

Gut microbiota modulation with norfloxacin and ampicillin enhances glucose tolerance in mice

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ABSTRACT Recent data suggest that the gut microbiota plays a significant role in fat accumulation. However, it is not clear whether gut microbiota is involved in the pathophysiology of type 2 diabetes. To assess this issue, we modulated gut microbiota *via* antibiotics administration in two different mouse models with insulin resistance. Results from dose-determination studies showed that a combination of norfloxacin and ampicillin, at a dose of 1g/L, maximally suppressed the numbers of cecal aerobic and anaerobic bacteria in *ob/ob* mice. After a 2-wk intervention with the antibiotic combination, both *ob/ob* and diet-induced obese and insulin-resistant mice showed a significant improvement in fasting glycemia and oral glucose tolerance. The improved glycemic control was independent of food intake or adiposity because pair-fed *ob/ob* mice were as glucose intolerant as the control *ob/ob* mice. Reduced liver triglycerides and increased liver glycogen correlated with improved glucose tolerance in the treated mice. Concomitant reduction of plasma lipopolysaccharides and increase of adiponectin further supported the antidiabetic effects of the antibiotic treatment in *ob/ob* mice. In summary, modulation of gut microbiota ameliorated glucose tolerance of mice by altering the expression of hepatic and intestinal genes involved in inflammation and metabolism, and by changing the hormonal, inflammatory, and metabolic status of the host.—Membrez, M., Blancher, F., Jaquet, M., Bibiloni, R., Cani, P. D., Burcelin, R. G., Corthesy, I., Macé, K., Chou, C. J. Gut microbiota modulation with norfloxacin and ampicillin enhances glucose tolerance in mice. *FASEB J.* 22, 000–000 (2008)

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THE HUMAN DIGESTIVE TRACT contains a significant amount of microorganisms, bacteria being the most dominant. The composition and functionality of the microbiota in each section of digestive tract has been an area of active research for many years. Several studies, based on the variations of 16S rRNA gene sequences derived from clone libraries, have made

comprehensive surveys on the profile of the microbiota in different parts of the digestive tract. These studies, utilizing culture-independent methods, have revealed unique microbiota profiles in the periodontal pocket (1), the distal esophagus (2), the stomach (3), and the colon (4). Especially in the gut, microbial communities have been shown to play a critical role in maturation (5, 6), development of innate immunity (7), production of essential vitamins (8), and the biotransformation of endogenous and exogenous compounds (9).

Recently, gut microbiota has been shown to affect fat storage and energy harvesting, which suggests that intestinal microorganisms may play a direct role in the development of obesity. Bäckhed *et al.* (10) demonstrated that germ-free mice had defects in storing fat in white adipose tissue, and that this was due to higher amounts of circulating lipoprotein lipase inhibitor Fiaf produced by the gut. In support of this, germ-free Fiaf knockout mice gained more weight than their germ-free wild-type littermates when all mice were fed a Western diet, confirming the protective role of Fiaf on body fat accumulation (11). Also, the composition of cecal microbiota in obese and insulin-resistant *ob/ob* mice differed from lean controls, with a higher ratio of Firmicutes/Bacteroidetes found in the *ob/ob* mice (12). Metagenomic analyses revealed that the cecal microbiota in the *ob/ob* mice was more capable of producing short-chain fatty acids by fermenting dietary fibers. The increased energy harvesting from dietary fibers may contribute partly to the excessive weight gain of the *ob/ob* mice (13). In humans, the fecal Firmicutes/Bacteroidetes ratio also decreased after obese individuals consumed different low-calorie weight loss diets, providing an association between gut microbiota profile and weight management (14). Thus, available evidence strongly suggests that gut microbiota could be a contributing factor to obesity.

In both *in vitro* and in animal models an increase in proinflammatory cytokines, such as TNF α , causes tissue insulin resistance (15, 16). When this systemic inflamma-

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tion is controlled by pharmaceutical interventions, the whole body insulin sensitivity is also improved in both mice and humans (17, 18). However, the source of this low-grade inflammation has not been clearly defined. Cani *et al.* (19) showed that subcutaneous infusion of a low dose of lipopolysaccharide (LPS), a component of gram-negative bacteria cell wall, leads to excessive weight gain and insulin resistance in mice. In the gut, pattern-recognition Toll-like receptors (TLRs) are important for host defense against bacterial infection and the development of innate immunity (20, 21), and specifically, TLR4 is responsible for recognizing bacterial LPS. On activation of TLR4, NF- κ B is translocated to the nucleus where it turns on the expression of inflammatory genes such as TNF- α and COX2 (22). Because of a large number of LPS containing gram-negative bacteria residing in the gut, chronic stimulation of intestinal TLR4 may exacerbate the low-grade inflammation associated with obesity and insulin resistance. To test this hypothesis, we eliminated most members of the gut microbiota in *ob/ob* and diet-induced obese and insulin resistant (DIO) mice using broad-range antibiotics. We postulated that insulin resistance can be reversed by removing or reducing the numbers of gut microbiota in the two animal models. Our data demonstrate that gut microbiota modulation improves whole body glucose tolerance and reduces hepatic steatosis, suggesting that controlling gut microbiota could be a novel therapeutic strategy in treating or managing type 2 diabetes.

MATERIALS AND METHODS

Dose-determination studies for selecting antibiotics for gut microbiota modulation

In vitro antibiotic susceptibility tests

Antimicrobial susceptibility of selected intestinal bacterial groups (lactobacilli, bifidobacteria, bacteroides, and enterobacteria) was determined by the strip test, according to the manufacturer's instructions (AB Biodisk, Solna, Sweden). Briefly, feces of *ob/ob* mice were diluted in Ringer/cystein solution and plated on specific media. One colony of each selected bacterial group was used to prepare the bacterial inocula for the susceptibility tests. The bacterial suspensions were swabbed onto specific media: *Brucella* agar for bacteroides, Mueller Hinton agar for enterobacteria, and MRS/cystein agar for lactobacilli and bifidobacteria. Antibiotic strips containing serial dilutions of antibiotics were applied to the agar surface, and plates were incubated at 37°C for 24 or 48 h in aerobic and anaerobic conditions, respectively. The preformed concentration gradient of antibiotic (amoxicillin, ampicillin, or norfloxacin) is immediately transferred to the agar medium and forms a symmetrical inhibition ellipse centered along the strip. The minimum inhibitory concentration is read directly from the scale at the point where the edge of inhibition ellipse intersects the strip. Bacterial groups were considered sensitive (S) or resistant (R) to the antibiotic according to a scale provided by the manufacturer.

In vivo dose-determination study in *ob/ob* mice

Eight- to 10-wk-old male *ob/ob* mice (Charles River Laboratories, L'Arbresle, France) were treated with a combination of

norfloxacin and ampicillin at 0, 0.2, 1, or 2 g/L for each antibiotic in their drinking water for 14 days ($n=6$ /group). All of the mice were housed individually and provided with a γ -irradiated sterile chow diet (diet 3434, Kliba Nafag, Basel, Switzerland) and sterile water *ad libitum*. At the end of the treatment, mice were sacrificed at 9 AM without food deprivation. Blood samples were collected for biochemical analyses. Liver and jejunum were collected for gene expression analyses. Liver total triglycerides and glycogen contents were also determined, while cecal content was exclusively used for the assessment of total bacteria. Body weight and food and water intake were monitored daily during the antibiotic treatment period. Only a given dose of norfloxacin and ampicillin with the lowest cecal bacteria count and minimal effect on food intake and body weight was selected for the future efficacy study.

