 doi:10.20944/preprints201912.0315.v1

Title: **Compound *Lactobacillus sp*. Administration Ameliorates Stress and Body Growth Through Gut Microbiota Optimization on Piglet Model.**

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Running title: Lactobacillus sp. Improve Stress and Body Growth

The word count for the entire manuscript (introduction through discussion): 3817

The number of figures (to print, not Supplementary data): 12.

The number of tables (to print, not Supplementary data): 3.

 doi:10.20944/preprints201912.0315.v1

**Abstract**

The composition of bacteria in the gastrointestinal tract of piglets is easily affected by environmental changes, particularly during the weaning period. Compound strains of *Lactobacillus reuteri* and *Lactobacillus salivarius* were supplemented to piglets during pre- and post-weaning to determine their effects in improving the growth performance and ameliorating the diarrhea rate and stress caused by antioxidation in piglets. A larger number of *L. reuteri* and *L. salivarius* colonized the distal segment of the ileum and the total numbers of *Lactobacillus* spp. and *Bifidobacterium* were higher in the ileal mucous membrane and cecal lumen with probiotics supplementation. The numbers of antioxidative and immune molecules were increased in the plasma following compound bacteria (CL) supplementation, whereas cortisol and endotoxin levels were lower and growth hormone and insulin-like growth factor 1 were higher. Spearman's and KEGG analysis of the bacterial operational taxonomic unit and antioxidative and immune indices and metabolic genes indicated that the body growth modulation by CL supplementation could be attributed to optimization of the intestinal bacterial composition. Collectively, these results suggest that supplementation with CL could reduce stress and improve the growth performance of piglets during weaning by optimizing the intestinal bacterial composition.

**Keywords:** *Lactobacillus*; piglet; weaning; gut microbiota; growth enhancement.

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## 1. Introduction

The microbiota of the mammalian gastrointestinal tract (GIT) plays evolutionarily conserved roles in the metabolism, immunity, development, and behavior of the host, and the microbiota composition is significantly altered during body growth and by changes in the diet and environment [1-3]. Because of the societal pressure to decline the number of non human primates such as dogs and monkeys employed in biomedical research leading to an increase use of pigs. Pigs are easy to fed and collecting samples are simple [4], 5].

The GIT microbiota of piglets is easily altered during the weaning period when the feed is altered or when the piglets are isolated from sows or transferred between groups, all of which cause physiological and psychological stress [5]. Hence, pathogens can infect piglets more easily during weaning, leading to high morbidity and low growth performance [6]. In addition, low levels of digestive enzymes and hydrochloric acid in the gastric juice secretions of piglets lead to insufficient feed digestion [7]. Together, these factors impede the growth performance of piglets during the pre- and post-weaning and group transfer periods. Some genera or strains of bacteria are conserved across animals of different age groups and species as core members of the GIT. *Lactobacillus* is a core genus in growing pigs and some other mammals [8]. Some strains of *Lactobacillus* compose the native bacteria of the mucous membrane of the GIT; the native bacteria are known to play important roles in maintaining the balance of the intestinal milieu, which influences the bacterial colonization at different mucosal sites in the GIT and the secretion of organic acids, digestive enzymes, and bio-active peptides [8, 9]. Additionally, some strains of lactobacilli, such as *Lactobacillus reuteri*, *Lactobacillus fermentum*, *Lactobacillus delbrueckii*, and *Lactobacillus rhamnosus* can influence the composition of the gut microbiota, which in turn modulates hormonal secretion through the brain-gut axis to reduce body stress and anxiety [10, 11]. Therefore, dietary supplementation of weaning piglets with *Lactobacillus* spp. could optimize the composition of the gut microbiota by increasing the number of lactic acid bacteria and promoting diversity, thereby improving digestive function and alleviating stress. *Lactobacillus reuteri* and *Lactobacillus salivarius* are two probiotic bacterial strains that reside in the mucous membrane of the mammalian alimentary tract and are known to exert health benefits, such as optimization of the GIT microbiota composition, inhibition of pathogens, enhancement of intestinal barrier function, improvement of host immunity, and improvement of host digestive function via secretion of organic acids, peptides, and digestive enzymes [8, 12]. Lactobacilli have strain-specific characteristics with diverse effects and interactions in the host. Therefore, while screening for probiotic GIT bacteria for use as supplements in animals, it is important to note that

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homogeneous origin of the isolated strains is crucial for host colonization.

Two strains of *Lactobacillus* sp., *L. reuteri* KT260178, and *L. salivarius* MH 517354, were isolated from the distal segment of ileal content of healthy local Wei finishing pigs from the Anhui province of China and stored in the China Center for Type Culture Collection. In our previous study, we found that *L. reuteri* KT260178 is a candidate probiotic bacterium based on its effects on the growth performance of neonatal piglets upon colonization of the mucous membrane of the distal jejunum and ileum [14]. The two strains of *L. reuteri* KT260178 and *L. salivarius* MH 517354 strongly affected the secretion of organic acids and digestive enzymes and inhibited pathogenic bacteria *in vitro*. Considering physiological characteristics of *L. reuteri* KT260178 and *L. salivarius* MH 517354, these strains would more useful to the weaning piglets in combination than as single supplementations to promote the organic acid secretion and optimize the bacterial composition. Many studies have confirmed that the beneficial effects of probiotics on health, immunity, pathogen defense, and other biological functions are much stronger when using multiple strains compared with using single-strain administration. This finding could be attributed to the symbiotic relationships and synergism among strains [12, 14, 15].

In the study, we combined two strains of swine origin, *L. reuteri* KT260178 and *L. salivarius* MH 517354, and administered these to piglets between the pre- and post-weaning period to evaluate their effects on the intestinal bacterial composition and digestive function and their effectiveness in ameliorating the stress, morbidity, and decreased growth performance, which set an example to human beings. The structure and anatomy of the digestive tract neonatal pigs are similar to those of infant; hence, they are commonly used as an animal model in biochemical research [16]. Supplementation with compound homologous probiotic bacteria could be extended to human infants to improve health.

## 2. Materials and Methods

### *2.1. Animal studies*

The experimental protocols in this study, including those related to animal husbandry and slaughter were approved by the Institution of Animal Science and Welfare of Anhui Province (no. IASWAP2017056937). The experimental guidelines and treatment, housing, and husbandry conditions conformed to the Institutional Animal Care and Use Committee of China [17]. A total of 45 sows (Landrace breed) with the same parity and body weight were randomly allocated to three groups (n = 10 each). The sows were artificially inseminated by six Duroc boars of similar body weight and age. The expected date of piglet birth of the selected sows was the same. Thirty sows that gave birth on the same day were selected. The

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number of neonatal piglets was adjusted to 12 for each sow (initial average birth weight 1.37 kg). When the neonatal piglets reached the age of 14 days, all 30 sows with 300 piglets (10 piglets with each sow, body weight nearly 3.0 kg, piglets with highest and lowest body weight were eliminated) were allocated to three groups: control, compound *L. reuteri* KT260178 and *L. salivarius* (CL), and aureomycin (antibiotic) supplementation groups. The experiment was carried out for 42 days. The feed duration was 28 days, from May 15 to June 12, 2017. Sows and piglets were individually housed in farrowing pens (2.2 × 1.8 m) with crates and slatted floors. Heating pads were additionally installed for the piglets. The farrowing room temperature was maintained at 20℃. All 300 piglets were separated from their sows at 21 days, and groups were transferred to the nursery house at day 28 after birth. Piglets were administered iron supplements by intramuscular injection of iron dextran at days 3 and 10 after birth. All piglets had free access to water and creep feed without any probiotics, antibiotics, or other medicines added during the experiment. A standard immune procedure was implemented throughout the trial.

*2.2. Diet for each group*

*Lactobacilli reuteri* KT260178 and *L. salivarius* MH 517354 were isolated from the ileum of healthy local Wei pigs in the Anhui province of China by our research group and were stored in the China Center for Type Culture Collection, Wuhan University, Wuhan, China. The two strains of *Lactobacilli sp.* were mixed and grown in de Man, Rogosa, and Sharpe medium after inoculation at 1%:1%. The bacterial cells were harvested after approximately 18h of fermentation. The second solid fermentation was used to increase the live number of bacterial cells, in which a mixture of corn and soybean meal powder was employed to incorporate the suspended fermented liquid containing the live bacterial cells; the ratio of mass to volume was 1:0.6. To obtain a solid direct microbial additive, the mixture was dried at a constant temperature of 45℃; the numbers of live *L. reuteri* and *L. salivarius* were $6.0 \times 10^8$ and $3.0 \times 10^8$ colony-forming units per gram (CFU/g), respectively. Piglets in the control group were fed the basal diet. The diets fed during the suckling and nursery stage were different. The composition and nutrient analysis results for the basal diet of suckling piglets and nursery pig are shown in supplementary material, both of diet fulfil requirements of National Research Council [18]. In the CL group, 0.5 kg of dried feed additive CL was added to a 99-kg basic mass feed. A blender was used to mix the additives uniformly for 10 min to attain a homogeneous mixture. After inoculation in the CL group, live *L. reuteri* and *L. salivarius* reached $3.0 \times 10^6$ and $1.5 \times 10^6$ CFU/g, respectively. The diet with aureomycin supplementation was prepared in the same manner as for the CL groups, with 150 μg/kg

aureomycin in the feed. All food fed to the piglets was prepared once every 7 days. The compositions of the basic diets are shown in supplementary material. The sows were individually fed and had free access to water throughout the experimental period. The diets for lactating sows were identical and formulated according to National Research Council requirements for gestating and lactating sows [18]; this diet contained no probiotics, antibiotics, or other medicine.

