the western diet's effect in mice. Data from humans also showed the concordant association between these microbes and obesity. Supplementation of Lactobacilli in WD-fed mice improved the fatty acid composition in the liver and systemic glucose metabolism, which led us to explore the effect of Lactobacilli in the liver. Gene expression and electron microscopy of the liver showed that *Lactobacilli* can act on hepatic mitochondria and improves hepatic beta-oxidation, lipid composition, and systemic glucose metabolism. We then performed a metabolomics study of serum collected from mono-colonized mice with Lactobacillus or germ-free mice. This analysis revealed glutathione as a major metabolome that mediates the beneficial effect of Lactobacillus. We also established an *in-vitro* system and validated that glutathione, indeed, upregulates the well-established genes associated with mitochondrial functions and homeostasis such as mt-Atp6, Ndufv1, Mfn1, Foxo3, Gabpa, Usp50, Ifitm3 & Rai12. Their expression was also upregulated in the liver of mice supplemented with *Lactobacilli.* Hence, this study identified probiotic strains that can prevent T2D and established a mechanistic insight into the mode of action. Overall, our studies on host-microbiota interactions in diet induced diabetic mouse model identified microbiota-mitochondria crosstalk as one of the mechanisms by which the commensal bacteria promote beneficial effects on the host. Our work

also identified a pathobiont, *Rombutsia ilealis*, that promotes glucose intolerance potentially via different mechanism than *Lactobacilli.* These findings from our studies echoed the idea that targeted microbial therapies rather than attempting to restore overall composition of microbiota could be an effective way to develop microbiota based therapeutics.

Harnessing the Potential of Gut Microbiome in the Context of Type 2 Diabetes

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I understand that my dissertation will become part of the permanent collection of Oregon State University libraries. My signature below authorizes release of my dissertation to any reader upon request.

Manoj Gurung, Author

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CONTRIBUTION OF AUTHORS

Chapter 2: Manoj Gurung, Dr. Zhipeng Li, Hannah You conceived the idea, performed literature review, prepared figures/tables, wrote the manuscript, reviewed and approved the final draft. Dr. Richard Rodrigues reviewed the literature, prepared fig 1, reviewed manuscript, approved the final draft. Dr. Donald B Jump reviewed the manuscript, approved the final draft. Dr. Andrey Morgun and Dr. Natalia Shulzhenko conceived the idea, supervised literature revision, authored/reviewed manuscript, edited manuscript and approved the final draft.

Chapter 3: All authors reviewed the manuscript and approved the final draft. Manoj Gurung and Dr. Zhipeng Li, conceived, designed and performed the experiments, analyzed data, prepared figures/tables, authored the manuscript. Dr. Richard Rosario Rodrigues analyzed sequencing data, performed network analysis, prepared figures, tables, authored manuscript. Dr. Renee Greer, Hyekyoung You, Jacob W. Pederson, Stephany Vasquez-Perez, Kimberly D. White, Briana Frink performed mouse and cell culture experiments. Dr. Christopher Gaulke and Dr. Thomas J. Sharpton analyzed human microbiome data. Dr. Benjamin Philmus and Dr. Manuel Garcia-Jaramillo performed metabolomics studies and analyzed the data. Dr. Donald B. Jump performed/supervised fatty acid quantification experiments, provided hepatic cell lines. Dr. Giorgio Trinchieri and Dr. Amiran Dzutsev design experiments, analyzed data, edited the manuscript. Dr. David Berry and Franziska Bauchinger performed keystoneness analysis. Dr. Andrey Morgun and Dr. Natalia Shulzhenko acquired funding, provided resources, conceived original ideas, developed and supervised overall study design/experiments, analyzed data, drafted and edited the manuscript.

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DEDICATION

To my parents for their endless love, support, patience, and encouragement

CHAPTER 1: INTRODUCTION

1.1 Type 2 Diabetes

Type 2 Diabetes mellitus (T2D) is a chronic, multi-organ, multifactorial metabolic disease. It is characterized by the body's inability to uptake glucose (hyperglycemia) as a result of defects in insulin secretion, insulin action (insulin resistance) or combination of both. T2D individuals have insulin resistance or relative insulin deficiency. Environmental and genetic predispositions are the major cause of T2D. Sedentary lifestyle, overnutrition of unhealthy diet leading to obesity are the main environmental reasons of T2D and with the increasing prevalence of obesity, the cases of T2D are also rising exponentially. Pathogenesis of T2D is multifactorial due to alternations in various gene products[1], which progressively impairs insulin secretion by pancreatic β-cells.

Hyperglycemia in T2D is preceded by a condition known as prediabetes. This condition is characterized by impaired fasting glucose, impaired glucose tolerance or increased glycated hemoglobin A1c[2]. The levels of these parameters in prediabetes are more than the normal individual but not enough to reach the diagnostic levels of T2D. If untreated, 7% of prediabetic people will progress to diabetes every year[3]. However, early diagnosis of prediabetes and taking preventive measures like regular exercise, healthy eating to improve basal mass index (BMI) and taking anti-T2D drugs like metformin can minimize the chances of transition from prediabetes to T2D.

As per International Diabetes Federation, about 463 million adults (20-79 years) had diabetes in 2019, which is estimated to reach 700 million in 2045. Diabetes alone caused US 760 billion dollars in healthcare expenses, making it one of the costliest diseases. About 90% of diabetes cases are Type 2 diabetes. Among diabetic adults, 79% were living in low- and middle-income countries. Though obesity (BMI > 30 kg/m^2) is considered the single most common risk factor of T2D, certain regions of South Asia and Africa have diabetic population with lower BMI or even normal BMI[4] leading to more complexities in T2D. More recently, more sub-types of T2D were described, some predominantly insulin-resistant while others primarily insulin-deficient adult-onset diabetes[5].

Along with obesity, another triggering factor of T2D is hereditary with a positive family history of T2D increasing the disease risk by 2.4 fold[1,6], with the highest relative risk of T2D in individuals with at least two siblings affected by T2D[7]. The odd ratio of T2D or impaired glucose tolerance for offspring of diabetic father was 3.5 and 2.7, respectively as compared to 3.4 and 1.7 of diabetic mother[8]. Whereas among those with both parents with T2D, the odd ratio of T2D or impaired glucose tolerance was 6.1 and 5.2, respectively[8].

1.2 Glucose Homeostasis

The human body maintains its blood glucose levels via very tight and complex networks of hormones, receptors, proteins and various tissues. Glucose homeostasis can be defined as the continuous process in which blood glucose levels are maintained in a narrow range of 70-170mg/dl for normal functions during the postprandial state (0-6 hours after taking meal), intermediate fasting (12-14 hours) or during prolonged fasting periods (>24 hours). Postprandial glucose homeostasis involves the processes to reduce the elevated level of glucose after taking the meal. In the 4-5 hours of the postprandial period, about 75% of the plasma glucose is from the meal[9]. This increase in plasma glucose promotes insulin secretion from the pancreatic β-cell which increases the glucose transport in the skeletal muscle permitting glucose entry and glycogen synthesis. Insulin activates insulin receptor tyrosine kinase and phosphorylation of insulin receptor substrate 1 (IRS1) and IRS2 and activates Akt2. The increase in insulin promotes glycogen synthesis in the liver and de novo lipogenesis and inhibits gluconeogenesis, decreasing endogenous glucose production. In the white adipose tissue, insulin suppresses lipolysis and promotes lipogenesis. A certain amount of glucose (around 20%) is also consumed by the brain as the production of endogenous glucose, which it generally uses, is reduced during this period[9]. The hepatic glucose absorption into the hepatocytes involves glucose transporters, SLC2A2/GLUT-2 being the predominant one[10]. The glucose absorbed by hepatocytes is phosphorylated to glucose-6-phosphate (G-6-P) via glucokinase which undergoes several metabolic pathways depending on the enzymes involved. Most of the G-6-P gets converted into glycogen via glucose-1-phosphate and UDP-glucose[10]. G-6-P also undergoes another pathway producing fructose-6-phosphate (F-6-P), which produces Acetyl-coA and Fatty acids via glycolysis. The final pathway that G-6-P undergoes

is the pentose phosphate pathway by which Ribose 5-phosphate or Xylulose 5-phosphate is formed[10]. In summary, hepatic glucose metabolism is responsible for part of postprandial glucose homeostasis by increasing glycogenesis and reducing gluconeogenesis.

During intermediate fasting state (e.g., 14-16 hours of overnight fasting), the blood glucose level is approximately in a steady state, with the level going down during the last hours of fasting. This step involves both glucose utilization and glucose production with decreased production of insulin. The major organs that utilize glucose during this period in the order of consumption are brain, skeletal muscle, kidney, blood cells, splanchnic organs, and adipose tissue[11]. While brain is the main organ for glucose utilization, liver is the primary organ for glucose production via glycogenolysis & gluconeogenesis. While glycogenolysis is the predominant source of glucose during initial fasting, as the glycogen source starts depleting, gluconeogenesis starts to be more dominant source. Liver is responsible for the producing approximately 50% of total glucose after overnight fasting and almost all of the total glucose after 42 hours of fasting[12]. Kidneys also perform gluconeogenesis from glutamine. The major substrates for gluconeogenesis in liver are lactate, pyruvate, propionate, glycerol and amino acids and stimulated by glucagon, growth hormone, cortisol and epinephrine. Lactate, which is the primary substrate, gets converted to pyruvate in the cytoplasm, which then gets transported into the mitochondria. In the mitochondria, the pyruvate gets carboxylated to oxaloacetate (OAA) by the enzyme pyruvate carboxylase (PC). The OAA can either directly converted to phosphoenolpyruvate (PEP) by mitochondrial PEP carboxykinase[13,14] or gets reduced to malate, released into the cytosol and then decarboxylated and phosphorylated into PEP by cytosolic PEP carboxykinase[15,16]. The PEP enters gluconeogenic cycle where fructose-6-phosphate is formed first (via fructose 1,6-biphosphate catalyzed by fructose 1,6-biphosphatase) and then converted into glucose-6-phosphate (by phosphoglucoisomerase). Glucose-6-phosphate is dephosphorylated to glucose by glucose-6-phosphatase. Glucagon, epinephrine or cyclic adenosine monophosphate can increase the rate of glucose synthesis via gluconeogenesis from lactate[17], whereas insulin suppresses it. Other hormones from the gastrointestinal tract (GIT) also control glucose metabolism which will be discussed in the following section.

1.3 Insulin Resistance (IR)

The hallmark of Type 2 diabetes is insulin resistance in which even with normal or above normal insulin level, there is the presence of fasting hyperglycemia owing to reduced effects of insulin in metabolic organs. Defects in insulin action, insulin secretion and the feedback loop of hyperglycemia to address hepatic glucose production are present in T2D[18]. Dysregulation of free fatty acids (FFAs) metabolism is the single most important factor of IR along with genetic predisposition. IR can occur years before fasting hyperglycemia and T2D[19]. All metabolic organs (liver, skeletal muscle, adipose tissue), kidneys, and brain can have IR[2].

1.3.1 Insulin Signaling

Insulin is a peptide hormone released by β cells of Islet of Langerhans in the Pancreas. Insulin singling takes place via insulin receptors located on the cell membrane. Insulin binds to the extracellular α-subunits of the receptor, bringing the two α-subunits closer together and leading to the binding of ATP to the β-subunit[20]. This binding promotes auto-phosphorylation and the activation of receptor tyrosine kinase which then phosphorylates insulin receptors substrates (IRS). Among the four types of IRS, IRS-1 and IRS-2 have shown to be the main components of insulin signaling as their depletions promote insulin resistance and pancreatic dysfuntion[21,22,22,23,24] with IRS-1 functions being predominant in skeletal muscle and IRS-2 in the liver[20]. Phosphorylated IRS proteins bind and activate phosphatidylinositol three dependent kinases (PI-3-kinases), PDK1 and PDK2, and phosphatidylinositol 3,4,5-triphosphate (PIP3) is formed. PIP3 binds to protein kinase B/Akt protein and translocate to the cell membrane and gets phosphorylated and causes translocation of GLUT4-containing storage vesicles (GSVs) to the cell membrane[25] via phosphorylation of AS160 (160kDA Akt substrate) and inhibition of Rab-GAP (Rab-GTPase-activating protein) activity. This translocation leads to glucose uptake by skeletal muscle and adipocytes.

1.3.2 Mechanism of Insulin Resistance

Transient insulin resistance takes place in certain stages of life (pregnancy, puberty, during aging)[26]. The main culprit of induced insulin resistance is obesity. Obesity can be defined as

the ectopic lipid accumulation like in skeletal muscle and liver with the increased amount of plasma free fatty acids. Reduction in the number of surface insulin receptors and insulin receptor substrates, defects in insulin binding to the receptors and defects in singling pathways following the binding are the major reasons for insulin resistance[9], with the latter contributing to the majority.

In the skeletal muscle, triacylglycerol (TAG) is converted into diacylglycerol (DAG) by adipose triglyceride lipase. DAGs activate the protein kinase C family (PKCθ), which reduces the activity of IRS[27]. Along with TAG and DAGs, ceramides and long-chain FA coenzyme A (FA-CoA) are also accumulated inside myocytes during obesity which can dysregulate insulin singling cascade in muscle leading to impaired insulin-stimulated glucose uptake[28]. In the liver, DAGs activate protein kinase C ε (PKCε) and inhibit IRS and downstream insulin singling. Both in muscle and liver and also in adipose tissue, FA-CoA can produce ceramides that increase Phosphoprotein Phosphatase PP2A which, impairs insulin action via dephosphorylation of Akt2[29,30]. Impairment of Akt2 in muscle limits GSVs translocation, whereas in the liver, gluconeogenic enzymes are activated and glycogen synthesis decreases. In addition, obesity increases the number of pro-inflammatory macrophages in the adipose tissue which produce inflammatory factors responsible for reduced insulin sensitivity and resistance. These macrophages are the primary source of tumor necrosis factor (TNF)-α, which inhibits insulin-stimulated glucose uptake in adipocytes via decreasing phosphorylation of insulin receptors and IRS1[31,32]. TNF-α also impairs insulin singling pathways in skeletal muscle and glucose uptake via reduction of tyrosine phosphorylation of IRS1 and impaired phosphorylation of AS160 needed for translocation of GLUT4[33]. Interlukin-6 (IL-6), one of the main proinflammatory cytokines elevated during obesity which reduces tyrosine phosphorylation association of IRS-1 with PI3 kinase and inhibits glycogen synthesis in hepatocytes which ultimately contribute to insulin resistance[34].

1.4 Mitochondrial dysregulation in T2DM

Mitochondria is a multifunctional organelle with its roles in several pathophysiological events like oxidative phosphorylation (OXPHOS), DNA repair, tricarboxylic acid cycle (TCA), urea cycle etc [35]. Mitochondrial dysregulation in organs like liver, skeletal muscle and adipose

tissue can impair fatty acid metabolism, and intracellular accumulation of fatty acids like diacylglycerol, triacylglycerol, and ceramides which in turns worsen insulin resistance.

It was reported that an imbalance between glycolytic enzymes and oxidative enzymes in muscle contributes to T2DM[36]. Insulin-resistant individuals also have a lesser number of mitochondria as compared to healthy individuals. Similarly, citrate synthase & oxidoreductase activities are reduced in T2D and obese individuals when compared to lean individuals[37]. Likewise, the skeletal muscle of T2D patients also possesses mitochondria of smaller size and presence of large vacuoles and disarrangement of internal membrane structure and cristae[37] suggesting mitochondrial impairment. These structural and functional alternations in the mitochondria of skeletal muscle contribute to insulin resistance and T2D. The enzymes responsible for TCA cycle are also reduced in the skeletal muscle of T2D patients[38], along with increased deletions of mitochondrial DNA in the skeletal muscle of people with impaired glucose tolerance[39]. Diet induced diabetic animal model also shows increased reactive oxygen species (ROS), dysregulation of mitochondrial biogenesis, & impairment of mitochondrial structure[40].

Another vital metabolic organ, liver, plays a major role in glucose homeostasis via hepatic gluconeogenesis, fatty acid oxidation & glycogen synthesis. Accumulation of lipids and decreased insulin sensitivity are hallmarks of the diabetic liver. The high fat diet causes oxidative stress in the liver with an increased level of malondialdehyde and a reduced glutathione level along with inhibition of mitochondrial respiratory complexes I and III[41]. In the insulin-resistant liver, maximal respiration & ATP production are reduced[42]. Pancreatic mitochondrial functions are also affected in diabetes. In diabetic pancreatic islets, there is downregulation of genes and proteins related to oxidative phosphorylation, reduced mitochondrial metabolism, reduced ATP synthesis, increased expression of uncoupling protein 2, & mutations in mitochondrial DNA.[43,44,45,46]. Similarly, mitochondrial dysregulation also occurs in white adipose tissue of diabetic subjects. In mitochondria of such tissue, increased production of ROS, accumulation of malondialdehyde, reduction of antioxidant agents like glutathione peroxidase, reduction of the amount of mitochondrial DNA, decreased level of mitochondrial proteins like ATP synthase & OXPHOS, impairment in mitochondrial morphology, & downregulation of electron transport chains genes are reported[47,48,49].

1.5 Gastrointestinal tract (GIT) and glucose homeostasis

The gastrointestinal tract plays an important role in glucose homeostasis due to its function in nutrients digestion, absorption and release of intestinal hormones (incretions) in response to nutrients. These hormones are released from enteroendocrine cells and play an essential role in insulin action and glucose homeostasis. The Gastric inhibitory polypeptide (GIP)/glucose-dependent insulinotropic peptide and glucagon-like peptide-1 (GLP-1) are two major incretions released by K and L cells of the enteroendocrine system of the intestine. GIP is a potent insulinotropic hormone that responds to glucose produced during absorption of nutrients, and enhances insulin secretion[50,51,52]. The expression of *gip* gene that codes for GIP protein increases due to the presence of nutrients in the gut[53]. GIP receptors (GIPR), in which GIP binds to elicit its effects, are distributed in α & β cells of the pancreas, GI tract, adipose tissue and several other organs[54,55,56,]. In the pancreas, GIP once binds to its receptor on β cells in response to increased glucose concentration after a meal, promotes insulin secretion via several signaling pathways, and direct exocytosis[52,57]. The signaling cascades activated by this binding are MAPK, PI3K, protein kinase B/Akt, phospholipase A2, phosphor kinase A, and increased intracellular Ca^{2+}[58]. GIP also promotes proliferation and survival of β cells[58,59]. The anti-apoptotic action of GIP on β cells has been found to be due to increased activity of PI3K/PKB/Foxo1 signaling cascade and decreased expression *bax* gene, a pro-apoptotic target of Foxo1 and downregulation of Bcl-2 gene, an antiapoptotic gene[59].

Another predominant incretin, GLP-1, is produced from the processing of proglucagon and alike GIP, released in the gut in response to nutrients intake. The L cells from which GLPs are released are located in the distal ileum and colon[60]. GLP-1 receptors (GLP-1R), 463 amino acid G protein-coupled receptors, are located in various target organs including, the GI tract, islets of the pancreas (mainly β cells), kidneys, heart, and tissues of the nervous system. GLP-1 has both insulinotropic and glucagonostatic actions and depends on the plasma glucose level[51]. In pancreatic β cells, the action of GLP-1 is either protein kinase A (PKA) dependent or independent. In PKA-dependent action, binding of GLP-1 to its receptors increases cAMP, causing PKA activation, whereas in independent action, cAMP-regulated guanine nucleotide exchange factors (*Epac*) expressed in β cells are regulated by GLP-1[61,62]. Activation of PKA

and Epac cause an increase in intracellular concentration of Ca^{2+}. The elevated concentration of Ca^{2+} promotes the fusion of insulin-containing granules with β cells plasma membrane, causing the release of insulin[62]. GLP-1 also upregulates proinsulin gene expression via β cells transcription factor, Pdx-1[63,58]. Besides the glucose-mediated release of insulin, GLP-1 also inhibits glucagon secretion from pancreatic alpha-cells leading to the lowering of glucose. GLP-1 also promotes β-cell proliferation, increases its and islet mass by enhancing cellular differentiation, inhibition of apoptosis and β-cell neogenesis[64,60,65]. Actions of GLP-1 go beyond the pancreas with ultimate effects in glucose maintenance. It inhibits gastric emptying and regulates gastric acid secretion[66,67,68] via the involvement of vagal afferent nerves[69,70]. Brain GLP-1 is also known to control the whole body glucose without the involvement of muscle insulin receptors[71].

1.6 Genes of T2D

T2D is a polygenic disease. Use of Genome Wide Association Study (GWAS), transcriptomic analysis, gene deletion methods and network analysis have helped identify numerous genetic factors involved in the pathophysiology of T2D. GWAS studies performed in subjects from Iceland, Europe and Hong Kong [72], UK[73], Finland and Sweden[74], various European sources[75], Finland[76] , France[77] in the initial studies of GWAS identified variants in or near the genes CDKAL1, TCF7L2, IGF2BP2, CDKN2A, CDKN2B, SLC30A8, HHEX, PPARG, KCNJ11, FTO, KCNQ1, MMP26, KCTD12, NGN3, CXCR4, LOC38776, CAMTA1, LDLR, EXT2, LOC646279 and HNF1A. Similarly, a study done in Italian subjects replicated some of the previously mentioned genes along with new genes (CAT, FTO, UCP1, ADIPOQ) associated with T2D and related micro and macrovascular complications[78].

