


Effect of exposure to antibiotics on the gut microbiome and biochemical indexes of pregnant women

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ABSTRACT

Introduction Exposure to antibiotics (ABX) during pregnancy can have a systematic effect on both fetal and maternal health. Although previous biomonitoring studies have indicated the effects on children of extensive exposure to ABX, studies on pregnant women remain scarce. To explore the effect on pregnant women of environmental exposure to ABX through accidental ingestion and identify potential health risks, the present study investigated 122 pregnant women in East China between 2019 and 2020.

Research design and methods The presence of six categories of ABX (quinolones, sulfonamides, lincosamides, tetracyclines, amide alcohol ABX, and β -lactams) in plasma samples taken from the pregnant women was investigated using an ABX kit and a time-resolved fluorescence immunoassay.

Results All six ABX were detected in the plasma, with a detection rate of 17.2%. It was discovered that the composition of intestinal flora in pregnant women exposed to ABX was different from that of pregnant women who had not been exposed to ABX. The intestinal flora of pregnant women exposed to ABX also changed at both the phylum and genus levels, and several genera almost disappeared. Furthermore, the metabolic levels of glucose and insulin and the alpha diversity of pregnant women exposed to ABX were higher than those of pregnant women not exposed to ABX.

Conclusion Pregnant women are potentially at higher risk of adverse microbial effects. Glucose metabolism and insulin levels were generally higher in pregnant women exposed to ABX than in unexposed women. Also, the composition and color of the gut microbiome changed.

INTRODUCTION

As a group of pharmaceuticals and personal care products, antibiotics (ABX) are emerging environmental contaminants, and there is concern about the threat they pose to both aquatic life and human health.¹ Since the 1950s, growth-promoter ABX administered in low doses have been widely used in the agricultural industry to promote the growth of farm animals and aquatic life, resulting in an increased weight gain in the animals of as much as 15%.² The effect of this low dose

Significance of this study

What is already known about this subject?

- ▶ Antibiotic (ABX) administration and dietary changes have been associated with changes in the population structure of the gut microbiome. The effects on pregnant women of exposure to subtherapeutic ABX dosages in food have not been previously studied.

What are the new findings?

- ▶ The concentration of six different ABX in the plasma of pregnant women were detected, with tetracyclines found to be the most common.
- ▶ It was also found that gut microbiome composition and coloration changed as a result of exposure to antibiotics.
- ▶ The levels of glucose metabolism and insulin in pregnant women who had been exposed to ABX were generally higher than in those who had not.

How might these results change the focus of research or clinical practice?

- ▶ This study explored the environmental exposure of pregnant women to ABX through accidental ingestion by examining the approximate concentration of ABX in their plasma. Few previous studies have done this. Plasma ABX can indicate the exposure of pregnant women to ABX in food and drinking water, so the findings of the present study complement those of previous studies that are based on urinary antibiotic concentration, questionnaires, or prescription examination.

of ABX is broad across vertebrate species, including cattle, pigs, sheep, chickens, and fish. Seafood, including fish and shellfish, is a major source of dietary proteins, especially elements and omega-3 fatty acids,³ which are very important for the health of pregnant women.

The effects observed with many categories of antibacterial agents, such as macrolides, tetracyclines, and sulfonamides, indicate that the side effects are not agent specific and are not the same as those observed with

antifungal or antiviral agents. Antimicrobial agents are used in animal husbandry to treat disease, to control infections and prevent them from spreading in herds and flocks, and to promote animal growth.⁴ Two safety precautions have been implemented for antimicrobial agents: the first relates to their use in animals, which can lead to the development of antimicrobial-resistant bacteria and resistance determinants, which can be passed on to humans via the food chain or zoonotic spread; the second addresses the potential impacts on the human gut microbiome of ingesting animal-derived foods (such as meat, milk, eggs, and edible tissues) containing antimicrobial residue from the metabolism of a parent drug or other compounds used to treat the animal.⁵ In the USA, the most widespread use of low doses of ABX in animals is in farming for food production, where the ABX increase the animals' weight gain. The effects of these ABX follow oral administration, either in feed or water, indicating that the microbiota of the gastrointestinal (GI) tract is a major target.⁶

The vertebrate GI tract contains an exceptionally complex and dense microbial environment, with the gut microbiota influencing the immune response of populations of reactive host cells⁷ and stimulating a rich matrix of effector mechanisms involved in innate and adaptive immune responses.⁸ The GI tract also produces hormones, including those involved in energy homeostasis—insulin, glucagon, leptin, and ghrelin, for example—and growth factors, such as glucose-dependent insulinotropic polypeptide and glucagon-like peptide 1.⁹ Alterations in the populations of the gut microbiota may change the intra-community metabolic interactions.¹⁰ However, therapeutic doses of antibiotic treatments alter both the composition of the gut microbiota and the host responses to specific microbial signals.¹¹

Intrapartum antibiotic prophylaxis (IAP) disrupts the colonization of the neonatal gut flora, potentially further altering its development pattern. In addition, significant disruption of intestinal flora, including a decrease in bacterial diversity and a change in taxonomic distribution, has been observed after neonatal antibiotic treatment at birth.^{12–13} Previous studies have also identified a sustained drop in *Bifidobacterium* for up to 7 days after antibiotic treatment for group B streptococcus during pregnancy.¹⁴ Another study found that IAP can reduce the richness of intestinal microflora, decrease the abundance of *Bacteroidetes*, and increase the abundance of *Firmicutes* in 3-month-old newborns.¹⁵ Similar effects were observed within 8 weeks of intravenous β -lactam combination therapy in neonates or perinatal ABX in mothers.¹³ Furthermore, one study found that the use of ABX during delivery or after an emergency cesarean section changes the composition of neonatal intestinal flora, such as increased *Clostridiales* and decreased *Bacteroidaceae*, but that breast feeding narrows the differences in this composition.¹⁵

The use of ABX during pregnancy can also have effects on children later in life. One study found that children

were overweight at the age of 7 if their mother received broad-spectrum ABX, such as ampicillin and amoxicillin, in the second trimester of her pregnancy.¹⁶ This finding is supported by three studies that reported larger body size among children exposed to ABX in utero.^{17–19}

Antimicrobial agents of different classes and varying activity are effective across several vertebrate species; however, antibiotic administration and dietary changes have been associated with changes in the population structure of the gut microbiome.²⁰ The effects on pregnant women of exposure to subtherapeutic ABX dosages in food have not been previously studied. Therefore, the present study hypothesized that such subtherapeutic administration alters the composition of the gut microbiota and metabolic capabilities of pregnant women.

