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1 **Comparative genomic analyses of *Lactobacillus rhamnosus* isolated**  
2 **from Chinese subjects**

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18 Running title: *L. rhamnosus* comparative genomics

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25 **Abstract**

26 *Lactobacillus rhamnosus* has been found in many niches, including human intestine, vagina,  
27 mouth and dairy products. To intensively investigate the genomic diversity of this species, draft  
28 genomes of 70 *L. rhamnosus* strains isolated from different Chinese subjects were sequenced and  
29 further investigated. The pan-genome of *L. rhamnosus* was open. And gene-trait matching (GTM)  
30 was done to explore the carbohydrate utilization ability and antibiotic resistance, and to establish a  
31 pattern of gene existence/absence and growth/absence. There were no significant correlations  
32 between genetic diversity of the strains and the age or region of the donors. The current results  
33 extend the understanding of *L. rhamnosus*, which could be used as a reference for subsequent  
34 research as well as mining and application of the species.

35

36 **Keywords:** *Lactobacillus rhamnosus*; comparative genomics; carbohydrate utilization; antibiotics  
37 resistance

## 38 1. Introduction

39 *Lactobacillus* is the most diverse genus of lactic acid bacteria (LAB), and is usually found in  
40 fermented foods, feeds, oral and gastrointestinal tracts (Barrons and Tassone, 2008). *Lactobacillus* in  
41 the gut have an important role, for example, immunity regulation, cholesterol control, and  
42 gastrointestinal function improvement, on the intestinal micro-ecological balance and health of hosts  
43 (Damodharan et al., 2016; Reuman et al., 1986). In addition, *Lactobacillus* have an important role in  
44 food production and processing, such as fermented dairy products (Todorov, 2010).

45 *L. rhamnosus* is a Gram-positive bacterium that exists in a variety of niches, such as the human  
46 intestine and vagina (Pascual et al., 2008). *L. rhamnosus* GG is one of the best commercialized strain  
47 among *L. rhamnosus*. It has a strong resistance to gastrointestinal digestion and potential probiotic  
48 characteristics. Previous studies on *L. rhamnosus* focused on its functional benefits including various  
49 diarrhea treatments, immunity improvement, and respiratory infections prevention (Barbieri et al., 2017;  
50 Evans et al., 2016). Phenotype studies were carried out, such as acid tolerance, bile salt tolerance,  
51 carbohydrate utilization and adherence to epithelial cells (Ceapa et al., 2015; 2016; Succi et al., 2005;  
52 Tripathi et al., 2013).

53 To explore the metabolism, evolution and speciation, high-throughput methods (e.g., site sequence  
54 typing (MLST) and OmniLog (Biolog) phenotyping (Di Cagno et al., 2010) have been used to analyze  
55 genotype and phenotype (Bao et al., 2016). Comparative genomic analysis is a common tool in  
56 bioinformatics (Kant et al., 2011) that is able to identify the association between strains and their  
57 origins as well as to evaluate the gene distribution of specific species (Kelleher et al., 2017). It is  
58 significant for strain characteristics such as combining phenotypic profiles with strain-specific genetic  
59 diversity allows the assignment of unknown functions to specific genetic loci and to determine  
60 interactions with the hosts (Siezen et al., 2010). It also provides a new approach to effectively assess  
61 the diversity of strains. Genetic diversity of several species among the *Lactobacillus* genus had been  
62 carried out, such as *L. plantarum* (Duar et al., 2017; Martino et al., 2016), *L. casei* (Broadbent, 2012), *L.*  
63 *reuteri* (Zheng et al., 2015) and *L. salivarius* (Raftis et al., 2011). Current research on the *L. rhamnosus*  
64 genome was primarily concerned with the use of carbohydrates. Ceapa et al. (2015; 2016) predicted  
65 that *L. rhamnosus* had a number of carbohydrate transport and utilization genes, combined with  
66 phenotypic analysis, with which the adhesion-related functional genes were predicted. To investigate  
67 the genomic diversity of *L. rhamnosus*, Douillard et al. (2013b) sequenced and compared the genome

68 and phenotype validation of 100 *L. rhamnosus* strains from different niches.

69 To further learn about the genetic diversity of *L. rhamnosus* from Chinese subjects, 70 *L.*  
70 *rhamnosus* strains were isolated, draft-genome sequenced and analyzed with comparative genomics  
71 approaches as well as gene-trait matching analysis for both carbohydrate utilization and antibiotic  
72 resistance based on the genotype-phenotype combination.

## 73 **2. Methods**

### 74 **2.1 Isolation of strains**

75 One hundred and twenty fecal samples were collected from different regions of China. The fecal  
76 samples were mixed with 30% sterile glycerin solution (China National Medicines Corp. Ltd., Beijing,  
77 China), stored temporarily at 4°C, and stored at -80°C within 48 h for a maximum of 8 wk. One g of  
78 each stool sample was blended with 9 ml sterile physiological saline (China National Medicines Corp.  
79 Ltd.) (Ingham, 1999). Serial dilution and plating were done in an anaerobic workstation (AW400TG,  
80 Electrotek Scientific Ltd., Shipley, West Yorkshire, UK). For selection of lactobacilli, 100 µl of diluent  
81 was plated on *Lactobacillus* selective agar (LBS) (China National Medicines Corp. Ltd.) (Ingham,  
82 1999), and 50 U/ml nystatin (Sangon Biotech Co., Ltd., Shanghai, China). Agar plates were cultured in  
83 the anaerobic workstation flushed with 80% N<sub>2</sub>, 10% CO<sub>2</sub> and 10% H<sub>2</sub> at 37°C for 72 h. For each  
84 sample, colonies on LBS plates were counted. Colonies were selected at random and re-streaked onto  
85 LBS agar for purity. The final pure culture was cultured in LBS at 37°C for 24 h and preserved in 30%  
86 glycerol (China National Medicines Corp. Ltd.) at -80°C (Bottacini et al., 2018).

87 DNA was extracted from each strain using the Rapid Bacterial Genomic DNA Isolation Kit  
88 (Sangon Biotech Co., Ltd.) according to the manufacturer's instructions. The identity of each putative  
89 *Lactobacillus* isolate was confirmed using 16S rRNA sequence analysis. A 1.5-kb 16S rRNA gene  
90 fragment was generated using bacterial universal primers (27F: 5'-AGA GTT TGA TCC TGG CTC  
91 AG-3' and 1492R: 5'-ACG GCT ACC TTG TTA CGA CTT-3'). Each PCR mixture (25 µl) contained  
92 1.5 mM of MgCl<sub>2</sub> (Takara, Dalian, Liaoning, China), 20 mM of Tris-HCl (Takara), 50 mM of KCl  
93 (Takara), 200 µM of each deoxynucleoside triphosphate (Takara), 25 pmol of each of the two primers,  
94 1 U of Taq DNA polymerase (Takara), and 50 ng of DNA template. Each PCR (T100™ Thermal Cycle,  
95 BioRad, Hercules, California, USA) cycling program consisted of an initial denaturation step of 10 min  
96 at 95°C, followed by amplification for 35 cycles as follows: denaturation (30 s at 95°C), annealing (40  
97 s at 58°C), and extension (1 min at 72°C). The PCR was completed with a single elongation step (5 min

98 at 72°C). PCR fragments were purified using the PCR purification kit (Sangon Biotech Co., Ltd.)  
99 according to the manufacturer's instructions and subsequently sequenced by BGI (Shenzhen, Guangdong,  
100 China). Strains were assigned to a particular species following comparison of the 16S rRNA sequences  
101 using the Genbank database (<http://www.ncbi.nlm.nih.gov/BLAST/>) to assign to a particular species.

## 102 **2.2 Sequencing and draft genome assembly**

103 Draft-genome sequencing of all the strains were done using the Illumina HiSeq PE150 platform  
104 (Beijing Novogene Bioinformatics Technology Co., Ltd., Beijing, China) and strains were sequenced to  
105 a coverage depth no less than 100 ×. A-tailed, ligated to paired-end adaptors and PCR amplified with a  
106 350 bp insert were used for the library construction. The reads were assembled using SOAPdenovo  
107 software (<https://omictools.com/soapdenovo-tool>), the optimal Kmer value was selected to obtain the  
108 splicing sequence (Duranti et al., 2016; Tettelin et al., 2008), and local inner gaps were filled by using  
109 the software GapCloser (<https://sourceforge.net/projects/soap-denovo2/files/GapCloser/>) (Luo et al.,  
110 2012).

## 111 **2.3 Genome features prediction**

112 The G+C content and start codon of each genome were predicted with Glimmer 3.02  
113 (<http://ccb.jhu.edu/software/glimmer/index.shtml>) (Delcher et al., 2007). Transfer RNA (tRNA) was  
114 identified using tRNAscan-SE 2.0 (<http://lowelab.ucsc.edu/tRNAscan-SE/>) (Lowe and Eddy, 1997).  
115 Open Reading Frame (ORF) prediction using Glimmer3.02 and ORF were annotated using BLASTP  
116 analysis against the non-redundant protein databases created by BLASTP based on the National Center  
117 for Biotechnology Information (NCBI) (<https://www.ncbi.nlm.nih.gov/>) (Hyatt et al., 2010). Genemark  
118 (<https://www.genemarks.com/>) was used to predict the genetic structure of the spliced sequence and to  
119 generate amino acid and nucleotide sequences. The amino acids sequences were subjected to gene  
120 annotation using a Swiss-Prot (<https://www.uniprot.org/>), COG (<https://www.ncbi.nlm.nih.gov/COG/>),  
121 and NR database (<https://www.ncbi.nlm.nih.gov/protein>) (Lugli et al., 2017).

