Multi-omics analysis of gut-organ axes reveals the environmental adaptation mechanisms in Tibetan chicken

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Abstract

Background

As a representative extreme ecosystem, the Qinghai-Tibet Plateau is subject to special climatic conditions, that require unique adaptations for organisms living in the region. In addition to genetic characteristics, the gut microbiota in animals can regulate the environmental adaptation of hosts through various "gut-organ" axes. The Tibetan chicken is the principal poultry species raised on the Qinghai-Tibet Plateau, however there is limited understanding of the interactions of its gut microbiota and host gene expression, as well as their associations with the plateau’s hypoxic conditions. In this study, a multi-omics approach was used to investigate the gut microbiota of Tibetan chickens and to explore the environmental adaptation mechanisms involved in the "gut-organ" axes.

Results

Significant differences in gut microbiota were found among different chicken populations sampled from across China, governed by variations in habitat species pools and species turnover. A more complex and stochastic-dominated gut microbiota with higher functional redundancy was observed in the Tibetan chicken population living in the plateau environment. Furthermore, Tibetan chickens also had more effective fatty acid degradation capacity, corresponding to their hypoxic environment. In contrast, chickens living in lowland breeding farms showed stronger immune system responses for defense against frequent health threats. These environmental adaptation strategies were found to be regulated by core gut microbes taxa in the phylum Firmicutes.

Conclusions

Our findings demonstrate the roles of breed and habitat in gut microbiota assembly of chickens, and clarifies the adaptation strategies of chickens to environmental changes via gut microbiota-driven "gut-organ" axes.

Background

The Qinghai-Tibet Plateau has been described as the “third pole on earth” for its extreme environmental conditions, such as low levels of oxygen, high levels of UV radiation, and cold temperatures, which challenge the survival of many species [1]. Tibetans, as well as Tibetan wild and domesticated animals living in the Qinghai-Tibet plateau have adapted to the hypoxic environment and have developed unique lifestyles, dietary habits, and genetic characteristics [2–5]. Modern genome analyses have identified unique genetic variations (EPAS1, EGNL1) of indigenous animal in terms of adaptation to the hypoxic environment at high altitudes [6–8]. Other key aspects such as gut microbiota have also been a topical research direction for animal adaptation to hypoxic environment [9]. Gut microbiota have been
conclusively shown to be closely related to both host health and host environmental adaptation [10, 11]. Recent studies have found significant altitude-correlated differences in the diversity of gut microbiota between high and humans [12], Tibetan pigs (*Sus domesticus*) [13], Tibetan wild asses (*Equus kiang*) [14] and yaks (*Bos grunniens*) [15], suggesting that the gut microbiota of mammals living at high altitudes may constitute part of their hosts’ adaptation the prevailing conditions. An understanding of the specific functional roles of gut microbiota in humans and animals living on the plateau is essential for elucidating the underlying adaptive mechanism to a hypoxic environment.

The Tibetan chicken (*Gallus gallus*) is a unique breed native to the Qinghai-Tibet Plateau that shows distinctive adaptation to the high-altitude environment acquired over thousands of years [16]. Unlike chicken breeds from lowland plains regions, Tibetan chickens have a higher venous CO$_2$ partial pressure, blood with a higher hemoglobin concentration, and hemoglobin with a stronger affinity for oxygen [17]. These unique characteristics, as well as its origin, suggest that the breed makes an interesting case study for understanding the adaptations of Tibetan gut microbiota to the plateau hypoxic environment. It has been reported that differences in the cecal microbiota of Tibetan chickens from five geographic regions were correlated with the chickens’ respective environments [18]. Another study found that the composition and diversity of gut microbes in Tibetan chickens changed after they were introduced to lower altitudes [19]. To our knowledge, these are the only two studies to date to have analyzed the characteristics of gut microbes in Tibetan chickens. Comprehensive comparisons of Tibetan chicken’s gut microbiota to low altitude populations with respect to diversity, composition, and assembly mechanisms have not yet been carried out. Multiple studies have uncovered the important role of gut microbiota on host physiology via various "gut-organ axes", in which gut microbiota regulate the gene expression of organs through their metabolites [20, 21]. There is also as yet no information about the interactions of gut microbiota and host gene expression in Tibetan chickens and their associations to the plateau hypoxic adaptation.

In this study, we selected six chicken populations from five representative Chinese chicken breeds based on geographical differences in altitude, consisting of one high-altitude Tibetan chicken population (In-TC, Tibet Plateau), one transitional chicken population relocated from high altitude to low altitude (Ex-TC, Jiangsu; descended from *ex situ in vivo* conservation for nearly 20 years), and four low-altitude chicken populations (BY, Beijing; LS, Jiangsu; QY, Guangdong; CH, Yunnan) (Fig. 1a). The In-TC population represents the unique breed native to the Qinghai-Tibet Plateau and shows distinctive genetic adaptation to high-altitude environmental conditions such as low oxygen, low temperature, and high ultraviolet exposure. The Ex-TC population is a descendant of In-TC, but has been raised in a low-altitude environment for 20 years. The other four populations are native breeds of lowland plains environments. We compared their gut microbiota via high-throughput sequencing to characterize the variations in diversity, composition, and assembly mechanisms related to the high-altitude features. Metabolites produced by the gut microbiota that could be associated to host adaptation were also identified by non-targeted metabolomics. RNA-seq was applied to depict the gene expression profiles of organs among
Results

