Dietary Exposure to Antibiotic Residues Facilitates Metabolic Disorder by Altering the Gut Microbiota and Bile Acid Composition

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Letter

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Dietary Exposure to Antibiotic Residues Facilitates Metabolic Disorder by Altering the Gut Microbiota and Bile Acid Composition

Low dose antibiotic residues in food potentially contribute to obesity and metabolic dysfunction\(^1\). However, the effect of chronic exposure to very low-dose antibiotic residue (~1000-fold lower than the therapeutic dose) on gut microbiota and host metabolism is poorly understood. Herein the effect of exposure to a residual dose of tylosin—an antibiotic growth promoter—on host metabolism and gut microbiota is explored in a mouse model. Theoretical maximal daily intake (TMDI) dose of tylosin facilitates high-fat diet-induced obesity, induces insulin resistance, and perturbs gut microbiota composition. Moreover, obesity-related phenotypes are transferrable to germ-free recipient mice following fecal microbiota transplantation. Tylosin TMDI exposure restricted to early life is sufficient to induce metabolic complications, alter the abundance of specific bacteria related to host metabolic homeostasis later in life, and modify the composition of short-chain fatty acids and bile acids. Finally, tylosin TMDI exposure induces lasting metabolic consequences via elevating the ratio of primary to secondary bile acids and its downstream FGF15 signaling pathway. Hence, exposure to very low doses of antibiotic residues, whether continuously or in early life, can exert long-lasting effects on host metabolism by altering gut microbiota and their metabolites.

Antibiotics used as growth promoters in livestock and animal husbandry can be detected in animal-derived food\(^2,5\). Although the maximum residue limits (MRLs) of these veterinary antibiotics has been evaluated, these estimated levels do not consider the effects of antibiotic residues on gut microbiota and microbial metabolites, or the effects of altered gut microbiota on host metabolism\(^6\). Recent studies have established
an association between exposure to veterinary antibiotics and children obesity\textsuperscript{1,7}.

Additionally, animal studies have indicated that exposure to sub-therapeutic doses of antibiotics leads to obesity and NAFLD\textsuperscript{8-10}. In this study, we aimed to further elucidate whether chronic exposure to acceptable residual amounts of antibiotic, which doses are hundred-fold lower than the sub-therapeutic doses used in previous studies, could cause obesity complications. Thus, C57BL6J mice were fed an acceptable daily intake (ADI, 0.37 mg/kg/day) and theoretical maximum daily intake (TMDI, 0.047 mg/kg/day) dose of tylosin (Fig. 1a). The ADI dose is the maximum daily dose with no adverse effect\textsuperscript{6}, while the TMDI dose is an estimate of maximum dietary intake level obtained for MRLs and the sum of average daily per capita consumption for each food product\textsuperscript{6}, which was used for simulating the ingestion of antibiotic residues through food consumption in the present study. Additionally, a normal-chow diet and high-fat diet were provided to assess the potential synergistic effects of antibiotics and diet on host metabolism.

Compared with NCD-CON (normal chow diet/control) mice, NCD-ADI and NCD-TMDI mice showed significantly greater weight gain from weaning to 5 weeks of age. NCD-ADI mice also exhibited higher weight gain from weeks 13 to 15 (Fig. 1b) with no significant changes detected among the NCD groups in relative fat or lean mass (Extended Data Fig. 1a, b). In contrast, high-fat diet (HFD)-ADI and HFD-TMDI mice exhibited significantly increased weight gain compared with HFD-CON mice from weaning to 17 weeks of age (Fig. 1c). HFD-ADI and HFD-TMDI mice had increased relative fat mass at weeks 8, 12, and 17, compared with HFD-CON mice (Fig. 1d), whereas HFD-TMDI mice had decreased relative lean mass (Fig. 1e). Likewise, HFD-TMDI mice had increased visceral fat, including epididymal and perinephric adipose tissues (Fig. 1f, g and Extended Data Fig. 1 c, d). HFD-ADI and HFD-TMDI
mice also exhibited adipocyte hypertrophy (Fig. 1h) and elevated adipocyte size (Fig. 1j) compared with HFD-CON mice. Hence, tylosin-induced adiposity is evident early in life for NCD and HFD mice; however, the continuous effects of tylosin-induced adiposity were preferentially observed in HFD-fed mice.

We next sought to investigate whether exposure to residual tylosin dose is causally associated with metabolic complications, including hepatic abnormalities and glucose homeostasis. Given that tylosin increased the fat mass in HFD-fed mice, not NCD-fed mice, additional investigations were conducted in the HFD-fed mice. Histological examination of the liver revealed that tylosin-treated mice exhibited increased lipid droplet formation (Fig. 1i), more inflammatory foci, and higher fatty liver scores (Fig. 1k), suggesting that the presence of residual tylosin caused more severe NAFLD. Both HFD-ADI and HFD-TMDI mice exhibited a trend toward higher plasma glucose during the oral glucose tolerance test (OGTT) (Extended Data Fig. 2a) and fasting glucose test (Extended Data Fig. 2b), with an increased OGTT AUC found in HFD-TMDI mice (Fig 2l). The fasting insulin level and homeostasis model assessment of insulin resistance (HOMA-IR) index were also elevated in HFD-ADI mice, compared with HFD-CON mice (Fig. 2m, n). Hence, a residual dose of tylosin can exacerbate HFD-induced adverse effects on metabolic complications. Remarkably, although the tylosin ADI dose is defined as the maximum antibiotic dose that can be ingested daily without health risk, it facilitated obesity-related metabolic disorders in our mice model. As such, the tolerance level of antibiotics may need to be re-evaluated in consideration of chronic metabolic diseases which were emerging in western lifestyle.