Culture-based microbial analysis of cecal contents

Total aerobic and anaerobic bacteria were enumerated in selective media and incubation conditions according to Schumann *et al.* (23). In brief, cecal samples were diluted in Ringer medium, and total aerobic and anaerobic bacteria were investigated by plating onto nonselective media: TSS medium (Biomerieux, Lyon, France) for 24 to 48 h at 37°C in aerobic and anaerobic conditions. Bacterial numbers were expressed as colony forming units (CFU)/mg cecal content.

Main study: efficacy of gut microbiota modulation on glucose tolerance

Ob/ob mice: animals and diet

Thirty-six 8- to 10-wk-old male *ob/ob* mice (Charles River Laboratories) were fed a sterile chow diet (diet 3434, Kliba Nafag) and housed individually. After 2 wk of habituation, mice were randomized based on their body weight and blood glucose concentrations into 3 groups ($n=12$ /group): 1) the control group, with free access to sterile food; 2) the antibiotic-treated group, given norfloxacin and ampicillin in drinking water (1 g/L each), with free access to sterile food and water; and 3) the pair-fed group, consuming the same amount of food as the antibiotic-treated group. Throughout the 17-day treatment period, body weight, water intake, and food intake were recorded daily. Pair-feeding was accomplished by measuring the 24-h food intake of each antibiotic-treated mouse throughout the experimental period. The following day, each pair-fed mouse received the same amount of food as its matched mouse in the antibiotic-treated group consumed. The food was divided into two equal portions, which were provided at 8 AM and 5 PM. Oral glucose tolerance tests (OGTTs) were conducted in overnight-fasted (15 h) mice on day 13. On day 17, overnight-fasted mice were sacrificed for tissue sample collection. Blood glucose concentrations were measured before anesthesia by tail incision using an Ascensia Elite XL glucometer (Bayer AG, Zurich, Switzerland). Epididymal and retroperitoneal adipose tissues, liver, pancreas, gastrointestinal (GI) tract from stomach to anus, and cecal content were collected from animals immediately after exsanguination *via* cardiac puncture, then flash-frozen in liquid nitrogen. One lobe of the liver and one part of the jejunum were preserved in buffered formaldehyde and embedded in paraffin. Five-micrometer sections were cut and stained with hematoxylin and eosin. All procedures were approved by the Office Vétérinaire Cantonal Vaudois (Lausanne, Switzerland).

Diet-induced obese and insulin-resistant mice: animal and diet

Male C57BL/6J mice, 6–7 wk old, were ordered from Charles River Laboratories. All mice were housed individually with access

to a standard rodent chow and water *ad libitum*. After a 2-wk habituation, all mice were switched to a high-fat diet (D12492 with 60% calories from fat; Research Diets, New Brunswick, NJ, USA) sterilized with γ -irradiation (25 kGy) for the rest of study. After 10 wk of the high-fat diet, mice were randomized to two groups ($n=12$ /group). Norfloxacin and ampicillin (1 g/L each) were added to the drinking water for 2 wk. Body weight, food intake, and water intake were monitored daily. Body composition of each animal was measured in a conscious state on day 12 using EchoMRI 3-in-1 (Echo Medical Systems, Houston, TX, USA). At the end of treatment, all mice were fasted for 6 h before the OGTTs.

Quantification of cecal enterobacteria

For quantitative and qualitative determination of *Enterobacteriaceae*, cecal content was diluted in 10 ml Ringer medium, shaken, and centrifuged at 10,000 rpm for 10 min. The pellet was resuspended in 1 ml of Ringer medium, and the diluted bacterial cells were spread onto Drigalski (Bio-Rad, Hercules, CA, USA) and EMB plates (Merck KGaA, Darmstadt, Germany) as described by Levine (24). Results were reported as CFU/mg of cecal content. In parallel, 100 μ l of washed cell suspension was inoculated into E.E. broth (Oxoid LTD, Basingstoke, England) for 24 h at 37°C, as described previously (25). Enterobacteria were enumerated by plating onto EMB agar, and the macroscopic characteristics of all of the colonies were investigated using API 32E tests (Biomerieux) as described by Murray *et al.* (26).

OGTTs

An OGTT was performed at the end of the treatment period. After measuring fasting glucose concentration in blood taken by tail incision, using an Ascensia Elite XL glucometer (Bayer AG), the animals were given 1.5 g/kg body weight of glucose by gavage at time 0. Blood glucose was measured after 15, 30, 60, and 120 min. Blood was also collected in EDTA-coated tubes for insulin analyses at 0, 15, and 60 min.

Liver glycogen measurements

Flash-frozen liver (50 to 100 mg) was incubated in 2 ml 30% KOH for 15–30 min in a boiling water bath. After homogenization, glycogen was precipitated with 3 ml 96% ethanol followed by centrifugation at 5000 rpm for 10 min. Pellets were resuspended in 1 ml distilled water for the amyloglucosidase digestion. Two-hundred-microliter samples were incubated in 1.8 ml of lyophilized amyloglucosidase dissolved in acetate buffer (0.2 M, pH 4.8 at a final concentration of 10 U/ml) for 2 h at 40°C. The resulting glucose solution was then phosphorylated and oxidized by hexokinase and glucose-6-phosphate dehydrogenase, respectively (1 U/ μ l each) in TEA buffer (0.3 M triethanolamine; 4 mM MgSO₄, pH 7.6; 1 mM NADP; 0.75 mM ATP). After 5–10 min, the absorbance at 340 nm was measured, and the glucose concentration was determined using a standard glucose dilution curve.

Determination of liver triglycerides

The lipids in 200 mg frozen liver were extracted according to Folch *et al.* (27). Triglycerides were first hydrolyzed in a basic solution (0.5 N KOH in ethanol) and then measured using a commercial enzymatic triglyceride analysis kit (PAP 150, BioMérieux, Marcy l'Etoile, France), following the manufacturer's instructions.

Measurements of plasma parameters

Plasma triglyceride (Roche Diagnostics, Basel, Switzerland), free fatty acid (Wako, Neuss, Germany), insulin (IBL, Hamburg, Germany), cholesterol (Roche Diagnostics), and adiponectin (Linco, Labodia, Switzerland), levels were measured using commercial kits. Plasma alanine transaminase activity was measured using a commercial kit following manufacturer's instructions (BioMérieux). Plasma lipopolysaccharide concentrations were determined using a kit based on *Limulus* amoebocyte extract (LAL kit, Cambrex BioScience, Walkersville, MD, USA).