*2.3. Growth performance and sample collection*

Piglets in every replicate from each treatment group were weighed both on the transfer days (experimental day 14, ED 14) and at the end of the experiment (ED 28). Average daily gain (ADG) was calculated according to the formula: ADG = body increase (g)/number of days (ED 14 or 28). The number of piglets with diarrhea in each group was recorded to calculate the diarrhea rate according to the following formula: diarrhea rate = sum of number in each group for 28 days/(20 × 11 × 28) × 100%. At the end of the experiment, one piglet from every replicate was selected, and 10 mg/kg ketamine hydrochloride was intramuscularly injected. Blood samples were collected from sacrificed piglets by bleeding of the carotid artery. Tissues of the distal ileum and cecum were removed under aseptic conditions, stored in sterile plastic tubes in boxes packed with ice, and immediately transported to our laboratory for microbiota quantification by the plate-counting method.

*2.4. Plate-counting of microbiota assay*

The samples of the distal ileum were cut open and washed with sterilized physiological saline (pH 7.0). Samples of ileal mucous membrane were scratched from the ileum using a slide. The ileal mucous membrane and cecal lumen contents (0.4 g each) were prepared and 10-fold dilutions were prepared with sterilized saline. The bacterial composition of the ileal mucous membrane and cecal lumen contents in all groups was determined using the plate method. Eosin methylene blue agar was used for total *Escherichia coli* (which mainly resides in cecal lumen or contents, abundant number or transfer of *E. coli* into other segment of intestine caused illness), a *Salmonella-Shigella* agar plate was used for *Salmonella* (pathogenic bacteria, resides in intestine and content), and MRS agar was used for total *Lactobacillus* [13, 19]. The assays were repeated three times.

*2.5. Quantitative real-time PCR assay*

*L. reuteri* KT260178 and *L. salivarius* MH 517354 were fermented in MRS medium respectively. Colonies were counted with the plate method under a microscope to obtain samples of $1\times10^4$, $10^5$, and $10^6$. Then, a tenfold dilution series ($1\times10^4$, $10^5$, and $10^6$) of *L. reuteri* and *L. salivarius* was prepared. Total RNA in each dilution was extracted. The

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standard curve of serious dilution was generated based on mean cycle threshold values of quantitative real-time polymerase chain reaction. The method was carried out according to our previous study (Yang et al., 2019a). The primer sequences were as follows: *L. reuteri* KT260178: forward 5′ AACTCCCTGAAATGACAGTGAAG 3′, reverse 5′ TGACTGAACACTAACCCGAACCT 3′. *L. salivarius* MH 517354: forward 5′ GACTCACTGACATGACAGTGACG 3′, reverse 5′ ACGCTGAGCACTAACGCGACAC 3′. Samples (0.2 g) of ileal mucous membrane were prepared to extract total RNA to evaluate colonization by *L. reuteri* KT260178 and *L. salivarius* MH 517354 cells according to our previously reported method (Yang et al., 2019a). Reverse transcription was performed with a GoScript Reverse System (Invitrogen, Carlsbad, CA, USA). First-strand cDNA was synthesized by incubating a reaction mixture containing 11 μL RNA and 1 μL RNase-free $dH_2O$ at 70℃ for 3 min, followed by 0℃ for 5 min. A dNTP mixture (1 μL; 10 mmol/L), 4 μL GoScript 5X reaction buffer, 1 μL GoScript reverse transcriptase, 1.5 μL $Mg^{2+}$ (25 mM), and 0.5 μL RNase inhibitor were combined in a total volume of 20 μL and incubated at a 37℃ in a water bath. Primers were designed according to the 16S ribosomal RNA (16S rRNA) of *L. reuteri* KT260178 and *L. salivarius* MH 517354 submitted to NCBI. Amplification was performed in a 20-μL mixture containing 10 μL of 2×qPCR SYBR Premix Ex-Taq, 2 μL template cDNA, 0.5 μL each primer (10 μmol/L), and 7 μL PCR-grade water. The cycling protocol was as follows: 95℃ for 30 s, followed by 40 cycles of 95℃ for 5 s and 60℃ for 30 s, and one cycle for melting curve analysis consisting of 95℃ for 60 s, 65℃ for 60 s, and 95 ℃ for 1 s. The amplification curve was generated based on the dilution of the standard curve of the *L. reuteri* KT260178 and *L. salivarius* MH 517354 cultures.

*2.6. DNA extraction, PCR amplification, and sequencing*

Samples (0.25 g) of the ileal mucous membrane and cecal lumen were prepared. Microbial DNA was extracted from these samples using the E.Z.N.A.® soil DNA Kit (Omega Bio-tek, Norcross, GA, USA) according to the manufacturer's protocol. Eight samples of each group were chosen, with two remained as standby to instead of the unqualified DNA. The final DNA concentration and purification were determined with a NanoDrop 2000 UV-vis spectrophotometer (Thermo Scientific, Waltham, MA, USA), and DNA quality was checked by 1% agarose gel electrophoresis. The V3-V4 hypervariable regions of the bacteria 16S rRNA gene were amplified with primers 338F (5′-ACTCCTACGGGAGGCAGCAG-3′) and 806R (5′-GGACTACHVGGGTWTCTAAT-3′) with a thermocycler PCR system (GeneAmp 9700, Applied Biosystems, Foster City, CA, USA). PCR was conducted as follows: 3 min of denaturation at 95℃, 27 cycles of 30 s at 95℃, 30 s for annealing at 55℃, and 45 s for

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elongation at 72℃, and a final extension at 72℃ for 10 min. PCR was performed in triplicate 20-μL mixtures 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, and 10 ng of template DNA. The resulting PCR products were extracted from a 2% agarose gel and further purified using the AxyPrep DNA Gel Extraction Kit (Axygen Biosciences, Union City, CA, USA) and quantified using QuantiFluor™-ST (Promega, Madison, WI, USA) according to the manufacturer's protocol. Purified amplicons were pooled in equimolar and paired-end sequenced (2 × 300) on an Illumina MiSeq platform (Illumina, San Diego, CA, USA) according to standard protocols.

*2.7. 16S rRNA amplicon data processing and statistics*

Diversity metrics were calculated using the core-diversity plugin within QIIME2. Feature level alpha diversity indices and operational taxonomic units (OTUs) were used to estimate the microbial diversity within an individual sample. Beta diversity distance measurements were performed with unweighted UniFrac to investigate the structural variation in the microbial communities across samples and then visualized via principal coordinate analysis (PCoA). Co-occurrence analysis was performed by calculating Spearman's rank correlations between predominant *Lactobacillus* and the network plot. Additionally, the potential KEGG Ortholog (KO) functional profiles of microbial communities were predicted with PICRUSt.

*2.8. Determining of plasma antioxidants, immune-related molecules, and hormones*

Blood samples were collected in 5.0-mL sterile heparinized tubes and then centrifuged at 3000 ×*g* for 10 min to collect the plasma for immune assays. Plasma levels of antioxidant factors, including malondialdehyde (MDA), total antioxidant capacity (T-AOC), superoxide dismutase (SOD), and glutathione peroxidase (GPX), and immune-related molecules, interferon-α (INF-α) and interferon-β (IFN-β), were measured with enzyme-linked immunosorbent assay (ELISA). All ELISA kits of MDA, T-AOC, SOD, GPX, INF-α and IFN-β were purchased from Nanjing Jiancheng Bioengineering Institute, Nanjing, China. Plasma hormonal levels of cortisol, endotoxin, growth hormone, and insulin-like growth factor 1 were evaluated by ELISA (kits were purchased from Nanjing Jiancheng Bioengineering Institute).

*2.9. Statistical analysis*

Statistical analyses of the data were performed using SPSS 16.0 software (SPSS, Inc., Chicago, IL, USA). Data are presented as the mean values with their standard errors. Differences between groups were compared by analysis of variance. Differences between means were assessed by Tukey's honestly significant difference test for post hoc multiple comparisons. A t-test was used to identify taxonomic features of microbiological DNA

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sequence. A P value < 0.05 was considered statistically significant. Spearman's correlation analysis was used to determine relationships between parameters. STAMP software was applied to detect differentially abundant Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways among groups with false discovery rate correction. A P-value (corrected) < 0.05 was considered to indicate statistical significance.

