1   **Unraveling the gut microbiota of Tibetan chickens: insights into highland**

2   **adaptation and ecological advantages**

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4   **Running title：Microbiome Signatures and adaptive advantages of Tibetan chicken**

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27 **ABSTRACT**

28   Tibetan animals have several unique advantages owing to the harsh ecological

29 conditions under which they live. Tibetan mammals have been studied extensively.

30 However, understanding of the advantages and underlying mechanisms of the

31 representative high-latitude bird, the Tibetan chicken (*Gallus gallus*, TC), remains

32 limited. The gut microbiota of animals has been conclusively shown to be closely related

33 to both host health and host environmental adaptation. This study aimed to explore the

34 relationships between the cecal microbiome and the advantages of TCs based on

35 comparisons among three populations: native TCs residing on the plateau, domestic TCs

36 living in the plain, and one native plain species. Metatranscriptomic sequencing revealed

37 a significant enrichment of active Bacteroidetes but a loss of active Firmicutes in native

38 TCs. Additionally, the upregulated expression of genes in the cecal microbiome of native

39 TCs showed enriched pathways related to energy metabolism, glycan metabolism, and

40 the immune response. Furthermore, the expression of genes involved in the biosynthesis

41 of short-chain fatty acids (SCFAs) and secondary bile acids (SBAs) was upregulated in

42 the cecal microbiome of native TCs. Data from targeted metabolomics further confirmed

43 elevated levels of certain SCFAs and SBAs in the cecum of native TCs. Based on the

44 multi-omics association analysis, we proposed that the higher ratio of active

45 Bacteroidetes/Firmicutes may be attributed to the efficient energy metabolism and

46 stronger immunological activity of native TCs. Our findings provide a better

47 understanding of the interactions between gut microbiota and highland adaptation, and

48 novel insights into the mechanisms by which Tibetan chickens adapt to the plateau

49 hypoxic environment.

50 **IMPORTANCE**

51 The composition and function of the active cecal microbiome were significantly different

52 between the plateau Tibetan chicken population and the plain chicken population. Higher

53 expression genes related to energy metabolism and immune response were found in the

54 cecal microbiome of the plateau Tibetan chicken population. The cecal microbiome in the

55 plateau Tibetan chicken population exhibited higher biosynthesis of short-chain fatty and

56 secondary bile acids, resulting in higher cecal content of these metabolites. The active

57 Bacteroidetes/Firmicutes ratio in the cecal microbiome may contribute to the high-

58 altitude adaptive advantage of the plateau Tibetan chicken population.

59

60 **KEYWORDS**

61 Multi-omics; active microbiome; innate immune activity; short-chain fatty acids;

62 secondary bile acids; active Bacteroidetes/Firmicutes ratio.

63

64 **INTRODUCTION**

65　The Tibetan Plateau is the largest uplifted crust on Earth, with an average elevation of

66 4000 m above sea level. The plateau has a highland continental climate and complex

67 topography with significant variations, rendering it a unique and challenging environment

68 for its inhabitants (1). The harsh ecological conditions in the Tibetan Plateau make it a

69 unique "natural laboratory" for investigating organism adaptations to extreme

70 environments (2). For example, plateau animals have acquired the ability to adapt to

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71 extreme cold, low oxygen levels, and strong UV radiation (3). In addition, certain species

72 display distinct behaviors, such as seasonal migration to lower elevations to locate food

73 and seek refuge from inclement weather, as well as burrowing to protect themselves from

74 cold and wind (4). High-altitude organisms have developed effective energy metabolism

75 and immunological capabilities under harsh environmental conditions, enabling them to

76 maintain their health despite harsh external stressors (5, 6). Understanding the

77 mechanisms underlying effective energy conversion and higher stress tolerance in

78 Tibetan animals could potentially contribute to the advancement of the global livestock

79 industry beyond the plateau. However, these mechanisms are not fully understood.

80   A strong association has been established between gut microbiota and host health as

81 well as host adaptation to the environment (7). Studies conducted on the Tibetan Plateau

82 have revealed the crucial function of gut microbiota in facilitating high-altitude

83 adaptation and health maintenance in both humans and animals in this challenging

84 environment (8). The gut microbiota of native Tibetans and Tibetan pigs varies between

85 high- and low-altitude habitats, indicating the influence of the plateau environment on gut

86 microbiota (5). In addition, a comparative analysis of the gut microbiota in native Tibetan

87 and Han populations residing at different altitudes revealed notable disparities in the

88 compositional and metabolic characteristics of the gut microbial communities. These

89 differences could have implications for health and disease susceptibility (9, 10). In

90 particular, the mutual interaction between host health and gut microbiota is primarily

91 influenced by metabolites produced by symbiotic microorganisms, such as short-chain

92 fatty acids (SCFAs), volatile fatty acids, secondary bile acids (SBAs), and vitamins (11).

93 The role of SCFAs in host immunity has been studied extensively. SCFAs can regulate

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94   the epithelial barrier function (12), increase resistance to enteric pathogens (13), and

95   serve as an energy source for colonocyte activity (14). The metabolite succinate, which is

96   primarily derived from dietary fermentation by the gut microbiota, has garnered

97   significant attention. It functions as an intermediate in the TCA cycle, maintains

98   metabolic homeostasis, and acts as a receptor ligand to activate the innate immune

99   response (15). Moreover, SBAs, such as lithocholate (LCA) and deoxycholate (DCA),

100   have been proven to be key innate immune modulators. These metabolites are exclusively

101   produced from primary bile acids (BAs) derived from the host by the symbiotic

102   microbiome (16). Despite numerous valuable findings, research in this field is ongoing,

103   and the intricate relationships between the gut microbiota, health, and highland

104   adaptation should be investigated further.

105   Tibetan chickens (*Gallus gallus*, TCs) are a unique breed native to the Tibetan Plateau.

106   They have demonstrated remarkable adaptations to high-altitude environments for

107   thousands of years (17, 18). TCs, the most important poultry breed in the Tibetan Plateau,

108   possess several advantages, including excellent disease resistance, medicinal value, and

109   high nutritional content. Moreover, this traditional food is known for its natural and

110   healthy qualities, making it a sought-after product with limited availability and high

111   market price (19). TCs were introduced to plain areas several years ago for large-scale

112   intensive breeding, driven by market demand and economic benefits. However, their

113   introduction into plain areas has resulted in the disappearance of certain valuable traits

114   (17, 20). The gut microbiota of animals is influenced by the microorganisms in their

115   living environment and diet (21). Variations in the cecal microbiome of TCs from five

116   geographic regions have been documented, and the degree of similarity has been

117   correlated with the geographic environment (22). One study found that once TCs were

118   introduced into plain areas, the composition and diversity of the cecal microbiome

119   changed (23). One possible explanation for the physiological changes in TCs living in

120   plains could be alterations in the gut microbiota. Previous studies on the symbiotic

121   microbiomes of TCs have primarily focused on DNA levels using techniques such as 16S

122   rRNA sequencing or metagenomics (24, 25). However, these methods only allow the

123   identification of microorganisms and do not provide information regarding their activity

124   (26). Metatranscriptomics offers valuable insights into microbial gene expression because

125   expressed transcripts serve as surrogates for the actual phenotype (27). With continued

126   advancements in tools and algorithms for analyzing metatranscriptomic data (28), there is

127   an opportunity to delve into the functional activity of the cecal microbiome of TCs.

