Vancomycin-Induced Modulation of Gram-Positive Gut Bacteria And Metabolites Remediate Insulin Resistance In INOS Knockout Mice

Hobby Aggarwal

CSIR-Central Drug Research Institute

Priya Pathak

CSIR-Central Drug Research Institute

Vishal Singh

The Pennsylvania State University

Yashwant Kumar

Translational Health Science and Technology Institute

Manoharan Shankar

Indian Institute of Technology, Jodhpur

Bhabatosh Das

Translational Health Science and Technology Institute

Kumaravelu Jagavelu

CSIR-Central Drug Research Institute

Madhu Dikshit (✉ drmadhudikshit@gmail.com)

Translational Health Science and Technology Institute <https://orcid.org/0000-0002-2777-0940>

Research

Keywords: iNOS-/- mice, Dyslipidemia, Insulin resistance, Obesity, Gut microbiota, Metabolome analysis

Posted Date: September 15th, 2021

DOI: <https://doi.org/10.21203/rs.3.rs-826534/v1>

License: © ⓘ This work is licensed under a Creative Commons Attribution 4.0 International License.

[Read Full License](#)

Abstract

Background: Inducible nitric oxide synthase (iNOS) has emerged as a crucial regulator of host metabolism and gut microbiota activity. The present study examines the role of the gut microbiome in determining host metabolic functions in absence of iNOS.

Results: Insulin resistant and dyslipidemic iNOS^{-/-} mice displayed reduced microbial diversity, with a higher relative abundance of the gram-positive bacteria, *Allobaculum* and *Bifidobacterium*, and altered serum metabolites linked with the metabolic dysregulation. Vancomycin, which largely depletes gram-positive bacteria, reversed the insulin resistance (IR), dyslipidemia, and related metabolic abnormalities in iNOS^{-/-} mice. Such correction in metabolic markers was accompanied by reduced the expression of genes involved in fatty acid synthesis in liver and adipose tissue, decreased lipid uptake by adipose tissue and enhanced lipid efflux by liver and intestine. Rescue of IR in vancomycin treated iNOS^{-/-} mice was associated with the alterations in the select serum metabolites such as 10-hydroxydecanoate, indole-3-ethanol, allantoin, hippurate, sebacic acid, aminoadipate, and ophthalmate, along with improvement in phosphatidylethanolamine to phosphatidylcholine (PE/PC) ratio.

Conclusions: Vancomycin-mediated depletion of gram-positive bacteria negates the detrimental metabolic effect, dyslipidemia and IR, observed in iNOS^{-/-} mice.

Background

Type 2 diabetes is a multitudinous metabolic disorder that arises from a complex interaction among genetic and environmental elements including dysregulated microbiota composition and function [1]. Insulin resistance (IR) or dyslipidemia is a key attribute of obesity and diabetes due to metabolic disruptions [2]. Gut microbiota dysbiosis, specifically changes in Firmicutes to Bacteroidetes ratio has been linked to a cluster of chronic metabolic diseases including IR, diabetes, and obesity in both humans and mice [3]. Prebiotics (such as inulin), probiotics, and other microbiota-targeted bacteriotherapy including antibiotics interventions are actively being explored to prevent and manage metabolic disorders [4]. Treatment with antibiotics reduces bacterial diversity in the host with reduced resistance to colonization of non-autochthonous microbes [5,6]. Thus, antibiotics adds an interesting dynamism to the host-microbiome association [7]. Antibiotics-induced depletion of the short chain fatty acids (SCFA)-producing gut microbes, reduced the levels of microbial derived-colonic SCFA in children and enhanced the likelihood to develop obesity and metabolic syndrome later in life [8–10]. On the contrary, antibiotic-induced microbiota depletion (AIMD) by the antibiotic cocktail or vancomycin provides protection against diet or genetically inherited obesity and glucose dysmetabolism [11–13] by reducing inflammation, oxidative stress and improving metabolic homeostasis [14]. Studies in humans have implied both Gram-positive and negative bacteria in the metabolic perturbations and obesity [15–18]. These studies thus indicate the strong association of gut microbiota with obesity and related metabolic disorders [19].

Nitric oxide (NO), has a pivotal role in the regulation of cardiovascular and metabolic functions. Among the different NOS isoforms, importance of iNOS has been predominantly investigated in the inflammatory and infectious diseases [20,21], but is now emerging as an important metabolic regulator [22–27]. iNOS-derived NO controls the growth of certain microorganisms as an adaptive response of host defense, while survival of certain microbes also seems to be facilitated [28]. iNOS^{-/-} mice are protected from LPS-induced IR [29], cardiovascular [30] and endothelial dysfunction [31]; and also displayed reduced inflammatory cytokines [22,23] with enhanced survival during septicemia [32]. Moreover, previous studies from our lab [24,25,27] and others [26,33] had demonstrated IR and disrupted metabolic homeostasis in iNOS^{-/-} mice fed on chow diet, LFD or HFD which was reversed by enhancing NO bioavailability *via* nitrite treatment [27] suggesting the importance of redox status in host metabolism. As oxidative stress and changes in gut microbiota could play a role in IR [34], iNOS^{-/-} mice might have dysbiosis due to the absence of iNOS-derived NO.

Therefore, our present study explored the contribution of gut microbiota in regulating the metabolic abnormality observed in mice lacking iNOS. We demonstrate that iNOS^{-/-} mice exhibit an altered gut microbial community with enhanced enrichment of gram-positive bacteria. Vancomycin (largely depletes gram-positive bacteria) induced depletion of gut bacteria establish that such enrichment of vancomycin sensitive microbes are vital contributors to metabolic abnormalities found in iNOS^{-/-} mice.

Materials And Methods

Mice and Diet

Twelve weeks old, age matched male C57BL/6 (WT) and iNOS knockout (iNOS^{-/-}) (Jackson Laboratory, USA; 002609) mice on C57BL/6J background were bred and maintained in IVC cages (Tecniplast, Italy) at 24 ± 2 °C. All procedures were approved by Institutional Animal Ethics Committee of CSIR-CDRI (IAEC/2014/43) in accordance with CPCSEA guidelines. Mice (WT and iNOS^{-/-}) were maintained on chow diet (1320, Altromin, Germany) and water *ad libitum*. Antibiotics- Vancomycin (0.5g/l) and Antibiotic cocktail (Abx) comprising of Ampicillin (1g/L), Neomycin (1g/L), Metronidazole (1g/L) and Vancomycin (0.5g/L) were administered *via* drinking water for 4 weeks for depletion of gram-positive anaerobic bacteria and majority of gut microbiota respectively [35,36].

Body weight, BMI and food consumption

Body weight was measured weekly from day zero to the completion of study at 4 weeks. The body length was also measured from nose tip to the tail's base of each mouse. Body mass index (BMI) was calculated using the formula body weight (g) divided by the square of nose to anus length (cm) as previously described [27] at the end of 4 weeks of antibiotic treatment. The weekly food consumption was measured by housing 2-3 mice per cage and adding the pre-weighed food pellets to each cage and

measuring the food left over on a sensitive weighing balance twice weekly. Average weekly food consumption and average food intake/day/mice during the study period was calculated.

Tolerance tests

Mice were administered 2 g/kg D-Glucose, 2 g/kg sodium pyruvate or 0.6 IU/kg insulin (Human insulin R, Eli Lilly) by intraperitoneal (i.p.) route to perform glucose (GTT), pyruvate (PTT) or insulin tolerance test (ITT) respectively after 6 hours fasting. Blood glucose was monitored using Accu-Chek glucometer (Roche Diagnostics, India) at 0, 15, 30, 60 and 120 minutes after administration of glucose, pyruvate or insulin and area under the curve (AUC) was calculated as described previously [24].

Body composition analysis

Body composition (fat and lean mass) was analyzed in live, conscious mice by echo MRI (E26-226-RM Echo MRI LLC, USA) allowing limited horizontal and vertical movements by applying radio frequency pulses at a distinct static magnetic field [37].

In vivo gut permeability assay

FITC-labelled dextran (4kDa) was used to assess in vivo intestinal permeability [38]. Mice were fasted for 4 hours with free access to water. 0.8 mg/ml FITC-dextran tracer was giving orally in 200 µl PBS followed by removal of both food and water after the gavage. Blood was collected retro-orbitally after 4 hours, serum separated and fluorescence intensity was measured using an excitation wavelength of 493 nm and an emission wavelength of 518 nm.

Serum biochemistry

Retro-orbital blood was collected from 6 hours fasted mice. Estimation of lipids like total cholesterol (TC), triglycerides (TG), low and high density lipoproteins (LDL and HDL), non-esterified fatty acids (NEFA) were performed in the serum using kits [25] (Randox, UK). Insulin was measured using kit from Crystal Chem, USA. Indices of IR- HOMA-IR and insulin sensitivity- QUICKI were calculated from fasting blood glucose and serum insulin as per the formulae used by other investigators [39].

Total nitrite estimation

The animals were sacrificed to retrieve the tissues (liver, epididymal white adipose tissue and small intestine). Tissues (liver, eWAT and intestine, 50 mg) were homogenised in 500 µl of hypotonic TKM buffer [25 mM Tris-HCl (pH-7.4), 2 mM MgCl₂, 5 mM KCl and 1% NP-40] followed by sonication. Supernatant was obtained by centrifugation at 15000 g at 4°C for 20 minutes. Total nitrite (nitrate and nitrite) was estimated in serum (100 µl) and tissues homogenates using Griess reagent by reducing nitrate to nitrite using pre-activated cadmium pellets followed by deproteinization in tissue homogenates with 3% trichloroacetic acid [24].

Tissue biochemistry

Liver tissue (50 mg) was processed as described previously [27] for the estimation of hepatic total cholesterol, triglycerides and free fatty acids (FFA) using Randox kit. Briefly, for TC estimation, samples were homogenized on ice in 1ml hexane:isopropanol mixture (3:2 ratio) followed by centrifugation, supernatant collection and drying in Centrivap concentrator (Labconco, USA). Samples were homogenised in 1ml of 1% triton-X-100 in chloroform on ice, centrifuged and lower phase dried in Centrivap for FFA estimation. The dried products for TC and FFA estimation were redissolved in ethanol: NP40 (9:1). For triglycerides estimation, samples were homogenized in 500 µl of 5% NP40 on ice, boiled for 5 minutes in water bath at 80-100°C and then cooled and reheated 3 times, centrifuged and supernatant collected.

Hepatic Glycogen

Insulin was administered at a dose of 0.6 IU/kg i.p. and animals were sacrificed after 30 minutes to collect liver for insulin stimulated glycogen estimation along with unstimulated controls in both WT and iNOS^{-/-} mice with or without vancomycin and Abx treatment. 50 mg liver tissue was homogenized in 500 µl distilled water on ice, boiled for 10 minutes, centrifuged to remove insoluble substances followed by supernatant collection and glycogen estimation using kit from Biovision (K646-100) as per the manufacturer's protocol.

Alcian blue (AB) staining

Formalin fixed, paraffin embedded small intestinal and colonic tissue was sectioned into 5 µm thin serial slices and stained with 1% Alcian blue solution (in 3% acetic acid, pH 2.5) for morphological examination of acid mucosubstances and acetic mucins and counterstained with 0.1% nuclear Fast Red solution [40]. Alcian blue stained area (%), villi and crypt lengths were calculated using Image J software.

Real time PCR

Quantitative gene expression analysis was performed using SYBR Green as described previously [37]. Briefly, total RNA was extracted using TRIZOL reagent followed by cDNA synthesis using RevertAid first strand cDNA synthesis kit using manufacturer's protocol. Real time PCR was performed using LightCycler 480II Real-Time PCR system (Roche Applied Science, Indianapolis, IN) with primers listed in Table S1. 18S rRNA was used as reference gene for normalization in liver and adipose tissue and RPL10 in intestinal tissue to calculate the expression of candidate genes. Relative fold change was calculated from mean normalised gene expression between different groups as compared to WT mice.