1.7 Animal model of T2D

Several animal models are available to study different aspects of T2D. Obese and non-obese, genetically manipulated, or diet-induced models are available. The earliest mono-genetically manipulated obese rodent models include *Lep*$^{ob/ob}$, *Lepr*$^{db/db}$, Zucker diabetic fatty (ZDF) rat, where either Leptin or leptin receptors are mutated, resulting in hyperphagia, obesity, and

ultimately diabetes. Another monogenic obese model is heterogeneous KK-A^y in which mice are obese due to hyperphagia leading to dysregulation of β-cells[79,80,81]. Polygenic obese models include male TALLYHO/JngJ mice, male NONcNZO10 mice, KK mice, OLETF rats, and NZO mice. Nongenetic animal models of T2D are achieved by feeding animals with high fat (mostly 60%) or high-fat high carbohydrate diets for several weeks. The effects of diet depend on many factors, including background, sex, and microbiota of the animal model being used. Several non-obese diabetic (NOD) models are also available or proposed. HND, Me1/Grb10 mice, GK rats, hIAPP mice are some of the examples of such models[82,83,80].

1.8 Microbiota and health

The last two decades have seen tremendous growth in microbiome research with many potential implications in human, animal, and plant health. The first phase of the Human Microbiome Project started in 2007, which pioneered the large-scale analysis of the human microbiome residing in five different body parts[84]. Microbial cells contribute to about half the cell population in a human body, whereas the number of microbial genes exceeds almost 100 times the host genes. With the advancement and decreasing cost of sequencing and increasing availability of robust computational tools, the importance of microbiota is being explored for numerous diseases leading to many ongoing clinical trials. Almost all diseases cause an imbalance in the composition of microbiota, termed as 'dysbiosis'. Gut microbiota has been implicated in conditions like Inflammatory Bowel Disease (IBD), several cancer types, Crohn's disease, obesity, diabetes, autism, aging, cardiovascular diseases (CVD), fatty liver disease, immune-deficiency conditions and so on. *Ruminococcus gnavus* (in IBD)[85], *Akkermansia muciniphila*, Lactobacillus species, *R. gnavus*, *Bifidobacterium longum*, *Bacteroides fragilis* (in obesity, type 2 diabetes)[86,87,88,89,90,91], *Acinetobacter baumanii* (in common variable immune-deficiency)[92], *Faecalibacterium prausnitzii*, *Bacteroides fragilis*, *Fusobacterium nucleatum*, *Prevotella bivia* (in cancer)[93,94,95,96,97], Bifidobacteria, Prevotella, *Eisenbergiela tayi* (in autism)[98,99], *Akkermansia muciniphila*, *Lactobacillus plantarum* (in CVD)[100,101] are among several microbes whose importance have been shown in various diseases. There are several ongoing clinical trials using either specific microbes or microbial products for the treatment of different diseases like colorectal cancer, epilepsy, NASH,

ulcerative colitis, C. difficile infection, obesity, cirrhosis, and so on. So, implications of microbiota in human health have a tremendous scope but come with several challenges.

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CHAPTER 2: ROLE OF GUT MICROBIOTA IN TYPE 2 DIABETES PATHOPHYSIOLOGY

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Review

Role of gut microbiota in type 2 diabetes pathophysiology

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Abstract
A substantial body of literature has provided evidence for the role of gut microbiota in metabolic diseases including type 2 diabetes. However, reports vary regarding the association of particular taxonomic groups with disease. In this systematic review, we focused on the potential role of different bacterial taxa affecting diabetes. We have summarized evidence from 42 human studies reporting microbial associations with disease and have identified supporting preclinical studies or clinical trials using treatments with probiotics. Among the commonly reported findings, the genera of *Bifidobacterium, Bacteroides, Faecalibacterium, Akkermansia and Roseburia* were negatively associated with T2D, while the genera of *Ruminococcus, Fusobacterium,* and *Blautia* were positively associated with T2D. We also discussed potential molecular mechanisms of microbiota effects in the onset and progression of T2D.
Keywords: Type 2 diabetes, Microbiota, 16S rRNA, Metagenomics, Insulin resistance

1. Introduction

The microbiome has been associated with pathophysiology of most chronic diseases. Type 2 diabetes (T2D) is no exception to this rule. Indeed, there is evidence for the effects of microbiota on glucose metabolism in both preclinical animal models of T2D and in healthy animals. Therefore, there is considerable interest in potential use of microbiota in clinical applications for understanding and treating T2D. At first glance, however, the microbiome literature on T2D appears chaotic and concerns have been raised about variability of the results. Different taxa are reported to be associated with T2D in different studies. Furthermore, a recent large study observed that different microbes were found associated with the same metabolic outcomes in different geographical areas [[1]]. While this might appear somewhat discouraging it is important to remember that discrepancies between results and disagreements about interpretations are common features of any emerging field in science. As a research community, we should not shy away from these problems, rather understand which aspects of the current literature are robust and which ones are not. A key issue moving forward is to identify properties of the microbiome and T2D that contribute to this apparent lack of reproducibility. In this review, we researched recent literature regarding microbiome in type 2 diabetes patients and summarize the most reliable findings.

2. Bacteria involved in T2D

Out of 42 human observational studies that investigated T2D and the bacterial microbiome, the majority of studies reported associations between specific taxa and disease or its phenotypes (see Supporting Table 1 and "Search strategy and selection criteria" below). However, only a handful reported similar results. Among the commonly and consistently reported findings, the genera of *Bifidobacterium, Bacteroides, Faecalibacterium, Akkermansia and Roseburia were negatively associated with T2D, while the genera of Ruminococcus, Fusobacterium,* and *Blautia* were positively associated with T2D (Fig. 1). *Lactobacillus* genus, while frequently detected and reported, shows the most discrepant results among studies. Interestingly, different macro-metrics of microbial communities, such as several indexes of diversity and the Bacteroidetes/Firmicutes ratio that have been previously suggested as markers of metabolic disease did not show consistent associations with T2D (Table 1).

Bacteroides and *bifidobacterium* represent beneficial genera most frequently reported in studies of T2D.

Bifidobacterium appears to be the most consistently supported by the literature genus containing microbes potentially protective against T2D. Indeed, nearly all papers report a negative association between this genus and T2D [2, 3, 4, 5, 6, 7, 8, 9]; while only one paper reported opposite results [[10]]. Furthermore, some studies also found a negative association between specific species such as *B. adolescentis, B. bifidum, B. pseudocatenulatum, B. longum, B. dentium* and disease in patients treated with metformin or after undergoing gastric bypass surgery [[6],[11]]. According to our literature search, *Bifidobacterium* has not been used alone as probiotics for T2D. However, almost all animal studies that tested several species from this genus (*B. bifidum, B. longum, B. infantis, B. animalis, B. pseudocatenulatum, B. breve*) showed improvement of glucose tolerance [12, 13, 14, 15, 16]. Thus, animal studies strengthen the idea that *Bifidobacterium* naturally habituating the human gut or introduced as probiotics play protective role in T2D.

The second most commonly reported genus was ***Bacteroides***. Eight studies have reported associations between the abundance of this genus and T2D. Among these, five cross-sectional studies [[3],17, 18, 19, 20] show negative associations with disease while three other studies [[6],[11],[21]] that involved some type of treatment reported positive

associations. This apparent inconsistency can be explained by previously reported antibiotic effect of metformin [[22]] and/or potential feedback mechanisms on gut icrobiota resulting from improved human physiology. Interestingly, in He et al. [[1],[23]] 21 out of 23 OTUs of Bacteroides detected in their study were negatively associated with T2D. Accordingly, in investigations that analyzed this genus on the species level, *Bacteroides intestinalis, Bacteroides 20–3 and Bacteroides vulgatus* were decreased in T2D patients and *Bacteroides stercoris* were enriched after sleeve gastrectomy (SG) surgery in T2D patients with diabetes remission [[5],[11],[17],[24]]. We also found only two experimental animal studies testing the ability of *Bacteroides* to treat diet induced metabolic disease. In these studies, administration of *Bacteroides acidifaciens* [[25]] and *Bacteroides uniformis* [[26]] improved glucose intolerance and insulin resistance in diabetic mice. Together, these studies indicate that *Bacteroides* plays a beneficial role on glucose metabolism in humans and experimental animals.

While ***Roseburia, Faecalibacterium,*** **and** ***Akkermansia*** were not reported as frequently as the two genera above mentioned (Bifidobacterium, Bacteroides) in the 42 studies we reviewed, but those genera were also found to be consistently negatively associated with T2D in human studies.

In five case-control studies *Roseburia* was found in lower frequencies in T2D group than in healthy controls [[3],[17],27, 28, 29]. Accordingly, investigations that were able to assign *Roseburia* to a species level also reported a negative association with disease for *Roseburia inulinivorans, Roseburia_272,* and one unclassified OTU from this genus [[11],[17],[24]]. Only one paper reported an opposite result for *Roseburia intestinalis* [[17]].

Table 1 Number of reports examining association between T2D and diversity of microbiota or Bacteroides/Firmicutes ratio.

Index		# Reports	No association	References (PMID)	Positive	References (PMID)	Negative	References (PMID)
Alpha diversity	Shannon	13	9	24013136, 29998997, 29280312, 29922272, 29596446, 27151248, 26756039	2	30397356, 26941724	2	27974055, 27151248
	Chao1	8	6	24013136, 29998997, 29280312, 29922272, 26756039, 27151248	2	26941724, 29789365	0	
	Simpson	3	1	29998997	1	26941724	1	29789365
Beta diversity		8	7	24988476, 28530702, 24997786, 29280312, 29922272,	0		1	27974055

Index	# Reports	No association	References (PMID)	Positive	References (PMID)	Negative	References (PMID)
			29596446, 27151248				
acteroides/Firmicutes ratio	14	6	24013136, 26756039, 29789365, 29434314, 29657308, 29998997	3	20140211, 29434314, 23032991	4	23657005, 27974055, 26919743, 22293842

Two case-control studies reported lower frequencies in the disease group for *Faecalibacterium* [[2],[28]]. Nevertheless, this genus was also found to be decreased after different types of antidiabetic treatments ranging from metformin and herbal medicine [[30]] to bariatric surgery [[11]]; only one study reported an opposite effect [[31]]. Moreover, studies that were able to analyze this genus at species level usually detected *F. prausnitzii.* This species was found to be negatively associated with T2D in four out five human case control studies [[17],[24],32, 33, 34]. While it is a popular probiotic for colitis [[35]], there were few attempts to use *F. prausnitzii* as a probiotic for metabolic disease. Interestingly, in one study the administration of *F. prausnitzii* resulted in improvement of hepatic function and decreased liver fat inflammation in mice with diet-induced metabolic disease without affecting blood glucose [[36]]. Finally, it was also shown that another species of this genus, *Faecalibacterium cf,* was associated with remission of diabetes after bariatric surgery [[11]].

Akkermansia muciniphila is a relatively recently discovered member of commensal microbiota [[37]]. Its beneficial effect on host glucose metabolism was first reported in animal models [[38],[39]]. In agreement with animal studies, the negative association between the abundance of this bacterium and T2D has been reported in human studies [[17],[38]].

In summary, a decrease in at least one of these five phylogenetically distant genera (*Bacteroides, Bifidobacterium, Roseburia, Faecalibacterium, and Akkermansia*) in patients was found in approximately half of T2D microbiome studies suggesting their potential role beyond serving as a biomarker. Supporting this notion, the majority of these bacteria have been tested as probiotics for metabolic disease in mice, but more rarely in humans [12, 13, 14, 15, 16,[25],[26],36, 37, 38, 39, 40, 41, 42]. The potential mechanisms of interaction between these microbes and mammalian organisms are discussed later in this paper.

Lactobacillus genus presents a complex case of apparently discordant results when considering all association studies, i.e. including those that analyzed changes after treatments (Fig. 1). However, cross-sectional studies of patients versus controls reported positive association between abundances of this genus and T2D in five out of six papers

[3, 4, 5,[29],[43]]. Furthermore, several associations of this genus tend to be species-specific. For example, while *L. acidophilus* [[34]], *L. gasseri* [[24]], *L. salivarius* [[24]] were increased, *L. amylovorus* [[29]] was decreased in T2D patients suggesting a high diversity in functional impact on host metabolism by bacteria from this genus. Moreover, several species from this genus have been also tested as probiotics. Experimental studies in mice show mostly beneficial effects in the models of T2D such as *L. plantarum* [44, 45, 46, 47], *L. reuteri* [[48]], *L. casei* [[49]], *L. curvatus* [[50]], *L. gasseri* [[51]], *L. paracasei* [[52]], *L. rhamnosus* [[53]], *L. sakei* [[54]]. More importantly, twenty-five human clinical trials [55, 56, 57, 58, 59, 60, 61, 62, 63, 64, 65, 66, 67, 68, 69, 70, 71, 72, 73, 74, 75, 76, 77, 78, 79] employed twelve different species of *Lactobacillus* with ten of those studies [55, 56, 57, 58, 59, 60, 61, 62,[64],[79]] adding other probiotics. Out of eleven studies [58, 59, 60, 61, 62, 63, 64,[72],[76],[77],[79]] that showed some protective effect, the majority combined other genera, most frequently *Bifidobacterium* [58, 59, 60, 61, 62,[64],[79]], suggesting that *Lactobacillus* and *Bifidobacterium* may work in a synergistic manner. Species *L. sporogenes* [[76],[77]], *L. casei Shirota* [[63]], *L. reuteri* [[72]] used as mono-probiotics have been reported to improve T2D related symptoms in humans.

L. plantarum, bacteria found in fermented food products, is intensively studied in animal systems, with many studies showing that *L. plantarum* improves glucose metabolism in diet-induced and genetic models of T2D [44, 45, 46, 47] mice; only one reported with no significant effect of this treatment [[80]]. However, this species had no significant effect on glucose metabolism in four clinical trials [68, 69, 70, 71]. Thus, it seems that *Lactobacilli* anti-diabetic effect is seen more frequently when they are a part of probiotic cocktail rather than administered individually [[58],[61],[62],[64]].

Overall, *Lactobacillus*genus is highly diverse and contains the highest number of OTUs in the human gut among potentially probiotic bacteria. Its effects on T2D seems to be species-specific or even strain-specific, which might explain why genus level analysis lacks consistency amongst studies using this bacteria (Fig. 1).

Fewer studies (11 out of 42) reported positive associations (increase in disease) of microbiota with T2D and/or hyperglycemia. Specifically, ***Ruminococcus,***

Fusobacterium**,* and ***Blautia have been reported in a positive association with T2D. On one hand, consistent findings have been reported in 5 studies on *Ruminococcus* genus [[3],[17],[28],[31],[81]] and 3 studies on *Fusobacterium* [[2],[4],[6]]. On the other hand, the studies reporting species levels of these bacteria reported conflicting results [[6],[11],[34]]. For example, while one study demonstrated that Ruminococcus sp. SR1/5 enriched by metformin treatment [[6]], another found *Ruminococcus bromii* enriched and *Ruminococcus torques* decreased after bariatric surgery and diabetes remission [[11]]. It is possible that different types of treatments might be a major reason for the inconsistences between results of these studies.

Blautia genus has been found increased in disease groups in three out of four cross-sectional studies for T2D [[17],[18],[82],[83]] and reduced after bariatric surgery [[31]]. Disagreeing with these reports, Blautia spp. were reported to increase after treatment with metformin in another study [[30]]. Importantly, results by He et al. 2018 [[1]], are concordant with the genus level analyses demonstrating positive associations between T2D and several OTUs of all three of these genera. The question still remains whether these bacteria play a causal role in T2D since there are no studies investigating these potentially harmful bacteria in animal models of T2D.

In summary, our review of literature regarding overall diversity and other macro-metrics of microbial communities failed to show a relation to diabetes (Table 1). However, some taxa have been systematically implicated in T2D. Surprisingly, some taxa are consistently associated with protection from T2D at genus level (e.g. *Bacteroides, Bifidobacterium*, etc.) or even phylogenetically at higher levels (e.g. Actinobacteria [[7],[17]]) whereas others (e.g. *Lactobacilli*) show only species- or strain-specific effects. This phenomenon might be to be associated with a diversity of a given genus habituating the human gut (i.e. the larger a number of strains of a given genus found in human gut, the more strain-specific effects are observed). Importantly, several of these microbes are currently tested as probiotics in mouse and human studies.

3. Potential mechanisms of microbiota effects on metabolism in the T2D patient

Multiple molecular mechanisms of gut microbiota contribution to metabolic disease and T2D have been recently reviewed elsewhere [[84]]. Microbiota modulates inflammation, interacts with dietary constituents, affects gut permeability, glucose and lipid metabolism,

insulin sensitivity and overall energy homeostasis in the mammalian host (Fig. 2). Herein, we summarize the mechanisms whereby specific taxa highlighted earlier in this review can affect T2D.

3.1 Modulation of inflammation

Overall, T2D is associated with elevated levels of pro-inflammatory cytokines, chemokines and inflammatory proteins. While some gut microbes and microbial products especially lipopolysaccharides (LPS) promote metabolic endotoxemia and low-grade inflammation, others stimulate anti-inflammatory cytokines and chemokines. For example, induction of IL-10 by species of *Roseburia intestinalis, Bacteroides fragilis, Akkermansia muciniphila, Lactobacillus plantarum, L. casei* [[37],85, 86, 87, 88] may contribute to improvement of glucose metabolism since overexpression of this cytokine in the muscle protects from ageing-related insulin resistance [[89]]. *R. intestinalis* can also increase IL-22 production, an anti-inflammatory cytokine [[90],[91]] known to restore insulin sensitivity and alleviate diabetes [[92]]. It can also promote T regulatory cell differentiation, induce TGF-β and suppress intestinal inflammation [[85],[90],[91]]. Likewise, *Bacteroides thetaiotaomicron* induces expression of T regulatory cell gene expression [[90]].

Inhibition of pro-inflammatory cytokines and chemokines is another route used by beneficial microbes to prevent inflammation. Various species of Lactobacillus (*L. plantarum, L. paracasei, L. casei*) can decrease IL-1β, Monocyte Chemoattractant Protein-1, Intercellular adhesion molecule-1, IL-8, CD36 and C-reactive protein [[93],[94]]. *L. paracasei* and *B. fragilis* inhibit expression of IL-6 [[86],[95]]. Similarly, Lactobacillus, Bacteroides and Akkermansia have been found to suppress TNF-α [[96],86, 87, 88,[95],[97],[98]]. *L. paracasei* and microbial anti-inflammatory molecule from *F. prausnitzii* inhibit the activity of NF-kB [[95],[99]]. Similarly, Roseburia and Faecalibacterium are butyrate producing bacteria and butyrate is also known to inhibit the activity of NF-kB [[100],[101]]. *Lactobacillus casei* and *Roseburia intestinalis* decrease another pro-inflammatory cytokine IFN-γ [[90],[91],[102]] whereas *Roseburia intestinalis* can inhibit IL-17 production [[90],[91]]. *Bacteroides thetaiotaomicron* reduces Th1, Th2 and Th17 cytokines in mono-associated mice [[90]].

Potentially detrimental microbes in T2D (pathobionts), like *Fusobacterium nucleatum* and *Ruminococcus gnavus* can increase several inflammatory cytokines, albeit in other inflammatory diseases [[103],[104]].