## 122 **2.4 Phylogenetic comparison**

123 Cluster analysis was done based on orthologous genes of *L. rhamnosus* using Orthomcl-v2.0.9  
124 software (<http://orthomcl.org/common/downloads/software/v2.0/>) (Kelleher et al., 2017). All the  
125 orthologous genes were extracted, then a phylogenetic tree was constructed using MAFFT alignment  
126 (<https://mafft.cbrc.jp/alignment/server/>) based on the orthologous genes and neighbor-joining (NJ)  
127 algorithm was used for evolutionary analysis (Mailund et al., 2006).

## 128 **2.5 Pan-genome and core-genome analysis**

129 To predict possible variation in the genome, the size of the pan-genome, core genome and unique  
130 genes were calculated. Based on the genomic sequence of all the strains, PGAP-1.2.1  
131 (<https://sourceforge.net/projects/pgap/files/PGAP-1.2.1>) was used for pan-genome calculations (Zhao  
132 et al., 2012). The core-genome was measured using the CD-HIT cluster analysis  
133 (<http://weizhongli-lab.org/cd-hit/>). Amino acids had a 50% pairwise identity and 0.7 length difference  
134 cut-off threshold (Harris et al., 2017). Then the Venn diagram was drawn to show the relationships  
135 among samples. In addition, the average nucleotide identity (ANI) values of each pair of genomes were  
136 calculated using ANI Perl (<https://github.com/chjp/ANI/blob/master/ANI.pl>) (Goris et al., 2007).

## 137 **2.6 Genotype/phenotype association applied to carbohydrate metabolism**

138 All the genomes were annotated using the HMM method in HMMER-3.1 (hmmer.org), and the  
139 enzymes involved in carbohydrate metabolism were analyzed using the carbohydrate-active enzymes  
140 (CAZY) database (<http://www.cazy.org/>) (Besemer et al., 2001; Lombard et al., 2014). Seven different  
141 carbohydrates including xylooligosaccharides (XOS), D-galactose, sucrose, D-trehalose, L-fucose,  
142 D-lactose and D-xylose (Sangon Biotech Co., Ltd.) were further selected for carbohydrate utilization  
143 analysis. A 10% (w/v) fresh aqueous solution of those carbohydrates were prepared and filtered through  
144 a 0.22 µm sterile membrane filter (Saigon Biotech Co., Ltd.). The utilization assay medium was freshly  
145 prepared with the same content as de Man, Rogosa and Sharpe (MRS) medium (China National  
146 Medicines Corp. Ltd.) (Ingham, 1999) except glucose, and two drops of 1.6% (w/v) bromocresol purple  
147 alcoholic solution (Sangon Biotech Co., Ltd.) were added, the latter as an indicator. After autoclaving  
148 and cooling, the sterile carbohydrate was added into the medium at 1% final concentration. To test the  
149 utilization capacity of each strain, after being sub-cultured twice in MRS medium, a 1% culture was  
150 inoculated into the growth media, each of which was supplemented with a different sugar instead of  
151 glucose. The utilization was observed as a color change and measured with a microplate reader at  
152 OD<sub>600nm</sub> (Varioskan Lux, Thermo, Waltham, MA, USA) after anaerobic culture at 37°C for 24 h (Hyatt  
153 et al., 2010). The test were done in triplicate.

## 154 **2.7 Antibiotic resistance gene and tolerance**

155 The antibiotic-resistant genes were analyzed using the comprehensive antibiotic resistance  
156 database (CARD) (<https://card.mcmaster.ca>) (Jia et al., 2017), to obtain information of predicted  
157 antibiotic resistance genes encoded by each genome.

158 According to ISO10932:2010 standard “Milk and Milk Products-Determination of the Minimal  
159 Inhibitory Concentration (MIC) of Antibiotics Applicable to Bifidobacteria and Non-Enterococcal  
160 Lactic Acid Bacteria (LAB)”  
161 ([http://www.iso.org/iso/iso\\_catalogue/catalogue\\_tc/catalogue\\_detail.htm?csnumber=46434](http://www.iso.org/iso/iso_catalogue/catalogue_tc/catalogue_detail.htm?csnumber=46434)), the MIC  
162 of 6 antibiotics (streptomycin, erythromycin, clindamycin, chloramphenicol, tetracycline, and  
163 trimethoprim), purchased from Sangon Biotech Co., Ltd., were analyzed for all the strains. Microbial  
164 dilutions were incubated in an anaerobic environment at 37°C for 48 h, measured with a microplate  
165 reader (Thermo) at OD<sub>625nm</sub>. And the threshold values for each antibiotic resistance issued by the  
166 European Food Safety Authority (EFSA) was used (FEEDAP, 2012).

167

### 168 3. Results

#### 169 3.1 Isolation of strains

170 The donors came from 18 different regions in China and their ages varied from 2 d to 102 yr (35  
171 samples of infants < 1 yr; 9 samples from 2-16 yr; 10 samples from 27-54 yr; 10 samples from 60-79 yr;  
172 7 samples from 82-102 yr). Most of donors were located in three cities (Wuhu, Anhui Province; Wuxi,  
173 Jiangsu Province; Wusu, Xinjiang Uygur Autonomous Region). A total of 600 isolates were isolated  
174 from the LBS agar, and all of them were species identified with 16S rRNA sequencing. Among them,  
175 168 isolates from 70 samples were confirmed as *L. rhamnosus*. Only one isolate from each sample was  
176 used for genome sequence to analyze the genetic features of the *L. rhamnosus* species (Table 1).

#### 177 3.2 General genome features

178 The draft genome of 70 strains isolated from Chinese subjects were sequenced and compared with  
179 *L. rhamnosus* GG. For all strains, the genome size ranged from 2.77 Mb for FXJWS25L4 to 3.10 Mb  
180 for FAHWH35L1. The quantity of genomic ORF ranged from 2676 to 3024 with an average of 2840.  
181 And only three strains had tRNA genes <40 tRNA genes. Furthermore, the GC content differed from  
182 46.6 to 47.5% for all strains (Table 1).

#### 183 3.3 Phylogenetic analyses of *L. rhamnosus*

184 Based on orthologous genes, a phylogenetic tree was constructed to evaluate the evolution of the  
185 species. The results showed that the species consisted of 1870 orthologous genes, which were shared  
186 among all the sequenced strains. According to the clustering relationship, all the 71 strains assessed  
187 were divided into 5 clusters. Focused on the strains isolated from different regions, there was no

188 significant regional correlation in clustering, although the strains isolated from Xinjiang Uygur  
189 Autonomous Region were relatively clustered (Figure 1). In the age distribution, the strains from  
190 infants <1 yr were located in the same branch, but there was no significantly clustering relationship  
191 when the age was subdivided. Interestingly, two pairs of strains were evolutionarily in the same small  
192 branch, which were FHNFQ3L5 and FHNFQ4L1 isolated from a mother and daughter pair, and  
193 FQHYN3M6 and FQHYN4M2 isolated from the samples with a father-son relationship. The results  
194 indicated that the clustering of strains was independent of age and region of the donors and might be  
195 related to the family relationship or daily diet of people from whom the samples were obtained.

#### 196 **3.4 Pan-genome and core genome analysis of *L. rhamnosus***

197 The 71 genomes of *L. rhamnosus* strains were used to calculate the pan-genome, and the resultant  
198 pan-genome was 8395 (Figure 2A). The pan-genome curve showed an upward trend (Figure 2A), while  
199 the power exponent in the pan-genome formula was >0.5 (Bosi et al., 2016). These results indicated  
200 that the pan-genome would increase if more genomes were included. However, new genes gradually  
201 decreased from the initial 190 to the last group at 54.3.

202 Based on the clusters of orthologous genes (COGS), the core-genome of *L. rhamnosus* was  
203 evaluated. The core genome correlation curve decreased with the addition of genomes, and was  
204 gradually stable after the 50th genome was included (Figure 2A). Ultimately the core genome of *L.*  
205 *rhamnosus* was 1835. The major role of the core genes were diverse, including energy production and  
206 amino acid metabolism (Figure 2C). In addition, the number of specific genes in the strains ranged  
207 from 5 to 146 (Figure 2B). In terms of gene content, FBJSY31L2 has the highest number of specific  
208 genes, which was highly diverse compared to the other strains.

209 To analyze the *L. rhamnosus* species and potential subspecies, ANI analysis was carried out. The  
210 ANI value of 70 strains as well as *L. rhamnosus* GG was 97.2-99.9%. It was greater than the threshold  
211 of 95%, (Richter and Rossello-Mora, 2009) which proved they were the same species without any  
212 subspecies. The ANI values of the FHNFQ3L5 and FHNFQ4L1 and the FQHYN3M6 and  
213 FQHYN4M2 were > 99%, which indicated that those two pair of strains from one family, might have a  
214 closer genetic relationship. However, clustering based on ANI has no significantly correlation with age  
215 or region of the donors (Figure 3).

#### 216 **3.5 Genotype/phenotype association applied to carbohydrate metabolism**

217 Carbohydrate-active enzymes were predicted using the CAZY database. The pan-genome of *L.*

218 *rhamnosus* contained genes encoding 27 glycosyl hydrolases (GH) families, 14 glycosyl transferases  
219 (GT) families, 8 carbohydrate esterases (CE) families, 8 carbohydrate binding modules (CBM) families,  
220 two polysaccharide lyases (PL) families and two activities (AA) families (Figure 4A). The heat-map of  
221 predicted GH family genes was constructed using HemI (Figure 4A), and the number of GH1, GH13,  
222 GH109 and GH25 genes was significantly greater than that of other members of the GH families. On  
223 the other hand, the number of genes for GH15, GH23, GH43, GH88, GH115 and GH126 were less than  
224 that of other members of the GH families. According to the evolutionary tree, the 71 strains tested were  
225 divided into 5 clusters. Some genes of the GH family had clustering differences in distribution, such as  
226 the GH115 family only existed with cluster-5, and the GH43 family existed in cluster-4 and -5.

