Time-course Alterations of Gut Microbiota and Short-chain Fatty Acids After Short-term Lincomycin Exposure in Young Swine

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Research

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Abstract

Background: Increasing evidence suggests that antibiotic administration causes gut injury, nutrient digestion, immune regulation, and colonization resistance against pathogens due to the disruption of gut microbiota. However, the time-course effects of therapeutic antibiotics on alterations of gut microbes and short-chain fatty acid in young swine are still unknown. In this study, twenty piglets were assigned into two groups and fed commercial diets with or without lincomycin in the first week for a 28-day trial period.

Results: Data showed that one-week lincomycin exposure did reduce the body weight on day 14 ($p = 0.0450$) and 28 ($p = 0.0362$). The alpha-diversity notably reduced after one-week lincomycin treatment, then gradually raised and reached the control group level in the second week on cessation of lincomycin exposure, indicated by the variation of Sobs, Chao, Shannon, and ACE index ($p < 0.05$). Beta-diversity analysis revealed that the distinct microbial cluster existed persistently for the whole trial period between two groups ($p < 0.001$). The relative abundance of most microbes including fiber-degrading (e.g., *Agathobacter*, *Coprococcus*, etc.), beneficial (e.g., *Lactobacillus*, *Mitsuokella*, etc.), or pathogenic bacteria (e.g., *Terrisporobacter*, *Lachnoclostridium*, etc.) decreased and the concentration of short-chain fatty acids also diminished in the feces of one-week lincomycin-administrated young swine (LDA score > 3). After the lincomycin administration stopped, the state of gut dysbiosis gradually recovered and formed new gut-microbe homeostasis distinct from microbial homeostasis of young pigs unexposed to lincomycin. The increased presence of potential pathogens, such as *Terrisporobacter*, *Negativibacillus*, *Escherichia-Shigella*, etc. and decreased beneficial bacteria such as *Lactobacillus*, *Agathobacter* were observed in new homeostasis reshaped by short-lincomycin administration (LDA score > 3 or $p < 0.05$), adversely affecting gut development and health of young pigs.

Conclusion: Collectively, these results suggested that severe disruption of the commensal microbiota occurred after short-term lincomycin exposure or termination of lincomycin exposure in young swine. Our study depicts the change rule of gut microbiota in young animals under the condition of short-term lincomycin treatment, providing basic data for evaluating the harmful impact on gut microbe of young animals.

Background

Due to the bacteriostatic and bactericidal characteristics of antibiotics against pathogenic bacteria, antibiotics have made tremendous contributions to public health and low dose of antibiotics are also widely employed to promote growth and reduce costs of animal husbandry in animal production over the past decades. Recently, increasing evidence has demonstrated that antibiotics administration has been documented to threaten animal health, injuring intestinal morphology and barrier, resulting in the dysfunction of intestinal immunity [1–3]. Besides, the hazardous effect of antibiotic exposure on host health, such as *Clostridioides difficile* infection-associated diarrhea [4], antibiotic-related diarrhea and indigestion [5], was also obviously observed in previous studies. Moreover, indiscreet uses of

antimicrobials lead to the production of superbugs with high antibiotic resistance [6, 7]. All these limitations of antibiotic use cause people to reconsider and restrict the application of antibiotics.

Despite increasing knowledge regarding the shortcomings of antibiotics use, the administration of antibiotics is commonly practiced for therapeutic treatment of mammals, especially young animals. Administration of antibiotics, which work via killing or suppressing pathogenic bacteria, induces imbalance of gut microbiota, causing the loss of colonization resistance [8], and antibiotic-induced dysbiosis enables the expansion of pathogenic bacteria, which infect healthy hosts. The microbes, ecologically inhabiting the mammalian intestinal tract, can not only contribute to the maintenance of metabolic functions in the digestive process but also interact with the host to adjust the physiological and immunological states in digestive tract [9, 10]. Also, the critical roles of commensal microbiota in maintaining epithelial integrity through providing colonization resistance against pathogens is reported [8]. Emerging evidence has displayed that early-life antibiotic exposure adversely affected the intestinal immunity, such as dysregulated vaccine immune response [11], declined resistance to subsequent bacterial infection [12] and altered maturation of innate intestinal immunity [13], which was directly related to antibiotic-associated gut dysbiosis. Mammals have co-evolved with intestinal microbial communities that can shape development and adapt to environmental changes, including antibiotic perturbation or nutrient flux.[14] However, knowledge of the dynamic alterations of gut microbial communities in early-life, particularly in young animals after cessation of antibiotic administration, remains limited and requires further investigation.