Gut microbiota sequencing of chicken

A total of 60 cecum contents from six chicken populations (10 for each) were sequenced, and a total of 704,194 high quality reads (average 12,986 for each sample) clustering to 619 amplicon sequence variants (ASVs) were obtained. All ASVs were successfully annotated for bacterial phyla with ~90% taxonomic annotation at the level of genus, but only 34.41% of ASVs were assigned to bacterial species (Figure S1). A total of 15 phyla, 20 classes, 39 orders, 65 families, 118 genera, and 95 species were identified, with clearly greater numbers in the In-TC group compared to other lowland plains populations (Table S1). The rarefaction curves of all six populations tended to be horizontal (Figure S2), indicating that the sequencing depth could reflect the complete gut microbiota. Significantly higher Chao1 indices of gut microbiota was found in In-TC samples compared to plains populations (Tukey's HSD test, \( p < 0.05 \)), while no difference was observed among plains populations (Fig. 1b). In addition, the Chao1 index for Ex-TC samples also showed obvious differences between the plateau and plains populations of Tibetan chicken (Fig. 1b). The Pd_faith index of gut microbiota in In-TC samples was detected to be significantly higher than that in plain populations, including the Ex-TC group (Tukey's HSD test, \( p < 0.05 \), Fig. 1c). In contrast, no significant variation was found in Shannon and Pielou_J index values between any two populations (Tukey's HSD test, \( p > 0.05 \), Figure S3). These results suggest a higher richness and phylogenetic diversity in gut microbiota of chicken populations from the plateau compared to those from lowland plains.

Mechanisms of diversity variations of gut microbiota among chicken populations

Based on the results of alpha diversity, Unifrac distance was applied to assess the beta-diversity of chicken gut microbiota due to it embraced the phylogenetic changes [22]. Both unweighted and weighted Unifrac distances of chicken gut microbiota was calculated, which respectively characterized the effects of local species pool and species relative abundance according to their formula modes [23]. PCoA based on the unweighted Unifrac distance showed a distinct separation of the In-TC group from the plain populations (including Ex-TC) along the PC2 axis, explaining 17% of the total variation (Fig. 2a). The Adonis test confirmed the significant explanatory power of population identity on gut microbiota composition (\( R^2 = 0.178, p < 0.05 \)). Using the weighted Unifrac distance, samples from the In-TC group were again clustered separately from other groups (Adonis test, \( R^2 = 0.200, p < 0.05 \), Fig. 2b). No significant differences in intra-group variation among different chicken populations were found based on the unweighted Unifrac distance (Fig. 2c), however variation was significantly smaller in the In-TC group than in the plain populations (Fig. 2d). These results indicate the presence of effects of both the local species pool and relative species abundance on the variation in gut microbiota among the different populations. The results of this multi-omics approach provide new insight into the mechanisms of how Tibetan chickens adapt to the plateau hypoxic environment via the "gut-organ axes".
chicken populations. The local species pool appeared to contribute more to the variations between
groups, and relative species abundance might be more influential regarding variations within a single
population.

The beta-diversity of communities may be divided into nestedness- or turnover-driven forms [24]. The
former implies that changes in the community derive from the emergence or loss of species, while the
latter is driven by the replacement of species between different communities [25]. In both unweighted and
weighted Unifrac distance analysis, the contributions of turnover were a more important mechanism than
nestedness (Student's t-test, \( p < 0.05 \), Fig. 2e and 2f). However, no difference was observed among
different populations no matter for nestedness or turnover and no matter for unweighted or weighted
Unifrac distances (Figure S4). We further quantified the relative contributions of alpha- and beta-diversity
to gamma-diversity (Fig. 2g). The alpha-diversity of the In-TC population contributed about 97% variation
in the gut microbiota at this level, which was significantly more than the plains populations (averaging
75.3–84.0%). In contrast, the contributions of beta-diversity exhibited the opposing trend, as here the
plains populations outweighed the In-TC group (Fig. 2g). These findings suggest a fundamental role of
host population and living environment in governing succession in gut microbiota in these chicken
populations.

Differences in gut microbiota composition among chicken populations

According to the taxonomic analyses, Firmicutes (58.2%) was the most abundant bacterial phylum in
chicken gut microbiota, followed by Proteobacteria (17.3%), Bacteroidota (16.9%), and Actinobacteriota
(4.0%) (Figure S5). The relative abundances of Proteobacteria and Actinobacteriota were significantly
lower in the In-TC samples compared to the plain population samples (including the Ex-TC group)
(Tukey's HSD test, \( p < 0.05 \), Figure S6). In contrast, several bacterial phyla with considerable abundances
in the In-TC group (such as Defferribacterota, Desulfobacterota, Fusobacteriota, and Verrucomicrobiota)
were almost absent in the lowland populations (Figure S6). Among bacterial genera, \textit{Lactobacillus}
(12.3%) was the most dominant. Other genera present at proportions of > 5% included \textit{Ligilactobacillus},
\textit{Escherichia-Shigella}, and \textit{Limosilactobacillus} (Figure S7). The bacterial genera \textit{Bacteroides},
\textit{Anaerosporobacter}, \textit{Desulfovibrio}, and \textit{Phascolarctobacterium} were found to be specifically enriched in
gut microbiota of the In-TC samples (Figure S8).

