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ORIGINAL ARTICLE

Bacterial lactase genes diversity in intestinal mucosa of mice with dysbacterial diarrhea induced by antibiotics

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Abstract

The current study aimed at exploring the diversity of bacterial lactase genes in the intestinal mucosa of mice with dysbacterial diarrhea induced by antibiotics and to provide experimental basis for antibiotics-induced diarrhea. Mice model of dysbacterial diarrhea was established by gastric perfusion with mixture of cephradine capsules and gentamicin sulfate (23.33 mL kg^{-1} d^{-1}), twice a day and continuously for 5 days. Intestinal mucosa from jejunum to ileum was collected, and bacterial metagenomic DNA was extracted for Miseq metagenome sequencing to carry out diversity analysis. The results showed that specific operational taxonomic units (OTUs) were 45 in the control group and 159 in the model group. The Chao1, ACE, Shannon and Simpson indices in model group were significantly higher ($P < 0.01$ or $P < 0.05$) than control group. Principal component analysis (PCA) and box chart of the control group were relatively intensive, while in the model group, they were widely dispersed. Furthermore, the inter-group box area was higher than that in the intra-group. Compared with the model group, the abundance of bacterial lactase genes in Proteobacteria from the intestinal mucosa of the control group was higher, but lower in Actinobacteria and unclassified bacteria. At the genus level, the relative abundance of bacterial species and taxon units in model group was obviously increased ($P < 0.05$). Our results indicate that antibiotics increased the diversity and abundance of bacterial lactase genes in the intestinal mucosa, as the abundance of *Betaproteobacteria*, *Cupriavidus*, *Ewingella*, *Methyloversatilis*, *Rhodocyclaceae* and *Rhodocyclales*. In addition, antibiotics become an additional source for lactase genes of *Ewingella*, *Methyloversatilis*, *Mycobacterium*, *Microbacterium*, *Beutenberqia* and *Actinomyces*.

Keywords Dysbacterial diarrhea · Lactase gene · Intestinal mucosa · Gene diversity · High-throughput sequencing · Antibiotics

Introduction

A large number and wide variety of microorganisms inhabit in human and animal intestinal tract. Intestinal microbiotas in the intestinal contents and mucosa are mainly composed of bacteria and fungi, and they are the main contributor to various reactions in the intestine (Cao et al. 2014). Usually, the intestinal microbiota lives in a dynamically balanced ecological environment and plays an irreplaceable role in antagonizing pathogens, regulating immunity, maintaining intestinal mucosal barrier integrity and so on (Guo 2001; Li et al. 2007; Lu 2001).

Animal intestinal mucosa is the direct contact surface to intestinal material and also the main interface between the immune system and external environment. Intestinal mucosa plays a major role in the absorption of nutrients and acts as an important barrier in the prevention of inflammation by preventing intestinal pathogens infection. Clinically, various

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symptoms are associated with intestinal mucosal immune system, such as various pathogen infections, inflammatory bowel disease and celiac disease (Merga et al. 2014; Parvin et al. 2013). The immune defense mechanism of intestinal mucosal is tightly related to intestinal microbiota, which promotes the production and maturation of intestinal mucosa lymphoid tissue, boosts the synthesis of secretory immunoglobulin A (sIgA), participates in the development of intestinal mucosal immune system and interacts with intestinal immune cells to maintain the stability of the intestinal environment (Hong and Zhan 2014). For example, intestinal symbiotic microbiota inhibit the growth of pathogens by competing consumption of nutrients and restrain the translocation of toxin in the intestine by decomposing metabolic carbohydrate to obtain short-chain fatty acids (mainly acetic acid) (Fukuda et al. 2011).