As sub-therapeutic doses of antibiotic treatments have been shown to disrupt the development and maturation of gut microbiota with metabolic consequences, we next
asked whether ADI and TMDI doses of tylosin alter gut microbiota composition. To
this end, fecal 16S rRNA sequencing was performed in mice at 3 (weaning), 8, and 17
weeks of age to elucidate changes in the gut microbiota. Compared with HFD-TMDI
and HFD-CON mice, HFD-ADI mice had significantly reduced Shannon indices at
week 3, which markedly increased during weeks 3–17 (Fig. 2a). Principal coordinate
analysis (PCoA) based on Bray-Curtis dissimilarity further revealed that the
microbiomes of NCD and HFD mice clustered separately (Extended Data Fig. 3a),
indicating that diet may represent the most critical factor influencing the composition of
the gut microbiota, while residual tylosin dose exhibited a secondary effect in shifting
the gut microbiota in a dose-dependent manner according to the PCoA2 axis (Extended
Data Fig. 3a).

Further, we analyzed the subsets of NCD (Extended Data Fig. 3b) and HFD-
fed mice (Fig. 2b) separately to observe the effect of tylosin on age-related microbiota
development. In both groups, ADI dose of tylosin substantially influenced the gut
microbiota composition, while TMDI dose of tylosin showed a lesser impact. Notably,
the gut microbiota of tylosin-treated mice was obviously changed at the early-life stage
(week 3) in both NCD and HFD groups, with gradually shifted toward the control group
at weeks 8 and 17, respectively. Bray-Curtis distances from HFD-CON mice to HFD-
ADI or HFD-TMDI mice were also shortened as the mice aged (Fig. 2c). These
findings suggest that gut microbiota has increased susceptibility to the perturbation
caused by residual tylosin in early life, yet susceptibility may decrease at later stages,
possibly due to the establishment and maturation of gut microbiota.

To further investigate differentially abundant bacteria modified by tylosin TMDI
dose, we identified significantly-altered bacteria at the amplicon sequence variants
(ASV) level (defined as log2-fold change >1, p values < 0.05; Fig. 2d, Extended Data
Oscillibacter, Blautia and Parasutterella, which are associated with obesity and inflammatory bowel disease\textsuperscript{12-14}, were significantly enriched in HFD-TMDI mice, whereas Bifidobacterium was significantly reduced (Fig. 2d). TMDI dose of tylosin may decrease beneficial bacteria while enriching pathogenic bacteria.

We next assessed the association between obesity-related features of 17-week-old HFD-fed mice and their gut microbiota composition by the envfit function in the vegan R package, the result is displayed in PCoA (Fig. 2e). The results showed that vectors indicating both obesity-related biomarkers (the blue arrows) and antibiotic exposure (the red arrow) were arrowed toward the mice treated with tylosin in the same direction, indicating the obesity outcomes were associated with the tylosin-shifted gut microbiome. We further performed Spearman's correlation between PCoA1 and PCoA2 of gut microbiota against individual obesity-related parameters (Supplementary Table. 1) and found that the PCoA2 was significantly correlated with obesogenic phenotypes (fat gain, fat mass, and relative fat mass), fatty liver score, and insulin resistance parameters (fasting insulin and HOMA-IR).

To understand the overall obesity phenotype and its relationship with tylosin altered-microbiome, we performed a dimension reduction of the obesity and metabolic disorders related biomarkers by using the principal component analysis (PCA). The result showed that the obesity phenotypes in tylosin-treated mice were significantly different from control mice ($p < 0.0001$; Extended data Fig. 5) We subsequently calculated a correlation between PC1 obesity biomarkers and the PCoA2 microbiota which displaying a positive correlation ($r = 0.71$, $p = 0.003$).

Given the above association between the metabolic disorder phenotypes and tylosin-altered gut microbiota, we conducted a fecal microbiota transplantation (FMT) study to investigate their causative role. Since a TMDI dose can adequately simulate
human exposure to antibiotics in food, feces from HFD-TMDI mice were transplanted to germ-free mice. Compared with germ-free mice that received feces from HFD-CON mice (FMT-CON), the HFD-TMDI recipient mice (FMT-TMDI) showed higher body weight at 11, 12, 13, 14 weeks of age (Fig. 2g), increased weight gain 2 weeks post-FMT (Fig. 2h), and slightly elevated fat mass at week 20 (Fig. 2i), implying that the microbiome from HFD-TMDI mice increased the adiposity of the recipient mice. Additionally, HFD-TMDI mice exhibited increased plasma glucose levels in OGTT\textsubscript{AUC}, and a higher HOMA-IR index (Fig. 2j, k). Thus, a TMDI dose of tylosin-altered microbiota induced obesity and insulin resistance in germ-free recipients, indicating that residual tylosin exposure facilitates metabolic disorders through the alternation of gut microbiota.