Only 21 bacterial ASVs were found to be shared among gut microbiota of the studied chicken
populations (Fig. 3a). These belonged to 4 phyla and 10 genera, and showed a composition similar to
that of the complete gut microbiota (Fig. 3b). The total abundance of these shared ASVs was only 10% in
the In-TC group compared to ~ 50–75% in the plain populations (Fig. 3c). These outcomes further
validate the finding of the beta-diversity analysis that differences in the local species pool were drivers of
the inter-population variations in chicken gut microbiota.

Assembly mechanisms of chicken gut microbiota
We used the betaNTI index to evaluate the gut microbiota assembly in different chicken populations. Median values of betaNTI were between −2 and 2, with the exception of the LS group, which had a value slightly above 2 (Fig. 3d). While these values indicate that stochastic processes governed the assembly of a majority of these microbiotas, there were still population-specific differences. Drift was the dominant stochastic process and heterogeneous selection the dominant deterministic process for gut microbiota assembly in the In-TC group (Fig. 3e). While this was also the case for the plain populations, specific contributions varied among these. The relative importance of drift was lower in the plain populations (26.67–53.33%) than in the In-TC group (60%), but the additional stochastic process of dispersal limitation emerged here (4.44–11.11%) (Fig. 3e). For deterministic processes, the contribution of heterogeneous selection was similar or greater in plains populations (26.67–55.56%) than in the In-TC group (31.11%), and homogeneous selection was added (4.44–11.11%) (Fig. 3e).

**Effects of habitat on gut microbiota of Tibetan chicken**

To enhance our understanding of the characteristics of Tibetan chicken in particular, we further compared the gut microbiota of the In-TC and Ex-TC groups, which had the same ancestors but had been raised in different habitats for more than twenty years. We observed significant inter-group differences in evolutionary diversity and composition structure of gut microbiota (Fig. 1c and 2). A total of 110 ASVs were found to be shared between these two groups (Figure S9), which was substantially higher than the proportion shared among all six studied populations (Fig. 3a). However, the sum abundance of these shared ASVs also made up a more limited proportion of gut microbiota in the In-TC samples (~35%) than in the Ex-TC samples (~75%) (Figure S10). *Bacteroides* was significantly enriched in the In-TC group, while *Limosilactobacillus* and *Lactobacillus* were more abundant in Ex-TC individuals (Figure S11). The predicted functions of microbiota indicated a higher abundance of functions involved in cellular processes in the In-TC group, and more abundant functions related to human diseases in the Ex-TC group (Figure S12).

Co-occurrence networks of gut microbiota from the In-TC and Ex-TC groups were constructed (Fig. 4a). The In-TC group featured a substantially more complex network, based on the comparison of topological parameters (Table S2). In contrast, significantly higher N:P cohesion was detected in the Ex-TC network (Student’s t-test, Fig. 4b), indicating lesser stability of gut microbiota in the In-TC group. Based on the results of the null model analysis, the gut microbiota assembly appeared to have changed from a stochastic-driven state in the In-TC group to a deterministic-driven state in the Ex-TC population (Fig. 4c). Comparison of the betaMNTD between the In-TC and Ex-TC groups revealed a higher betaMNTD among samples of the Ex-TC population than those in the In-TC population (Fig. 4d). Function prediction indicates that 5,538 function terms were present in gut microbiota in the In-TC group, implying a greater degree of redundancy among functions, while less than half his number (2,150) was found in the Ex-TC group (Fig. 4e). Significant positive correlations were found between the betaNTI and FRI in both groups (linear regression, $p < 0.05$, Fig. 4f). The slope for correlation of betaNTI and FRI in the In-TC group was higher than that in the Ex-TC group, implying a closer correlation between gut microbiota assembly and their functional redundancy in the In-TC population. Taken together, individuals of the In-TC population
had predominantly stochastic gut microbiota with greater complexity, lower stability, greater functional redundancy, and narrower phylogenetic distribution compared to those in the Ex-TC population.

**Relationships between gut microbiota and its metabolites**

The gut metabolome of three populations (native Tibetan chicken, In-TC; Tibetan chicken raised in the plains, Ex-TC; and a native plains chicken breed, QY) was investigated to explore the potential mechanisms behind the plateau adaptability of the In-TC population. Approximately 1500 metabolites were identified from the chicken intestinal tract based on ion current profiles. PCoA showed separate clusters of different populations with an obviously smaller intra-group variations for the In-TC group (Fig. 5a). A total of 389, 406, and 181 metabolites were identified as DAMs in comparisons between the In-TC vs Ex-TC, In-TC vs QY, and Ex-TC vs QY, respectively (Figure S13). The number of DAMs between the native Tibetan chicken and either plains population were higher than that for the two plains populations, and most of this increase was contributed by the up-regulated DAMs (286 and 324 vs 103, Figure S13).