RESEARCH DESIGN AND METHODS

Study population and sample collection

A total of 122 pregnant women who were given a 75 g oral glucose tolerance test between 24 and 28 weeks of pregnancy at Shanghai General Hospital, Shanghai Jiao Tong University School of Medicine, between September 2019 and June 2020, were studied. Based on their homogenization of age, pre-pregnancy body mass index, gestational age, blood pressure, race, diet, and living environment, 37 of the patients were included in the study. These patients were separated into two cohorts: those patients without any detectable ABX in circulation (controls, n=25) and those patients who had detectable ABX in circulation (ABX detected, n=12).

Inclusion criteria:¹ residents in Songjiang District of Shanghai had a normal diet;² they did not have diabetes or impaired glucose tolerance before pregnancy.

Exclusion criteria:¹ multiple births;² diabetes, hypertension, thyroid disease, gastrointestinal disease, or cardiovascular disease before pregnancy;³ use of assisted reproductive technology;⁴ all patients consuming a “prescribed antibiotics”;⁵ active smoking.

Most maternal characteristics, fasting plasma glucose (FPG) levels, 1-hour plasma glucose (1 h PLG), 2-hour plasma glucose (2 h PLG), fasting insulin levels (FINS), 1-hour plasma insulin (1 h PIN), 2-hour plasma insulin (2 h PIN), glycosylated hemoglobin (HbA1c), homeostasis model assessment (HOMA-IR), triglyceride (TG) levels, total cholesterol (TC), high-density lipoprotein (HDL), and low-density lipoprotein (LDL) levels were obtained from the patients' medical records. All serum aliquots and fresh feces were collected and immediately stored at -80°C prior to DNA extraction.

DNA extraction

A frozen aliquot (200 mg) of each fecal sample was suspended in 250 μL of guanidine thiocyanate (Sigma-Aldrich, GER), 0.1 M tris (pH 7.5) (Amresco, USA), and 40 μL of 10% *N*-lauroyl sarcosine (Sigma-Aldrich, GER). Total bacterial genomic DNA samples were extracted from 53 samples using a QIAamp DNA stool mini kit

(Qiagen, California, USA). A NanoDrop 2000 spectrophotometer (Thermo Scientific, USA) was used to detect the concentration of the extracted DNA. Any samples that did not meet the detection standards were removed.

16S ribosomal RNA (16S rRNA) amplicon pyrosequencing

The V3 and V4 variable regions of 16S rRNA were specifically amplified by PCR with the forward primer 338F (5'-ACTCCTACGGGAGGCAGCAG-3') and the reverse primer 806R (5'-GGACTACHVGGGTWTCTAAT-3'), where the barcode was an eight-base sequence unique to each sample. PCR was performed by DNA Extraction Kit (OMEGA-soil DNA Kit; Omega Bio-Tek, USA) that in a triplicate 20 µL mixture containing 4 µL of 5× FastPfu Buffer, 2 µL of 2.5 mM dNTPs, 0.8 µL of each primer (5 µM), 0.4 µL of FastPfu Polymerase (FastPfu Polymerase; TransGen, China), and 10 ng of template DNA, and it was conducted under the following conditions: initial denaturation (95°C, 2 min), 25 cycles at 95°C (30 s), annealing at 55°C (30 s), extension at 72°C (30 s), final extension at 72°C (5 min), 10°C until halted by user. The PCR products were extracted from 2% agarose gels (Biowest agarose; Biowest, Spain) and purified using an AxyPrep DNA gel extraction kit (Axygen Biosciences, Union City, CA, USA) according to the manufacturer's instructions. They were then quantified using a QuantiFluor-ST fluorimeter (Promega, USA) before being sequenced. A database was established using an Illumina MiSeq instrument (Illumina, San Diego, California, USA) at SHBIO Biotechnology Corporation (Shanghai, China).

Sequence analysis

Raw FASTQ files were demultiplexed and quality filtered using QIIME V.1.9.1 (Knight and Caporaso Labs, USA) with default parameters. Operational taxonomic units (OTUs) were clustered with 97% similarity cut-off using UPARSE (V.7.1, <http://drive5.com/uparse/>), and chimeric sequences were identified and removed using UCHIME (usearch 8.1.1861, Robert C. Edgar lab, independent scientist, USA). The taxonomy of each 16S rRNA gene sequence was analyzed using the Ribosomal Database Project classifier (<http://rdp.cme.msu.edu/>) against the SILVA (SSU123) 16S rRNA database, using a confidence threshold of 70%.

Detection of ABX in the serum aliquots

Six major categories of ABX (quinolones, sulfonamides, lincosamides, tetracyclines, amide alcohol antibiotics, and β-lactams) were studied using a time-resolved fluorescence immunoassay (version SS-030, Shanghai Tracing-Bio Technology Co., Ltd., Shanghai, China). The serum aliquots, which had been stored at -80°C, and the detection ABX kits (Antibiotics, Shanghai, China) were returned to room temperature. A total of 150 mL diluent was then fully mixed and dissolved into the lyophilized sample for a tetracycline drug test. Next, 10 µL of serum sample was added to the lyophilized product and mixed well, and a reagent strip was inserted vertically and the

time counted. After 6 min, the reagent strip was removed and placed in the time-resolved immunoassay analyzer for the results. The limit of positive judgment for tetracycline concentration was 15 ng/mL. A + indicated that the concentration of tetracycline was between 15 and 60 ng/mL, while ++ indicated that the concentration was greater than 60 ng/mL. The concentration limits of β-lactams, quinolones, sulfonamides, lincosamides, and amide alcohol ABX were 4, 25, 4, 10, and 1 ng/mL, respectively.