Short-chain fatty acids (SCFAs), primarily produced via microbial carbohydrate fermentation in large gut, are considered as beneficial metabolites interacting with intestinal microbiota to affect gut physiological functions, inflammation, and systemic metabolism [15]. Previous studies exhibited that antibiotic administration attenuated the concentration of acetate, propionate and butyrate in intestinal digesta or feces, responding to the alteration of gut microbiota [2, 16]. However, a detailed understanding of dynamic changes of microbiota-derived SCFAs after cessation of antibiotic administration is still unclear. Lincomycin, a broad-spectrum antimicrobial that acts through inhibiting bacterial protein synthesis, is widely used in clinical practice for young children or in animal husbandry for diarrhea and fever of young animals. Since the limited information concerning the dynamic alteration role of gut microbiota with antibiotic exposure ceased is available, we objected to explore the time-course changes in fecal microbial composition and microbiota-derived SCFA after one-week lincomycin exposure. Microbiome analysis and SCFA profiling revealed that one-week lincomycin administration remarkably induced gut dysbiosis including reduced beneficial or pathogenic bacteria, then microbiota reshaped and developed to the distinct cluster after lincomycin administration stopped.

Materials And Methods

Experimental pigs and study design

Twenty weaned pigs (21-day old) housed at trial base of Kangjin town, Hulan district, Harbin, were randomly allocated into two groups (ten pigs in each group). The pigs in the test group (LM group) were administered with 1000 mg/kg lincomycin in feeding for the first week, while the control group (Con group) pigs were without. After 3-day adaptation period, the animals were given *ad libitum* access to clean drinking water and commercial feed without any antibiotics for 28-day experimental period. The amount of feed intake was recorded every day and body weight of each pig was record on day 0, 14 and 28 of the whole experiment. On the day 7, 14, 21 and 28 of the entire experiment, the feces of two groups' pigs were aseptically collected in 2-ml sterile tubes, immediately frozen in liquid nitrogen, and then stored at -80°C for sequencing of microbial 16S rDNA and analysis for SCFA quantification. The timeline was shown in Fig. 1A.

DNA extraction, amplification and sequencing

Microbial community genomic DNA was extracted from feces samples using the E.Z.N.A.[®] soil DNA Kit (D5625-02, Omega Bio-Tek, Norcross, GA, USA) according to the manufacturer's instructions. The quality of extracted DNA was checked on 1% agarose gel (Fig. S1A), and DNA quantity was determined with NanoDrop2000 spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA). The extracted genomic DNA displayed suitable ratios of A260:A280 (1.8-2.0) and DNA concentration (20-300 ng/μL), meeting the requirements for subsequent sequencing.

The hypervariable region V3-V4 of the bacterial 16S rRNA gene was amplified by an ABI GeneAmp[®] 9700 PCR thermocycler (ABI, CA, USA) with primer pairs 338F (5'-ACTCCTACGGGAGGCAGCAG-3') and 806R (5'-GGACTACHVGGGTWTCTAAT-3'). The reaction parameters of PCR amplification was performed as follows: initial denaturation at 95 °C for 3 min, followed by 27 cycles of denaturing at 95 °C for 30 s, annealing at 55 °C for 30 s and extension at 72 °C for 45 s, and single extension at 72 °C for 10 min, and end at 4 °C. The PCR mixtures contain 5 × FastPfu buffer 4 μL, 2.5 mM dNTPs 2 μL, forward primer (5 μM) 0.8 μL, reverse primer (5 μM) 0.8 μL, FastPfu Polymerase 0.4 μL, BSA 0.2 μL, template DNA 10 ng, and finally ddH₂O up to 20 μL. PCR reactions were performed in triplicate. The PCR product was extracted from 2% agarose gel (Fig. S1B) and purified using the AxyPrep DNA Gel Extraction Kit (Axygen Biosciences, Union City, CA, USA) according to manufacturer's instructions and quantified using Quantus[™] Fluorometer (Promega Corporation, Madison, WI, USA).

Purified amplicons were pooled in equimolar and paired-end sequenced on an Illumina MiSeq platform (Illumina, San Diego, USA) according to the standard protocols by Majorbio Bio-Pharm Technology Co. Ltd. (Shanghai, China). The raw reads were deposited into the NCBI Sequence Read Archive (SRA) database.

Processing of sequencing data

The raw 16S rRNA gene sequencing reads were demultiplexed, quality-filtered by fastp version 0.20.0 [17] and merged by FLASH version 1.2.7 [18] with the following criteria: (i) the 300 bp reads were truncated at any site receiving an average quality score of < 20 over a 50 bp sliding window, and the truncated reads

shorter than 50 bp were discarded, reads containing ambiguous characters were also discarded; (ii) only overlapping sequences longer than 10 bp were assembled according to their overlapped sequence. The maximum mismatch ratio of overlap region is 0.2. Reads that could not be assembled were discarded; (iii) Samples were distinguished according to the barcode and primers, and the sequence direction was adjusted, exact barcode matching, 2 nucleotide mismatch in primer matching. Operational taxonomic units (OTUs) with 97% similarity cutoff [19, 20] were clustered using UPARSE version 7.1 [19], and chimeric sequences were identified and removed. The taxonomy of each OTU representative sequence was analyzed by RDP Classifier version 2.2 [21] against the 16S rRNA database (Silva v138) using a confidence threshold of 0.7.

