

# An in-depth analysis of the intestinal microbiome & metabolites of rats under high-altitude hypoxic-condition

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## Research article

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# Abstract

## Background

Under anoxic conditions, the body will undergo a series of changes to compensate for the low oxygen environment. Changes in gut microbes are an important part of this. Currently, there are relatively few studies on the changes of intestinal microorganisms and metabolites in the low-oxygen environment of plateau. This study employed a non-targeted metabolomics approach coupled with shotgun metagenomic sequencing to analysis the changes of intestinal microorganisms and metabolites in rats under hypoxic environment.

## Results

Significant difference in biodiversity were observed. At the genus level, the relative abundance of *Lactobacillus*, *Alistipes* in the hypoxic cohort were significantly increased, while *Flavonifractor*, *Faecalibacterium* and *Dorea* were significantly decreased ( $P < 0.05$ ). Non-targeted metabolomics analysis showed significant differences in 4 metabolic pathways (pyrimidine metabolism, aminoacyl-tRNA biosynthesis, nicotinate and nicotinamide metabolism, thyroid hormone synthesis) between T group and C group ( $P < 0.05$ ). Furthermore, correlation analysis of metagenomic and metabolomic data sets showed that there was a certain relative quantitative relationship between some bacterial genera and differential metabolites.

## Conclusions

Our results indicate that a plateau hypoxic environment can significantly alter the composition of intestinal microbes in the host. Compared with the plain control group, the relative increase of some bacteria genera was accompanied by the increase of related metabolites, suggesting that these bacteria genera and metabolites were closely related to the low-oxygen environment in the plateau. It provides a certain foundation for the next research.

## Background

The climatic conditions in the plateau or high-altitude area, such as low oxygen partial pressure, low temperature, strong winds & ultraviolet radiation, which are ill-suited for human habitats, results in the sparsely populated plateau area. The past few years have witnessed an eventual rise in the societal affluence exhibited by increased frequency of travel and recreational activities in high-altitude areas. These changes, coupled with advancement in the transportation facility, has encouraged a large group of the population not only to travel but to migrate to the high-altitude areas [1, 2]. The ascent in the altitude from plains to plateau areas necessitates a physiological adjustment by the human body as a part of the adaptation process. For instance, high-altitude hypoxic conditions critically impact the individual's

metabolite levels, vital enzymes, and metabolic pathways involved in inflammation and energy metabolism[3]. Besides, the intestinal microflora also changes as a result of the major shift in the external atmospheric conditions at the same time[4].

The human microflora acts as a key player in numerous physiological processes[5]. Change in the external environmental conditions impacts microbial consortium of the gut. This, in turn, affects the intestinal physiological functions such as metabolism, protection & maintenance of structural integrity of the intestine[6]. Intestinal microbes play a crucial role in the maintenance of overall health and disease manifestation in individuals. Apart from the immediate physiological response, prolonged exposure to the high-altitude hypoxic conditions results in complex time-dependent changes in the lowlanders ascended to the high-altitudes[7]. Previous studies state that the low-oxygen environmental conditions persisting in the high-altitude areas lead to a significant difference in the intestinal microbiome[8].

In this study, to evaluate the changes associated with the intestinal microbiome, we employed a non-targeted metabolomics (LC-MS) approach[9, 10] coupled with metagenomic analysis. Results showed that on day 28 after hypoxia exposure, the feces of rats in two groups showed different microbial community structure and metabolite composition, and there was a certain relative quantitative change relationship between these. It provides a microbiological entry point for the future studies of metabolic changes in the body under the low-oxygen environment at the plateau. Future researches on the related specific genera will help to figure out the mechanism of metabolic changes in the host under the low-oxygen environment, and help the host to adapt to the low-oxygen environment as soon as possible.