Metabolomics analysis

Sample preparation

100 µl serum was lyophilised and stored at -80°C until further processing. The sample was reconstituted in 200 µl methanol, 50 µl water and 870 µl Methyl tert-butyl ether (MTBE) and vortexed for 1 hour to extract the metabolites. Organic and aqueous phase separation was induced by adding 250 µl water and centrifuged at 15000g for 15 minutes at 4°C. Upper organic and lower aqueous layer (100 µl each) were vacuum dried in SpeedVac concentrator and stored till further analysis at -80°C. Samples were reconstituted in 50 µl 15% methanol and kept in ice for 30 minutes, vortexed for another 30 minutes, centrifuged at 15000g for 15 minutes at 4°C and supernatant collected and were subjected to metabolomic analysis by using LC-MS platform.

Metabolomics measurement

The metabolomics data was acquired on the orbitrap fusion mass spectrometer (Thermo Scientific, USA) equipped with heated electrospray ionization (HESI) source. Data were acquired on positive and negative mode at 120,000 mass resolution in MS mode and 30000 resolution in data dependent MS2 scan mode. Spray voltage of 4000 and 35000 volt were used for positive and negative mode respectively. Sheath gas and auxiliary gas was set to 42 and 11 respectively. Mass scan range of 50-1000 m/z, automatic gain control (AGC) target at 200000 ions and maximum injection time was 80 ms for MS and AGC target was 20000 ions and maximum injection time 60 ms for MSMS was used. Extracted metabolites were separated on UPLC ultimate 3000 using HSS T3 column (100x2.1 mm i.d, 1.7 µm, waters) maintained at 40°C temperature. The mobile phase A was water with 0.1% formic acid and mobile phase B was acetonitrile with 0.1% formic acid. The elution gradient used is as follows: 0 min, 1% B, 1 min, 15% B, 4 min, 35% B, 7 min, 95% B, 9 min, 95% B, 10 min, 1% B and 14 min, 1% B. The flow rate was 0.3mL/min and sample injection volume was 5 µl. Pool quality control (QC) sample was prepared by collecting 10 µl from each sample and was run after every five samples to monitor retention time shift, signal variation and drift in mass error [41].

Data Processing

All acquired data has been processed using Progenesis QI software (Water Corporation) using default setting. The untargeted metabolomics workflow of Progenesis QI was used to perform retention time alignment, feature detection, elemental composition prediction and database search. Identification of metabolite was done on the basis of in house metabolite library with accurate mass, retention time and fragmentation pattern information match. Additionally, spectral data matching with mzcloud and mass bank for the fragmentation match for identification of metabolites were also used. Metabolomics data were normalized by sum and pareto scaled before multivariate analysis. Relative fold-change values in metabolite expression analysis were calculated for each treated samples with respect to the untreated time-matched control (WT) for two sets of experiments and then differential analysis was performed on the pooled fold change data. Fold change values were log transformed for clearer representation in the heat map analysis. Statistical analysis were performed by multiple t-tests followed by a two-stage linear step-up procedure of Benjamini, Krieger, and Yekutieli to correct for multiple comparisons by controlling the False Discovery Rate (<0.05).

16S rRNA gene sequence analysis

Genomic DNA from approximately 200 mg mice stool samples was extracted by a optimized enzymatic, chemical, physical, and mechanical lysis method as described previously [42]. V3-V4 region of 16S rRNA gene was amplified and gel extracted for metagenomic sequencing as described previously [43]. Metagenome libraries were prepared using V3-V4 region-specific primers. Approximately, 40 ng of DNA samples were amplified for 26 cycles of round 1 PCR using KAPA HiFi Hot-Start PCR Kit (KAPA Biosystems Inc., USA). The forward and reverse primers were used at the concentration of 5 μ M each. The amplicons were analyzed on 1.2% agarose gel. 1 μ l of diluted round 1 PCR amplicons were used for Indexing PCR (Round 2). Here, the round 1 PCR amplicons were amplified for 10 cycles to add Illumina sequencing barcoded adaptors (Nextera XT v2 Index Kit, Illumina, USA). Round 2 PCR amplicons (sequencing libraries) were analyzed on 1.2% agarose gel. Illumina Adapter Sequences: 5'-AATGATACGGCGACCACCGAGATCTACAC[i5]TCGTCTGCGCAGCGTC and 5'-CAAGCAGAAGACGGCATACGAGAT[i7]GTCTCGTGGGCTCGG where [i5, i7] was unique dual index sequence to identify sample-specific sequencing data. Further, the samples were loaded into an Illumina MiSeq v3 600 cycles flow cell (Illumina, CA, USA) and the sequencing run was performed according to standard Illumina protocol.

Data Analysis

The Illumina paired-end raw reads having V3-V4 primer sequence and high-quality bases ($>Q30$) were selected. Bcl2fastq software v2.20 was used for data demultiplexing and FastQ files were generated based on the unique dual barcode sequences. FastQC v0.11.8 software was used to assess the sequencing quality. The adapter sequences were trimmed and bases above Q30 were considered. During

read pre-processing, low quality bases were filtered off and then data is used for downstream analysis. The reads were further stitched using Fastq-join. These stitched reads were subjected to QIIME pipeline for microbiome analysis. The query sequences were clustered using UCLUST method against a curated chimera free 16s rRNA database, Greengenes v 13.8. The taxonomies were assigned using RDP classifier to these clusters at $\geq 97\%$ sequence similarity against the reference Greengenes database. PCA and alpha diversity analysis was performed using MicrobiomeAnalyst. The raw reads data was normalised and relative abundance in % was calculated. For comparative analysis minimal data filtering was done and features containing non-zero values in less than $\sim 10\%$ samples were removed. Statistical analysis at the level of phylum, family and genus were performed by multiple t-tests with assumption that all rows are sample from populations with the same scatter (SD) followed by a two-stage linear step-up procedure of Benjamini, Krieger, and Yekutieli to correct for multiple comparisons by controlling the False Discovery Rate (< 0.05). Due to taxonomic resolution limit of the partial 16S rRNA gene sequencing based microbiome study the abundance difference of the bacterial species in WT and insulin resistant $iNOS^{-/-}$ mice has not been included in the present study.

Statistical Analysis

Data has been presented as Mean $\pm$ SEM. Independent unpaired Student t test was used for comparisons as appropriate using GraphPad Prism 8 software. More than two groups were compared by one way analysis of variance (ANOVA) followed by post hoc Tukey's multiple comparison test or Dunnett's test. Differences were considered statistically significant at $p < 0.05$. For the correlation analysis, Pearson correlation coefficients were calculated and p value was corrected according to the Benjamini–Hochberg correction for multiple comparisons, with a false discovery rate < 0.05 .

Results

Insulin resistant $iNOS^{-/-}$ mice display atypical gut microbiota with gram-positive bacteria dominance and altered serum metabolome

Chow fed $iNOS^{-/-}$ mice were glucose intolerant (Fig. S1A, B) and systemic insulin resistant (Fig. S1F, G), hyperglycemic and hyperinsulinemic as compared to WT (Fig. S1C-D), alongwith enhanced HOMA-IR (Fig. S1E) and decreased QUICKI (Fig. S1H). $iNOS^{-/-}$ mice also displayed enhanced gluconeogenesis as evident by PTT (Fig. S1K, L). Circulating total cholesterol, triglycerides (Fig. S1I, J), LDL and NEFA were significantly more in $iNOS^{-/-}$ mice while HDL levels were comparable to WT mice (Fig. S1M-O). The microbial alpha-diversity was reduced in insulin resistant $iNOS^{-/-}$ mice in the faecal samples as compared to WT (reduced observed, Shannon and Chao1 diversity indices). However, no significant difference was

observed in the Simpson diversity index (Fig. 1A-D). Multidimensional scaling analysis through principal coordinate plot represents that the bacterial communities between the WT and iNOS^{-/-} mice varied significantly (Fig. 1E). At the phylum level, relative abundance of Firmicutes was decreased with increased Verrucomicrobia. At the family level, Erysipelotrichaceae, Bifidobacteriaceae and Verrucomicrobiaceae were increased, while, Lactobacillaceae and Ruminococcaceae were decreased. *Allobaculum*, *Bifidobacterium* and *Akkermansia* were increased significantly, while, *Lactobacillus* genus was reduced in insulin resistant iNOS^{-/-} mice as compared to WT (Fig. 1F) indicating differential gut microbiome with major changes in gram-positive bacteria. In PCA score scatter plot from serum metabolomics, distinctly separated clusters between the WT and iNOS^{-/-} mice in ESI positive mode were seen (Fig. 1G) with significantly differential metabolites (p<0.05) being visualized through volcano plot (Fig. 1H). iNOS^{-/-} mice displayed enhanced purine and pyrimidine metabolites, PE lipids, PE to PC ratio, 10-hydroxydecanoate, 3-nitrotyrosine, cysteamine, cysteate, carbohydrate metabolites, indole-3-ethanol, diosmetin and phosphonoacetate. PC, PA and PS lipids, laurate, lauroyl carnitine, anthranilate and cystathionine were decreased in iNOS^{-/-} mice suggesting altered metabolic profile as compare to WT (Fig. 1I).

Vancomycin-induced modulation of gut microbiota rescue iNOS^{-/-} mice from systemic IR and dyslipidemia

Subsequently, we used vancomycin to deplete the gram-positive and antibiotic cocktail to deplete both gram-positive and negative gut microbiota in iNOS^{-/-} mice to assess the correlation, if any, between gut microbiome and altered metabolic homeostasis in iNOS^{-/-} mice. Vancomycin (Fig. 2A-D) and Abx (Fig. S3A-D), as expected, further decreased the microbial diversity in insulin resistant iNOS^{-/-} mice (decreased observed, Shannon, Simpson and Chao1 α -diversity indices) confirming the depletion of gut microbiota. Abx treated iNOS^{-/-} mice showed the lowest alpha-diversity among all the groups. Bacterial communities between vancomycin and Abx treated WT (Fig. S2E, S5F) and iNOS^{-/-} mice (Fig. 2E, S3E) vary distinctly and significantly from the untreated controls with markedly different clusters in PCA analysis. At the phylum level, Bacteroidetes and Actinobacteria were decreased significantly in vancomycin treated WT and iNOS^{-/-} mice with increased Proteobacteria. Verrucomicrobia was increased significantly by vancomycin in iNOS^{-/-} mice. Bacterial families- Bifidobacteriaceae, Ruminococcaceae, S24-7 were decreased significantly in vancomycin treated WT and iNOS^{-/-} mice with increased Veillonellaceae, Verrucomicrobiaceae and Enterobacteriaceae. Erysipelotrichaceae was decreased with increased Lactobacillaceae, Staphylococcaceae and Porphyromonadaceae in vancomycin treated iNOS^{-/-} mice. Lactobacillaceae and Lachnospiraceae were decreased in vancomycin treated WT mice. At the genus level, *Bifidobacterium* was decreased with increased *Akkermansia* and *Veillonella* by vancomycin treatment in WT and iNOS^{-/-} mice. Vancomycin decreased *Allobaculum* and increased *Lactobacillus*, *Staphylococcus* and *Parabacteroides* in iNOS^{-/-} mice; while decreased *Lactobacillus* and *Oscillospira* in WT (Fig. S2H, 2F). Abx decreased Firmicutes and Bacteroidetes with increased Proteobacteria in WT and iNOS^{-/-} mice depleting majority of gut microbiota. Actinobacteria and