Quantification of Short-fatty acids

Gas chromatography (GC) was utilized to quantify feces SCFAs (including acetate, propionate, butyrate, valerate, isobutyrate and isovalerate) according to the methods described by Tang et al. [22]. SCFA in the feces was extracted by ultrapure water, and 25% metaphosphoric acid was mixed with the extracts at a ratio of 1:9. After centrifugation, the mixture was passed through the 0.45- μ m Milled-LG filter (Millipore, Billerica, MA, USA) and subjected for SCFA analysis with the Agilent 7890N gas chromatograph (Agilent, Santa Clara, CA, USA).

Statistical analysis

Student's t-test of the data on body weight, bacterial alpha-diversity indices (Sobs, Shannon, ACE, and Chao) and SCFAs were performed using the JMP software (JMP Version 10.0.0, SAS Institute, Cary, NC, USA) for Windows. $p < 0.05$ was regarded as statistically significant, while $0.05 < p < 0.1$ was set as significant trend.

The principal coordinate (PCoA, beta-diversity) analysis based on the Bray-Curtis distance and ANOSIM test were employed using Majorbio I-Sanger Cloud Platform (www.i-sanger.com). The significant difference between the Con group and the LM group on different time points was tested by linear discriminant analysis effect size (LDA Effect Size, LEfSe), and the threshold of LDA score was 3.0. Statistical differences of key altered genera between two groups were calculated by the Mann-Whitney U test (nonparametric test) with $p < 0.05$ representing statistically significant and $0.05 < p < 0.1$ representing significant trend.

Results

Growth performance

The initial body weight (5.47 ± 0.17 kg) of two groups' pigs was comparable at the beginning of this study. After 7 days lincomycin exposure, the body weight was approximately 5.42% or 9.23% lower than the Con group pigs on day 14 ($p = 0.0450$) or day 28 ($p = 0.0362$) (Fig. 1C). Besides, lincomycin exposure attenuated the average daily feed intake (Fig. 1B).

Evaluation of sequencing data

A total of 2355031 valid sequences from 64 feces samples were obtained with sequences ranging from 22225 to 53516 per sample after assembling and quality filtering. The normalized depth of 22225 reads based on the minimum number of sample sequences was clustered into 1109 OTUs for all samples at a 97% sequence similarity value, further clustered into 239 genera, 96 families, 55 orders, 23 classes and 15 phyla. The excellent coverage (> 0.999) and rarefaction curves (Fig. S1C-E) indicated that most of the microbial diversity and bacterial communities in feces samples had been sufficiently captured.

Variation in alpha-diversity

Alpha-diversity is presented in Fig. 2 and Fig. S2. The results showed that the microbial alpha-diversity (including Sobs, Chao, ACE and Shannon indexes) in piglet feces did not change on the first three weeks of nursery period ($p > 0.05$), whereas the alpha-diversity (Sobs, $p < 0.0001$; Chao, $p = 0.0002$; ACE, $p = 0.0003$; Shannon, $p < 0.0001$) notably rose on the fourth week of nursery period (Fig. 2A-B and Fig. S2A-B). One-week lincomycin exposure enormously decreased the microbial alpha-diversity (including Sobs, Chao, ACE and Shannon indexes) in piglet feces ($p < 0.0001$), and the lower Sobs, Chao, ACE and Shannon indexes were still observed after one week on cessation of lincomycin exposure ($p < 0.0001$) (Fig. 2A-B and Fig. S2A-B). Yet no alterations of microbial alpha-diversity (including Sobs, Chao, ACE and Shannon indexes) in feces between LM group pigs and Con group pigs were found after two weeks or three weeks on cessation of lincomycin exposure ($p > 0.05$, Fig. 2A-B and Fig. S2A-B). After termination of lincomycin exposure, the Sobs, Chao, ACE and Shannon indexes of fecal microbiota in LM group pigs gradually ascended with the time passing and reached a significant level ($p < 0.05$, Fig. 2A-B and Fig. S2A-B).

Variation in beta-diversity

The PCoA results based on the Bray-Curtis distance and ANOSIM test at OTU level revealed that community structure of fecal microbiota was no clear difference on the first three weeks of pig nursery period, but distinct microbial community on the fourth week of nursery period was formed ($R = 0.4385$, $p < 0.0010$, Fig. 3B and A). Compared with Con group pigs, one-week lincomycin-exposed pigs exhibited a distinct cluster in feces microbiota ($R = 0.5123$, $p < 0.0010$, Fig. 3D and A). Even on the first ($R = 0.6295$, $p < 0.0010$, Fig. 3E and A), second ($R = 0.5020$, $p < 0.0010$, Fig. 3F and A) and third weeks ($R = 0.6052$, $p < 0.0010$, Fig. 3G and A) of cessation of lincomycin exposure, the fecal microbiota of the two groups still demonstrated two notably distinct cluster. After termination of lincomycin exposure, the community structure of fecal microbiota in LM group pigs gradually shifted with the time passing and reached a significant level ($R = 0.6779$, $p < 0.0010$, Fig. 3C and A).