Growing evidence indicates that antibiotic exposure during infancy, which has been considered the critical window of gut microbiota development, is associated with increased risk of being overweight and obese. More specifically, antibiotic exposure during early life, even at sub-therapeutic levels, disturbs the colonization and maturation of the intestinal microbiota, leading to lasting effects on the metabolism of the host. Accordingly, an early-exposure experiment was conducted to investigate the influence of early-life exposure to a TMDI dose of tylosin on obesity-related phenotypes and the gut microbiota (Fig. 3a).

Cont-TMDI mice, which were continuously exposed to TMDI dose of tylosin throughout the experimental period, displayed continuously elevated body weight, relative fat mass, visceral fat mass, and OGTT\textsubscript{AUC}, compared with HFD-CON mice (Fig. 3b-e). Interestingly, Early-TMDI mice, which were exposed to tylosin TMDI during pregnancy and the nursing period, also exhibited consistently elevated body weight, relative fat mass, fasting insulin, and HOMA-IR index values after cessation of
tylosin exposure (Fig. 3b-g). These findings suggest that exposure to antibiotic residue in food products during early life is sufficient to induce long-lasting effects on metabolism and lead to obesity. Compared with previous studies showing that antibiotic exposure limited to early life induces elevated adiposity later in life\(^8\), our findings further demonstrate that metabolic complications can emerge later in life even with a food-grade low dose of antibiotic residue.

The overall gut microbiota composition based on PCoA of Bray-Curtis distances showed that tylosin influenced the gut microbiota composition at weeks 5 and 20 (\(p < 0.05\); Fig. 3h). Compared with CON mice, Cont-TMDI mice showed a greater difference than Early-TMDI mice, suggesting that continuous tylosin exposure has a more significant effect compared to exposure restricted to early life (Fig. 3h). Furthermore, the Early-TMDI and CON mice exhibited greater differences at week 5 than week 20, possibly due to establishment of the gut microbiota (Fig. 3h).

Interestingly, despite Early-TMDI mice exhibited a more similarly resembled microbial community to that of CON mice at week 20 than at week 5 (Fig. 3h), their fat mass at week 20 was more significantly increased than at week 5 (Fig. 3c). This finding suggests that the impaired metabolic phenotype can persist despite resilience of the microbiota. That is, minor disruption of the microbiota in early life caused by antibiotic residue appears to be sufficient for inducing significant adiposity\(^{18,19}\).

Studies have indicated that microbial colonization can be perturbed by exposure to antibiotic early in life, which, in turn, contributes to metabolic disorders in adulthood\(^{20,21}\). Thus, we proposed that although the overall gut microbiota composition can restore in later life, the abundance of specific bacteria associated with metabolic homeostasis of the host are persistently depleted. To investigate this hypothesis, we identified 32 bacterial genera significantly altered by early or continuous exposure to
TMDI dose of tylosin (Fig. 3i), then examined the association between these bacterial genera and obesity-related phenotypes (Fig. 3j). Bacterial genera increased in both Early-TMDI and Cont-TMDI mice, including *Anaerofustis*, and demonstrated a significant positive correlation with obesity-related phenotypes (Fig. 3i). In contrast, genera that were depleted in Early-TMDI and Cont-TMDI mice, including bacteria belonging to the *Lachnospiraceae* and *Ruminococcaceae* families, exhibited a significantly negative correlation with obesity-related phenotypes (Fig. 3i). Previous studies have also reported that these bacteria are related to obesity. *Anaerofustis*, the tylosin-enriched bacterium is increased in obese humans, while tylosin-depleted *Ruminococcaceae* and *Lachnospiraceae* are associated with lower long-term weight gain. Notably, despite the discontinuation of tylosin exposure in Early-TMDI mice at 3 weeks of age, several bacterial genera that are negatively correlated with obesity-related phenotypes remained diminished at week 20. Consistent with our hypothesis, although early exposure to residual dose of antibiotics may not cause a significant impact on the overall microbial composition in later life, early-depletion of certain bacteria, that continued to be lifelong lost, may contribute to metabolic dysfunctions in later life.

Given that antibiotics reportedly alter short-chain fatty acid (SCFA), bile acids composition and their signaling pathways, thereby leading to metabolic consequences, we next investigated the effect of TMDI dose of tylosin on these two major metabolites. SCFA analysis showed that propionic acid and butyric acid, two main SCFAs associated with enhanced intestinal barrier function and insulin sensitivity, exhibited decreasing trends in Cont-TMDI mice (Fig. 4a). Isovaleric acid, a branched SCFA reported to improve insulin-stimulated glucose uptake and enhance insulin sensitivity, was significantly decreased in Cont-TMDI mice (Fig. 4a).
Reduction of SCFAs could be attributed to the reduction of *Lachnospiraceae* and *Ruminococcaceae* in Early-TMDI and Cont-TMDI mice ([Fig. 3i](#))\(^{29,30}\).