Verrucomicrobia were decreased by Abx in iNOS^{-/-} mice. Bacterial families- Ruminococcaceae, Lactobacillaceae, S24-7 were decreased with increased Enterobacteriaceae in Abx treated WT and iNOS^{-/-} mice. Bifidobacteriaceae, Erysipelotrichaceae, Verrucomicrobiaceae and Bacteroidaceae were decreased in Abx treated iNOS^{-/-} mice with increased Brucellaceae in WT. At the genus level, *Lactobacillus*, *Bifidobacterium*, *Akkermansia* were decreased by Abx treatment in WT and iNOS^{-/-} mice with increased *Klebsiella*. Abx decreased *Allobaculum* and increased *Serratia* in iNOS^{-/-} mice; while increased *Pseudomonas*, *Elizabethkingia* and *Ochrobactrum* in WT (Fig. S5H, S3F). Both, vancomycin (Fig. 2G-L) and Abx (Fig. S3G-L) reversed the systemic glucose intolerance, IR and enhanced gluconeogenesis in iNOS^{-/-} mice as seen by decrease in AUC during GTT, ITT and PTT respectively. The increase in the circulating level of glucose and insulin in iNOS^{-/-} mice was decreased by both the treatments (Fig. 2M, N; S3M, N). HOMA-IR was decreased by antibiotics treatment with increased QUICKI reversing the IR phenotype observed in iNOS^{-/-} mice (Fig. 2O, P; S3O, P). The enhanced circulating TC, TG, LDL and NEFA in iNOS^{-/-} mice were reversed by the antibiotics treatment, thus rescuing the dyslipidemia in iNOS^{-/-} mice (Fig. 2Q-T; S3Q-T). The body weight (Fig. S6A, B) and food intake (Fig. S6D, E) however remained unaltered in obese iNOS^{-/-} mice upon vancomycin and Abx treatment. Body composition and BMI also remained unaltered in vancomycin treated iNOS^{-/-} mice with increased fat mass in Abx treated iNOS^{-/-} mice (Fig. S6C, F). Liver, adipose tissue, and heart weight ratio was decreased in vancomycin and Abx treated iNOS^{-/-} mice with increased weight and length ratio of small intestine and caecum (Fig. S6G, H).

Vancomycin-induced alterations in serum metabolites in iNOS^{-/-} mice

The PCA score plots of vancomycin (Fig. 3A) and Abx (Fig. S4A) treated and untreated iNOS^{-/-} mice and vancomycin (Fig. S2A) and Abx (Fig. S5A) treated and untreated WT mice in ESI (+) mode showed very distinct separations. Significantly differential metabolites ($p < 0.05$) between untreated iNOS^{-/-} and iNOS^{-/-} treated with vancomycin (Fig. 3B) or Abx (Fig. S4B) were visualized through volcano plots. Nucleic acid metabolites- allantoin and pyrimidines were decreased by vancomycin (Fig. 3C) and Abx (Fig. S4H) in iNOS^{-/-} mice with decreased methylthioadenosine by Abx. Vancomycin and Abx treatment did not decreased other purine metabolites in WT and iNOS^{-/-} mice. Methylthioadenosine and allantoin were also decreased in WT by vancomycin (Fig. S2C) and Abx (Fig. S5D) along with decreased pyrimidines by Abx. 15-Methyl PGA₂ and corticosterone was increased by both vancomycin (Fig. 3D) and Abx (Fig. S4F) in iNOS^{-/-} mice. Serotonin was increased by vancomycin treatment in iNOS^{-/-} mice with decreased thyrotropin-releasing hormone. Bile acid-cholate and cofactor-Pyridoxate was decreased by Abx (Fig. S5B, S4F) in WT and iNOS^{-/-} mice but not by vancomycin (Fig. S2B, 3D). Glycolysis and Krebs cycle intermediates were decreased in vancomycin (Fig. 3E) and Abx (Fig. S4D) treated iNOS^{-/-} mice while glyceraldehyde was increased. Vancomycin in WT (Fig. S2F) did not decreased the carbohydrate metabolites to a significant extent as compared to Abx treatment (Fig. S5C). Oxoproline, 2-methylhippuric acid, hippurate, indole-3-ethanol, ophthalmate, mevalonate and aspartame were decreased by vancomycin and Abx in WT (Fig. S2D, S5E) and iNOS^{-/-} mice (Fig. 3F, S4C). 4-Acetamidobutanoic acid,

indole-3-methyl acetate, sebacic acid, 4-chlorophenol and isopentenyl-adenine-7-glucoside were decreased by vancomycin and Abx in iNOS^{-/-} mice while formate was increased by vancomycin suggesting the modulation of bacterial derived/dependent metabolites by antibiotics. Majority of amino acids were significantly decreased in vancomycin and Abx treated WT (Fig. S2G, S5G) and iNOS^{-/-} mice (Fig. 3G, S4E) including aminoadipate except, 3-nitrotyrosine, cysteamine and cysteate. Aromatic amino acid metabolites- tryptophan was increased in iNOS^{-/-} mice while 3-methoxytyrosine and N-acetyl phenylalanine was increased in WT by Abx (Fig. S5G, S4E). Spermidine was increased by vancomycin and Abx in WT mice (Fig. S2G, S5G). Many lipid species- PE, PC, PA, PS, PG, ceramides and 10-Hydroxydecanoate were decreased by vancomycin and Abx treatment in iNOS^{-/-} (Fig. 3H, S4G) and WT mice (Fig. S2I, S5I); while palmitate was increased suggesting improved lipid metabolism along with improvement in PE to PC ratio. Laurate and lauroyl carnitine were decreased in iNOS^{-/-} mice and were increased by vancomycin but not by Abx. These results suggest that vancomycin-induced gram positive bacteria depletion improved the metabolic perturbations in iNOS^{-/-} mice.

Improvement in the disrupted lipid and glucose homeostasis in liver in iNOS^{-/-} mice following treatment with vancomycin

Hepatic TC, TG and FFA levels were reduced in the vancomycin (Fig. 4A-C) and Abx (Fig. S7A-C) treated iNOS^{-/-} mice. FFA levels were also decreased in vancomycin and Abx treated WT (Fig. 4C, S7C). The enhanced expression of lipid synthesis genes (PPAR γ , SREBP-1c and ACC1) in the liver of iNOS^{-/-} mice were decreased by vancomycin (Fig. 4F) and Abx (Fig. S7F) with unchanged LXR α , LXR β , and HMGCR in liver. FAS and SREBP-2 expression was decreased significantly by vancomycin in iNOS^{-/-} mice, but not by Abx. Expression of LXR α , LXR β and HMGCR were increased in liver of vancomycin treated WT (Fig. 4F). PGC-1 β expression was decreased in liver of vancomycin (Fig. 4G) and Abx (Fig. S7G) treated WT and iNOS^{-/-} mice. PPAR α and UCP2 expressions were decreased in liver of Abx treated WT and vancomycin treated iNOS^{-/-} mice. Liver PGC-1 α expression was increased in vancomycin treated WT. ACC2 expression remained unchanged in liver of vancomycin and Abx treated iNOS^{-/-} mice. Expression of genes involved in the hepatic lipid uptake (CD36, LPL) were unaltered in iNOS^{-/-} mice following vancomycin (Fig. 4H) and Abx (Fig. S7H) treatment with decreased SR-1B. CD36 and LPL expression was increased in vancomycin treated WT. LDLR expression was reduced in iNOS^{-/-} mice and was unaltered by vancomycin and Abx. The expression of genes involved in hepatic lipid efflux- ABCG5 and ABCG8 was decreased in iNOS^{-/-} mice and was increased significantly in Abx treated iNOS^{-/-} mice (Fig. S7I) and were comparable to WT in vancomycin treated iNOS^{-/-} mice (Fig. 4I). Hepatic PC expression was decreased by vancomycin with decreased G6PC expression by vancomycin (Fig. 4E) and Abx (Fig. S7E) in iNOS^{-/-} mice along with unchanged FOXO1 and PEPCK. Hepatic glycogen levels also remained unaltered upon vancomycin treatment (Fig. 4D), while insulin stimulated glycogen levels were increased significantly by Abx (Fig. S7D).

Improvement in the disrupted lipid and glucose homeostasis in adipose tissue and intestine in iNOS^{-/-} mice following treatment with vancomycin

The enhanced expression of lipid synthesis genes (PPAR γ , SREBP-1c, FAS and ACC1) in adipose tissue of iNOS^{-/-} mice were decreased by vancomycin, while, Abx treatment decreased SREBP-1c and FAS. LXR α expression was increased in eWAT of vancomycin (Fig. 5C) and Abx treated iNOS^{-/-} mice (Fig. S8C). PGC-1 α and UCP2 expressions in adipose tissue were increased in Abx treated iNOS^{-/-} mice, while, PGC-1 β , ACC2 and PPAR α remains unchanged by vancomycin (Fig. 5D) or Abx (Fig. S8D). The enhanced expression of CD36 and LPL in adipose tissue of iNOS^{-/-} mice was decreased by vancomycin (Fig. 5E) and Abx treatment (Fig. S8E). PEPCK and G6PC was decreased in adipose tissue by vancomycin (Fig. 5A) and Abx (Fig. S8A) in iNOS^{-/-} mice with unchanged FOXO1 and PC. Decreased Akt2 gene expression was increased in white adipose tissue of vancomycin treated WT and iNOS^{-/-} mice suggesting improved insulin signaling upon gram-positive bacteria depletion. The glucose transporters in adipose tissue remained unaltered upon vancomycin (Fig. 5B) or Abx treatment (Fig. S8B).

The LXR α , HMGCR and CYP7A1 expressions were decreased in small intestine of vancomycin treated iNOS^{-/-} and WT mice (Fig. 5F). Abx decreased LXR α expression in iNOS^{-/-} and WT mice (Fig. S8F). Expression of most of the lipid uptake genes in the small intestine (CD36, NPC1L1, FABP1, LDLR, ApoE, FFAR1, FFAR2) was not altered in the vancomycin (Fig. 5G) and Abx treated iNOS^{-/-} mice (Fig. S8G), except SR-1B, which was reduced. Further, expression of CD36, NPC1L1, LDLR, FFAR1 and FFAR2 was reduced in the vancomycin treated WT mice (Fig. 5G). ABCG5 expression was decreased in small intestine in iNOS^{-/-} mice and was increased by vancomycin (Fig. 5H) and Abx treatment (Fig. S8H) with unchanged ABCG8 and ABCA1.

The morphometric calculations (Fig. S9A, D) displayed increased intestinal villi and colonic crypt length in iNOS^{-/-} mice (Fig. S9C, F). The barrier functionality of the gut remained unchanged in iNOS^{-/-} mice as assessed by in vivo FITC-dextran gut permeability assay (Fig. S9G) and Alcian blue staining in intestinal (Fig. S9A, B) and colonic tissues (Fig. S9D, E). The gene expression of tight junction protein- Claudin-2 was increased with decreased ZO-1 and unaltered occludin in iNOS^{-/-} mice. Mucins (Muc-2 and Muc-5AC) remained unaltered in iNOS^{-/-} mice with reduced expression of antimicrobial peptide Reg3 γ (Fig. S9H). The FITC-dextran gut permeability assay showed significant reduction in the intestinal permeability following vancomycin and Abx treatment in WT and iNOS^{-/-} mice as compared to untreated controls (Fig. S9G). The mRNA expression for tight junction proteins, mucins and antimicrobial peptide in small intestine largely remained unchanged in antibiotics treated iNOS^{-/-} mice. Claudin-2 mRNA expression was increased in Abx treated WT and iNOS^{-/-} mice (Fig. S9H). Further, status of NO was checked and the decreased total nitrite levels in iNOS^{-/-} mice were not altered in serum, adipose tissue and intestine after antibiotics treatment. In liver, the total nitrite levels were further decreased in iNOS^{-/-} mice by antibiotics (Fig. S9I-L). nNOS expression remained unchanged in the liver and intestine, and was decreased in adipose tissue upon antibiotics treatment in iNOS^{-/-} mice. Decreased eNOS expression was rescued in the

adipose tissue and intestine by antibiotics and remained unaltered in liver (Fig. S9M-O).