Metabolome enrichment analyses were further performed, with DAMs in the comparison of In-TC and Ex-TC significantly enriched in pathways of purine metabolism, isoflavonoid biosynthesis, flavonoid biosynthesis, and biosynthesis of unsaturated fatty acids (Fig. 5b). Several pathways were also detected to be significantly enriched in the comparison of In-TC and QY populations, including isoflavonoid biosynthesis, glycine, serine and threonine metabolism, glucosinolate biosynthesis, cyanoamino acid metabolism, and aminoacyl-tRNA biosynthesis (Fig. 5b). In contrast, only one pathway (biosynthesis of unsaturated fatty acids) was observed to be enriched in the comparison of the two plains populations. We expected that enriched pathways present in comparisons between the plateau and plains populations, but not the two plains populations, might be important for the plateau adaptability of Tibetan chicken. Only one pathway, isoflavonoid biosynthesis, met this requirement (Fig. 5b). Five DAMs were identified in this pathway (liquiritigenin, daidzein, glycine, naringenine and genistein), which were all down-regulated in the In-TC group compared to the plain populations (Figure S14).

Among the DAMs identified in all three comparisons, 213 up- and 44 down-regulated DAMs were identified as potential key metabolites for plateau adaptability, using the same criterion as for enriched pathways (Table S3). These potential key metabolites were further correlated to the gut microbiota through a network analysis approach, and 16 gut-bacterial ASVs were found to be significantly correlated to these metabolites (Fig. 5c). Among these, seven ASVs (ASV53, ASV65, ASV73, ASV100, ASV125, ASV155, and ASV191) were correlated principally to up-regulated DAMs, and three of these (ASV100, ASV125, and ASV191) were also associated to the down-regulated DAMs (Fig. 5c). We also assessed correlations between these key gut-bacterial ASVs and DAMs involved in the isoflavonoid biosynthesis pathways (Fig. 5d). Seven ASVs (three Desulfovibrio, one Phascolarctobacterium, one Faecalibacterium, and two uncultured bacteria) were negatively correlated to all five DAMs, while one bacterial ASV (Limosilactobacillus) was positively correlated to all five DAMs (Spearman's rank correlation, \( p < 0.05 \)). Interestingly, the ASVs significantly correlated to all five isoflavonoid biosynthesis DAMs all belonged to the phylum Firmicutes.
Effects of gut microbiota on host gene expression

The transcriptome of three different tissues (lung, heart, and liver) potentially related to the plateau adaptation of the Tibetan chicken was investigated and compared among different populations. The comprehensive transcriptome of all three tissues varied significantly among the In-TC, Ex-TC, and QY populations (Adonis test, $p < 0.05$). According to the clustering patterns in the PCoA plots, the transcriptome was similar for the two plains populations (Ex-TC and QY) but separated from the In-TC group (Figure S15). A total of 2211, 1936, and 537 genes in lung were recognized as DEGs in comparisons between In-TC vs Ex-TC, In-TC vs QY, and Ex-TC vs QY, respectively (Table S4). For heart, the number of DEGs was 1336 (In-TC vs Ex-TC), 1084 (In-TC vs QY), and 590 (Ex-TC vs QY) (Table S4). DEG numbers in liver were similar to those in heart (1296 for In-TC vs Ex-TC, 1045 for In-TC vs QY, and 489 for Ex-TC vs QY) (Table S4). For all three tissues, DEG numbers between the native Tibetan chicken and the plains populations were higher than between the two plains populations, and the variation level in the transcriptome of lung was higher than in heart and liver.

As in the metabolome analysis, we assumed that DEGs detected in comparisons between the plateau and plains populations but not between the two plains populations could be important for the plateau adaptability of Tibetan chicken. According to this criterion, in lung a total of 410 up- and 915 down-regulated DEGs were recognized as potential key DEGs; in heart, 172 (up) and 387 (down); and in liver, 269 (up) and 386 (down) (Table S5). Correlations between the gut microbiota and these potential key genes in lung were obtained by network analysis, and only a few key DEGs showed significant correlations to gut bacterial ASVs (Fig. 6a). For lung, there were twelve candidates, among which two (ASV7 and ASV76) were only correlated to down-regulated genes, three (ASV15, ASV81, and ASV214) were only correlated to up-regulated genes, and the others were correlated to both. A KEGG pathway, viral protein interaction with cytokine and cytokine receptor, was found to be potentially important due to its enrichment in the In-TC group but not in the Ex-TC and QY populations (Fig. 6b). Most DEGs involved in this pathway were significantly down-regulated in In-TC lung compared to Ex-TC and QY samples (Figure S16). Interestingly, the expression levels of DEGs in this pathway were only significantly correlated to the relative abundances of the twelve potential key gut bacterial ASVs noted above (Fig. 6c). These results indicate the important role of a "gut-lung" axis in the plateau adaptation of Tibetan chicken.