Comparison of microbial composition at phylum and genus levels

At phylum level, most abundant phyla were Firmicutes and Bacteroidota, which consists of more than 90% of total phyla with Actinobacteriota in fecal microbiota (Fig. S3A). One-week lincomycin exposure

increased the relative abundance of Firmicutes and decreased Bacteroidota in fecal microbiota, but none of them reached a significant level ($p > 0.05$, Fig. S3A-C). The increment of Firmicutes ($(p = 0.0181)$) and decrement of Bacteroidota ($(p > 0.0136)$) were still observed on the third week after the termination of lincomycin exposure, which reached a significant level (Fig. S3A-B and D).

At genus level, the top ten abundance genera in pig feces were *Prevotella*, *Clostridium_sensu_stricto_1*, *Subdoligranulum*, *Blautia*, *Ruminococcus_torques_group*, *Prevotellaceae_NK3B31_group*, *norank_f_muribaculaceae*, *norank_f_norank_o_Clostridia_UCG-014*, *Olsenella* and *Terrisporobacter*, which consists of ~50% of total genera (Fig. 4A). The alterations of top 50 genera in relative abundance attracted more attention. Some relative abundance of genera notably modified after one-week lincomycin exposure, but those genera restored normal mass after three weeks on cessation of lincomycin exposure (e.g., *norank_f_Butyricicoccaceae*, *norank_f_Ruminococcaceae*, *Catenibacterium*, *Catenisphaera*, *Etc.*; Fig. 4A-B). Although another part of genera did not alter after lincomycin exposure, the relative abundance of those genera was varied remarkably after three weeks on the termination of lincomycin exposure (e.g., *Ruminococcus_torques_group*, *norank_f_Muribaculaceae*, *Olsenella*, *Prevotella*, *Lachnospiraceae_NK4A136_group*; Fig. 4A-B). The relative abundance of most genera obviously diminished due to the lincomycin exposure, among which most of those genera achieved augment level (e.g., *Clostridium_sensu_stricto_1*, *Terrisporobacter*, *Collinsella*, *UCG-0058*, *etc.*; Fig. 4 A-C) and others still did not return to normal abundance (e.g., *norank_f_norank_o_Clostridia_UCG-014*, *Lactobacillus*, *Faecalibacterium*, *unclassified_f_Prevotellaceae*, *Etc.*; Fig. 4A-C) after three weeks on cessation of lincomycin exposure. Besides, some bacterial genera always remained high relative abundance after lincomycin exposure and three weeks on end of lincomycin exposure (e.g., *Blautia*, *norank_f_Eubacterium_coprostanoligenes_group*, *UCG-002*, *Ruminococcus*, *etc.*; Fig. 4A-B).

Alteration of specific microbiota

LEfSe was employed to identify the specific taxa at genus level in the fecal microbiome that was notably associated with lincomycin exposure and the threshold of LDA score was 3.0. A total of 26, 43, 28 or 31 microbiotas were identified in feces of pigs exposed to lincomycin on day 7, 14, 21 or 28, respectively (genus level, LDA score > 3, Supplementary file 1, Fig. 5A and 6A). The top five abundant microbiotas of identifies taxa in feces on day 7 were *Blautia* (6.3%), *Prevotellaceae_NK3B31_group* (4.43%), *Lactobacillus* (4.30%), *Clostridium_sensu_stricto_1* (4.09%) and *Coprococcus* (2.51%) (Fig. 5C). As shown in Fig. 5A-B, most abundant microbiotas of identified taxa at genus level remarkably declined after one-week lincomycin exposure, among which ten microbiotas (*Agathobacter*, *Megasphaera*, *Phascolarctobacterium*, *Succinivibrio*, *Mitsuokella*, *Enorma*, *Terrisporobacter*, *Clostridium_sensu_stricto_1*, *Clostridium_sensu_stricto_6* and *norank_f_Oscillospiraceae*) with a 10-fold decrement were observed. In contrast, a small number of bacteria with a significant increase in relative abundance varied from 3.4 to 8.4-fold, whereas the augmented level of *Enterococcus* was more than 40 times. More than 80% of altered microbiotas in feces on day 7 were derived from Firmicutes phylum, and most of them were from Clostridia class (Fig. 5B).