128   This study aimed to investigate and compare the active cecal microbiome and its gene

129   expression profiles between chickens residing in plateaus and plains, using

130   metatranscriptomic technology. Additionally, variations in the synthesis capacity of

131   SCFAs and innate immune factors, which are influenced by the cecal microbiome in

132   different chicken populations, were explored. Furthermore, the levels of key metabolites

133   produced by the cecal microbiome, such as SCFAs and BAs, were measured using

134   targeted metabolomics. Through combined analysis of the metatranscriptome and

135   metabolome, our study probed the associations between the unique characteristics of TCs

136   and their cecal microbiome.

137

138   **RESULTS**

Active microorganisms and functions in the cecum of chickens

Metatranscriptomics were applied to three chicken populations, yielding an average of 105,456,908 reads per sample. The effective ratio was 99.79%, with 3.17% rRNA (Table S1). The In-TC samples yielded an average of 77,827 contigs, with an N50 length of 899.25 bp and 111,490 ORFs (Table S2). In contrast, the Ex-TC and QY samples had a lower number of contigs, with averages of 11,767 and 7,179, respectively. The N50 lengths for these samples were 735 and 811 bp, respectively (Table S2). The host taxonomy of these transcripts was assigned and compared among the three chicken populations. The results showed that Bacteroidetes and Firmicutes were the dominant active cecal bacterial phyla in chickens residing on the plateau (In-TC) and plain (Ex-TC and QY), respectively (Fig 1A). In addition, the active cecal microbiome had a relatively stable composition in the In-TC samples, whereas significant individual differences were observed in the Ex-TC and QY populations (Fig 1A). These findings were further confirmed by the significantly reduced Bray-Curtis distance of active cecal microbiomes in the In-TC group compared to those in the Ex-TC and QY groups (Tukey's HSD test, p < 0.05, Fig S1). Moreover, comparison of the active cecal bacterial genera among different chicken populations revealed higher activities of *Alistipes*, *Bacteroidetes*, *Desulfovibrio*, *Mediterranea*, and *Prevotella* in the In-TC samples (Tukey's HSD test, p < 0.05, Fig 1B). In contrast, only *Lactobacillus* and *Streptococcus* were more active in the Ex-TC and QY individuals (Tukey's HSD test, p < 0.05, Fig 1B).

Based on the comparison of transcripts belonging to different COG terms, the expression levels of genes involved in multiple functions were upregulated in the cecal microbiome of the In-TC group compared to those in the Ex-TC and QY groups (Tukey's

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162   HSD test, $p < 0.05$, Fig 2A). The functions of the In-TC samples included cell division,

163   cell motility, defense mechanisms, energy conversion, signal transduction, and secondary

164   metabolite conversion (Fig 2A). Furthermore, we also observed significantly higher

165   expression of genes related to carbohydrate metabolism in the cecal microbiomes of In-

166   TC samples compared to those of the Ex-TC and QY groups (Tukey's HSD test, $p < 0.05$,

167   Fig 2B).

168   **Identification of DEGs and their functions in the cecal microbiome of chicken**

169   **populations**

170   The PCoA plot revealed distinct clusters of gene expression profiles between the cecal

171   microbiomes of chickens residing in the plateau (In-TC) and plain (Ex-TC and QY)

172   regions (Fig S2A). The Adonis test also confirmed significant differences in the gene

173   expression profiles of the cecal microbiomes among the three chicken populations

174   studied ($p < 0.05$). The In-TC populations had a total of 95,455 and 96,014 DEGs in their

175   cecal microbiomes compared to the Ex-TC and QY populations, respectively (Fig S2B

176   and S2C). Most of these DEGs (92,347 and 90,267, respectively; Fig S2B and S2C) were

177   upregulated in the In-TC group, accounting for over 95% of the DEGs. In contrast, only

178   6151 genes were found to be differentially expressed in the cecal microbiomes between

179   the Ex-TC and QY populations (Fig S2D). The number of DEGs in the comparison of the

180   Ex-TC and QY groups significantly decreased, primarily because of the decrease in

181   upregulated genes (2635 compared to more than 90,000).

182   Considering those chickens from the Ex-TC and QY populations both lived in plain

183   environments, we identified potentially critical genes for the high-latitude adaptability of

184   TCs by examining DEGs that were present in the comparisons of In-TC with Ex-TC or

185   QY, but not in the comparison of Ex-TC with QY. Based on Venn diagrams, we

186   identified 89,971 upregulated and 440 downregulated genes that were differentially

187   expressed in the cecal microbiomes of chickens residing in the plateau and plain regions

188   (Fig 3A and 3B). The upregulated genes were enriched in several KEGG pathways

189   related to energy metabolism, glycan metabolism, and immune response (Fig 3C). No

190   enriched pathways were identified for the downregulated genes.

191   **Powerful biosynthesis of SCFAs and immune activity in the cecal microbiome of In-**

192   **TCs**

193   Regarding genes responsive to the synthesis of SCFAs, *pct* and *ACSS1_2* for acetate

194   and propionate biosynthesis, respectively, and *buk* and *atoAD* for butyrate biosynthesis

195   showed significantly higher expression in the cecal microbiome of In-TC individuals than

196   in Ex-TC and QY samples (Fig 4A). The hosts of the *pct* gene in all three populations

197   belonged to the phylum Firmicutes (Fig 4B), indicating that discrepancies in gene

198   expression could be attributable to host differences at the fine taxonomic level. Further

199   research revealed that the *Anaerotignum* genus was the primary host of the *pct* gene in In-

200   TC samples, whereas the *Ruminococcus* genus was the primary host in Ex-TC and QY

201   samples (Fig S3). In all three populations, the host of the *ACSS1_2* gene was the

202   *Desulfovibrio* genus belonging to the phylum *Proteobacteria* (Fig 4B and Fig S4). The

203   abundance of active *Desulfovibrio* was significantly higher in the In-TC samples than in

204   the Ex-TC and QY samples (Fig 1B). This finding was consistent with the upregulated

205   expression of the *ACSS1_2* gene in In-TC individuals. The hosts of *buk* and *atoAD* genes

206   in the studied samples were mostly from the Bacteroidetes phylum (Fig 4B), particularly

207   in the In-TC samples (100%). The higher expression levels of *buk* and *atoAD* genes in the

9

208   In-TC population were consistent with the higher abundance of active Bacteroidetes in

209   the cecal microbiome of In-TC individuals (Fig 1A).