Long-term antibiotic exposure can induce damage in intestinal mucosal barrier by leading to intestinal dysbacteriosis, which is manifested in the abnormal changes in number, type, proportion, location and biological characteristics of intestinal microbiota (Tan et al. 2013; Liu et al. 2015). On the other hand, dysbacteriosis will cause body diarrhea by affecting the body's absorption of nutrients, inhibiting intestinal mucosal lactase activity and weakening intestinal mucosal barrier (Chen et al. 2014; Liu et al. 2015). A variety of diarrhea can be treated by supplementing lactic acid bacteria or lactase (Luo et al. 2016). Lactase is an important functional enzyme in the intestine associated with diarrhea, distributed in the intestinal contents and mucosa, and can be secreted through intestinal microorganisms and intestinal mucosa. Many bacteria living in the intestinal tract also produce lactase, such as *Lactobacillus* sp., *Bifidobacterium* sp., *Bacillus* sp., *Escherichia coli* (Juajun et al. 2011; Rhimi et al. 2009). The diversity of lactase-producing bacteria leads to the change of lactase gene diversity and activity.

In our preliminary research, we found that antibiotics reduced the diversity of bacterial lactase genes in intestinal contents and transformed their structure (Long et al. 2017a). The current research aimed to further reveal the correlation between diarrhea and bacterial lactase genes in intestinal mucosa, to explain the mechanism of antibiotics-associated diarrhea treated with traditional Chinese medicine, and more importantly, to understand the mucosal bacterial composition in detail. Therefore, we shall investigate the diversity of bacterial lactase genes in intestinal mucosa of mice with dysbacterial diarrhea induced by antibiotics.

Materials and methods

Materials

Animals

Twelve mature Kunming mice (six males and six females) weighing 20 ± 2 g were purchased from Hunan Slaccas Jingda Laboratory Animal Company (Hunan, China) with license number SCXK (Xiang) 2013-0004. All procedures involving animals were performed according to protocols approved by the Institutional Animal Care and Use Committee of Hunan University of Chinese Medicine.

Reagents

Cephradine capsules were purchased from Suzhou Zhonghua Pharmaceutical Industry Co., Ltd, product batch number: 151101. Gentamicin sulfate injection was purchased from Yichang Renfu Pharmaceutical Co., Ltd, product batch number: 5150307. Solutions like protease K, lysozyme, TE buffer and Tris-saturated phenol–chloroform–isoamyl alcohol (25:24:1) were purchased from Beijing Ding-guo Biotechnology Co., Ltd. Other solutions such as 10% SDS, 0.1 mol L^{-1} PBS buffer, CTAB/NaCl and 5 mol L^{-1} NaCl were prepared in the laboratory.

Methods

After 2 days of adaptive feeding, mice were randomly selected as control group (lcm) and model group (lmm), six mice (three males and three females) in each group. The mice in lmm were administered 0.35 mL antibiotics mixture at the concentration of 62.5 g L^{-1} composed of gentamycin sulfate and cefradine (Zeng et al. 2012; Zhang et al. 2014). The mice in lcm were treated with 0.35 mL sterile water, twice a day for 5 days. Mice in both lmm and lcm were maintained under controlled conditions (23–25 °C, humidity 50–70%). When symptoms, such as fecal material wet, cold limbs, curled up, arched back trembling and poor appetite appear in mice, it indicates the success of establishing diarrhea model. Then the mice were killed using cervical dislocation; test specimens from jejunum to ileum were selected. On a sterile operation platform, after squeezing out the chymus, cutting open the intestinal tract and cleaning the intestinal wall with saline, intestinal mucosa was scraped with coverslips and added 2 times weight of saline, then intestinal mucosa samples were immediately frozen and stored at 4 °C for DNA extraction (Jin et al. 2012).