The most abundant microbiota of identified taxa were *Prevotella* (28.72%), *Clostridium_sensu_stricto_1* (5.29%) and *Lactobacillus* (4.93%), which consists of ~50% of total microbiotas at genus level with *Agathobacter* (3.62%), *unclassified_f_Prevotellaceae* (2.02%), *norank_f_Muribaculaceae* (1.56%), *Terrisporobacter* (1.42%), *unclassified_f_Lachnospiraceae* (1.32%) and *Alloprevotella* (0.91%) on day 28 of whole trial (Fig. 6C). After three weeks on cessation of lincomycin exposure (day 28), only the relative abundance of two microbiotas (*Eubacterium* and *Christensenellaceae_R-7_group*) ascended by more than 10-fold, and other identified bacteria varied from 1.2-fold to 7.12-fold (Fig. 6A-B). After filtering out the bacteria with an abundance of less than 0.1% among the identified differential bacteria, we observed that 10 bacteria in the feces of lincomycin-exposed pigs were potentially beneficial bacteria (most of them were SCFA producers), included 5 down-regulated genera (*Agathobacter*, *Prevotella*, *Mitsuokella*, *Prevotellaceae_UCG-001* and *Lactobacillus*) and 5 up-regulated genera (*Eubacterium*, *norank_f_Muribaculaceae*, *Romboutsia*, *unclassified_f_Lachnospiraceae* and *Christensenellaceae_R-7_group*) (Fig. 6B). Besides, 6 pathogenic microbiotas (*Oscillospira*, *Terrisporobacter*, *Negativibacillus*, *Clostridium_sensu_stricto_1*, *Alistipes* and *Alloprevotella*) were remarkably altered at genus level in the feces after three weeks on cessation of lincomycin exposure (day 28, Fig. 6B). Approximate 90% of altered microbiotas in feces on day 28 were derived from Firmicutes (71%) and Bacteroidota (19%) phylum, and most of them were from Clostridia (58%) and Bacteroidia (19%) class (Fig. 6B).

The nonparametric test was utilized to further analyze the alterations of the well-recognized and relatively high abundant bacteria at each time point. One-week lincomycin exposure noticeably reduced the relative abundance of *Terrisporobacter* and *Clostridium_sensu_stricto_1* ($p < 0.001$), whereas the relative abundance remarkably ascended on day 14, 21 and 28 with lincomycin exposure ceased ($p < 0.05$) (Fig. 6D). Besides, one-week lincomycin exposure augmented the relative abundance of *Escherichia-Shigella* ($p = 0.0339$) and *Negativibacillus* (not reach statistical significance level), and the upward trend of these two bacteria had decreased in the first week after the termination of lincomycin exposure; the relative abundance of *Escherichia-Shigella* and *Negativibacillus* descended in the second week after stopping lincomycin exposure, but the decline of those two bacteria notably reversed in the third week with lincomycin exposure ceased ($p < 0.01$) (Fig. 6D). The diminished *Lactobacillus* and *Agathobacter* were obviously observed after one-week lincomycin exposure ($p < 0.05$), and the attenuated abundance of these two bacteria continued until the third week on cessation of lincomycin exposure (Fig. 6D).

Predicted function of microbiota analysis

To better comprehend the effect of lincomycin exposure on fecal microbiota in pigs, Tax4Fun analysis was used to predict the functional role of altered microbiota in feces. The results of predicted function demonstrated that 55, 4, 5 or 68 functional pathways at level 3 were obviously altered between LM and Con group pigs on day 7, 14, 21 or 28, respectively ($p < 0.05$, Supplementary file 2). The relative abundances of genes involved in metabolism-related pathways were noticeably altered after one-week lincomycin exposure (day 7), such as amino acid metabolism (e.g., alanine, arginine, histidine, valine, glutathione, etc.), carbohydrate metabolism, lipid metabolism, nucleotide metabolism (e.g., purine and pyrimidine), protein digestion and absorption (Fig. S4A, Supplementary file 2). Carbohydrate metabolism-

related pathways or lipid metabolism-related pathways were regulated in the first or second week on cessation of lincomycin exposure (Supplementary file 2). In contrast, we observed that metabolism-related pathways, such as amino acid metabolism, carbohydrate metabolism, energy metabolism, and lipid metabolism, were remarkably regulated in third week with lincomycin exposure ceased (Fig. S4B, Supplementary file 2).

Short-fatty acid production

Based on the changes of fecal microbiota, further investigation of SCFA quantification on different time points after one-week lincomycin exposure was completed and depicted in Fig. 7. One-week lincomycin exposure did diminish the concentration of acetate ($p = 0.0391$), valerate ($p < 0.0001$), isovalerate ($p = 0.0079$), isobutyrate ($p = 0.0060$), total SCFA ($p = 0.0162$) and decreased trend in butyrate level ($p = 0.0639$) was obviously found in the lincomycin exposed pigs. Despite the lower level of propionate from the trend point of view in lincomycin exposed pigs on day 7, no significant difference was found ($p = 0.2162$). The concentration of isovalerate and isobutyrate restored normal level on cessation of lincomycin exposure, so no alteration was detected among the two groups on day 14, 21 or 28 ($p > 0.05$). In contrast, the lower concentration of acetate (D14, $p = 0.0238$; D21, $p = 0.0644$), propionate (D14, $p = 0.0431$; D21, $p = 0.0186$) or total SCFA (D14, $p = 0.0273$; D21, $p = 0.0866$) from feces of lincomycin exposed pigs was still maintained in the first or second week after the end of lincomycin exposure, but the downward trend of these SCFAs disappeared in the third week with lincomycin exposure ceased (D28, $p > 0.05$). The concentration of butyrate ($p = 0.0893$) and valerate ($p = 0.0793$) had a declined trend in the first week with lincomycin exposed ceased, and the decrement was abolished in the next two weeks ($p > 0.05$).