## 3. Results

### *3.1. Body growth*

All piglets were weighed in both groups on ED 14 and ED 28. The average body weights of piglets with supplemented with CL were 0.52 kg higher than those of controls on ED 14 and 0.93 kg higher than those of controls (P < 0.01) on ED 28, revealing no significant differences from those of the antibiotic group (P > 0.05; Fig. 1A). Additionally, the results of ADG were consistent with those for body weight, and CL-supplemented piglets showed a higher average daily gain of body weight compared those in the control group both at ED 14 (P < 0.05; Fig. 1B) and ED 28 (P < 0.01; Fig. 1B). The diarrhea rate in the groups was CL group < antibiotic group < control group (P < 0.01; Fig. 1C) at both ED 14 and ED 28.

### *3.2. Colonization of Lactobacillus sp.*

The probiotic *Lactobacillus* sp. can colonize the mucous membrane of the ileum, as demonstrated by the qPCR assay. A larger number of *L. reuteri* and *L. salivarius* was detected in the ileal mucous membrane samples both on ED 14 and 28 (Fig. 2A and 2B) in probiotics supplementary group than those of control and antibiotc groups (P < 0.01). The number of *L. reuteri* and *L. salivarius* in antibiotic group showed decreased tendency compared with control, without significance (P > 0.05). The total number of *Lactobacillus* sp. and *Bifidobacterium* in the ileal mucous membrane and cecal content was the highest in all groups (P < 0.01) (Table 1). In contrast, the number of *Lactobacilli* sp. and *Bifidobacterium* in the antibiotic-supplemented group was lower than that in other groups, and the other enumerated bacteria, such as *Escherichia coli* (conditionally pathogenic) and *Salmonella* (pathogenic), were significantly reduced compared to that in the control. Consistent with this result, *Salmonella* and *E. coli* counts were minimally reduced in the CL-supplemented group.

### *3.3. Overall bacterial community structure*

The bacterial composition in the ileal mucous membrane and cecal content of piglets in the antibiotic group were significantly influenced by aureomycin supplementation. We compared the CL and control groups on ED 14 and ED 28 to determine the effects of supplementation with the two *Lactobacillus* spp. and whether the influence of antioxidation on the composition of the microbiota. The composition of microbiota of the CL and control groups was analyzed

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by sequencing of the bacterial 16S rDNA V3 and V4 regions. High-throughput pyrosequencing of the samples (n = 6) produced a total of 1,365,826 raw reads. After removing low-quality sequences, 468,232 clean tags were identified as a total of 484 OTUs in six samples. This sequencing depth nearly reflected the total microbial species richness, and most OTUs were present at low abundance (Fig. 3A). In total, 365 and 387 OTUs were obtained from the ileal mucous membrane and 392 and 413 OTUs from the cecal content in the control and CL groups, respectively. The microbiota in the ileal mucous membrane of piglets was mostly comprised by three phyla: Bacteroidetes, Firmicutes, and Proteobacteria, which collectively accounted for 98.92% and 98.57% of the bacterial abundance on ED 14 and ED 28 (Fig. 3B). The colonic microbiota of piglets on ED 14 and ED 28 was also chiefly constituted by Bacteroidetes, Firmicutes, and Proteobacteria, which accounted for more than 90% of the total microbiological abundance. Actinobacteria, Fusobacteria, Verrucomicrobia, and Saccharibacteria constituted the remaining 10% abundance of the microbiota (Fig. 3B).

*3.4. Differences in diversities of bacteria in ileum and cecum*

To determine the phylogenetic variations caused by CL supplementation and age of piglets, we measured unweighted UniFrac distances. Analysis of similarities of the UniFrac-based principal coordinate analysis revealed significant differences in the bacterial community structure among different groups (illustrated in the PCoA plot in Fig. 4). In the PCoA of bacterial OTUs, the samples of cecal content in the CL group both in ED 14 and ED28clustered together more than in control group. The situation in the ileal mucous membrane was different than in the cecum. The samples of bacterial OTUs in the two groups clustered adjacently. However, the cluster of samples in CL groups at two time points ED 14 and ED 28 was closer than that in the control group. The longer distance among spots of samples indicated that their bacterial communities were more different and unstable.

To further elucidate the dynamics and differences between the gut bacterial communities of the CL-supplementation and control groups, the OTUs at the phylum and genus levels were characterized (Fig. 3). At the phylum level, CL supplementation increased Bacteroidetes population in the ileal mucous membrane on ED 14 (CL group: 35.14% and control group: 30.05%) (Fig. 3A) ($P < 0.01$). Similarly, the relative abundances of Proteobacteria (CL group: 10.45% and control group: 7.46%) and Firmicutes in the CL group was higher than that in the control group on ED 14 (CL group: 53.32% and control group: 61.08%) (Fig. 3B). The same results were observed for the cecal content. The relative abundances of Bacteroidetes and Proteobacteria in the CL group were significantly decreased compared to those in the control, and the abundance of Firmicutes was improved ($P < 0.01$). On ED 28, the relative abundances

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of Bacteroidetes (22.06%) and Proteobacteria (3.57%) in the ileal mucous membrane were reduced compared with those on ED 14 in both groups (19.06% and 2.76%, respectively). The relative abundances of Bacteroidetes and Proteobacteria in CL were significantly higher than those in the control. In contrast, the abundances of Firmicutes in the two groups on ED 28 were increased compared to those on ED 14. The results of microbiological composition of the cecal content were correlated with those of the ileal mucous membrane. The main phyla were Firmicutes, Bacteroidetes, and Proteobacteria, accounting for 67.52%, 21.82%, and 4.78% of the relative abundances in the CL group and 71.41%, 19.32%, and 3.28% in the control group on ED 14, respectively. The results on ED 28 showed that Firmicutes, Bacteroidetes, and Proteobacteria comprised 72.59%, 16.29%, and 3.82%, respectively, of the CL group microbiota and 77.93%, 12.29%, and 2.63%, respectively, of the control group microbiota (Fig. 5A). CL-supplemented piglets both on ED14 and 28 showed higher microbial diversity than control group piglets. Actinobacteria, Fusobacteria, Verrucomicrobia, and Saccharibacteria were more abundant in the CL group, showing high microbial proportions (Fig. 5B).

We further compared the microbial community at the genus level. The main genera were *Lactobacillus*, *Escherichia-Shigella*, *Peptoclostridium*, *Acinetobacter*, *Ruminococcaceae* UCG-014, *Streptococcus*, *Clostridium sensu stricto* 1, *Bacteroides*, *Roseburia*, and *Helicobacter* in the ileum and cecum, accounting for 99% of the relative abundance (Fig. 5A). Piglets in the CL group showed higher relative abundances for the genera *Lactobacillus*, *Acinetobacter*, *Ruminococcaceae* UCG-014, *Bacteroides*, and *Helicobacter*. However, lower relative abundances of *Escherichia-Shigella*, *Streptococcus*, *Peptoclostridium*, *Clostridium sensu stricto* 1, *Fusobacterium*, *Roseburia*, and *Veillonella* were observed. *Lactobacillus* and *Escherichia-Shigella* were opposite in their ratio. The proportions of *Lactobacillus* in the ileal mucous membrane at both experimental time points were 20.14% and 16.14%, showing higher values than in the control (12.70% and 11.23%; $P < 0.01$) (Fig. 5B). The proportion of *Escherichia-Shigella* decreased by 2.78% and 4.61% compared to in the control ($P < 0.01$). The relative abundances of *Escherichia-Shigella* in the cecal content on ED 14 and ED 28 in the CL group were 0.12% and 2.74% and in the control group were 0.31% and 12.63%, respectively (Fig. 5B). The relative abundance of *Lactobacillus* in the cecal content of piglets with CL supplementation was significantly increased at both time points.

*3.5. Antioxidant and immune-related molecule levels*

To investigate the protective roles of CL on oxidative molecules caused by stress, MDA, SOD, T-AOC, and GPX in the plasma were measured on ED 14 and ED 28 (Table 2). With

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antibiotic and CL supplementation, the concentration of MDA in the plasma was decreased significantly compared to in the control at both time points ($P < 0.01$). The MDA content in the CL group was the lowest, showing values 4.16 and 6.13 μmol/L lower than in the control on ED 14 and ED 28, respectively. SOD, CAT, and GPX activities in CL-supplemented piglets were significantly higher than in the control ($P < 0.05$); there were no differences in SOD and CAT activities between the antibiotic and control groups ($P > 0.05$). The plasma levels of immune-related molecules are shown in Table 3. IFN-α levels in the CL group were higher than that in the aureomycin supplementation group ($P < 0.01$) and higher than in control piglets ($P < 0.01$). Similar results were obtained for IFN-β, with the highest level detected in CL-supplemented piglets ($P < 0.01$), showing the highest value among the three groups.