mRNA expression analysis

Total RNAs were prepared from 50 to 100 mg liver or jejunum using the Nucleospin RNAII kit (Macherey Nagel, Duren, Germany) according to the protocol provided by the manufacturer. Reverse transcription was performed on 1.5 μ g total RNA using the first-strand cDNA synthesis kit for real-time PCR (AMV, Roche Biomedical, Basel, Switzerland) with oligo d(T)₁₅ as primer. Real-time reverse transcription-PCR analyses were performed in a fluorescent temperature cycler (GeneAmp[®] PCR 5700 Sequence Detection System; Applied Biosystems, Foster City, CA, USA). The following primer sequences were used for mouse genes: PEPCK: forward, 5'-CCACAGCTGCTGCAGAACAC-3'; reverse, 5'-GGGTCCGATGGCAAAGG-3'. G6P: forward, 5'-GAGGAAAGAAAAAGCCAAACGTATG-3'; reverse, 5'-CCCAGAATCCCAACCACAAG-3'. FAS: forward, 5'-CCCCCTCAAGTGCACAGTGT-3'; reverse, 5'-AGTGT-TCCCCGTGAGCCATGTA-3'. ACC1: forward, 5'-TGTTGAG-ACGCTGGTTTGTAGAA-3'; reverse, 5'-GGTCCTTATTATTGTCCCAGACGTA-3'. SGLT-1: forward, 5'-GCGTCATCACATCTTGATCA-3'; reverse, 5'-ACAGGTGCACGTCCTGGAATG-3'. TLR4: forward, 5'-CCAGATTTTTCCCCAGGAAGT-3'; reverse, 5'-TCTCCACAGCCACCAGATCT-3'. TNF- α : forward, 5'-ACCGTCAGCCGATTTGCTAT-3'; reverse, 5'-TTGACGGCAGAGAGGAGGTT-3'. RPL-p0: forward, 5'-AAAGGAAGAGTCCGAGGAATCAG-3'; reverse, 5'-TGGCGGGATTAGTCGAAGAG-3'. Values were normalized to ribosomal large protein-P0 (RPL-p0) expression. The effects of treatments on gene expression were evaluated by calculating the relative expression level as 2^x, where x = mean Ct genes of interest – mean Ct RPL-p0, using the raw cycle-threshold (Ct) values.

Statistical analysis

Statistical analyses were performed using the software R2.3.1 (Foundation for Statistical Computing, Vienna, Austria). Because of the presence of some outliers and the different distribution of the data between groups, nonparametric tests were used. Kruskal-Wallis test followed by Wilcoxon tests for paired comparisons were performed. For the results of gene expression data, analysis of variance was applied. When the test was significant, Fisher's least significant difference tests were applied for paired comparisons. Tests were considered significant at values of $P < 0.05$.

RESULTS

Combination and dose determination of antibiotics for microbiota modulation

A combination of norfloxacin and ampicillin was selected for gut microbiota modulation based on results obtained from *in vitro* antibiotic susceptibility tests

TABLE 1. Results of in vitro antibiotics screening

Bacteria	Amoxicillin	Ampicillin	Norfloxacin
<i>Enterobacteriaceae</i>	R(0/19)	S/R(13/19)	S(19/19)
<i>Bacteroides spp.</i>	S/R(14/16)	S(16/16)	R(0/16)
<i>Lactobacillus spp.</i>	S(33/33)	S(33/33)	S/R(25/33)
<i>Bifidobacterium spp.</i>	S(3/3)	S(3/3)	R(0/3)

Results are presented as sensitive (S) or resistant (R) to antibiotic treatment. Degree of effectiveness is presented as (sensitive/total). Different bacteria were isolated from fecal samples of *ob/ob* mice by selective culture media.

(Table 1). Norfloxacin was the only antibiotic capable of killing fecal enterobacteria, while ampicillin was the most efficient antibiotic in eliminating bacteroides. For lactobacilli and bifidobacteria, both ampicillin and amoxicillin were equally effective.

To determine the most efficient dose of the antibiotic combination for modulating gut microbiota, a dose-response study using different concentrations (0, 0.2, 1 and 2 g/L) of the norfloxacin and ampicillin combination in *ob/ob* mice was performed. At the end of the treatment period, cecal samples were collected and cultured in aerobic and anaerobic conditions. As illustrated in Fig. 1A, B, treatments with norfloxacin and ampicillin dose-dependently suppressed the numbers of cecal aerobic and anaerobic bacteria in *ob/ob* mice, and the highest level of suppression was achieved with 1 g/L dose. Body weight of mice was not affected by the treatment regardless of the treatment concentration, but food intake was significantly affected by the highest doses (2 g/L) of antibiotic treatment (Table 2). In addition, metabolic parameters were included as supporting criteria for the dose determination. As shown in Table 2, blood glucose and liver triglycerides were reduced, and liver glycogen was increased after the 2-wk antibiotic treatment, and only the 1 g/L dose improved all three metabolic parameters. On the basis of results of cecal bacteria culturing and metabolic parameters, the combination of norfloxacin and ampicillin at 1 g/L dose was selected for gut microbiota modulation. With this dose, although not statistically significantly different, a 20% reduction of cumulative food intake did occur (98.0 ± 9.6 g in control *vs.* 78.5 ± 8.3 g in 1g/L group) during the 2-wk antibiotic treatment period, which could affect the insulin-resistant phenotype of *ob/ob* mice.

Gut microbiota modulation improved oral glucose tolerance of *ob/ob* mice

On the basis of the notable reduction in food intake during the dose-response study, we designed a pair-feeding study to control for potential effects caused by the antibiotic treatment. Figure 2A, B illustrates the body weight and food intake of the *ob/ob* mice. The treated mice consumed less food during the first 3 days but quickly returned to the same level of food intake as the control group on day 4. After 13 days of treatment, the whole body glucose tolerance of the mice was tested after an overnight fast. Illustrated in Fig. 3A, B, *ob/ob*

mice received the antibiotic treatment were more glucose tolerant, and corresponding plasma insulin concentrations during the OGTT were also significantly lower in the treated than in control or pair-fed mice (Fig. 3C, D). In addition, fasting blood glucose (Fig. 3E) and insulin (Fig. 3F) concentrations before the OGTT were also much reduced ($P < 0.01$) in the treated mice. The improved oral glucose tolerance was unlikely due to a defect in glucose absorption, as the expression of sodium glucose cotransporter 1 (SGLT-1) and glucose transporter 2 (GLUT2) in jejunum (data not shown) was

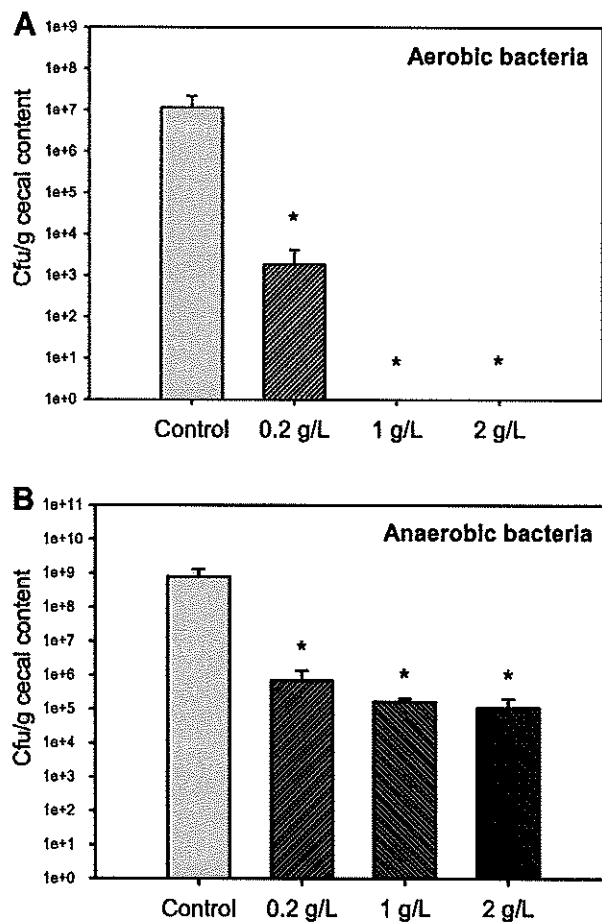


Figure 1. Cecal samples were cultured in aerobic (A) and anaerobic (B) environments as described in Materials and Methods. Aerobic bacteria counts went below the detection limit in the groups treated with the combination of norfloxacin and ampicillin at 1 and 2 g/L. Data are expressed as median \pm SE; $n = 6$. * $P < 0.05$ *vs.* control.