227 The GH analysis showed the presence of the predicted  $\alpha$ -trehalase (GH15, GH65), fucosidase  
228 (GH1, GH29, GH30), galactosidase (GH1, GH2, GH4, GH31, GH35, GH36, GH59), mannose  
229 glycosidase (GH2, GH31, GH38) and xylanase (GH3, GH31, GH39, GH43). Genotype-phenotype  
230 correlations were carried out to identify the characteristics of *L. rhamnosus*. Based on the prediction of  
231 metabolic related genes, the 7 types of sugars were selected for the *in vitro* utilization assay. All the  
232 strains could use and grow well with D-galactose, sucrose, D-trehalose and L-fucose as the sole carbon  
233 source. Whereas the utilization of the other four carbohydrates differed among the strains assessed.  
234 D-lactose as the carbon source could support the growth of 43 strains. Only four could use D-xylose,  
235 while 20 strains could use XOS (Figure 4B).

236 Based on the genotype and phenotypic analysis, all the strains utilized D-galactose, sucrose,  
237 D-trehalose and L-fucose, indicating that the genotype was consistent with the phenotypic results.  
238 However, for some strains, the genotype and phenotype was not consistent. For instance, the gene  
239 encoding xylosidase was found in all 71 strains, but only four could utilize D-xylose, which only had a  
240 5.6% genotype-phenotype correlation. The presence of the predicted gene encoding lactase positively  
241 correlated with the glycometabolism phenotype for 43 strains (60.6% totally). Particularly, 28 strains  
242 having lactase in their genomes could not utilize D-lactose. For XOS, twenty strains could utilize  
243 D-xylose with a 28.2% genotype-phenotype correlation. There was no significant correlation between  
244 the carbohydrate utilization and the donor's age and region in both genotypes and phenotypes. The  
245 genes involved in D-lactose and L-fucose utilization were analyzed in detail for each strain and the  
246 results showed that the strains with the utilization ability had the whole related operons (Figure 4C).

### 247 **3.6 Genotype/phenotype association of antibiotic resistance**

248 The antibiotic resistant genes were analyzed using CARD, and based on the prediction, the heat  
249 map was constructed. These results showed that there were no resistance genes for gentamicin,  
250 streptomycin and neomycin in all 71 strains, but all of them contained the resistance gene for  
251 kanamycin, erythromycin, clindamycin, chloramphenicol, tetracycline, trimethoprim, ampicillin,  
252 ciprofloxacin, amoxicillin, vancomycin and rifampicin. The number of resistant genes for erythromycin,  
253 tetracycline and ciprofloxacin were significantly higher than for the other antibiotics. However, there  
254 was no significant difference in the number of genes of the same antibiotics among the strains. In  
255 addition, there was no significant correlation between the resistance gene number and the donor's age  
256 or region. The difference in the number of genes only reflected the differences among individual strains  
257 (Figure 5).

258 The MIC value of each strain was compared with the drug resistance threshold reported by EFSA  
259 to determine whether the strain was resistant or sensitive (FEEDAP et al., 2012). According to the  
260 genotype, six antibiotics were selected for the susceptibility test. Among the 6 antibiotics, no  
261 streptomycin resistance gene was predicted in the 71 strains, but the other five antibiotic resistance  
262 genes could be found among all the strains. However, all 71 strains showed different resistance to those  
263 antibiotics. The MIC of streptomycin for all the strains tested differed from 1 to 32  $\mu\text{g/ml}$  and mostly  
264 were 2 to 8  $\mu\text{g/ml}$  (Figure 6A). According to EFSA's guidance, all the strains were sensitive. The MIC  
265 of erythromycin for the strains ranged from 0.0625 to 8  $\mu\text{g/ml}$  (Figure 6B), which indicated 69 strains  
266 were sensitive. In addition, only 4.23% of the strains were resistant to tetracycline (Figure 6C). And  
267 among the 71 strains, the resistance rate for clindamycin reached 9.8% (Figure 6D). Compared with the  
268 first four antibiotics, the overall resistance rate for chloramphenicol was 76.1% (Figure 6E). *L.*  
269 *rhamnosus* has a conserved resistance gene for trimethoprim, therefore, there was no accurate threshold.  
270 The range of MIC of trimethoprim was 8-64  $\mu\text{g/ml}$ , in which 54 strains had a MIC value of 64  $\mu\text{g/ml}$   
271 (Figure 6F). These results showed that the MIC was not related to the subjects region and age, and was  
272 independent of the clustering of the strain.

273 All 71 strains were sensitive for streptomycin, which indicated that there was a certain correlation  
274 between genotype and phenotype. The resistance gene for erythromycin was observed in all 71 strains.  
275 However, only one strain was actually resistant to erythromycin, and the genotype-phenotype  
276 correlation rate was 2.8%. Three strains were resistant for tetracycline with 4.23% genotype-phenotype  
277 correlation. The resistance genes for clindamycin were observed in all 71 strains with only 9.8%

278 genotype-phenotype correlation. All the strains were resistant to trimethoprim with 100%  
279 genotype-phenotypic correlation.

280

#### 281 **4. Discussion**

282 With sequencing technology improvements, more researches involving the genome of the strain *L.*  
283 *rhamnosus* contained a 3.0 Mb genome, which was one of the largest for LAB (Lebeer et al., 2008).  
284 Several genomic sequences of *L. rhamnosus* have been identified to date, including human intestinal  
285 derived *L. rhamnosus* GG (ATCC 53103) (Hidetoshi et al., 2009), cheese-derived *L. rhamnosus* LC705  
286 (Prisciandaro et al., 2011) and soil derived *L. rhamnosus* CASL (Yu et al., 2011). In the current study,  
287 70 strains of *L. rhamnosus* were successfully isolated from different Chinese subjects from different  
288 regions, and their draft-genomes were sequenced to determine the genetic diversity and phylogenetic  
289 correlations within the species. The genomic characteristics of 70 Chinese *L. rhamnosus* strains were  
290 similar to previous literature (Kant et al., 2014). The GC content was significantly higher than that of *L.*  
291 *salivarius* and the average value of the *Lactobacilli* was ~42.4% (Harris et al., 2017).

292 Phylogenetic trees of all the 71 strains (including *L. rhamnosus* GG) based on orthologous genes  
293 showed no significant correlation between strain clustering and age or region of the donors (Odamaki  
294 et al., 2018). Furthermore, ANI is a classic index to distinguish whether particular strains belong to the  
295 same species, usually with a threshold of 95% as the species boundary (Sun et al., 2015). The ANI  
296 value of all the strains was >97%, and the similarity of most strains was as high as 99%. The clustering  
297 of strains was similar to that of phylogenetic trees established based on orthologous genes, but there  
298 were differences, which may be the result of different algorithms and the high similarity among  
299 different strains. From the phylogenetic trees constructed using the two methods, the clustering of *L.*  
300 *rhamnosus* strains had no significant correlation with age or region of donors. FHNFQ3L5 and  
301 FHNFQ4L1, isolated from two samples with a mother-daughter relationship, living in the same family  
302 and sharing the same daily diets, were evolutionarily in the same subgroup. And similar results were  
303 observed between FQHXN3M6 and FQHXN4M2, which were isolated from a father-son pair. The  
304 reduced differences of strains derived from one family indicated that similar diets and environments  
305 would decrease the genetic diversity of strains.

306 The pan-genome was mainly composed of core genes, accessory genes and specific genes, which  
307 represent the whole genome of a species (Borneman, 2012). The trend of the pan-genome in *L.*

308 *rhamnosus* was temporarily open, which was significantly higher than that of 13 strains of *L.*  
309 *rhamnosus* reported by Kant (Kant et al., 2014). When the strains increased, the pan-genome showed  
310 an upward trend. Core genes are in charge of the basic biological functions and the main phenotypic  
311 characteristics of the species (Inglin et al., 2018). The core-gene number in the current work was 1835,  
312 less than previous report, indicating when the number of strains increases, the core genes would decline.  
313 In the *Lactobacillus* genus, the size of core genes of *L. rhamnosus* was similar to that of *L. paracasei*  
314 (Smokvina et al., 2013), while higher than that of *L. salivarius* (Harris et al., 2017). Non-essential  
315 genes and specific genes are mainly involved in secondary metabolic pathways (Mols et al., 2010), and  
316 the number of specific genes of each strain was independent of the donor's region and age.