*3.6. Hormonal levels*

The plasma hormonal levels of cortisol, endotoxin, growth hormone, and insulin-like growth factor 1 are shown in Table 3. At ED 14 and ED 28, the plasma level of cortisol in piglets with CL supplementation was significantly lower than those in the two groups without *Lactobacilli* sp. supplementation ($P < 0.05$). There were no differences between the control and antibiotic groups ($P > 0.05$). The level of plasma endotoxin in the CL group was very significantly reduced compared to in the antibiotic group ($P < 0.01$), which was lower than in the control group ($P < 0.05$) at both time points. The levels of growth hormone and insulin-like growth factor 1 in the CL-supplemented group were significantly increased compared to in the control and antibiotic groups at both times points, which were 1.13 and 59.92 ng/mL higher than in the control group, respectively, on ED 28 ($P < 0.01$). There were no differences between the control and antibiotic groups ($P > 0.05$).

*3.7. Comparison of metabolic pathway abundance*

To determine the relationship between bacteria colonizing the ileal mucous membrane and antioxidant and immune-related molecule levels, Spearman's correlation analysis was performed. The antioxidant and bacterial abundance in ileal mucous membrane samples on ED 28 is shown in Fig. 6. The strains of *L. delbrueckii*, *L. salivarius*, *L. formicilis*, *L. reuteri*, and *L. mucosae* were positively correlated with plasma antioxidation; SOD, GPX, and CAT levels and negatively correlated with the MDA concentration. However, the strains of *L. agilis* and *L. pontis* were diverse and negatively correlated with the levels of SOD, GPX, and CAT. The strains of *L. delbrueckii*, *L. salivarius*, and *L. formicilis* were positively correlated with IFN-β levels. However, *L. agilis* was negatively correlated with INF-α and IFN-β levels. Additionally, *L. pontis* was negatively correlated with INF-α levels. *L. salivarius* was

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positively correlated with both the antioxidative and immune indices.

To further study which metabolic genes were altered after treatment with probiotics, KEGG pathways among the samples between the CL and control groups on ED 28 were analyzed. In terms of metabolic pathways, genes regulating the metabolism of cofactors and vitamins ($P < 0.01$), other amino acids ($P < 0.01$), lipid ($P < 0.01$), terpenoids and polyketides ($P < 0.05$), transport and catabolism ($P < 0.01$) were more abundant in the CL supplemented group than in the control group (Fig. 7A, 7B). However, the genes related to membrane transport ($P < 0.01$), replication and repair ($P < 0.01$) were less abundant in the CL group than those in the control group.

## 4. Discussion

Probiotic *Lactobacillus* in multi-strain combining strains capable of reducing antigen-load, improving the intestinal barrier, and eliciting a regulated immune response could potentially have stronger overall effects than single-strain on gastrointestinal barrier function, homeostasis and restore the ecological balance, during antibiotic administration and other unfriendly conditions [20]. In use of improved and stress and growth performance of body, different studies have confirmed positive effects on health when multi-strain probiotics are used, due to the symbiosis among strains [21].

The composition of bacteria in the gastrointestinal tract of piglets is easily affected by environmental changes, particularly during the weaning period. Both physiological and psychological stresses can cause oxidative damage in the body. The free radical chain reaction is the most widely accepted theory of inflammation [22]. Free radicals, other reactive oxygen species, and toxic products produced by oxidation can attack biological molecules, causing serious damage, including cellular damage. The MDA level is a marker of oxidative stress [23]. Enzymes such as SOD, GPX, and CAT contribute to antioxidant defense and therefore can serve as biomarkers for evaluating the efficacy of nutritional interventions [24].

A usable strain of isolated *Lactobacillus* must be able to survive in and effectively colonize the GIT, particularly, the intestinal mucous [13, 25]. Our qPCR results indicated that both *L. reuteri* and *L. salivarius* colonized the mucous membrane of the distal segment of the ileum, and the finding is supported by previous plate assays [26, 27]. Once *L. reuteri* and *L. salivarius* colonize the ileal mucous, they can utilize nutrition in the intestine for propagation. The probiotic induced the secretion of metabolic enzymes, small peptides, and organic acids; optimized the composition of the gut microbiota; and promoted various beneficial interactions within the host piglets. These effects collectively improve nutrient absorption and alleviated stress through the brain gut axis [28, 29]. The growth performance of the piglets

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supplemented with CL improved, while and diarrhea rate decreased, partly because of the probiotic effects of *L. reuteri* and *L. salivarius*. This result agrees with the findings of our previous study [12].

The small and large intestine play key roles in digestion and nutrient absorption. The composition of the microbiome of the ileum and cecum contributes to various metabolic reactions, including the synthesis of amino acids, lipids, carbohydrates, vitamin B, and short-chain fatty acids [30, 31]. An abundant and diverse intestinal microbiota improves health by alleviating the oxidation caused by stress [32, 33].

Aureomycin was chosen as the positive control to compare the growth performance with the compound *L. reuteri* and *L. salivarius* supplementation, which was to determine the efficient of compound use of those two strains. While, the bacterial composition conducted by the plate method assay indicated that the number of tested bacteria reduced significantly compared with the control. Considering the adverse effect of aureomycin supplemented on composition of bacteria, bacterial composition between the probiotic supplementation and the control was compared. The OTUs of the bacterial community of the ileal mucous membrane and cecal content indicated that the microbiological composition of the CL group was diverse. The PCoA results of the unweighted UniFrac distance metric on bacterial community showed that the OTUs from piglets supplemented with CL clustered together at both ED 14 and ED 28. However, OTUs from the control showed dispersion at both time points. The main influence on bacterial diversity is exerted by CL supplementation and age [34, 35]. These results indicate that piglets in the CL group were significantly affected by age or CL feeding during the experimental period. Additionally, the structure of the microbial community in the ileum and cecum varied widely, indicating their different functions in the body. *Lactobacillus* and *Escherichia-Shigella* had the greatest influence, consistent with the results of the plate method to evaluate the ileal mucous membrane and cecal contents. *Escherichia coli* as a colibiota constitute normal GIT flora, which mainly reside in the cecal lumen or contents and whose abundant number in the cecum or transfer into other segment of the intestine can cause illness [36]. Salmonella is a pathogenic bacteria that reside in the intestine and cecal contents. In this study, the number of *Escherichia coli* and Salmonella was significantly reduced in the ileal mucous membrane and cecal lumen contents with compounds *L. reuteri,* and *L. salivarius* supplemented, and the number of total *Lactobacillus* and *Bifidobacterium* significantly increased indicated by the plate method assay. The results, coupled with the sequencing assay, suggested that the optimized bacterial flora was achieved via *Lactobacillus* supplementation.

Antioxidative and immune functions were found to be related to strains of *L. delbrueckii*, *L.*

 doi:10.20944/preprints201912.0315.v1

*salivarius*, *L. formicilis*, *L. reuteri*, and *L. mucosae.* In our results, greater colonization by *L. salivarius* and *L. reuteri* colonization in the ileal mucous membrane contributed to lower abundances of conditional and pathogenic bacteria owing to the optimized the bacterial composition [37-40]. The results suggested that more probiotic *Lactobacillus* resided in the mucosal membrane contributing to body antioxidation and immunity, which reduce the damage caused by stress.

The supplemented effects of *L. reuteri* and *L. salivarius* were cumulative to body. It is true that in both experimental point ED 14 and ED 28, number of *Lactobacillus* colonized were significantly improved. Thereafter, certain biochemical indexes showed significantly compared with control especial in longer duration. In order to confirm and highlight the probiotic effect of *L. reuteri* and *L. salivarius* the experimental point ED 28 was chosen to carry out Spearman's correlation analysis. In the KEGG pathway analysis of samples on ED 28, the genes encoding molecules involved in regulating the metabolism of piglets supplemented with CL were significantly enriched [41, 42]. The results of study suggested that genes regulating the metabolism of cofactors and vitamins , other amino acids, lipid, terpenoids and polyketides, transport and catabolism were more enhanced with CL supplemented. Both *L. reuteri* and *L. salivarius* secrete related metabolic bioactive peptides and lactate and hydrogen peroxide, can colonize in distal segments of the ileum. The development of microstructures in the small intestine, such as intestinal villi and crypt of piglets was enhanced with CL supplementation at the concentration of $10^6$-$10^7$CFU/g. The intestinal bacterial structure is a crucial producer of vitamins that play a key role in host health [43], implying the importance of increased gut bacterial vitamin B metabolism [35]. All of these factors were helpful for body nutrition absorption. Molecules related to lipid and amino acid metabolism, which influence the secretion of hormones involved in growth, such as cortisol, endotoxin, growth hormone, and insulin-like growth factor 1, were increased, thereby improving growth performance and reducing stress [44, 45]. The colonization of *L. reuteri* and *L. salivarius* in the ileal mucous membrane reduced the infection of pathogen and optimized the commensal bacterial structure, which alleviated stress and enhancement of immunity and antioxidation. These benefits could contribute to a lower diarrhea rate in CL supplementary piglets in weaning and group transferred periods.