TABLE 2. Dose-dependent effects of the antibiotic treatment in *ob/ob* mice observed in a nonfasting state

Dose (g/L)	Body weight (g)	Cumulative food intake (g)	Total antibiotic intake (mg)	Blood glucose (mg/dl)	Liver glycogen (mg/g liver)	Liver triglycerides (μ g/g liver)
0	47.9 \pm 1.6	98.0 \pm 9.6	0.0 \pm 0.0	158.5 \pm 43.8	37.5 \pm 6.1	162.8 \pm 7.9
0.2	46.3 \pm 1.0	80.6 \pm 6.3	17.1 \pm 1.3	133.0 \pm 17.1	49.9 \pm 2.7*	134.3 \pm 6.1*
1.0	47.2 \pm 2.0	78.5 \pm 8.3	64.6 \pm 1.4	92.5 \pm 4.6*	58.9 \pm 4.6*	117.1 \pm 0.8*
2.0	45.9 \pm 2.5	75.7 \pm 3.8*	136.0 \pm 3.6	109.0 \pm 15.5	47.7 \pm 2.8	131.2 \pm 13*

Norfloxacin and ampicillin were added to the drinking water at the concentrations indicated. Body weight, blood glucose, liver glycogen, and liver triglycerides were determined in a nonfasting state. Data are expressed as median \pm SE; $N = 6$. * $P < 0.05$ vs. control.

not affected by gut microbiota modulation. In addition, plasma adiponectin concentrations were slightly higher in treated *ob/ob* mice (Table 3), which also positively contributes to the improved oral glucose tolerance observed in the treated mice. In the present study, neither body weight nor food intake could be used to explain the improved oral glucose tolerance in the antibiotic treated *ob/ob* mice. The body weight of mice on the day of OGTT was similar in all groups (48.8 \pm 0.6 g in control vs.

47.6 \pm 0.4 g in antibiotic treated vs. 46.0 \pm 0.7 g in pair-fed), and food intake among all mice was nearly identical since day 4. Our data clearly indicate that modulating the gut microbiota by the antibiotic treatment improved the oral glucose tolerance in *ob/ob* mice.

Dyslipidemia is commonly associated with insulin resistance. In the present study, the abnormal concentrations of plasma triglycerides, free fatty acids, and cholesterol were not affected by the antibiotic treatment (Table 3).

Liver glycogen is often lower in patients with type 2 diabetes, and restoration of liver glycogen storage is associated with increased hepatic insulin sensitivity (29). Indeed, in the nonfasting state, the control *ob/ob* mice had significantly less liver glycogen than the antibiotic-treated mice (Table 2). With the dose of 1 g/L, hepatic expression of glucose-6-phosphatase (G6P) showed a 60% suppression ($P = 0.037$) vs. control mice (data not shown). An overnight fast in *ob/ob* mice depleted liver glycogen in the control, antibiotic treated and pair-fed *ob/ob* mice (data not shown), and the expression of gluconeogenic genes such as phosphoenol pyruvate carboxykinase (PEPCK) and G6P was comparable in all 3 groups (data not shown). Our data suggest that a restoration of hepatic glycogen storage in the fed state contributed to the improvement of nonfasting blood glucose concentrations observed in the *ob/ob* mice with modulated gut microbiota.

Gut microbiota modulation improved lipid metabolism in the liver

Hepatic triglyceride accumulation is commonly found in mouse models with liver insulin resistance (28), and *ob/ob* mice have elevated hepatic microvesicular and macrovesicular steatosis (Fig. 4A). When compared with the control group, gut microbiota modulation by the antibiotic treatment alleviated the excessive fat storage in the liver of overnight-fasted *ob/ob* mice (Fig. 4A, B). Similar, but to a lesser extent, levels of liver triglycerides in treated mice were lower than in the pair-fed mice. In the treated mice, the expression of Cyp4A10, a marker for microsomal fatty acid oxidation, was significantly augmented (Fig. 4C), and both control and pair-fed mice had a similar and lowered level of Cyp4A10 expression. The lipogenic gene acetyl CoA carboxylase 1 (ACC1) was only increased in the pair-fed mice (Fig. 4D). As shown in Table 2, the same dose of

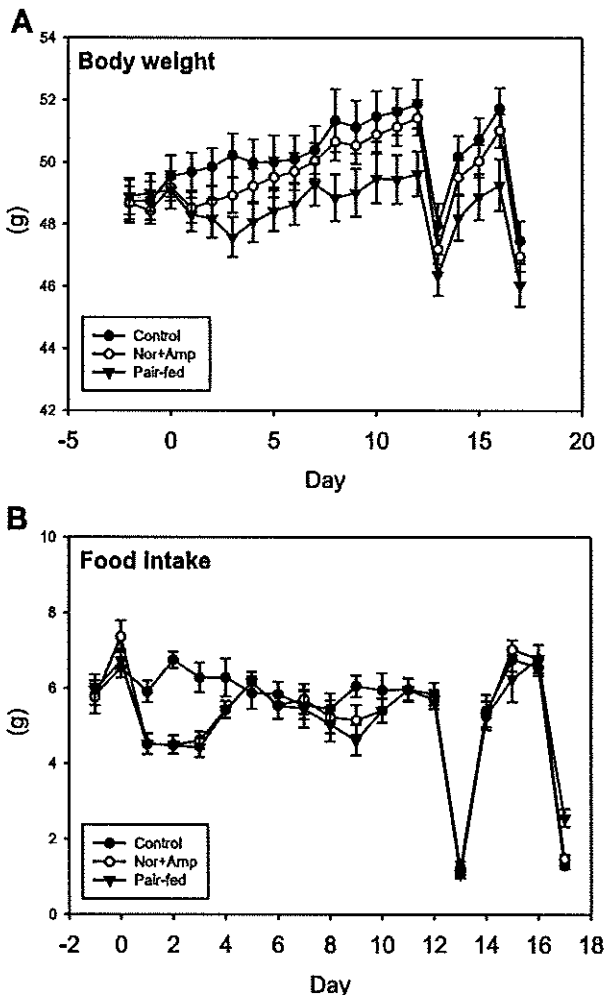


Figure 2. Records of daily body weight (A) and food intake (B) during the period of antibiotic treatment. Notable reductions in body weight and food intake on days 13 and 17 are due to overnight fasting. Data are expressed as median \pm SE; $n = 12$