Discussion

The present study targeted to seek the dynamic alterations of gut microbiota after therapeutic doses of lincomycin administration, and we found one-week lincomycin exposure induced the imbalance of fecal microbiota, leading to shaping distinct microbial clusters on cessation of lincomycin exposure. Our present results demonstrated that descent of body weight of young pigs in the first or third week on cessation of one-week lincomycin exposure (1 g/kg feeding) was notably observed, in accordance with previous study that one-week lincomycin administration (1 g/L water) reduced the body weight of young mice [2]. In addition to the decrement of feed intake in this study, the reason for weight loss might be that therapeutic dose (high dose) of lincomycin administration injured jejunum morphology and reduced the villi height of young animals [2], negatively affecting the absorption and transportation of nutrient.

Gut dysbiosis, also called imbalance of gut microbiota, is mainly caused by loss of microbial diversity and alterations in microbial composition, bacterial activity or microbial distribution [8, 23]. Previous researches report that the loss of microbial diversity and the changes of microbial composition and relative abundance were induced by lincomycin treatment [2, 24, 25]. In accordance with previous studies, present results displayed that therapeutic lincomycin administration attenuated microbial diversity and

the fecal microbiota obviously separated into distinct cluster in LM pigs compared with Con pigs, indicating that the microorganism environment was disrupted by lincomycin and gut dysbiosis might happen. A large and rapid population of the inhibited or destroyed gut microbiota increased with lincomycin exposure ceased and the alpha-diversity was restored to normal level after two weeks on cessation of lincomycin exposure. Nevertheless, the obviously continuing changes of microbial composition and microbial abundance had happened, which suggested that gut microbes were reshaped by short-term lincomycin administration in young animals. Altogether the time-course variation of fecal microbes served as an evident characteristic shift pattern of microbes after short-term therapeutic dose of lincomycin exposure in young animals.

At genus level, the relative abundance of most microbiota was remarkably reduced after short-term therapeutic dose of lincomycin exposure in young animals, no matter beneficial (e.g., *Lactobacillus*, *Agathobacter*, *Coprococcus*, etc.) or harmful bacteria (e.g., *Terrisporobacter*, *Clostridium_sensu_stricto_1*, *Clostridium_sensu_stricto_6*, etc.). Among these notably declined microbes, genera *Agathobacter* [26, 27], *Coprococcus* [28], *Megasphaera* [29, 30], *Phascolarctobacterium* [31, 32], *Prevotellaceae_NK3B31_group* [33, 34], *Succinivibrio* [35, 36] and *Mitsuokella* [37, 38] were widely known symbionts for the degradation of dietary fiber, which is vital to gut health and immunity. On the one hand, colonization resistance of gut fiber-degrading bacteria constituted the first line of defense against invading pathogens, positively affecting gut health and immunity [39]. On the other hand, SCFAs, the usual product of fiber degradation, not only could supply energy to reduce the waste of nutrients but also served as signaling metabolites derived from microbiota interacting with microbiota to affect gut health, inflammation and metabolic homeostasis [40, 41]. Our study exhibited that the lower concentration of SCFAs (including acetate, butyrate, valerate, isobutyrate and isovalerate) was observed in young pigs exposed to short-term lincomycin and similar results also observed in mice [2]. Among which, the decrement of acetate closely related to the decreased acetate-producing bacteria *Prevotellaceae_NK3B31_group* [33, 34] and *Succinivibrio* [35, 36], whereas the descended butyrate was strongly associated with the descent of butyrate-producing bacteria *Agathobacter* [26, 27], *Coprococcus* [28] and *Megasphaera* [29, 30]. Besides, the relative abundance of high abundant and beneficial microbiota *Lactobacillus*, also known as acetate-producing bacteria, was enormously diminished, which responded to the lower acetate concentration. These negative results indicated that the attenuation of fiber-degrading bacteria and *Lactobacillus* induced by short-term lincomycin exposure in young animals was harmful to gut health and the maintenance of immune function.