Bioinformatics and statistical analysis

The QIIME (V.1.9.1) and R packages were used for gut microbiota sequence analyses. Alpha diversity indices, such as Chao richness estimator, Shannon diversity index, observed species, and coverages, were calculated using the OTUs table in QIIME to investigate gut microbiota community richness. Beta diversity was measured using the Bray-Curtis dissimilarity and unweighted and weighted UniFrac distance.²¹ Beta diversity analysis, including principal co-ordinates analysis based on UniFrac distance matrix analysis and visualized via non-metric multidimensional scaling, was used to evaluate the structural variation of microbial communities. The total microbial composition difference between the two groups was indicated by permutational multivariate analysis of variance.²² Tax4Fun,²¹ an R package obtained from the SILVA dataset version 132, was used for functional profiling. Pattern recognition analysis based on forward feature selection and linear discriminant analysis (LDA) was performed using R V.3.5.1.²³ Unique OTUs and those shared between samples were illustrated using a Venn diagram that was created in the R package "Venn Diagram". The relative abundance of taxa at all levels was statistically compared between the two groups using the Kruskal-Wallis test in the R stats package, and the microbiota correlation network was constructed using the SparCC algorithm²⁴ and visualized with Cytoscape V.3.4.0.²⁵

Normal distributed continuous variables were presented as mean±SD and analyzed using Student's t-test, while non-normal distributed continuous variables were reported as median with IQRs (Q1–Q3) and analyzed using either the Wilcoxon signed-rank test or the Mann-Whitney U test, which were conducted in SPSS V.23.0 (SPSS Inc., Chicago, IL, USA). A p value <0.05 was considered statistically significant.

For dichotomous variables, the McNemar χ^2 test, Pearson's χ^2 test, or Fisher's exact test was applied. LDA effect size²⁶ with a p-value cut-off of 0.05 and an LDA score cut-off of 2 was used to obtain the differential taxa and functions of the two groups. Spearman's rank correlation was used for correlation analysis between the groups.

RESULTS

General characteristics

The characteristics of the participants are presented in [table 1](#). There were no differences between the two

Table 1 Maternal characteristics and biochemical data

Maternal characteristics and biochemical variables	Controls (n=25)	ABX detected (n=12)	P value
Age (years)	30.6±4.85	31±5.31	0.839
Pre-BMI (kg/m ²)	22.5±4.98	21.3±2.0	0.442
Gestational age (weeks)	25.7±1.22	25.2±1.11	0.208
SBP (mmHg)	107.4±10.0	110.7±10.4	0.347
DBP (mmHg)	70.2 (66~74)	67.8 (65~70.7)	0.282
Gravidity	1.8 (1~2.5)	2.1 (1.3~3)	0.184
Parity	0.5 (0~1)	0.6 (0~1)	0.657
Fasting glucose (mmol/L)	4.89 (4.56~4.96)	4.94 (4.76~4.95)	0.411
1 h glucose (mmol/L)	7.61±2.17	7.89±1.15	0.746
2 h glucose (mmol/L)	6.69±2.11	6.09±0.60	0.335
HbA1c (mmol/L)	4.94±0.57	4.99±0.44	0.773
Fasting insulin (μU/L)	50.1 (33.1~62.1)	52.4 (34.3~63.0)	0.546
1 h insulin (μU/L)	319.0±170.7	397.7±139.1	0.176
2 h insulin (μU/L)	279.1±159.2	208.2±68.8	0.152
HOMA-IR	1.56 (0.99~2.01)	1.65 (1.01~2.18)	0.535
Triglyceride (mmol/L)	2.71±0.84	2.24±0.77	0.114
Total cholesterol (mmol/L)	6.38±1.07	5.64±1.11	0.064
HDL (mmol/L)	2.05 (1.72~2.15)	2.01 (1.68~2.31)	0.687
LDL (mmol/L)	3.13±0.89	2.69±1.01	0.184

Clinical characteristics, biochemical and hormonal variables of antibiotic exposure and normal healthy pregnant women at 24–28 weeks' gestation are presented as mean±SEM when normally distributed or median with 25–75th IQR when non-normal distributed. Statistically significant difference between the GDM and normoglycemic women group are highlighted (*p<0.05, **p<0.01). HOMA-IR, HOMA-IR=FPG (mmol/L) × FINS (μU/mL)/22.5.

ABX, antibiotics; BMI, body mass index; DBP, diastolic blood pressure; FINS, fasting insulin levels; FPG, fasting plasma glucose; HbA1c, glycosylated hemoglobin; HDL, high-density lipoprotein; HOMA-IR, homeostasis model assessment; LDL, low-density lipoprotein; SBP, systolic blood pressure.

groups in markers of age, pre-pregnancy body mass index, gestational age, systolic blood pressure, diastolic blood pressure, gravidity, or parity. The patients in the ABX detection group had higher FPG, 1 h PLG, HbA1c, FINS, 1 h PIN, and HOMA-IR levels than those in the control group, although there were no statistically significant differences. The two groups had similar 2 h PLG, 2 h PIN, TG, TC, HDL, and LDL levels.

Exposure to antibiotics

Of the 122 patients initially studied, 21 tested positive for ABX in venous blood tests (table 2). One patient was exposed to β-lactams, one to quinolones, one to amide alcohol ABX, three to lincosamides, four to sulfonamides, and eighteen to tetracyclines. Six of the patients had two or more ABX in their blood. The antibiotic exposure rate was 17.2%.

The limit of positive judgment for detected tetracycline concentration was 15 ng/mL. The detected concentration limit of β-lactams was 4 ng/mL. The detected concentration limit of quinolones was 25 ng/mL. The limit of positive judgment for sulfonamide detected concentration was 4 ng/mL. The limit of positive judgment for lincosamides detected concentration was 10 ng/mL. The limit of positive judgment for amide alcohol ABX detected concentration was 1 ng/mL.