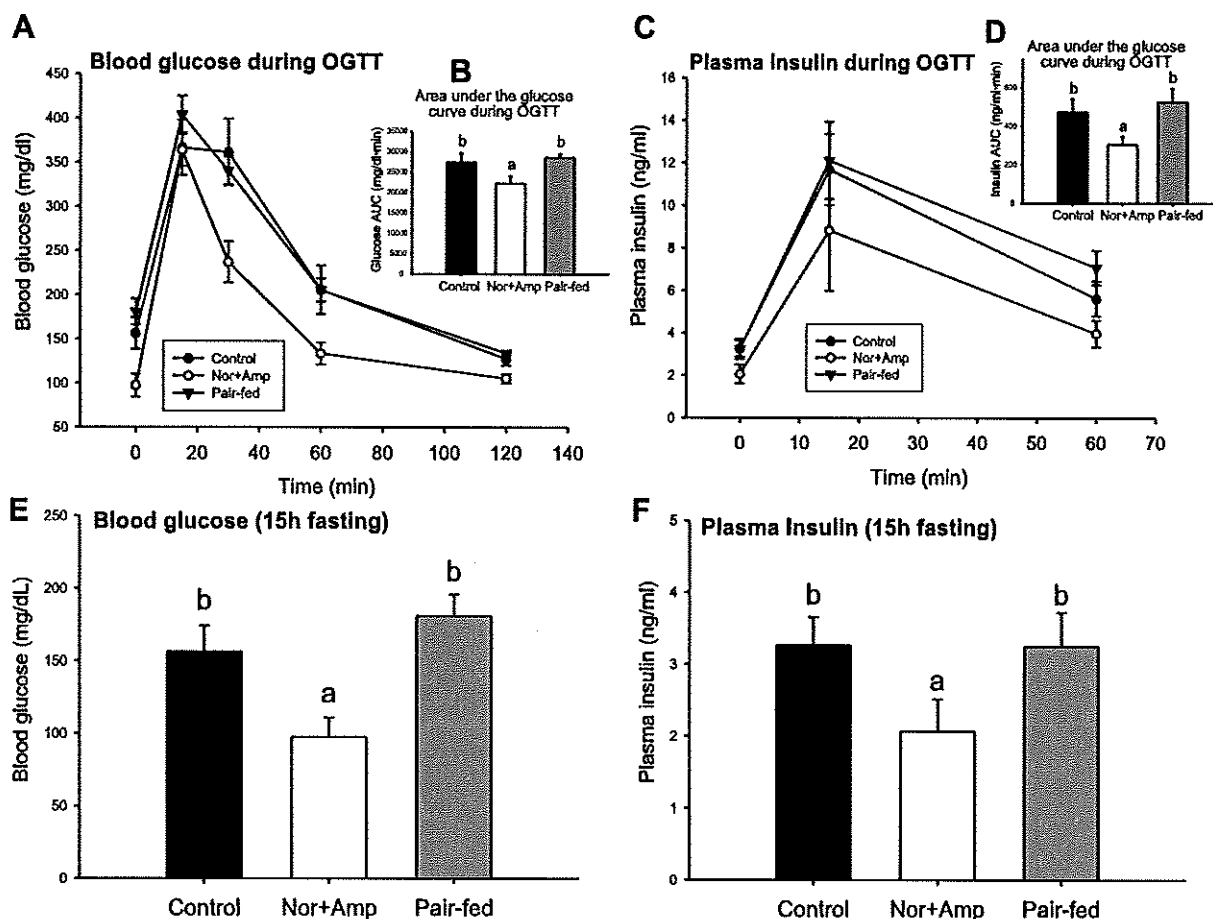


Figure 3. Results of blood glucose (A) and the area under glucose curves (B) during OGTTs in *ob/ob* mice. All mice were fasted overnight (15 h) before the OGTT. Plasma insulin concentrations at OGTT (C), the area under the insulin curves during OGTT (D), and baseline blood glucose (E) and insulin concentrations (F) are illustrated. Data are expressed as median \pm SE; $n = 12$. Different superior letters represent statistical significance ($P < 0.05$) using Kruskal-Wallis test followed by Wilcoxon tests for paired comparison.

antibiotic combination also reduced hepatic steatosis in a nonfasting state. In this condition, the expression of peroxisome proliferator-activated receptor gamma (PPAR γ) and lipogenic genes ACC1 and fatty acid synthase (FAS) showed a 1.8-fold ($P = 0.014$), 4.7-fold ($P = 0.027$), and 7.6-fold ($P = 0.054$) reduction in the treated mice. Increased fatty acid oxidation in the fasting state, and reduced lipogenesis in the fed state, can explain the reduced hepatic steatosis in the treated *ob/ob* mice. The reduced liver fat is also associated with improved liver function. The activity of plasma ALT activity, a marker of liver function, had a

40% and 28% reduction in the treated mice when comparing with the control and pair-fed mice, respectively (Fig. 4E).

Gut microbiota modulation suppressed plasma endotoxemia and intestinal inflammatory responses

Endotoxins such as LPS from gram-negative bacteria in the gut has been shown to play an important role in the development of insulin resistance (19) and nonalcoholic fatty liver disease (30). To examine the contribu-

TABLE 3. Plasma parameters in *ob/ob* mice treated with antibiotics

Plasma parameter	Control	Antibiotic treated	Pair-fed
Triglycerides (mmol/L)	0.72 \pm 0.04	0.88 \pm 0.10	0.81 \pm 0.06
Free fatty acids (mmol/L)	0.83 \pm 0.06	0.82 \pm 0.08	0.74 \pm 0.08
Cholesterol (mg/dl)	148.7 \pm 9.0	132.1 \pm 4.0	143.0 \pm 14.8
Adiponectin (μ g/ml)	15.8 \pm 0.57 ^b	18.2 \pm 0.78 ^a	15.9 \pm 1.21 ^{a,b}

Blood and plasma samples were collected after 15 h of fasting. Data are expressed as median \pm SE; $N = 12$. Different superior letters indicate statistical significance ($P < 0.05$) using Kruskal-Wallis test followed by Wilcoxon tests for paired comparison.