317 *Lactobacillus* has the great ability to ferment different carbohydrates and subsequently obtain  
318 metabolic energy (Ceapa et al., 2015; 2016). To analyze the carbohydrate fermentation capacity of *L.*  
319 *rhamnosus*, the carbohydrate-utilization related genes were predicted using the CAZY database, which  
320 contained GH, GT, PL, CE, CBM and AA (Lugli et al., 2017). GH are responsible for the hydrolysis (or  
321 modification) of carbohydrate glycoside bonds, which were mainly analyzed *in silico*. All the strains  
322 assessed contained  $\alpha$ -trehalase (EC 3.2.1.28), xylosidase (EC 3.2.1.37), fucosidase (EC 3.2.1.38),  
323 fructan  $\beta$ -(2,1)-fructosidase/1-exohydrolase (EC 3.2.1.153), fructan dfructosidase/6-exohydrolase (EC  
324 3.2.1.154), lactase (EC 3.2.1.108) and  $\alpha$ -L-arabinofuranosidase (EC 3.2.1.55), which have important  
325 roles in sugar metabolism. The *in vitro* carbohydrate utilization ability of the 71 strains showed that all  
326 the strains could use D-galactose, sucrose, D-trehalose and L-fucose, while only 5.6% could use  
327 D-xylose, which were consistent with the results in Bergey's Manual of Systematics of Archaea and  
328 Bacteria (Hammes and Hertel, 2015). Invertase (EC 3.2.1.26), also known as sucrase or  
329 fructofuranosidase, is an important enzyme for the irreversible hydrolysis of sucrose to glucose and  
330 fructose (Yu et al., 2017). This enzyme and the corresponding PTS system were present in all the  
331 strains indicating that all of them could utilize sucrose. Lactose is hydrolyzed by  $\beta$ -galactosidase (EC  
332 3.2.1.23) to give  $\alpha$ -D-glucose and D-galactose, which are utilized by the corresponding PTS pathways.  
333 Another pathway for lactose hydration is catalyzed by lactose phosphotransferase (EC 2.7.1.207) and  
334 6-phospho-beta-galactosidase (EC 3.2.1.85) with lactose-6-phosphate, galactose-6-phosphate,  
335 following the tagatose-6-phosphate pathway to further catabolism (Kankainen et al., 2009). All of the  
336 71 strains tested contained galactosidase, of which 28 strains were not able to metabolize D-lactose,  
337 including the reference strain LGG, and the results were consistent with the previous literature

338 (Kankainen et al., 2009). All 71 strains had the genes for the lactose PTS pathway (*lacFEG*) and  
339 tagatose-6-phosphate pathway (*lacABCD*), while further analysis indicated that antiterminator (*lacT*)  
340 and 6-phospho- $\beta$ -galactosidase (*lacG*) genes were altered in some of those strains, resulting in the loss  
341 of lactose utilization capacity (Douillard et al., 2013a; Kankainen et al., 2009). All the strains in  
342 cluster-4 and -5 (Figure 4B) could use lactose and were far away from LGG in their evolutionary  
343 relationship, indicating that there was a certain relationship between gene expression and strain  
344 clustering. Another typical example is L-fucose, all 71 strains including LGG could use L-fucose.  
345 When the L-fucose operon was analyzed it showed that the whole L-fucose catabolic pathway  
346 consisting of  $\alpha$ -L-fucosidase (*fucA*, EC 3.2.1.51), *fucU*, *fucI* and *fucS* was present similar to that in  
347 LGG (Becerra et al., 2015; Douillard et al., 2013a). Carbohydrate metabolism is not only related to the  
348 related hydrolases, but to the corresponding metabolic pathways and transport proteins. For example,  
349 the metabolism of xylose is related to the intracellular pentose phosphate pathway (PPP) as well as  
350  $\beta$ -xylosidase. Xylose isomerase could directly convert xylose to xylulose, which could be further  
351 phosphorylated by xylulose kinase to form 5-oxokinose-5-phosphate, which eventually enters the  
352 glycolysis pathway to produce alcohol by the intermediates 6-phosphate glucose and 3-phosphate  
353 glyceraldehyde (Yu et al., 2017). The genetic prediction results showed that the lack of xylose  
354 isomerase and xylulose kinase might result in no xylose entering the cell to participate in the PPP,  
355 consequently the strain could not metabolize xylose. It was confirmed that only four strains could use  
356 D-xylose *in vitro*, which might result from potential horizontal transfer of genes and was consistent  
357 with previous results that few strains could use D-xylose (Douillard et al., 2013b). XOS is a functional  
358 polymeric sugar composed of 2-7 xylose molecules bound by  $\beta$ -1,4 glycosidic bonds, usually requiring  
359 the ABC transport system to transfer to the intracellular space for hydrolysis. During the metabolism,  
360  $\alpha$ -L-arabinofuranosidase (EC 3.2.1.55) hydrolyzes the arabinose side chain in the XOS component, and  
361 xylosidase acts on the end of the XOS to release xylose (Andersen et al., 2013; Arboleya et al.,  
362 2018). All of the strains had an  $\alpha$ -arabinofuranosidase and a xylosidase, possibly lacking the  
363 corresponding transport system. Therefore, 51 strains were unable to use XOS. In addition, the gene  
364 involved in glucose metabolism had no correlation with the age and regional origin of the donors, and a  
365 similar conclusion was reached for sugar fermentation *in vitro*. Instead, they were consistent with the  
366 phylogenetic tree classification.

367 Antibiotic resistance could be divided into natural resistance and acquired resistance. The innate

368 resistance gene inherent to the genus or species could be inherited (Campedelli et al., 2019; Karapetkov  
369 et al., 2011;). The 71 strains were innately resistant to vancomycin (data not shown) and had a related  
370 resistance gene, consistent with the results of Korhonen et al. (2010). D-alanine residues at the  
371 pentapeptide end of the cell wall of *L. rhamnosus* were replaced by D-lactic acid, which prevented the  
372 binding of vancomycin. Thus, the strains were resistant to vancomycin, and same resistance was  
373 observed in *L. casei*, *L. plantarum* and *L. acidophilus* (Hamilton-Miller and Shah, 1998; Korhonen et  
374 al., 2010). The resistance gene of trimethoprim is *dfr*, a dihydrofolate reductase encoded by the  
375 integron originally found in *Vibrio cholera*, which inhibited the synthesis of tetrahydrofolate and  
376 exerted antibacterial activity. A congenital resistance gene to trimethoprim was observed consistent  
377 with previous results in *L. casei* and *L. plantarum* (Roberts et al., 1981). Most of the strains were  
378 sensitive to macrolide antibiotics (erythromycin and tetracycline), and only two strains (FAHWH5L1,  
379 FHeNJZ4L2) were resistant to erythromycin. Among the 71 strains, the ATP-binding cassette (ABC)  
380 antibiotic efflux pump for multi-drugs was observed to prevent the resistance for macrolides and  
381 tetracyclines (Comunian et al., 2010). A reasonable explanation for FAHWH5L1 and FHeNJZ4L2  
382 erythromycin resistance was that an A-to-G conversion point mutation on the 23S rRNA resulted in the  
383 binding of ribosomes to erythromycin or the A to G transition resulted in a lack of binding to the  
384 ribosome, making *L. rhamnosus* resistant to macrolide antibiotics (Begovic et al., 2009). All 71 strains  
385 of *L. rhamnosus* had the ABC-F ATP binding cassette ribosome protective protein and ABC antibiotic  
386 efflux pump involving chloramphenicol (Schwarz et al., 2004). Antibiotics are transported out of cells  
387 by efflux, which reduces intracellular antibiotic concentration and improves the drug resistance of the  
388 strains. Therefore, 76.1% of the strains were resistant to chloramphenicol. No target sites for the action  
389 of streptomycin antibiotics and drug efflux pumps were observed. In the streptomycin resistance test,  
390 none of the 71 strains were resistant, i.e., the genotype and phenotype were consistent. The genotype  
391 and phenotype were combined to analyze the resistance of the strain, and it was observed that the  
392 number of genes had no obvious relationship with the phenotypic results. In addition, genotypes and  
393 phenotypes were independent of the source of the strain, reflecting individual differences in the strain.

394

## 395 5. Conclusion

396 Seventy strains of *L. rhamnosus* were isolated from Chinese infants and adults and their  
397 draft-genome sequenced. Combined with LGG, the genome and biological properties of all the strains

398 were analyzed. The results showed that the pan-genome of *L. rhamnosus* was 8395 and core genome  
399 was 1835. All the strains could utilize D-galactose, sucrose, D-trehalose and L-fucose and only a few  
400 strains could use XOS and D-xylose, consistent with their genotype. For antibiotics resistance, most  
401 genotypic results of *L. rhamnosus* were consistent with phenotypic results. This study increased the  
402 genomic information and phenotypic study of *L. rhamnosus*, and provided reference value for future  
403 research on *L. rhamnosus*. The results will provide information on the meanings of the genetic diversity  
404 observed in this species, especially the carbohydrate utilization capabilities and antibiotic resistance,  
405 and can be applied to the further application of characterization of strains for probiotic additives.

406

#### 407 **Conflict of interest**

408 All authors declared no conflict of interest.

409

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415

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**Table 1** *L. rhamnosus* genomes sequenced and analyzed in this study.