Metagenome extraction and purification

Metagenome DNA was extracted from intestinal mucosal microorganism according to our previous publication (Wu et al. 2012; Long et al. 2017b). Two grams of intestinal mucosa was collected in a sterile environment, and homogenized in 5 mL of 0.1 mol L^{-1} phosphate buffer solution (PBS), followed by centrifugation at 200*g* for 2 min. After being washed twice with PBS, the supernatant was transferred into new germ-free tubes and centrifuged for 8 min at 10,000*g*. The new sediments were then collected, washed once with PBS, twice with acetone and three times with PBS, then resuspended in 4 mL TE buffer. After sample pretreatment, 500 µL of spreadhead were added with 45 µL TE buffer, 20 µL lysozyme and 5 µL proteinase K and homogenized in 1.5 mL germ-free Eppendorf tubes. Samples were incubated at 37 °C for 30 min and mixed with 30 µL of 10% SDS, followed by incubation at 37 °C for 40 min, with vortexing once every 10 min. Then 80 µL of CTAB/NaCl and 100 µL of 5 mol L^{-1} NaCl were mixed. The mixture was vortexed at 65 °C for 10 min. An equal volume of Tris-saturated phenol–chloroform–isoamyl alcohol (25:24:1) then was added to the sample, mixed well and centrifuged at 10,000*g* for 3 min. The supernatant was transferred to new germ-free tubes, mixed with an equal volume of chloroform–isoamyl alcohol (24:1), centrifuged at 10,000*g* for 3 min. The supernatant was transferred into fresh germ-free tubes and mixed with an equal volume of chloroform–isoamyl alcohol (24:1) again. After centrifugation at 10,000*g* for 3 min, the supernatant was transferred into fresh germ-free tubes; 10^{-1} volume of 3 mol L^{-1} sodium acetate and double volume of absolute ethyl alcohol were added, and precipitated at − 20 °C for about 12 h. Samples were centrifuged at 10,000*g* for 3 min. The acquired sediment were washed with 70% ethanol, dried and eventually dissolved in 50 µL TE buffer for DNA metagenome extraction.

PCR amplification and Miseq metagenome sequencing

A pair of degenerate primers was designed to amplify the DNA according to the conserved region of the beta-galactosidase (lacZ) gene nucleotide sequence of *Lactobacillus* and *E. coli* reported by NCBI. The upstream primer was: 5′-TRRGCAACGAATACGGSTG-3′ and the downstream primer was: 5′-ACCATGAARTTSGTGGTSARCGG-3′. Universal primers were designed and synthesized by Shanghai Personal Biotechnology Co., Ltd. PCR mixture (25 µL) contained 5× reaction buffer 5.0 µL, 5× GC buffer 5.0 µL, 10 mmol L^{-1} dNTP 0.5 µL, 10 µmol L^{-1} forward primer 1.0 µL, 10 µmol L^{-1} reverse primer 1.0 µL, DNA template 1.0 µL, sterilized ddH_2O 11.25 µL, Q5 high-fidelity DNA polymerase 0.25 µL. The PCR conditions were as follows: initial denaturation at 98 °C for 30 s, followed by denaturation at 98 °C for 15 s, annealing at 46 °C for 30 s, extension at 72 °C for 30 s and then at 72 °C for 5 min, repeated for 32 cycles (Long et al. 2017b). Miseq metagenome sequencing was completed by Shanghai Persional Biotechnology Co., Ltd.

Bioinformatic and statistical analysis

Alpha diversity analysis which contained Chao1, ACE, Simpson, and Shannon indices was applied to identify the population of intestinal mucosal bacterial lactase genes by determining operational taxonomic units (OTUs) (Shannon 1997; Mahaffee and Kloepper 1997; Pitta et al. 2010, 2014). Chao1 and ACE abundance indices, Simpson and Shannon diversity indices were calculated using Qiime (v1.8.0, http://qiime.org/) software (Caporaso et al. 2010). Principle component analysis (PCA) (Ramette 2007) and Lefse analysis (Segata et al. 2011) were used to analyze the main distribution characteristics of community samples based on linear discriminant analysis (LDA) effect size. Uclust sequence alignment tool in Qiime software was applied to classify high-quality sequences and divide OTUs (Blaxter et al. 2005) according to similarity over 97%. The SPSS 21.0 software (IBM Corp, Armonk, NY, USA) was used to analyze the measurement data. Pairwise samples *t* test was applied to compare the statistical significance of differences, with $P < 0.05$ or $P < 0.01$.