## Methods

### Experimental setup and sample collection

Female SD rats were randomly divided into two groups: the control group (n = 10) and the high-altitude group (n = 10). The control group of rats was housed in animal facilities with a plain environment. The high-altitude group of rats was housed in the controlled low-pressure chamber (08-Y1300-004, 1.3 × 1.3 × 1.7 m, Yantai Hongyuan Oxygen Industry Co., Ltd., Yantai, China) at an altitude of 6,000 meters and exposed for 8 hours per day for 28 days. All rats were maintained on autoclaved food and water. We euthanized the rats at the end of the 28th day of hypoxic exposure, under inhaling ether as anesthesia. It was followed by the blood sample collection through cardiac puncture. We carried out the hematological and biochemical analysis of the sample was by a fully automated blood cell analyzer (BM-800, Beijing). The MDA and GSH levels of the sample were assessed with the help of kit (Nanjing Jiancheng Biotechnology Research Institute Co., Ltd, Nanjing). Fresh stool samples were collected on days 28, and transferred to a 2 ml sterile centrifuge tubes and immediately cryopreserved stored at -80°C freezer.

### Untargeted Metabolomics Analysis

#### Preparation of quality control (QC) samples and data quality control

Before starting with the experiments, all fecal samples were thawed on ice, and a "quality control" (QC) sample was prepared by mixing and blending the equal volumes of each fecal sample. The QC sample was detected on the LC-MS/MS: before the test sample injection, during the test sample injection, and after the test sample injection. The QC sample injection before the test sample injection was used to monitor the state of the instrument before the test sample injection and to equilibrate the chromatography-mass spectrometry system. The QC sample injected in the middle of the test samples to evaluate the system stability and to conduct the sample correlation analysis during the whole experiment. The QC sample was injected after the end of the test samples for the qualitative analysis of metabolites by secondary mass spectrometry.

## **Metabolomic analysis**

Firstly, the original files (.raw) obtained by the mass spectrometric analysis were imported into the Compound Discoverer 3.0 (CD) software. It was followed by the spectrum processing and database searches to obtain the qualitative and quantitative results of the metabolites. Later the data were quality-controlled to ensure the accuracy and reliability of the results. Subsequently, we performed the multivariate statistical analysis of metabolites, which included the principal component analysis (PCA) and the partial least squares discriminant analysis (PLS-DA), and revealed the differences in metabolites in T & C-group on day 28. Using hierarchical clustering (HCA) and metabolite correlation analysis, the relationship between the samples and metabolites were revealed. Finally, the functional analysis of the metabolic pathways was used to explain the biological significance of metabolites.

# **Metagenomic Sequencing**

## **DNA sample detection and library construction**

We used agarose gel electrophoresis (AGE) to analyze the purity and integrity of the DNA, and the DNA concentration was quantified with the help of Qubit 2.0. The qualified DNA samples were randomly broken into fragments of about 350 bp by Covaris sonicator, and the whole library was prepared by the terminal repair, the addition of A tail & sequencing linker, & the purification of the product followed by the PCR amplification. After the library construction, preliminary quantification of the product was performed using Qubit 2.0, and the library was diluted to a final concentration of 2 ng/ $\mu$ l. Later on, the insert size of the library was detected by Agilent 2100. Once we got the expected insert size, the effective concentration of the library (> 3 nM) was determined by Q-PCR for the accurate quantification to ensure the library quality.

## **Sequencing and data quality control**

The library was sequenced on an Illumina PE150 platform. The raw data obtained by sequencing acquired by sequencing contained the low-quality reads as well. In order to ensure the accuracy and reliability analysis, the raw data was quality-controlled and host-filtered to obtain high-quality data. The

high-quality data for each sample was then used to perform macrogene assembly, and the unused reads in each sample were put together for mixed assembly to find the low-abundance species information in the sample.

### **Gene prediction and species annotation**

MetaGeneMark was used for the gene prediction of assembled scaffolds. Each sample and mixed assembly predicted genes were put together for de-redundancy to construct gene catalog. The clean data were combined to obtain the abundance information of the gene catalog in each sample. It was then compared with the NCBI's NR (Version: 2018.01) database to obtain the species annotation information of Unigene, and the species abundance table of different classification levels by combining the gene abundance table.

## **Correlation Analysis Between Bacteria And Metabolites**

The correlations within differential genera and differential metabolites were examined using Pearson statistical method, which was conducted using R software (version 3.5.2) with "corrplot" packages.