Strains	Age	Region	Accession	Size (Mb)	ORF	tRNA	GC%
LGG (Kankainen et al., 2009)	<1 yr	North Carolina	ASM2650v1	3.01	2985	57	46.7
FAHWH26L1	6d	Anhui	PRJNA558200	2.88	2734	51	46.8
FAHWH30L1	7d	Anhui	PRJNA558202	2.90	2902	52	46.7
FAHWH2L1	93yr	Anhui	PRJNA558204	2.94	2856	57	47.3
FAHWH5L1	90yr	Anhui	PRJNA558208	2.91	2874	30	47.3
FAHWHD30L7	86yr	Anhui	PRJNA558583	2.89	2806	56	47.5
FAHWH35L1	70yr	Anhui	PRJNA558584	3.10	3024	53	47.4
FAHWH38L5	77yr	Anhui	PRJNA558585	2.92	2866	54	47.4
FBJCY2L1	9m	Beijing	PRJNA558586	2.93	2818	47	46.6
FBJCY3L1	11m	Beijing	PRJNA558587	2.86	2738	39	46.7
FBJSY7L3	62yr	Beijing	PRJNA558588	3.02	2986	56	47.2
FBJSY31L2	54yr	Beijing	PRJNA558589	2.94	2914	50	46.7
FBJSY60L1	64yr	Beijing	PRJNA558590	2.95	2833	58	47.3
FBJSY66L1	60yr	Beijing	PRJNA558592	2.90	2829	54	47.4
FFJND15L1	6m	Fujian	PRJNA558593	2.92	2795	52	46.7
FFJLY7L1	11yr	Fujian	PRJNA558594	2.94	2876	56	47.3
FGSZY12L6	84yr	Gansu	PRJNA558595	2.94	2865	54	47.3
FHeNJZ4L2	<1yr	Henan	PRJNA558596	2.95	2857	52	46.8
FHeNJZ7L1	6m	Henan	PRJNA558597	2.90	2801	38	46.8
FHeNJZ8L1	7m	Henan	PRJNA558599	2.91	2758	48	46.8
FHNFQ3L5	27yrs	Henan	PRJNA558600	2.95	2914	56	47.4
FHNFQ4L1	8m	Henan	PRJNA558601	2.96	2923	56	47.4
FHNFQ14L7	61yr	Henan	PRJNA558602	3.00	2970	55	47.3
FH28-1	102yr	Hubei	PRJNA558603	2.86	2743	40	46.7
FNMGEL5-1	7m	Inner Mongolia	PRJNA558604	2.90	2700	50	46.8
FNMGHLBE6L3	29yr	Inner Mongolia	PRJNA558605	3.02	2990	58	47.3
FNMGHLBE18L5	70yr	Inner Mongolia	PRJNA558606	2.95	2830	58	47.3
FJSWX1L3	6m	Jiangsu	PRJNA558607	3.02	2980	56	47.3
FJSWX2L6	4m	Jiangsu	PRJNA558608	2.94	2869	54	47.3
FJSWX3-L2	6m	Jiangsu	PRJNA558609	3.00	2926	50	46.6
FJSWX9L1	9m	Jiangsu	PRJNA558610	2.82	2739	54	47.4
FJSWX22-4	10m	Jiangsu	PRJNA558611	2.93	2815	51	46.6
FJSWX24-1	6m	Jiangsu	PRJNA558613	2.92	2789	43	46.7
FJSWX28L2	11m	Jiangsu	PRJNA558614	2.89	2787	54	47.4
FWXBH7L3	79yr	Jiangsu	PRJNA558615	2.94	2830	58	47.3
FJSYC1-5	<1yr	Jiangsu	PRJNA558616	2.81	2681	50	46.8
FJSYC4-1	<1yr	Jiangsu	PRJNA558617	2.90	2765	52	46.7
FJSNJ1-1-M2	1yr	Jiangsu	PRJNA558618	2.86	2732	40	46.7
FJSSZ2L1	<1yr	Jiangsu	PRJNA558620	2.94	2829	53	46.6
FJSZJ2-1	11m	Jiangsu	PRJNA558621	2.90	2762	61	46.7
FJXSRPYH4L2	72yr	Jiangxi	PRJNA558622	2.95	2829	59	47.3
FNXYCHL9M3	82yr	Ningxia	PRJNA558623	3.02	2977	55	47.2

FQHXN3M6	36yr	Qinghai	PRJNA558624	2.94	2827	56	47.3
FQHXN4M2	7yr	Qinghai	PRJNA558626	2.94	2830	56	47.3
FSHMX1-2	7m	Shanghai	PRJNA558627	2.92	2817	51	46.6
FSHMX3-1	3m	Shanghai	PRJNA558628	2.93	2817	48	46.6
FSDLZ7M12	85yr	Shandong	PRJNA558639	2.83	2676	52	46.7
FPAL5	<1yr	Shanxi	PRJNA558640	2.86	2730	51	46.7
FTJDG4G3	3d	Tianjin	PRJNA558641	2.93	2807	51	46.6
FTJDG9L1	4d	Tianjin	PRJNA558642	2.97	2842	44	46.7
FTJDG10L2	2d	Tianjin	PRJNA558643	2.96	2844	52	46.7
FTJDG11L1	2d	Tianjin	PRJNA558644	2.96	2864	60	46.7
FSCYA1L2	8m	Sichuan	PRJNA558646	2.87	2732	49	46.8
FXJWS2M1	43yr	Xinjiang	PRJNA558647	3.01	2970	55	47.2
FXJWS3M4	5m	Xinjiang	PRJNA558648	2.89	2786	54	47.4
FXJWS10M1	6m	Xinjiang	PRJNA558649	2.95	2833	58	47.3
FXJWS12L6	7yr	Xinjiang	PRJNA558650	2.95	2832	56	47.3
FXJWS13L6	16yr	Xinjiang	PRJNA558651	2.95	2832	56	47.3
FXJWS19L2	10yr	Xinjiang	PRJNA558652	2.96	2904	57	47.4
FXJWS25L4	40yr	Xinjiang	PRJNA558654	2.77	2730	47	47.2
FXJWS27L1	10yr	Xinjiang	PRJNA558655	2.95	2830	57	47.3
FXJWS37M4	40yr	Xinjiang	PRJNA558656	2.92	2868	54	47.2
FXJWS38L2	28yr	Xinjiang	PRJNA558657	2.95	2828	56	47.3
FXJWS44-L2	38yr	Xinjiang	PRJNA558658	2.95	2831	58	47.3
FXJSW6-1	31yr	Xinjiang	PRJNA558659	2.94	2828	58	47.3
FXJSW24M2	2yr	Xinjiang	PRJNA558661	2.96	2841	57	47.3
FZJHZ4L6	<1yr	Zhejiang	PRJNA558662	2.94	2824	58	47.3
FZJHZD11L1	10yr	Zhejiang	PRJNA558663	2.95	2830	56	47.3
FZJHZ14L3	8d	Zhejiang	PRJNA558664	2.94	2877	54	47.3
FZJZH6L2	4m	Zhejiang	PRJNA558665	2.86	2791	56	47.4
FZJTZ46L6	67yr	Zhejiang	PRJNA558666	3.04	3017	54	47.2

626 **Figure legends**

627 **Fig. 1 Phylogenetic analysis of *L. rhamnosus*.**

628 The triangle indicated donor's age with color-coded as follows: yellow for yr 0-1, grey for yr 2-16,  
629 orange for yr 27-54, pink for yr 60-79, light-blue for yr 82-102. The star indicated the region of sample  
630 collected, and the same area was represented by one color. Less than two samples in a region are  
631 represented in white. Red for Anhui, green for Beijing, blue for Henan, pink for Jiangsu, purple for  
632 Inner Mongolia, light green for Qinghai, light blue for Fujian, orange for Shanghai, grey for Xinjiang,  
633 yellow for Zhejiang, dark red for Tianjin. The strip divided the cluster into 5 large clusters, which are  
634 cluster 1 (blue), cluster 2 (green), cluster 3 (yellow), cluster 4 (orange), and cluster 5 (red).

635

636 **Fig. 2 Pan- and core genes of *L. rhamnosus*.**

637 (A) Pan-genome and core genome. The equation for calculating the pan-genome and core genome is  
638  $y=317.5*x^{0.677}+2706$  and  $y=573.3*e^{(-0.07*x)}+1830$ , respectively, in which  $x$  is the genome number of *L.*  
639 *rhamnosus* assayed.

640 (B) Venn diagram displaying the unique and core genes.

641 (C) Functional assignment of the core genome based on the COG database.

642

643 **Fig. 3 Pairwise average nucleotide identity (ANI) analysis of *L. rhamnosus*.**

644 The darker the color, the greater the similarity.

645

646 **Fig. 4 Evaluation of carbohydrate utilization in *L. rhamnosus*.**

647 (A) The predicted glycometabolism gene. A gradation of color from blue to white to red represents an  
648 increasing number of genes.

649 (B) The growth performance of the strains on different carbon sources. The red indicated growth, while  
650 the blue represented no growth.

651 (C) Gene cluster for D-lactose and L-fucose utilization.