210   The expression levels of several genes involved in the biosynthesis of innate immune

211   factors were further extracted from metatranscriptome data and compared across different

212   populations. Secondary bile acid biosynthesis gene, *cbh*, and succinate synthesis genes,

213   *SDH* and *aarC,* were found to be significantly upregulated in the cecal microbiome of In-

214   TC individuals compared to Ex-TC and QY samples (Fig 4C). In the In-TC populations,

215   the Bacteroidetes phylum was the primary host for these three genes, whereas the

216   Firmicutes phylum was the primary host in the Ex-TC and QY groups (Fig 4D). These

217   findings corroborated the increased activity of Bacteroidetes and decreased activity of

218   Firmicutes in the cecal microbiome of In-TC individuals (Fig 1A).

219   **Correlations between the cecal microbiome and metabolome in chickens**

220   Target metabolomics data revealed that the cecum of different chicken populations

221   displayed distinct compositions of SCFAs and Bas, as indicated by the PCoA and adonis

222   test (Fig 5A and 5B). In addition, the cecum of In-TC individuals exhibited a

223   significantly higher concentration of acetic acid than that of the Ex-TC and QY groups

224   (Fig 5C). Moreover, the cecum of In-TC individuals showed a higher abundance of

225   multiple BAs than the Ex-TC and QY populations, including UDCA, UCA, isoLCA,

226   GUDCA, GLCA, GHDCA, GDCA, beta-UDCA, beta-MCA, and 7-ketoLCA (Fig 5C).

227   In contrast, only norDCA decreased in the cecum of In-TC individuals (Fig 5C).

228   The above results indicated that the abundance of Bacteroidetes and Firmicutes in the

229   cecal microbiome could contribute to the high-altitude adaptive advantage of TCs. The

230   ratio of Bacteroidetes/Firmicutes in the gut microbiota has been proven to be an indicator

231   of health in humans and rats (29, 30). In this study, we analyzed the ratio of active

232   Bacteroidetes/Firmicutes in the cecal microbiome of chickens and compared them

233   between populations residing in the plateau and plain regions. A significantly higher ratio

234   of active Bacteroidetes/Firmicutes was observed in the cecal microbiome of In-TC

235   individuals than that in the Ex-TC and QY samples (Tukey's HSD test, $p < 0.05$, Fig 6).

236   In all analyzed samples, we found significant positive correlations between the ratio of

237   active Bacteroidetes/Firmicutes and the expression levels of SCFA biosynthesis genes

238   (*pct*, *atoAD*, and *buk*) (Fig 6). They were further positively correlated with propionic

239   acid, acetic acid, and butyric acid contents in the cecum of chickens (Fig 6). Furthermore,

240   significant positive correlations were observed between the ratio of active

241   Bacteroidetes/Firmicutes and expression levels of genes involved in the biosynthesis of

242   innate immune factors (*SDH*, *aarC*, and *cbh*) (Fig 6). These genes were positively

243   correlated with the content of diverse BAs in the cecum of chickens (Fig 6).

244

DISCUSSION

246   This is the first study to use metatranscriptomic technology to investigate the active

247   microorganisms and their physiological functions in the cecum of TCs. We observed

248   significant variations in the composition and function of the active cecal microbiome

249   between chickens residing in the plateau and plain regions, based on comparisons

250   between the In-TC, Ex-TC, and QY populations. In contrast, the active cecal microbiome

251   of TCs became remarkably similar to that of other plain populations once introduced into

252   the plains. Previous studies have indicated that both genetic and environmental factors

253   influence the symbiotic microbiome of chickens (31, 32). In the present study, the In-TC

254   and Ex-TC populations had similar genetic backgrounds. Thus, the differences in the

255   active cecal microbiome between the two groups could be attributed to habitat factors,

256   including environmental conditions and dietary differences. Given that the Ex-TC and

257   QY populations in our study resided on the same farm, shared a consistent diet, and were

258   exposed to similar environmental factors, the presence of a similar active cecal

259   microbiome in both groups highlights the significant influence of habitat on genetic

260   constraints. These findings indicate that maintaining the advantages related to the cecal

261   microbiome in TCs became challenging once they were introduced into plain areas.

262   At the taxonomic level, *Alistipes*, an active cecal bacterial genus abundant in the In-TC

263   population, has been associated with potential benefits in protecting the human

264   gastrointestinal tract against colitis, autism, and cirrhosis (33). In addition, another In-

265   TC-enriched active cecal bacterial genus, *Prevotella*, has been found to be more abundant

266   in Tibetan animals, which benefits the host by maintaining gut energy homeostasis and

267   adapting to hypoxic pressure (34, 35). *Streptococcus*, an active cecal bacterial genus

268   enriched in chicken populations residing in plains, is a pathogen that causes various

269   infections in humans (36). Streptococcal bacteria are highly contagious and can easily

270   spread through droplets produced when an infected person coughs or sneezes, as well as

271   through shared food or drinks (37). At the functional level, the expression levels of all

272   carbohydrate-active enzymes were higher in the In-TC population. These enzymes

273   degrade carbohydrates in food to generate energy (38). The upregulated DEGs in the In-

274   TC cecal microbiome were enriched in various energy metabolism-related pathways.

275   These pathways include fatty acid and saccharide metabolism as well as the TCA cycle.

276   These findings indicate that TCs residing on the plateau use energy more efficiently and

277   pose relatively low human risks.

278   *Alistipes* species, in addition to their role in disease protection, are indole-positive,

279   which affects the serotonin precursor tryptophan (33). These factors are closely linked to

280   host innate immunity (11). Several glycan-related pathways were enriched in the active

281   cecal microbiome of the In-TC population. Glycans are complex carbohydrates that play

282   crucial roles in the regulation of various aspects of human physiology and pathology.

283   These functions include signal transduction and defense against microbial infections (39).

284   Glycans, in particular, act as molecular patterns recognized by glycan-binding proteins

285   and hence play a crucial role in various cellular processes, including cell-cell interactions

286   and immunological responses (40). Additionally, we observed that some immune-related

287   pathways were enriched in the active cecal microbiome of the In-TC population,

288   including pathways for immunological signal transduction (AMPK and HIF-1) and

289   biosynthesis of antibiotics that can effectively eliminate pathogens (prodigiosin and

290   vancomycin). Furthermore, our findings revealed higher contents of acetic acid and

291   several SBAs in the cecum of In-TC individuals. Acetate, an SCFA, is produced by

292   symbiotic microorganisms and plays a crucial role in maintaining the overall health of the

293   host (41). Acetate may affect gut health by improving intestinal barrier function via

294   cross-feeding mechanisms (42). Acetate has also been associated with epithelial

295   maintenance, wound healing, and improved barrier function in the gastrointestinal tract.

296   Research has demonstrated that ursodeoxycholic acid (UDCA) regulates the defense

297   mechanisms of antimicrobial peptides, protects epithelial cells, and exerts anti-

298   inflammatory effects in the cecum of the In-TC population (43). Another enriched SBA,

299 isoLCA (isolithocholic acid), has been found to be one of the most abundant BAs in the

300 gastrointestinal tract and is regarded as an essential modulator of immunological

301 homeostasis (44). These results suggest that a higher level of immunological activity is

302 mediated by the cecal microbiome in TCs residing on the plateau.

303   Our study combined metatranscriptomics and targeted metabolomics technologies to

304 explore the underlying mechanisms of the active cecal microbiome that contribute to the

305 advantages of TCs living in the Tibetan Plateau. Association analysis yielded a

306 framework that could explain the higher immunological activity of TCs residing on the

307 plateau. The ratio of active Bacteroidetes/Firmicutes increases in the cecal microbiome of

308 TCs residing in the plateau, which induces the powerful biosynthesis of SCFAs and

309 SBAs to support increased immunological activity. In contrast, the ratio of active

310 Bacteroidetes/Firmicutes was lower in TCs and QYs living in the plain, resulting in

311 reduced SCFA and BA content in the cecum. Notably, a recent analysis of the gut

312 microbiota of Tibetan Plateau animals revealed contradictory results to our findings, with

313 the authors obtaining a lower Bacteroidetes/Firmicutes ratio in higher-altitude mammals

314 than in lower-altitude mammals (8). As the dominant phyla of the mammalian gut

315 microbiota, the relative abundance of Bacteroidetes and Firmicutes can serve as

316 indicators of obesity, with a higher abundance of Firmicutes and a lower abundance of

317 Bacteroidetes in obese individuals (29, 45). This is consistent with the fact that high-

318 altitude animals require additional body fat to resist cold. However, the higher ratio of

319 active Bacteroidetes/Firmicutes observed in the In-TC samples contradicts previous

320 findings. One possible explanation for this conflict could be the variation in the research

321 methods. Our study measured active microorganisms based on microbial RNA

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322 sequencing, which differs from previous studies that relied on microbial DNA. Another

323 potential explanation could be the physiological differences between mammals and birds.