## **Results**

### **Hematological and biochemical assessment**

In this study, we examined the hematological and other related determinants between the hypoxic (T group) and control (C group) of rats at the end of the hypoxic exposure, i.e., on day 28. We found that the hemoglobin and the red blood cell value of the T-group of rats housed at 6000 meters, increased significantly as compared to the C-group of rats (Fig. 1). We also evaluated the levels of Glutathione (GSH) and malondialdehyde (MDA) levels in both groups of rats. We observed a significant variation between the levels of GSH and MDA in both groups. We found a lower antioxidant capacity and a higher lipid membrane peroxidation capacity in the T-group of rats as compared to the C-group of rats, which demonstrated the effectiveness of the 28-days hypoxic exposure (Fig. 1). Apart from this, the pathological manifestations of high-altitude polycythemia (HAPC) were surfaced in the T-group of rats.

## **Metabolomics Results**

### **Metabolic profiling of the rat feces**

We performed a non-targeted metabolomics analysis of fecal samples from the T-group of rats. Firstly, we evaluated the stability and reproducibility of the current data by measuring the Quality Control (QC) samples throughout the experiment. The peak area value thus obtained was used to analyze the Pearson correlation coefficient between the QC samples. A higher correlation value of the QC samples indicated

better stability of the whole detection process, and the higher the data quality. The results are presented in Supplementary Fig. 1 and demonstrate excellent stability, higher reproducibility, and data quality in our current metabolomics data set.

A quality control check qualified a total of 4,360 peaks in positive ion modes and 4,973 peaks in negative ion modes from the stool samples of rats, which were further annotated with the help of ChemSpider and mzCloud databases comparison (Supplementary Table 1).

## Metabolic Findings In Rat Feces

We employed the partial least squares discriminant analysis (PLS-DA)[11] to establish a model of the relationships between the metabolite expression and sample grouping for sample size prediction (Supplementary Fig. 2). Subsequently, we sorted the model to verify if the model was “over-fitting.” The results demonstrated that in the PLS-DA model of each group, R2Y was close to 1, which indicated that each group could explain the grouping well. At the same time, based on the results of the sort verification (Supplementary Fig. 3), we can say that the grouping model was not “over-fitting.” Thus, we can conclude that the experimental results are reliable, and the data set can be subsequently analyzed.

Based on this analysis, we used the Variable Importance in the Projection (VIP) value of the first principal component of the PLS-DA model to represent the contribution rates of metabolite differences in different groups. The difference multiple (Fold Change, FC) was the ratio of the mean of all biological replicate quantitative values for each metabolite in the two groups, and the p-value of the t-test was used to find the differentially expressed metabolites (Table 1).

Compared Samples	Num. of Total Ident.	Num. of Total Sig.	Num. of Sig.Up	Num. of Sig.down
DAY 28_pos	4360	101	41	60
DAY 28_neg	4973	130	25	105

**Table.1 The number of differential metabolites between the experimental group and the control group on day 28.** We have set the threshold value to VIP > 1.0, FC > 2.0 or FC < 0.5 and p-value < 0.05, and screened the differential metabolites between the experimental group and the control group.

We performed the hierarchical cluster on each group of differential metabolites. And it was followed by pathway analysis where exact mass and mass spectrometry fragment patterns were searched in the KEGG database. Then a hypergeometric test was applied, as shown in the supplementary Fig. 4, to find pathways enriched in the differential metabolites compared to all identified metabolite backgrounds (With P-value < 0.05 as the threshold). The differential metabolites identified in our study were mapped to 4 metabolic pathways of the KEGG database. They were pyrimidine metabolism pathway, aminoacyl-tRNA biosynthesis pathway, nicotinate and nicotinamide metabolism pathway, and thyroid hormone synthesis

pathway. The metabolites in these pathways were found to be significantly different in T group compared with C group (Table 2).