Altered gut microbiota in pregnant women exposed to ABX

In the control group, the total number of OTUs in common was 4392, while the ABX detection group had 6393 OTUs in common; 1013 OTUs were shared between the two groups (figure 1A). Alpha diversity analysis (figure 1B) indicated that the antibiotic exposure of the ABX detection group presented higher

Table 2 Holistic situation of antibiotic exposure in 122 pregnant women at 24–28 weeks' gestation

β-Lactams	Sulfonamides	Quinolones	Amide alcohol antibiotics	Tetracyclines	Lincosamides
n=1	n=4	n=1	n=1	n=18	n=3

n represents the number of pregnant women whose ABX examination was positive.
ABX, antibiotics.

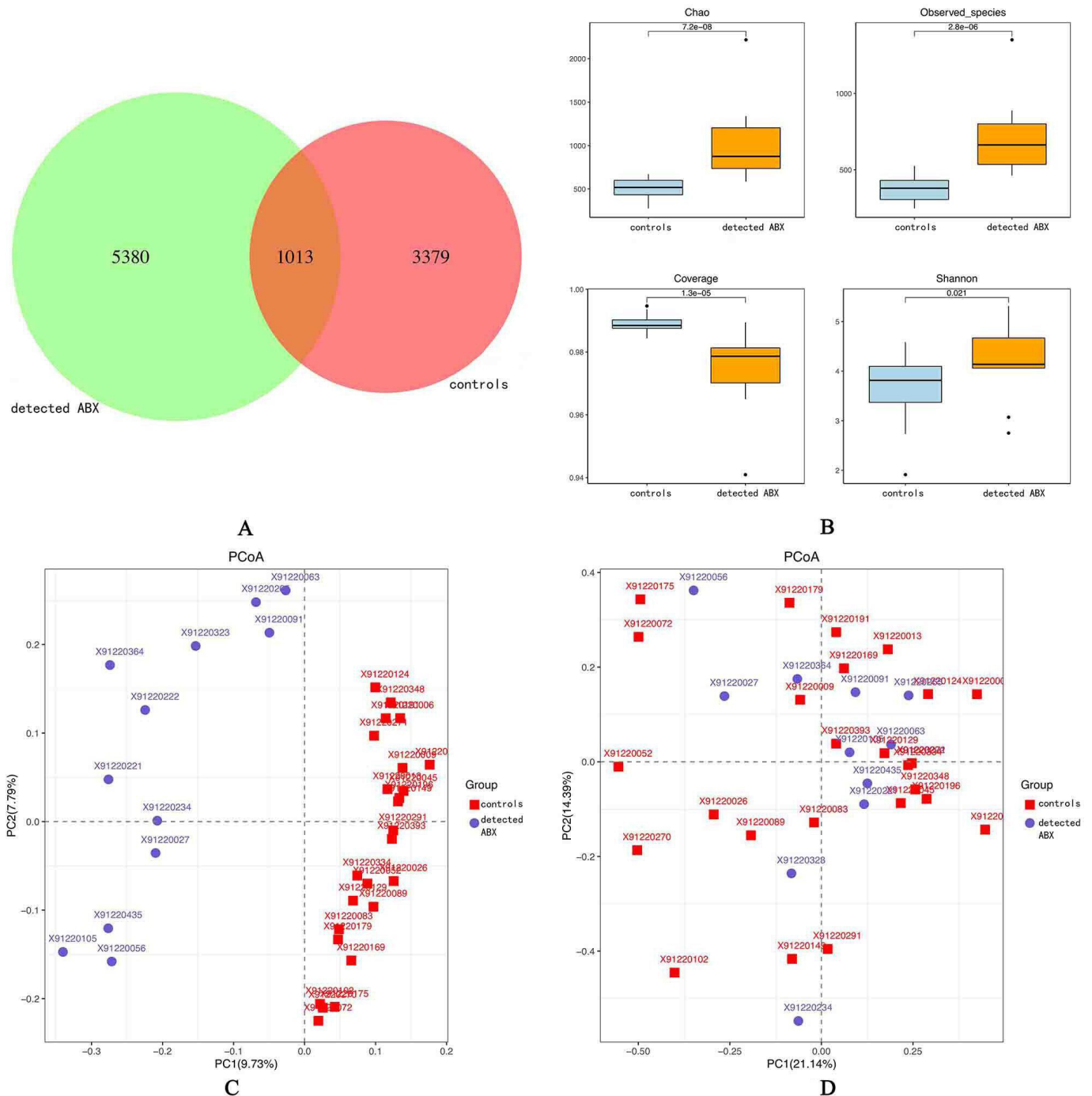


Figure 1 Richness and diversity of the gut microbiota in pregnant women who had been exposed to antibiotics (ABX) and those who had not. (A) Venn diagram of exposure to ABX in pregnant women; controls had not been exposed to ABX while detected ABX had. Detected ABX had more operational taxonomic units than controls. (B) Alpha diversity was calculated using QIIME2 V.2018.2 based on a sequence similarity level of 100%, including the Chao richness estimator, observed species, coverage, and the Shannon diversity index. Detected ABX showed higher alpha diversity than controls. Principal coordinates analysis based on the unweighted UniFrac matrix (C) and weighted UniFrac matrix (D) showed that the overall fecal microbiota composition was different between the two groups. Each point represents one sample of antibiotic exposure (red, controls, n=25; blue, detected ABX, n=12). The distances between different samples reflect the comparability of the two groups.

richness (higher Chao estimator, $p=7.2e-08$; higher observed species, $p=2.8e-06$) and higher diversity (lower coverage $p=1.3e-05$; higher Shannon index, $p=0.021$) than that detected in the control group. Principal coordinate analysis was conducted based on unweighted UniFrac distance to evaluate the overall

diversity of microbial composition. Permutational multivariate ANOVA demonstrated significant differences in gut taxonomic composition between the two groups (figure 1C). The differences in microbial community between the two groups were also significant for weighted UniFrac distance (figure 1D).