The total amount of bile acids and the ratio of conjugated to unconjugated bile acids were not significantly altered by tylosin exposure ([Extended Data Fig. 6a, b](#)). Notably, the ratio of primary bile acids (PBA) to secondary bile acids (SBA) was significantly increased in Cont-TMDI and Early-TMDI mice ([Fig. 4b](#)). Indeed, a decreased PBA/SBA ratio in NAFLD patients who have undergone bariatric surgery is associated with improved insulin sensitivity, indicating that an increased PBA/SBA ratio tends to induce metabolic disorder\(^{31,32}\). To further investigate the effect of tylosin on the conversion of PBA to SBA, the detected bile acids were classified into non-12-OH bile acids ([Fig. 4c](#)), muricholic acids (MCA; [Fig. 4d](#)), and 12-OH bile acids ([Fig. 4e](#)) based on their metabolic pathway\(^{33}\). The levels of PBAs, namely, chenodeoxycholic acid, α-MCA, β-MCA, and cholic acid ([Fig. 4c-e](#)) were significantly increased, whereas those of the SBAs, including ursodeoxycholic acid (UDCA) and ω-MCA ([Fig. 4c, d](#)), were decreased in tylosin-treated mice, possibly due to inhibition of bacteria associated with epimerization and dehydroxylation of PBAs, such as *Clostridia*, *Peptostreptococcus*\(^{34}\) ([Extended Data Fig. 6c, d](#)).

Antibiotics have also been found to increase the PBA/SBA ratio with a subsequent decrease in plasma fibroblast growth factor 19 (FGF19; human orthologue of FGF15). FGF19 is an insulin-like hormone secreted by intestinal epithelium to regulate hepatic lipid and glucose metabolism by binding to FGFR4 in the liver, thereby decreasing peripheral insulin sensitivity\(^{24,35,36}\). Moreover, *Parasutterella*, the genus enriched in tylosin-treated mice ([Fig. 2e](#) and [Extended Data Fig. 4a](#)), reportedly increases β-MCA and decreases ileal *Fgf15* gene expression\(^{13}\). Hence, the FGF15/FGFR4 signaling pathway was further explored. Tylosin reduced ileal FGF15
expression (Fig. 4g) and portal vein FGF15 levels (Fig. 4f) with subsequent reduction in hepatic FGFR4 levels (Fig. 4h). Collectively, tylosin TMDI-treated mice showed an increased PBA/SBA ratio, as well as lower FGF15 levels in the ileum and portal vein, and decreased expression of hepatic FGFR4, which may cause metabolic disorders by affecting metabolism-related signaling pathways in the liver\textsuperscript{35-37}.

This study has certain limitations. First, we only investigated the \textit{in vivo} effects of one antibiotic (tylosin), whereas exposure to other antibiotics may lead to different outcomes owing to the specific antimicrobial action and spectrum of each antibiotic. Second, the human dietary pattern is dynamic, with daily exposure to multiple types of antibiotics, and even pesticides. Future research is warranted to investigate the effects of other antibiotics at residual amounts, as well as the combination of different antibiotics to better reflect real-life conditions.

In conclusion (Fig. 4i), tylosin at ADI and TMDI doses, which are generally regarded as harmless, facilitated HFD-induced metabolic complications and perturbated the gut microbiota composition. Moreover, the altered gut microbiota was critical for tylosin TMDI-induced metabolic consequences, suggesting that continuous exposure to very low doses of antibiotic residues in food can affect human metabolism by altering gut microbiota. Since the gut microbial community is dynamic and susceptible to environmental shifts in early life, early exposure to residual doses of tylosin was sufficient to persistently deplete specific bacteria involved in the metabolic homeostasis of the host, which may induce lasting metabolic consequences. Finally, we demonstrated that residual tylosin altered the conversion of bile acids with downstream effects on the FGF15 signaling pathway, a possible mechanism that participates in the lasting metabolic disorders. Taken together, these findings indicate that permissible
exposure levels of antibiotic residues should be re-established while considering its impact on the gut microbiota, for which this study provides valuable insights.
Materials and Methods

Antibiotic selection and dose calculation
Tylosin was selected as a model antibiotic growth promoter due to its high annual consumption as a veterinary antibiotic (Bureau of Animal and Plant Health Inspection and Quarantine, 2014) and its presence in animal-derived food38-40. The ADI and TMDI doses were obtained from the World Health Organization Technical Report Series41.

Experimental design of the animal studies
Animal experiments were performed with permission from the Institutional Animal Care and Use Committee of National Taiwan University (approval number: NTU-106-EL-051 and NALC 107-0-006-R2). All mice were purchased from the National Laboratory Animal Center (Taipei City, Taiwan). For animal studies I and III, each experimental group comprised 3 C57BL/6J mother mice. The number of mice in each experimental group varied according to the number of offspring of the mother mice.