Results

Effects of antibiotics on the OTUs number of bacterial lactase genes in the intestinal mucosa

In total, 270,653 effective sequences were obtained, over 90% of them were high-quality sequences. Among the high-quality sequences, their sequence length was concentrated at 361 bp, which can be used for subsequent information analysis. Based on sequence homogeneity, the sequences with over 97% similarity were aligned and grouped into individual OTUs using Qiime software. There were 339 and 225 OTUs expressed in mice from model group and control group, respectively. Among them, 180 were identical. In addition, the numbers of unique OTUs identified from model group and control group were 159 and 45 (Fig. 1). It indicates that there was significantly difference in intestinal

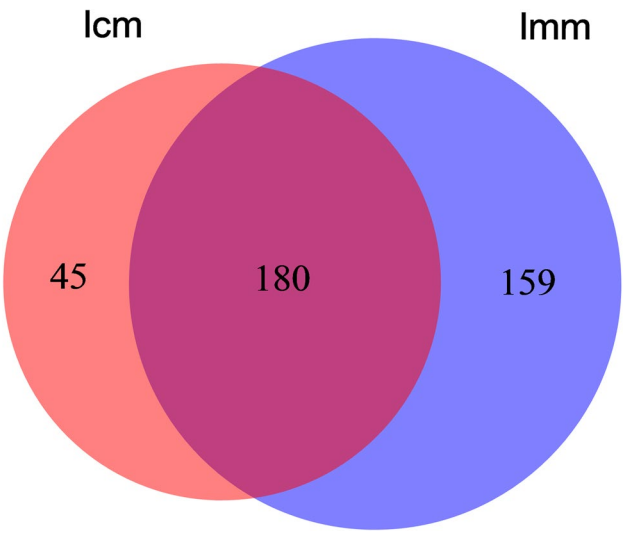

Fig. 1 Effects of antibiotics on the intestinal mucosa bacterial lactase genes OTUs number. *lcm* control group, *lmm* model group

mucosa bacteria lactase genes between model group mice and control group mice.

Effect of antibiotics on the diversity of bacterial lactase genes in the intestinal mucosa

Alpha diversity was estimated by four indices which contained Chao1, ACE, Simpson and Shannon. According to the definition, a higher value of the Chao1 or ACE index indicates greater abundance of a bacterial population, and higher Shannon and Simpson indices suggests a more diverse bacterial population. We found that Chao1, ACE, Simpson and Shannon indices were significantly higher in the model group than those in the control group ($P < 0.01$ or $P < 0.05$; Fig. 2), indicating that antibiotics increased the abundance and diversity of bacterial lactase genes in the intestinal mucosal community.