Pathways	Metabolites	VIP	FC	P.value	Up.Down
Pyrimidine metabolism	Uridine	2.78	0.35	5.79E-03	down
	Pseudouridine	2.75	0.36	7.67E-03	down
	Thymidine*	3.31	0.20	2.30E-02	down
		4.22	0.11	4.15E-02	down
	Cytidine	1.88	0.47	6.93E-03	down
Aminoacyl-tRNA biosynthesis	L-Aspartic acid	2.07	2.28	5.95E-04	up
	L-Tyrosine	2.04	0.39	4.72E-02	down
Nicotinate and nicotinamide metabolism	L-Aspartic acid	2.07	2.28	5.95E-04	up
	Maleic acid	1.97	2.02	3.39E-02	up
Thyroid hormone synthesis	Glutathione disulfide	1.84	2.00	7.86E-03	up

**Table.2 Major differential metabolic markers and the enriched pathways on day 28.** \*The expression of thymidine was different in both positive and negative ion modes

## Microbial Community Analysis

We used Illumina HiSeq sequencing platform for sequencing, and obtained 112,695.48 Mbp of Raw Data. After single sample assembly and mixed assembly, a total of 2,231,469,534 BP scaffolds were obtained. We got 2,746,053 open reading frames (ORFs) by using MetaGeneMark. Then, the blastp algorithm was used to compare with the MicroNR Library, and annotated species with LCA algorithm. The proportions of Genera and Phyla were 52.90% and 78.65%, respectively. The core-pan genetic analysis (Supplementary Fig. 5) denoted that samples tended to saturate the platform, which indicates that the sequence coverage was sufficient to capture the diversity of bacterial communities in the sample.

### Beta diversity of gut microbiota between the two groups with multivariate statistics analysis

We used Non-Metric Multi-Dimensional Scaling (NMDS) as a simple method of visual interpretations to compare the overall structure of fecal microbiota between two samples (Fig. 2A). NMDS was performed using the Bray-Curtis similarity index based on the ORFs. Moreover, we also described the use of analysis of similarity (Anosim) to statistically test the significant difference between groups (Fig. 2B). Analysis of similarity (ANOSIM) revealed that significant differences were observed between T group and C group in microbiota community structure (R = 0.344; P = 0.006).

**Fig 2. Non-Metric Multi-Dimensional Scaling (NMDS) plot (A) and ANOSIM analysis (B).** NMDS is a simple method for visual interpretations to compare the overall structure of fecal microbiota between two samples while ANOSIM is used to statistically test the significant difference between groups.

## Fecal Microbiota Composition Between Two Groups

Bacterial communities with inter-group differences were analyzed at the genus level. Metastat analysis showed that the relative abundance of genus *Lactobacillus*, *Alistipes* in the experimental group were significantly increased than that in the control group, while genus *Flavonifractor*, *Faecalibacterium* and *Dorea* were decreased.

Table 3

**Metastat analysis between two groups at genus levels.** The mean of group indicates the relative abundance of this genus, the screening threshold are *P* value less than 0.05 and relative abundance greater than 0.1%.

Taxa	Mean(T group)	Mean(C group)	<i>P</i> value
Genus <i>Lactobacillus</i>	2.24%	0.97%	1.07E-02
Genus <i>Alistipes</i>	1.20%	0.58%	8.52E-04
Genus <i>Flavonifractor</i>	0.23%	0.55%	1.68E-02
Genus <i>Faecalibacterium</i>	0.14%	0.46%	4.06E-03
Genus <i>Dorea</i>	0.11%	0.30%	3.47E-02

### Association of the intestinal microbial species with the metabolites.

In order to explore the correlations between differential genera and differential metabolites. We used Pearson statistical method to calculate the correlation coefficients rho and *P* values ( $p \leq 0.05$ ) between the relative abundance of each genus and metabolites. The results are shown in Fig. 3. Genus *Alistipes* has significant positive correlations with glutathione disulfide, L-aspartic acid, and maleic, and negative correlations with uridine, pseudouridine, cytidine and L-tyrosine; Genus *Dorea* has significant positive correlations with metabolites thymidine and L-tyrosine; Genus *Faecalibacterium* has a significant positive correlation with pseudouridine, and negative with glutathione disulfide; Genus *Flavonifractor* has a significant negative correlation with the metabolite L-aspartic acid; Genus *Lactobacillus* has no significant correlation with these differential metabolites.