The results in accordance with expectation was that the restraint of well-recognized pathogenic bacteria *Terrisporobacter* [42], *Lachnoclostridium* [43, 44], *Clostridium_sensu_stricto_1* [33, 45], *Clostridium_sensu_stricto_6* [46] and unknown functional bacteria *norank_f__Oscillospiraceae*, *unclassified_f__Prevotellaceae*, etc. were notably observed after short-term lincomycin treated. Nonetheless, the relative abundance of potentially pathogenic bacteria *Enterococcus* was actually higher after lincomycin administration, which usually occurred in repeated antibiotic treatments, negatively affecting gut health of young animals [47, 48]. Consistently, further Tax4Fun analysis for functional prediction of microbiota revealed that the enriched pathways related to carbohydrate (including fiber

degradation), amino acid (e.g., Arg, Glu, Ala, Asp, *etc.*), lipid, nucleotide metabolism obviously altered after lincomycin administration. Many amino acids considered as signaling molecules were required to maintain gut health, regulating intestinal immunity, such as arginine synthesized from glutamine could enhance intestinal epithelial barrier function [49]. All these data indicated that the gut dysbiosis (including decreased fiber-degrading bacteria and *Lactobacillus*) and the attenuation of microbiota-derived SCFA might be harmful to gut health after short-term lincomycin administration.

After lincomycin administration ceased, we continued to detect the changes in fecal microbiota and microbiota-derived SCFA for three weeks. The interaction of different microbes is critical to the recovery of gut ecosystems with severe perturbations. Typically, some keystone species are needed to trigger a chain of food-net interactions, resulting in the recovery of gut microbiota and a previous study claimed that key species related to specific carbohydrate-degradation and energy-production pathways supported gut ecosystems recovery [50]. Our results demonstrated that the enormous fold-change of altered microbiota (including SCFA-producing bacteria) and lower SCFA concentration induced by lincomycin exposure gradually became small over time after lincomycin exposure stopped, but compared with Con pigs, LM pigs showed the distinct microbial community on cessation of lincomycin exposure, indicating that the process of initial gut ecosystems recovery to reshape new gut homeostasis happened with lincomycin exposure ceased. Following microbial changes after the end of lincomycin exposure could reflect the microbial adaptation to the new gut environment [51]. Confirming this possibility, a recent study depicted a model that showed for the recovery of the *Palleja et al.* microbial communities from antibiotics among which there was a transition to a “new alternative stable state” [52].

Nevertheless, the new host-microbe homeostasis reshaped by short-term lincomycin administration does seem unfriendly to gut health. Genus *Agathobacter* can utilize acetate as a substrate to produce butyrate [26, 27] and genus *Lactobacillus* produces numerous advantageous organic acids that can be converted into butyrate [53], which have beneficial effects on the host. Our results displayed that the attenuation of these two beneficial bacteria was observed in the whole experimental time. *Clostridium_sensu_stricto_1*, generally considered as pathogenic bacteria, is associated with inflammation [33] and pediatric diarrhea, as well as mucosal injuries in the colon [45], and *Terrisporobacter* is an emerging anaerobic pathogen and acetogenic bacterium [42], which are harmful to the gut. One-week lincomycin treatment notably diminished genera *Clostridium_sensu_stricto_1* and *Terrisporobacter* level in feces of young pigs. However, the relative abundance of this two pathogenic bacteria immediately rebounded and continued to keep high abundance as soon as the lincomycin treatment disappeared. Besides, pathogenic bacteria *Negativibacillus* and *Escherichia-Shigella* associated with gut dysbiosis and the pathogenesis of inflammatory bowel disease [54, 55] were dramatically ascended in this research after lincomycin treatment at first, the cessation of lincomycin disturbed their relative abundance. After the dynamic variation, the relative abundance of *Negativibacillus* and *Escherichia-Shigella* undergo drastic ascent in the third week on cessation of lincomycin administration. Moreover, further Tax4Fun analysis suggested that predicted functional alteration of fecal microbial communities with time on cessation of lincomycin administration was related to lipid, carbohydrate and amino acid metabolism pathways. Collectively, our data indicated that gut dysbiosis

induced by lincomycin exposure still had relatively long-term adverse effects on gut health after stopping lincomycin exposure.

Intriguingly, the alterations of two body weight-related microbiotas were identified, which might partly explain the decrement of body weight after lincomycin administration. Pathogenic inflammation-related bacteria *Oscillospira* appreciably enhanced in the third week on cessation of lincomycin treatment, which was strongly associated with host metabolic disorders and body weight changed [56, 57]. Simultaneously, the effect of *Christensenella*, the low abundant (less than 0.01%) and highly heritable (transmissible from parent to offspring) bacteria, on the mitigation of body weight gain has been proved [58], confirming that low abundant microbiota can impact host physiology [59], and genus *Christensenella* also notably augmented with low body weight in this trial on day 28.