## 5. Conclusions

In this study, we investigated the effects of compound strains of *L. reuteri* and *L. salivarius* supplemented to piglets during pre- and post-weaning for improving the growth performance and ameliorating the diarrhea rate and stress caused by antioxidation. The results revealed that

colonization of *L. reuteri* and *L. salivarius* in the distal segment of the ileum improved the development of small intestine and gut bacterial composition, which reduced the damage of stress caused by oxidation and improved immunity. The research revealed that probiotic *L. salivarius* and *L. reuteri* ameliorated stress and improved growth performance of weaning piglets via gut microbiota optimization. This study also suggested that functional strains of *L. delbrueckii*, *L. salivarius*, *L. formicilis*, *L. reuteri*, and *L. mucosae* were positively correlated with body antioxidation and immunity with CL supplementation, the strains of *L. agilis* and *L. pontis* were diverse and negatively correlated. However, the related metabolic signals and molecular pathways affected with CL supplementation need investigation in the future. The benefits of probiotic *L. salivarius* and *L. reuteri* were elaborated and can be used as an alternative to antibiotic drug in the breed of weaning piglets and infant. These discoveries can inform strategies for consumption and can have important economic implications.

**Author contributions:** JY designed the study, fed the piglets and recorded the growth data, wrote the paper, established the qRT-PCR assay. MZ and XP measured the levels of plasma antioxidant, immunity and hormonal indexes, and mRNA level, JW analyzed the data of plasma antioxidant, immunity and hormonal indexes, CW and KH was involved in technical direction. We would like to thank Editage (www.editage.cn) for English language editing.

**Funding:** This study was financially supported by the fund of Anhui Academy of Agricultural Sciences Key Laboratory Project (No.2019YL021), Anhui Science and Technology Key Project (No.17030701008), Anhui Swine Industry Technology System Project (No.AHCYTX-05-09), National Key Research and Development Program of China (2016YFD0500509) and the Science and Technology Program of Anhui Province (No.1704A07020066)

**Confict of interest:** All authors declare that they have no conflict of interest.

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**Figure Legend**

Figure 1. Body weight, average daily gain and diarrhea rate in ED 14 and ED 28 of three groups.

Figure 2. The total number of *L. reuteri* and *L. salivarius* in ileal mucous membrane of three groups measured by qPCR .

Figure 3 and 5. The OTU of bacterial community of three groups in phylum level.
The weight of 0.25g samples of ileal mucous membrane and cecal lumen contents were prepared. Microbial DNA was extracted from samples of ileal mucous membrane and cecal lumen contents, which were prepared with PCR reactions. Purified amplicons were pooled in equimolar and paired-end sequenced (2×300) on an Illumina MiSeq platform (Illumina, San Diego, USA). The data was collected and drawn in Excel.

Figure 4. PCoA analysis of UniFrac distance metric of bacterial OTUs. The figure was drawn by the ANOSIM of UniFrac distance metric.

Figure 6. Heatmap of the Spearman rank correlations between the significantly modified *Lactobacillus* with antioxidative and immune parameters in weaning piglets.

Figure 7. KEGG pathways among samples between CL and control groups on ED 28. STAMP software was applied to detect the differentially abundant Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways among groups with false discovery rate correction.

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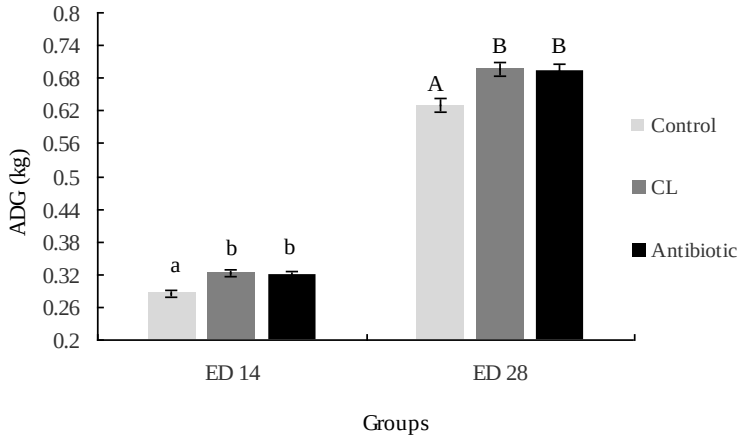


Figure 1A Body weight of piglets in three groups

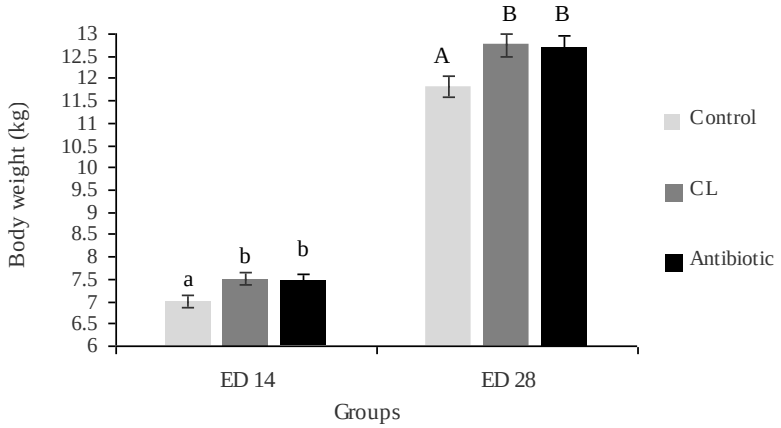


Figure 1B ADG of piglets in three groups

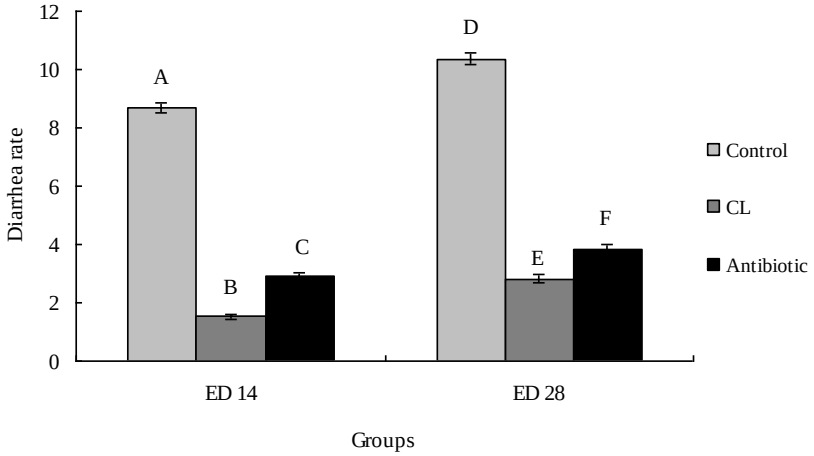


Figure 1C Diarrhea rate of piglets in three groups

Figure 1 Growth performance of three groups. The different superscript capital letters in the same classification of column mean significant difference at 0.01 levels ($P<0.01$), the different superscript lower case letters mean significant difference at 0.05 levels ($P<0.05$).

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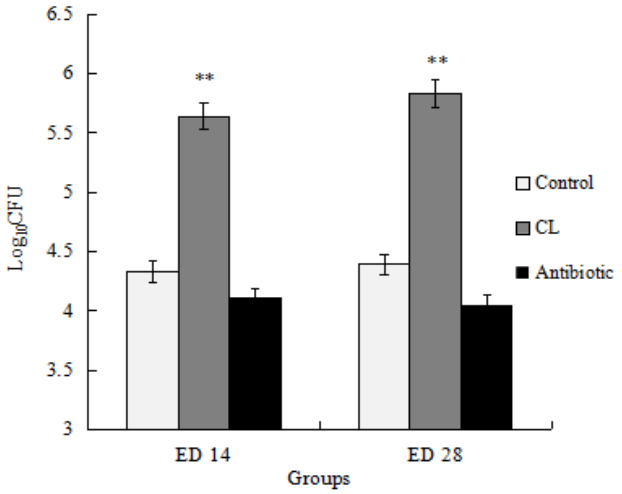


Figure 2A The total number of *L. reuteri* in ileal mucous membrane

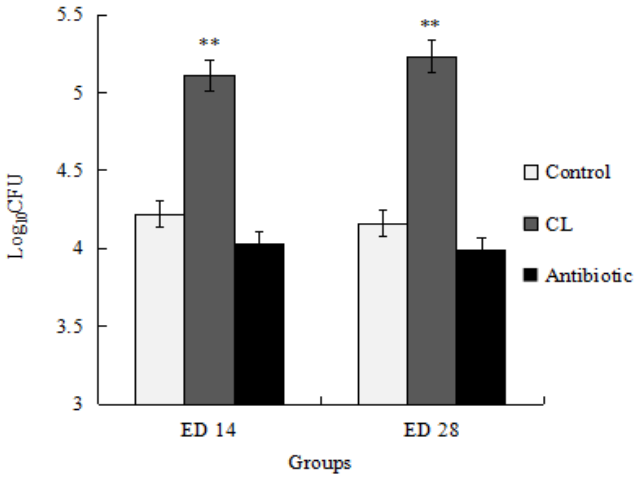


Figure 2B The total number of *L. salivarius* in ileal mucous membrane

Figure 2 The total number of *L. reuteri* and *L. salivarius* in ileal mucous membrane of three groups measured by qPCR . **mean significant difference at 0.01 levels (P<0.01).