Table 3 Microbiome composition in phylum and genera levels of antibiotic exposure and normal healthy pregnant women are different at 24–28 weeks' gestation

	Controls (n=25)	ABX detected (n=12)	P value
<i>Chao</i>	681.8±140.2	1537.3±564.6	<0.01
<i>Shannon</i>	3.82±0.67	4.41±0.73	0.022
OTUs	459.7±85.0	899.0±284.5	<0.01
Phylum			
<i>Firmicutes</i>	0.589±0.192	0.682±0.138	0.145
<i>Bacteroidetes</i>	0.229±0.155	0.204±0.126	0.639
<i>Proteobacteria</i>	0.119 (0.025–0.160)	0.073 (0.021–0.080)	0.119
<i>Actinobacteria</i>	0.033 (0.013–0.044)	0.012 (0.015–0.021)**	0.004
<i>Verrucomicrobia</i>	0.017 (0–0.015)	0.003 (0–0.002)	0.135
<i>Tenericutes</i>	0.006 (0–0.005)	0.003 (0–0.001)	0.289
<i>Other</i>	0.002 (0–0.002)	0.006 (0.0002–0.008)	<0.01
Genera			
<i>Bacteroides</i>	0.184 (0.066–0.240)	0.160 (0.072–0.208)	0.721
<i>Faecalibacterium</i>	0.162 (0.072–0.222)	0.189 (0.188–0.247)	0.119
<i>Other_A</i>	0.137 (0.071–0.161)	0.161 (0.094–0.219)	0.256
<i>Megamonas</i>	0.034 (0–0.011)	0.018 (0–0.004)	0.088
<i>Lachnospiraceae</i>	0.020 (0.004–0.024)	0.020 (0.008–0.031)	0.721
<i>EscherichiaShigella</i>	0.029 (0.001–0.041)	0.008 (0–0.006)	0.098
<i>Ruminococcaceae_UCG014</i>	0.051 (0–0.05)	0.016 (0–0.037)	0.459
<i>Incertae_sedis</i>	0.004 (0–0.004)	0.042 (0.002–0.025)*	0.019
<i>Eubacterium_coprostanoligenes_group</i>	0.03 (0.005–0.050)	0.007 (0–0.007)**	0.009
<i>Ruminococcaceae_UCG002</i>	0.028 (0–0.017)	0.021 (0–0.023)	0.407
<i>Christensenellaceae_R7_group</i>	0.026 (0.005–0.035)	0.004 (0–0.008)**	0.001
<i>Dialister</i>	0.017 (0–0.011)	0.033 (0–0.048)	0.060
<i>Bifidobacterium</i>	0.013 (0.002–0.016)	0.010 (0.001–0.015)	0.338
<i>Hafnia</i>	0.011 (0–0)	0 (0–0)**	0.845
<i>Subdoligranulum</i>	0.024 (0–0.029)	0.006 (0–0.01)*	0.024
<i>Ruminococcus_2</i>	0.015 (0.001–0.024)	0.006 (0–0.016)*	0.031
<i>Prevotella_9</i>	0.001 (0–0)	0.008 (0–0.020)	0.017
<i>Lachnospiraceae_UCG004</i>	0.003 (0–0.003)	0.006 (0.004–0.009)	0.004
<i>Rhodococcus</i>	0.008 (0.007–0.007)	0.0002 (0–0.0003)**	<0.01

Significant differences between two groups show in **/*, *p<0.05,**p<0.01, the diversity and microbiome composition are presented as mean±SEM when normally distributed or median with 25–75th IQR when non-normal distributed the decreased genera in GDM group are in boldface.

ABX, antibiotic; OTU, operational taxonomic unit.

The diversity of the fecal microbiome in the two groups was found to be mainly related to six phyla—*Firmicutes*, *Bacteroidetes*, *Proteobacteria*, *Actinobacteria*, *Verrucomicrobia*, and *Tenericutes*—and those under the category, “Other” (table 3). The 10 most common phyla in the two groups are shown in figure 2A. The predominant genus in both groups was *Firmicutes* (control group: 58.9%; ABX detection group: 68.2%), and there was no significant difference between the groups. *Actinobacteria* was lower in the ABX detection group than in the control group, and the difference was statistically significant (control group:

3.3% vs ABX detection group: 1.2%; p=0.004). The other four genera were lower in the ABX detection group than in the control group but with no statistically significant differences (*Bacteroidetes*, control group: 22.9% vs ABX detection group: 20.4%; *Proteobacteria*, control group: 11.9% vs ABX detection group: 7.3%; *Verrucomicrobia*, control group: 1.7% vs ABX detection group: 0.3%; *Tenericutes*, control group: 0.3% vs ABX detection group: 0.6%).

To explore the altered gut microbiota in pregnant women exposed to antibiotics, the 16 most common