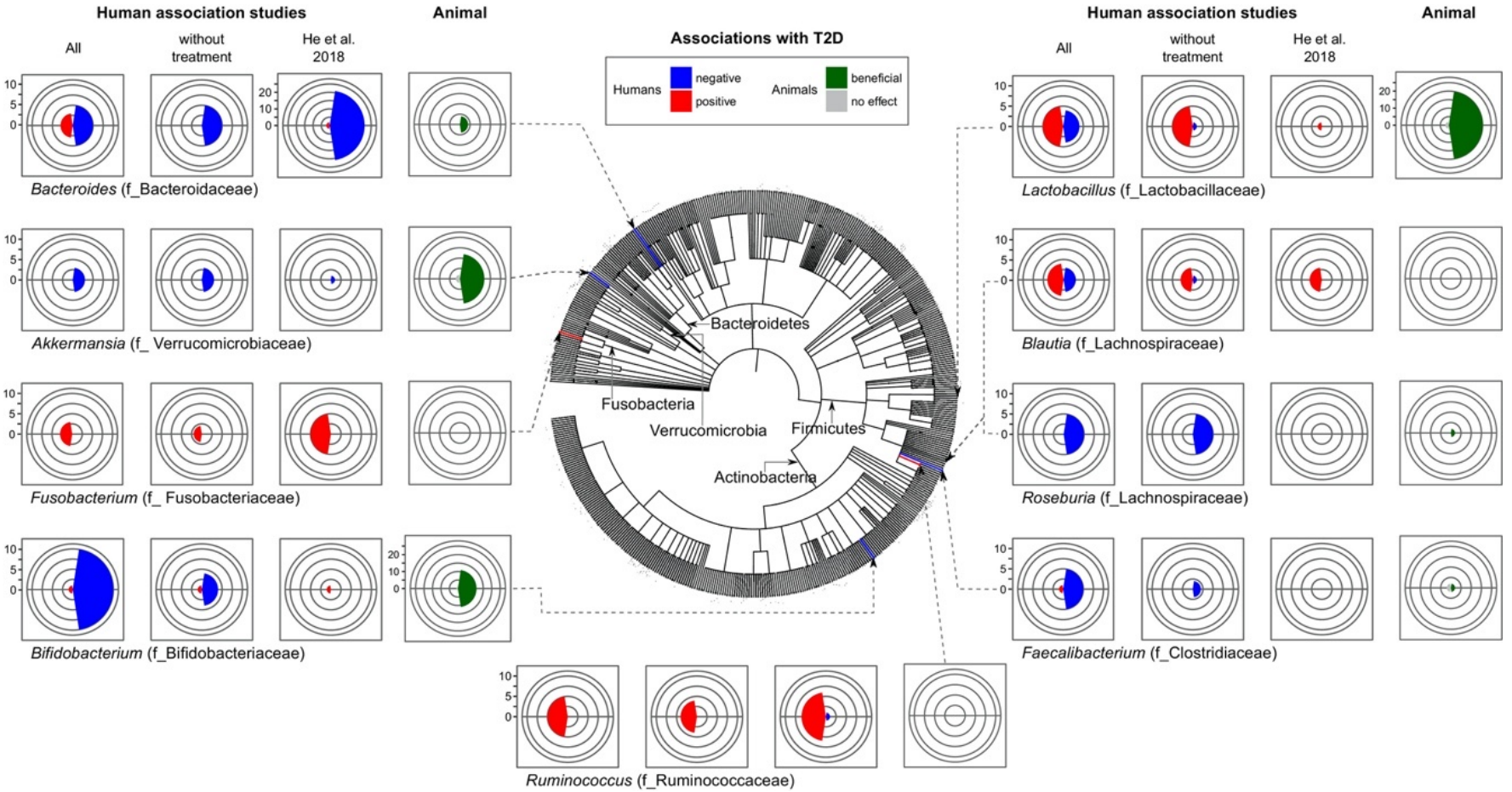
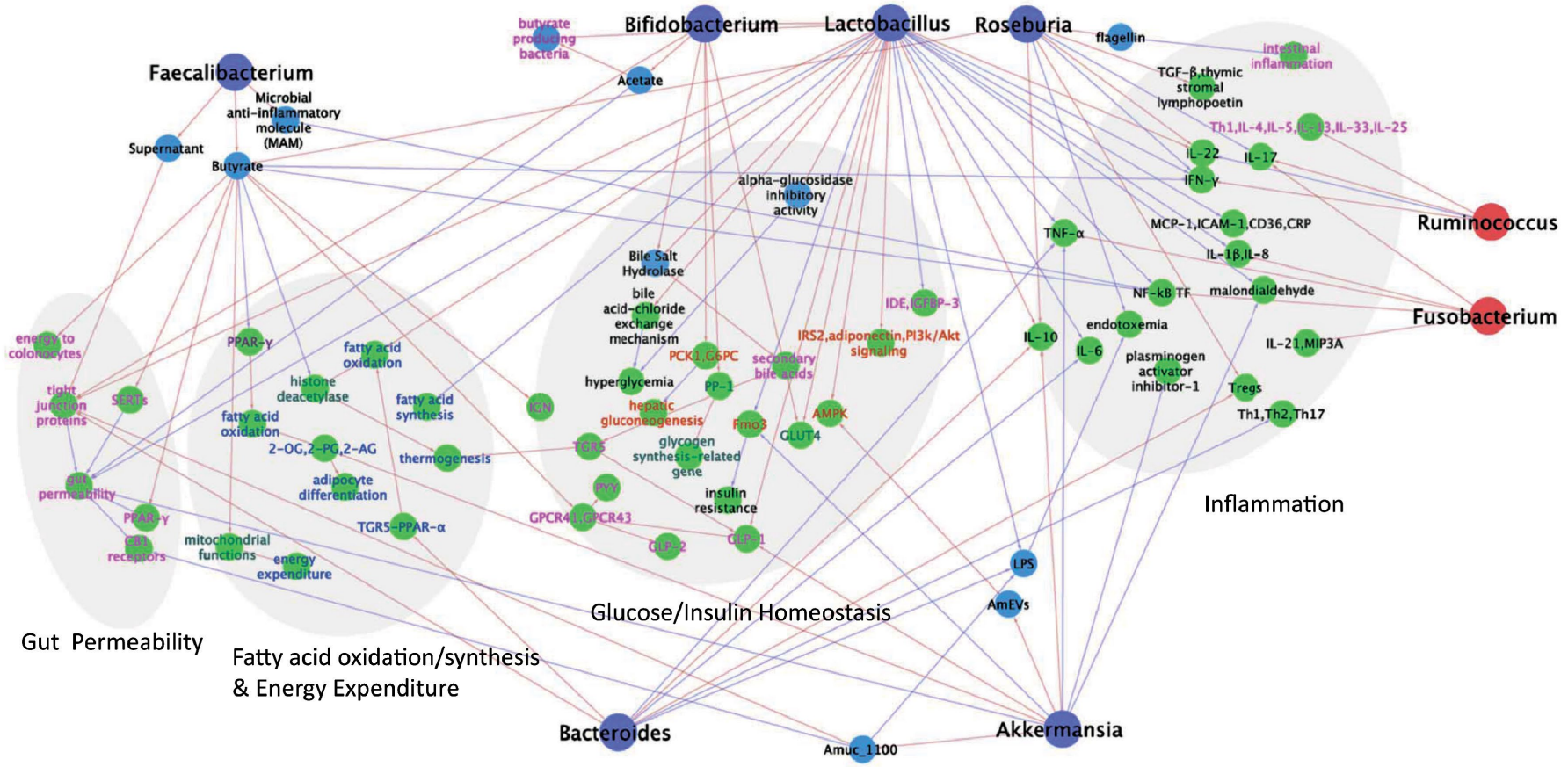


Fig. 1 Microbial genera most frequently found to be associated with T2D.

Number of studies reporting one of the indicated genera in association with T2D (without treatment), and including anti-diabetic therapy (All) in addition to the largest human study by He et al., 2018 [[1]].



Color of labels in the node represents different organs where microbiota potentially elicit its effect: adipose tissue, gut, liver, liver&adipose tissue, muscle, systemic
Color of nodes: host features/products; microbial products; microbe positively associated with disease, microbe negatively associated with disease
Color of edge: blue, negative association between nodes, red, positive association between nodes

Fig. 2. Literature-based network analysis of potential effects on metabolism of bacterial taxa consistently found in association with human T2D (shown in Fig. 1). References corresponding to each edge can be found in the text.

3.2 Gut permeability

Increased intestinal permeability is a characteristic of human T2D. It results in translocation of gut microbial products into the blood and causes metabolic endotoxemia [[105]]. Two species (*Bacteroides vulgatus* and *B. dorei*) from the potentially beneficial for T2D genera have been found to upregulate the expression of tight junction genes in the colon leading to reduction in gut permeability, reduction of LPS production and amelioration of endotoxemia in a mouse model [[106]]. Another probiotic bacterium, *Akkermansia muciniphila,* decreased gut permeability using extracellular vesicles which improve intestinal tight junctions via AMPK activation in epithelium [[42]]. The outer membrane protein (Amuc_1100) of this bacterium enhances the expression of occludin and tight junction protein-1 (Tjp-1) and improves gut integrity [[37]]. Amuc_1100 also inhibits cannabinoid receptor type 1 (CB1) in the gut, which in turn, reduces gut permeability and systemic LPS levels [[37]]. While a specific bacterial component was not determined for *Faecalibacterium prausnitzii*, it was shown that the supernatant from the cultured bacterium enhances the expression of tight junction proteins improving intestinal barrier functions in colitis model [[107]]. Finally, butyrate, produced by *Faecalibacterium, Roseburia*, also have potential to reduce gut permeability through serotonin transporters and PPAR-γ pathways [[101]].

3.3 Glucose metabolism

Gut microbiota may also affect T2D by influencing glucose homeostasis and insulin resistance in major metabolic organs such as liver, muscle and fat, as well as by affecting digestion of sugars and production of gut hormones that control this process. For example, one of the potential probiotics discussed above (*Bifidobacterium lactis*) can increase glycogen synthesis and decrease expression of hepatic gluconeogenesis-related genes [[108]]. In the same report, *B. lactis* improved the translocation of glucose transporter-4 (GLUT4) and insulin-stimulated glucose uptake.

Lactobacillus gasseri BNR17 also increases GLUT-4 expression in the muscle with potential anti-diabetes effect [[109]]. *Akkermansia muciniphila* and *Lactobacillus plantarum* reduce the expression of hepatic flavin monooxygenase 3 (Fmo3) [[37],[93]], a key enzyme of xenobiotic metabolism, whose knockdown has been found to prevent development of hyperglycemia and hyperlipidemia in insulin resistant mice

[[110]]. *Lactobacillus casei* can ameliorate insulin resistance by increasing the mRNA level of phosphatidylinositol-3-kinase (PI3K), insulin receptor substrate 2 (IRS2), AMPK, Akt2 and glycogen synthesis in the liver [[97],[111]]. The effect of this particular microbe is not limited to the effects on liver. Indeed, *L. casei* also reduces hyperglycemia via a bile acid-chloride exchange mechanism involving the up regulation of multiple genes, i.e., ClC1-7, GlyRα1, SLC26A3, SLC26A6, GABAAα1, Bestrophin-3 and CFTR [[112]]. It also decreases the insulin-degrading enzyme (IDE) in the caco-2 cells and insulin-like growth factor binding proteins-3 (IGFBP-3) in the white adipose tissue [[97],[111],[113]]. *L. rhamnosus*, another lactobacillus species, increases adiponectin level in the epididymal fat, thus, improving insulin sensitization [[98]].

Some species of Lactobacillii and *Akkermansia muciniphila* possess potent alpha-glucosidase inhibitory activity that prevents the breakdown of complex carbohydrates and reduces postprandial hyperglycemia [[52]]. Microbiota and their products can modulate gut hormones and enzymes and improve insulin resistance and glucose tolerance. Butyrate can act as ligand for G-protein coupled receptors (GPCR41 and GPCR43) in the gut and promotes the release of gut hormones GLP-1, PYY and GLP-2 from entero-endocrine l-cells (reviewed in [[114],[115]]). *Bifidobacterium* and *Lactobacillus* produce bile salt hydrolases, which convert primary conjugated bile salts into deconjugated bile acids (BA) that are subsequently converted into secondary BA. Secondary BAs activate the membrane bile acid receptor (TGR5) to induce the production of GLP-1 (reviewed in [[114]]).

3.4 Fatty acid oxidation, synthesis and energy expenditure

Increasing fatty acid oxidation and energy expenditure and reducing synthesis of fatty acids ameliorates obesity and consequently T2D [[116]]. *Akkermansia muciniphila, Bacteroides acidifaciens, Lactobacillus gasseri* and short chain fatty acids have been reported to increase fatty acid oxidation in the adipose tissue.

For example, *Akkermansia muciniphila* has been found to increase the levels of 2-oleoyl glycerol (2-OG), 2-palmitoylglycerol (2-PG), 2-acylglycerol (2-AG) in the adipose tissue which increase the fatty acid oxidation and adipocyte differentiation [[39]]. Furthermore, *Bacteroides acidifaciens* also improves fatty acid oxidation in the adipose tissue via TGR5-PPAR-α pathway [[25]]. Likewise, butyrate can promote fatty acid oxidation and thermogenesis by inhibiting the histone deacetylation process in the muscle which increases energy expenditure partially by promoting mitochondrial functions in the

muscle [[117]]. In liver and adipose tissue, butyrate and other two SCFAs, propionate and acetate, decrease the expression of PPAR-γ [[118]] which in turns increases fatty acid oxidation. *Lactobacillus gasseri* has been shown to reduce obesity by increasing the fatty acid oxidation genes and reducing fatty acid synthesis related genes [[109]]. Serum level of malonidialdehyde, a marker of oxidative damage of lipids, has been found to be reduced by *Akkermansia muciniphila* and *Lactobacillus casei*in diabetic rodents [[87],[96]]. Hence, members of microbiota with beneficial effect on T2D modulate fatty acid metabolism and associated energy expenditure in the host that results in alleviation of obesity and accompanying T2D.

3.5 Combined effects of bacteria

Besides the above-mentioned mechanisms, some microbes can also affect the host physiology by increasing other potential beneficial microbiota or by cross-feeding. Several species of Bifodobacterium were shown to have cross feeding interaction with other microbiota like Faecalibacterium and Roseburia [[119],[120]]. *Lactobacillus rhamnosus* can increase Bifidobacterium abundance in the cecum of rats [[98]]. *L. casei* has been found to increase the butyrate producing bacteria [[97],[111]].

4. Contribution of microbiota to the success of drug therapy for T2D

The interplay of drugs and gut microbiota is receiving much-deserved interest (reviewed in [[121]]). It is well known that antibiotics [[122],[123]], non-antibiotic drugs [[124]] and anti-diabetic drugs (Table 2) can modulate microbiota and improve diabetes. Similarly, the baseline microbiota can positively and negatively affect the pharmacokinetics and pharmacodynamics of drugs and numerous chemicals via a variety of mechanisms (reviewed in [[125]]). Fewer studies, however, have examined how altering gut microbiota (via pre- and/or probiotics) changes the effects of anti-diabetic drugs.One recent study examined effects of a probiotic *Bifidobacterium animalis* ssp. *lactis* 420, prebiotic polydextrose and their combination with sitagliptin in diabetic mice [[126]]. The combination of sitagliptin with pre- and probiotics was effective in reducing several T2D parameters. A similar study in Zucker diabetic rats observed that combining prebiotic polysaccharide with the antidiabetic drugs metformin and sitagliptin reduced hyperglycemia and adiposity compared to using only the drugs [[127]].

In another study, streptozotocin-induced diabetic mice were treated with a combination of a prebiotic and metformin. Improvements in fasting blood glucose, glucose tolerance and insulin resistance were observed with the combined therapy, as compared to metformin or MOS alone [[128]]. Thus, a new direction in the microbiome research has emerged focused on the interaction between anti-diabetic drugs and microbiota. These studies should answer important questions such as (1) how different anti-diabetic drugs affect microbiota; (2) which characteristics of gut microbiota are underlying different responses to anti-diabetic drugs; and (3) which co- pre- and probiotics are needed to improve response to medication.

5. Outstanding questions

T2D is a multi-organ, heterogeneous, multi-factorial disease making the dissection of causative microbes from the gut microbiome challenging. In human studies, confounding factors like geographic location, race, culture, health status and drug-use lead to inconsistency in identifying microbiota associated with T2D [[1]]. Moreover, due to challenges in sampling from the intestine of humans, most studies use stool samples for microbiota analysis. However, the stool microbiota profile does not fully reflect the gut microbiome. Furthermore, most studies focused on genomics, rarely studying the transcriptome, proteome or metabolome. Even at the genomic level, deep shotgun sequencing is expensive, making marker-based amplicon sequencing such as 16S rRNA gene prevailing. Further, the existing sequencing and analysis technologies rarely identify (annotate) microbes at species or strain levels. Considering that the functional capacity varies between strains from the same species, identification of microbes and microbial genes associated with disease is challenging.

A significant problem in the field is that the majority of human association studies do not attempt to infer microbes that may have contributing and/or causal role in T2D. Although inference of causality is a complex statistical problem, it is possible for host-microbiome interactions. Indeed, new approaches, such as Transkingdom Network Analysis [[122]] and novel application of Mendelian Randomization methods [[129]], have been recently developed and validated to answer which microbes and microbial genes/pathways are in control of host physiological processes.

Table 2 Contribution of microbiota to the success of therapy of T2D.

Anti-diabetic Drug	**Effects on Microbiota**	**References (PMID)**		**References (PMID)**
	Promotes		**Reduces**	
Biguanides (Metformin)	*Akkermansia muciniphila, Escherichia,Bifidobacterium adolescentis, Lactobacillus,Butyrivibrio, Bifidobacterium bifidum, Megasphaera, Prevetolla, Escherichi-Shigella, Erysipelotrichaceae incertate sedis, Fusobacterium, Flavonifractor, Lachnospiraceae, Lachnospiracea incertae sedis, and Clostridium XVIII and IV*	23804561, 28530702, 25038099, 27999002, 29056513, 30261008, 30815546, 29789365	*Intestinibacter, Romboutsia, Peptostreptococcaceae unclassified, Clostridiaceae 1 unclas sified, Asaccharospora, Alistipes, Oscillibacter, Bacteroides, Parabacteroides, un-Ruminococcaceae*	28530702, 30261008, 29789365
Alpha-glucosidase Inhibitors (eg. Acarbose, voglibose, miglitol)	Lactobacillus, Faecalibacterium,Dialister,Subdoligranulum,Allisonella , *Megasphaera,* Bifidobacterium, Enterococcus, faecalis	28130771, 29176714, 25327485	*Butyricicoccus, Phascolarctobacterium, Ruminococcus, Eggerthella, Bacteroides, Oribacterium, Erysipelotrichaceae, Coriobacteriaceae*, Bacteroides	28130771, 28349245, 29176714, 25327485

Anti-diabetic Drug	**Effects on Microbiota**	**References (PMID)**		**References (PMID)**
	Promotes		**Reduces**	
GLP-1 Receptor agonist(eg. Liraglutide)	*Akkermansia muciniphila, Bacteroides acidifaciens, Lachnoclostridium, Flavonifractor, Ruminococcus gnavus,Allobaculum, Turicibacter, Anaerostipes, Lactobacillus, Butyricimonas, Desulfovibrio*	30815546, 30292107, 29171288, 27633081	*Helicobacter, Prevotella, Ruminococcaceae, Christensenellaceae, Roseburia, Candidatus Arthromitus, Marvinbryantia,Incertae Sedis*	30292,107,29 171288, 27633081
Thiazolidinediones (Pioglitazone)			Proteobacteria	27751827
DPP-4 Inhibitors (Vildagliptin,sitagliptin,saxagliptin)	Lactobacillus, *Streptococcus, Bacteroides acidifaciens, Streptococcus hyointestinalis, Erysipelotrichaceae, Allobaculu, Turicibacter,Roseburia*	29797022, 29036231, 27633081, 27631013	Oscillibacter, Ruminiclostridium_6, *A naerotruncus, Kurthia,Christensenellaceae, Prevotellaceae, Bacteroides,Prevotella,Blautia,*	29797022, 29036231, 27633081, 27631013
SGLT2 Inhibitors (eg.Dapagliflozin)	Akkermansia, Enterococcus	29703207	*Oscillospira*	29703207

Finally, challenges related to animal studies testing effects of microbiota on diabetes hamper progress. First, discrepancies between results caused by differences between microbiomes of otherwise genetically identical animals is one problem. Second, current advanced technologies in gnotobiotics such as studies of germfree and mono/oligo-colonized animals are currently incompatible with functional metabolic studies employing metabolic cages and hyperinsulinemic-euglycemic clamp techniques. Our research community should overcome these technical challenges and develop robust experimental systems to validate predictions coming from human studies and investigate mechanisms of host-microbiota interactions in metabolic diseases.

Future research is needed to develop new diagnostic, preventive and therapeutic microbiota tools for personalized/precision medicine of T2D. First, design of microbiome studies will need to account for clinical, molecular, and genetic as well as drug response diversity of T2D patients stratifying patient populations for analyses. Second, non-invasive approaches to collect microbiota samples from different sites of intestinal tract are needed as fecal material is limited in representation of gut microbiota. Third, while it is easier to focus on individual causal microbes, identifying combination of microbes is required to truly capture the community-level dynamics of the gut microbiota. In addition to taxon-based analysis, grouping microbes by function regardless of taxonomic similarity and function-based analysis should be pursued. Accordingly, we anticipate development of a new generation of analytical methods that will model cause-effect relationships and infer targets of therapeutic interventions. Finally, in order to test new drugs and probiotics as well as drug-microbiota interactions, well-defined gnotobiotic models, specifically humanized microbiota, will become a main tool in animal studies.

6. Conclusion

Despite multiple studies supporting the importance of gut microbiota in pathophysiology of T2D, the field is in early stage. Currently, we have reached a point in our understanding that some microbial taxa and related molecular mechanisms may be involved in glucose metabolism related to T2D. However, the heterogeneity of T2D and redundancy of gut microbiota do not promise simple interpretations (e.g. low diversity) and easy solutions (such as fecal transplant from non-diabetic/non-obese donor). In contrast, we should work

towards precision/personalized medicine selecting anti-diabetics and probiotics for a given patient based on the combination of her/his mammalian and microbial genomes.

7. Search strategy and selection criteria

PubMed and Google Scholar literature searches were performed. To identify gut microbiome composition of T2D patients, articles between 2006 and 2018 were included with combinations of the terms “T2D”, “Glucose”, “gut” “Microbiome” “16S rRNA”, “metagenomics”, and “sequencing”. Additional papers relevant to our research were manually sought through bibliography search. Inclusion criteria in our review were (1) Human case-controlled studies; (2) articles focused on T2D (3) gut microbiota quantified from stool samples; (4) Glucose testing performed during the study (5) Either 16S rRNA gene sequencing or metagenomic sequencing performed in stool samples.