I. Antibiotic residue exposure model
C57BL/6J mother mice received tylosin at ADI (0.37 mg/kg) and TMDI (0.047 mg/kg) doses 10 days before giving birth, and their offspring were continuously administered the antibiotic. Tylosin was administered through drinking water. Control mice (CON) did not receive antibiotics. To investigate the syngenetic effect of tylosin and diet, the offspring were randomly divided into NCD (MFG, Oriental Yeast Co., Ltd., Tokyo, Japan) and HFD (60% kcal from fat) groups (D12492, Research Diets, New Brunswick, NJ, USA). Body composition was measured at 8, 12, and 17 weeks. The metabolic measurements and OGTT were performed at 20 weeks prior to sacrifice. After euthanasia by carbon dioxide inhalation, blood and tissue samples were collected and stored at −80 °C.

II. Fecal microbial transplantation study
Eight-week-old C57BL/6 germ-free mice were randomly divided into fecal microbiota transplantation-control (FMT-CON) and FMT-TMDI groups, which were transplanted with fecal microbiota from HFD-CON and HFD-TMDI mice, respectively. After FMT, the recipient mice were housed in two independent isolators and fed with irradiated HFD until 20 weeks of age. Before the recipient mice were euthanized, the body composition analysis and OGTT were performed.

**III. Early-life exposure model**

The mice were divided into three groups: CON, feeding conditions were the same as those of HFD-CON; Early-TMDI, exposure duration was limited to the gestation and lactation period; Cont-TMDI, feeding conditions were the same as those of HFD-TMDI. TMDI administration of tylosin started on day 10 of gestation. Before the mice were weaned, both Early-TMDI and Cont-TMDI mice were exposed to a TMDI dose of tylosin. After weaning, only the Cont-TMDI mice were continuously exposed to tylosin through drinking water. Body composition was measured at 5, 10, 15, and 20 weeks. The OGTT was performed before euthanizing the mice at 20 weeks.

**Body composition analysis**

Body composition was determined using Minispec LF50 TD-NMR Body Composition Analyzer (Bruker, Billerica, MA, USA), which provides the measurement of body weight and lean and fat mass. The relative fat mass was calculated as the fat mass (g)/body weight (g) ratio.

**Glucose and insulin sensitivity**

For the OGTT, mice were deprived of food for 5 h. Blood was collected from the submandibular vein, and the glucose levels were measured using a glucometer (Roche, Basel, Switzerland) at 0, 15, 30, 60, 90, and 120 min after oral administration with 2 g/kg glucose. The fasting insulin levels were detected by an enzyme-linked
The HOMA-IR index was calculated using the formula: fasting glucose (nmol/L) × fasting insulin (µU/mL)/22.5 \(^{42}\).

**Histopathological analysis of liver and adipose tissue**

Liver and adipose tissue sections were collected and fixed in 10% formalin solution. Histopathological analysis was performed by the formalin-fixed, paraffin-embedded, and hematoxylin and eosin (H&E)-stained slide method. The fatty liver score was estimated by a pathologist as previously described\(^ {43}\). The fatty liver score included the evaluation of steatosis (macrovesicular, microvesicular, and hypertrophy) and inflammation (number of inflammatory foci). The visceral adipocyte quantification was performed by HCImage Live software (HCImage, Sewickley, PA, USA).

**Quantification of plasma lipopolysaccharides**

Plasma samples were centrifuged and added to HEK-Blue™ mTLR4 cell lines in the HEK-Blue™ Detection medium (InvivoGen, San Diego, CA, USA). After 24 h of incubation, the color of secreted embryonic alkaline phosphatase (SEAP) released by the reporter cells was measured by a spectrophotometer at 620 nm \(^ {44}\).

**Metabolic measurements**

Metabolic measurements (food intake, locomotor activity, VO\(_2\) consumption, and VCO\(_2\) production) were made using the Promethion metabolic phenotyping system (Sable Systems, Las Vegas, NV, USA). Monitoring was performed for 24–48 h with *ad libitum* access to food and water after mice had been acclimatized to cages for 6–12 h.

**Fecal microbiota extraction and 16S rRNA gene sequencing**

The mice in each group were randomly selected for fecal microbiota analysis. Mice feces were collected and frozen immediately at −80 °C. Fecal DNA extraction and library preparation and sequencing were performed according to the protocol provided
by QIAGEN (QIAGEN, MD, USA). Briefly, fecal microbial DNA was isolated by using QIAamp 96 PowerFecal QIAcube HT Kit (QIAGEN, Germantown, MD, USA). The 16S V4 region was amplified by the forward primer (F515, 5ʹ-GTGCCAGCMG CCGCGGTAA-3ʹ) and the reverse primer (R806, 5ʹ-GGACTACHVGGGTWTCTAAT-3ʹ). The reaction conditions were as follows: 3 min at 95 °C, followed by 25 cycles of 95 °C for 30 s, 55 °C for 30 s, 72 °C for 30 s, and 5 min at 72 °C for a final extension.

For library construction, the amplified PCR product was attached with Illumina sequencing adapters by Nextera XT Index Kit then purified using AMPure XP beads. The library quantification was conducted using the DNA 1000 kit and 2100 Bioanalyzer Instrument (Agilent Technologies, Santa Clara, CA, USA). The sequencing (paired-end reads, 2 × 150 bp) was performed by Illumina NextSeq (Illumina, San Diego, CA, USA).