324 Previous research in Arbor Acres broiler chickens indicated that, unlike in mammals, the

325 Bacteroidetes/Firmicutes ratio in birds cannot be used as an indicator of gut health (46).

326 Our findings suggest that the presence of active Bacteroidetes/Firmicutes could serve as a

327 significant marker of innate immunological activity mediated by the cecal microbiome in

328 chickens. However, further large-scale population comparisons and validation

329 experiments are necessary to confirm the accuracy and feasibility of regulating active

330 Bacteroidetes/Firmicutes to improve immunity in chickens.

331

332 **CONCLUSION**

333 In this study, we present an initial investigation of the active cecal microbiome and its

334 potential advantages in plateau-dwelling TCs over plain-dwelling TCs. The composition

335 and function of the active cecal microbiome vary significantly across different chicken

336 populations. In particular, the higher expression of functional genes in the cecal

337 microbiome suggests that TCs residing on the plateau have more effective energy

338 metabolism and a stronger innate immune response than individuals residing on the

339 plains. Moreover, the cecal microbiome in the native TC population has an increased

340 ability to produce SCFAs and SBAs, resulting in higher cecal content of these

341 metabolites. Finally, the advantages of native TCs are linked to the active

342 Bacteroidetes/Firmicutes ratio in the cecal microbiome. Our findings deepen the

343 understanding of the interactions between gut microbiota and highland adaptation and

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344 provide valuable insights into the mechanisms by which Tibetan chickens adapt to the

345 hypoxic environment of the plateau.

346

347 **METHODS**

348 **Study animals and sample collection**

349 Two different chicken species, TCs and Qingyuan (QY) chickens, were used in our

350 study. Two different populations were selected from the TC individuals. One population

351 included TCs that always resided on the Tibetan Plateau after birth (In-TC), and the other

352 was a transitional TC population residing in plain environments ex situ over 20 years

353 (Ex-TC). The QY species was a native breed in plain environments. The Ex-TC and QY

354 populations were raised in the same environment to exclude the influence of diet and

355 breeding environment. This sample setting considered both genetic and environmental

356 variations in the population, enabling a more accurate assessment of the uniqueness of

357 TCs. Twelve individuals were selected from each population for sample collection. All

358 study animals were approximately 300 days old, the chickens were healthy, and had not

359 received any medications for at least three months prior to sampling. The cecal content of

360 each individual was collected under sterile conditions. Subsequently, the contents of three

361 individuals from the same population were combined and added to 1.5 ml sterile

362 polypropylene tubes. All samples were immediately snap-frozen in liquid nitrogen until

363 further analysis.

364 **RNA extraction and metatranscriptomic sequencing**

365   Total RNA was extracted from the cecal contents using TRIzol Reagent (Invitrogen,

366   Carlsbad, USA), and genomic DNA was removed using DNase I (TAKARA, Dalian,

367   China). Next, the quality of the extracted RNA was assessed using an Agilent 2100

368   Bioanalyzer and NanoDrop ND-2000. Metatranscriptome libraries (450 bp) were

369   constructed using the TruSeqTM Stranded Total RNA Sample Preparation Kit (Illumina,

370   San Diego, CA, USA) and sequenced on the Illumina NovaSeq 6000 platform with a

371   pair-end 150 bp strategy at Shanghai BIOZERON Co., Ltd. (Shanghai, China). The raw

372   reads underwent quality control using Trimmomatic with a sliding window (4:15) and

373   minlen (75) settings. Next, the clean reads were aligned to the chicken reference genome,

374   and the mapped reads were removed. The rRNA reads from the clean reads were further

375   removed by alignment to the SILVA database using SortMeRNA software (47). The

376   MegaHit algorithm (48) was used to assemble the remaining mRNA reads into contigs,

377   and open reading frames were predicted using METAProdigal (49). The CD-HIT method

378   (50) was used to remove redundancy and construct a non-redundant gene catalog with

379   95% identity and 90% coverage. All unique genes were searched against the eggNOG

380   (51), CAZyme (52), and KEGG (53) databases for functional annotation using BLASTp

381   with a cut-off E-value less than 1e-5. The genes were further aligned to the NR database

382   using BLASTp to assign host taxonomy. The expression level of each gene in each

383   sample was calculated using Salmon (54). Three comparisons were conducted to identify

384   differentially expressed genes (DEGs): In-TC vs. Ex-TC, In-TC vs. QY, and Ex-TC vs.

385   QY. Based on the results obtained from the "edgeR" package (55), genes with a log-trans

386   fold change greater than 2 and a p-value adjusted by the false discovery rate method less

387   than 0.5 in each comparison, were recognized as DEGs.

388   The biosynthesis-related functional analyses in this study primarily focused on SCFAs

389   and certain innate immune factors generated by microbes. Specifically, SCFAs include

390   formate, acetate, propionate, and butyrate. Additionally, innate immune factors include

391   indole-3-acetic acid (IAA), indole-3-aldehyde (IAId), kynurenine, SBAs, succinate, and

392   trimethylamine N-oxide (TMAO). The genetic potential of the gut microbiota was

393   analyzed by sorting marker genes based on the KEGG database. The detailed marker

394   genes and abundance calculation processes used in this study are listed in Supplementary

395   Data 1. Based on the above KEGG annotations, nucleotide sequences of genes encoding

396   the selected marker genes were searched against the NCBI NR database using BLASTX.

397   The top 50 hits with E-values < 0.001 were imported into MEGAN, and the taxonomy

398   was assigned using the lowest common ancestor algorithm.

399   **Targeted metabolomic analysis**

400   Standards for SCFAs and BAs were obtained from Sigma-Aldrich (St. Louis, MO,

401   USA). Targeted metabolome analysis involved resuspending the cecum digesta in liquid

402   nitrogen and diluting the sample by vigorous vortexing in water. Next, a 50 μL fraction

403   was homogenized with 200 μL of acetonitrile/methanol (1:1) containing the appropriate

404   internal standards. The mixture was vortexed thoroughly to ensure homogenization. The

405   mixture was then placed on ice for 30 min. The mixture was then centrifuged at 12,000

406   rpm for 10 min. An ultra-high-performance liquid chromatography coupled with tandem

407   mass spectrometry (UHPLC-MS/MS) system (ExionLC™ AD UHPLC-QTRAP 6500+,

408   AB SCIEX Corp., Boston, MA, USA) was used to quantify the SCFAs and AAs.

409   Separation was carried out on an ACQUITY UPLC BEH Amide column (2.1 × 100 mm,

410   1.7 μm) that was maintained at 50 °C. The mobile phase, consisting of 0.1% formic acid

411   in 5 mM ammonium acetate (solvent A) and 0.1% formic acid in acetonitrile (solvent B),

412   was delivered at a flow rate of 0.30 mL/min. The solvent gradient was set as follows:

413   initial 80% B, 0.5 min; 80-70% B, 2 min; 70-45% B, 4 min; 45-80% B, 6.01 min; 80% B,

414   9 min. The mass spectrometer was operated in the positive multiple reaction mode

415   (MRM). The parameters used were as follows: IonSpray Voltage (5500 V), Curtain Gas

416   (35 psi), Ion Source Temperature (550 °C), and Ion Source Gas of 1 and 2 (50 and 60 psi,

417   respectively).