Google scholar and PubMed found 42 papers relevant to our focus. Articles were rejected if it was determined from the title and the abstract that the study failed to meet the inclusion criteria. Any ambiguities regarding the application of the selection criteria were resolved through discussions between at least 3 researchers involved. Each publication was an academic and peer-reviewed study.

Majority (79%) of studies utilized 16S rRNA gene sequencing with V3 and V4 regions most frequently (33% and 42%, respectively) targeted for sequencing (Supporting Table 1). Human subjects across all studies had mean age of 53 years (standard deviation 10 years) and were equivalently distributed between sexes. On average, patients had body mass index 28.3 ± 3 whereas controls 25.8 ± 4.

We searched for mouse colonization studies for the top 8 microbes found in the human-case studies. Articles between 1997 and 2018 were included with combinations of the terms “Mouse”, “Glucose”, “[*selected microbe*]”. Selected microbes included: Bacteroides, Bifidobacterium, Lactobacillus, Blautia, Faecalibacterium, Ruminococcus, Roseburia, and Fusobacterium. Inclusion criteria were (1) Mouse colonization studies; (2) Articles focused on T2D; (3) Glucose testing performed during the study. We also analyzed the literature on *Akkermansia muciniphila*, though it is in the species level, because of its recent emergence as an important potential probiotic microbe.

Similar to the mouse colonization study literature search, we searched for results from clinical trials with microbes/probiotic supplementation. Inclusion criteria were: (1) Human

Clinical study w/ microbes/probiotic supplementation; (2) Glucose testing performed during the study; and (3) Microbes or Probiotics from genera identified in our papers as frequently found in human association studies.

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Supplementary materials

Supplementary material associated with this article can be found in the online version at doi:10.1016/j.ebiom.2019.11.051.

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CHAPTER 3: TRANSKINGDOM INTERACTIONS BETWEEN *LACTOBACILLI* AND HEPATIC MITOCHONDRIA ATTENUATE WESTERN DIET INDUCED DIABETES

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Transkingdom interactions between *Lactobacilli* and hepatic mitochondria attenuate western diet-induced diabetes

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3.1 Abstract

Western diet (WD) is one of the major culprits of metabolic disease including type 2 diabetes (T2D) with gut microbiota playing an important role in modulating effects of the diet. Herein, we use a data-driven approach (Transkingdom Network analysis) to model host-microbiome interactions under WD to infer which members of microbiota contribute to the altered host metabolism. Interrogation of this network pointed to taxa with potential beneficial or harmful effects on host's metabolism. We then validate the functional role of the predicted bacteria in regulating metabolism and show that they act via different host pathways. Our gene expression and electron microscopy studies show that two species from *Lactobacillus* genus act upon mitochondria in the liver leading to the improvement of lipid metabolism. Metabolomics analyses revealed that reduced glutathione may mediate these effects. Our study identifies potential probiotic strains for T2D and provides important insights into mechanisms of their action.

3.2 Introduction

Increasing evidence underscores the importance of the microbiome in human metabolic health and disease[1]. One of the most prevalent metabolic diseases, type 2 diabetes (T2D), is now a global pandemic and the number of patients that will be diagnosed with this disease is expected to further increase over the next decade[2]. The so called "western diet" (WD, a diet high in saturated fats and refined sugars) has been recognized as one of the major culprits of T2D with gut microbiota playing an important role in modulating effects of diet[3,4]. Thus, there is an urgent need to elucidate the contributions of gut microbiota to metabolic damages caused by WD and to identify preventive approaches for T2D.

On the one hand, it is believed that complex changes in the structure of gut microbial communities resulting from interactions of hundreds of different microbes, also called dysbiosis, underlies metabolic harm to the host[5]. On the other hand, some reports claim that individual members of the microbial community changed by the diet might have a significant impact on the host[6]. Although these two points of view are not necessary mutually exclusive, it is still unclear which hypothesis is more credible[7].

Herein, we used a data-driven systems biology approach (Transkingdom Network analysis) to model host-microbe interactions under WD and to investigate whether individual members of microbiota and/or their interactions contribute to altered host metabolism induced by the WD.

The interrogation of the Transkingdom Network pointed to individual microbes with potential causal effects on host's lipid and glucose metabolism. Furthermore, the analysis also enabled inference of whether microbes might elicit beneficial or harmful effects on the host. Additionally, we detected associations between the frequencies of these microbes and obesity in humans. We then validated the functional role of the predicted bacteria in regulating metabolism by supplementing mice with these microbes. Next, gene expression, electron microscopy and multi-omics network pointed to a novel finding that these two *Lactobacilli* may act by boosting mitochondrial health in the liver leading to the improvement in hepatic lipid and systemic glucose metabolism. Finally, the metabolomics analysis revealed few metabolites (e.g. reduced glutathione) that may mediate beneficial effects of probiotics.

3.3 Results

3.3.1 Transkingdom Network predicts beneficial and harmful microbes

We started by inducing T2D-like metabolic disease in C57BL/6 mice by feeding them a western diet, which prior work has found to yield murine phenotypes that mimic human T2D[8,9,10]. As expected, when compared to mice receiving a control (normal) diet, the mice fed the WD exhibited glucose intolerance and insulin resistance (Figure 1a, Fig. S1). The observed phenotypic changes were consistent at 4 and 8 weeks, as well as between replicate experiments. These results align with previous studies showing metabolic changes in male C57BL/6J mice fed WD[9,10]. Concurrently, the gut (ileum and stool) microbial communities were altered because of diet (Figure 1b). While gut location explained the majority of the variation in the microbial communities as expected[11,12] we observed robust changes in microbiota associated with feeding WD [8,13]. Interestingly, the overall composition of the gut microbiota was similar at 4 and 8 weeks of WD (Supplementary Data 1a).

Previous studies showed associations between ecological properties of microbial community (e.g. Shannon diversity) and host metabolism[14,15]. Therefore, we analyzed the association between several community parameters (Supplementary Data 1b) and host phenotypes altered by WD. However, analysis of data from two separate times points (4 and 8 weeks of WD) and microbiome results from intestinal and fecal samples did not find any correlations that showed significant associations in both independent experiments (Supplementary Data 1c). Thus, it does not seem that general dysbiosis explains metabolic alterations in this experimental system.

Next, we sought to identify specific microbes regulating metabolic parameters using a Transkingdom (TK) network approach; this approach has been successfully used to identify key microbiota associated with various disease states, including human disease[16,17]. Towards this end, we created a TK network by integrating microbial abundances with systemic measurements of host metabolic parameters changed by the WD (Figure 1c, Supplementary Data 2). The TK-network contained 1009 edges between 226 nodes (6 metabolic parameters and 220 microbial OTUs).

The node degree distribution of the TK-network followed the power law function (Figure 1c), supporting that the TK-network captures a cross-regulatory nature of the gut microbiota and host phenotypic ecosystem as power law had been shown as a critical property of biological networks[18,19]. Thus, the TK-network provided an opportunity to infer microbes responsible for controlling the overall composition of the microbial community (i.e. keystone species) as well as those that may control host phenotypes.

To identify microbes that likely contribute to T2D related systemic changes in metabolism, we calculated a network property, called bi-partite betweenness centrality (BiBC) that measures the frequency with which a node connects other microbe and host nodes in the graph[20]. We then integrated BiBC scores of each OTU with the WD-induced changes in abundance of ileal microbiota. A microbe was considered to be potentially beneficial (T2D *improver*) if it had a high-BiBC score and a lower abundance in the ileum of WD-fed mice (Supplementary Data 3). Conversely, a microbe was considered to be potentially *harmful* (i.e., a T2D worsener) if it had a high-BiBC score and a higher abundance in the ileum of mice fed WD (Supplementary Data 4).

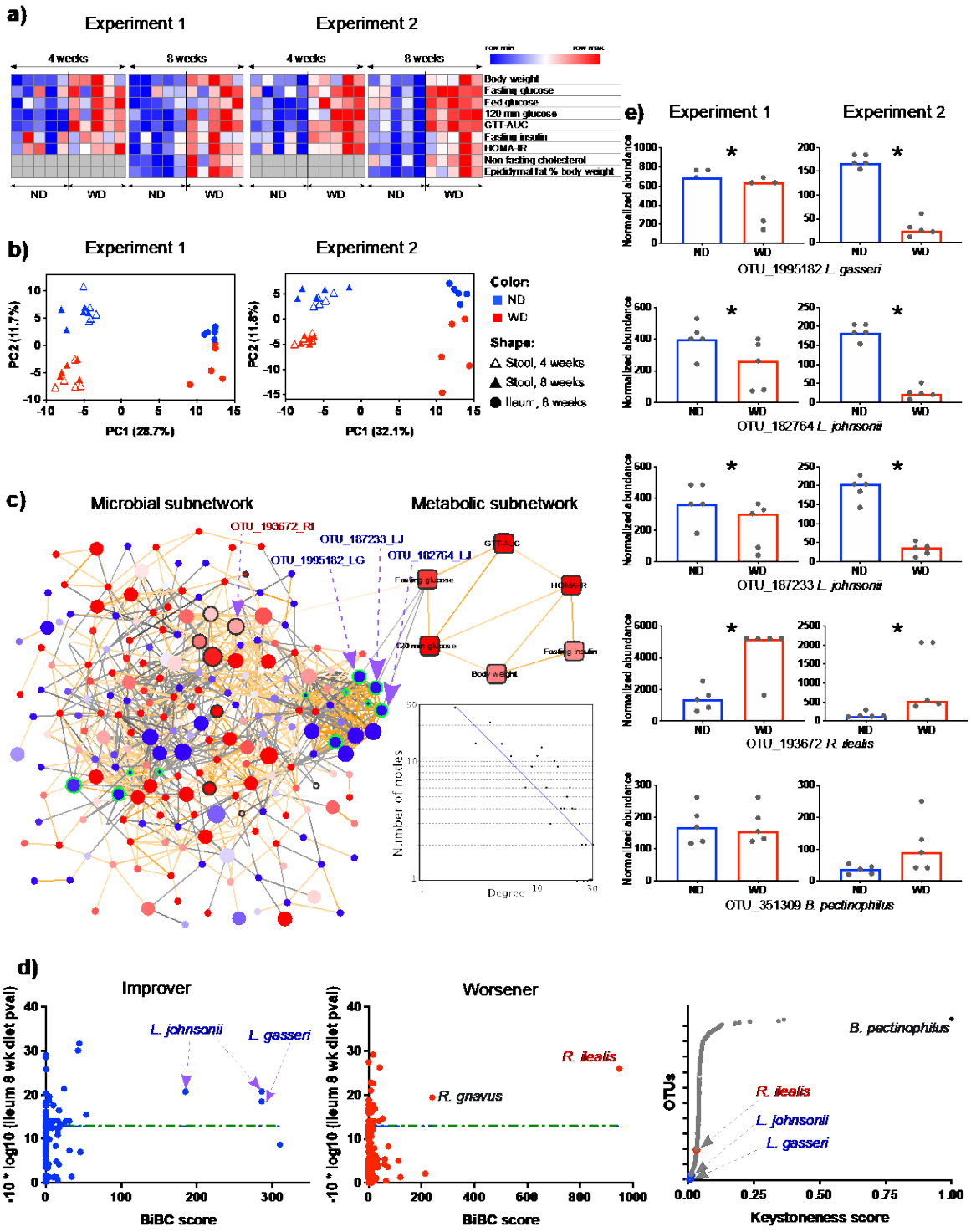

Fig. 1 Inference of gut microbes affecting glucose metabolism in the host.

a. The red and blue colors indicate higher and lower levels of metabolic parameters measured in mice fed normal diet (ND) or western diet (WD) at 4 and 8 weeks. Source data are provided as a Source Data file. **b.** Principal Component Analysis of stool (triangle) and ileal (circle) microbial communities of mice on

ND (blue) or WD (red). Source data are available at https://www.ncbi.nlm.nih.gov/sra/?term=PRJNA558801. **c.** The microbe and host parameter nodes are represented by circles and squares, respectively, in the transkingdom (TK) network. Red and blue colors of nodes indicate increased and decreased (WD/ND) fold change, respectively, whereas the size of circle represents frequency of microbe in stool of WD mice. The black and green node borders indicate the microbes were significantly increased or decreased, respectively, in ileum of WD mice compared with ND (Fisher's p value across experiments <0.05). The orange and black edges indicate positive and negative correlations, respectively. The degree distribution of the TK-network follows a power law. The blue line indicates the fitted line. Source data are available at https://tinyurl.com/TK-NW-Fig-1C. **d.** The left two figures allow inference of microbial candidates that are potentially improvers (left figure) or worseners (middle figure) using high values of TK-network property (bipartite betweenness centrality (BiBC) on the x axis) and significance of change in ileal (WD vs ND) abundance of microbes (log transformed Fisher's p value across experiments on y axis). The horizontal green line indicates a log transformed value for Fisher's p value of 0.05. The right figure shows the keystoneness score (x axis) of the microbial nodes (y axis). Source data are provided as a Source Data file. **e** Ileal abundance of potential candidate and keystone microbes in ND and WD-fed mice at 8 weeks. Asterisk indicate the change in abundance passed statistical significance threshold (two-tail Mann–Whitney p value <0.2 in each experiment, Fisher's p value across experiments <0.05, and FDR < 10%. Each dot represents a mouse, bars present median of the group. Source data same as for **b**.

As a result of these analyses, we identified four OTUs predicted to regulate glucose metabolism, which corresponded with high similarity to four bacterial species *Lactobacillus johnsonii, Lactobacillus gasseri, Romboutsia ilealis* and *Ruminococcus gnavus* (Figure 1d, 1e; Supplementary Data 16). The first two microbes were considered potentially beneficial (i.e., T2D phenotype *improvers*). The other two (*Romboutsia ilealis* and *Ruminococcus gnavus)* were predicted to be worseners. Notably, *Ruminococcus gnavus* has been previously shown to be associated with obesity[21,22]. Overall, these results indicate that individual microbes and/or their interactions and not community level dysbiosis (Figure 1, Supplementary Data 1) could be key players in T2D.

It was proposed that keystone species have significant influence on the rest of gut microbiota, also characterized by a high number of connections within a network[23,24]. Therefore, we asked whether microbes with characteristics of keystone species in our network are among microbes that are predicted to influence host metabolic parameters. Using an approach developed by Berry and Widder[24], we investigated the microbial network and found one microbe with the closest match to *Bacteroides pectinophilus*, with a prominent keystoneness score, followed by few other microbes that also might qualify as keystone species (Figure 1d, 1e, Supplementary Data 5, Supplementary Data 16). Notably, the candidate microbes predicted to affect the host had a low keystoneness score suggesting that microbes

with potentially high effect on the host do not necessarily play a central role in regulating the microbial community (Figure 1d, Supplementary Data 5).

3.4.2 Inferences from mice are validated by associations in humans

To check the relevance of the candidate microbes in humans we identified a human study of a clinical population that consumes a WD-like diet and used the data to computationally evaluate our predictions[25]. In agreement with inferences from mouse data, we found correlations between body mass index (BMI) and the abundance of these microbial candidates (Figure 2) in obese humans[25]. Specifically, the abundance of improvers was negatively correlated with BMI, whereas the abundance of the worsener was positively correlated. Furthermore, we found *R. ilealis* to be present in over 80% of obese patients, suggesting that this microbe could be a prevalent pathobiont in obese humans. While the result for *R. ilealis* seemed to be more robust we observed only trend of positive association for *R. gnavus* that concurs with much smaller BiBC score for this bacterium (Figure 1, S2). Altogether, these observations provide further support for the predictions resulting from our analyses in the WD-fed mouse model.

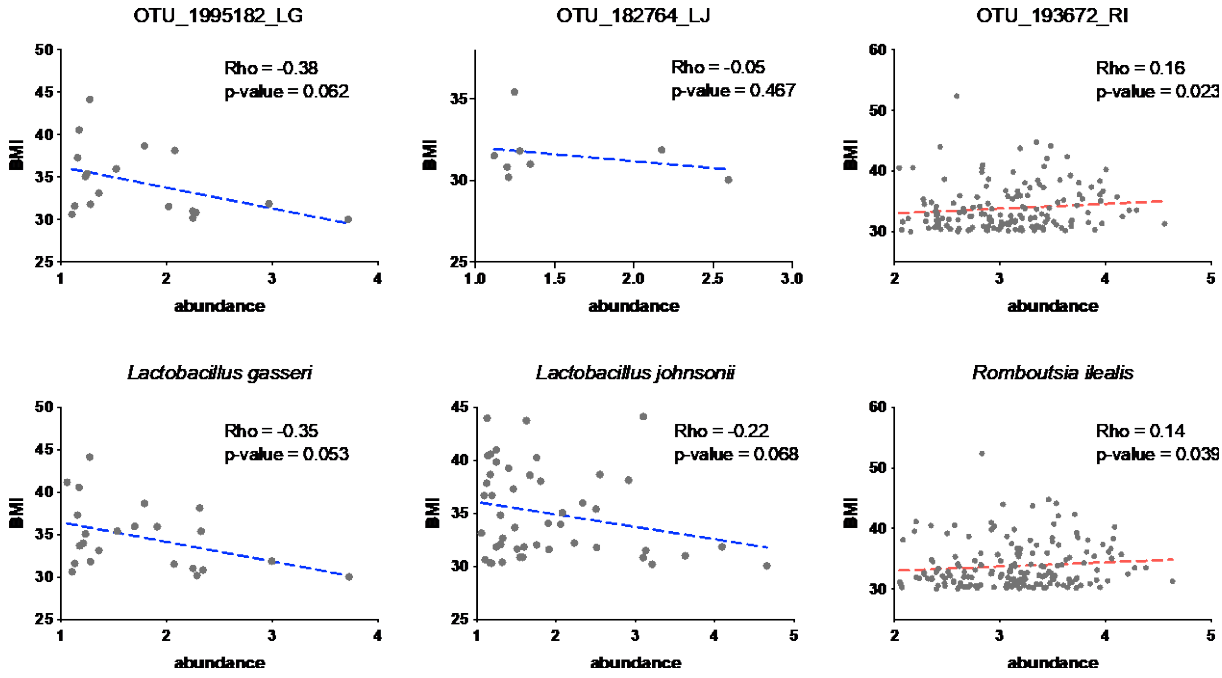

Fig. 2 Computational verification of predicted microbes in human data from the literature[26]

Each scatter plot shows the abundance of the microbes (X axis) in stool versus the BMI of obese humans (Y axis). The dotted line indicates the fitted line. The Spearman rho correlation co-efficient and one-tail p-value is shown. Data retrieved from www.ebi.ac.uk/metagenomics/studies/ERP015317

3.4.3 Lactobacilli improve and *Romboutsia* worsen glucose metabolism

Encouraged by the support of our inferences in human data, we proceeded to test the role of *L. gasseri, L. johnsonii* and *R. ilealis* in *in vivo* experiments designed according to predicted functional effects on the host. We anticipated that potential metabolic improvers (*L. gasseri, L. johnsonii)* would ameliorate metabolism damaged by western diet whereas the potential pathobiont (*R. ilealis*) would worsen metabolism in mice fed with normal diet. As predicted, WD-fed mice administered *L. gasseri* or *L. johnsonii* showed improved glucose tolerance (AUC and 120 min glucose levels) compared to mice on WD (Figure 3a, Figure S3). In addition, supplementation with *L. gasseri* ameliorated the established glucose intolerance in mice (Figure S4). Conversely, mice supplemented with *R. ilealis* showed impaired glucose tolerance (15 mins. glucose levels in GTT) and reduced fasting insulin compared to mice fed with normal diet (Figure 3a, S3). Accordingly, HOMA-B, the index that reflects pancreatic beta-cell function, was also reduced by supplementation with *R. ilealis* (Fig S3). These results suggest that the worsener/pathobiont and improver/probiotic microbes modulate the host systemic phenotypes likely via different mechanisms. Indeed, while higher levels of glucose early after glucose injection are most probably explained by decreased production of insulin in *R. ilealis* supplemented mice, *L. gasseri* and *L. johnsonii* improve glucose tolerance without altering insulin levels. Furthermore, while adiposity was not altered by *R. ilealis,* it was reduced in mice supplemented with improvers (*L. gasseri* or *L. johnsonii*) (Figure 3a).