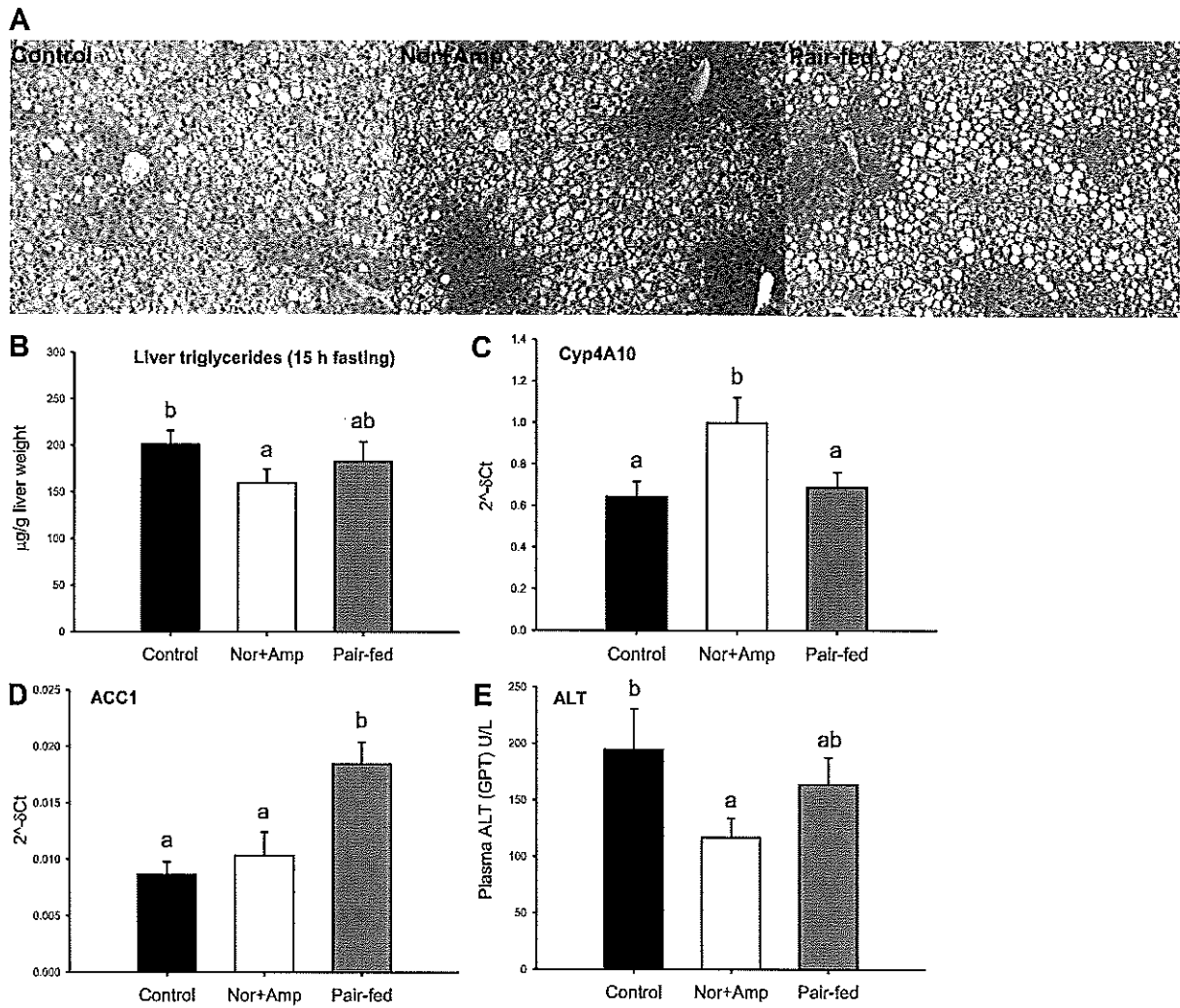


Figure 4. Histology of liver by hematoxylin-and-eosin staining after the antibiotic treatments, $\times 100$ (A), and the quantification of liver triglyceride levels (B) are shown. Data are expressed as median \pm SE; $n = 12$. Hepatic Cyp4A10 (C) and ACC1 mRNA levels (D) and plasma ALT activity (E) are also illustrated. Data are expressed as means \pm SE; $n = 10$. Different letters represent statistical significance ($P < 0.05$) using Kruskal-Wallis test followed by Wilcoxon tests for paired comparison.

tion of endogenous LPS on the regulation of whole body insulin sensitivity, we measured the plasma endotoxin concentrations in the control, antibiotic treated,

and pair-fed *ob/ob* mice. Figure 5A illustrates that plasma LPS levels were significantly lower in the antibiotic treated as compared to the control and pair-fed

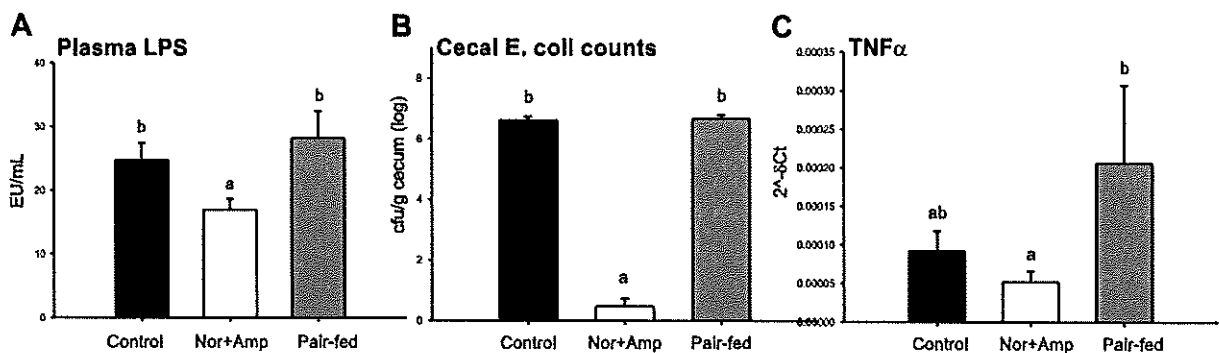


Figure 5. Plasma LPS concentrations (A), cecal *E. coli* counts (B) and jejunal TNF- α expression (C). Data are expressed as median \pm SE; $n = 12$. Different letters represent statistical significance ($P < 0.05$) using Kruskal-Wallis test followed by Wilcoxon tests for paired comparison.

mice. Similar to plasma LPS concentrations, the number of cecal *E. coli* showed a significant 5-log reduction in the treated mice (Fig. 5B), and total cecal bacterial DNA concentration was reduced below the level of detection (data not shown). The expression of jejunal TNF- α level was lower in the treated than in the pair-fed mice (Fig. 5C), suggesting that modulating gut microbiota by norfloxacin and ampicillin diminished the inflammatory status in the intestine of *ob/ob* mice.

Gut microbiota modulation did not affect adiposity in *ob/ob* mice

As shown in Fig. 2, the body weight of pair-fed mice was not identical to the antibiotic-treated mice, but at sacrifice, all mice weighed similarly (Table 4). However, the weight of total fat pad, which is a sum of epididymal, mesenteric, and retroperitoneal fat pads, was higher in the control than in the treated and pair-fed *ob/ob* mice (Table 4). These data indicate that the amount of food intake, rather than modulation of gut microbiota by antibiotics, determined the adiposity of the *ob/ob* mice. In addition, the weight of gut was much higher in the treated than in the control and pair-fed mice (Table 4), and this increased weight in the gut was mainly due to the enlargement of cecum, a feature also found in germ-free rodents.

Our data strongly support the direct effect of gut microbiota modulation, rather than possible influences of fat mass or changes in circulating lipids on the improvement of whole body glucose regulation in *ob/ob* mice.