418   **Statistical analysis**

419   All statistical analyses were conducted using the R v4.2.2 platform and the results were

420   visualized using the "ggplot2" package. Variations in the abundance of active cecal

421   microorganisms, COG terms, and CAZyme categories in cecal microbiomes among the

422   three chicken populations were analyzed using Tukey's HSD test ("multcomp" package).

423   PCoA and adonis test based on the Bray-Curtis distance ("vegan" package) was

424   performed to explore the differences in the composition of and gene expression profiles

425   of active cecal microbiome as well as the compositions of SCFAs and BAs in the cecum

426   across three different chicken populations. A Venn diagram ("VennDiagram" package)

427   was used to identify the potential key genes related to the high-altitude adaptability of

428   TCs based on the recognized DEGs. KEGG enrichment analysis of these putative key

429   genes was performed using the KOBAS tool (56). Differences in the expression levels of

430   genes related to SCFA biosynthesis and innate immune factors in the cecal microbiomes

431   and the contents of SCFAs and BAs in the cecum among the three chicken populations

432   were also compared using Tukey's HSD test. Tukey's HSD test was also used to evaluate

433   variations in the ratio of active Bacteroidetes/Firmicutes in the cecal microbiomes among

434 the three chicken populations. The correlations between the ratio of active

435 Bacteroidetes/Firmicutes, gene expression levels related to SCFA biosynthesis and innate

436 immune factors, and the contents of SCFAs and BAs were assessed using Spearman

437 correlation ("psych" package).

438

439 **SUPPORTING INFORMATION**

440 **Table S1.** Statistics of metatranscriptome sequencing.

441 **Table S2.** Statistics of assembly and gene recognition.

442 **Fig S1.** Differences in the inter-individual variations of active microbiome among

443 different populations based on the Bray-Curtis distance.

444 **Fig S2.** Identification of DEGs in the cecal microbiome of chicken populations. (A)

445 PCoA illustrating the differences in gene expression profiles of the cecal microbiome

446 among the three studied chicken populations. Volcano plots were used to identify the

447 DEGs in cecal microbiome in the comparisons of In-TC vs. Ex-TC (B), In-TC vs. QY

448 (C), and Ex-TC vs. QY (D), respectively.

449 **Fig S3.** Host composition of the pct gene in cecal microbiome of chickens.

450 **Fig S4.** Host composition of the *ACSS1_2* gene in cecal microbiome of chickens.

451 **Supplementary Data 1.** Key genes for biosynthesis of nutrients and innate immune

452 factors in gut microbiota of animals.

453 **AUTHOR CONTRIBUTIONS**

454 Tao Zeng, Lizhi Lu and Wei Han planned the project. Tao Zeng, Lizhi Lu, Wei Han,

455 Yong Tian and Peishi Feng designed the research. Tao Zeng, Yongqing Cao, Jianmei

456 Yin, Hanxue Sun, Tiantian Gu, Xueying Ma and Li Chen prepared the research materials.

457 Tao Zeng, Yongqing Cao, Yibo Zong, Zelong Zhao and Wenwu Xu performed the

458 experiments for sequencing and the data analyses. Tao Zeng drafted the manuscript. Lizhi

459 Lu and Wei Han revised the manuscript. All authors read and approved the final

460 manuscript.

CONFLICT OF INTEREST STATEMENT

468 The authors declare that they have no competing interests.

DATA AVAILABILITY STATEMENT

470 The raw reads of metatranscriptomic sequencing for all samples have been deposited in

471 the NCBI Sequence Read Archive (SRA) under the accession number of

472 PRJNA1060358. Targeted metabolomics data of SCFAs and Bas have been deposited in

473 the OMIX, China National Center for Bioinformation / Beijing Institute of Genomics,

474 Chinese Academy of Sciences (https://ngdc.cncb.ac.cn/omix: accession no.OMIX

475 005604 and OMIX 005605).

ETHICS STATEMENT

477   Animals used in this study were raised in accordance with the national standard of

478   Laboratory Animal Guidelines for ethical review of animal welfare. All experiment

479   procedures were approved by the Animal Use Committee of Zhejiang Academy of

480   Agricultural Sciences (No. 20-022).

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672
673

674 **FIG LEGENDS**

675 **FIG 1** The composition of the active cecal microbiome between the plateau Tibetan

676 chicken population and the plain chicken population. (A) Relative abundance of

677 dominant active cecal bacterial phyla among the samples studied. (B) Differences in the

678 relative abundance of active cecal bacterial genera among the three chicken populations.

679 The size and color of the points represent the average abundance of active cecal bacterial

680 genera in samples from the same population. Different lowercase letters inside the points

681 in the same column represent significant differences (p-value of Tukey's HSD test < 0.05)

682 between different populations.

683 **FIG 2** Expression levels of COG terms and CAZymes in the cecal microbiome between

684 the plateau Tibetan chicken population and the plain chicken population. Differences in

685 the expression levels of COG terms (A) and CAZymes (B) in the cecal microbiome of the

686 three chicken populations. Different lowercase letters above the boxes in the same subFig

687 represent significant differences (p-value of Tukey's HSD test < 0.05) between different

688 populations.

689 **FIG 3** DGEs and enrichment analysis in the cecal microbiomes between the plateau

690 Tibetan chicken population and the plain chicken population. Venn diagrams for

691 upregulated (A) and downregulated (B) DEGs among the three comparisons. (B) KEGG