Association of serum metabolites with metabolic profile of iNOS^{-/-} mice upon treatment with antibiotics

The phenotypic, biochemical, functional, metabolic and molecular analysis suggests that iNOS^{-/-} mice displayed IR and dyslipidemia along with altered gut microbiome as compared to WT mice. Gut microbiota depletion/ modulation by antibiotics showed marked improvement in metabolic parameters with precise alterations in the serum metabolites. Pearson's correlation was used to identify the serum metabolites which strongly correlated with the metabolic biomarkers quantified in control (WT), insulin resistant (iNOS^{-/-}) and antibiotics (vancomycin or Abx) treated WT and iNOS^{-/-} mice. Lipid species- MGDGs, PEs, PAs, PSs, PGs, ceramides and 10-hydroxydecanoate positively correlated with bacterial diversity, dyslipidemia and IR; and negatively with caecum weight. Laurate and palmitate were negatively correlated liver lipids and bacterial diversity (Fig. 6A). Majority of amino acids positively correlated with biomarkers of IR, dyslipidemia and α -diversity and negatively correlated with HDL levels and caecum weight (Fig. 6B).

Indole-3-methyl acetate, 5-oxoproline, 2-methylhippuric acid, hippurate, indole-3-ethanol, ophthalmate, mevalonate, 6-carboxyhexanoate, sebacic acid, 4-chlorophenol, δ -undecalactone and aspartame positively correlated with IR, dyslipidemia and α -diversity and negatively correlated with caecum weight. S-carboxymethylcysteine and formate were negatively correlated with dyslipidemia and α -diversity (Fig. S10A). Nucleic acid metabolites- methylthioadenosine, xanthine, allantoin, thymine, cytosine and uracil positively correlated with biomarkers of IR and dyslipidemia and negatively correlated with caecum weight. Glycolysis and Krebs cycle intermediates were correlated positively and glyceraldehyde negatively with liver lipids and α -diversity. 4-Pyridoxate and melatonin were positively correlated with glucose intolerance and dyslipidemia. Bile acids and bile pigments were positively correlated with IR, dyslipidemia and negatively correlated with caecum weight (Fig. S10B). These results suggest that serum metabolites exhibited strong association with specific metabolic biomarkers and were modulated by microbiota depletion by antibiotics.

Association of gut microbiota with metabolic parameters in iNOS^{-/-} mice following treatment with antibiotics

Further, association between antibiotics induced compositional changes in microbiota with the alterations in metabolic parameters, was investigated. Phylum- Bacteroidetes and Actinobacteria were positively associated with IR, dyslipidemia and α -diversity, while Proteobacteria correlated negatively. Firmicutes and Verrucomicrobia were negatively correlated with caecum weight and positively correlated with dyslipidemia (Fig. 7A). Bifidobacteriaceae (Actinobacteria), Erysipelotrichaceae (Firmicutes) and S24-7 (Bacteroidetes) were positively correlated with IR, dyslipidemia and α -diversity and negatively with caecum weight. Microbial families- Ruminococcaceae, Eubacteriaceae, Lachnospiraceae, Dehalobacteriaceae, Mogibacteriaceae, Lactobacillaceae (Firmicutes); Coriobacteriaceae

(Actinobacteria); Verrucomicrobiaceae (Verrucomicrobia) were positively correlated with dyslipidemia and α -diversity and negatively correlated with caecum weight. Weeksellaceae, Sphingobacteriaceae (Bacteroidetes); Enterococcaceae, Bacillaceae, Tissierellaceae, Leuconostocaceae (Firmicutes); Microbacteriaceae, Micrococcaceae, Gordoniaceae, Nocardaceae, Nocardiaceae, Promicromonosporaceae, Beutenbergiaceae, Streptomyetaceae, Pseudonocardiaceae, Brevibacteriaceae, Geodermatophilaceae (Actinobacteria) and many families of Proteobacteria including Enterobacteriaceae were negatively correlated with serum NEFA and positively correlated caecum weight (Fig. S11).

Bifidobacterium (Actinobacteria); *Allobaculum* and *Ruminococcus* (Firmicutes) were positively correlated with IR, dyslipidemia and α -diversity and negatively with caecum weight. *Anaerofustis* (Firmicutes) and *Adlercreutzia* (Actinobacteria) were positively correlated with serum NEFA and α -diversity and negatively correlated with caecum weight. *Pediococcus*, *Bacillus*, *Enterococcus*, *Dialister* (Firmicutes); *Sphingobacterium*, *Elizabethkingia* (Bacteroidetes); *Gordonia*, *Aeromicrobium*, *Streptomyces*, *Rhodococcus*, *Mycetocola*, *Salana*, *Microbacterium*, *Luteimicrobium*, *Nesterkonja*, *Saccharopolyspora*, *Brevibacterium* (Actinobacteria) and many genus of Proteobacteria phylum including *Enterobacter* were negatively correlated with NEFA and positively correlated with caecum weight. (Fig. 7B). These results suggest that Gram-positive bacteria depletion (*Allobaculum*, *Bifidobacterium* and *Ruminococcus*) following treatment with vancomycin is directly correlated with the improvement in metabolic biomarkers.

Gut microbiota association with serum metabolites in iNOS^{-/-} mice upon antibiotics treatment

Pearson's correlation coefficients were calculated between serum metabolites and microbial taxa, which positively correlated with blood glucose and glucose intolerance in WT and iNOS^{-/-} mice without or with antibiotics (vancomycin or Abx) treatment to gain better insights into the host metabolism. Interestingly, most of these microbial taxa linked to impaired glucose metabolism are gram-positive bacteria. Lipid species- MGDG, PE, PC, PA, PS, PG, ceramide, 10-hydroxydecanoate, dodecanedioic acid and myristate were positively correlated and palmitate negatively correlated with taxa directly associated with glucose intolerance (Actinobacteria, Bacteroidetes, Erysipelotrichaceae, Bifidobacteriaceae, S24-7, Ruminococcaceae, *Allobaculum*, *Bifidobacterium*, *Ruminococcus*) (Fig. 8A). Most amino acids metabolites including anthranilate, histidine, carnosine, serine, N-formylmethionine, N-acetyl cysteine, N-acetyl norvaline, carnitine, amino adipate, pipecolinic acid, methyl glutarate, glutamate, N-acetyl ornithine, betaine, creatine, creatinine, proline, leucyl proline and 3-amino-4-hydroxybutyric acid were positively correlated with taxa associated with glucose intolerance (Fig. 8B).

Metabolites- Indole-3-methyl acetate, 5-Oxoproline, 2-methylhippuric acid, hippurate, indole-3-ethanol, ophthalmate, mevalonate, 6-carboxyhexanoate, sebacic acid, 4-chlorophenol, δ -undecalactone, aspartame and bis(2-ethylhexyl)phthalate were positively correlated and S-carboxymethylcysteine and formate were negatively correlated with taxa associated with glucose intolerance (Fig. 8C). Purines (allantoin, methylthioadenosine) and pyrimidines (uracil) correlated positively with taxa associated with

glucose intolerance. Lactic acid, N-acetyl neuraminate, isocitrate and citramalate were positively correlated, while, glyceraldehyde was negatively correlated with taxa associated with glucose intolerance. 4-Pyridoxate, melatonin, bile acids and pigments- cholate, deoxycholate and bilirubin were positively correlated with taxa associated with glucose intolerance (Fig. 8D). These results suggest that metabolites involved in lipids, amino acids, bile acids, nucleic acids, carbohydrate, bacterial derived/dependent metabolism exhibited significant correlation with gram-positive bacterial taxa linked to IR and dyslipidemia in iNOS^{-/-} mice.

Discussion

Involvement of both gram-positive and negative bacteria have been reported in diabetes and metabolic syndrome, however, most of the studies on gram-negative bacteria linked LPS mediated inflammation with metabolic dysregulation [15–18]. iNOS^{-/-} mice are however protected against LPS-induced inflammation and the associated metabolic perturbations [29–31]. iNOS^{-/-} mice were insulin resistant and dyslipidemic on chow, LFD, and HFD diets, which was improved substantially by nitrite supplementation [24, 25, 27, 44]. To investigate further the role of gut bacteria in metabolic perturbations in iNOS^{-/-} mice we checked the microbial diversity in fecal samples. Reduced bacterial diversity in iNOS^{-/-} mice correlated with less diverse microbial communities in obese subjects [45]. Similar to that reported in ileum [46], we observed increased relative abundance of *Bifidobacterium* and *Allobaculum* in faeces belonging to the phylum Actinobacteria and Firmicutes, respectively in iNOS^{-/-} mice. Actinobacteria are augmented in obese subjects, and in patients with liver diseases and diabetes [45]. *Allobaculum*, the efficient energy harvesters and active assimilators of glucose [47] were enhanced in LFD and HFD fed mice [48], during metabolic dysbiosis [49], and in diabetic ZDF rats [50]. *Allobaculum* is a secondary degrader and seems to be dependent on primary degraders like *Bifidobacterium* for the utilization of complex polysaccharides for their growth. We and others [50, 51] did find a positive correlation between *Bifidobacterium*, *Ruminococcus*, and *Allobaculum* in the hyperglycemic condition. Gut barrier dysfunction and increased availability of microbial products in the systemic circulation, commonly known as metabolic endotoxemia, are considered as key drivers of obesity and metabolic syndrome [52]. Surprisingly, iNOS-deficient did not display alterations in gut permeability, possibly due to an increase in the relative abundance of *Akkermansia muciniphila*, a gram-negative bacteria that is shown to maintain and restore mucosal barrier integrity in obese and diabetic mice and in inflammatory conditions [53],[54]. Intestinal villi and colon crypt length were also increased in iNOS^{-/-} mice and this could be an adaptation for improved absorption of nutrients under hyperglycemic conditions [55].

As iNOS^{-/-} mice exhibited a higher abundance of gram-positive bacteria *Allobaculum* and *Bifidobacterium*, we employed vancomycin, which largely depletes gram-positive bacteria to study their association with IR and dyslipidemia. The vancomycin intervention altered the gut microbiome profiles as reported earlier [56, 57] in both WT and iNOS^{-/-} mice with decreased bacterial alpha diversity and

increased relative abundance of Proteobacteria [56], the signature markers of antibiotics treatment. Proteobacteria exhibit metabolic plasticity [58] and are reservoirs of several horizontally acquired antimicrobial resistance genes [59] leading to their expansion after antibiotics treatment. Intriguingly, vancomycin-induced reduction of *Allobaculum*, *Bifidobacterium*, and *Ruminococcus* was linked with improved IR and dyslipidemia in iNOS^{-/-} mice. Antibiotic strengthens the intestinal integrity [13, 57] with no distinguishable change in mucins and tight junction proteins [56, 57] as was also observed in iNOS^{-/-} mice. The depletion of microbiota by antibiotics treatment in WT [35, 60, 61] and in diet induced obese mice [11, 12, 14, 62] improved glucose tolerance and IR as found by us in the iNOS^{-/-} mice. *Veillonella* has recently been reported to improve cardiovascular health and exercise performance by utilising lactic acid and converting it into propionate [63] which might have symbiotic relation with lactic acid producing *Lactobacillus*. This might have been the reason for the expansion of both *Veillonella* and *Lactobacillus* in vancomycin treated iNOS^{-/-} mice. Susceptibility of *Lactobacilli* to vancomycin varies as *Lactobacillus acidophilus* and *Lact. delbreuckii* are vancomycin-sensitive, while *Lact. rhamnosus* are vancomycin-resistant [64]. We also used broad-spectrum antibiotic cocktail to deplete the majority of gut gram-positive and negative bacteria so as to further assess the role of gut microbiota in iNOS^{-/-} mice. As expected, *Allobaculum*, *Bifidobacterium*, *Lactobacillus* and *Akkermansia* were also depleted by Abx, with metabolic improvements along with reversal of IR and dyslipidemia. *Lactobacillus rhamnosus* and *Lactobacillus casei* improved glucose tolerance and IR in db/db mice [65], HFD fed obese mice [66] and diabetic mice [67], however, Abx treatment in this study also reduced *Lactobacillus*. We therefore failed to notice any additional advantage in IR and metabolism. Captivatingly, vancomycin and antibiotic cocktail reshaped gut microbiome and correlation analysis suggest importance of gram-positive bacteria- *Allobaculum*, *Bifidobacterium* and *Ruminococcus* depletion in the reversal of IR and dyslipidemia in iNOS^{-/-} mice with seemingly less important role of gram-negative bacteria in the redox imbalanced state.