Although many human studies did not detect significant changes in fecal microbiota after probiotic administration[26-28], there were recent reports concerning the possible damaging effects of probiotics on the upper intestinal microbiota[29,30]. Therefore, we sequenced 16S rRNA gene in ileum and fecal samples from mice supplemented with three candidate bacteria. Very few changes were observed in the ileal and stool microbiota composition due to supplementation by these microbes (Figure 3b, Fig. S5a, Supplementary Data 6). In hindsight, these results agree with the low keystoneness score of all three tested microbes that have indicated their little influence on the rest of bacterial community (Figure 1d). Furthermore, we

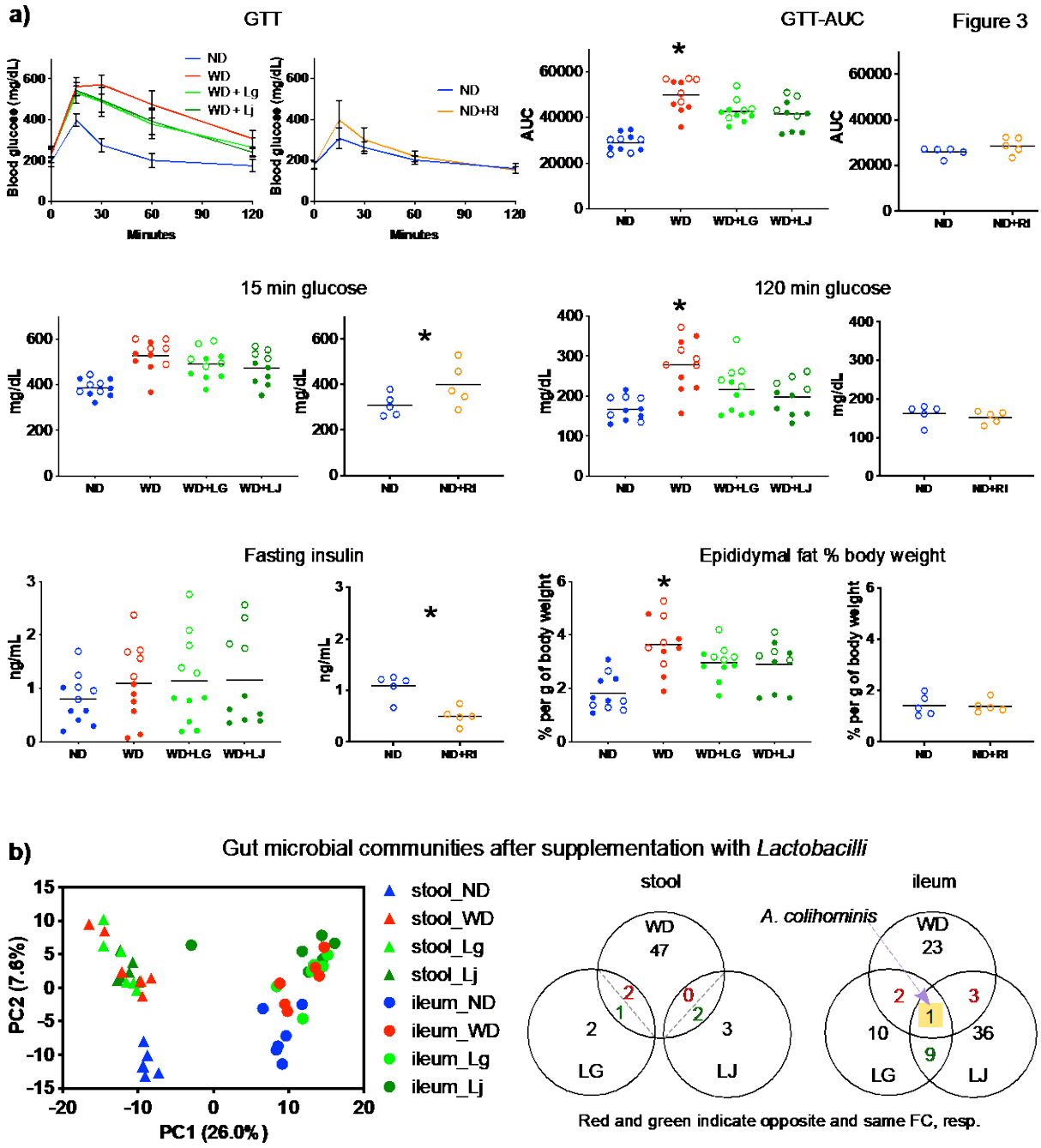

Fig. 3 Experimental validation of microbial candidates

a) Metabolic parameters in mice given control diets and supplemented with or without the indicated microbe. Glucose tolerance test (GTT) curves show the mean and SD of blood glucose over time. Open and closed circles indicate two independent experiments; * indicates statistically significant differences in levels of the parameter between control group (WD for *Lactobacilli*, ND for *R. ilealis*) versus those supplemented with bacteria (one-tail t-test p-value <0.05 with FDR <15%). Blue, ND; red, WD; light green WD with L. gasseri (WD+LG); dark green, WD with *L. johnsonii* (WD+LJ); orange, *R. ilealis* (ND+RI), respectively. Source

data are provided as a Source Data file. b) Principal Component Analysis of stool (triangle) and ileal (circle) microbial communities and Venn diagram of microbes changed in mice on ND, WD, WD+LG or WD+LJ and with >0.1% median abundance in at least one group across experiments (Fisher's p-value <0.05 calculated using two-tail Mann-Whitney per experiment). For Lactobacilli supplementation experiments, n = 11 mice for ND, WD and WD + Lg groups, n = 10 mice for WD + Lj group. For *R. ilealis* (ND and ND+RI), n = 5 mice per group.

did not find differences for individual taxa in stool samples in mice supplemented with bacteria. In the ileum, only one bacterium, *Anaerotruncus colihominis* (Supplementary Data 16), was reduced due to western diet and increased by both *L. gasseri* and *L. johnsonii* (Fig. S5b). In agreement with our result, a study of gut microbiota from the Old Order Amish sect found this microbe to be negatively correlated with BMI and serum triglycerides[31]. Altogether, however, minimal alterations in microbiota induced by *L. gasseri* and *L. johnsonii* supplementation did not explain restoration of glucose metabolism promoted by these bacteria.

3.4.4 Lactobacilli improve hepatic mitochondria and lipid metabolism

Besides identifying effective probiotics for obesity/diabetes, it is critical to establish the host pathways through which these microbes exert their effect. Therefore, we next investigated two major target organs (intestine and liver) upon which both *Lactobacilli* might be acting to improve systemic metabolism. For a comprehensive evaluation of these organs we first analyzed global gene expression altered by *L. gasseri* and *L. johnsonii* supplementation. To identify common mechanisms by which *L. gasseri* and *L. johnsonii* improve metabolism, we focused on the genes that responded similarly to both microbes by identifying genes differentially expressed between both *L. gasseri* and *L. johnsonii* comparing to WD. The transcriptome of the ileum and liver showed distinct changes in response to supplementation by these bacteria (Figure 4a). In striking contrast to the number of genes differentially expressed in the ileum (152, FDR < 10%), there were much higher numbers of genes differentially expressed in the liver (654, FDR < 10%) (Supplementary Data 7-8). Furthermore, the great majority (638/654) of these genes were upregulated by *Lactobacilli* supplementation.

Functional enrichment analysis showed that genes that were changed in the ileum were enriched for only a few categories with the circadian rhythm function as the main one (Supplementary Data 9). Notably, one of the genes was Nfil3, which was downregulated in the ileum of *L. gasseri* or *L. johnsonii* supplemented mice as compared to the WD mice (Supplementary Data 7). In agreement with our results, the knockout of this gene in the

intestinal epithelium had been shown to prevent mice from obesity, insulin resistance and glucose intolerance[32].

Pathway enrichment analysis in liver, however, showed that multiple categories, and processes related to mitochondrial functions were over-represented among genes upregulated by *L. gasseri* and *L. johnsonii* (Figure 4b, S6, Supplementary Data 10). In addition, further analysis demonstrated that genes belonging to all five mitochondrial complexes of the oxidative phosphorylation pathway (Figure 4c) were upregulated in the liver of *L. gasseri* and *L. johnsonii* supplemented mice (Supplementary Data 8). There was also a group of genes coding for large and small subunits of mitochondrial ribosomal proteins with increased levels of expression in the *L. gasseri* and *L. johnsonii* group. Furthermore, genes involved in mitochondrial fusion were upregulated by the *Lactobacilli* including mitofusin 1 and 2 (Mfn1, Mfn2), mitoguardin 2 (Miga2), and optic atrophy 1 (Opa1) (Supplementary Data 8).

Hepatic mitochondrial functions are well known to be dysregulated in T2D[33-35]. Overall, our results suggest that in addition to mitochondrial functions, these probiotic bacteria induced structural/morphological changes in liver mitochondria. Thus, we performed electron microscopy of the livers from mice fed with WD and supplemented or not with each *Lactobacilli* (i.e. WD, WD+LG, WD+LJ) (Figure 4d). While there was no difference in the number of mitochondria, overall area occupied by mitochondria was larger in WD group mice than in *L. gasseri* or *L. johnsonii* (Figure 4e) suggesting increased size of mitochondria in livers of WD as compared to mice supplemented by *Lactobacilli*. This result indicates that mitochondrial swelling caused by WD, a phenomenon that can perturb proper functioning of mitochondria[36-38], was ameliorated by probiotic supplementation.

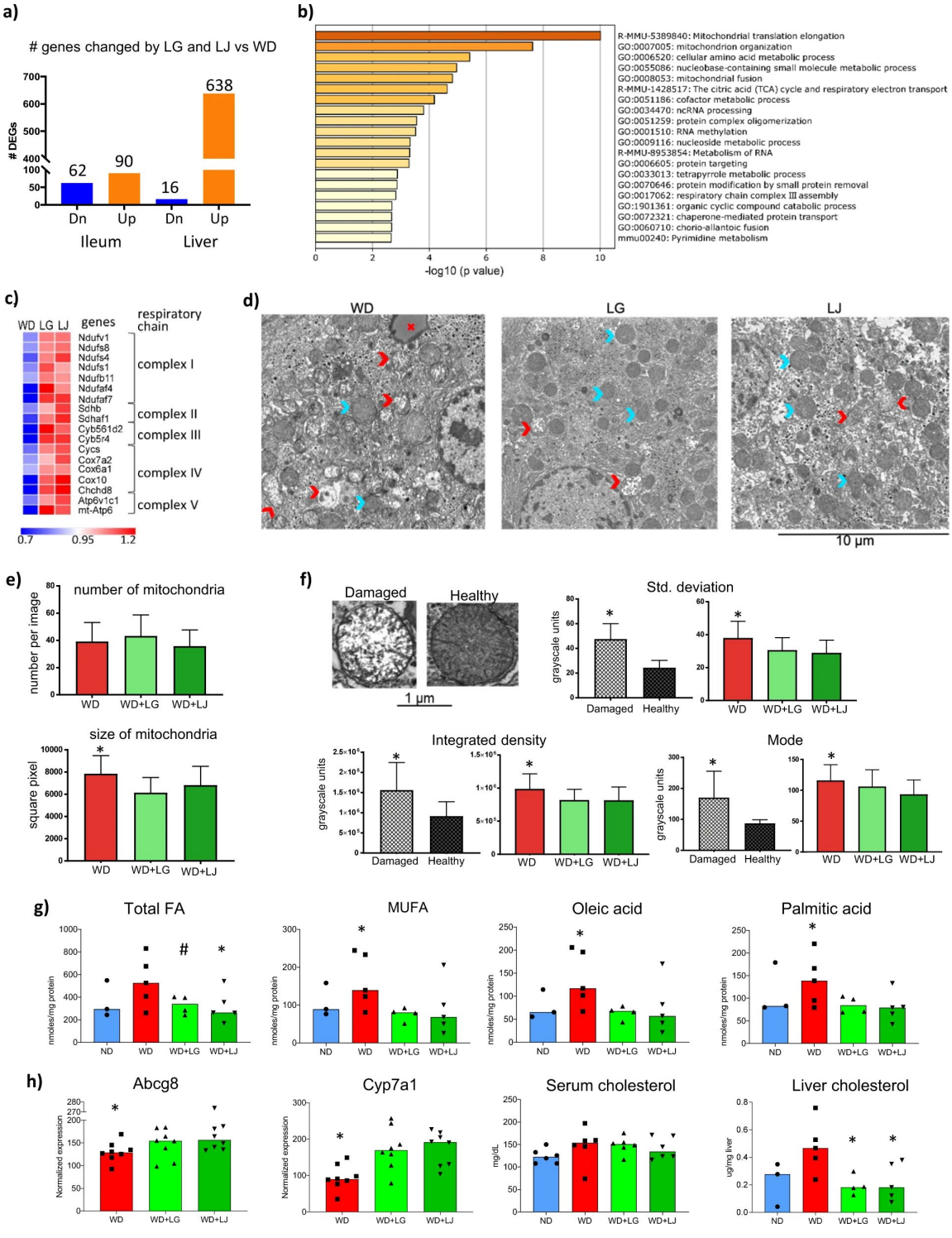

Fig. 4 Transcriptome analysis, liver mitochondria and lipids after supplementation with *L. gasseri* or *L. johnsonii.*

a) Number of differently expressed genes (#DEGs, two-sided t-test p-value <5% in each Lactobacilli, Fisher's p-value < 5% calculated over both *Lactobacilli*, and FDR <10%) regulated by *L. gasseri* and *L. johnsonii* in the same direction comparing to western diet. b) Over-represented processes in the genes of the network in a) of mice supplemented with *Lactobacilli*. c) A heatmap showing the median expression of genes from the respiratory chain process in the livers of mice. d) Representative electron microscope images of liver cells. The blue and red arrows indicate healthy and damaged mitochondria, respectively. e), f) various metrics of mitochondria in the liver of mice; *statistically significant differences between control and groups supplemented with bacteria (one-sided t-test p-value < 5%). Data are presented as mean ± s.d. (n = 40 images for WD, n = 35 images for WD + LG and n = 37 images for WD + LJ groups; n = 60 mitochondria for healthy and n = 61 for damaged mitochondria). Source data are provided as a Source Data file. g) Levels of long chain fatty acids, h) expression of cholesterol metabolism genes in livers of mice fed WD and supplemented with or without *Lactobacilli*. Each symbol represents one mouse, bars are median values. Source data are provided as a Source Data file; n=3-5 mice per group (except serum cholesterol where n=10-11 mice per group); * indicates statistically significant differences in WD vs WD+ LG or LJ (one-sided t-test p-value <5%); # indicates p=0.065.

Next, we undertook quantitative evaluation of mitochondrial ultrastructural changes. Current agreement in the field is that healthy and damaged mitochondria correspond to dark, electron-dense and lucent, fragmented cristae images, respectively[37,39]. According to those criteria, we first identified a set of healthy and damaged mitochondria within individual images (Figure 4f). Next, we estimated, in an unbiased manner (i.e. comparing healthy and good mitochondria within a given sample), which image parameters discriminated between the two types of mitochondria. We found lower values of standard deviation, integrated density, and the density mode in healthy compared to damaged mitochondria (note, in grayscale, white is 255 and black is 0) (Supplementary Data 11). Comparison between the three groups of mice showed significantly lower levels of these parameters in *L. gasseri* and *L. johnsonii* groups than in WD (Figure 4f), pointing to healthier mitochondria in the former two groups of mice. Overall, these results support the prediction derived from gene expression data and indicate that *L. gasseri* and *L. johnsonii* supplementation prevented hepatic mitochondrial damage induced by western diet.

One of the important consequences of improved mitochondrial health is a restoration of fatty acid beta-oxidation. This process decreases build-up of detrimental fatty acids in the liver leading to improved systemic glucose metabolism[40,41]. In our data, among 19 regulated genes from the beta-oxidation gene subset, 18 genes were upregulated by supplementation of probiotic strains (Supplementary Data 12). Among upregulated genes were those involved in fatty acid transport (Slc25a17, Slc27a2), oxidation (Acads, Acadl) and hydration (Echs1) of

fatty acyl representing major steps of beta-oxidation. These results pointed to possible increase in catabolism of fatty acids by *Lactobacilli* supplementation. Indeed, we found overall reduction of total hepatic lipids including several most abundant fatty acids known to have damaging effects on metabolism associated with T2D [42] such as monounsaturated fatty acids, oleic and palmitic acids (Figure 4g, S5c, Supplementary Data 13). Overall, these results are in accordance with the idea that changes in the liver fat are central to development as well as reversion of T2D[43].

Besides fatty acids metabolism, two genes with well-established functions in cholesterol metabolism were also upregulated by both *Lactobacilli:* Abcg8, (hepatic cholesterol efflux[44]) and Cyp7a1, (conversion of cholesterol into bile acids[45]) (Fig. 4h). Therefore, we measured cholesterol in liver and serum samples. Although there was no change in serum cholesterol, there were reduced levels of liver total cholesterol in mice supplemented with *L. gasseri* or *L. johnsonii* (Figure 4h). These results agree with an idea that alterations in the liver might precede lipid alterations detectable in serum[43].

3.4.5 Multi-omic network infers key liver genes for effects of *Lactobacilli*

To identify potential mechanisms by which *Lactobacilli* alter lipid and glucose metabolism, we created a multi-omic network by integrating the gene expression changed by *Lactobacilli* and lipid profile from the liver with systemic measurements of metabolic parameters changed by the WD (Figure 5a). The multi-omic network contained 1776 edges connecting 380 nodes. The node degree distribution of this network followed the power law function (Figure S7), a critical property of biological networks[18,19]. Furthermore, while over half of differentially expressed genes made into the multi-omic network, the enrichment analysis showed similar results with mitochondrial translation, fusion, organization and autophagy formations being top enriched functions in this network (Figure 5b). Next, we interrogated this network to infer genes regulated by *Lactobacilli* and potentially responsible for changing the systemic phenotypes. Specifically, we used the degree (local network property counting the immediate neighbors) and BiBC[20], which is a global network property that measures the overall frequency with which a node connects to the nodes of other omics-type in the graph. Noteworthy, we found that gene expression nodes were predominantly connected to GTT, fasting glucose and 120 min glucose, two of which were significantly decreased by *Lactobacilli* supplementation (Figure 5a, 5c). Furthermore, Ifitm3, Usp50, Rai12 (Elp5) and Snap47, which are known to be

involved in the maintenance of functional mitochondria[46-48], were found as key genes connecting expression alterations with systemic glucose metabolism (Figure 5c). Interestingly, epididymal fat (also decreased in mice by *Lactobacilli*) was highly connected to liver fatty acids and to only one gene (Mfsd3), which codes for a solute carrier previously found in association with palmitic acid levels in a genome-wide association study[49].

Thus, the network analysis further suggested that expression of genes responsible for mitochondrial organization and maintenance in the liver is the primary driver of improved systemic glucose metabolism.

3.4.6 L. gasseri and *L. johnsonii* increase serum glutathione and bilirubin

Next, we applied a metabolomics approach to identify potential mechanisms responsible for improved hepatic mitochondrial health evoked by *Lactobacilli*. First, we established which metabolites were specifically increased by these bacteria in the serum of mice that did not contain other microbes. For this, germfree mice fed WD were monocolonized or not with *L. gasseri* for 2 weeks and mouse serum was subject to metabolite profiling. Out of 133 metabolites that were identified (Supplementary Data 14a), 12 were increased after monocolonization, ranging from two-fold for 8-iso-15-keto-PGF2a to 48 for bilirubin (Figure 5d, Supplementary Data 14b). After this pre-selection in mono-colonized mice, we compared abundance of the 12 metabolites between pools of sera of SPF mice supplemented with *L. gasseri* or *L. johnsonii* in three independent experiments (see details in Methods). We found that reduced (but not oxidized) glutathione (GSH) increased about 4 times, and bilirubin showed a trend to increased levels (FDR=0.12), while two tauro-conjugated bile acids and 3-hydroxytetradecanedioic fatty acid showed various levels of decrease in *Lactobacilli* supplemented SPF mice (Figure 5e, Supplementary Data 14c).