692 enrichment analysis of upregulated genes in the cecal microbiomes of chickens residing
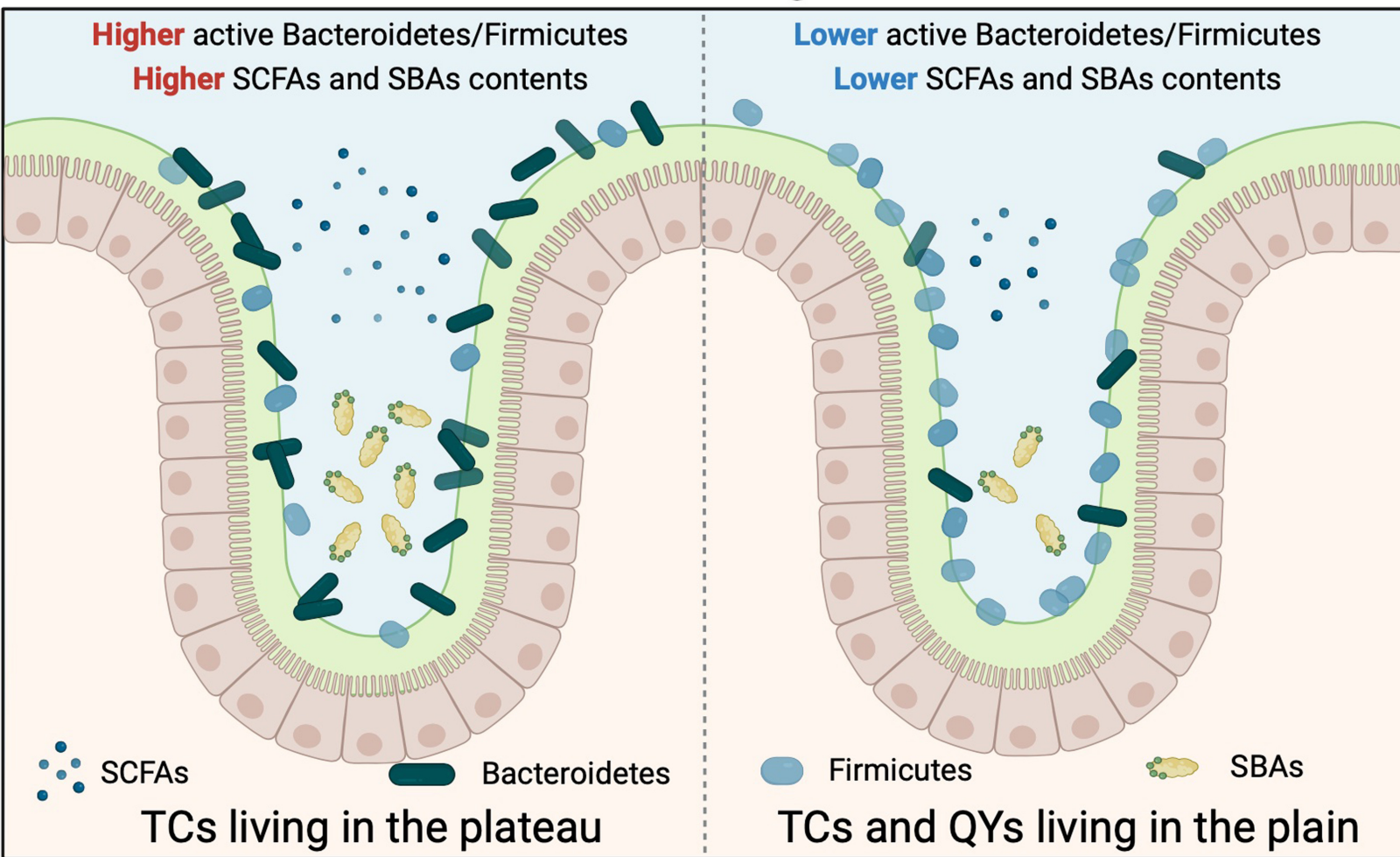
693 in the plateau and plain regions.

694 **FIG 4** Biosynthesis of SCFAs and immune activity in the cecal microbiome of In-TCs.

695 (A) Expression levels of genes associated with SCFA biosynthesis in the cecal

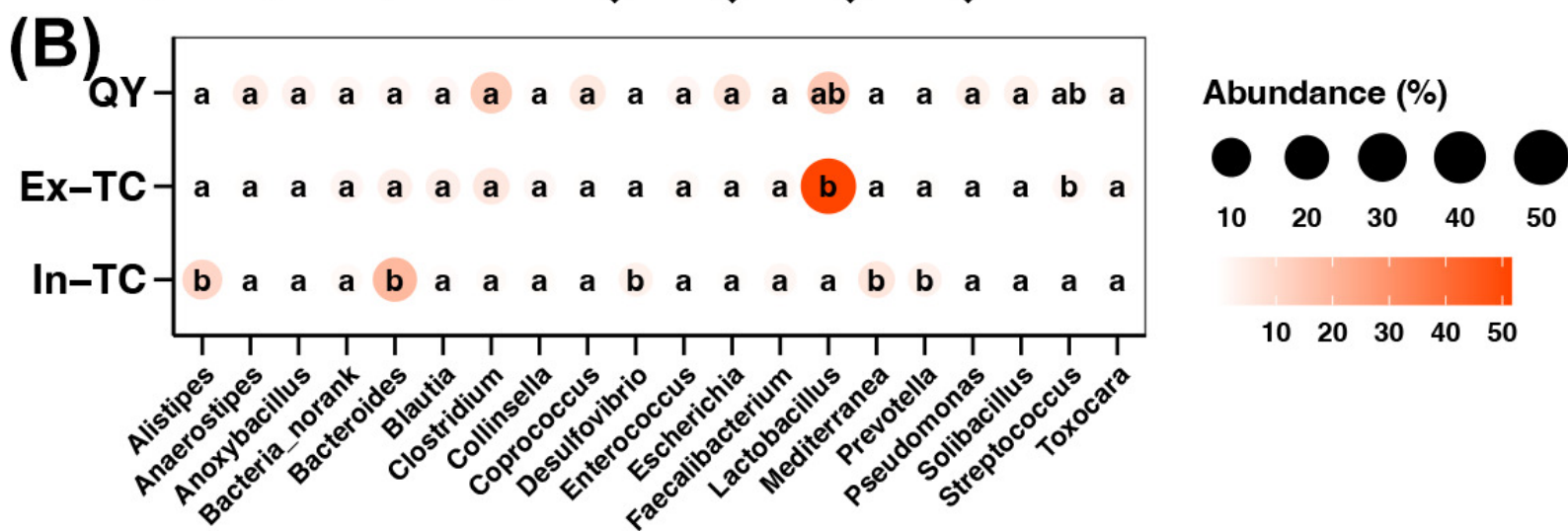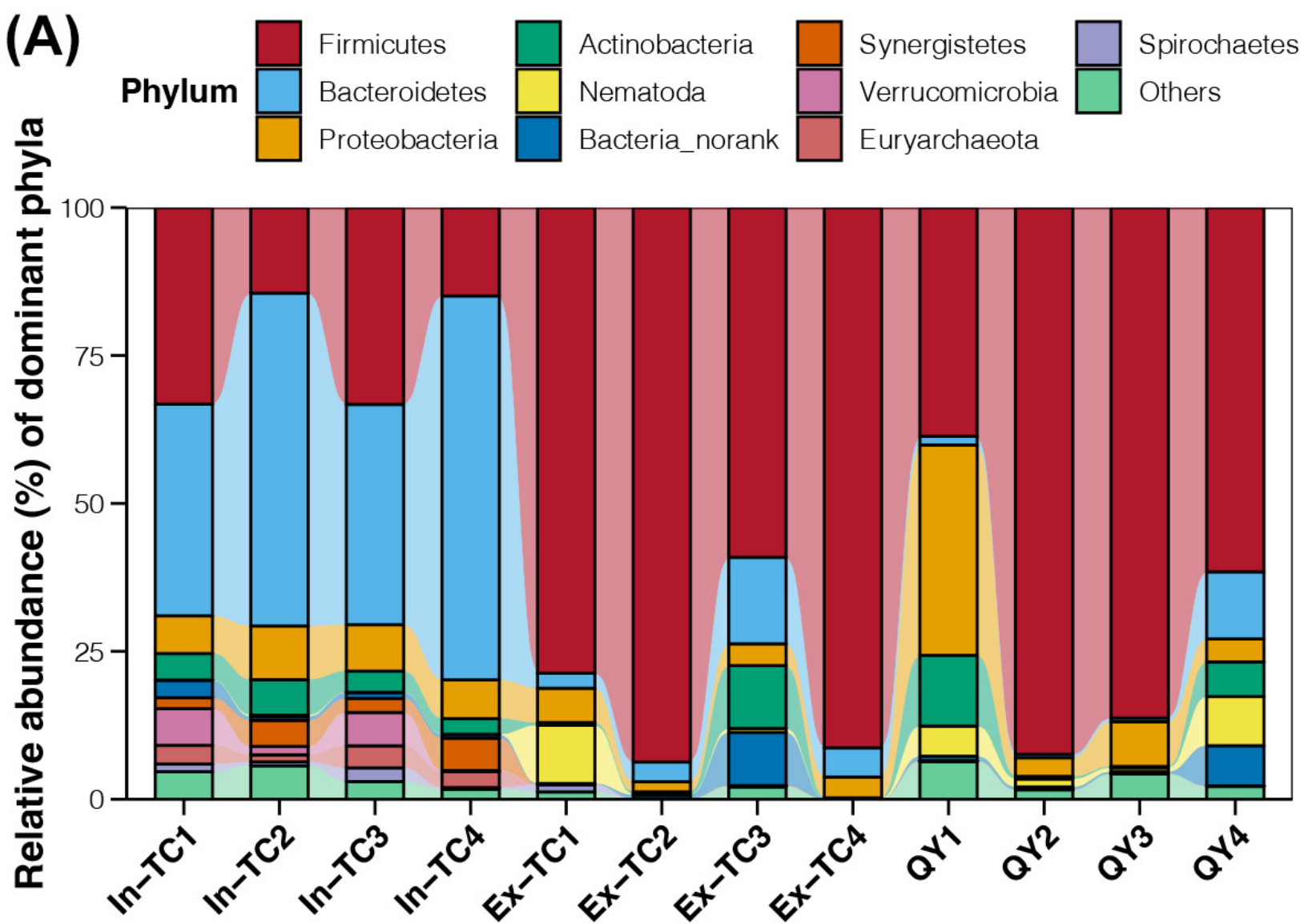696 microbiome of different chicken populations. (B) Host composition of SCFA