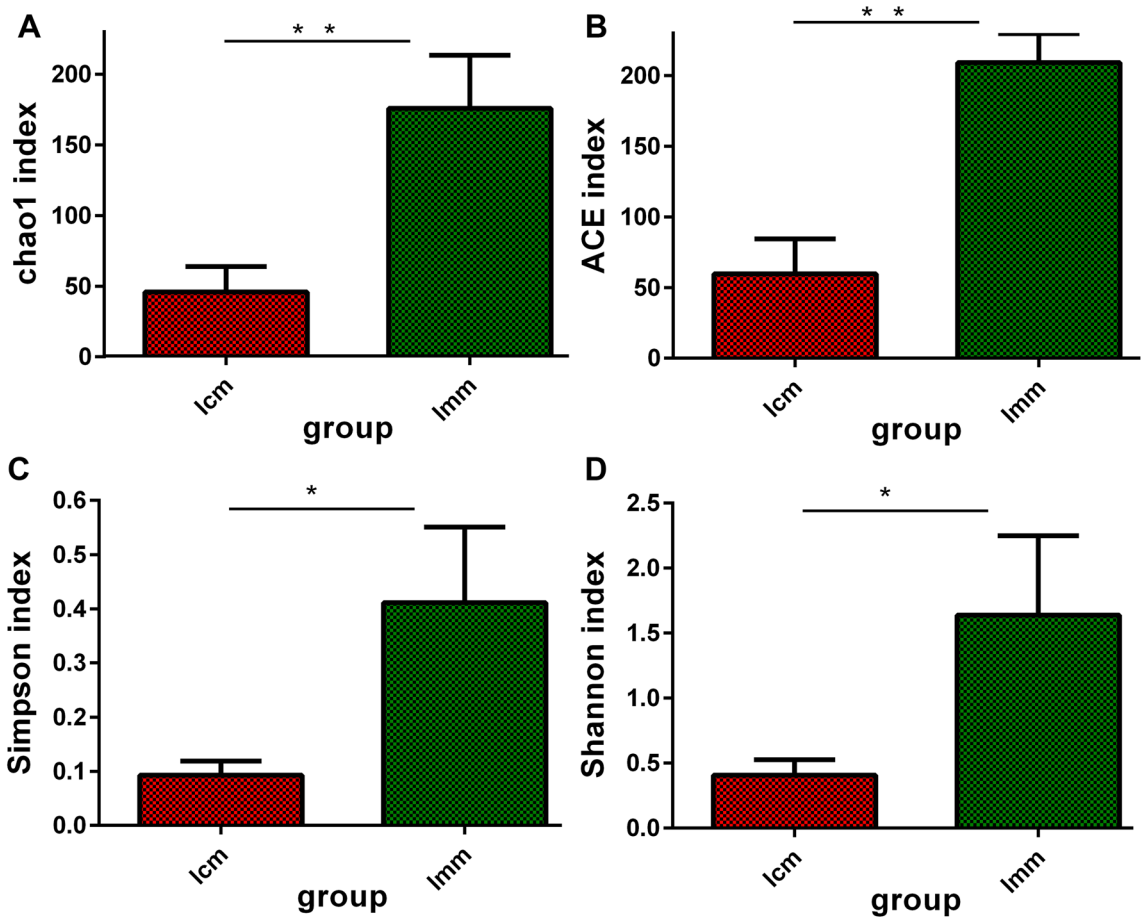

Fig. 2 Effects of antibiotics on the intestinal mucosa bacterial lactase genes diversity $^{*}P < 0.05$, $^{**}P < 0.01$

Effects of antibiotics on the similarity of bacterial lactase genes in the intestinal mucosa

A box plot for multi-group comparison can present the specific distribution characteristics of each sample data according to minimum value, lower quartile, median value, upper quartile and maximum value based on UniFrac distance. The abscissa indicates the comparisons in inter-group or in intra-group between two samples, and the ordinate represents the corresponding distance values, while the size of the box is in response to the difference size of sample gene sequence. In general, the distance between samples at the same group reflects the difference in inter-group. The differences in intra-group is significantly higher than in inter-group, indicating that there is statistical difference between samples of two groups. The results showed that the difference in inter-sample was minimum in the control group, and the differences in intra-group was higher than that in inter-group (Fig. 3a).

Each point represented one sample, and the points with identical color belonged to the same group. The closer the distance between two points was, the higher the similarity of gene sequence between two samples, and the smaller the difference was. The distance of samples from the control group was tightly concentrated compared to the samples from the model group (Fig. 3b). It indicates that the similarity of lactase genes sequence in control group mice is high, and antibiotics may destroy the structure of intestinal mucosa lactase genes.

Effects of antibiotics on the abundance of bacterial lactase genes in the intestinal mucosa

The intestinal mucosa bacterial lactase genes were mainly originated from Proteobacteria, Actinobacteria and unclassified bacteria at the phylum level, and the Proteobacteria was the most abundant. After being induced by antibiotics, the relative abundance of Actinobacteria and unclassified bacteria increased and the abundance of Proteobacteria decreased (Fig. 4a). At the genus level, based on the source of lactase genes, the quantity of them was lower in the control group than in the model group. Except for *Stenotrophomonas*, the relative abundance of other bacterial lactase genes increased in the model group. Furthermore, *Ewingella*, *Methylversatilis, Mycobacterium, Microbacterium, Beutenberqia* and *Actinomyces* were only found in the model group (Fig. 4b). In Fig. 4c, the ordinate indicates taxonomic units with significant differences between control group and model group. The abscissa shows the logarithm scores of the LDA difference analysis corresponding to the taxonomic units based on bar graphs and sorted by score values to describe the difference between groups. The longer the bar length, the more the corresponding difference of taxonomic unit became. As indicated in Fig. 4c the abundance of *Betaproteobacteria,*