The same type of network and KEGG enrichment analyses were also performed for heart and liver samples. In heart, a relatively large number of gut bacterial ASVs was significantly correlated to potential key DEGs (Fig. 7a). Notably, most of these gut bacterial ASVs were significantly correlated to two up-regulated DEGs (FGA and CL2), while another five (ASV53, ASV59, ASV65, ASV67, and ASV131) were correlated to diverse DEGs (Fig. 7a). Four KEGG pathways were enriched in heart samples of the In-TC group compared to those of the plain populations; these were Th1 and Th2 cell differentiation, T cell receptor signaling pathway, natural killer cell mediated cytotoxicity, and cytokine-cytokine receptor interaction (Fig. 7b). For liver, 10 gut bacterial ASVs were found to be significantly correlated to potential key DEGs (Fig. 7c), and six KEGG pathways (biosynthesis of antibiotics, biosynthesis of secondary metabolites, fatty acid degradation, fatty acid metabolism, phagosome, and PPAR signaling) were
enriched in the In-TC group compared to the plain populations (Fig. 7d). It is noteworthy that five gut bacterial ASVs (ASV53, ASV59, ASV65, ASV67, and ASV131) were identified as potential key gut bacterial in all three investigated axes —"gut-lung", "gut-heart", and "gut-liver"— of the Tibetan chicken.

Discussion

We found significant variations in the diversity, composition, and assembly mechanism of gut microbiota among the different chicken populations. Inter-population variations in gut microbiota have been reported in some organisms including humans [26], pigs [27], and poultry [28]. Increased species turnover over time was found in the gut microbiome of diverse human populations [29]. Our results also revealed that species turnover was the dominant mechanism driving population variations in gut microbiota of chickens. Previous studies have also shown that host genetic variation can shape the composition of gut microbiota in chickens [30]. The effects of population variations in gut microbiota of hosts mainly manifested in constraining colonization by specific microorganisms [31]. This mechanism could partially explain the significant richness differences among gut microbiota of chicken populations and the dominant roles of several levels of assembly diversity seen in our results. However, there are many biotic and abiotic factors in addition to host genetic variation that contribute to variation in gut microbiota, such as age, health status, diet, geography, and environmental exposure, etc. [32, 33]. The individuals used in our study were all healthy and of the same age, which allowed us to exclude some physiological host factors that could influence gut microbiota. More importantly, all five plains populations in this study were raised on the same farm with consistent diet and environmental exposure. The comparatively minuscule differences in gut microbiota between these plain populations indicate that living conditions could normally constitute a more powerful factor than breed in determining the gut microbiota assembly of chickens.

The In-TC and Ex-TC populations shared the same genetic background but were raised in different geographic locations. Differences in gut microbiota between these two populations could reveal the effects of habitat factors [34]. Our results indicate that variation in local species pool was the dominant driver of inter-population differences in chicken gut microbiota. The environmental microbiome is known to act as a source pool for gut microbiota of animals [35]. The geographical and climatic characteristics of the Qinghai-Tibet Plateau have created a microbial community that is distinct from that of lower elevations [36], which can result in significant differences in local species pools between plateau and plains habitats. In addition, different diets for In-TC and Ex-TC populations also could introduce different microbes into the intestinal tract [37]. A relatively narrow phylogenetic distribution of gut microbes was observed in the In-TC samples. This may also be attributable to the differences in local species pools between the plateau and plains habitats, as specific microbes with adaptability to low temperatures and low oxygen levels could be selectively enriched in plateau habitats.

Knowledge of assembly mechanisms of gut microbiota in animals is important for improving our understanding of the relationships between gut microbiota and host health state [38]. From a meta-community perspective, the biotic and abiotic factors that influence the community assembly can be
classified into deterministic and stochastic processes [39]. Our findings showed a gut microbiota dominated by stochastic processes in Tibetan chicken living in the plateau environment. Such an assembly may represent a more balanced state that enhances gut microbiota resistance to external stresses [40]. In addition, a more complex gut-microbial network was found in the In-TC population compared to the Ex-TC individuals. High microbiota network complexity often indicates greater robustness against external perturbations [41]; an example are the changes in gut microbiota of healthy pigs with increasing age [42]. We also found higher functional redundancy of gut microbiota in the In-TC population compared to the Ex-TC samples, which is considered indicative of a greater ability to resist strong external stress [43]. Taken together, it can be concluded that the gut microbiota in the Tibetan chicken population raised on the plateau possessed greater resistance to the external stresses imposed by the plateau ecosystem.

One of the objectives of this study was to explore the characteristics of environmental adaptation in Tibetan chickens. Two important genes (CL2 and FGA) were found in heart of Tibetan chickens that were significantly down-regulated in the In-TC samples compared to samples from the plain populations. The FGA gene encodes the alpha component of fibrinogen, which in turn regulates the formation of blood clots [44]. CL2 encodes the protein collectin-11, which is involved in the complement pathway and forms oligomers and deposits [45, 46]. This suggests that Tibetan chickens living in high-altitude areas may have reduced formation of polymers in the blood, lower blood viscosity (Fig. 8), and increased blood oxygen transfer efficiency, and gain other benefits to their adaptation to hypoxic plateau conditions. In addition, a significant enrichment of the fatty acid degradation pathway was detected in liver of the In-TC group, with a significant up-regulation of all DEGs (Figure S22-S23). The liver is the central organ in the fatty acid metabolism, in which ingested fatty acids are oxidized to produce ATP [47]. A strong fatty acid degradation ability in liver of Tibetan chickens may imply more effective energy absorption, which might increase survival in harsh conditions (Fig. 8).