**Bioinformatic analysis for microbial taxonomic profiling**

The amplicon sequences were processed by a QIIME 2 pipeline (version 2019.10, https://qiime2.org). The primer sequences of raw reads were trimmed using the cutadapt plugin. The trimmed single-end (forward) sequences were subsequently denoised with the DADA2 plugin of QIIME2. To obtain qualified data, we truncated reads to 130 bp from the 3ʹ end based on the quality score. The DADA2 outputs of high confident ASVs went through quality filtering, denoising, and chimeric-read removing. The classify-consensus-vsearch plugin was applied for taxonomy assignment by aligning against SILVA 132 99% 16S rDNA sequences, and identity cutoff was set at ≥80% sequence similarity by default. The vegan package in R was used to calculate Shannon index and perform principal coordinate analysis (PCoA) based on the Bray–Curtis distance. The permutation multivariate analysis of variance (ANOVA) using distance matrixes (adonis) was performed to calculate the significance of microbiota composition among
groups. The PCoA plot of gut microbiota with an association between obesity-related features of 17-week-old HFD-fed mice was performed by the envfit function in the vegan R package, displaying as the vectors (p < 0.05).

**Correlation analysis**

The Spearman’s correlation analysis between PCoA1 and PCoA2 of gut microbiota against individual obesity-related parameters was performed with FDR-adjusted p-value. The correlation coefficient and p-value of Spearman’s correlation between PC1 obesity biomarkers (representing the overall obesity phenotype) and PCoA2 microbiota (representing the gut microbiota composition) was calculated.

**Principal component analysis (PCA) of obesity biomarkers**

PCA was used for dimension reduction of obesity biomarkers, including body weight at 17 weeks of age, fat mass at 17 weeks of age, relative fat mass at 17 weeks of age, weight gain between 3 to 17 weeks of age, fat mass gain between 8 to 17 weeks of age, fasting glucose, OGTT \( \text{AUC} \), fasting insulin, HOMA-IR, weight of eWAT, and fatty liver score.

**Fecal short-chain fatty acid analysis**

The SCFAs we analyzed included acetic acid (C2), propionic acid (C3), butyric acid (C4), isobutyric acid (C4), valeric acid (C5), and isovaleric acid (C5). Briefly, 20 mg of the raw feces was dissolved in 500 \( \mu \)L of a 0.5% \( \text{H}_3\text{PO}_4 \) aqueous solution and homogenized with Geno/Grinder® at 1,000 rpm for 2 min. The sample solutions were then centrifuged at 18,000 \( \times \) g for 10 min at 4 °C to separate depositions. Afterwards, 285 \( \mu \)L of the supernatant was collected in a clean centrifuge tube, and 15 \( \mu \)L of acetate-d3 was added. For the liquid–liquid extraction of SCFAs, 300 \( \mu \)L of butanol and the final solution were mixed and centrifuged. Last, 20 \( \mu \)L of internal standard propionate-d5 was added to 180 \( \mu \)L of the upper organic layer. GC–MS analysis was
performed using an Agilent 7890A gas chromatograph (Agilent Technologies) coupled with a Pegasus 4D GC × GC–TOF–MS system (Leco Corporation, St. Joseph, MI, USA) using an VF-WAXms capillary column.  

**Fecal bile acid analysis**

The bile acid quantification included four primary bile acids (α-MCA, β-MCA, CA, and UDCA), four secondary bile acids (ω-MCA, CDCA, DCA, and LCA), and seven conjugated bile acids (Tβ-MCA, TUDCA, TCA, GCA, TCDCA, TDCA, TLCA).

Briefly, 20 mg of raw feces was dissolved in 200 μL of 70% d3-cholic acid aqueous solution containing 2-ppm d4-cholic acid as internal standard, and it was homogenized using an ultrasonicator for 30 min. The sample solutions were then centrifuged at 18,000 × g for 5 min. LC–MS analysis was performed using UltiMate 3000 liquid chromatography system (Thermo Fisher Scientific, Dreieich, Germany) coupled with a high-resolution Q Exactive Plus instrument equipped with the ESI source (Thermo Fisher Scientific), and an Acquity HSS T3 (2.1 × 100 mm, 1.7 μm) column (Waters, Milford, MA, USA).