Gut microbiota modulation improved glucose tolerance in DIO mice

To confirm the effect of gut microbiota modulation on improving glycemic control, we treated DIO mice with the same antibiotic combination, as described previously. After 10 wk of consuming a high-fat diet, all mice developed hyperglycemia (Fig. 6A). Over the 2 wk of antibiotic treatment, cumulative food intake was similar between the control group (20.6 ± 0.9 g) and the antibiotic-treated DIO mice (18.7 ± 0.7 g). Similar to the results observed in the previous experiment with *ob/ob* mice, blood glucose concentrations were markedly reduced after a 2-wk treatment with norfloxacin and ampicillin (Fig. 6A). Oral glucose tolerance was also significantly improved in the treated DIO mice, indi-

cating the robust effect of the gut microbiota modulation (Fig. 6B, C). The basal insulin concentration (1.3 ± 0.15 ng/ml in control *vs.* 1.62 ± 0.22 ng/ml in the treated DIO mice) and insulin response in the first 60 min during the OGTT (101.77 ± 16.18 ng/ml \cdot min in control *vs.* 138.65 ± 26.71 ng/ml \cdot min in the treated DIO mice) were not different between the control and treated DIO mice. The improved glycemic control was independent of body weight (37.4 ± 1.4 g in control *vs.* 37.3 ± 1.3 g in treated mice), body fat mass measured by NMR (14.7 ± 1.1 g in control *vs.* 14.2 ± 1.1 g in treated mice), or plasma adiponectin concentration (27.2 ± 1.4 μ g/ml in control *vs.* 25.3 ± 1.4 μ g/ml in the treated mice).

DISCUSSION

Recent data showed that the gut microbiota is involved in the development of obesity. In the present study, we demonstrated that modification of gut microbiota by norfloxacin and ampicillin ameliorated oral glucose tolerance and reduced hepatic steatosis in *ob/ob* mice. Furthermore, decreased plasma LPS concentrations and jejunum TNF- α mRNA expression correlated with the reduction of cecal bacteria induced by the treatment. In comparison, the pair-fed and control *ob/ob* mice had almost identical insulin-resistant phenotypes. On the basis of the results, we conclude that gut microbiota is a contributing factor to whole body insulin sensitivity, but not obesity, in mice.

Previous data show that oxytetracycline treatment ameliorated diabetic symptoms in BB rats (31) and *ob/ob* mice (32, 33). However, the beneficial effects of the oxytetracycline treatment did not only depend on modulation of the gut microbiota, as tetracycline has also been shown to regulate insulin secretion (34). Both norfloxacin and ampicillin have good bioavailability and can easily be absorbed by the gut. Consequently, the antibiotics may directly influence the status of insulin sensitivity *via* unpredicted pleiotropic effects instead of gut microbiota modulation. Norfloxacin has been shown to antagonize GABA_A receptor (35). Activation of GABA_A receptors on the membrane of pancreatic α -cells suppressed the release of glucagon, but it is not clear whether an inhibition of GABA_A receptor by norfloxacin can affect the secretion of glucagon and consequently regulate hepatic glucose output. Nor-

TABLE 4. Body weight, fat pad weight, and gut weight of *ob/ob* mice treated with antibiotics

Group	Body weight, final (g)	Total fat pad weight (g)	Gut weight (g)
Control	47.8 ± 0.9	5.95 ± 0.18^b	4.03 ± 0.13^b
Nor + Amp (1g/L)	47.2 ± 0.6	5.43 ± 0.11^a	6.00 ± 0.05^c
Pair-fed	46.1 ± 0.9	5.46 ± 0.11^a	3.59 ± 0.13^a

Body weight, fat pad weight, and gut weight were measured during the sacrifice. Total fat pads include epididymal, retroperitoneal, and mesenteric fat pads. Gut weight includes the weight of stomach, duodenum, jejunum, ileum, cecum, and colon. Data are expressed as median \pm SE; $N = 12$. Different superior letters indicate statistical significance ($P < 0.05$) using Kruskal-Wallis test followed by Wilcoxon tests for paired comparison.

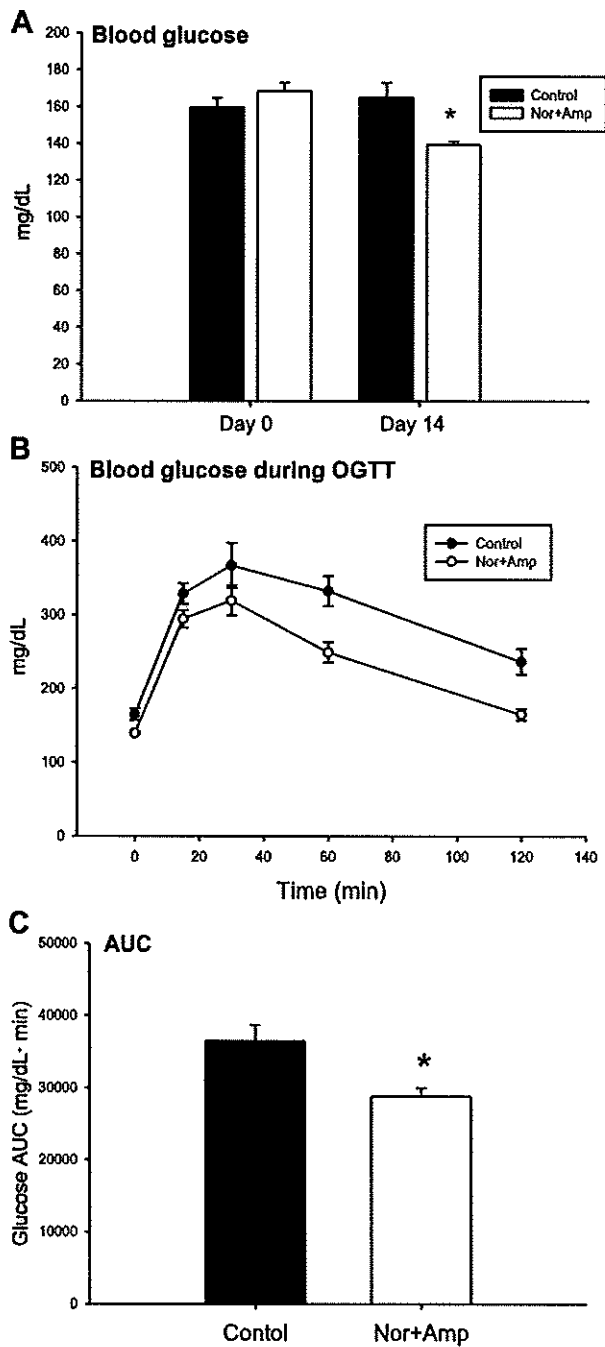


Figure 6. Blood glucose levels (A) in DIO mice were determined after 6 h fasting (8 AM to 2 PM) before (day 0) and after (day 14) of gut microbiota modulation. The treatment also improved the profile of blood glucose (B) and areas under the curve (C) in oral glucose tolerance tests. Data are expressed as median \pm SE; $n = 12$. * $P < 0.05$ vs. control

floxacin has also been shown to function as a weak inhibitor for ATP-sensitive K^+ (K^{ATP}) channel (36). However, during the OGTT, treated mice had either the same or a lower amount of insulin than the untreated mice, suggesting that stimulation of insulin secretion by inhibiting K^{ATP} channel in the pancreas would not be the cause for improved glucose tolerance.

To the best of our knowledge, there is no report indicating that ampicillin is capable of interfering with the pathophysiology of insulin resistance.

Constitutive androstane receptor (CAR) is a xenobiotic sensor and a transcription factor for activating many genes involved in hepatic drug metabolisms (37). Recently, data showed that activation of CAR by phenobarbital suppressed the expression of hepatic PEPCK (38), suggesting that activation of CAR may enhance insulin sensitivity by reducing hepatic gluconeogenesis. But in the present study, the expression of CAR mRNA in liver and jejunum of the treated *ob/ob* mice was similar regardless of the treatment (data not shown), and the CAR target gene, *Cyp2B10*, in both organs was also not affected by the treatment (data not shown). On the basis of the available evidence, we conclude that the improved glycemic control in the antibiotic-treated *ob/ob* mice was not due to the activation of CAR.