It would generally be assumed that Tibetan chickens surviving in harsh environments have unique evolutionary advantages that enable them to outcompete other populations. If this is correct, however, why would the breed display long-term adaptations to domestication when raised at lower altitudes? Some findings of this study may shed some light on the evolutionary perspective of this situation. We found that several isoflavonoids were more abundant in the intestinal tract of plains chicken populations than that of In-TC individuals. These isoflavonoids are related to resistance to invasion by pathogens and elimination of excessive oxygen stress [48, 49]. More importantly, the transcriptome results of heart, liver and lung all showed that multiple adaptive immune-related pathways (connected to cytokines and T cell activation) were enriched and that most of the DEGs among these were significantly up-regulated in the plains populations compared to the In-TC individuals (Figure S16–S21). High cytokine and T cell activity is beneficial for pathogen resistance and maintenance of host health [50]. Our results indicate that the plains populations had more effective immune systems than the plateau population (Fig. 8). The impacts of urbanization and human activities on the vast and sparsely populated Qinghai-Tibet Plateau are much less severe than on other more populous locations such as coastal cities. Thus, chickens living in urban farms could face more frequent and severe health threats, and the enhanced activation of the immune
system may be a response strategy to these threats. This strategy is determined by environmental adaptability rather than innate genetic characteristics, just as humans who have been vaccinated can more effectively resist epidemic diseases [51].

Finally, we detected five core gut microbes that were significantly correlated to the immune activities involved in the "gut-organ" axis in all populations, all of which belonged to the phylum Firmicutes. In a healthy state, the host's immune response to intestinal microbiota is orchestrated to maintain key features of host-microbe symbiosis [52]. Firmicutes in gut microbiota have been reported to be related to the host immune system. Colonization by Firmicutes was found to mediate the gut immune maturation in human and mouse [53]. Another study revealed that Bacteroidetes and Firmicutes have distinct effects on intestinal immunity by differentially inducing primary and secondary responses [54]. However, the relationship between Firmicutes and the host immune system is complex, and the specific mechanisms underlying this interaction are not clear. Host adaptive immunity has also been observed to alter the gut microbiota by causing an enrichment of Firmicutes [55]. To fully understand the role of Firmicutes in host adaptation, further studies are required to explore the molecular mechanisms behind this interaction. The core gut microbes identified in this study may also provide candidate resources for screening probiotics to improve the extreme environments resistance of chickens. In future work, the isolation of the functional microorganisms and the development of effective food additives are worth investigating at greater depth.

Conclusions

We carried out a multi-omics study to explore the mechanisms of environmental adaptation in chickens, with special focus on the Tibetan chicken breed. We found significant effects of population origin and habitat on gut microbiota, in which the local species pool and species turnover acted as the dominant drivers of variation in gut microbiota. When compared with populations living in a lower-altitude plains environment, Tibetan chickens were found to possess a gut microbiota with a more powerful capacity to resist the external stresses of the plateau environment. Our study also elucidated the environmental adaptation strategies of chickens via the "gut-organ" axes, as exemplified by effective energy absorption in chickens living on the plateau and stronger immune activities in chickens living in plains habitats. These findings may contribute valuable insights into the adaptation of gut microbiota to high-altitude environments and provide candidate resources for the future exploitation of probiotic products to improve the hypoxic environmental resistance.

Materials and methods

Study animals and sample collection

In order to exclude the influence of diet and breeding environment, the cohorts of all five chicken populations from lowland plains environments were raised in the same low-altitude location, while the In-TC population was raised on the plateau. All study animals were approximately 300 days old, healthy, and had not received any medication for at least 3 months prior to sampling. Ten individuals were picked
from each population and their cecum contents, heart, liver, and lung tissues were collected in sterile conditions. Samples were added separately to 1.5 ml sterile polypropylene tubes and immediately snap-frozen in liquid nitrogen until further analysis.

DNA extraction and gut microbiota sequencing

The microbial DNA of each cecum content sample was extracted using a QIAGEN DNA Stool MiniKit (QIAGEN, Venlo, Netherlands) according to the operating instructions. Agarose gel electrophoresis (1.5%) was performed to validate successful DNA extraction. A NanoPhotometer Classic instrument (Implen, Munich, Germany) was used to measure the purity and concentration of extracted DNA. Primers for the full-length of bacterial 16S rRNA gene (27F and 1492R) were applied to amplify the target fragment from the successfully extracted DNA. PCR amplification, product purification, and amplicon library construction followed Gao et al. [56]. Amplicon sequencing was carried out using a PacBio Sequel platform by Shanghai Biozeron Biotechnology Co., Ltd. (Shanghai, China). PacBio raw reads were processed to obtain demultiplexed circular consensus sequence (CCS) reads using SMRT Link Analysis software (v 9.0). After assigning the CCS reads to specific samples according to the unique barcode, data processing (quality control, paired end read assembly, clustering, and counting) was executed using QIIME2 software based on the amplicon sequence variant (ASV) strategy [57]. ASVs with only one read number (singletons) were removed, and the remaining ASVs were taxonomically annotated according to the SILVA database (Release 138) [58]. Finally, the ASV abundance table was normalized based on the lowest read number among all samples.