**Biochemical analysis of the FGF15–FGFR4 pathway**

For western blotting, frozen tissues were lysed in lysis buffer containing 7-M urea, 2-M thiourea, 2% CHAPS, 0.002% bromophenol blue, 60-mM DTT, and a protease and phosphatase inhibitor cocktail. The cell lysates were sonicated for 5 min and centrifuged at 17,500 × g for 30 min at 4 °C. Total protein content was measured based on the Bradford assay with a Bradford reagent (Bioshop Canada Inc., Burlington, Canada) and an ELISA reader. For the loading buffer, 62.5-mM Tris-HCl, 10% glycerol, 2% SDS, and 0.01% bromophenol blue were mixed with the protein samples then heated at 95 °C for 10 min for protein denaturation. Proteins were separated by 10% or 12% SDS–polyacrylamide gel electrophoresis, they were then transferred onto
polyvinylidene difluoride membranes (Millipore, Burlington, MA, USA). After blocking with 5% bovine serum albumin in tris-buffered saline with Tween 20, the membranes were incubated with rabbit monoclonal anti–GAPDH antibody (1:5000; Catalog No. 5174; Cell Signaling, Danvers, MA, USA), or rabbit polyclonal anti–FGF15 antibody (1:500; Catalog No. ab229630; Abcam, Cambridge, UK), or rabbit polyclonal anti–FGFR4 antibody (1:500; Catalog No. ab119378; Abcam) at 4 °C for 16 h and subsequently incubated with HRP-linked anti-rabbit IgG antibody (1:3000; Catalog No. 7074; Cell Signaling). The protein expression signal was captured and quantified using the BioSpectrum AC imaging system (UVP, Upland, CA, USA) and ImageJ (version 1.53; National Institutes of Health, Bethesda, MD, USA), respectively. Portal FGF15 levels were quantified using an ELISA kit (Mercodia, Uppsala, Sweden).

**Statistical analysis**

Data were represented as mean ± standard deviation (SD) or mean ± standard error of the mean (SEM). One-way ANOVA and Tukey’s range test or Student’s t-test were applied for intergroup comparisons. Statistical assessment of the gut microbiome, SCFAs, and bile acids was performed using Kruskal–Wallis with/without false discovery rate or one-way ANOVA with Tukey’s range test. All statistical data were analyzed using GraphPad Prism software (version 9.2.0; GraphPad Software, San Diego, CA, USA) or RStudio (version 1.2.5001, RStudio, Boston, MA, USA).
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Author contributions: R.A.C. designed and performed the animal study, performed metabolomic, bioinformatics, and statistical analysis, and drafted the manuscript; W.K.W. proposed, designed, and instructed the study; S.P. assisted the experiments and bioinformatics analysis; P.Y.L. instructed the bioinformatics analysis; H.L.C. performed the germ-free mice study and assisted the metabolic measurement; Y.H.C. assisted the germ-free animal study and performed the LPS analysis; Q.L. instructed the bile acid analysis; H.C.H. and H.B.Z. performed SCFA analysis and assisted with mass spectrometry analysis; T.L.L. instructed the LPS analysis; Y.T.Y. conducted the PCR and library preparation for 16S rRNA sequencing; H.S.H. and Y.E.L. instructed the western blotting; S.P., T.C.D.S., W.K.W., P.Y.L. and L.Y.S. critically revised the manuscript; W.K.W., L.Y.S., C.C.H., M.S.W., H.C.L., C.C.C. and C.T.H., provided professional insights, techniques, and relevant resources for the study.

Data availability: The raw 16s rRNA sequencing data are accessible at the National Center for Biotechnology Information Short Read Archive (BioProject: PRJNA715326).

Competing interests: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.
References


Fig. 1 | Residual dose of tylosin facilitates HFD-induced obesity and metabolic disorders.

a, Experimental design of antibiotic residue exposure model. b, Body weight changes in NCD and c, HFD mice (n = 8–12/group). d, Relative fat mass and e, relative lean mass changes in HFD mice (n = 8–12/group). f, Weight of epididymal adipose tissue and g, perinephric adipose tissue (n = 8–12/group). h-i, histological features of H&E stained in epididymal adipose tissue (h) and liver (i) (n = 8–12/group). j, Adipocyte diameter of epididymal adipose tissue (n = 6/group). k, Fatty liver score including steatosis (macrovesicular, microvesicular, and hypertrophy) and inflammation (number of inflammatory foci) (n = 8–12/group). l, Area under the curve (AUC) derived from the OGTT (n = 7–8/group). m, Plasma insulin level after overnight fasting (n = 7–8/group). n, HOMA-IR index represented as an indicator of insulin resistance (n = 7–8/group).

Data are expressed as mean ± SD. For b-e, and j, Statistical analyses were performed by one-way ANOVA with Tukey’s range test within diet groups (NCD or HFD) as
follows: CON vs ADI (\(^{\#}p < 0.05; \^{\#\#}p < 0.01\)); CON vs TMDI (*p < 0.05; **p < 0.01; ***p < 0.001). For f-g, and k-n, one-way ANOVA with Tukey’s range test (*p < 0.05; **p < 0.01; ***p < 0.001). Abbreviations: ADI, acceptable daily intake; AUC, area under curve; CON, control; HFD, high-fat diet; HOMA-IR, homeostatic model assessment of insulin resistance; NCD, normal chow diet; OGTT, oral glucose tolerance test; TMDI, theoretical maximum daily intake.
Fig. 2 | Residual dose of tylosin remodels the gut microbiota composition, and their microbiota shift verifies its pathogenesis on generating the obesogenic and metabolic phenotype in germ-free mice.