Conclusions

In conclusion, the imbalance of gut microbes and microbiota-derived SCFA caused by short-term lincomycin exposure occurred in young pigs, demonstrating the drastic decrement in the relative abundance of numerous microbiotas including fiber-degrading bacteria (e.g., *Agathobacter*, *Coprococcus*, etc.), beneficial bacteria (e.g., *Lactobacillus*, *Mitsuokella*, etc.) or pathogenic bacteria (e.g., *Terrisporobacter*, *Lachnoclostridium*, etc.). After the lincomycin exposure stopped, the state of gut dysbiosis gradually recovered and formed new gut-microbe homeostasis distinct from microbial homeostasis of young pigs unexposed to lincomycin. The increased presence of potential pathogens, such as *Terrisporobacter*, *Negativibacillus*, *Escherichia-Shigella*, etc. and decreased beneficial bacteria such as *Lactobacillus*, *Agathobacter* were observed in new homeostasis reshaped by short-lincomycin administration, adversely affecting gut development and health of young pigs. Our study depicts the change rule of gut microbiota in young animals under the condition of short-term lincomycin treatment, providing basic data for evaluating the harmful impact on the young animal gut microbe. The limitation of this research is that we discontinuously follow the young animal to adulthood after three weeks on cessation of lincomycin exposure to observe the longer-term effects.

Declarations

Ethics approval and consent to participate

All procedures used in this study were granted by the Experimental Animal Welfare and Ethical Committee of Institute of Animal Science of Chinese Academy of Agricultural Sciences (IAS2020-107).

Consent for publication

Not applicable

Availability of data and materials

The datasets used during the current study are available from the corresponding author on reasonable request. The raw data on fecal microbiota generated from sequencing during the current study are available in the NCBI Sequence Read Archive (SRA) database (Accession Number: PRJNA750589).

Competing interests

The authors declare that they have no competing interests

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Authors' contributions

S.T., R.Z., and H.Z. designed the research; S.T., R.Z., S.Z., and D.S. conducted the research; S.T. and L.C. analyzed the data; S.T. and R.Z. wrote the paper and had primary responsibility for the final content; L.L. and B.X. provided the animals and expertise. All authors read and approved the final manuscript.

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Figures

Figure 1

Growth performance. Timeline of lincomycin exposure trial (A); changes of feed intake (B) and body weight (n = 10 pigs/group) (C) of each pig exposed to lincomycin or not. Data are expressed as mean $\pm$ SE in body weight and expressed as mean in feed intake. * indicates p < 0.05.

Figure 2

Alpha-diversity. The box plot of Sobs (A) and Chao (B) index (OTU level) in feces after lincomycin exposure. *** indicates $p < 0.001$ between Con and LM group ($n = 8$ pigs/group). Boxes (x, y or a, b, c, d) of Con or LM group with different letters are significantly different at $p < 0.05$ among different time points, respectively.

Figure 3

Beta-diversity. PCoA (OTU level) of community membership based on the Bray-Curtis distance and ANOSIM test in all groups (A), Con (B) or LM group (C) at different time points, Con vs. LM on day 7 (D) or day 14 (E), and Con vs. LM on day 21 (F) or day 28 (G). (n = 8 pigs/group)

Figure 4

Community composition analysis. Time-course effects of the lincomycin on the relative abundance (A) or trends change (B) of the bacterial genus (top 50) in feces; the specifically altered genera in feces (C). The value was displayed as the mean with eight pigs per group.

Figure 5

Differential genera analysis on day 7. Cladogram in Con vs. LM group on day 7 (A); fold change and taxonomy of differentially abundant genera from feces in Con vs. LM group on day 7 (B). Linear discriminate analysis effect size (LEfSe) was performed to determine the difference in abundance at genus level, and the threshold of LDA score was 3.0 ($n = 8$ pigs/group). Positive fold changes indicate that a genus is enriched in the LM group, while negative fold changes suggest that a genus is enriched in the Con group. Differential genera with a relative abundance of less than 0.1% do not show the fold change. The composition of differential genera in Con pigs on day 7 (C) and only genera with a relative ratio greater than 0.8% were presented.

Figure 6

Differential genera analysis on day 28. Cladogram in Con vs. LM group on day 28 (A); fold change and taxonomy of differentially abundant genera from feces in Con vs. LM group on day 28 (B). Linear discriminate analysis effect size (LEfSe) was performed to determine the difference in abundance at genus level, and the threshold of LDA score was 3.0 ($n = 8$ pigs/group). Positive fold changes indicate that a genus is enriched in the LM group, while negative fold changes indicate that a genus is enriched in the Con group. Differential genera with a relative abundance of less than 0.1% do not show the fold change. The composition of differential genera in Con pigs on day 28 (C) and only genera with a relative ratio greater than 0.8% were presented. The key altered genera in feces at each time point (D). Statistical differences of key altered genera between two groups were calculated by the Mann-Whitney U test

(nonparametric test). *, ** or *** indicates $p < 0.05$, $p < 0.01$ or $p < 0.001$ between Con and LM group, respectively.

Figure 7

Concentrations of short-chain fatty acids. Alterations of acetate (A), propionate (B), butyrate (C), valerate (D), isovalerate (E), isobutyrate (F) and total SCFA (G) in feces after one-week lincomycin on day 7, 14, 21 or 28. Data are expressed as min to max showing all points ($n = 7-8$ pigs/group), * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$.

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