## Discussion

Previous studies have stated the consequences of high-altitude hypoxic exposure on human intestinal microbes, but the outcomes of these studies were influenced by external factors such as diet, lifestyle, and genetics of local inhabitants[12, 13]. In the current study, we constrained the interference of these determinants by limiting their intervention in the animal model, & tried to maintain the high-altitude hypoxic conditions as the only variable. We used metagenomic and metabolomic analysis to compare the intestinal microbiome and the metabolites between the test group exposed to the high-altitude hypoxic condition and the control group.

The metagenomic analysis of the rat feces revealed that the acclimatization to the hypoxic conditions will lead to a significant difference in microbial community. It suggests that the high-altitude hypoxic conditions impact the intestinal microbiome of the rats. At the genus level, we found 5 differential genera: genus *Alistipes*, *Lactobacillus*, *Flavonifractor*, *Faecalibacterium* and *Dorea*. Previous studies have shown that genus *Alistipes* may be associated with severe intestinal diseases and immune system maturation [14, 15], and shows notable presence in patients with myalgic encephalomyelitis/chronic fatigue syndrome, irritable bowel syndrome and depression [16, 17]. A study by Fremont et al., suggests that the increase in the genus *Alistipes* population can be a plausible reason behind the hypoxia-induced intestinal disorders [18]. Genus *Lactobacillus* utilize carbohydrates fermentatively and produce lactic acid as a major end-product[19]. They are facultatively anaerobic, catalase-negative, Gram-positive, non-spore-forming rods that often grow better under microaerophilic conditions. This may explain the increase of Genus *Lactobacillus* in low oxygen environment. *Lactobacilli* represent the types of microorganisms that mammalian immune systems have learned not to react to, which is recognized as a potential driving force in the evolution of the human immune system[20]. *Flavonifractor* was a bacterial genus that may induce oxidative stress and inflammation in its host[21]. Members of genus *Faecalibacterium* are commensal bacteria, ubiquitous in the gastrointestinal tracts of animals and humans. Within the human colon, this taxon is the main member of the Clostridium leptum cluster, and comprises the second-most common representative in fecal samples, after Clostridium coccoides[22, 23]. *Faecalibacterium prausnitzii* is the only identified species in the genus *Faecalibacterium* and this species is a functionally important member of the microbiota and likely has an impact on the physiology and health of the host[24]. *Dorea* is the main aerogenic bacteria in human intestine, which produces gas using carbohydrates, and it may have association with the persistent allergy[25].

Our metabolomic analysis inferred a significant variation between the metabolic pathways on day 28, the onset of HPAC. The main metabolic differential pathways identified between T and C, were pyrimidine metabolism, aminoacyl-tRNA biosynthesis, nicotinate and nicotinamide metabolism, thyroid metabolism pathways. The pyrimidines are required for the synthesis of DNA, RNA, and other metabolites. They are the essential structural components of a broad spectrum of key molecules that participate in diverse cellular functions[26]. Numerous studies provide an insight into the biological, chemical and pharmacological aspects of pyrimidine metabolism across the species[27, 28]. Niacin and thyroid hormone levels can influence the critical determinant of the hematopoietic microenvironment- the

grapevine transporter (GTP) [29–31], and we think it may affect the occurrence of HAPC. Besides, relevant studies have shown that the altered thyroid cells lead to enhanced production of IL-6 [32], which in turn leads to increased levels of hypererythremia-related factors (such as HIF-1 $\alpha$ , EPO, and oxygen free radicals, and so on) [33, 34]. Thus we can conclude that the over-expression of the thyroid metabolic pathway may act as a critical component in the pathogenesis of HAPC.

Furthermore, we analyzed the association between the different genera and metabolites. We observed that genus *Alistipes* was most closely related to the differential metabolites. It was positively correlated with L-aspartic acid, maleic acid, glutathione disulfide, and negatively correlated with uridine, pseudouridine, cytidine, L-tyrosine. L-aspartic acid and maleic acid play a vital role in the metabolism of nicotinic acid and nicotinamide, which are dehydrogenated and hydrogenated in the bio-oxidation process and participates in the body events such as glucose glycolysis, fat metabolism, stress and immune response[35–38]. Glutathione disulfide is involved in the thyroid hormone biosynthesis, which regulates the nervous system induced excitability and accelerates the breathing and heart rate along with the body heat production. It was shown that in response to the high-altitude exposure, the restoration process in the human body escalates the level of thyroid hormone[39]. From this, we think that during the onset of HAPC, bacteria (especially genus *Alistipes*) played a certain role in body metabolism.