a, Change in Shannon diversity index in 3-, 8-, 17-week-old HFD-fed mice (n = 6/group). b, Gut microbiota composition as represented by principal coordinate analysis (PCoA) of Bray-Curtis distances for 3-, 8-, and 17-week-old HFD-fed mice (n = 6/group). c, Bray-Curtis dissimilarity index comparing distances of tylosin-treated groups to CON at different time points (n = 6/group). d, Volcano plot showing the enriched or decreased bacteria based on log2 fold change >1 and significant difference (p < 0.05) using the Wilcoxon signed-rank test (n = 6/group). e, Bray-Curtis distances-based PCoA of 17-week-old HFD-fed mice's gut microbiota and the fitted antibiotic exposure/obesity-related variables which significantly correlated to the shifted microbiome (p < 0.05) by using the envfit package in R (n = 5-6/group). f, Spearman's
correlation of PCoA microbiota and PC1 obesity biomarkers (n = 5-6/group). g, Body weight change in mice transplanted with feces of HFD-CON and HFD-TMDI mice (n = 10/group). h, Body weight change at 2 weeks after transplantation (ΔBW (W10-W8)) (n = 10/group). i, Relative fat mass at 20 weeks of age (n = 10/group). j, AUC derived from the OGTT (n = 9/group). k, HOMA-IR index (n = 9/group). Data are expressed as mean ± SD. For a, Statistical analyses were performed by Wilcoxon signed-rank test with FDR-adjust as follow: HFD-CON vs HFD-ADI (##p < 0.01); HFD-CON vs HFD-TMDI (**p < 0.01). For e, One-way ANOVA with Tukey’s range test (**p < 0.01; ***p < 0.001; ****p < 0.0001). For e, Wilcoxon signed-rank test with FDR-adjust. For g-k, unpaired t-test (*p < 0.05; ****p < 0.0001). Abbreviations: AUC, area under curve; CON, control; FMT, fecal microbiota transplantation; HOMA-IR, homeostatic model assessment of insulin resistance; OGTT, oral glucose tolerance test; TMDI, theoretical maximum daily intake.
Fig. 3 | Early-life exposure of tylosin residue sufficiently induces obesity and metabolic disorder disease and modifies gut microbiota composition by enhancing the re-structuring of obesity-related genera with deep-rooted change.

a, Experimental design of early-life exposure model. b, Body weight change (n = 8-11/group). c, Relative fat mass change (n = 8-11/group). d, Weight of visceral adipose tissue (n = 8-11/group). e, AUC derived from the OGTT (n = 7-8/group). f, Insulin level after overnight fasting (n = 7-8/group). g, HOMA-IR index (n = 7-8/group). h, PCoA based on Bray-Curtis distances of gut microbiota at 5 and 20 weeks of age (n = 8/group). i, Heatmap showing 32 bacteria with significant differences (q < 0.05) among groups and j, their Spearman's correlation with obesity-related variables (n = 8/group). Data are expressed as mean ± SD. For b-e, Statistical analyses were performed by one-way ANOVA with Tukey’s range test as follow: CON vs Early-TMDI (#p < 0.05 and ##p < 0.01), CON vs Cont-TMDI (*p < 0.05; **p < 0.01; ***p < 0.001), or Cont-TMDI
vs Early-TMDI (†p < 0.05). For **d-g**, One-way ANOVA with Tukey’s range test (*p < 0.05; **p < 0.01). For h, Adonis was performed to test the difference among groups. For i, Kruskal-Wallis test with FDR-adjusted p-value (q < 0.05). For j, Spearman’s correlation with FDR-adjusted p-value (* q < 0.1; **q < 0.05). Abbreviations: AUC, area under curve; CON, control; HFD, high-fat diet; HOMA-IR, homeostatic model assessment of insulin resistance; OGTT, oral glucose tolerance test; TMDI, theoretical maximum daily intake.
Fig. 4 | The modification of fecal primary-secondary bile acids ratio by gut microbiota and downregulates FGF15 signaling pathway is involved in the consequences of obesogenic and metabolic dysfunctions caused by tylosin residue exposure at TMDI dose.

a, Fecal short-chain fatty acids levels (n = 7/group). b, Ratio of fecal primary bile acids to secondary bile acids (n = 7-11/group). c, Levels of non-12-OH bile acids, d, muricholic acids and e, 12-OH bile acids (n = 7-11/group). f, Portal FGF15 levels (n = 6-11/group). g, Western blotting of ileal FGF15 expression normalized to GAPDH (n = 4/group). h, Western blotting of hepatic FGFR4 level normalized to GAPDH. Data are presented by mean ± SD (n = 4/group). For a-h, Statistical analyses were performed by one-way ANOVA with Tukey’s range test (*p < 0.05; **p < 0.01; ***p < 0.001).

Abbreviations: AGP, antibiotic growth promoter; α-MCA, α-muricholic acid; β-MCA, β-muricholic acid; ω-MCA, ω-muricholic acid; CON, control; CDCA,
chenodeoxycholic acid; FGF15, fibroblast growth factor 15; FGFR4, fibroblast growth factor receptor 4; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; LCA, lithocholic acid; PBA, primary bile acid; SBA, secondary bile acid; T-β-MCA, tauro-beta-muricholic acid; TCDCA, taurochenodeoxycholic acid; TLCA, tauro lithocholic acid; TMDI, theoretical maximum daily intake; TUDCA, tauroursodeoxycholic acid; UDCA, ursodeoxycholic acid.
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