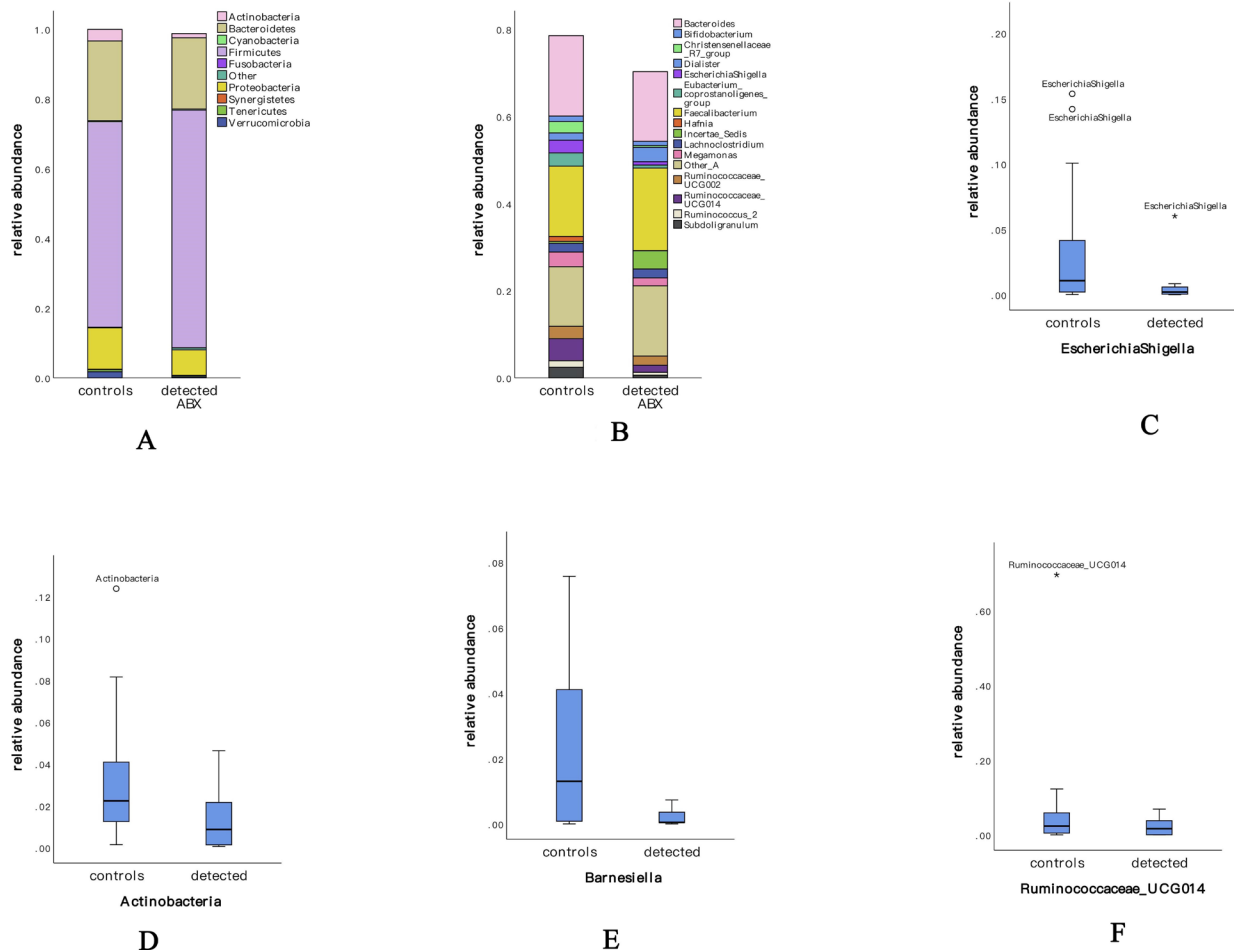


Figure 2 Composition of the gut microbiome at phylum and genera levels and several almost-disappeared genera in the second trimester. (A, B) The composition of the gut biome microflora of the two groups, indicating the dominant microflora. (C) The genera of *Escherichia–Shigella*, which are sensitive to sulfonamide antibiotics, almost disappeared in the gut microbiomes of pregnant women who had been exposed to antibiotics. (D) The phylum of Actinobacteria and (E) the genus of *Barnesiella* were sensitive to tetracycline antibiotics and declined or disappeared in pregnant women who had been exposed to antibiotics. (F) Ruminococcaceae UCG-014 was affected by both sulfonamides and tetracyclines and declined in women who had been exposed to antibiotics. ABX, antibiotics.

genera (figure 2B) were studied. The predominant genus in both groups was *Bacteroidetes*, although it was present in lower levels in the ABX detection group (control group: 18.4% vs ABX detection group: 16%; $p=0.721$). At the genus level, the ABX detection group showed a significantly lower abundance of the *Eubacterium coprostanoligenes* group ($p=0.009$), the *Christensenellaceae R7* group ($p=0.001$), *Subdoligranulum* ($p=0.024$), *Ruminococcus 2* ($p=0.031$), *Prevotella 9* ($p=0.017$), *Lachnospiraceae UCG-004* ($p=0.004$), and *Rhodococcus* ($p=0$). The relative abundance of *Megamonas*, *Escherichia–Shigella*, *Ruminococcaceae UCG-014*, and *Ruminococcaceae UCG-002* in the ABX detection group was also lower than in the control group, although there were no significant differences (table 3). These findings point to changes in the gut microbiota of pregnant women exposed to antibiotics.

Effects on the gut microbiome of exposure to ABX

Two sulfonamide and tetracycline ABX were found to have an influence on the gut microbiome (table 4).

Escherichia–Shigella ($p=0.034$), *Ruminococcus 2* ($p=0.016$), and *Alistipes* ($p=0.045$) were found to be sensitive to sulfonamide antibiotics, and their relative abundance decreased significantly in pregnant women whose blood samples were positive for sulfonamides. The presence of *Escherichia–Shigella* (figure 2C) could hardly be detected. The abundance of *Actinobacteria* ($p=0.035$) (figure 2D), the *Eubacterium coprostanoligenes* group ($p=0.034$), the *Christensenellaceae R7* group ($p=0.004$), and *Subdoligranulum* ($p=0.025$) in the intestines of pregnant women who were positive for tetracyclines was significantly decreased, while *Barnesiella* ($p=0.04$) (figure 2E) almost disappeared. *Ruminococcaceae UCG-014* declined in the ABX detection group (figure 2F) and was affected by both sulfonamides and tetracyclines.