652

653 **Fig. 5 Antibiotic resistance gene prediction in *L. rhamnosus*.**

654 The darker the color, the more the antibiotic resistance gene.

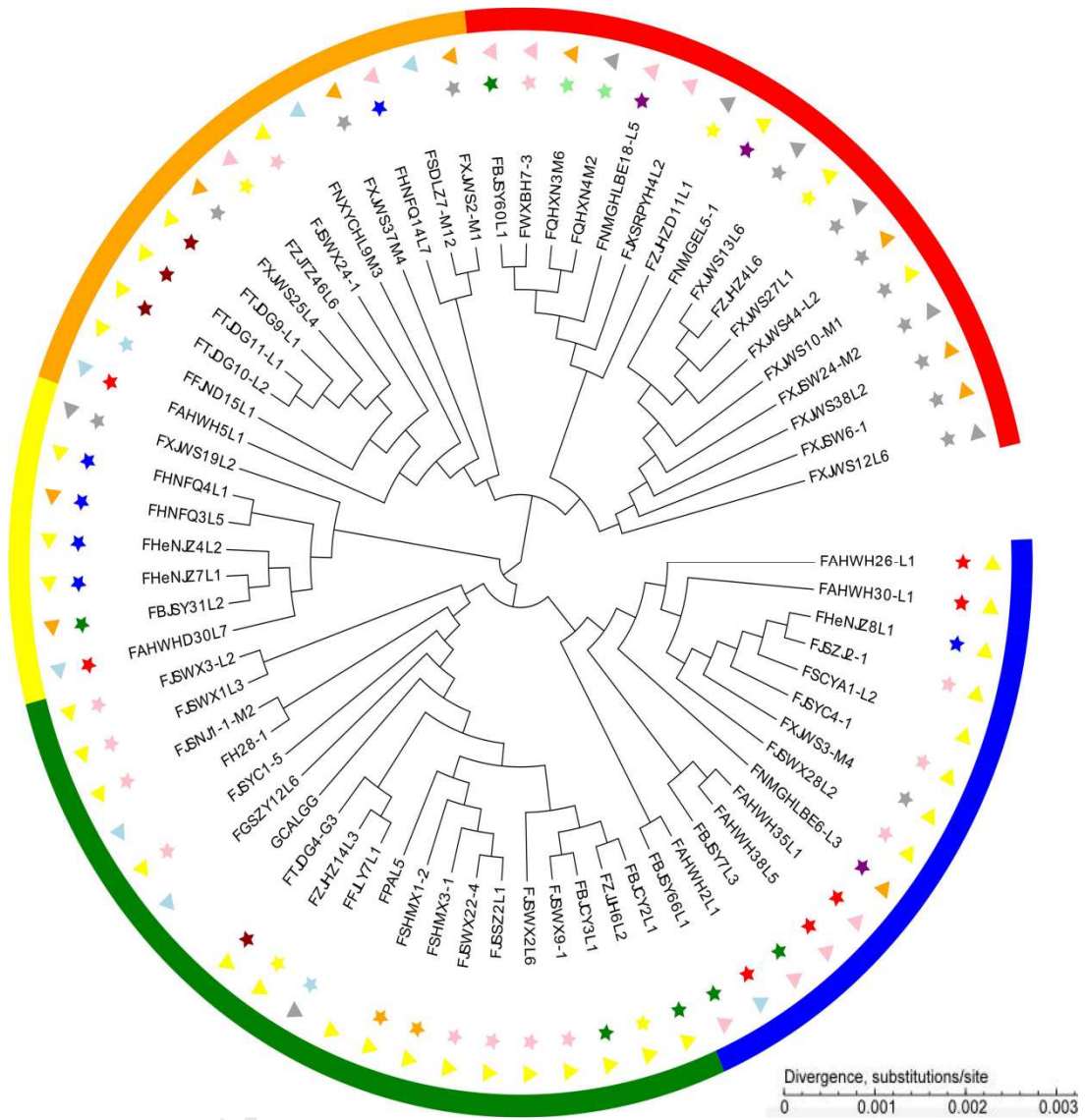
655

656 **Fig. 6 Microbiological cut-off threshold values.**

657 Distribution of cut-off threshold values for streptomycin (A), erythromycin (B), tetracycline (C),  
658 clindamycin (D), chloramphenicol (E), and trimethoprim (F) in all strains. The red dotted line  
659 represented the cut-off threshold value of EFSA.

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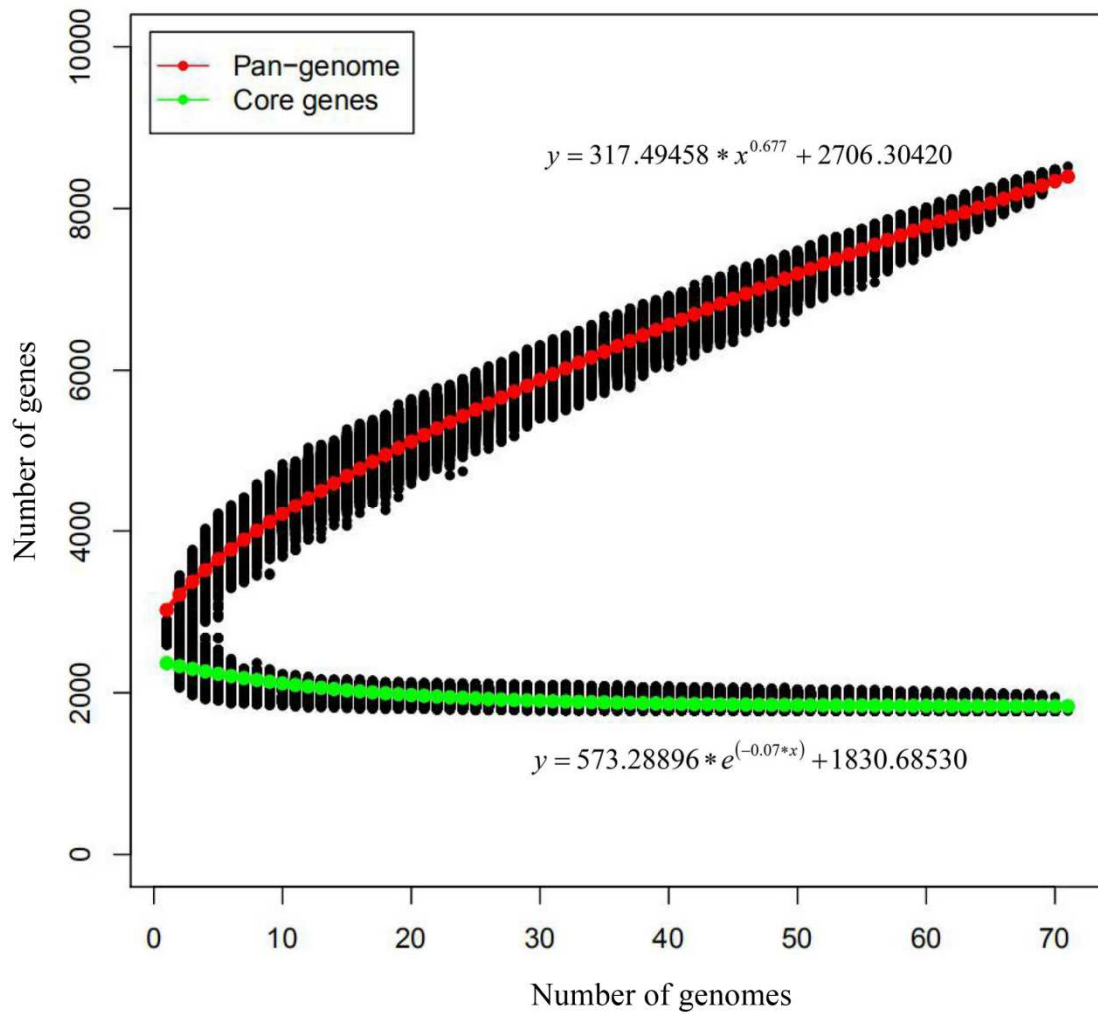
660 **Figure 1**



661

662 **Figure 2**

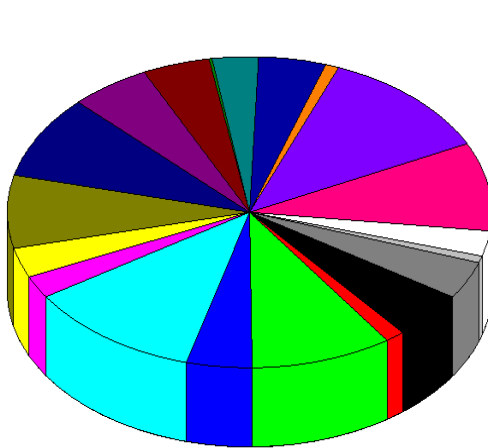
663 (A)



664



667 (C)