Reduced gluconeogenesis was evident by change in PTT and decrease in the expression of gluconeogenic enzymes in the liver and adipose tissue of iNOS^{-/-} mice after treatment with antibiotics [61, 62]. A decrease in the insulin-stimulated glycogen levels in iNOS^{-/-} mice suggests enhanced flux of glucose into lipogenic pathways and their storage [68], which was enhanced by Abx as reported previously in ob/ob mice [62]. Treatment with antibiotics improved insulin signaling in iNOS^{-/-} mice via Akt [11]. Interestingly, antibiotics treatment rescued dyslipidemia [60] in iNOS^{-/-} mice *via* reduction in the expression of genes involved in fatty acid synthesis [61] in the liver and adipose tissue and of lipid uptake in the adipose tissue; while expression of genes involved in the lipid efflux was augmented in the liver and intestine.

Systemic changes in metabolism were reflected by untargeted metabolomics in serum and revealed that purine and pyrimidine metabolites were elevated in iNOS^{-/-} mice as supported by other studies in diabetic human subjects and rodents [69, 70]. Bile acids and bile salts have been positively correlated with IR in humans [71] and also in iNOS^{-/-} mice; and were decreased upon gut microbiota depletion by antibiotics as reported previously in HFD fed mice [13, 61]. Lipids are the major source of energy metabolism and are independent predictors for impairment of insulin actions and progression to diabetes

[72]. The present study revealed that lipid metabolism seems to be the principal disordered pathway in $iNOS^{-/-}$ mice. Following vancomycin treatment dyslipidemia and IR was reversed suggesting an association of lipid species with glucose intolerance. It has been reported that in NAFLD/NASH in humans, PE concentration is increased relative to the disease progression [73] and NASH patients have a decreased ratio of PC to PE [74]. Nozaki *et. al*, observed higher propensity of fatty liver damage in $iNOS^{-/-}$ mice [76] and perturbed PE/PC ratio observed by us in $iNOS^{-/-}$ mice might provide a plausible explanation. Moreover, PE/PC ratio was improved upon antibiotics treatment in $iNOS^{-/-}$ mice similar to WT along with metabolic improvements by gram-positive bacteria depletion. Hydroxydecanoate, is enhanced in obesity-associated IR and type 2 diabetes [75]; we, similarly, observed an increase in this metabolite in $iNOS^{-/-}$ mice and reduction with vancomycin intervention. Laurate and lauroyl carnitine were decreased in $iNOS^{-/-}$ mice, which could be due to the enhanced oxidation of fatty acids, as they are suggested to be accumulated during defects in fatty acids oxidation in diabetic states [76, 77]. Interestingly, laurate and lauroyl carnitine were increased upon vancomycin treatment specifically and not by antibiotic cocktail in $iNOS^{-/-}$ mice suggesting the role of vancomycin-selective gram-positive bacteria in their metabolism.

Hippurate, a bacterial metabolite is an early biomarker of IR and diabetes [78]. Allantoin, an oxidative stress biomarker is the end product of the uric acid oxidation, and it is enhanced in streptozotocin-induced diabetic rats, db/db mice, obese dogs, and gestational diabetic-females [79]. Gram-positive bacteria are positive contributors of hippurate, allantoin, glucose, 2-oxoglutarate and glutamine as these were found to be present in the serum of mice infected with gram-positive bacteria [80]. In the present study following vancomycin-induced depletion of gram-positive bacteria, these metabolites were reduced in $iNOS^{-/-}$ mice. Formate, produced by gram-negative bacteria [80] was increased in vancomycin treated $iNOS^{-/-}$ mice but not by antibiotic cocktail treatment suggesting the differential metabolic response of gram-positive and -negative bacteria. Indole-3-ethanol, a bacterial derived metabolite of tryptophan [81] was increased in $iNOS^{-/-}$ mice. Sebacic acid was higher in the diabetic [82] and NAFLD patients [83]. Amino adipate, a diabetogen, is reported to be involved in glycemic perturbations [84]. Ophthalmate is a potential biomarker for oxidative stress and glutathione depletion [85]. These bacteria-derived metabolites were decreased upon microbiota depletion by antibiotics, suggesting their altered metabolism and possible association with IR in $iNOS^{-/-}$ mice. The dominance of gram-positive bacteria in insulin resistant $iNOS^{-/-}$ mice and improvement in metabolic markers with vancomycin intervention indicate that gram-positive bacteria are crucial drivers of observed metabolic phenotype in these mice. However, we do not completely rule out the role of gram-negative bacteria and other microorganisms such as archaea in regulating the metabolic functions of $iNOS^{-/-}$ mice, and this requires further investigations. Understanding the interplay between nutrition, genetics and microbial metabolism with redox imbalance is necessary to combat the scourge of IR.

Altogether, in the present study, we report that increased relative abundance of gram-positive *Allobaculum* and *Bifidobacterium* in redox imbalanced $iNOS^{-/-}$ mice led to metabolic perturbations with enhanced

PE/PC ratio, 10-hydroxydecanate and indole-3-ethanol levels. Vancomycin mediated depletion of *Allobaculum*, *Bifidobacterium* and *Ruminococcus*, was associated with the reversal of IR and dyslipidemia in iNOS^{-/-} mice. Vancomycin treatment also led to improvement in PE/PC ratio, 10-hydroxydecanoate, indole-3-ethanol, allantoin, hippurate, sebacic acid, amino adipate and ophthamate (Fig. 9). Overall, the results obtained in the present study demonstrate that gram-positive gut microbiota, the associated metabolites, and their crosstalk with the host drives dyslipidemia and insulin resistance in iNOS^{-/-} mice.

Declarations

Availability of data and materials

All data used in this study are present in the main text and supplementary materials. The raw 16S rRNA gene sequencing data has been deposited in the Sequence Read Archive (SRA) of the National Center for Biotechnology Information (NCBI) under accession number PRJNA740126.

Acknowledgments: The efforts of Dr. Gagandeep Kang (Former Executive Director, THSTI, India) in helping with the 16S rRNA gene sequencing are gratefully acknowledged. The help and critical inputs of Dr. Manoj Kumar Barthwal (Senior Principal Scientist, CSIR-CDRI, India) during the course of this study are gratefully acknowledged.

Fundings: The present study was supported by JC Bose National fellowship [SB/SE/JCB-017/2015] and THSTI core grant to Madhu Dikshit; and DST SERB Project [EMR/2017/003730] to Kumaravelu Jagavelu, CSIR-CDRI. Research fellowships to HA from Indian Council of Medical Research and PP from Council of Scientific and Industrial Research, India are acknowledged.

Author Contributions: HA performed most of the experiments and wrote the manuscript. PP performed some experiments. VS provided deeper understanding and critical suggesting during the study. SM provided critical inputs and interpretation for gut microbiome studies/data. YK performed the metabolomics experiments and analyzed the data. BD provided critical inputs regarding fecal DNA isolation and interpretation for gut microbiome studies/data. KJ provided the animals and critical suggestions during the study. MD conceptualized the whole project, designed, supervised the studies and has interpreted the data as being presented in the MS; she also finalized the manuscript.

Ethics approval and consent to participate: All animal experiments were approved by Institutional Animal Ethics Committee of CSIR-CDRI (IAEC/2014/43) in accordance with CPCSEA guidelines.

Consent for publication

Not applicable.

Competing Interests: The authors declare that they have no competing interests.

Data and materials availability:

All data needed to evaluate the conclusions in the paper are present in the paper and/or the Supplementary Materials. The raw 16S rRNA gene sequencing data has been deposited in the Sequence Read Archive (SRA) of the National Center for Biotechnology Information (NCBI) under accession number PRJNA740126.

References

1. Ahlqvist E, Storm P, Käräjämäki A, Martinell M, Dorkhan M, Carlsson A, et al. Novel subgroups of adult-onset diabetes and their association with outcomes: a data-driven cluster analysis of six variables. *Lancet Diabetes Endocrinol* [Internet]. 2018 [cited 2020 Apr 17];6:361–9. Available from: <http://www.ncbi.nlm.nih.gov/pubmed/29503172>.
2. Ormazabal V, Nair S, Elfeky O, Aguayo C, Salomon C, Zuñiga FA. Association between insulin resistance and the development of cardiovascular disease. *Cardiovasc Diabetol* [Internet]. BioMed Central; 2018 [cited 2020 Jun 9];17:122. Available from: <http://www.ncbi.nlm.nih.gov/pubmed/30170598>.
3. Eid HM, Wright ML, Anil Kumar NV, Qawasmeh A, Hassan STS, Mocan A, et al. Significance of Microbiota in Obesity and Metabolic Diseases and the Modulatory Potential by Medicinal Plant and Food Ingredients. *Front Pharmacol Frontiers*. 2017;0:387.
4. H S, J S, E E. Personalized microbiome-based approaches to metabolic syndrome management and prevention. *J Diabetes* [Internet]. J Diabetes; 2017 [cited 2021 Aug 16];9:226–36. Available from: <https://pubmed.ncbi.nlm.nih.gov/27787945/>.
5. Dethlefsen L, Huse S, Sogin ML, Relman DA. The pervasive effects of an antibiotic on the human gut microbiota, as revealed by deep 16s rRNA sequencing. *PLoS Biol* [Internet]. PLoS Biol; 2008 [cited 2020 Dec 8];6:2383–400. Available from: <https://pubmed.ncbi.nlm.nih.gov/19018661/>.
6. Sullivan Å. Effect of antimicrobial agents on the ecological balance of human microflora [Internet]. *Lancet Infect. Dis. Lancet Infect Dis*; 2001 [cited 2020 Dec 8]. p. 101–14. Available from: <https://pubmed.ncbi.nlm.nih.gov/11871461/>.
7. Jernberg C, Löfmark S, Edlund C, Jansson JK. Long-term impacts of antibiotic exposure on the human intestinal microbiota [Internet]. *Microbiology. Microbiology Society*; 2010 [cited 2020 Dec 8]. p. 3216–23. Available from: <https://www.microbiologyresearch.org/content/journal/micro/10.1099/mic.0.040618-0>.
8. Wilkins AT, Reimer RA. Obesity, Early Life Gut Microbiota, and Antibiotics. *Microorganisms* [Internet]. Multidisciplinary Digital Publishing Institute (MDPI); 2021 [cited 2021 Aug 16];9:1–20. Available from: [/pmc/articles/PMC7922584/](https://pmc/articles/PMC7922584/).