Non-targeted metabolomics

Based on the results of gut microbiota analyses, three populations (In-TC, Ex-TC, and QY) were selected to perform non-targeted metabolomics. To extract the metabolites, 100 mg chicken cecum contents of each individual were resuspended with 500 µL pre-chilled 80% methanol with 0.1% formic acid and well vortexed. The samples were then incubated at 4°C for 5 min and centrifuged at 15,000 × g for 20 min. The supernatant of each sample was diluted to a final concentration of 53% methanol by HPLC grade water, and then centrifuged again at 15,000 × g for 20 min. Supernatants were collected and injected into a Vanquish UHPLC system (Thermo Fisher Scientific, Waltham, MA, USA) with a Hypesil Gold column (100 mm × 2.1 mm; 1.9 µm) to separate metabolites. An Qrbitrap Q Exactive TMHF-X mass spectrometer (Thermo Fisher Scientific) was applied to detect metabolites eluting from the UPLC column. The detailed process of UHPLC-MS/MS followed a previous study [59]. Compound Discoverer v3.1 (Thermo Fisher Scientific) was employed to process peak information from the raw data to quantity metabolites. Peaks were matched with the mzVault and MassList database to obtain the accurate qualitative and quantitative results of each metabolite. Three comparisons of metabolites were carried out: In-TC vs Ex-TC, In-TC vs QY, and Ex-TC vs QY. Student's t-test (p-value < 0.05), VIP value (higher than 1) and fold change (FC, > 2 or < 0.5) were considered to identify differential abundant metabolites (DAMs) among different chicken populations. DAMs were annotated using the HMDB and KEGG databases based on searches for accurate DAM m/z values.

Transcriptome sequencing
Total RNA from the lung, heart, and liver of samples from the same three populations used in non-targeted metabolomics (In-TC, Ex-TC, and QY) were extracted using the trizol method for RNA-Seq [60]. Transcriptome libraries (paired end 150 bp) were constructed using the TruSeq™ RNA sample preparation Kit (Illumina, San Diego, CA, USA) and sequenced by Illumina NovaSeq 6000 platform at Shanghai BIOZERON Co., Ltd (Shanghai, China). Quality control for raw reads was executed by Trimmomatic with parameters of sliding window 4:15 and minlen 75 [61]. The processes of reference genome mapping and gene expression calculation were performed by HISTA2 [62] and HTSeq [63], respectively. Similar to the non-target metabolomics, three comparisons (n-TC vs Ex-TC, In-TC vs QY, and Ex-TC vs QY) were carried out to identify differentially expressed genes (DEGs). Based on the results obtained using the "edgeR" package [64] in R, genes with a log-trans fold change > 2 and a p-value < 0.5 (adjusted by the false discovery rate method) in each comparison were recognized as DEGs. Finally, the KEGG annotation of DEGs was obtained from the reference genome and enrichment analysis was performed using the KOBAS tool [65].

**Statistical analysis**

Diversity of chicken gut microbiota was assessed by four alpha diversity indices: Chao1, Shannon, Pielou_J, and Pd_faith. Difference in alpha diversity indices, bacterial abundances, and functional terms of chicken gut microbiota among different populations were tested using Tukey's honestly significant difference (HSD) test. Two types of distance (weighted and unweighted Unifrac) between pairs of microbiota were calculated, and variations in microbiota composition among different chicken populations were evaluate by Adonis test, Tukey's HSD test, and principal coordinate analysis (PCoA). The relative importance of nestedness and turnover on the variation of gut microbiota composition was determined using the betapart method [66]. Venn diagrams were generated to determine shared ASVs among different chicken populations, and their taxonomic composition was displayed by a Sankey diagram. Processes governing gut microbiota assembly were quantified by a null model method based on the β-nearest taxon index (βNTI) and the Raup-Crick metric (RC) [67].

To further decipher differences in gut microbiota between In-TC and Ex-TC populations, co-occurrence networks were constructed based on co-relations among gut ASVs. If the Spearman correlation coefficient was > 0.8 and the Benjamini-Hochberg adjusted p-value was < 0.01, a correlation between two ASVs was considered statistically robust. The obtained networks were visualized using the Gephi interactive platform and their topological parameters were extracted [68]. To assess the stability of microbiota communities, robustness, vulnerability and cohesion were calculated for co-occurrence networks of In-TC and Ex-TC samples, following a previous study [69]. Differences in these stability indices between the In-TC and Ex-TC groups were analyzed using Student's t-test. The functional redundancy index (FRI) of individuals from the In-TC and Ex-TC populations was determined using the Tax4Fun2 method [70]. Linear regression analysis (ordinary least-squares) was performed on the coordinated variations in FRI and betaNTI in In-TC and Ex-TC ponds.

PCoA was also carried out based on metabolite abundances or gene expression levels to evaluate differences in metabolite composition and transcriptome among different chicken populations. Volcano
plots were generated to filter DAMs based on log2(FC) and log10(p-value) of metabolites. Enrichment analysis was conducted based on the DAMs identified from each of the comparisons with the KEGG annotation. Venn diagrams were applied to explore the potential key metabolites involving in the environmental adaptation of chicken populations. Correlation networks were further created to illustrate the relationships between DAMs or DEGs with bacterial ASVs based on Spearman's rank correlation with the same thresholds for co-occurrence networks of gut microbiota. Finally, a heatmap was created to show the correlations between potential key metabolites or genes with key gut bacterial ASVs as determined from the correlation network, based on Spearman's rank correlation.