## Conclusions

With the help of non-targeted metabolomics and metagenomics techniques, this study demonstrated that the high-altitude hypoxic conditions affect the composition of the rat intestinal flora as well as the gut microbiota-related metabolism and metabolites. It provides a certain foundation for later researches. Future studies can focus on the three aspects of the current research for an in-depth investigation. Firstly, due to the differences between individual animals were still relatively large from the results, an increase in the sample size and exposure time will provide enough time for the enrichment of specific differential intestinal flora and metabolites, which can then be easily characterized in the analysis. Secondly, the underlying factors behind the variation of the metabolites involved in the significant metabolic pathways should be evaluated. Finally, the interaction mechanism between differential genera and metabolites should be studied.

## Declarations

### Ethics approval and consent to participate

We have obtained the approval of the laboratory animal ethics committee of the academy of military sciences before carrying out animal experiments

### Consent for publication

Not applicable.

## Availability of data and material

At present, due to the impact of the COVID-19 Situation, I cannot go to the laboratory to upload the sequencing data. Please kindly understand. If the article is finally accepted, I will upload the data and provide relevant information as soon as possible.

## Competing interests

The authors declare that they have no competing interests.

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## Authors' contributions

Sicong Su and Weili Liu contributed equally to this work.

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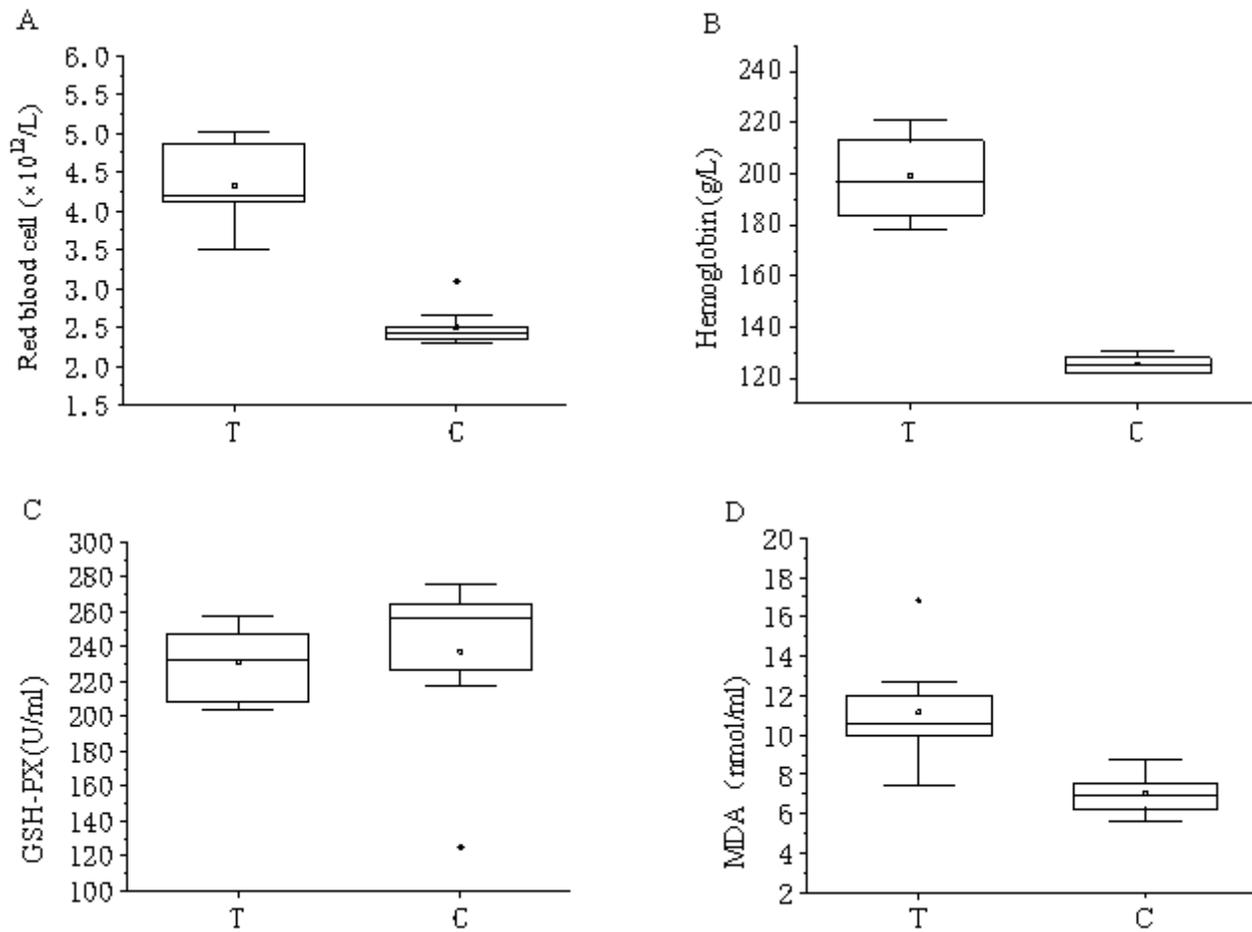
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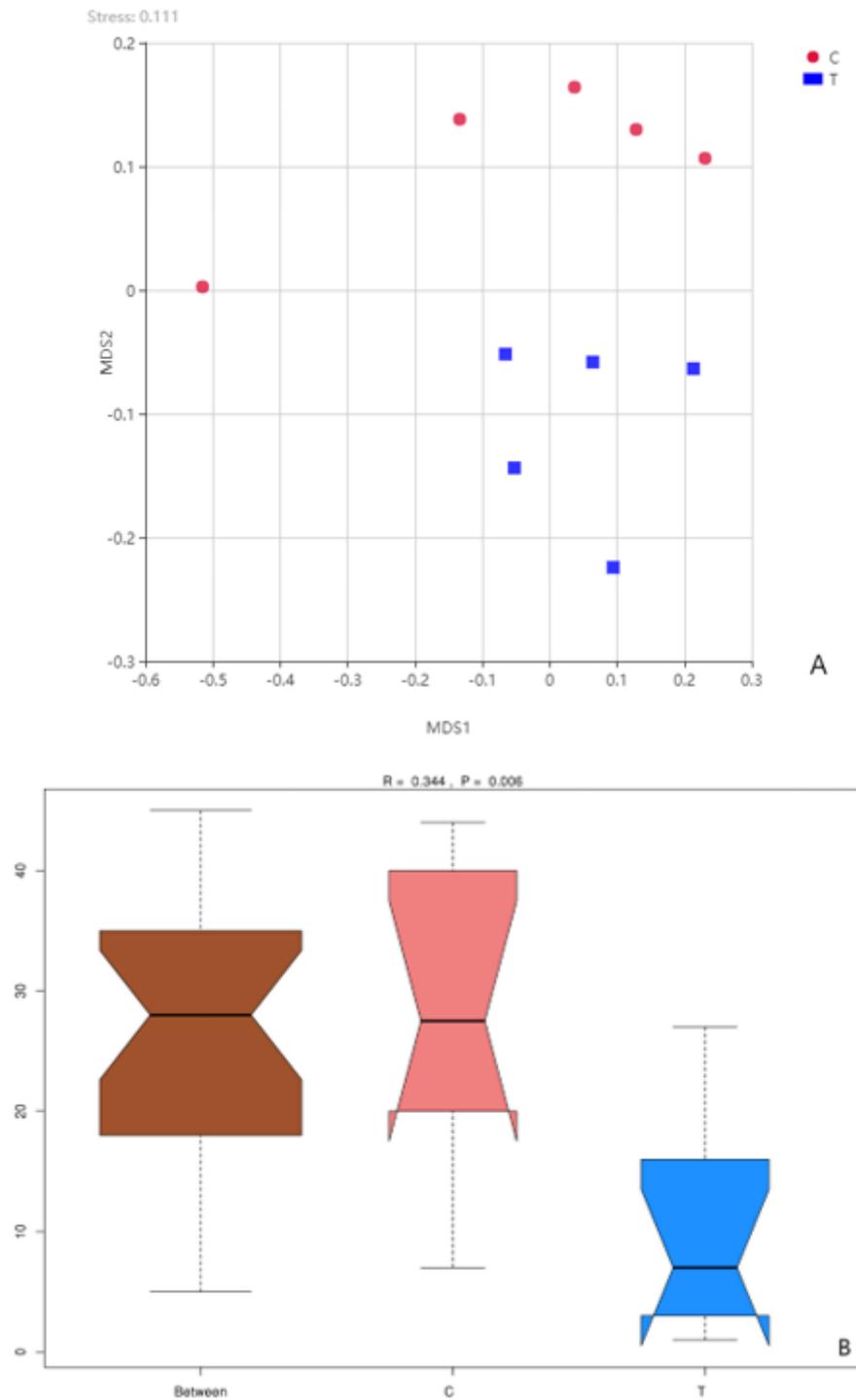
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## Figures