9. MA KK, K ZMK K, E S, A S, et al. Childhood BMI in relation to microbiota in infancy and lifetime antibiotic use. *Microbiome* [Internet]. *Microbiome*; 2017 [cited 2021 Aug 16];5. Available from: <https://pubmed.ncbi.nlm.nih.gov/28253911/>.
10. Li J, Yang K, Ju T, Ho T, McKay CA, Gao Y, et al. Early life antibiotic exposure affects pancreatic islet development and metabolic regulation. *Sci Reports* 2017 71 [Internet]. Nature Publishing Group; 2017 [cited 2021 Aug 16];7:1–12. Available from: <https://www.nature.com/articles/srep41778>.
11. Carvalho BM, Guadagnini D, Tsukumo DML, Schenka AA, Latuf-Filho P, Vassallo J, et al. Modulation of gut microbiota by antibiotics improves insulin signalling in high-fat fed mice. *Diabetologia* [Internet]. *Diabetologia*; 2012 [cited 2020 Dec 8];55:2823–34. Available from: <https://pubmed.ncbi.nlm.nih.gov/22828956/>.
12. Hwang I, Park YJ, Kim Y, Kim YN, Ka S, Lee HY, et al. Alteration of gut microbiota by vancomycin and bacitracin improves insulin resistance *via* glucagon-like peptide 1 in diet-induced obesity. *FASEB J* [Internet]. *FASEB*; 2015 [cited 2020 Dec 30];29:2397–411. Available from: <https://onlinelibrary.wiley.com/doi/abs/10.1096/fj.14-265983>.
13. Fujisaka S, Ussar S, Clish C, Devkota S, Dreyfuss JM, Sakaguchi M, et al. Antibiotic effects on gut microbiota and metabolism are host dependent. *J Clin Invest American Society for Clinical Investigation*. 2016;126:4430–43.
14. Cani PD, Bibiloni R, Knauf C, Waget A, Neyrinck AM, Delzenne NM, et al. Changes in gut microbiota control metabolic endotoxemia-induced inflammation in high-fat diet-induced obesity and diabetes in mice. *Diabetes* [Internet]. *Diabetes*; 2008 [cited 2020 Dec 8];57:1470–81. Available from: <https://pubmed.ncbi.nlm.nih.gov/18305141/>.
15. Radilla-Vázquez RB, Parra-Rojas I, Martínez-Hernández NE, Márquez-Sandoval YF, Illades-Aguilar B, Castro-Alarcón N. Gut Microbiota and Metabolic Endotoxemia in Young Obese Mexican Subjects. *Obes Facts* [Internet]. Karger Publishers; 2016 [cited 2021 Jul 30];9:1. Available from: </pmc/articles/PMC5644836/>.
16. Liu Y-X, Cao Q-M, Ma B-C. Pathogens distribution and drug resistance in patients with acute cerebral infarction complicated with diabetes and nosocomial pulmonary infection. *BMC Infect Dis* 2019 191 [Internet]. BioMed Central; 2019 [cited 2021 Jul 30];19:1–6. Available from: <https://bmcinfectdis.biomedcentral.com/articles/10.1186/s12879-019-4142-9>.
17. Rawat V, Singhai M, Kumar A, Jha PK, Goyal R. Bacteriological and Resistance Profile in Isolates from Diabetic Patients. *N Am J Med Sci* [Internet]. Wolters Kluwer – Medknow Publications; 2012 [cited 2021 Jul 30];4:563. Available from: </pmc/articles/PMC3503374/>.
18. Pushpanathan P, Srikanth P, Seshadri KG, Selvarajan S, Pitani RS, Kumar TD, et al. Gut Microbiota in Type 2 Diabetes Individuals and Correlation with Monocyte Chemoattractant Protein1 and Interferon Gamma from Patients Attending a Tertiary Care Centre in Chennai, India. *Indian J Endocrinol Metab* [Internet]. Wolters Kluwer – Medknow Publications; 2016 [cited 2021 Jul 30];20:523. Available from: </pmc/articles/PMC4911843/>.
19. Cox LM, Blaser MJ. Pathways in microbe-induced obesity. *Cell Metab. Cell Press*; 2013. p. 883–94.

20. Wong JM, Billiar TR. Regulation and Function of Inducible Nitric Oxide Synthase during Sepsis and Acute Inflammation. *Adv Pharmacol* [Internet]. Academic Press; 1995 [cited 2020 Jan 7];34:155–70. Available from: <https://www.sciencedirect.com/science/article/abs/pii/S1054358908610844>.
21. Kröncke KD, Fehsel K, Kolb-Bachofen V. Inducible nitric oxide synthase in human diseases. *Clin Exp Immunol* [Internet]. Wiley-Blackwell; 1998 [cited 2020 Jan 7];113:147–56. Available from: <http://www.ncbi.nlm.nih.gov/pubmed/9717962>.
22. Perreault M, Marette A. Targeted disruption of inducible nitric oxide synthase protects against obesity-linked insulin resistance in muscle. *Nat Med* [Internet]. 2001 [cited 2018 Jul 21];7:1138–43. Available from: <http://www.ncbi.nlm.nih.gov/pubmed/11590438>.
23. Cha H-N, Song SE, Kim Y-W, Kim J-Y, Won K-C, Park S-Y. Lack of inducible nitric oxide synthase prevents lipid-induced skeletal muscle insulin resistance without attenuating cytokine level. *J Pharmacol Sci* [Internet]. 2011 [cited 2018 Jul 26];117:77–86. Available from: <http://www.ncbi.nlm.nih.gov/pubmed/22001626>.
24. Kanuri BN, Kanshana JS, Rebello SC, Pathak P, Gupta AP, Gayen JR, et al. Altered glucose and lipid homeostasis in liver and adipose tissue pre-dispose inducible NOS knockout mice to insulin resistance. *Sci Rep* [Internet]. 2017 [cited 2018 Jul 28];7:41009. Available from: <http://www.nature.com/articles/srep41009>.
25. Pathak P, Kanshana JS, Kanuri B, Rebello SC, Aggarwal H, Jagavelu K, et al. Vasoreactivity of isolated aortic rings from dyslipidemic and insulin resistant inducible nitric oxide synthase knockout mice. *Eur J Pharmacol* [Internet]. 2019 [cited 2019 Aug 21];855:90–7. Available from: <http://www.ncbi.nlm.nih.gov/pubmed/31063772>.
26. Kakimoto PA, Chausse B, Caldeira da Silva CC, Donato Júnior J, Kowaltowski AJ. Resilient hepatic mitochondrial function and lack of iNOS dependence in diet-induced insulin resistance. *PLoS One* [Internet]. Public Library of Science; 2019 [cited 2019 Oct 9];14:e0211733. Available from: <http://www.ncbi.nlm.nih.gov/pubmed/30716103>.
27. Aggarwal H, Pathak P, Singh P, Gayen JR, Jagavelu K, Dikshit M. Systemic Insulin Resistance and Metabolic Perturbations in Chow Fed Inducible Nitric Oxide Synthase Knockout Male Mice: Partial Reversal by Nitrite Supplementation. *Antioxidants* [Internet]. MDPI AG; 2020 [cited 2021 Feb 5];9:736. Available from: <https://www.mdpi.com/2076-3921/9/8/736>.
28. Bogdan C. Nitric oxide synthase in innate and adaptive immunity: An update [Internet]. *Trends Immunol*. Elsevier Ltd; 2015 [cited 2020 Dec 3]. p. 161–78. Available from: <https://pubmed.ncbi.nlm.nih.gov/25687683/>.
29. Carvalho-Filho MA, Ueno M, Carvalheira JBC, Velloso LA, Saad MJA. Targeted disruption of iNOS prevents LPS-induced S-nitrosation of IR β /IRS-1 and Akt and insulin resistance in muscle of mice. *Am J Physiol Metab* [Internet]. 2006 [cited 2020 Jan 6];291:E476–82. Available from: <http://www.ncbi.nlm.nih.gov/pubmed/16638822>.
30. House li LM, Morris RT, Barnes TM, Lantier L, Cyphert TJ, McGuinness OP, et al. Tissue inflammation and nitric oxide-mediated alterations in cardiovascular function are major determinants of endotoxin-

- induced insulin resistance. 2012 [cited 2018 Aug 5]; Available from: <https://link.springer.com/content/pdf/10.1186/s12933-015-0223-2.pdf>.
31. SD C, RJ GSPAV, AJ M H, A A. Protection against lipopolysaccharide-induced endothelial dysfunction in resistance and conduit vasculature of iNOS knockout mice. *FASEB J* [Internet]. *FASEB J*; 2003 [cited 2021 Jul 30];17:773–5. Available from: <https://pubmed.ncbi.nlm.nih.gov/12586741/>.
 32. Hollenberg SM, Broussard M, Osman J, Parrillo JE. Increased microvascular reactivity and improved mortality in septic mice lacking inducible nitric oxide synthase. *Circ Res* [Internet]. Lippincott Williams and Wilkins; 2000 [cited 2021 May 21];86:774–9. Available from: <http://www.circresaha.org>.
 33. Nakata S, Tsutsui M, Shimokawa H, Suda O, Morishita T, Shibata K, et al. Spontaneous myocardial infarction in mice lacking all nitric oxide synthase isoforms. *Circulation* [Internet]. 2008 [cited 2019 Oct 15];117:2211–23. Available from: <https://www.ahajournals.org/doi/10.1161/CIRCULATIONAHA.107.742692>.
 34. Cui J, Le G, Yang R, Shi Y. Lipoic acid attenuates high fat diet-induced chronic oxidative stress and immunosuppression in mice jejunum: A microarray analysis. *Cell Immunol* [Internet]. 2009 [cited 2017 Sep 13];260:44–50. Available from: <http://linkinghub.elsevier.com/retrieve/pii/S0008874909001464>.
 35. Rodrigues RR, Greer RL, Dong X, DSouza KN, Gurung M, Wu JY, et al. Antibiotic-induced alterations in gut microbiota are associated with changes in glucose metabolism in healthy mice. *Front Microbiol* [Internet]. Frontiers Media S.A.; 2017 [cited 2020 Aug 28];8. Available from: <https://pubmed.ncbi.nlm.nih.gov/29213261/>.
 36. Hansen CHF, Krych L, Nielsen DS, Vogensen FK, Hansen LH, Sørensen SJ, et al. Early life treatment with vancomycin propagates *Akkermansia muciniphila* and reduces diabetes incidence in the NOD mouse [Internet]. *Diabetologia*. Springer; 2012 [cited 2020 Aug 28]. p. 2285–94. Available from: <https://link.springer.com/article/10.1007/s00125-012-2564-7>.
 37. Kanuri BN, Rebello SC, Pathak P, Agarwal H, Kanshana JS, Awasthi D, et al. Glucose and lipid metabolism alterations in liver and adipose tissue pre-dispose p47^{phox} knockout mice to systemic insulin resistance. *Free Radic Res* [Internet]. 2018 [cited 2018 Jul 29];52:568–82. Available from: <http://www.ncbi.nlm.nih.gov/pubmed/29544378>.
 38. Thevaranjan N, Puchta A, Schulz C, Naidoo A, Szamosi JC, Verschoor CP, et al. Age-Associated Microbial Dysbiosis Promotes Intestinal Permeability, Systemic Inflammation, and Macrophage Dysfunction. *Cell Host Microbe* [Internet]. 2017 [cited 2018 Oct 20];21:455–466.e4. Available from: <http://www.ncbi.nlm.nih.gov/pubmed/28407483>.
 39. Yokoyama H, Emoto M, Fujiwara S, Motoyama K, Morioka T, Komatsu M, et al. Quantitative insulin sensitivity check index and the reciprocal index of homeostasis model assessment in normal range weight and moderately obese type 2 diabetic patients. *Diabetes Care* [Internet]. 2003 [cited 2018 Oct 20];26:2426–32. Available from: <http://www.ncbi.nlm.nih.gov/pubmed/12882874>.
 40. Otsuka M, Kang YJ, Ren J, Jiang H, Wang Y, Omata M, et al. Distinct Effects of p38 α Deletion in Myeloid Lineage and Gut Epithelia in Mouse Models of Inflammatory Bowel Disease. In:

- Gastroenterology [Internet]. W.B. Saunders; 2010. [cited 2020 Aug 25];138:1255. Available from: /pmc/articles/PMC2846963/?report = abstract.
41. Kumar A, Kumar Y, Sevak JK, Kumar S, Kumar N, Gopinath SD. Metabolomic analysis of primary human skeletal muscle cells during myogenic progression. *Sci Rep* [Internet]. Nature Research; 2020 [cited 2020 Aug 26];10:11824. Available from: <https://doi.org/10.1038/s41598-020-68796-4>.
 42. Bag S, Saha B, Mehta O, Anbumani D, Kumar N, Dayal M, et al. An Improved Method for High Quality Metagenomics DNA Extraction from Human and Environmental Samples. *Sci Rep* [Internet]. Nature Publishing Group; 2016 [cited 2019 Aug 21];6:26775. Available from: <http://www.nature.com/articles/srep26775>.
 43. Tandon D, Haque MM, Saravanan R, Shaikh S, Sriram P, Dubey AK, et al. A snapshot of gut microbiota of an adult urban population from Western region of India. *PLoS One* [Internet]. Public Library of Science; 2018 [cited 2021 May 10];13:e0195643. Available from: <https://doi.org/10.1371/journal.pone.0195643>.
 44. Aggarwal H, Kanuri BN, Dikshit M. Role of iNOS in Insulin Resistance and Endothelial Dysfunction. *Oxidative Stress Hear Dis* [Internet]. Singapore: Springer Singapore; 2019 [cited 2020 Feb 18]. p. 461–82. Available from: http://link.springer.com/10.1007/978-981-13-8273-4_21.
 45. Turnbaugh PJ, Hamady M, Yatsunenko T, Cantarel BL, Duncan A, Ley RE, et al. A core gut microbiome in obese and lean twins. *Nature* [Internet]. Nature; 2009 [cited 2020 Dec 8];457:480–4. Available from: <https://pubmed.ncbi.nlm.nih.gov/19043404/>.
 46. Matziouridou C, Rocha SC, Haabeth OA, Rudi K, Carlsen H, Kielland A. iNOS- and NOX1-dependent ROS production maintains bacterial homeostasis in the ileum of mice. *Nat Publ Gr* [Internet]. 2017;11. Available from: www.nature.com/mi.
 47. Herrmann E, Young W, Rosendale D, Reichert-Grimm V, Riedel CU, Conrad R, et al. RNA-Based Stable Isotope Probing Suggests *Allobaculum* spp. as Particularly Active Glucose Assimilators in a Complex Murine Microbiota Cultured In Vitro. 2017; Available from: <https://doi.org/10.1155/2017/1829685>.
 48. Ravussin Y, Koren O, Spor A, Leduc C, Gutman R, Stombaugh J, et al. Responses of gut microbiota to diet composition and weight loss in lean and obese mice. *Obesity* [Internet]. Obesity (Silver Spring); 2012 [cited 2021 Jan 12];20:738–47. Available from: <https://pubmed.ncbi.nlm.nih.gov/21593810/>.
 49. Nobel YR, Cox LM, Kirigin FF, Bokulich NA, Yamanishi S, Teitler I, et al. Metabolic and metagenomic outcomes from early-life pulsed antibiotic treatment. *Nat Commun* [Internet]. Nature Publishing Group; 2015 [cited 2021 Jan 12];6. Available from: <https://pubmed.ncbi.nlm.nih.gov/26123276/>.
 50. Zhou W, Xu H, Zhan L, Lu X, Zhang L. Dynamic development of fecal microbiome during the progression of diabetes mellitus in Zucker diabetic fatty rats. *Front Microbiol* [Internet]. Frontiers Media S.A.; 2019 [cited 2021 Jan 12];10:232. Available from: /pmc/articles/PMC6382700/?report = abstract.
 51. Kim T, Holleman CL, Ptacek T, Morrow CD, Habegger KM. Duodenal endoluminal barrier sleeve alters gut microbiota of ZDF rats. *Int J Obes* [Internet]. Nature Publishing Group; 2017 [cited 2021 Jan 12];41:381–9. Available from: <https://pubmed.ncbi.nlm.nih.gov/27924082/>.

52. PD C, MA JA, C IMP K, D B, et al. Metabolic endotoxemia initiates obesity and insulin resistance. *Diabetes* [Internet]. *Diabetes*; 2007 [cited 2021 Aug 17];56:1761–72. Available from: <https://pubmed.ncbi.nlm.nih.gov/17456850/>.
53. Bian X, Wu W, Yang L, Lv L, Wang Q, Li Y, et al. Administration of *Akkermansia muciniphila* Ameliorates Dextran Sulfate Sodium-Induced Ulcerative Colitis in Mice. *Front Microbiol* [Internet]. *Frontiers Media S.A.*; 2019 [cited 2021 Jan 12];10. Available from: </pmc/articles/PMC6779789/?report=abstract>.
54. C AE, JP BLG, LB OCD. B, et al. Cross-talk between *Akkermansia muciniphila* and intestinal epithelium controls diet-induced obesity. *Proc Natl Acad Sci U S A* [Internet]. *Proc Natl Acad Sci U S A*; 2013 [cited 2021 Aug 17];110:9066–71. Available from: <https://pubmed.ncbi.nlm.nih.gov/23671105/>.
55. Isah MB, Masola B. Effect of oleanolic acid on small intestine morphology and enzymes of glutamine metabolism in diabetic rats. *Int J Physiol Pathophysiol Pharmacol* [Internet]. *E-Century Publishing Corporation*; 2017 [cited 2021 Jan 12];9:128–36. Available from: www.ijppp.org.
56. Ray P, Pandey U, Aich P. Comparative analysis of beneficial effects of vancomycin treatment on Th1- and Th2-biased mice and the role of gut microbiota. *J Appl Microbiol* [Internet]. *Blackwell Publishing Ltd*; 2020 [cited 2020 Dec 30];jam.14853. Available from: <https://onlinelibrary.wiley.com/doi/10.1111/jam.14853>.
57. Tulstrup MV-L, Christensen EG, Carvalho V, Linnings C, Ahrné S, Højberg O, et al Antibiotic Treatment Affects Intestinal Permeability and Gut Microbial Composition in Wistar Rats Dependent on Antibiotic Class. *Loh G, editor. PLoS One* [Internet]. *Public Library of Science*; 2015 [cited 2020 Dec 31];10:e0144854. Available from: <https://dx.plos.org/10.1371/journal.pone.0144854>.
58. Stecher B, Denzler R, Maier L, Bernet F, Sanders MJ, Pickard DJ, et al. Gut inflammation can boost horizontal gene transfer between pathogenic and commensal *Enterobacteriaceae*. *Proc Natl Acad Sci U S A* [Internet]. *National Academy of Sciences*; 2012 [cited 2020 Dec 9];109:1269–74. Available from: www.pnas.org/cgi/doi/10.1073/pnas.1113246109.
59. Jiang X, Ellabaan MMH, Charusanti P, Munck C, Blin K, Tong Y, et al. Dissemination of antibiotic resistance genes from antibiotic producers to pathogens. *Nat Commun* [Internet]. *Nature Publishing Group*; 2017 [cited 2021 May 18];8. Available from: <https://pubmed.ncbi.nlm.nih.gov/28589945/>.
60. Kuno T, Hirayama-Kurogi M, Ito S, Ohtsuki S. Reduction in hepatic secondary bile acids caused by short-term antibiotic-induced dysbiosis decreases mouse serum glucose and triglyceride levels. *Sci Rep* [Internet]. *Nature Publishing Group*; 2018 [cited 2021 Jan 1];8. Available from: <https://pubmed.ncbi.nlm.nih.gov/29352187/>.
61. Zarrinpar A, Chaix A, Xu ZZ, Chang MW, Marotz CA, Saghatelian A, et al. Antibiotic-induced microbiome depletion alters metabolic homeostasis by affecting gut signaling and colonic metabolism. *Nat Commun* [Internet]. *Nature Publishing Group*; 2018 [cited 2019 Mar 7];9:2872. Available from: <http://www.nature.com/articles/s41467-018-05336-9>.
62. Membrez M, Blancher F, Jaquet M, Bibiloni R, Cani PD, Burcelin RG, et al. Gut microbiota modulation with norfloxacin and ampicillin enhances glucose tolerance in mice. *FASEB J* [Internet]. *Wiley*; 2008

- [cited 2020 Dec 8];22:2416–26. Available from: <https://pubmed.ncbi.nlm.nih.gov/18326786/>.
63. Scheiman J, Lubner JM, Chavkin TA, MacDonald T, Tung A, Pham LD, et al. Meta-omics analysis of elite athletes identifies a performance-enhancing microbe that functions via lactate metabolism. *Nat Med* [Internet] Nature Publishing Group. 2019;25:1104–9. [cited 2021 Jan 13];. Available from: www.nature.com/naturemedicine1104.
64. Hamilton-Miller JMT, Shah S. Vancomycin susceptibility as an aid to the identification of lactobacilli. *Lett Appl Microbiol* [Internet]. Lett Appl Microbiol; 1998 [cited 2021 Jun 4];26:153–4. Available from: <https://pubmed.ncbi.nlm.nih.gov/9569701/>.
65. Park K-Y, Kim B, Hyun C-K. Lactobacillus rhamnosus GG improves glucose tolerance through alleviating ER stress and suppressing macrophage activation in db/db mice. *J Clin Biochem Nutr* [Internet]. The Society for Free Radical Research Japan; 2015 [cited 2019 Mar 7];56:240–6. Available from: <http://www.ncbi.nlm.nih.gov/pubmed/26060355>.
66. Kim SW, Park KY, Kim B, Kim E, Hyun CK. Lactobacillus rhamnosus GG improves insulin sensitivity and reduces adiposity in high-fat diet-fed mice through enhancement of adiponectin production. *Biochem Biophys Res Commun* [Internet]. Biochem Biophys Res Commun; 2013 [cited 2020 Dec 12];431:258–63. Available from: <https://pubmed.ncbi.nlm.nih.gov/23313485/>.
67. Li X, Wang E, Yin B, Fang D, Chen P, Wang G, et al. Effects of lactobacillus casei CCFM419 on insulin resistance and gut microbiota in type 2 diabetic mice. *Benef Microbes Wageningen Academic Publishers*. 2017;8:421–32.
68. Irimia JM, Meyer CM, Segvich DM, Surendran S, Depaoli-Roach AA, Morral N, et al. Lack of liver glycogen causes hepatic insulin resistance and steatosis in mice. *J Biol Chem* [Internet]. American Society for Biochemistry and Molecular Biology Inc.; 2017 [cited 2020 Dec 13];292:10455–64. Available from: <https://pubmed.ncbi.nlm.nih.gov/28483921/>.
69. Urasaki Y, Pizzorno G, Le TT. Chronic Uridine Administration Induces Fatty Liver and Pre-Diabetic Conditions in Mice. *PLoS One* [Internet]. Public Library of Science; 2016 [cited 2020 Dec 7];11. Available from: [/pmc/articles/PMC4720477/?report=abstract](http://pmc/articles/PMC4720477/?report=abstract).
70. Dudzinska W. Purine nucleotides and their metabolites in patients with type 1 and 2 diabetes mellitus. *J Biomed Sci Eng* [Internet]. Scientific Research Publishing, Inc.; 2014 [cited 2020 Dec 16];07:38–44. Available from: <http://dx.doi.org/10.4236/jbise.2014.71006>PublishedOnlineJanuary2014.
71. Cariou B, Chetiveaux M, Zar Y, Pouteau E, Disse E, Guyomarc'H-Delasalle B, et al. Fasting plasma chenodeoxycholic acid and cholic acid concentrations are inversely correlated with insulin sensitivity in adults. *Nutr Metab* [Internet]. BioMed Central Ltd.; 2011 [cited 2020 Dec 16];8. Available from: <https://pubmed.ncbi.nlm.nih.gov/21736725/>.
72. Zhao Q, Zhang A, Zong W, An N, Zhang H, Luan Y, et al. Exploring potential biomarkers and determining the metabolic mechanism of type 2 diabetes mellitus using liquid chromatography coupled to high-resolution mass spectrometry. *RSC Adv* [Internet]. Royal Society of Chemistry; 2017