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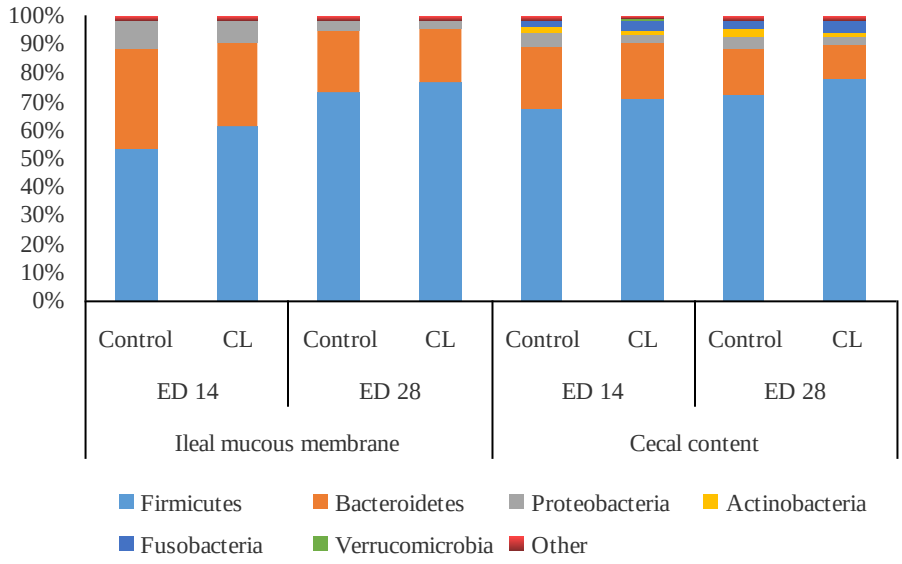


Figure 3A The bacterial community of three groups in phylum level

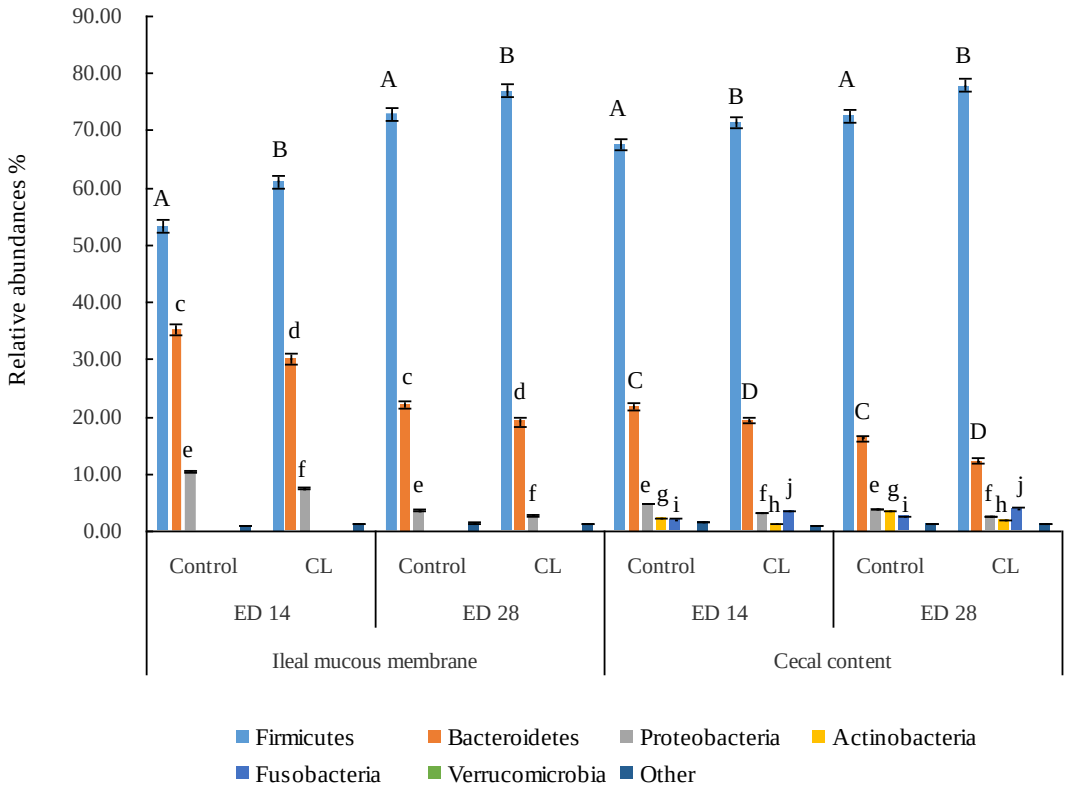


Figure 3B Differences in bacterial community of three groups in phylum level

Figure 3 The OTU of bacterial community of three groups in phylum level. The different superscript capital letters in the same colour of column in the same experimental duration mean significant difference at 0.01 levels ($P<0.01$), the different superscript lower case letters mean significant difference at 0.05 levels ($P<0.05$).

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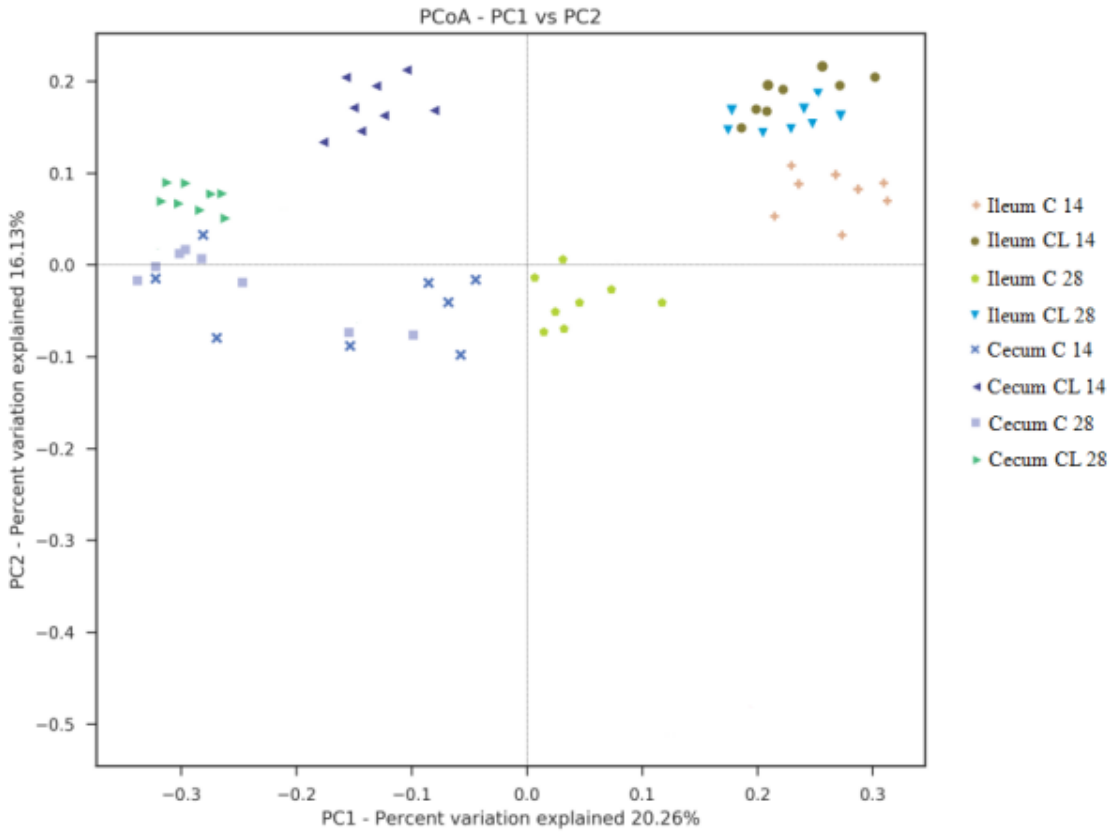