27

697   biosynthesis genes differentially expressed among different chicken populations. (C)

698   Expression levels of genes related to the biosynthesis of innate immune factors in the

699   cecal microbiome of different chicken populations. (D) Host composition of innate

700   immune factor biosynthesis-related genes differentially expressed among different

701   chicken populations. Color and values represent the fold change of gene expression levels

702   in each population compared to that in the In-TC group. Red points represent significant

703   differences in gene expression levels among the different groups.

704   **FIG 5** Targeted metabolomic analysis of SCFAs and BAs in the cecum among the three

705   chicken populations. PCoA showing differences in the composition of SCFAs (a) and

706   BAs (b) in the cecum among the three chicken populations studied. (c) Comparison of

707   SCFA and BA concentrations in the cecum of different chicken populations. Colors and

708   values represent the fold-change in metabolites in each population compared with those

709   in the In-TC group. The red points represent significant differences in metabolite

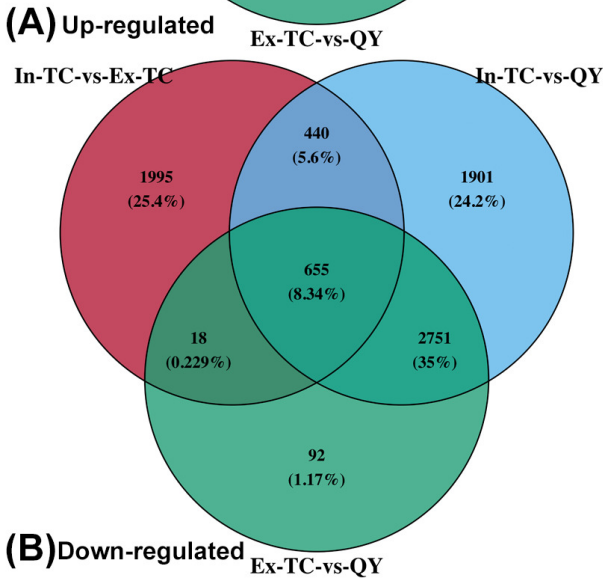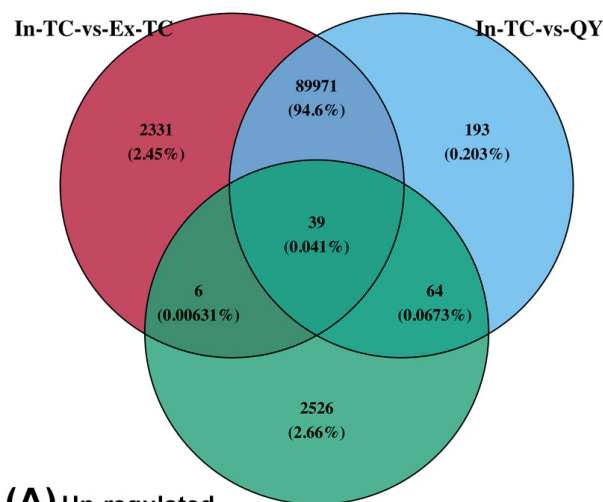710   concentrations among the different groups.

711   **FIG 6** Differences in the ratio of active Bacteroidetes/Firmicutes in the cecal microbiome

712   between chickens residing in plateau and plain areas and their correlations with gene

713   expression levels and metabolite contents related to SCFAs and BAs. Different lowercase

714   letters above the boxes in the boxplot represent statistically significant differences (p-

715   value of Tukey's HSD test < 0.05) between different populations. The color of the blocks

716   in the heatmaps represents the correlation coefficient of Spearman correlations. "+" and

717   "++" represent p-values Spearman correlations lower than 0.05 and 0.01, respectively.