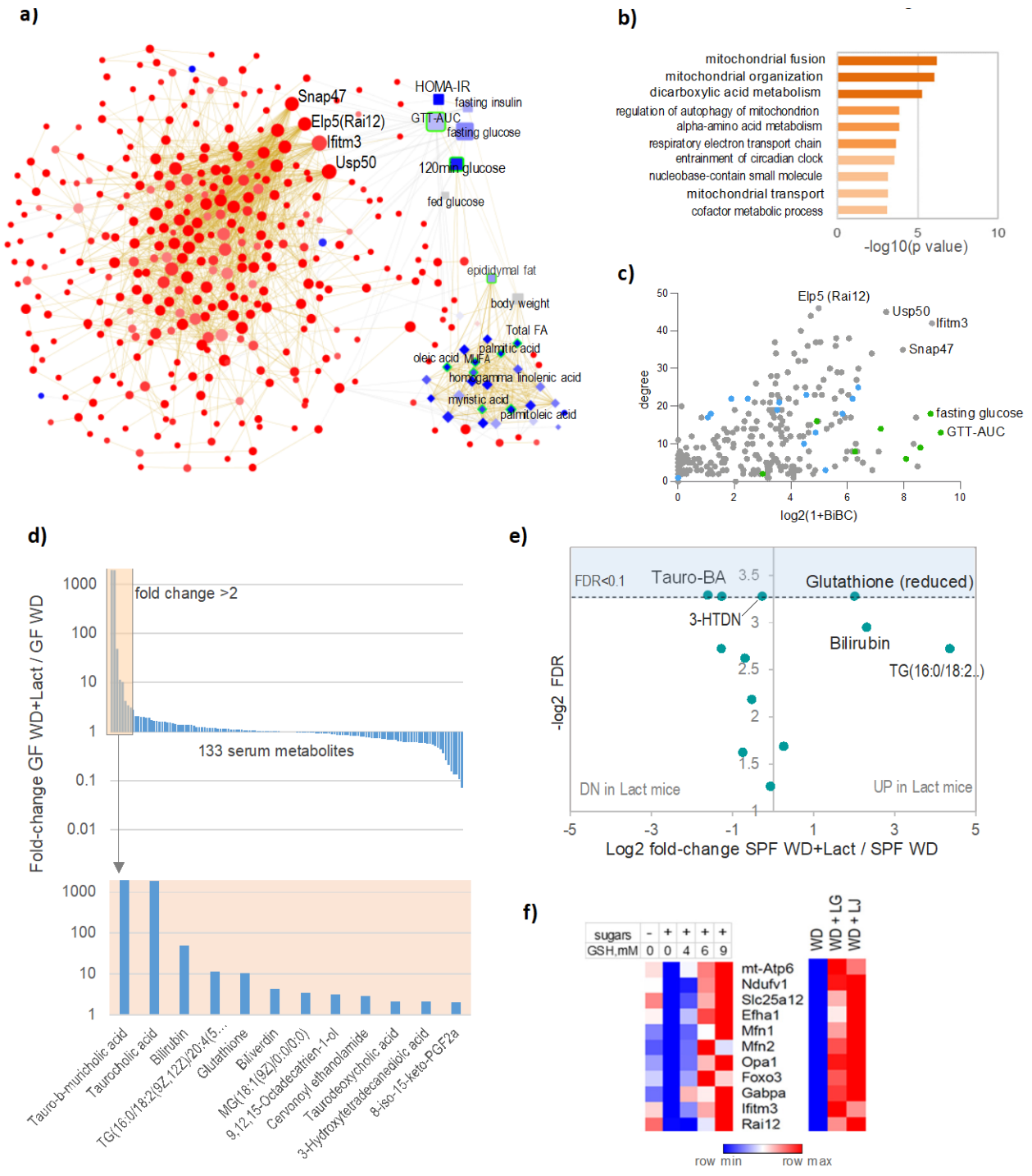

Fig. 5 Multi-omic network analysis, metabolomics in mice supplemented with *Lactobacilli* and validation of glutathione in vitro

a) Multi-omic network integrating gene expression of genes significantly regulated in liver by *Lactobacilli* (circles), liver lipid profile (diamonds), and systemic metabolic parameters (squares) with red symbols indicating up-regulated and blue are down in *Lactobacilli* supplemented mice. Green outline of nodes indicates significantly decreased lipid or phenotype; size of circle corresponds to the combined score of degree and bi-partite betweenness centrality (BiBC) in the network. The orange and black edges indicate positive and negative correlations, respectively. Genes with top degree and BiBC are indicated. Source data are available at https://tinyurl.com/multi-omic-NW-Fig-5A **b)** Gene ontology biological functions over-represented in the genes of multi-omic network. **c)** Scatterplot showing the degree and BiBC of all nodes in the multi-omic network with genes (grey), lipids (blue), phenotypes (green). **d)** Fold-changes of 133 serum metabolites in germfree (GF) mice fed western diet (WD) and colonized with *L. gasseri* for 2 weeks in comparison to GF mice on WD (n=2 per group). TG, Triacylglycerol (16:0/18:2(9Z,12Z)/20:4(5Z,8Z,11Z,14Z)); MG, Monoacylglycerol; 8-iso-15-keto PGF2α, 8-iso-15-keto Prostaglandin F2α. Source data are provided in Supplementary Supplementary Data S14. **e)** Changes in 12 metabolites identified in Fig. 5d in specific-pathogen mice (SPF) fed WD (data of serum pools of 4-6 mice in each pool per group), in 5 experiments of *Lactobacilli* supplemented mice, mean fold change across 5 experiments and FDR (false discovery rate) is plotted. Source data are provided in Supplementary Supplementary Data S14c. **f)** Left heat map shows the geometric mean of normalized gene expression in AML12 cells treated with either low sugar medium (glucose 17 mM), high sugar medium (glucose and fructose at 50 mM each) or high sugar medium supplemented with 4 mM, 6 mM or 9 mM of reduced glutathione (GSH) ethyl ester (5-6 independent experiments). The right heat map shows geometric mean of normalized gene expression from RNA-Seq in liver of western diet (WD) fed mice or WD fed mice supplemented with either *L. gasseri* or *L. johnsonii* (red, high; blue, low relative gene expression). Source data are provided as a Source Data file.

Although the mechanisms of GSH surge by *Lactobacilli* is not clear yet, this metabolite seemed to be a plausible candidate to cause hepatic mitochondrial improvement in mice as its antioxidant functions are well-established[50]. To test this hypothesis, we used AML-12 cell culture mimicking diabetic alterations in liver by adding high concentrations of fructose and glucose. Treatment of cells with different concentrations of GSH (in high sugar) enhanced expression of several genes with well-known mitochondrial functions such as mt-Atp6, Ndufv1, Mfn1, Opa1, Foxo3, Gabpa whose expression was also upregulated by *Lactobacilli* in the livers of mice (Figure 5f, Supplementary Data 15a). We further tested three genes (Usp50, Ifitm3, Rai12) predicted by the network analysis (Figure 5c) to play a key role in the control of mitochondrial health in liver and systemic glucose metabolism and have been previously shown to support mitochondrial homeostasis[47,48]. While we could not detect Usp50 in cell culture, the two other genes (Ifitm3, Rai12) showed increased expression in 6 and 9 mM GSH similar to other mitochondrial genes (Figure 5f). Thus, altogether these results indicate that an increase in GSH in the serum of mice is likely to be one of the important mechanisms used by *Lactobacilli* for boosting liver mitochondrial and anti-oxidant function, consequently improving systemic glucose metabolism.

3.5 Discussion

Our work provides further support for the hypothesis that variations in abundance of a few key (but not keystone) microbes rather than overall changes of the microbial community might explain microbiota-related damage caused by western diet in T2D. Indeed, administration of two bacteria (*L. gasseri* and *L. johnsonii*), decreased by western diet, improved systemic glucose metabolism. The fact that this improvement could be achieved by supplementation of single bacteria, however, does not eliminate a possibility of microbe-microbe interaction playing a role in this process. Furthermore, both *Lactobacilli* had very low keystoneness, and accordingly we did not detect strong alterations in the gut microbiota (fecal or ileal) of mice supplemented by these two microbes. This is in agreement with several human studies that used other strains of probiotic bacteria and largely did not observe changes in taxonomic composition of fecal microbiota[26-28]. In contrast, two recent reports showed alterations in human mucosal microbiota communities by probiotics and potential adverse side effects of probiotics, especially when used after antibiotics[29,30].

The two species of *Lactobacilli* we predicted and tested in mice fed WD, enhanced systemic glucose tolerance, decreased adiposity, reduced several "bad lipids" in the liver, which could be all a consequence of improved hepatic mitochondrial health. This thought is supported, on the one hand, by clinical studies that have shown that reduction in hepatic fat in animals and humans results in recovery from T2D[37,51,52]. On the other hand, impairment of liver mitochondrial function has been long known as an important contributor to metabolic disease[33-35,53]. Furthermore, it has been shown that both palmitic and oleic acids (decreased by *Lactobacilli*) can damage liver mitochondria[54,55,56]. Conversely, enhancement of mitochondrial functioning stimulates beta-oxidation resulting in the reduction of damaging fatty acids[57,58].

The multi-omic network analysis in our study further supported the central role of hepatic mitochondrial health. Specifically, it pointed to several genes (Figure 5a-c) involved in proper mitochondrial organization and mitochondrial autophagy (mitophagy) as the key players in relation to systemic glucose metabolism.

Investigations performed over the last decade have reported several mechanisms whereby microbiota can affect T2D including modulation of inflammation and immune mediators, gut hormones, mucosal permeability, insulin production among others[59]. Our present findings bring to the picture of host-microbiota interactions an intriguing link between

mitochondria (regarded as mammalian endosymbionts) and the symbiotic microorganisms in the gut. Interactions between mitochondria and microbiota is an emerging direction in microbiome research and have been implicated in Parkinson's disease[60], intestinal cell death by antibiotic-resistant microbiota[61] and longevity of *C. elegans*[62]. Metabolic health is synonymous with mitochondrial health where the ancestral mitochondrion-microbiome axis may play an important role[63].

Our investigation of serum metabolome pointed to several changes caused by *Lactobacilli.* While the fact that *Lactobacilli* supplementation can alter certain bile acids levels might not be surprising, a biological role of these alterations is uncertain. Furthermore, we were not able to follow up the detected changes by targeted metabolomics in this work, which can be a subject of future studies. However, two metabolites, glutathione and bilirubin, are known to play complementary antioxidant roles, which would improve mitochondrial respiration and other metabolic functions[64,65]. More recent reports demonstrated that deletion of biliverdin reductase A, which transforms biliverdin into bilirubin induced oxidative stress and lipid accumulation[66] and that bilirubin itself protects mitochondria via scavenging O_2^- [67]. Glutathione, however, uses somewhat different mechanisms of beneficial effects on mitochondria. For example, it was shown to improve mitochondrial fusion[68]. Indeed, we found that both *Lactobacilli* in *vivo* and GSH in *vitro* increased expression of three main GTPases (Mfn1, Mfn2, Opa1) required for this process.

Unlike bilirubin, which is produced by hepatocytes, glutathione origin is not limited to mammalian cells but it can also be produced by many bacteria. For example, some species of *Lactobacilli* are known to produce glutathione, which they utilize to protect themselves from bile salts, reactive oxygen species and other types of cellular damages[69,70]. Therefore, it is plausible that our observation of increased levels of glutathione is a result of simultaneous induction of its production by host cells[71] and by *Lactobacilli* itself. Although, further studies are warranted to identify the main source of glutathione, it is highly plausible that this metabolite is one of the main mediators of *Lactobacilli* effect on liver mitochondria.

In agreement with our result, it was reported that another strain of *L. johnsonii* may improve hepatic mitochondria[72]. Interestingly, these mitochondrial effects may not be limited to the liver, since another species of Lactobacilli *L. paracasei* attenuated cardiac mitochondrial

dysfunction in obese rats[73], and a different strain of *L. gasseri* increased resistance to mitochondrial dysfunction in aging *Caenorhabditis elegans*[74]. Notable, the two strains (*L. gasseri* and *L. johnsonii*) identified and tested in our study are also promising candidates for future testing in clinical settings of T2D as they would have minimal adverse effects on gut microbiota while improving glucose metabolism. Other strains of these two species of *Lactobacilli* have been tested in clinical trials for other diseases and in mouse models of diabetes[59,75] and thus might share critical mechanisms of effects on the mammalian host.

In conclusion, our study demonstrates that damaging effects of western diet on metabolism can be at least partially explained by decrease of beneficial microbes (e.g. *Lactobacilli*) and increase of pathobionts (e.g. *Romboutsia ilealis*) in gut microbiota, each of them acting via different host pathways. Furthermore, it revealed potential probiotic strains for treatment of T2D as well as critical insights into mechanisms of their action, offering an opportunity to develop targeted therapies of diabetes rather than attempting to restore "healthy" microbiota as a whole.

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Author contributions
Original idea and overall study design: AM, NS.

Design of individual experiments: RRR, MG, ZL, MGJ, RG, BP, DBJ, GT, AD, AM, NS.

Data generation: MG, ZL, MGJ, RG, HY, JWP, CG, SVP, KDW, BF, BP, DBJ, AD.

Data analysis: RRR, MG, ZL, MGJ, FB, TJS, CG, BP, DBJ, GT, DB, AD, AM, NS.

Drafting manuscript: RRR, MG, ZL, MGJ, FB, BP, AM, NS.

Editing manuscript: TJS, BP, DBJ, GT, DB, AM, NS.

Supervision of specific set of experiments and/or series of data analyses: TJS, BP, DBJ, GT, DB, AM, NS. Overall study leadership: AM, NS.

Competing Interests

The authors declare no competing interests.

3.6 METHODS

Mice and diets

Seven weeks old, C57BL/6 male mice were purchased from Jackson Laboratories (Bar Harbor, Maine) and housed at Laboratory Animal Research Center (LARC) at the Oregon State University. After 1 week of acclimatization, mice were either switched to western diet (WD) D12451 containing 45% lard and 20% sucrose or to a matched normal diet D12450K (ND) produced by Research Diets (New Brunswick, NJ). Mice were on these diets for 8 weeks. Two independent experiments were performed with 5 mice per group in each experiment. Ethical approval for this work was obtained from the Oregon State University Institutional Animal Care and Use Committee. The study complied with all relevant ethical regulations regarding the use research animals.

Bacteria

Lactobacillus gasseri ATCC 33323 were purchased from American Type Culture Collection (ATCC, Manassas, VA). *Lactobacillus johnsonii* NCC 533 were donated by Nestlé Culture Collection (Nestec Ltd., Nestlé Research Center Lausanne, P.O. Box 44, CH-1000 Lausanne 26). Both bacteria were grown anaerobically in MRS broth for 24 h at 37ºC, CFU was determined by serial dilutions, aliquoted in 15% glycerol stocks in cryovials and stored at -80ºC. Before the gavage, the bacterial glycerol stocks were thawed, spun down and resuspended in sterile PBS. For *Romboutsia* experiment, active culture of *Romboutsia ilealis* DSM 25109 were purchased from the German Collection of Microorganisms DMSZ.

Bacterial Supplementation Experiments

For the microbial supplementation experiments, 8 weeks old C57BL/6 mice were given either ND or WD or WD + *L. gasseri* (gavaged 1X10^9 CFU/mouse every other day) or WD + *L. johnsonii* (gavaged 1X10^9 CFU/mouse every other day) for 8 weeks. For the control, both ND and WD groups were gavaged with equal volume of PBS (0.2 ml per mouse). Two independent experiments were performed with 5-6 mice per group per experiment. For the treatment experiment, mice were fed ND or WD for 8 weeks when one group of WD mice was supplemented with *L. gasseri* (gavaged 1x10^9 CFU/mouse every other day). Glucose

tolerance test was performed at 8 weeks on WD and 4, 9 and 12 weeks on WD + *L. gasseri* (n = 5 per group). For *Romboutsia ilealis* supplementation experiment, after 1 week of acclimatization, all mice were switched to ND and were either given PBS or 1x10^9 CFU of *R. ilealis* every other day for 4 weeks (n=5). Metabolic measurements were done as described below except for *R. ilealis* experiment 1 mg/kg glucose was injected for IPGTT.

For gnotobiotic mouse experiment, germ-free mice on western diet were colonized with 1x10^9 CFU *L. gasseri* on Day 0, Day 2, Day 4 and Day 12 and sacrificed on D14 (n=2).

Intraperitoneal Glucose Tolerance Test (IPGTT)

Mice were fasted for 6 h during the light phase with free access to water. A concentration of 2 mg/kg glucose (Sigma-Aldrich) was injected intraperitoneally. Blood glucose was measured at 0 min (immediately before glucose injection), 15, 30, 60 and 120 mins with a Freestyle Lite glucometer (Abbot Diabetes Care).

Fasting insulin & fasting glucose

Mice were fasted for 6 hours with free access to water. Fasting blood was collected either via submandibular bleed or from the tail vein. Insulin and glucose levels in fasting plasma or serum was measured with Mouse Insulin ELISA Kit (Crystal Chem) and Glucose Colorometric Assay Kit (Cayman Chemical), respectively, according to manufacturer's protocol. HOMA-IR and HOMA-B were calculated according to equations (1) and (2), respectively:

$$\mathrm{HOMA-IR} = \frac{\mathrm{Glucose\ (mg/dL)} \times \mathrm{Insulin(\mu U/mL)}}{405} \quad (1)$$

$$\mathrm{HOMA-B} = \frac{360 \times \mathrm{Insulin}\left(\mu\frac{\mathrm{U}}{\mathrm{mL}}\right)}{\mathrm{Glucose}\left(\frac{\mathrm{mg}}{\mathrm{dL}}\right) - 63}\,\% \quad (2)$$

The heatmap of results of systemic measurements was created using Morpheus (https://software.broadinstitute.org/morpheus/).

Hepatic fatty acids and cholesterol

Hepatic fatty acids were quantified using established protocols[76]. Briefly, total lipid was extracted from liver in chloroform-methanol (2:1) containing 1 mM butylated hydroxytoluene. 7-Nonadecenoic acid (C19:1) was added as a recovery standard. Total protein was measured after the initial homogenization step by BCA assay (Bio-Rad, Hercules, CA). Fatty acids in the extracts were saponified in 80% methanol containing 0.4 M KOH. Afterward, saponified fatty acids were converted to fatty acid methyl esters (FAME) in methanol containing 1% of 24 M H_2SO_4 and then quantified by gas chromatography.

Hepatic total cholesterol in liver lipid extracts and in serum was measured using Amplex™ Red Cholesterol Assay Kit (Thermo Fisher Scientific) according to manufacturer's protocol.

RNA preparation and gene expression analysis

RNA was extracted using an OMNI Bead Ruptor and 2.8 mm ceramic beads (OMNI International) in RLT buffer followed by Qiashredder and RNeasy kit using Qiacube (Qiagen) automated extraction according to manufacturer's specifications. Total RNA was quantified using Quant-iT RNA Assay Kit (Thermo Fisher Scientific). Complementary DNA was prepared using qScript reverse transcription kit (Quantabio) and qPCR was performed using Perfecta SYBR mix (Quantabio) and StepOne Plus Real Time PCR system and software (Applied Biosystems). RNA libraries were prepared with QuantSeq 3'mRNA-Seq Library Prep Kit (Lexogen) and sequenced using Illumina NextSeq. Sequences were processed to remove adapter, polyA and low-quality bases by BBTools (https://jgi.doe.gov/data-and-tools/bbtools/) using bbduk parameters of k=13, ktrim=r, forcetrimleft=12, useshortkmers=t, mink=5, qtrim=r, trimq=15, minlength=20.

Reads were aligned to mouse genome and transcriptome (ENSEMBL NCBIM37) using Tophat (v2.1.1) [77]with default parameters. Number of reads per million for mouse genes were counted using HTSeq (v 0.6.0) [78] and quantile normalized. BRB-ArrayTools was used to identify genes differentially expressed in the liver and ileum when supplemented with or without the *Lactobacillus* candidates. Pathway enrichment was performed using Metascape[79].

DNA extraction and 16S rRNA gene libraries preparation

For microbial measurements, stool pellets were collected at T1 (4 weeks of diet) and stool pellets and terminal ileum contents were collected at T2 (8 weeks). To get microbial DNA, frozen fecal pellets and ileum with content were resuspended in 1.4 ml ASL buffer (Qiagen) and homogenized with 2.8 mm ceramic beads followed by 0.5mm glass beads using an OMNI Bead Ruptor (OMNI International). DNA was extracted from the entire resulting suspension using QiaAmp mini stool kit (Qiagen) according to manufacturer's protocol. DNA was quantified using Qubit broad range DNA assay (Life Technologies). The V4 region of 16s rRNA gene was amplified using universal primers (515f and 806r) as in[16]. Individual samples were barcoded, pooled to construct the sequencing library, and then

sequenced using an Illumina Miseq (Illumina, San Diego, CA) to generate pair-ended 250 bp reads.

16S rRNA gene sequencing data analysis

The samples were demultiplexed and forward-end fastq files were analyzed using QIIME v. 1.9.1[80]. The default quality filter parameters from QIIME's *split_libraries_fastq.py* were applied to retain high quality reads (Phred quality score > = 20 and minimum read length = 75% of 250 nucleotides). A closed reference OTU picking with 97% sequence similarity was performed using UCLUST[81] and Greengenes reference database v13.8 [82,83] to cluster 16S rRNA gene sequence reads into OTUs and assign taxonomy. The reference sequence of candidate OTUs from the Greengenes database was used to obtain species level taxonomic assignment using Megablast [84] (top hit using default parameters). A threshold of 99% cumulative abundance across all samples in an experiment was used to retain abundant microbes, thus removing OTUs with approximately <0.01% abundance across all samples in that experiment. The read counts were normalized using cumulative sum scaling[85], accounted for DNA quantity, followed by quantile normalization. The principal component analysis for the 16S sequencing data was created using Clustvis[86], GraphPad Prism software (version 7), R packages seqtime version 0.1.1, igraph version 1.2.5.

Network analyses

Transkingdom Network reconstruction and prediction of causal microbes

Spearman rank correlations were calculated between all pairs of microbes (OTUs) and metabolic parameters (phenotypes) in each group of both experiments. A combined Fisher's *p*-value was calculated for each pair from the correlation *p*-values from each experiment. A FDR was calculated on the combined *p*-values separately for the following correlations: (i) within metabolic parameters, (ii) within OTUs, and (iii) between OTUs and metabolic parameters. We retained edges that satisfied the following criteria: the sign of correlation coefficients in the two experiments consistent in stool of WD-fed mice at 4 weeks (n=35 per expt.), individual *p*-value of correlation within each experiment is <30%, combined Fisher's *p*-value of all experiments <5% and FDR cutoff of 10% for within edges (i and ii). Finally, the transkingdom network was generated [20,61,87-89] by adding microbe-phenotype edges where the microbe showed significant change in (WD vs ND) abundance in ileum at 8 weeks, edges showed consistent sign of per-group Spearman correlation coefficient between the two

experiments of three WD-fed groups (WD-stool 4 weeks, WD-stool 8 weeks, and WD-ileum 8 weeks), and satisfied principles of causality[90] (i.e., had concordance between fold change in WD vs. ND comparison and correlation sign between the two partners) in all three WD-fed groups. The network was visualized in Cytoscape and is available at https://tinyurl.com/TK-NW-Fig-1C.