All statistical analysis was performed in R v4.2.2 with the packages of "vegan", "multcomp", "GUniFrac", "ape", "betapart", "VennDiagram", "networkD3", "picant", "WGCNA", "Tax4Fun2", "LinkET", "pheatmap", and "ggplot2".

**Declarations**

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**Authors’ contributions**

TZ, LZ and WH planned the project. TZ, LZ, WH, YT and PS designed the research. TZ, YQ, JM, TT, HX, XY, GH, LC and QX prepared the research materials. TZ, YQ, ZL and WW performed the experiments for sequencing and the data analyses. TZ drafted the manuscript. LZ and WH revised the manuscript. All authors read and approved the final manuscript.

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**Availability of data and materials**

The raw reads of PacBio full-length 16S rRNA sequencing and transcriptome sequencing for all samples have been deposited in the NCBI Sequence Read Archive (SRA) under the accession number of PRJNA975664 and PRJNA950191. Metabolomics data has been deposited into China National GeneBank Sequence Archive (CNSA) of China National GeneBank DataBase (CNGBdb) under project CNP0004383.

**Ethics approval and consent to participate**
Animals used in this study were raised in accordance with the national standard of Laboratory Animal Guidelines for ethical review of animal welfare. All experiment procedures were approved by the Animal Use Committee of Zhejiang Academy of Agricultural Sciences (No. 20-022).

**Consent for publication**

Not applicable.

**Competing interests**

The authors declare that they have no competing interests.

**References**


Figures
Figure 1

(a) Schematic diagram for sample collection. (b) Differences in the Chao1 and (c) Pd_faith indices of gut microbiota among different chicken populations. Different lowercase letters above the boxes represent significant differences (Tukey’s HSD $p > 0.05$) between groups.
Figure 2

(a) PCoA and Adonis test of chicken gut microbiota based on unweighted and (b) weighted Unifrac distances, respectively. (c) Differences in the intra-variations of gut microbiota among different chicken populations according to the unweighted and (d) weighted Unifrac distances, respectively. (e) Relative importance of nestedness and turnover on beta-diversity of chicken gut microbiota on the basis of the unweighted and (f) weighted Unifrac distances, respectively. (g) Contributions of alpha- and beta-diversity to the variations in gamma-diversity of gut microbiota among different chicken populations. Different lowercase letters above the boxes represents significant differences (Tukey's HSD $p > 0.05$) between groups.
Figure 3

(a) PCoA and Adonis test of chicken gut microbiota based on unweighted and (b) weighted Unifrac distances, respectively. (c) Differences in the intra-variations of gut microbiota among different chicken populations according to the unweighted and (d) weighted Unifrac distances, respectively. (e) Relative importance of nestedness and turnover on beta-diversity of chicken gut microbiota on the basis of the unweighted and (f) weighted Unifrac distances, respectively. (g) Contributions of alpha- and beta-diversity to the variations in gamma-diversity of gut microbiota among different chicken populations. Different lowercase letters above the boxes represents significant differences (Tukey’s HSD $p > 0.05$) between groups.
Figure 4

(a) Co-occurrence networks of gut microbiota in the In-TC and Ex-TC group, respectively. Modules are labelled in different colors in the respective network. (b) Differences in negative:positive cohesion between gut microbiota in the In-TC and Ex-TC groups. (c) Ratio of relative contributions of deterministic and stochastic processes to the assembly of gut microbiota in the In-TC and Ex-TC groups. (d) Differences in phylogenetic distance between gut microbiota in the In-TC and Ex-TC groups. (e) Ratio of FRI between gut microbiota in the In-TC and Ex-TC groups. (f) Linear regression between betaNTI and FRI for gut microbiota in the In-TC and Ex-TC groups.
Figure 5

(a) PCoA showing the differences in intestinal metabolome among In-TC, Ex-TC, and QY populations. (b) KEGG enrichment analyses based on DAMs identified from different comparisons. (c) Correlation network of gut microbiota and potential key DAMs. (d) Heatmap showing correlations between key gut bacterial ASVs and DAMs involved in the isoflavonoid biosynthesis pathway. "++" and "+" represent $p$-values < 0.01 and < 0.05, respectively.
Figure 6

(a) Correlation network of gut microbiota and potential key DEGs in lung. (b) KEGG enrichment analyses based on DEGs identified from lung of different comparisons. (c) Heatmap showing the correlations between key gut bacterial ASVs and DEGs involving in the viral protein interaction with cytokine and cytokine receptor pathway. "++" and "+" represent $p$-values $< 0.01$ and $< 0.05$, respectively.
Figure 7

(a) Correlation network of gut microbiota and potential key DEGs in heart. (b) KEGG enrichment analyses based on DEGs identified from heart of different comparisons. (c) Correlation network of gut microbiota and potential key DEGs in liver. (d) KEGG enrichment analyses based on DEGs identified from liver of different comparisons.
Figure 8

Gut microbiota-centered conceptual framework for environmental adaptation of Tibetan chicken.

Supplementary Files

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