Figure 4 PCoA analysis of UniFrac distance metric of bacterial OTUs

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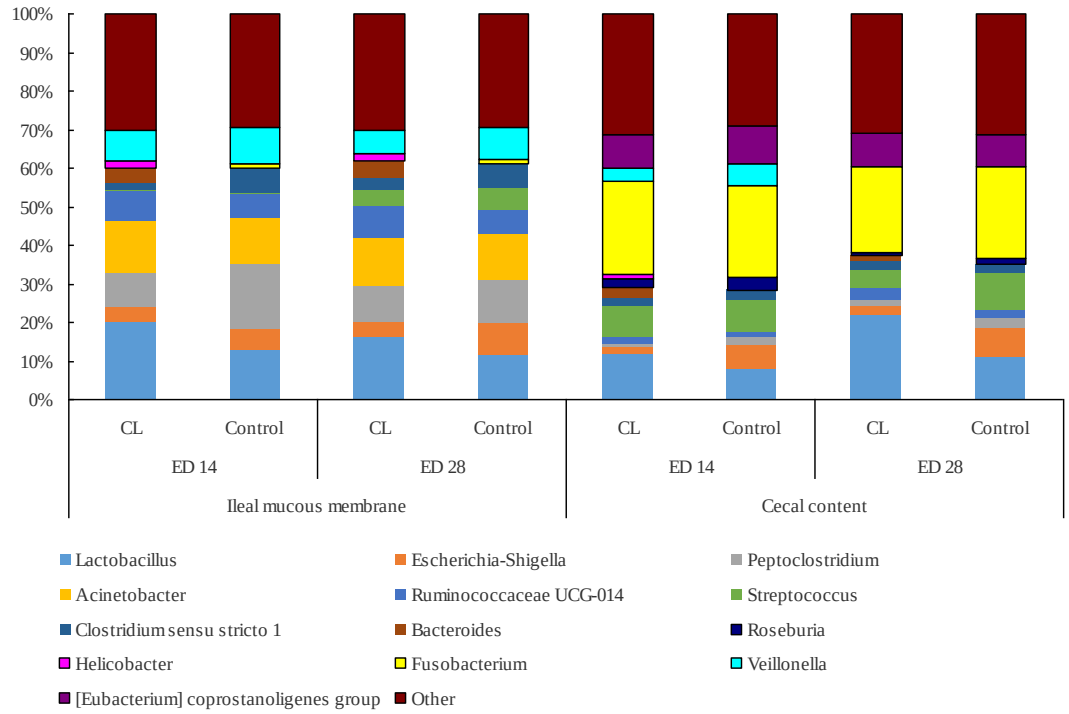


Figure 5A The bacterial community of three groups in genus level

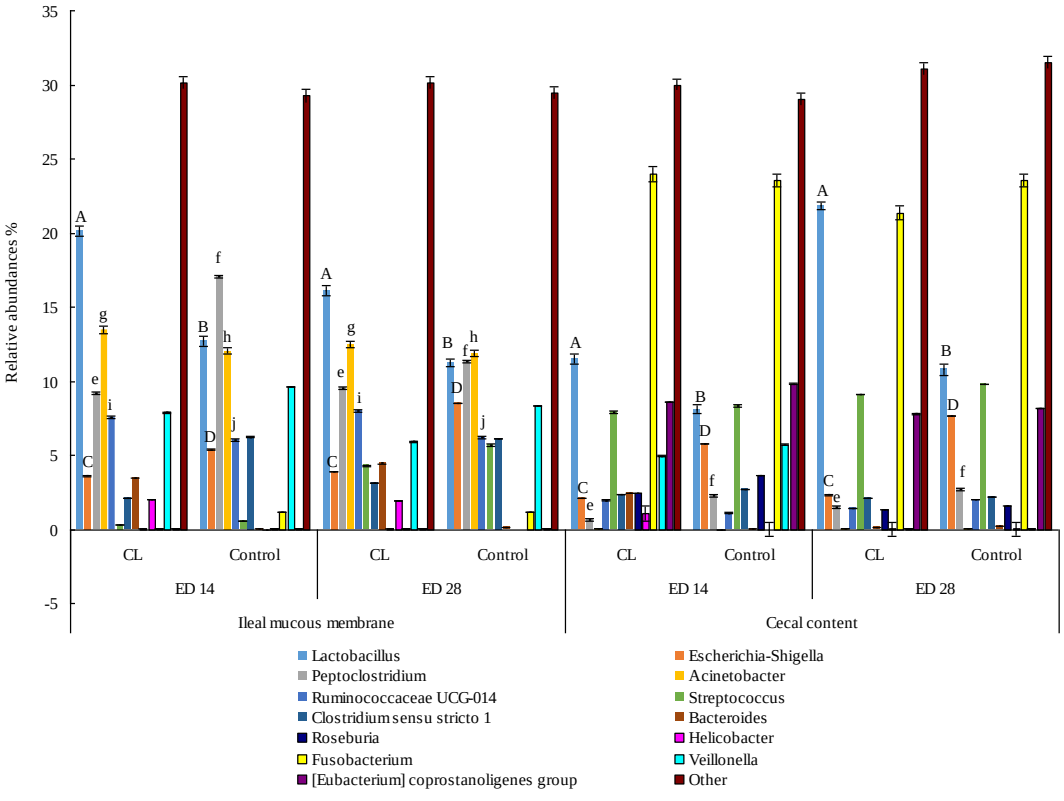


Figure 5B Differences in bacterial community of three groups in genus level

Figure 5 The different superscript capital letters in the same colour of column in the same experimental duration mean significant difference at 0.01 levels ($P<0.01$), the different superscript lower case letters mean significant difference at 0.05 levels ($P<0.05$).

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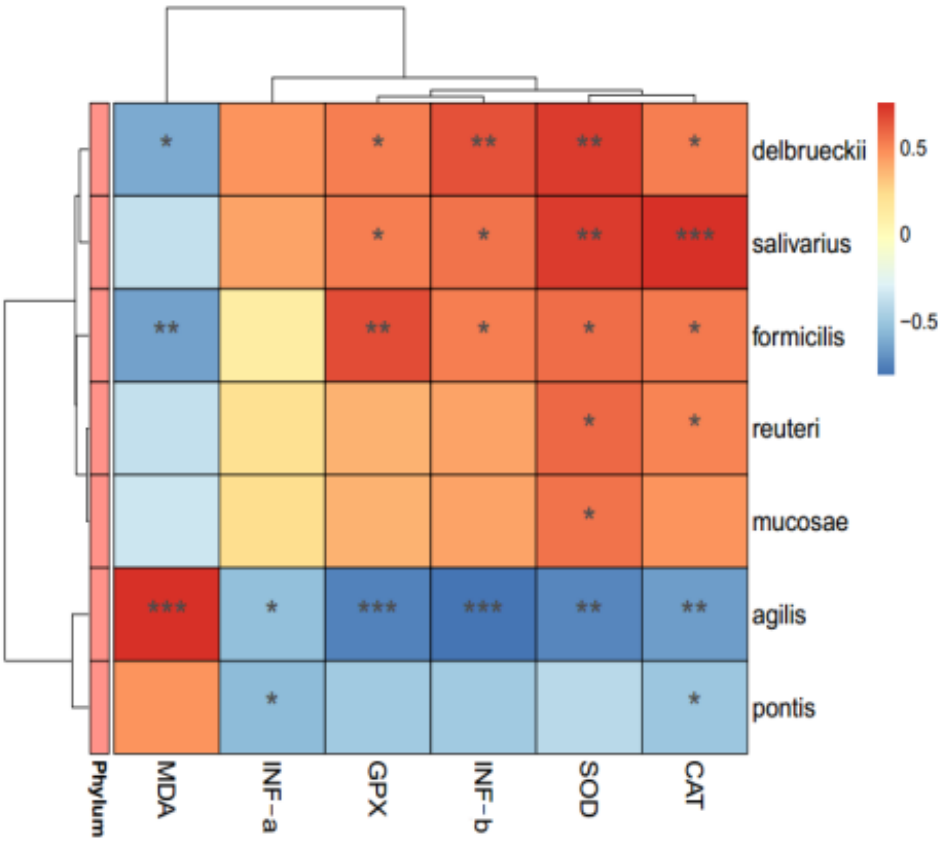


Figure 6 Heatmap of the Spearman rank correlations between the significantly modified bacteria with antioxidative and immune parameters in weaning piglets. Asterisks in different colors represent significant positive correlation. ***mean very strong correction.

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Figure 7A KEGG pathways among samples between CL and control groups on ED 28

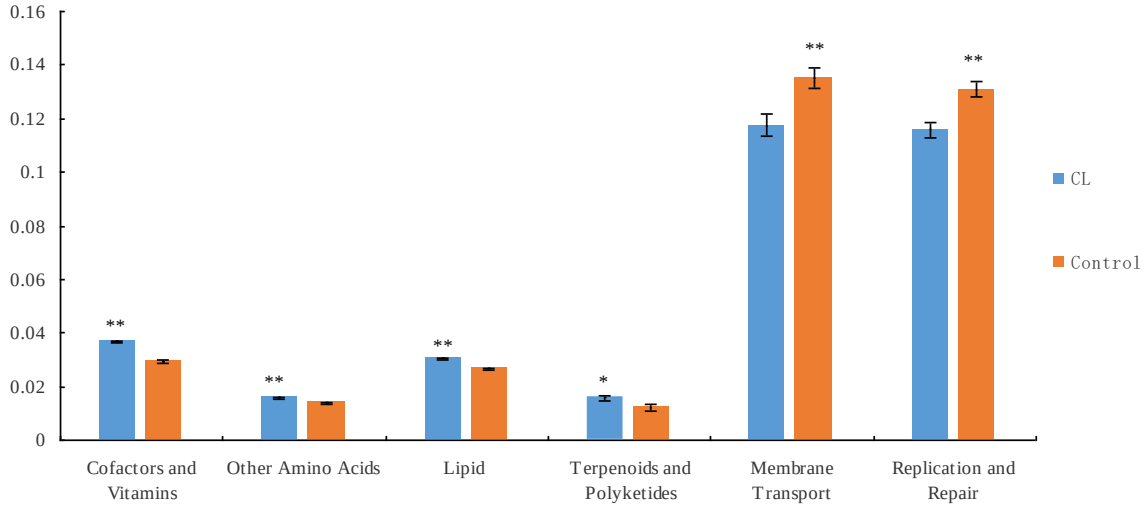


Figure 7B Differences in gene expression of three groups.