Identification of keystone microbes

Generation of training data was accomplished as follows: 100 instances of 542 generalized Lotka-Volterra models were run to steady state and steady state species abundances were considered individual samples. Those individual samples consisted of 10 to 100 species drawn from a model-specific species pool. The size of the species pool was determined by defining similarity in species composition between samples (between 0.4 and 0.95). The individual models further varied in the following parameters: Connectivity of the species interaction matrix (between 0.005 and 0.7), negative edge percentage of the species interaction matrix (0% - 100%), species-specific growth rates (between 0 and 1) and carrying capacities (between 0 and 100), as well as the topography of the species interaction matrix (interactions sampled from a uniform distribution or assigned according to the Klemm-Eguíluz model[91]. The R-package seqtime was used to generate the species interaction matrices[92].

Subsequently, each species included in a model was in turn removed from the community and a Canberra distance between original and sub-sampled community was calculated. 1000 iterations of this procedure were performed per species and the average Canberra distance induced by a species' absence was considered its keystoneness score.

For Model training, the data was split into training set and test set. The training set was used to train a linear model to predict keystoneness based on mean relative abundance and the following node parameters computed from a spearman correlation network: sum of absolute correlation strength, node degree, relative closeness centrality, betweenness centrality and eccentricity. With the exception of absolute correlation strength, the network parameters were calculated within the R-package igraph (http://igraph.org). This model was then used to predict keystoneness on the test set. A linear model between real and predicted keystoneness in the test set gave an adjusted R^2 of 0.4219, with a p-value < 2.2e-16.

The trained linear model was subsequently applied to the OTU abundance data and the previously computed correlation network to predict keystoneness scores for each OTU. Lastly,

keystoneness scores were scaled between 0 and 1 to remove negative values occurring as an artifact of the linear model.

Multi-omic Network analysis

Spearman rank correlations were calculated between all pairs of genes, lipids, and phenotypes. The phenotypic subnetwork was obtained from the transkingdom network. For gene subnetwork, correlation was calculated by pooling samples supplemented with the same Lactobacilli from both experiments. Edges were retained if they satisfy the following criteria: the sign of correlation coefficients in the two Lactobacilli groups should be consistent, individual p-value of correlation is <30%, combined Fisher's p-value over two Lactobacilli groups <5%, FDR cutoff of 5%, and satisfying principles of causality (i.e., satisfied fold change relationship between the two partners in the Lactobacilli vs. WD comparison). For the lipid subnetwork, correlations were calculated per experiment in the WD-groups of the three datasets (two WD vs ND experiments, and a Lactobacilli supplementation experiment). Edges were retained if the sign of correlation coefficients was consistent, Fisher's p-value <5%, FDR cutoff of 10%, and satisfied principles of causality.

For between-omics edges, correlations were calculated per experiment in the WD-groups of three datasets and a voting strategy was used for meta-analysis. Pairs were shortlisted if they had the same sign of correlation and p-values <10% in at least two datasets. If the p-value in the third dataset was over the threshold, the pair was retained but the third dataset was removed during calculation of Fisher p-value. The pair was kept if the p-value in the third dataset was under the threshold and the sign of correlation was same in all 3 datasets, else the pair was entirely removed. Edges with FDR <10% and satisfying principles of causality were added to the network.

Computational analysis using human datasets

Sequence read files of 1046 humans[25] were downloaded from European Bioinformatics Institute (https://www.ebi.ac.uk/), quality filtered and trimmed with ea-utils using default settings except the base removal quality threshold was set at < 20. Cleaned sequence reads were binned into Greengenes (v13_8) 97% identity operational taxonomic units (OTUs) using the QIIME 1.9 closed reference OTU picking workflow (pick_closed_reference_otus.py). Spearman correlations between BMI and microbial abundance of exact candidate OTU (or the sum of OTUs assigned to the bacterial species) were calculated in obese humans. To avoid

bias from outlier samples, a sample was considered only if had > 10 reads per million for *Lactobacillus* OTUs and > 100 reads per million for *Romboutsia* OTUs.

Transmission Electron Microscopy (TEM)

Frozen liver samples were prepared and fixed in 1.5% paraformaldehyde and incubated at 4ºC overnight[93], after which fixed tissues were processed usinf a protocol based on[94]. Specifically, the vibratome sectioned fixed tissues (~ $1mm^3$) were postfixed in solution containing 2% osmium tetroxide and 1.5% potassium ferrocyanide for 30 min at room temperature in dark. It was followed by staining with 0.2% tannic acid in water for 10 minutes, fixing in 1% osmium tetroxide for 30 minutes and staining in 1% thiocarbohydrazide (TCH) in water for 20 minutes at room temperature. The samples were then incubated with 1% osmium tetroxide for 30 minutes at room temperature. Then the samples were incubated with 0.5% uranylacetate in 25% methanol overnight at 4oC, which was followed by incubation in Walton's lead aspartate for 30 minutes at 60C. Then samples were dehydrated with graded series of ethanol, infiltrated with ethanol/epon mixture (1:1) for 1 hour at room temperature and 1:2 for 1 hour at room temperature. Ultramicrotome was done using a RMC PowerTome PC. Microscopy was done with a Helios 650 NanoLab (ThermoFisher). Scanning Transmission Electron Microscopy (STEM) mode was used for imaging. 10 – 12 images were taken per sample. The images were imported into FIJI (i.e. ImageJ) software (version 2.0.0-rc-69/1.52i). Each mitochondrion in the images was outlined and different attributes were measured using default "measure" option in the software.

In order to identify image parameters that discriminate between healthy and damaged mitochondria we used images representative of all analyzed groups. In each image, a pair of damaged (bright, lucent) and healthy mitochondria (dark, dense) were identified according to images in EM atlas (http://www.drjastrow.de/WAI/EM/EMAtlas.html). Next, we extracted quantitative data for 17 different image parameters (See Supplementary Data 11) and analyzed which of those differed between the two types of mitochondria. The selection has been performed "blindly" (i.e. the image analyst was unaware of treatment identity of samples. Among parameters that significantly differed between two types of mitochondria we chose less interdependent ones to compare different treatment groups. To establish whether the structure of mitochondria differs between groups supplemented or not with probiotic bacteria we

analyzed the above selected image parameters in 119 TEM images from liver samples of 9 mice totalizing 4709 mitochondria.

Un-targeted metabolomics

Serum samples used for metabolomics included the following: germfree mice fed WD for 2 weeks (n=2), mono-colonized for 2 weeks with *L. gasseri* fed WD (n=2); SPF mice supplemented or not with either L. *gasseri* or *L. johnsonii* (n=4-6 per group) and fed WD for 8 weeks in two experiments shown in Fig. 3; SPF mice first fed WD for 8 weeks, then supplemented (or not) with *L. gasseri* for additional 12 weeks along with WD (n=5 per group). For technical reasons, metabolomics was performed in pooled sera of each group of mice, which were run in a randomized manner as one batch.

An aliquot of 30 µl of pooled serum was processed following a protocol adapted from a published study[95]. Briefly, metabolites were extracted with 4 volumes of cold methanol/acetonitrile (1:1, v/v). To precipitate proteins, the samples were incubated for 1h at -20°C. After the samples were centrifuged at 4°C for 15 min at 15,871xg (13,000 rpm), the supernatant was collected and evaporated to dryness in a vacuum concentrator. The dry extracts were then reconstituted in 90 µL of acetonitrile/H2O (1:1, v/v) containing 10 ng/mL CUDA (12-(((cyclohexylamino)carbonyl) amino)-dodecanoic acid). This standard was used as a control to monitor platform stability along the fully randomized batch analysis, and to account for possible injection variabilities. A quality control (QC) pooled sample was prepared by combining, in a single vial, 10 µL of each sample. Pooled QC sample provided a 'mean' profile representing all analytes encountered during the analysis. To the QC sample a methanol solution containing verapamil and verapamil-D3 (Cayman Chemical, Ann Arbor, MI) was added at a final concentration of 0.1 ppm each. The ratio of their monoisotopic peaks was used to monitor quantification stability along the fully randomized batch analysis. The supernatant was then analyzed via LC-MS/MS (liquid chromatography with tandem mass spectrometry).

High-resolution mass spectrometry was performed using an Agilent 6545 Q-ToF downstream of an Agilent 1260 Infinity HPLC (high-performance liquid chromatography) system consisting of a degasser, quaternary pump, autosampler (maintained at 4°C) and column heater (maintained at 30°C). The Q-ToF machine was operated using MassHunter software and an analysis in positive and negative ionization mode was performed for each sample. Separation was achieved using an InfinityLab Poroshell EC-C18 column (100 x 3.0 mm, 2.7 µm, Agilent)

at a flow rate of 0.4 mL/min. Line A was water with 0.1% (v/v) formic acid and line B was methanol with 0.1% (v/v) formic acid, adapted from a previously described protocol[96]. The column was pre-equilibrated with 1% B. After injection (3 µL of the sample) this composition was held for 1 min and then changed to 30% B over the next 10 min using a linear gradient. The composition was then changed to 100% B over the next 14 min and then held at 100% B for 5 minutes. The mobile phase was then adjusted back to 1% B over two minutes and the column was re-equilibrated for 6 minutes prior to the next injection. The Agilent Q-ToF mass spectrometer was equipped with an Agilent JetSpray source operated with the following parameters: Auto MS/MS mode, Gas Temp, 325 °C; Drying gas, 10 L/min; Nebulizer, 20 psi; Sheath gas temp, 375 °C; Sheath gas flow, 12 L/min; Capillary Voltage (VCap), 4000 V; Nozzle voltage (Expt), 600 V; Fragmentor, 175 V; Skimmer, 65 V; Oct 1 RF Vpp, 750 V; Mass range, 100-3000 m/z; Acquisition rate, 10 spectra/s; Time, 100 ms/spectrum. The MS/MS spectra (Mass range, 50-3000 m/z; Acquisition rate, 10 spectra/s; Time, 100 ms/spectrum) were obtained by isolating the precursor ion with a medium isolation width (~4 m/z) summing spectra generated with collision energies of 15, 30, and 40 V. Blanks and QC samples were run before and after every four serum samples to ensure system equilibration. Based on the reproducibility of our QC and on the intensity of the CUDA, we can assume that the instrument was stable during the full randomized batch, and that intensity differences are due to biological differences and not to technical variation.

LC-MS/MS Data Processing

Raw data was imported into Progenesis QI software (Version 2.3, Nonlinear Dynamics, Waters) in order to perform data normalization, feature detection, peak alignment, and peak integration[97-99]. Metabolites were confirmed by MS, MS/MS fragmentation, and isotopic distribution using Metlin (Version 1.0.6499.51447, https://metlin.scripps.edu) and the Human Metabolome (HMBD, Version March 2020, https://hmdb.ca) databases as the reference[100]. The data acquired in both, electrospray ionization (ESI) negative and positive modes, which resulted in ESI + in 7,100 features with just MS information, 2,461 features with both MS and MS/MS information; serum ESI- gave 2,141 features with just MS information and 1,204 features with both MS and MS/MS information. Thus, a total of 3,665 features with both MS and MS/MS information was obtained. Next, a metabolite was sieved out when a match with a difference between observed and theoretical mass was less than 10 ppm and the molecular

formula of matched metabolites further identified by the isotopic distribution measurement. By doing so, the number of annotated compounds with a known identification was reduced to 133 metabolites, which had match score >35 (range 36.1-57.8), and isotope similarity between 67.8 and 99.1%). We chose to increase the confidence of our annotations, rather than increase the number of annotated compounds with a lower level of confidence. Zero values were assigned minimal values calculated as 3 STDEV of technical variation subtracted from the minimal measured level of a given metabolite in this study. Technical variation was defined by using CUDA and corresponded to STDEV of 0.135 and mean of 1.02. The level of metabolite identification was 2 for all compounds based on Sumner et al. (2007)[101]: level two refers to putatively annotated compounds (e.g. without chemical reference standards, based upon physicochemical properties and/or spectral similarity with public/commercial spectral libraries).

Cell Culture

AML12(ATCC CRL-2254) cells were grown in complete growth medium(DMEM:12 Medium(ATCC 30-2006) supplemented with 10% Fetal Bovine Serum (FBS), 10 µg/ml insulin, 5.5 µg/ml transferrin, 5 ng/ml selenium, 40 ng/ml dexamethasone and 1% Penicillin/Streptomycin) at 37°C in 5%CO2. After obtaining 80-85% confluency, 20,000 cells per well were seeded in complete growth medium in 96 well plate for 24 hours. After 24 hours of incubation, the medium was replaced either with low glucose medium (5.5 mM Glucose, 10% FBS, low sugar group) or mixture of 100mM Glucose and Fructose (1:1 ratio, with 10% FBS, high sugar group) alone or mixed with 4, 6 or 9 mM reduced glutathione ethyl ester (GSH, Sigma-Aldrich). After 6 hours of treatment, culture medium was removed, cells were lysed in RLT buffer (Qiagen) and RNA was extracted using RNeasy Mini kit (Qiagen). Total RNA was quantified using Quant-iT RNA Assay Kit (Thermo Fisher Scientific). Complementary DNA was prepared using qScript reverse transcription kit (Quantabio) and qPCR was performed using Perfecta SYBR mix (Quantabio) and StepOne Plus Real Time PCR system and software (Applied Biosystems). Polymerase (Polr2c) gene was used as the control gene. Primers used for qPCR are listed in the supplementary Supplementary Data 15b. Total six experiments were performed. The gene expression was normalized using the control group per experiment and per gene across the experiments, followed by log2 transformation. Control and treatment groups were compared using paired, one-sided parametric t-test.

Statistics and Reproducibility

Overall, the data was log transformed, checked for normality and an appropriate test was performed accordingly (i.e. parametric tests as default and non-parametric tests when distribution did not fulfil normality criteria), followed by Benjamini Hochberg false discovery rate correction. A two-sided test was used when there was no prior hypothesis of the expected direction of change; otherwise, one-sided test was used. For initial experiments, to capture the strongest and consistent signals across independent experiments (e.g. WD vs ND), non-parametric tests were used, and the meta-analysis was performed over experiments using Fisher's meta-analysis test. To achieve statistical power in the Lactobacilli supplementation experiments, the samples were normalized within each experiment to the mean of control group and analyzed together using parametric tests for host-derived variables. Meta-analysis was performed over the microbiome data. Gene enrichment analysis using Metascape software[79] that implements hypergeometric test. For metabolomics analysis, results of five lactobacilli supplementation from three experiments were normalized over corresponding controls with no probiotic supplementation. Log2 transformed ratios (lacto/control) for each metabolite were compared for deviation from 0 using parametric test. In experiments with interrelated data from two groups (e.g., AML-12 in-vitro experiment) we used paired test. Outliers (1%) were identified using ROUT method of GraphPad Prism 8.4.1 and removed (used only once in the whole study, one value was removed for one concentration of GSH treatment). Actual tests, cutoffs applied are mentioned in each figure caption, exact p values are available in supplementary data and source data files.

Data Availability

Data were submitted to NCBI SRA under submission PRJNA558801 (https://www.ncbi.nlm.nih.gov/sra/?term=PRJNA558801) for 16S rRNA, to GEO under GSE136033 (https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE136033), and to Metabolomics Workbench under ST001436 (https://www.metabolomicsworkbench.org/data/DRCCMetadata.php?Mode=Study&StudyID=ST001436)

Transkingdom network access: https://tinyurl.com/TK-NW-Fig-1C

Multi-omic network access: https://tinyurl.com/multi-omic-NW-Fig-5A

Source data are provided with this paper.

Code Availability

Custom codes available at https://github.com/richrr/TransNetDemo and https://github.com/fbauchinger/keystone_species_model

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CHAPTER 3
APPENDIX FIGURES
SUPPLEMENTARY FIGURES

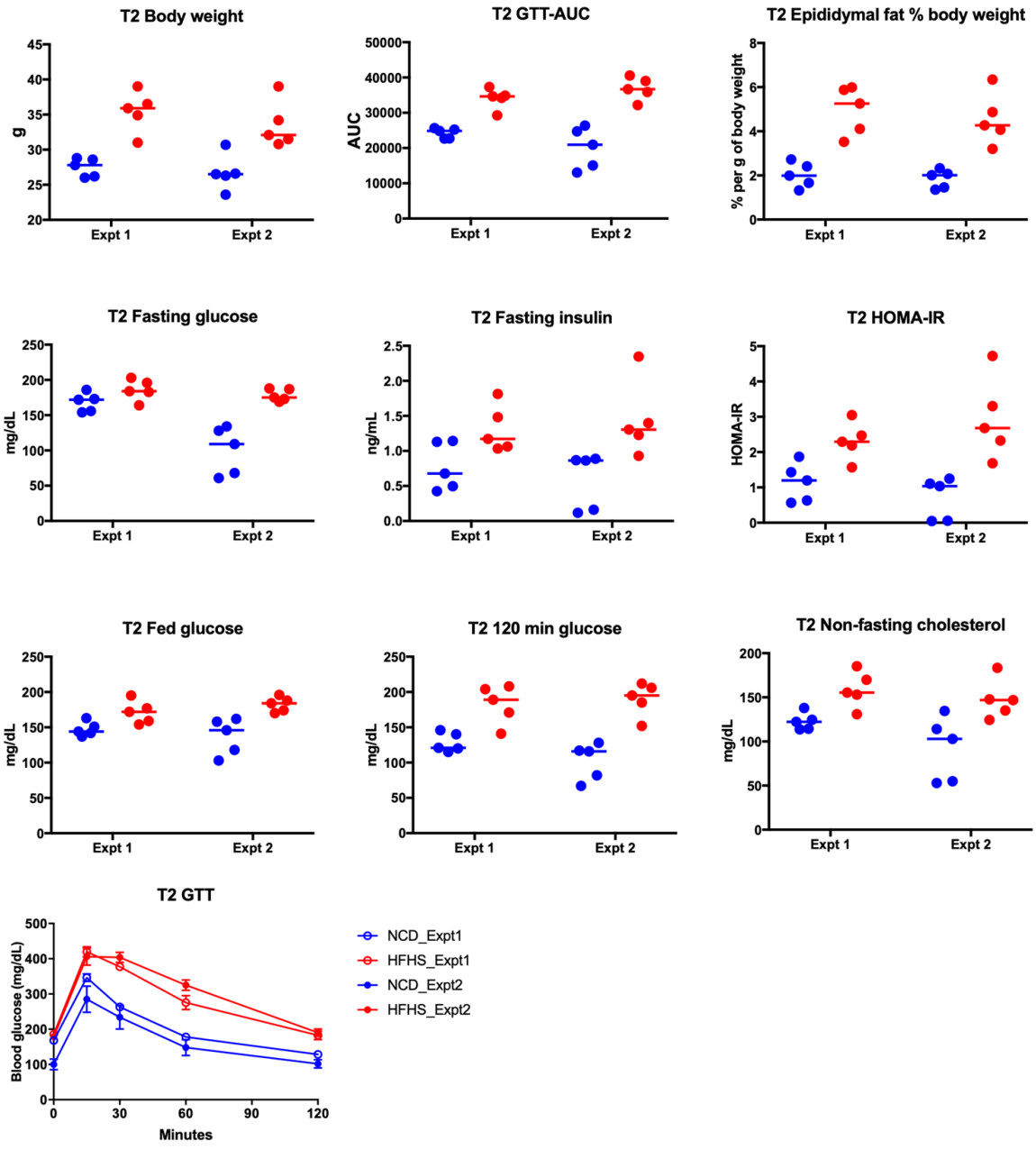

Fig. S1. Changes in metabolic parameters due to diet.

The blue and red colors indicate levels of metabolic parameters measured in mice fed normal diet (ND, blue) or western diet (WD, red) for 8 weeks. All these parameters are statistically significant (two-tail Mann-Whitney p-value < 20% in each experiment, Fisher's p-value over

experiments < 5%, and FDR < 10%). Each symbol represent mouse, lines are median values. Source data are provided as a Source D ata file.

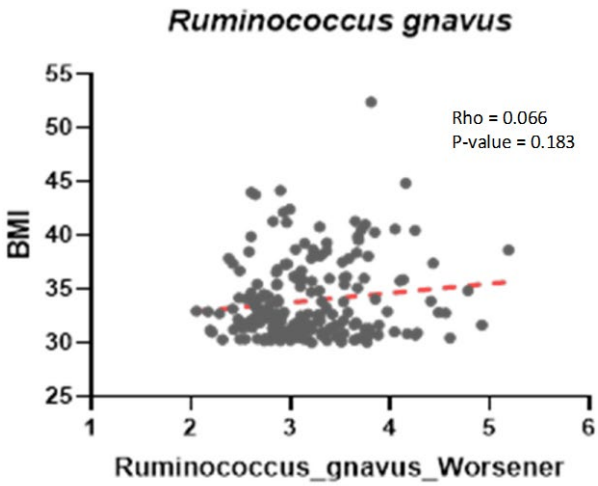

Fig. S2. Spearman correlation of *Ruminococcus gnavus* abundance in stool with BMI of obese human. The spearman rho correlation co-efficient and one-tail p-value is provided.