Ob/ob mice with gut microbiota modulation had normalized fasting and nonfasting blood glucose concentrations, suggesting improved insulin sensitivity. One possible link between gut microbiota and insulin sensitivity is LPS. An injection of a high-dose LPS caused acute whole body insulin resistance (39). Similarly, a low dose and chronic subcutaneous LPS administration *via* an osmotic minipump caused weight gain and insulin resistance (19). Our results show that the improvement of insulin sensitivity was associated with reduced plasma LPS concentrations supporting the possible role of bacterial LPS in causing insulin resistance. The second possible link between gut microbiota and insulin sensitivity is the amount of proinflammatory cytokine TNF- α produced by the gut. In the gut, LPS is a ligand for TLR4, and the activation of TLR4 leads to the expression TNF- α . In this study, the jejunum of antibiotic-treated *ob/ob* mice had normal expression of TLR4, CD14, MyD88 (data not shown), which suggests that the gut was fully capable of sensing bacterial LPS and producing TNF- α . In contrast, the expression of TNF- α in jejunum was higher in the pair-fed mice. However, more research has to be done to evaluate whether the production of intestinal TNF- α can affect hepatic insulin sensitivity. A transgenic animal model overexpressing TNF- α in the intestine would be a great tool to answer this question.

Improvement of hepatic insulin sensitivity leads to the suppression of hepatic glucose output and an increase in liver glycogen storage. In the antibiotics-treated *ob/ob* mice, the level of liver glycogen was markedly increased, and the expression of hepatic G6P was significantly reduced. Prolonged fasting induces gluconeogenesis and the expression of G6P, while refeeding promotes glycogen synthesis and diminishes the G6P expression. The dynamic range related to the expression of G6P in the transition from the fed to fasting state represents a state of metabolic flexibility. In the treated *ob/ob* mice, G6P mRNA levels were increased 21-fold after an overnight fast. In contrast, the G6P levels in control *ob/ob* mice only showed a 5-fold increase after fasting. A much improved transcriptional regulation of G6P gene or metabolic flex-

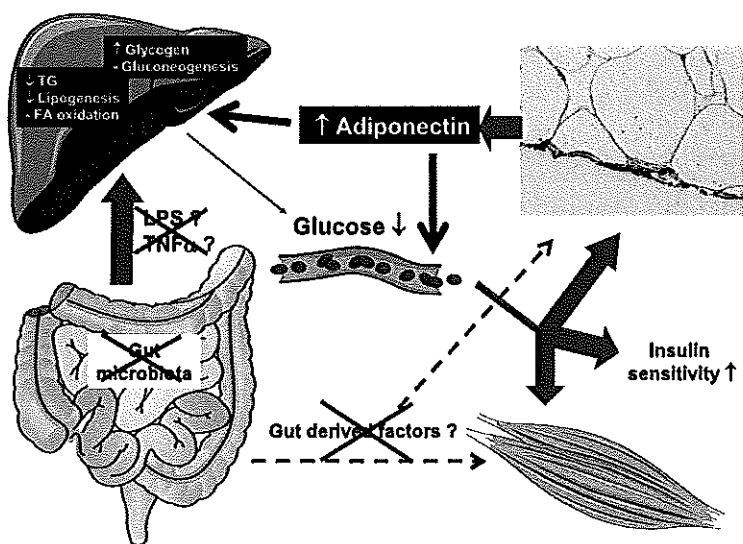


Figure 7. Gut microbiota modulation improved whole body glucose tolerance by reducing the population of gut microbiota and suppressing factors originating in the microbiota, including TNF- α and LPS. The liver responded to the changes by increasing glycogen storage and decreasing triglyceride accumulation. Elevated adiponectin levels might further enhance the insulin sensitivity. Together, gut microbiota might affect insulin sensitivity through multiple pathways.

ibility by the state of feeding suggests a restoration of hepatic insulin sensitivity. In addition, plasma adiponectin concentrations were elevated after antibiotic treatment. Adiponectin is known to alleviate alcohol and nonalcoholic fatty liver diseases in mice by 1) enhancing hepatic fatty acid oxidation and decreasing the activity of FAS and ACC1 and 2) lowering circulating TNF- α concentrations and the production of hepatic TNF- α (40). In fact, the amelioration of hepatic steatosis and reduction of ACC1 expression in the liver was observed in antibiotic-treated *ob/ob* mice. However, it is not clear whether the gut microbiota plays a role in the production of adiponectin in adipose tissue. It is also not known whether elevated adiponectin is required for the improvement of insulin sensitivity in different mouse models, because DIO mice treated with norfloxacin and ampicillin showed improved glucose tolerance without any change in plasma adiponectin level.

The observed improvements in glycemic control in the treated *ob/ob* mice were independent of body weight, body fat mass, and food intake. These results suggest that gut microbiota is a contributing factor in whole body glucose homeostasis but not in obesity. In contrast, results from studies comparing between germ-free and conventionalized mice revealed a significant role of gut microbiota in fat storage in the host. Germ-free mice eating either a chow diet (10) or a high-fat diet (11) were lean. Bäckhed *et al.* (10, 11) suggested that a high amount of circulating LPL inhibitor Fiaf, produced by the gut, contributed to the reduced fat mass found in germ-free mice. However, it is still not clear how germ-free mice were able to maintain negative energy balance, since they ate at least the same amount of food as conventional mice but weighed less (10, 11). Further research in the areas of energy expenditure and nutrient absorption are needed to address the pending question. Recent results show that germ-free mice have reduced capillary network in small intestinal villi (6), and a lower expression of intestinal SGLT-1 (5) when compared with conven-

tionalized mice. This indicates that germ-free mice have an immaturely developed gut. It is plausible that reduced SGLT-1 expression and gut immaturity limit the efficiency of glucose absorption, which may contribute to the lean phenotype of germ-free mice. In the case of the mouse model with gut microbiota modulation, mice ate and drank less due to the antibiotics in the drinking water. However, a small reduction in food intake during the first 4 days of treatment was unlikely to cause the improvement in insulin sensitivity, since the pair-fed *ob/ob* mice were insulin resistant. The advantage of using the mouse model with gut microbiota modulation is that gut maturity is not a confounder. The expressions of SGLT-1 and GLUT2 (data not shown) in jejunum were not altered by the antibiotic treatment.

In summary, gut microbiota modulation with norfloxacin and ampicillin reversed the insulin resistance characteristic of *ob/ob* mice *via* multiple pathways (Fig. 7). It is possible that the presence of certain bacteria in the gut might exacerbate the low-grade systemic inflammation, which further causes whole body insulin resistance. In the present study, mice treated with antibiotics showed improved glycemic control and yet had similar adiposity to those of the pair-fed mice, suggesting that gut microbiota influences whole body glucose homeostasis independent of obesity. In conclusion, our results support the idea that modulating gut microbiota could be beneficial for improving glycemic control. However, more work has to be done in order to prove that gut microbiota modulation is a safe and effective therapeutic strategy in treating or managing type 2 diabetes in humans. EJ

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