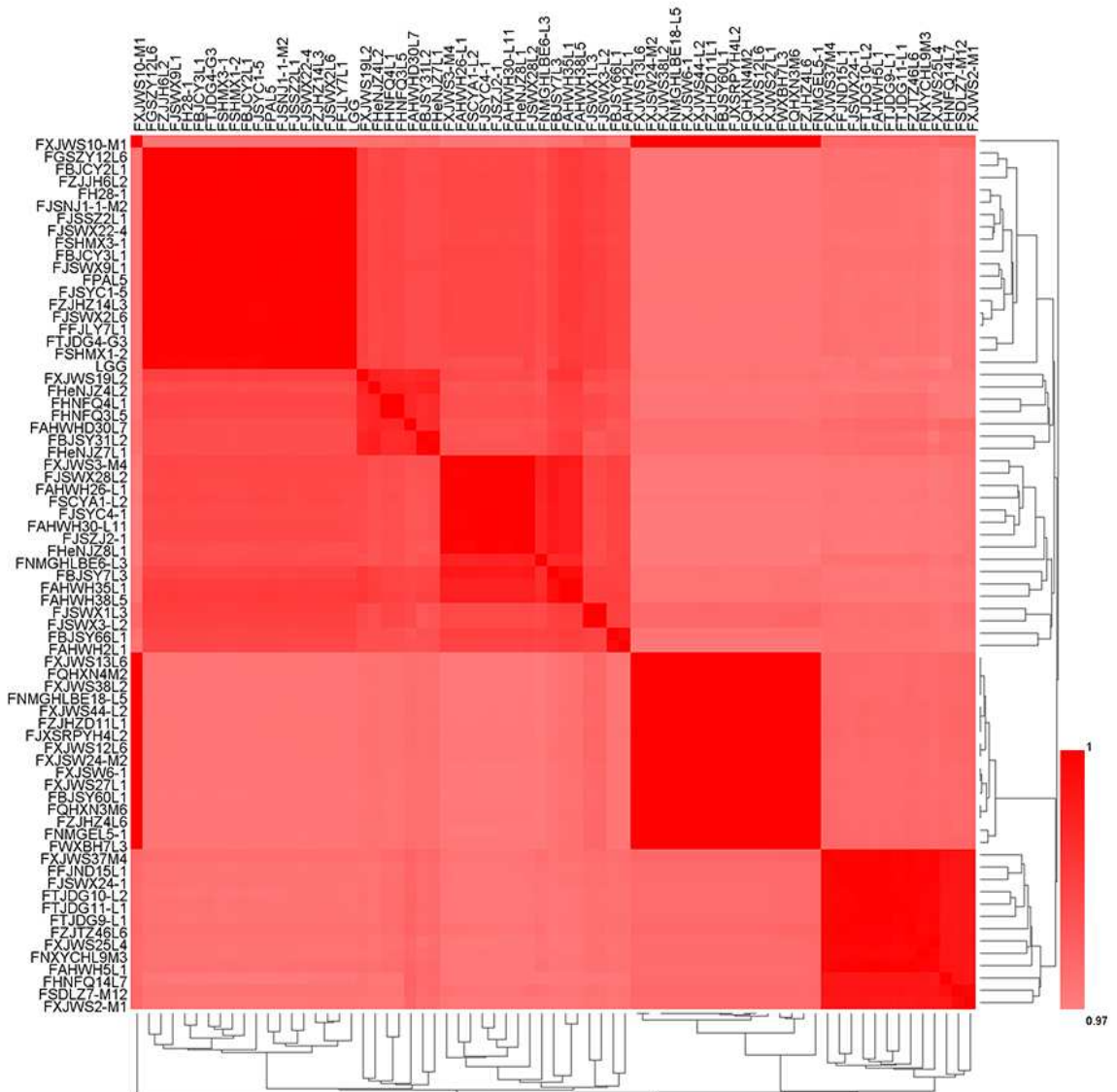


- [C] Energy production and conversion
- [D] Cell cycle control, cell division, chromosome partitioning
- [E] Amino acid transport and metabolism
- [F] Nucleotide transport and metabolism
- [G] Carbohydrate transport and metabolism
- [H] Coenzyme transport and metabolism
- [I] Lipid transport and metabolism
- [J] Translation, ribosomal structure and biogenesis
- [K] Transcription
- [L] Replication, recombination and repair
- [M] Cell wall/membrane/envelope biogenesis
- [N] Cell motility
- [O] Posttranslational modification, protein turnover, chaperones
- [P] Inorganic ion transport and metabolism
- [Q] Secondary metabolites biosynthesis, transport and catabolism
- [R] General function prediction only
- [S] Function unknown
- [T] Signal transduction mechanisms
- [U] Intracellular trafficking, secretion and vesicular transport
- [V] Defense mechanism

668  
669

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670 **Figure 3**

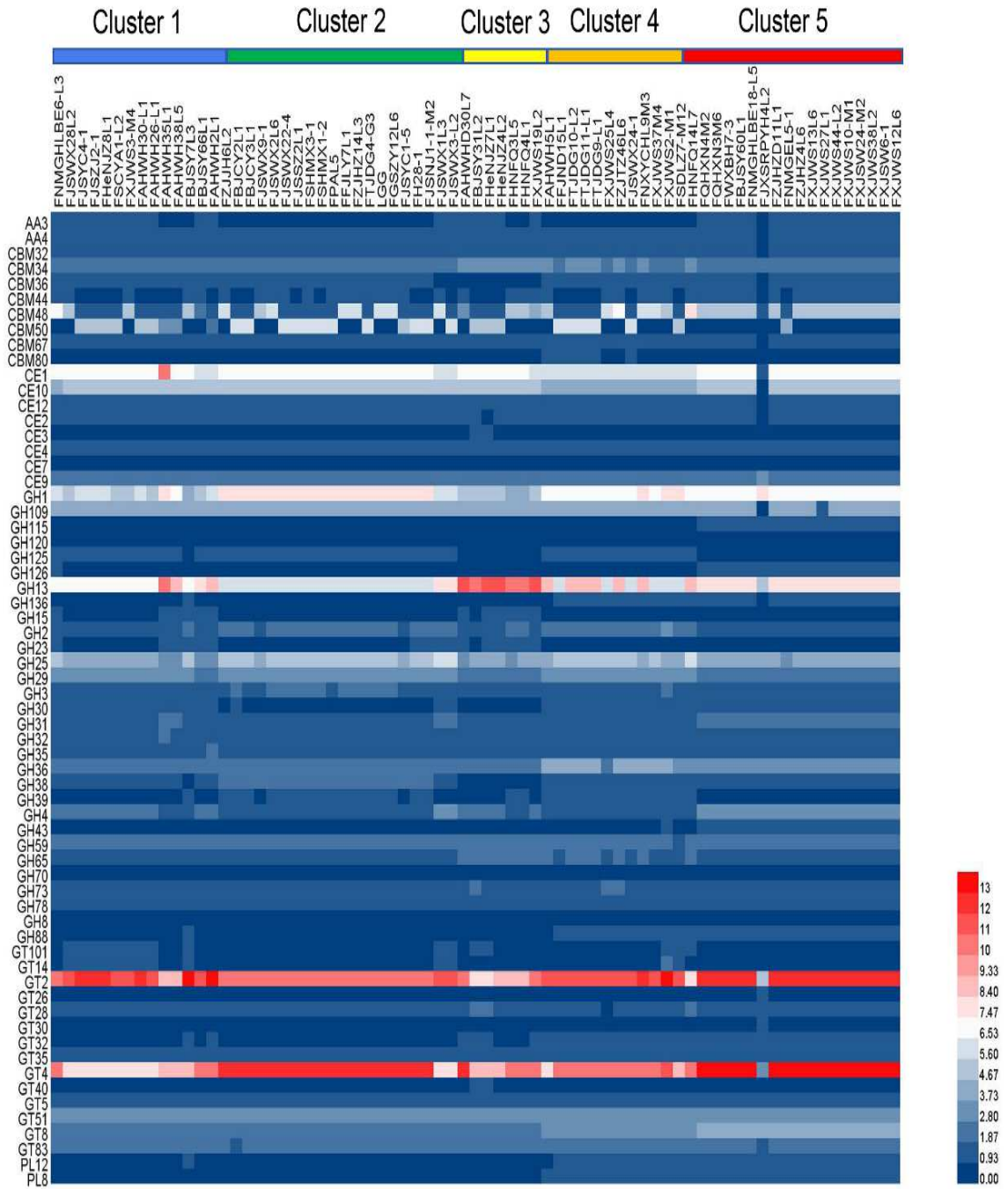


671

672

673 **Figure 4**

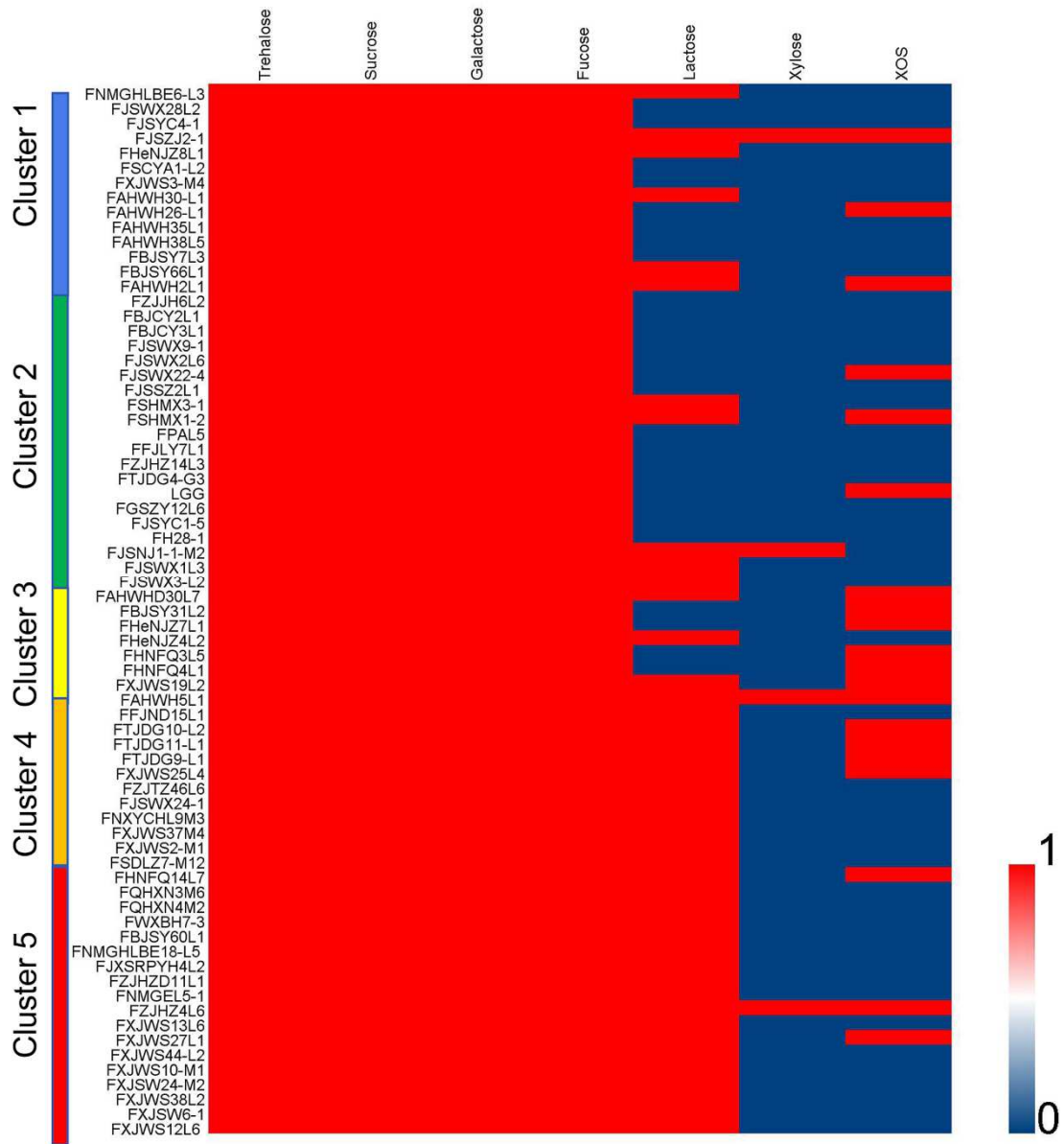
674 (A)



675

676

677 (B)