Figure 7 KEGG pathways among samples between CL and control groups on ED 28. * mean significant difference at 0.05 levels (P<0.05), **mean significant difference at 0.01 levels (P<0.01).

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Table 1 Effects of different treatments on ileal and caecal microflora $\log_{10}$ CFU·g$^{-1}$

| Groups | Escherichia coli | Staphylococcus | Lactobacillus | Bifidobacterium |
|---|---|---|---|---|
| | | ileal mucosa | | |
| Control | 8.48$^{A}$ | 8.39$^{A}$ | 9.01$^{A}$ | 8.04$^{A}$ |
| CL | 7.92$^{B}$ | 7.63$^{B}$ | 9.3$^{B}$ | 8.27$^{B}$ |
| Antibiotic | 7.46$^{C}$ | 7.29$^{C}$ | 8.23$^{C}$ | 7.56$^{C}$ |
| s.e.m.[1)] | 0.10 | 0.09 | 0.11 | 0.08 |
| P-value | 0.0016 | 0.0023 | 0.0021 | 0.0014 |
| | | cecal content | | |
| Control | 8.13$^{A}$ | 6.55$^{A}$ | 8.67$^{A}$ | 7.56$^{A}$ |
| CL | 7.78$^{B}$ | 5.12$^{B}$ | 9.19$^{B}$ | 8.24$^{B}$ |
| Antibiotic | 7.42$^{C}$ | 5.53$^{C}$ | 7.92$^{C}$ | 6.75$^{C}$ |
| s.e.m.[1)] | 0.05 | 0.04 | 0.06 | 0.05 |
| P-value | 0.0018 | 0.0022 | 0.0025 | 0.0024 |

Note: 1) s.e.m., standard error of mean. Data are means of ten piglets for each treatment. The different superscript capital letters in the same column A, B, C mean significant difference at 0.01 levels ($P<0·01$).

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Table 2 Indexes of plasma antioxidative and immune status in different groups

| Groups | MDA | T-AOC | T-SOD | GPX | INF-α | INF-β |
|---|---|---|---|---|---|---|
| | mmol/L | U/mL | U/mL | mol/L | pg/mL | pg/mL |
| | | | ED 14 | | | |
| Control | 10.06$^{A}$ | 13.67$^{a}$ | 89.47$^{a}$ | 505.25$^{a}$ | 5.64$^{A}$ | 216.79$^{A}$ |
| CL | 5.84$^{B}$ | 15.33$^{b}$ | 108.29$^{b}$ | 524.35$^{b}$ | 7.49$^{B}$ | 280.94$^{B}$ |
| Antibiotic | 8.11$^{C}$ | 14.93$^{b}$ | 109.19$^{b}$ | 516.75$^{ab}$ | 6.71$^{C}$ | 253.29$^{C}$ |
| s.e.m.[1)] | 0.31 | 0.42 | 4.76 | 8.91 | 0.29 | 7.88 |
| *P*-value | 0.0041 | 0.025 | 0.031 | 0.036 | 0.001 | 0.0006 |
| | | | ED 28 | | | |
| Control | 10.25$^{A}$ | 13.98$^{a}$ | 98.09$^{a}$ | 512.36$^{a}$ | 5.97$^{A}$ | 209.88$^{A}$ |
| CL | 4.12$^{B}$ | 16.15$^{b}$ | 115.89$^{b}$ | 535.86$^{b}$ | 7.88$^{B}$ | 282.45$^{B}$ |
| Antibiotic | 7.93$^{C}$ | 15.02$^{b}$ | 113.77$^{b}$ | 529.53$^{ab}$ | 6.92$^{C}$ | 246.29$^{C}$ |
| s.e.m.[1)] | 0.28 | 0.45 | 4.31 | 7.97 | 0.23 | 7.47 |
| *P*-value | 0.0039 | 0.023 | 0.030 | 0.033 | 0.006 | 0.007 |

Note: 1) s.e.m., standard error of mean. Data are means of ten piglets for each treatment. The different superscript capital letters in the same column A, B, C mean significant difference at 0.01 levels ($P<0·01$), different lower case letters a, b, c mean significant difference at 0.05 levels ($P<0·05$).

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Table 3 Indexes of plasma hormonal levels in different groups

| Groups | Cortisol | Endotoxin | Growth hormone | Insulin-like growth factor 1 |
|---|---|---|---|---|
| | ng/mL | | | |
| | | ED 14 | | |
| Control | 15.29$^{a}$ | 17.28$^{Aa}$ | 4.49$^{A}$ | 198.87$^{A}$ |
| CL | 12.18$^{b}$ | 11.25$^{B}$ | 5.62$^{B}$ | 258.79$^{B}$ |
| Antibiotic | 15.13$^{a}$ | 15.26$^{c}$ | 4.56$^{A}$ | 199.25$^{A}$ |
| s.e.m.[1)] | 0.65 | 0.39 | 0.16 | 5.91 |
| *P*-value | 0.041 | 0.0035 | 0.002 | 0.0036 |
| | | ED 28 | | |
| Control | 13.79$^{a}$ | 16.12$^{Aa}$ | 3.79A | 188.65$^{A}$ |
| CL | 11.01$^{b}$ | 10.99$^{B}$ | 4.92B | 231.36$^{B}$ |
| Antibiotic | 13.92$^{a}$ | 15.89$^{c}$ | 3.76A | 187.53$^{A}$ |
| s.e.m.[1)] | 0.57 | 0.37 | 0.14 | 5.22 |
| *P*-value | 0.038 | 0.0032 | 0.003 | 0.0029 |

Note: 1) s.e.m., standard error of mean. Data are means of ten piglets for each treatment. The different superscript capital letters in the same column A, B, C mean significant difference at 0.01 levels ($P<0{\cdot}01$), different lower case letters a, b, c mean significant difference at 0.05 levels ($P<0{\cdot}05$).

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Supplementary material

Nutrient analysis of the basic diet for sow and piglets

| Composition[a] | Percentage % |
|---|---|
| suckling piglets | |
| Crude protein | 20.05 |
| Carbohydrate | 18.89 |
| Crude fat | 9.82 |
| Calcium | 1.02 |
| Total phosphorus | 0.76 |
| Available phosphorus | 0.56 |
| Lysine | 1.55 |
| Methionine | 0.52 |
| Threonine | 0.95 |
| Tryptophan | 0.24 |
| Digestive energy(MJ/kg) | 15.12 |
| nursery pig | |
| Crude protein | 18.85 |
| Carbohydrate | 16.50 |
| Crude fat | 9.09 |
| Calcium | 0.82 |
| Total phosphorus | 0.71 |
| Available phosphorus | 0.51 |
| Lysine | 1.33 |
| Methionine | 0.48 |
| Threonine | 0.83 |
| Tryptophan | 0.21 |
| Digestive energy(MJ/kg) | 14.07 |
| lactating sows | |
| Crude protein | 16.25 |
| Carbohydrate | 14.50 |
| Crude fat | 6.89 |
| Calcium | 1.02 |
| Total phosphorus | 0.71 |
| Available phosphorus | 0.51 |
| Lysine | 0.71 |
| Methionine | 0.45 |
| Threonine | 0.75 |
| Tryptophan | 0.19 |
| Digestive energy(MJ/kg) | 13.68 |

Note: [a] vitamins and trace elements per kg diet: Vitamin A (retinyl acetate) 3, 450 IU, Vitamin $D_3$ (cholecalciferol) 300 IU, Vitamin E (DL-α-tocopheryl acetate) 80 IU, menadione 20 mg, Vitamin $B_{12}$ (cyanocobalamin) 0.2 mg, thiamin 30 mg, riboflavin 50 mg, biotin 0.55mg, folacin 0.05 mg, niacin 12 mg, pantothenic acid 12 mg, and pyridoxine 50 mg, manganese 100 mg, zinc 80 mg, iron 80 mg, copper 10 mg, iodine 0.15 mg, and selenium 0.15 mg, chromium 0.2mg.