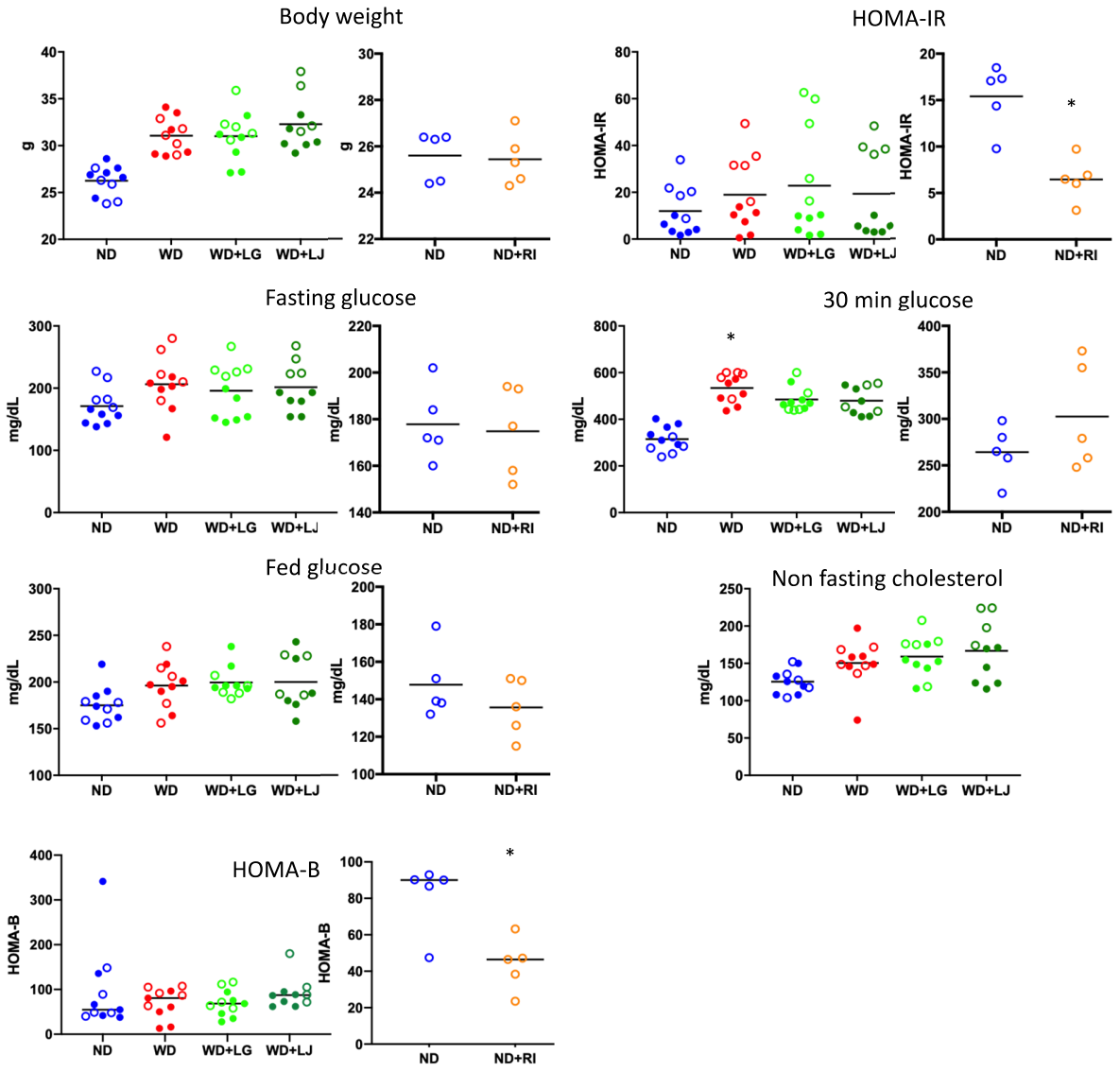

Fig. S3. Changes in metabolic parameters due to supplementation of candidate microbes.

The blue, red, light green, dark green, and orange colors indicate ND, WD, WD with *L. gasseri*(WD+LG), wd with *L. johnsonii* (WD+LJ), ND with *R. ilealis* (ND+RI), respectively. The metabolic parameters in mice supplemented with or without candidate microbe. Open and closed circles indicate 2 independent experiments. * indicates statistically significant differences in levels of the parameter between control group (WD for *Lactobacilli*, ND for *R. ilealis*) versus those supplemented with bacteria (one-tail t-test p-value < 5% with FDR <15%. Each symbol represents mouse, lines are median values, all replicates are shown. Source data are provided as a Source Data file.

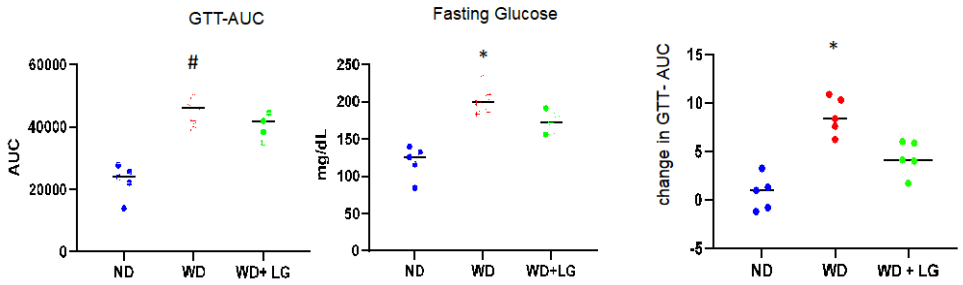

Fig. S4. Glucose tolerance test and fasting glucose in mice supplemented with L. gasseri (LG) after 8 weeks on WD.

The right figure shows the change in glucose tolerance test (AUC) after 12 weeks of WD (which was 4 wks of LG), calculated by subtracting the 12 week AUC of each mouse from 8 week AUC (when mice were on WD only) and normalizing by median of AUC change of ND group. * indicates significant change between control group (WD and WD + LG, one-sided p-value < 5%, Mann-Whitney test, median). # indicates p-value of 0.07. Each symbol represent mouse, lines are median values, all replicates are shown. Source data are provided as a Source Data file.

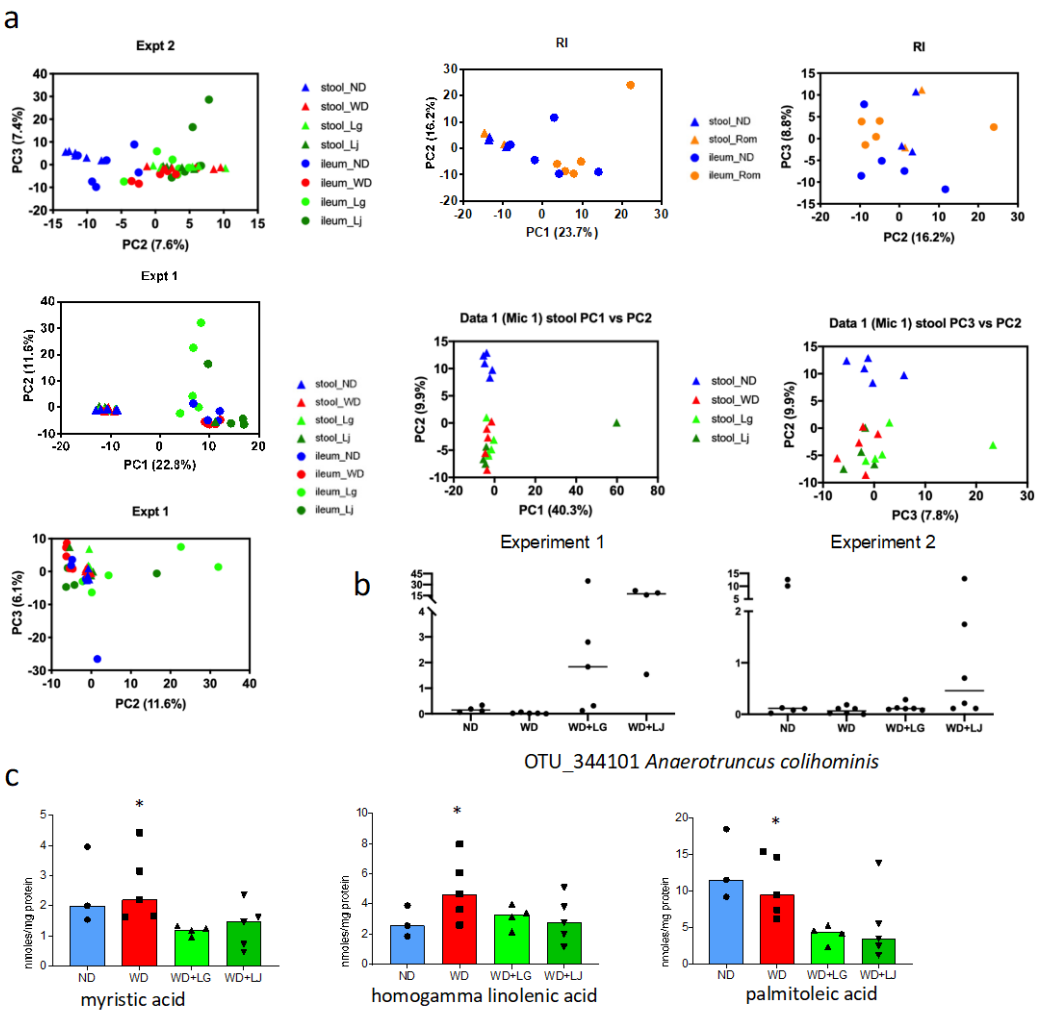

Fig. S5. Effect of microbial supplementation on microbial communities and fatty acids.

a) Principal Component Analysis of stool (triangle) and ileal (circle) microbial communities of mice on ND, WD, WD+LG, WD+LJ, or RI. b) The ileal abundance of *Anaerotruncus colihominis* in ND, WD, WD+LG, and WD+LJ fed mice. c) The levels of long chain fatty acids in liver of mice supplemented with or without *Lactobacillus* candidates. Asterisk indicates statistically significant differences between WD and groups supplemented with bacteria (one-sided t-test p-value < 5%, except for # p<8%). Each dot represents a mouse, all replicates are shown. Source data are provided as a Source Data file.

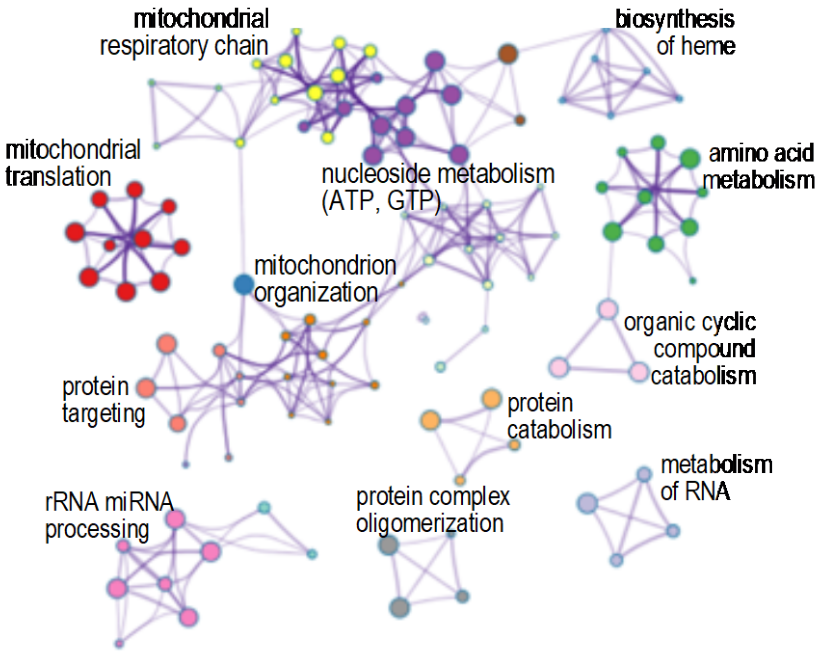

Fig. S6. Functional enrichment for the liver genes upregulated by the *Lactobacillus gasseri/johnsonii*. Pathways identified by metascape (http://metascape.org).

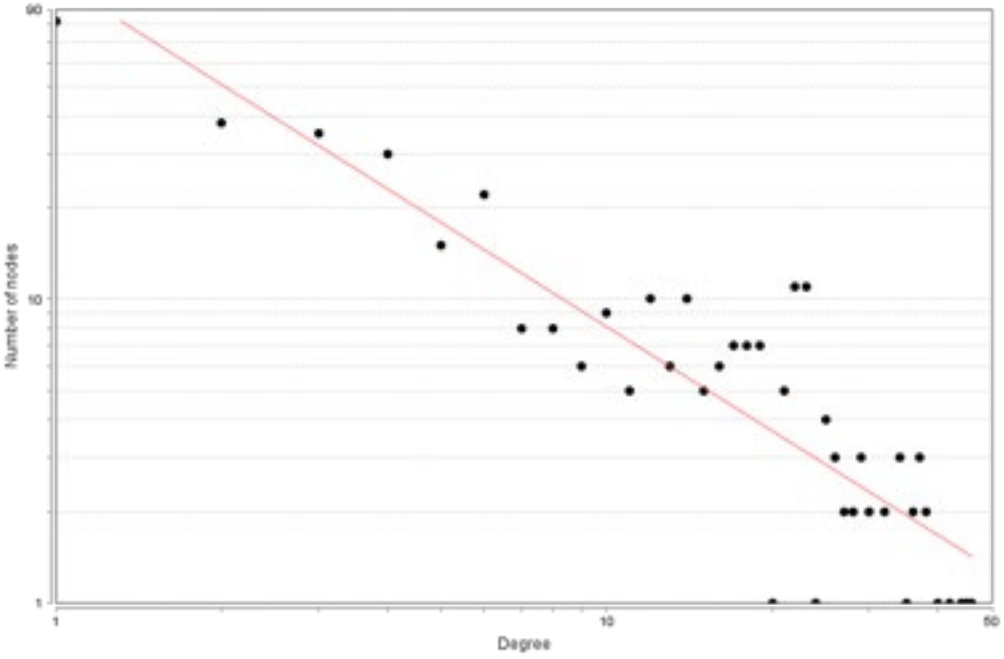

Fig. S7. Power law distribution of nodes in multi-omics network

Supplementary data 1A: Multivariate test to identify significant difference in the beta diversity of microbial community of different groups.

ANOSIM WITH BRAY-CURTIS INDEX FOR **BETA DIVERSITY**	**EXPT 1**		**EXPT 2**	
	R	**PVAL**	**R**	**PVAL**
TISSUE	0.7	< 0.001	0.82	< 0.001
TIME	0.05	< 0.206	0.1	< 0.073
DIET	0.4	< 0.001	0.3	< 0.002
GROUP	0.72	< 0.001	0.69	< 0.001

CHAPTER 4: CONCLUSIONS

Considering the exponential growth in the diabetic population and the increased cost of management of T2D, a novel approach to developing therapeutics is an urgent need. With the current focus on dysbiosis due to predisposing causes of chronic metabolic diseases like diets and other environmental factors, a better understanding of the role of the gut microbiome in T2D can facilitate microbiome-based therapeutics. One of the modifiable causes of T2D is obesity, characterized by ectopic accumulation of adipose tissue, and diets rich in sugar and processed fats (western diet) are the main culprits of the obesity epidemic. The western diet also causes an imbalance in the composition of gut microbiota. So, we hypothesized that the western diet reduces beneficial microbes or promotes pathobiont that exacerbates the effects of the western diet and targeting such microbes could alleviate T2D.

In chapter 2, we performed a comprehensive review of 42 human literature related to gut microbiome and obesity, insulin resistance, glucose metabolism, and T2D. From this review, we identified few microbiome genera which had consistent relation with T2D and related phenotypes. *Bifidobacterium*, *Bacteroides*, *Faecalibacterium*, *Akkermansia*, and *Roseburia* were the microbes negatively correlated with T2D, whereas *Ruminococcus*, *Fusobacterium*, and *Blautia* were positively correlated with T2D. Inhibition of pro-inflammatory modulations, upregulation of anti-inflammatory cytokines, reduction in gut permeability, improved butyrate production, decrease in LPS, increase in glycogen synthesis, reduction in hepatic gluconeogenesis, increased insulin sensitivity, reduced endotoxemia, increased fatty acid oxidation and energy expenditure were the most studied mechanisms by which beneficial microbiota affects metabolism in T2D.

In chapter 3, using system biology and causal inference analysis, we identified two species of *Lactobacillus* that can improve glucose homeostasis. We then used a mouse model of type 2 diabetes, ex-vivo and in-vitro assays; we validated that *Lactobacillus gasseri* and *Lactobacillus johnsonii* improve glucose metabolism via upregulation of glutathione production and improving hepatic mitochondrial health and reduction of hepatic fatty acid deposition. Supplementation of *Lactobacilli* in the western diet-fed mice did not change the overall composition of gut microbes along with minimal keystoneness of these microbes.

Our approach of using data-driven system biology to interrogate host-microbes interactions identified two commensals (*L. gasseri* and *L. johnsonii*) and a pathobiont (*R. ilealis*) in diet-induced metabolic disease. These findings suggest that the western diet induces the reduction of beneficial microbes or promotes pathobiont eliciting harmful health effects. Our studies also identified the gut microbiota- mitochondrial interactions as an essential pathway that affect metabolic health. In conclusion, our studies promote the idea of targeted therapies for particular microbes instead of restoration of the overall gut community as an approach to develop microbiota-based therapeutics. With the increasing research interests in the gut microbiota-host interaction in T2D, a robust system to identify beneficial/pathobiont can promote better reproducibility of the findings. Our approach of data-driven network analysis along with validation experiments using animal studies, in-vitro and ex-vivo systems could be a valuable tool in such direction.

CHAPTER 5: CURRICULUM VITAE

Education:

2015 – 06/2021	Oregon State University, Ph. D, Comparative Health Science
2011	Institute of Agriculture and Animal Science, Tribhuvan University, Bachelor of Veterinary Science & Animal Husbandry

Professional Experience:

Sep 2020 – April 2021	Co-op, Functional Genomics, Tissue and Cells Sciences GlaxoSmithKline, PA
Sep 2015 – Aug 2020	Graduate Research Assistant, Department of Biomedical Sciences, Oregon State University, OR
Feb 2014 – Aug 2015	Vet Technician, Clark N Oak Animal Health Center/VCA Lakeshore Animal Hospital, IL
June 2013 – Nov 2013	Veterinary officer, District livestock services office, Nepal
March 2012 – May 2013	Instructor, Veterinary technician program, Nepal

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Submitted/under preparation:

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Li Z#, **Gurung M**#, Rodrigues RR#, Newman N, Padiadpu J, Manes N, Vasquez-Perez S, You H, Greer RL, Fel A, Trinchieri G, Nita-Lazar A, Dzutsev A, Shulzhenko N, Morgun A. Dissecting effects of western diet and microbiota on metabolic disease reveals Mmp12 as a novel microbiota-dependent regulator of glucose metabolism (under preparation)

Logan, I.E., Shulzhenko, N., Sharpton, T.J.,Bobe, G., Liu, K., Nuss, S., Jones, M.L., Miranda, C.L.,Vasquez-Perez, S., Pennington, J.M., Leonard, S.W., Choi, J., Wu, W., **Gurung, M**., Kim, J.P., Lowry, M.B.,Morgun, A., Maier, C.S., Stevens, J.F., Gombart, A.F.

Xanthohumol Requires the Intestinal Microbiota to Improve Glucose Metabolism in Diet-Induced Obese Mice (Submitted)
# Equal contribution

Presentation:

Spotlight Talk: "Mmp12 is a microbiota-dependent link between adipose tissue inflammation and mitochondrial health in type 2 diabetes," 2020 Miami Winter Symposium, Molecular Mechanisms linking the Microbiome and Human Health. Miami, USA. January 26-29, 2020.

"Lactobacilli ameliorate western diet induced diabetes by preventing hepatic mitochondrial damage", CGRB Fall Conference. Corvallis, Oregon. September 20, 2019.

"Role of Gut Microbiota in a mouse model of Type 2 Diabetes". CGRB Spring Conference/Colloquium. Corvallis, Oregon. April 19, 2019. "Microbiome in a mouse model of Type 2 Diabetes". American Society of Microbiology Northwest Branch. Portland, Oregon. 2018.

"*Lactobacillus gasseri* ameliorates diet induced diabetes in mice via changing lipid metabolism gene expression in the gut". 7th Conference on Beneficial Microbes. Madison, Wisconsin. July 8-11, 2018.

"Transkingdom network analysis reveals the role of IgA and a pathobiont Acinetobacter in the Immunodeficiency-associated enteropathy". META CENTER for Systems Biology Symposium on Host-Microbe Systems Biology:From Models to Medicine. Eugene, OR. October 5, 2016.

Awards:

2021	Dissertation Completion Award, OSU Graduate School
2020	Graduate Research Award, CVM, OSU
2019	Best Poster Presentation Award, CGRB Fall Conference
2006-2011	Merit based scholarship for BVSc & AH, Tribhuvan University

Funding/Fellowship:

Accelerator Innovation and Development (AID) Fund of $15,000 from the OSU Venture Development Fund for commercialization and product development of a novel probiotic strain

Ruth L. Kirschstein Predoncotal Individual National Research Service Award – F31 (Applied not granted)

Technical skills:

Microbial analysis using next generation sequencing, immunological assays including FACS, ELISA, animal models including gnotobiotic mouse model, Fecal Microbiota Transplantation, qPCR, PCR, bacterial culture, primary immune cell culture, mammalian cell culture, immune cells isolation of intestine and lymph nodes, GraphPad Prism, FlowJo, BRB array tools, R/Linux, data analysis, gene knock down, dendritic cells differentiation