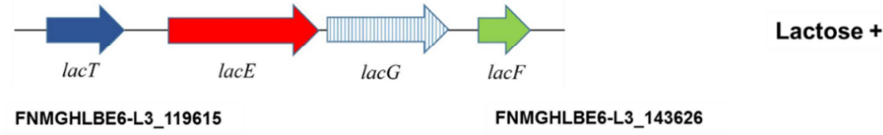
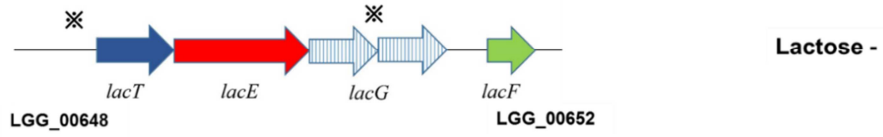


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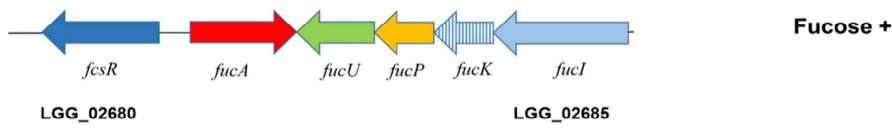
680 (C)

**Lactose utilization cluster**



681

**Fucose utilization cluster**

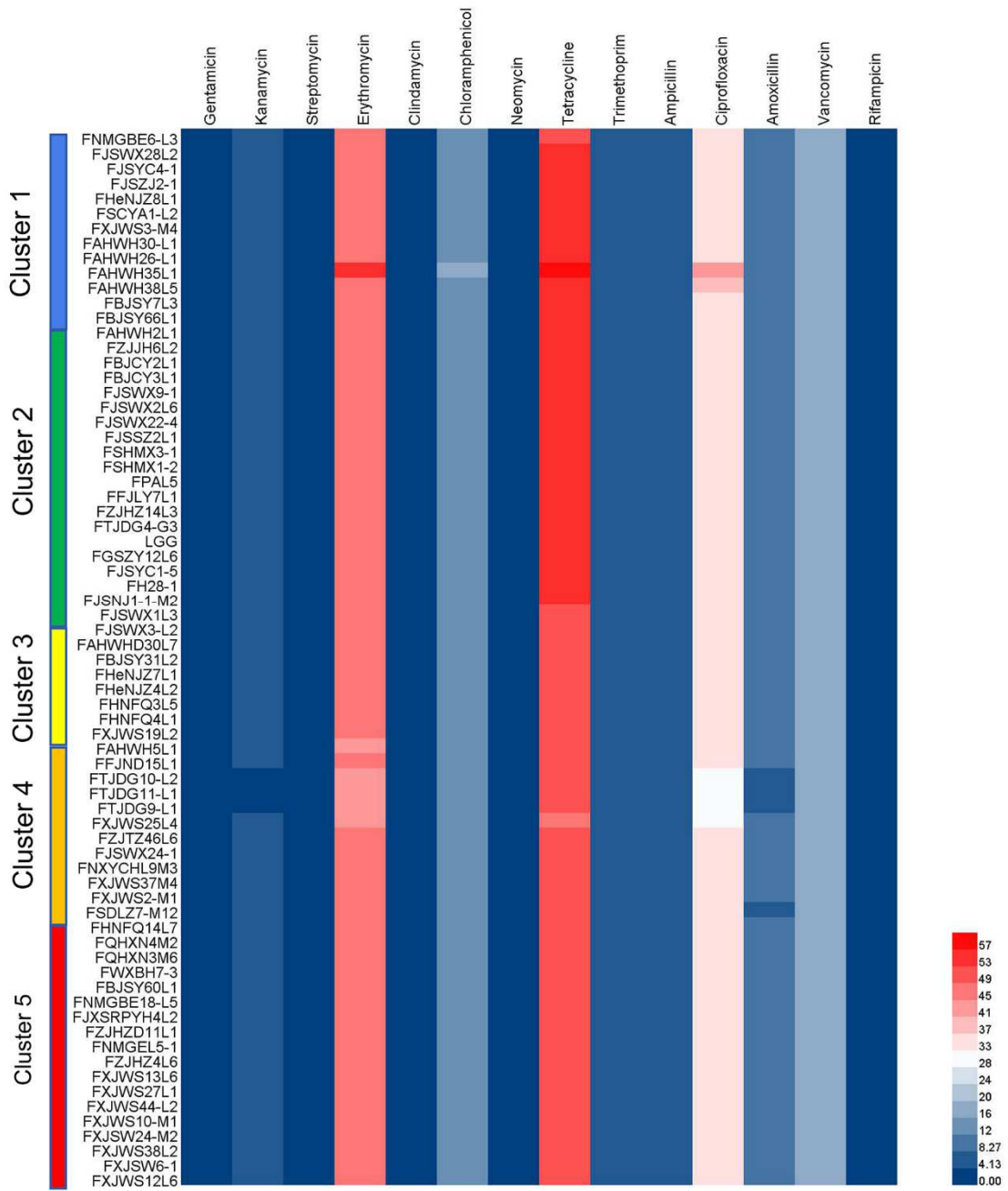


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684 **Figure 5**

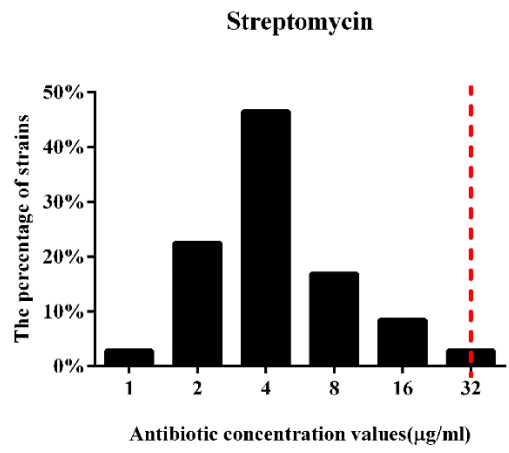


685

686 **Figure 6**

687

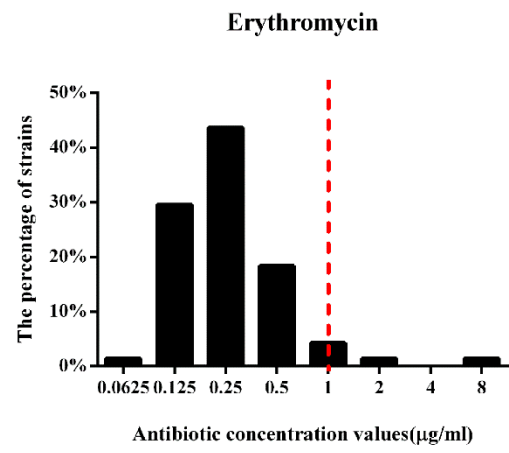
(A)



688

689

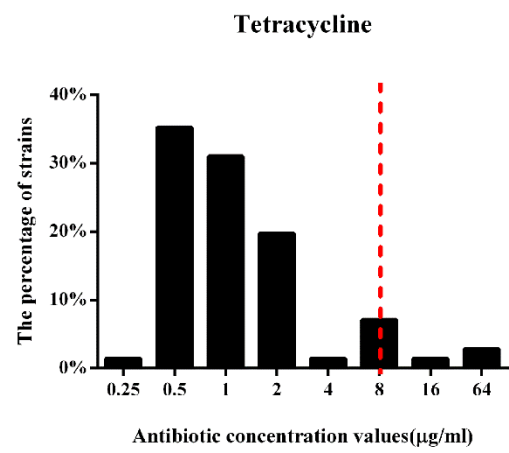
(B)



690

691

(C)

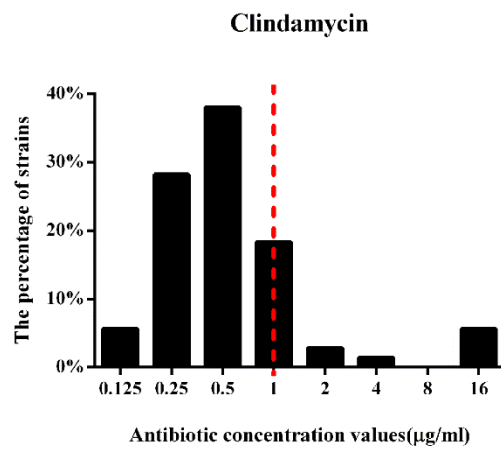


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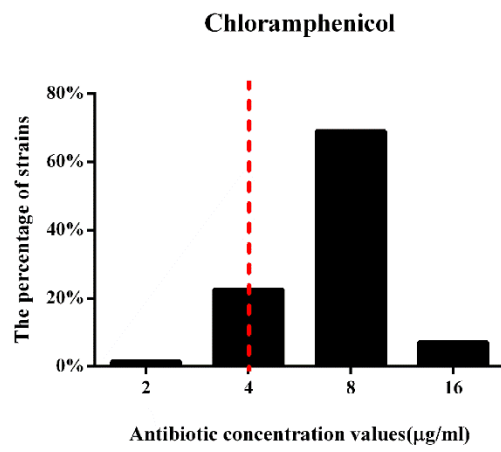
(D)



695

696

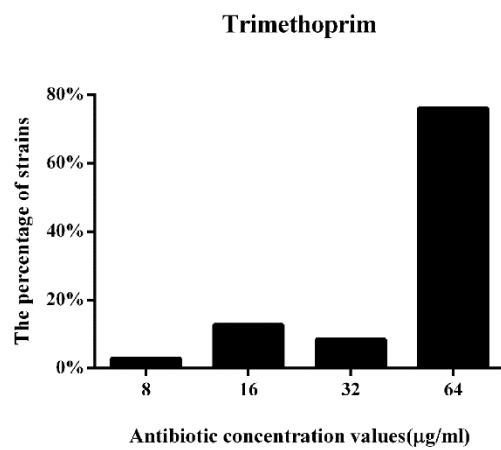
(E)



697

698

(F)



699

**Conflict of interest**

**All authors declared no conflict of interest.**

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