Association of microbial composition with glycaemic traits

Spearman correlation analysis was used to identify deeper-level taxa associated with glycaemic traits in pregnant women (table 5). It was found that *Actinobacteria* was

Table 4 Holistic situation of antibiotic exposure in 122 pregnant women at 24–28 weeks' gestation

	Sulfonamides		Tetracyclines	
	Controls	Detected ABX	Controls	Detected ABX
Phylum				
-	-	-	0.031 (0.012~0.044)	0.014 (0.001~0.02)
-	-	-	0.002 (0~0.002)	0.006 (0.002~0.008)
Genera				
<i>EscherichiaShigella</i>	0.024 (0.001~0.036)	0	0.028 (0.004~0.024)	0.008 (0.001~0.008)
<i>Ruminococcus_2</i>	0.027 (0.0015~0.015)	0.016 (0.027~0)	0.025 (0.004~0.03)	0.004 (0~0.007)
<i>Alistipes</i>	0.0096 (0.0012~0.012)	0	0.023 (0.004~0.029)	0.006 (0~0.007)
			0.012 (0~0.016)	0
				0.04

ABX, antibiotics.

not significantly positively or negatively associated with blood glucose and lipid metabolism-related indicators. *Escherichia-Shigella* was significantly affected by sulfonamides and was only found to be negatively correlated with HDL. *Barnesiella* was influenced by tetracyclines and had a negative correlation with HbA1c ($r = -0.330$, $p = 0.046$), FINS ($r = -0.421$, $p = 0.009$), and HOMA-IR ($r = -0.435$, $p = 0.007$). *Ruminococcaceae UCG-014* was lower in the ABX detection group than in the control group and had a negative association with HbA1c ($r = -0.397$, $p = 0.015$), 1 h PLG ($r = -0.490$, $p = 0.002$), FINS ($r = -0.396$, $p = 0.015$), 1 h PIN ($r = -0.362$, $p = 0.027$), and HOMA-IR ($r = -0.408$, $p = 0.012$).

DISCUSSION

This is the first study to explore the effect of environmental exposure to ABX through accidental ingestion on the gut microbiome of pregnant women. The blood concentration of ABX is very low—far lower than that of oral or intravenous antibiotics. The study population was strictly screened to exclude those who had used prescribed ABX, so it was concluded that the exposure to ABX was more likely to be from food or drinking water. ABX of accidental ingestion are emerging contaminants, and there is concern about their possible threat to human health and aquatic life.¹ ABX consumed in human and animal medicine can directly or indirectly enter the human body, other animals, food, drinking water, and the environment.²⁷ In humans, ABX entering the body through medical use, contaminated food, or the environment can reach the urinary and intestinal tracts through excretion in urine and feces, facilitating the emergence or immigration of resistant genes and bacteria in these sites. This poses a potential risk of infection by resistant bacteria.²⁸ Exposure to ABX may influence childhood-onset asthma, childhood obesity, and colorectal carcinoma.²⁹ Recently, it has been found that the disturbance of the gut microbiome through exposure to ABX has a profound influence on human physiology.³⁰

Compared with children and other adults, pregnant women have different requirements for dietary nutrients and are treated more prudently with regard to drug use; as such, they may have a different ABX exposure profile.³¹ The present study measured six types of antibiotics: β -lactams (which is the only one routinely given to pregnant women), tetracyclines, sulfonamides, quinolones, amide alcohol ABX, and lincosamides (which are rarely given to pregnant women). The study found that 21 of 122 pregnant women were positive for ABX in venous blood tests. Tetracyclines and sulfonamides were found to have the greatest effect on the gut microbiome; for example, *Escherichia-Shigella*, *Alistipes*, and *Barnesiella* almost disappeared in response to exposure to these antibiotics.

Because of the side effects of enamel hypoplasia and the irreversible staining of deciduous teeth,³² tetracyclines are not given to children under 8 years of age³³; instead, they are mainly used as a veterinary ABX.³⁴ In clinical

Table 5 Gut microbiota abundance (phylum and genus) and their correlation with clinical characteristics and biochemical variables in antibiotic exposure and normal health pregnant women

	HbA1c (mmol/L)		1 h plasma glucose (mmol/L)		Fasting insulin (μU/L)		1 h plasma insulin (μU/L)		HOMA-IR		HDL (mmol/L)	
	r value	P value	r value	P value	r value	P value	r value	P value	r value	P value	r value	P value
p_Actinobacteria	–	–	–	–	–	–	–	–	–	–	–	–
g_EscherichiaShigella	–	–	–	–	–	–	–	–	–	–	–0.345*	0.037
g_Barnesiella	–0.330*	0.046	–	–	–0.421**	0.009	–	–	–0.435**	0.007	–	–
g_Ruminococcaceae_UCG014	–0.397*	0.015	–0.490*	0.002	–0.396*	0.015	–0.362*	0.027	–0.408*	0.012	–	–

Data are Spearman correlation coefficients. Significant correlations showed. *p<0.05, **p<0.01. p_, phylum level; g_, genus level. HbA1c, glycosylated hemoglobin; HDL, high-density lipoprotein; HOMA-IR, homeostasis model assessment.

practice, sulfonamides have been largely replaced by the safer β-lactams or macrolides.^{35,36} Moreover, these two ABX are used in animals for growth promotion, disease prevention, as feed additives, and for the treatment of infection,³⁷ and most of them are frequently detected in surface water or food in China.^{38,39}

Given that ABX are extensively used in animal husbandry and aquaculture to prevent and treat disease or promote growth,⁴⁰ meat may have substantial antibiotic residues; a previous study found that fish, shrimp, pork, chicken, and milk are the most common sources of exposure to veterinary ABX.³¹ Based on the present study, it can be suggested that the presence of tetracyclines, quinolones, and sulfonamides in the plasma samples of pregnant women mainly resulted from contaminated environments or food.

A previous study⁴¹ analyzed the biological effect of low-dose ABX exposure on pregnant mice and its impact on the body-fat content of newborn mice. It found that (1) maternal low-dose antibiotic exposure in late pregnancy affected the intestinal flora structure of the offspring after birth and had a profound impact on their subsequent development process and health, (2) maternal low-dose antibiotic exposure led to a significant increase in the body weight and body fat of the offspring, and (3) maternal antibiotic exposure during pregnancy led to excessive growth of *Firmicutes* and *Bacteroidetes* in the intestinal flora of the offspring. An unbalanced *Firmicutes*-to-*Bacteroidetes* ratio causes intestinal flora disorder, indirectly promotes intestinal flora by affecting the short-chain fatty acids and blood-glucose metabolism, and eventually leads to gestational diabetes mellitus. Furthermore, Yoshimoto *et al* found that administration of low-dose ABX from 1 week before pregnancy to birth affected the composition of the gut microbiota and abdominal fat percentage of the pups. Microbiome changes mainly included the increased abundance of *Firmicutes* and *Clostridium*; *Clostridium IV* and *XIVa* were also positively associated with increased abdominal fat percentage, while *Firmicutes* and *Bacteroidetes* did not have any correlation with body-fat percentage.⁴¹ Chao *et al* reported that the penicillin, vancomycin, and chlor-tetracycline ABX treatment of pups at subtherapeutic levels for 7 weeks increased the abundance of *Firmicutes*,