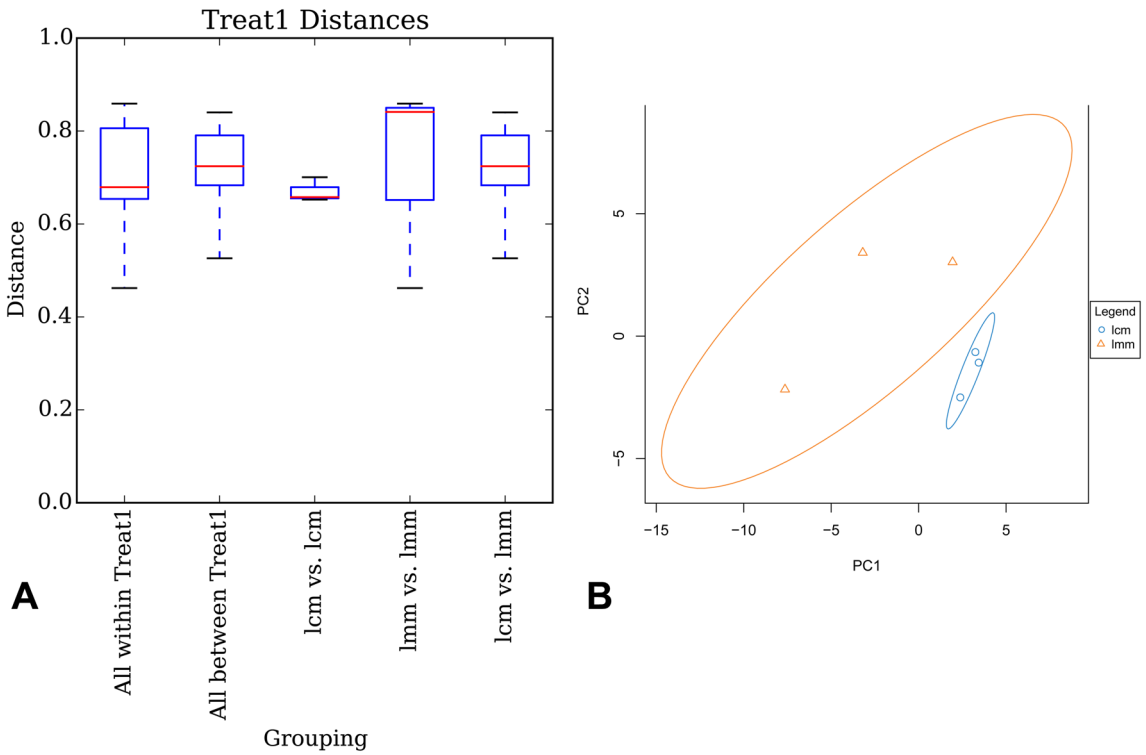

Fig. 3 Effects of antibiotics on the intestinal mucosa bacterial lactase genes similarity. *lcm* control group, *lmm* model group