718

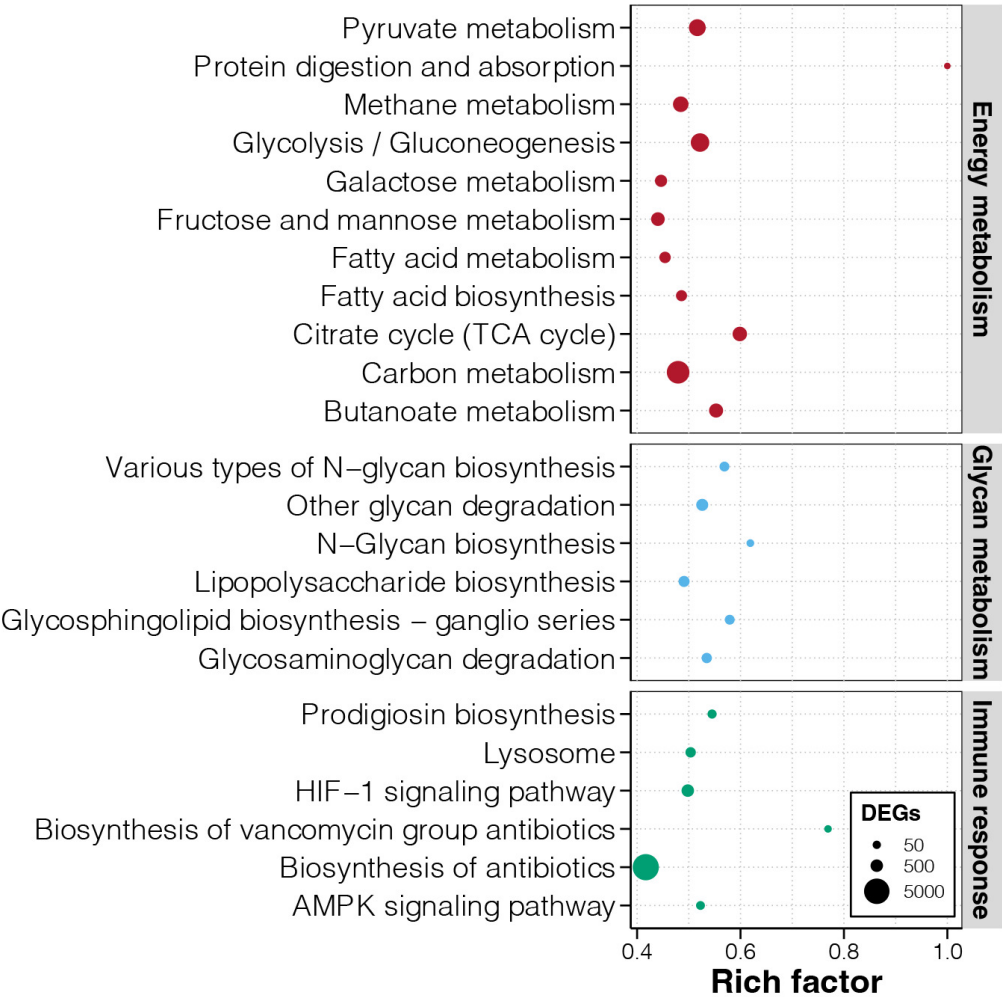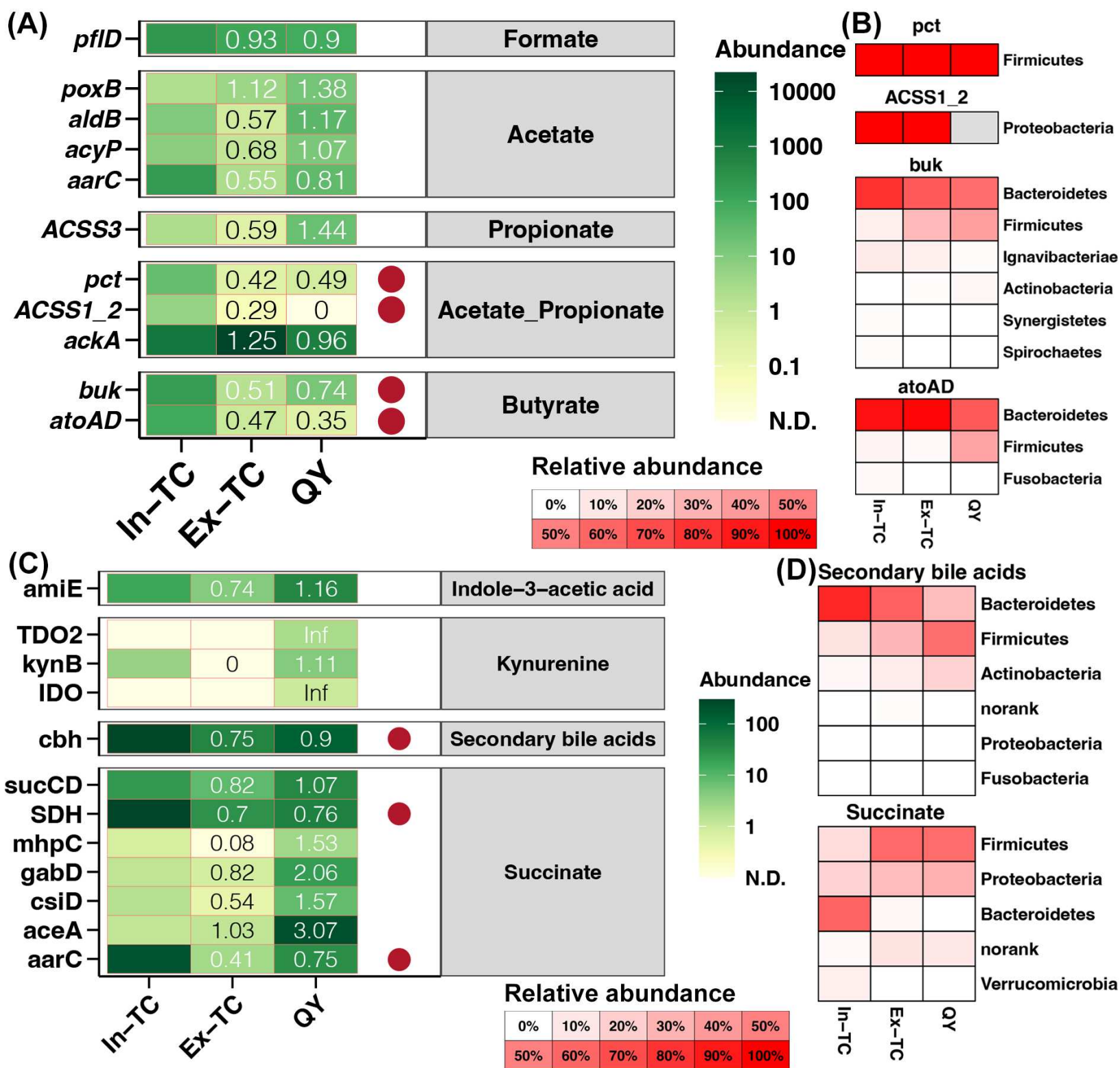# Active Bacteroidetes/Firmicutes regulating the synthesis of SCFAs and SBAs in gut of chickens
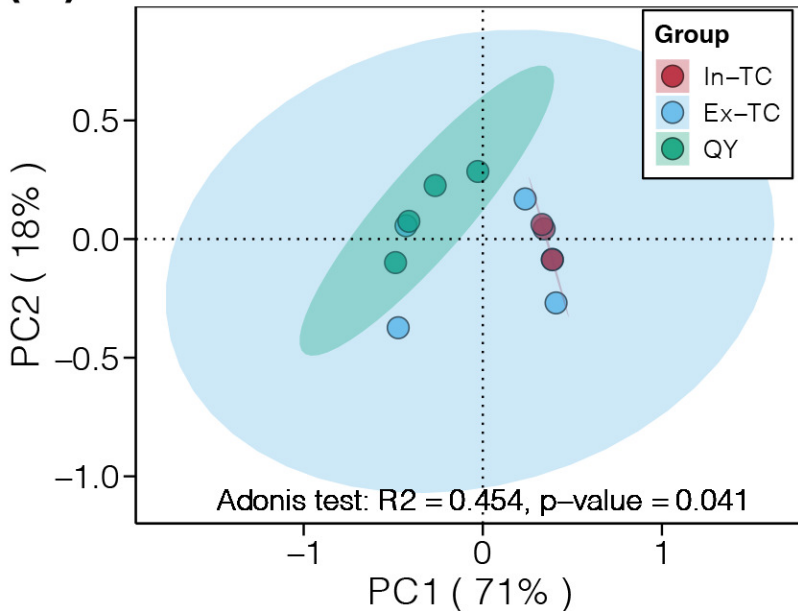
Higher active Bacteroidetes/Firmicutes
Higher SCFAs and SBAs contents

Lower active Bacteroidetes/Firmicutes
Lower SCFAs and SBAs contents

SCFAs   Bacteroidetes   Firmicutes   SBAs

TCs living in the plateau   TCs and QYs living in the plain