body-weight gain, and levels of colonic short-chain fatty acids.⁶ The present study found that the group of women exposed to ABX had a higher alpha diversity and more OTUs. Among the six most common phyla, the relative abundance of *Firmicutes*, which has been previously found to be associated with gestational weight gain at a nominal significance threshold,⁴² increased by 10%. *Bacteroides*, *Megamonas*, *Escherichia–Shigella*, *Ruminococcaceae UCG-014*, and *Rhodococcus*, however, all decreased. *Bacteroides*, which can harbor butyrate-producing members, has been previously found to be negatively associated with blood pressure, body weight, and fat mass.⁴³ The present study also found that the decreased abundance of *Escherichia–Shigella*, *Barnesiella*, and *Ruminococcaceae UCG-014* had negative associations with HbA1c, 1 h PLG, FINS (μU/L), 1 h PIN, and HOMA-IR. *Ruminococcaceae*, which can break down indigestible polysaccharides (ie, dietary fiber) into short-chain fatty acids, such as butyrate, have been previously found to have a strong positive correlation with adipokine levels.⁴⁴

One previous study assessed the effect of amoxicillin on the diversity of intestinal flora and found no effect.⁴⁵ Rifaximin, however, has been found to decrease the abundance of *Streptococcus* spp and *Veillonella* spp and increase the abundance of *Eubacterium* spp, although it has not been found to reduce the diversity of the intestinal flora.^{46,47} Other studies have found that minocycline can increase the relative abundance of *Alistipes* spp, *Bacteroides* spp, and *Marvinbryantia* spp. In addition, it has been found that the abundance reduction of *Anaerostipes* spp, *Bifidobacterium* spp, *Collinsella* spp, *Coprococcus* spp, *Dialister* spp, *Dorea* spp, and *Faecalibacterium* spp eventually reduced the diversity of the bacteria community.⁴⁵ Some studies have found that amoxicillin, which belongs to β-lactams, can increase the abundance of *Alistipes*, *Bacteroides*,⁴⁵ and *Ruminococcus*,⁴⁸ while clindamycin, which belongs to lincosamides, has been found to significantly reduce the abundance of *Alistipes*,⁴⁵ which is consistent with the findings of the present study. *Alistipes* and *Ruminococcus* can produce short-chain fatty acids, and butyric acid can prevent colon cancer and improve insulin sensitivity.⁴⁹

In a mouse model of autoimmune encephalomyelitis before delivery and during weaning, antibiotic exposure

increased the abundance of *Proteobacteria* and *Bacteroidetes* and decreased the abundance of *Firmicutes* and *Actinomyces*; this imbalance of field-intestinal flora coincided with high expression of pro-inflammatory factors, such as IFN- γ and IL-17.⁵⁰ Antibiotic-induced intestinal microflora imbalance can reduce corneal nerve density by reducing the production of CCR2-corneal macrophages, and it has been suggested that it can be reversed by fecal transplanting or practical probiotics.⁵¹ *Ruminococcaceae UCG-014* belongs to *Ruminococcaceae*, and both sulfonamides and tetracyclines can reduce its abundance. *Lachnospiraceae* and *Ruminococcaceae*, both belonging to *Firmicutes*, are dominant bacteria in energy metabolism, which decomposes dietary fiber to produce butyrate;⁵² they have been found to be negatively correlated with 1-hour blood glucose levels in an oral glucose tolerance test⁵³ and positively correlated with serum leptin levels.⁴⁴

This study explored the environmental exposure of pregnant women to ABX through accidental ingestion by examining the approximate concentration of ABX in their plasma. Few previous studies have done this. Plasma ABX can indicate the exposure of pregnant women to ABX in food and drinking water, so the findings of the present study complement those of previous studies that are based on urinary antibiotic concentration, questionnaires, or prescription examination. However, there are several limitations that should be considered when interpreting the findings of this study. First, pregnant women who undergo obstetric examination at a local health service organization may have different education and economic levels that influence their dietary habits and attitudes to the use of medicine. Second, due to the fact that most of the ABX examined in this study have a fast metabolic rate, with a half-life of less than 12 hours, it is difficult to capture every instance of exposure to ABX with one spot plasma sample; therefore, the actual level of exposure may be underestimated. Third, ABX were only detected in the patients' blood, and the intermediate metabolites of ABX were not tested. Lastly, the sample size and sampling location should be expanded in future studies to further validate the experimental conclusion.

CONCLUSION

The concentration of six different ABX in the plasma of pregnant women were detected, with tetracyclines found to be the most common. It was also found that gut microbiome composition and coloration changed as a result of exposure to antibiotics. The levels of glucose metabolism and insulin in pregnant women who had been exposed to ABX were generally higher than in those who had not. In view of the very low blood concentration of ABX and the rules regarding the use of ABX during pregnancy, it is suggested that the presence of ABX in the plasma of pregnant women may come from food and drinking water. In addition to the findings of this study, the effects on the growth and development of pregnant women and fetuses

need to be further studied and given greater attention by both medical staff and pregnant women.

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Contributors YS, X-MX, and X-PG conceived the idea and conceptualized the study. YS, H-HQ, and F-FL collected the data. X-MX, D-CC, and D-YZ analyzed the data. YS, X-MX, W-YL, and H-KW drafted the manuscript, then LC, J-FZ, and Y-NC reviewed the manuscript. All authors read and approved the final draft. X-MX is responsible for the overall content as guarantor. The guarantor accepts full responsibility for the finished work and/or the conduct of the study, had access to the data, and controlled the decision to publish.

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