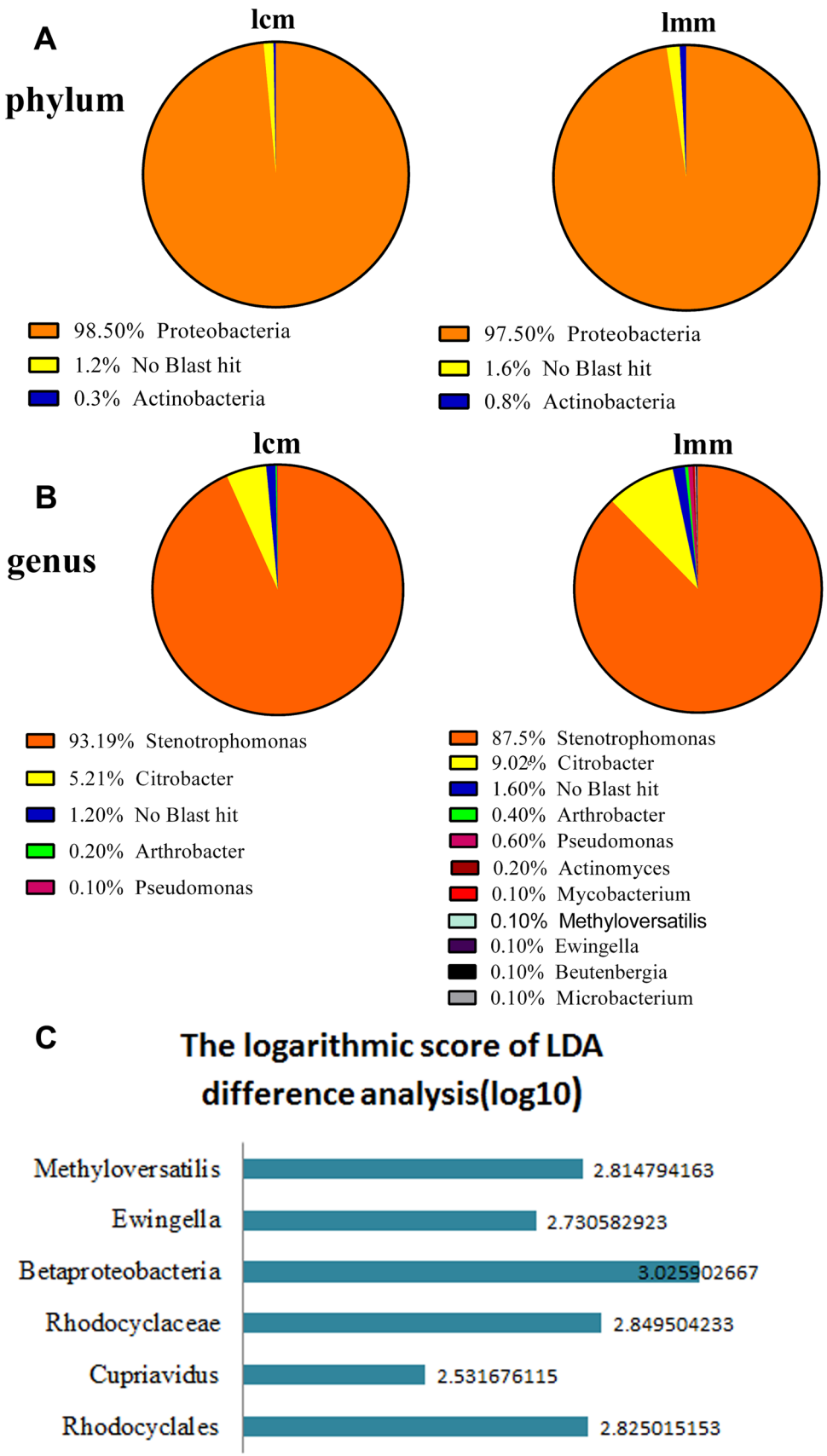

Fig. 4 Effects of antibiotics on the intestinal mucosa bacterial lactase genes abundance. *lcm* control group, *lmm* model group

Cupriavidus, Ewingella, Methyloversatilis, Rhodocyclaceae and *Rhodocyclales* in model group was significantly higher than that in control group.

Discussion

Antibiotics significantly increased intestinal mucosa bacterial lactase genes diversity

In recent years, the diversity of enzymes and their functional genes have drawn great attentions from scholars (Yang et al. 2015). It has become a hot issue to explore the microbial ecological function and community diversity from enzyme functional genes (Amann et al. 1995; Zhang et al. 2006; Edgar 2010). In addition to intestinal mucosa, intestinal microbes are also the main source of lactase. To investigate the diversity of bacterial lactase genes in the intestinal mucosa, we applied Miseq metagenome sequencing of beta-galactosidase gene from antibiotic-induced mice for the first time. The results show that the alpha diversity index of bacterial lactase genes in the intestinal mucosa was significantly increased after being induced by antibiotics ($P < 0.05$ or $P < 0.01$). From the total number of OTUs, there was a significant difference between control group mice and model group mice, indicating the poor similarity between them. These results are in sharp contrast to our previous study in intestinal contents, the number of OTUs, chao1 index and ACE index of bacterial lactase genes in the intestinal contents were significantly decreased in model group mice treated with antibiotics (Long et al. 2017a). A possible reason may be closely related to the different bacterial composition between intestinal contents and intestinal mucosa. In the known species, Firmicutes is a major phylum in intestinal contents (Zhao et al. 2016). However, there is few Firmicutes in intestinal mucosa.