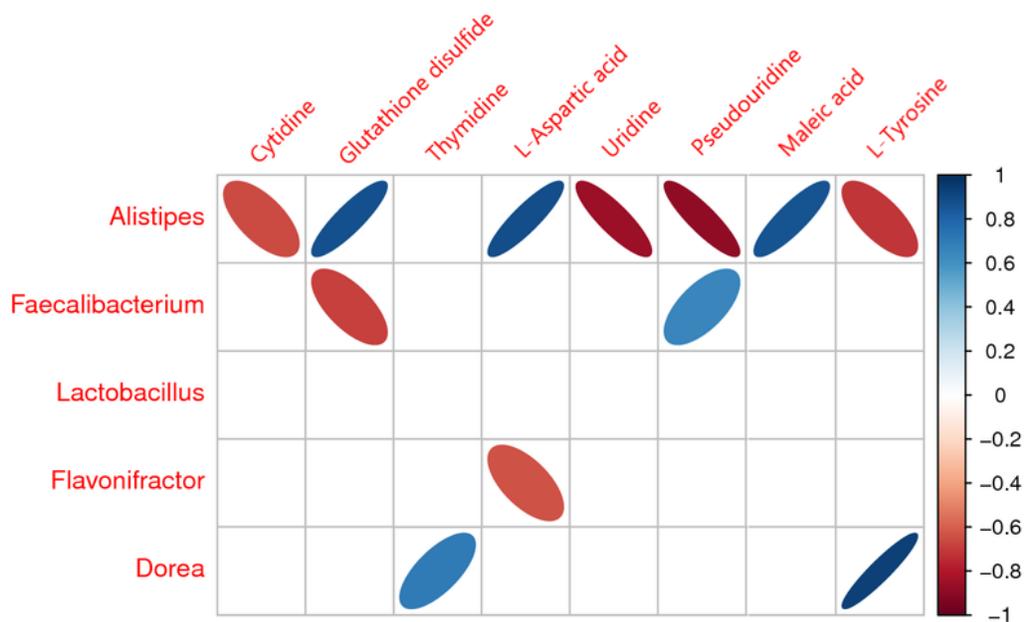
**Figure 1**

Blood routine and antioxidant index levels in rats-A: red blood cells; B: Hemoglobin; C: Glutathione (GSH); D: Malondialdehyde (MDA);  $P < 0.05$ . T vs. C-group of rats.



**Figure 2**

Non-Metric Multi-Dimensional Scaling (NMDS) plot (A) and ANOSIM analysis (B). NMDS is a simple method for visual interpretations to compare the overall structure of fecal microbiota between two samples while ANOSIM is used to statistically test the significant difference between groups.



**Figure 3**

Association analysis between differential genera and metabolites. Aabscissa is the differential metabolites in the two groups, left ordinate is differential genera and the correlation coefficient is on the right. Blue indicates positive correlation and red indicates negative correlation. Blank indicates no significant correlation between genus and metabolite.

## Supplementary Files

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