- [cited 2020 Dec 7];7:44186–98. Available from: <https://pubs.rsc.org/en/content/articlehtml/2017/ra/c7ra05722a>.
73. Ma DWL, Arendt BM, Hillyer LM, Fung SK, McGilvray I, Guindi M, et al. Plasma phospholipids and fatty acid composition differ between liver biopsy-proven nonalcoholic fatty liver disease and healthy subjects. *Nutr Diabetes* 2016 67 [Internet]. Nature Publishing Group; 2016 [cited 2021 Jul 12];6:e220–e220. Available from: <https://www.nature.com/articles/nutd201627>.
 74. Li Z, Agellon LB, Allen TM, Umeda M, Jewell L, Mason A, et al. The ratio of phosphatidylcholine to phosphatidylethanolamine influences membrane integrity and steatohepatitis. *Cell Metab Cell Press*. 2006;3:321–31.
 75. Al-Sulaiti H, Diboun I, Agha MV, Mohamed FFS, Atkin S, Dömling AS, et al. Metabolic signature of obesity-associated insulin resistance and type 2 diabetes. *J Transl Med* [Internet]. BioMed Central; 2019 [cited 2021 Jul 12];17. Available from: [/pmc/articles/PMC6805293/](https://pubmed.ncbi.nlm.nih.gov/31805293/).
 76. M M, A K, H L, L B. The pattern of urinary acylcarnitines determined by electrospray mass spectrometry: a new tool in the diagnosis of diabetes mellitus. *Anal Bioanal Chem* [Internet]. Anal Bioanal Chem; 2003 [cited 2021 Aug 11];375:200–10. Available from: <https://pubmed.ncbi.nlm.nih.gov/12560963/>.
 77. Nowak C, Hetty S, Salihovic S, Castillejo-Lopez C, Ganna A, Cook NL, et al. Glucose challenge metabolomics implicates medium-chain acylcarnitines in insulin resistance. *Sci Reports* 2018 81 [Internet]. Nature Publishing Group; 2018 [cited 2021 Aug 11];8:1–10. Available from: <https://www.nature.com/articles/s41598-018-26701-0>.
 78. Zhang L. A Systematic Review of Metabolite Profiling in Diabetic Nephropathy. *J Endocrinol Diabetes Symbiosis Group*. 2015;2:01–11.
 79. Hernandez-Baixauli J, Quesada-Vázquez S, Mariné-Casadó R, Cardoso KG, Caimari A, Bas JM, Del, et al. Detection of Early Disease Risk Factors Associated with Metabolic Syndrome: A New Era with the NMR Metabolomics Assessment. *Nutr* 2020, Vol 12, Page 806 [Internet]. Multidisciplinary Digital Publishing Institute; 2020 [cited 2021 Jul 12];12:806. Available from: <https://www.mdpi.com/2072-6643/12/3/806/htm>.
 80. Hoerr V, Zbytnuik L, Leger C, Tam PPC, Kubes P, Vogel HJ. Gram-negative and Gram-Positive Bacterial Infections Give Rise to a Different Metabolic Response in a Mouse Model. *J Proteome Res* [Internet]. American Chemical Society; 2012 [cited 2021 Jul 24];11:3231–45. Available from: <https://pubs.acs.org/doi/full/10.1021/pr201274r>.
 81. Roager HM, Licht TR. Microbial tryptophan catabolites in health and disease [Internet]. *Nat. Commun.* Nature Publishing Group; 2018 [cited 2021 Jan 10]. Available from: <https://pubmed.ncbi.nlm.nih.gov/30120222/>.
 82. Hameed A, Mojsak P, Buczynska A, Suleria HAR, Kretowski A, Ciborowski M. Altered Metabolome of Lipids and Amino Acids Species: A Source of Early Signature Biomarkers of T2DM. *J Clin Med* [Internet]. MDPI AG; 2020 [cited 2021 Jan 10];9:2257. Available from: <https://www.mdpi.com/2077-0383/9/7/2257>.

83. Liu L, Zhao J, Zhang R, Wang X, Wang Y, Chen Y, et al. Serum untargeted metabolomics delineates the metabolic status in different subtypes of non-alcoholic fatty liver disease. J Pharm Biomed Anal Elsevier. 2021;200:114058.

84. Wang TJ, Ngo D, Psychogios N, Dejam A, Larson MG, Vasan RS, et al. 2-Amino adipic acid is a biomarker for diabetes risk. J Clin Invest [Internet]. J Clin Invest; 2013 [cited 2020 Dec 7];123:4309–17. Available from: <https://pubmed.ncbi.nlm.nih.gov/24091325/>.

85. Mehta HH, Xiao J, Ramirez R, Miller B, Kim SJ, Cohen P, et al. Metabolomic profile of diet-induced obesity mice in response to humanin and small humanin-like peptide 2 treatment. Springer New York LLC; 2019. Internet. ; [cited 2021 Jan 10];15. Available from: [/pmc/articles/PMC6554247/?report = abstract](https://pubmed.ncbi.nlm.nih.gov/32544247/).

Figures

Figure 1

Insulin resistant iNOS-/- mice display atypical gut microbiota with gram-positive bacteria dominance and altered serum metabolome. Gut microbiota analysis in WT and insulin resistant iNOS-/- mice. α-diversity indices in stool samples (A) Observed, (B) Shannon, (C) Simpson and (D) Chao1 index. (E) β-diversity

analysis via Principal Coordinate Analysis (PCA) plot based on Bray-Curtis distance. Each dot represents an animal, projected onto first (horizontal axis) and second (vertical axis) variables. (F) Differentially abundant microbiota at the phylum, family and genus level. Serum metabolomic analysis in chow fed WT and iNOS^{-/-} mice in ESI (+) mode (G) PCA score plot and (H) Volcano plot of differential metabolites ($p < 0.05$) between WT and iNOS^{-/-} mice. Red in volcano plot indicates significantly up-regulated metabolites, green indicates the down-regulated metabolites, and grey shows no significant difference. (I) Heat map of differential metabolites found by metabolomics analysis in chow fed WT and iNOS^{-/-} mice. Data are represented as mean $\pm$ SEM ($n \geq 6$). * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$ vs WT. See also Figure S1.

Figure 2

Vancomycin-induced modulation of gut microbiota rescue iNOS^{-/-} mice from systemic IR and dyslipidemia. Gut microbiota analysis in untreated and vancomycin treated iNOS^{-/-} mice. α -diversity indices in stool samples (A) Observed, (B) Shannon, (C) Simpson and (D) Chao1 index. (E) PCA plot based on Bray-Curtis distance. (F) Differentially abundant microbiota at the phylum, family and genus level. Systemic IR analysis in vancomycin treated and untreated WT and iNOS^{-/-} mice. (G) Intraperitoneal glucose tolerance test (GTT), (H) Area under the curve (AUC) calculated from IPGTT data, (I) Intraperitoneal insulin tolerance test (ITT), (J) AUC calculated from ITT (K) Intraperitoneal pyruvate tolerance test (PTT), (L) AUC calculated from PTT, (M) Fasting blood glucose levels and (N) Fasting

serum insulin levels. Indices of insulin sensitivity (O) HOMA-IR and (P) QUICKI. Serum lipids (Q) Total cholesterol (TC), (R) Triglycerides (TG), (S) Low density lipoprotein (LDL) and (T) Non-esterified free fatty acids (NEFA). Data are represented as mean $\pm$ SEM (n $\geq$ 6). *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001 between indicated groups. #p<0.05, ##p<0.01, ###p<0.001 and ####p<0.0001 vs iNOS^{-/-}. See also Figure S2, S3, S5 and S6.

Figure 3

Vancomycin-induced alterations in serum metabolites in iNOS^{-/-} mice. Serum metabolomic analysis in in untreated and vancomycin treated iNOS^{-/-} mice in ESI (+) mode. (A) PCA score plot and (B) Volcano plot of differential metabolites between iNOS^{-/-} mice with or without vancomycin treatment. Red in volcano plot indicates significantly up-regulated metabolites, green indicates the down-regulated metabolites, and grey shows no significant difference. Heat map of differential metabolites found by metabolomics analysis in iNOS^{-/-} mice with and without vancomycin treatment related to (C) Nucleic acids metabolism, (D) Vitamins, hormones and bile acids metabolism, (E) Carbohydrate metabolism, (F) Miscellaneous/ microbiota derived metabolites, (G) Amino acid metabolism, and (H) Lipid metabolism. Data are represented as mean $\pm$ SEM (n $\geq$ 6). *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001 vs iNOS^{-/-}. See also Figure S4 and S5.

Figure 4

Improvement in the disrupted lipid and glucose homeostasis in liver in iNOS^{-/-} mice following treatment with vancomycin. Hepatic lipid levels in WT and iNOS^{-/-} mice with and without vancomycin treatment (A) TC, (B) TG and (C) FFA. (D) Hepatic glycogen levels with or without insulin stimulation in WT and iNOS^{-/-} mice with and without vancomycin treatment. Hepatic mRNA expression analysis of genes involved in (E) Gluconeogenesis, (F) Lipid synthesis, (G) Lipid oxidation, (H) Lipid uptake and (I) Lipid efflux. Data are represented as mean $\pm$ SEM (n $\geq$ 6). *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001 between indicated groups. See also Figure S7.

Figure 5

Improvement in the disrupted lipid and glucose homeostasis in adipose tissue and intestine in iNOS^{-/-} mice following treatment with vancomycin. Adipose tissue mRNA expression analysis of genes involved in (A) Gluconeogenesis, (B) Glucose homeostasis, (C) Lipid synthesis, (D) Lipid oxidation and (E) Lipid uptake. Intestinal tissue mRNA expression analysis of genes involved in (F) Lipid synthesis, (G) Lipid uptake and (H) Lipid efflux. Data are represented as mean $\pm$ SEM ($n \geq 6$). * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$ between indicated groups. See also Figure S8 and S9.

Figure 6

Association of serum metabolites with metabolic profile of iNOS^{-/-} mice upon treatment with antibiotics. Heat map of correlation analysis between metabolic biomarkers and serum metabolites in WT and iNOS^{-/-} mice treated with antibiotics- vancomycin or Abx along with untreated controls based on Pearson's correlation coefficients related to (A) lipid metabolism and (B) amino acid metabolism. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$ represent significant correlations between metabolic biomarker and serum metabolite. Green color represents negative and red positive correlations. See also Figure S10.

Figure 7

Association of gut microbiota with metabolic parameters in iNOS^{-/-} mice following treatment with antibiotics. Heat map of Pearson's correlation coefficients between changes in different metabolic parameters and microbial taxa at (A) Phylum and (B) Genus level caused by gut microbiota modulation by vancomycin and Abx in WT and iNOS^{-/-} mice. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$ represent

significant correlations between metabolic biomarker and bacterial taxa. Blue color represents negative and red positive correlations. See also Figure S11.

Figure 8

Gut microbiota association with serum metabolites in iNOS^{-/-} mice upon antibiotics treatment. Heat map of correlation analysis between gut microbiota and serum metabolites related to (A) Lipid metabolism, (B) Amino acids metabolism, (C) Miscellaneous/ microbiota derived metabolites and (D) Cofactors, hormones, bile acids, carbohydrate and nucleic acid metabolites in WT and iNOS^{-/-} mice treated with antibiotics- vancomycin or Abx along with untreated controls based on Pearson's correlation coefficients.

*p<0.05, **p<0.01, ***p<0.001, ****p<0.0001 represent significant correlations between bacterial taxa and serum metabolite. Blue color represents negative and orange positive correlations.

Figure 9

Vancomycin-induced depletion of gram-positive gut bacteria and modulation of associated metabolites rescues the IR and dyslipidemia observed in iNOS^{-/-} mice. Red up or down arrows or dash represent increase, decrease or no change in iNOS^{-/-} mice in comparison to WT. Violet up or down arrows or dash represent increase, decrease or no change in vancomycin treated iNOS^{-/-} mice respectively in comparison to untreated iNOS^{-/-}.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- [MicrobiomeMSSupplementaryinformation.docx](#)