Antibiotics increased intestinal mucosa bacterial lactase genes abundance

Bacterial lactase genes from the intestinal mucosa were mainly constituted of Proteobacteria, Actinobacteria and unclassified bacteria at the phylum level. Of them, Proteobacteria was the most abundant phylum, followed by unclassified bacteria and Actinobacteria. At the genus level, the difference was more apparent. For example, *Ewingella*, *Methyloversatilis*, *Mycobacterium*, *Microbacterium*, *Beutenberqia* and *Actinomyces* were commonly found in the intestinal mucosa of model mice. However, the lactase genes from these bacteria were not detectable in control mice. Furthermore, in the Lefse analysis, some critical microbial community of lactase-producing gene could be consistently detected in model mice treated with antibiotics including *Betaproteobacteria, Cupriavidus, Ewingella, Methyloversatilis, Rhodocyclaceae* and *Rhodocyclales*. And the abundance of these taxonomic units in model group was significantly higher than that in control group. In our previous study, bacterial lactase genes in intestinal contents were mainly derived from Actinobacteria, Probeobacteria, Firmicutes and unclassified bacteria. There were 79.33 ± 0.58 and 76.00 ± 4.58 different genera in the control group and model group, respectively (Long et al. 2017a), far beyond the genera of bacterial lactase from intestinal mucosa, indicating that there are more lactase-producing strains in the intestinal contents. Among the known intestinal microbiota, their relative proportions are very low in the intestinal contents (Long et al. 2017a). Moreover, *Streptococcus, Escherichia*, *Clostridium* and *Enterococcus* are the dominant genera in intestinal contents (Zhao et al. 2016). Maybe it is related to the liquidity of the contents and the effect of antibiotics on species activity. Moreover, we found difference in the main source of lactase-producing strains between contents and mucosa. In our opinion, when compared to intestinal contents, bacteria in intestinal mucosa are more stable. In contrast, bacteria in intestinal contents are more susceptible to be affected by external factors, such as diet and antibiotics. In addition, the intestinal mucosa has the function of self-immunity, which can maintain the stability of microbiota by self-regulation.

Antibiotics changed the community structure of intestinal mucosa bacterial lactase-producing genes

At present, a few researchers tried to explore the gastrointestinal bacterial community diversity of mammalian by using intestinal microbial function enzyme gene. The differences in enzyme gene diversity affect the activity level of functional enzyme, mainly in the type and quantity of functional enzyme strains, even the variation of functional enzyme gene structure coming from the same strain also has a certain relevance to the enzyme activity (Jiang et al. 2014; Tan and Shi 2008; Tan et al. 2009; Wang et al. 2014). From our results, there were significant differences in intestinal mucosa bacterial lactase genes between control and model groups by PCA analysis. In box plot, the size was smaller in inter-group and larger in intra-group, suggesting the poor similarity between control and model groups. We have got the same result in the intestinal contents, but different in forms. Abuse of antibiotics, which damaged intestinal mucosal brush-like lactase, affected the rapid regeneration of intestinal mucosa and decreased its activity, leading to diarrhea (Peng and Ren 2011). But for intestinal contents, some of the sensitive bacterial lactase genes were inhibited or killed, and insensitive genes were reproduced, mainly

changed the number, source and structure of lactase-producing strains, and decreased their activity, leading to diarrhea (Long et al. 2017a).

In summary, antibiotics-induced diarrhea increased the diversity and abundance of bacterial lactase genes in the intestinal mucosa and decreased some key lactase-producing strains activity by destroying the community structure of bacterial prolactinase genes or by undermining the encoding function of normal lactase gene, leading to diarrhea. Further study of gene expression would be needed for verifying it.

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Compliance with ethical standards

Conflict of interest The authors declare no conflict of interest related to this article.

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