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Title Page

Gut Microbiome Alterations in Pulmonary Hypertension in Highlanders and Lowlanders

Wenxue Dong[1*], Lifeng Ma[1*], Qiumin Huang[2*], Xu Yang[1], Zhendong Mei[2], Mengmeng Kong[2], Zhonghan Sun[2], Zhiying Zhang[1], Jing Li[1], Jiaojiao Zou[2], Dandan Chen[3], Yuxiang Dai[3], Daxin Zhou[3], Yan Zheng[2,3,4†], Longli Kang[1†]

[1] Key Laboratory for Molecular Genetic Mechanisms and Intervention Research on High Altitude Disease of Tibet Autonomous Region, School of Medicine, Xizang Minzu University, Xianyang, Shaanxi, 712000, China.

[2] State Key Laboratory of Genetic Engineering, School of Life Sciences and Human Phenome Institute, Fudan University, Shanghai 200433, China.

[3] Department of Cardiology, Shanghai Institute of Cardiovascular Disease, Zhongshan Hospital, Fudan University, 1609 Xietu Road, Shanghai, 200032 China.

[4] Yiwu Research Institute of Fudan University, Shanghai, 200032 China.

[*] These authors contributed equally.

[†] Correspondences:

Yan Zheng, School of Life Sciences, Fudan University Room C601, No. 2005 Songhu Road, Yangpu District, Shanghai 200433, China; Email: yan_zheng@fudan.edu.cn

Longli Kang, Key Laboratory for Molecular Genetic Mechanisms and Intervention Research on High Altitude Disease of Tibet Autonomous Region, School of Medicine, Xizang Minzu University, No.006 Wenhui East Road, Xianyang, Shannxi, 712082, China; Email: longli_kang@163.com

Short title

Pulmonary Hypertension and Gut Microbiota

Abstract

Background: Alterations in the gut microbiota have been observed in patients with pulmonary hypertension (PH), though whether the roles of the gut microbiota in PH at different altitudes are the same is unknown. This study aims to evaluate the associations of the gut microbiome with PH in the highlanders and lowlanders.

Methods: PH patients and controls were recruited from those who permanently live on the Tibetan plateau (highlanders) or the plains (lowlanders), and underwent transthoracic echocardiography close to their altitude of residence (at 5070m for highlanders *v.s.*6m for lowlanders). The gut microbiome was profiled using metagenomic shotgun sequencing.

Results: In total, 13 PH patients (46% highlanders) and 88 controls (70% highlanders) were included. The overall microbial composition was different in PH patients compared to controls (P=0.003). Notably, among lowlanders, a composite microbial score of pro-atherosclerotic trimethylamine-producing species was increased in PH patients compared with that in controls (P=0.028), while among highlanders no such difference was observed (P=0.087). Another composite gut microbial score including eight species of *Lactobacillus*, which has shown beneficial effects on cardiovascular functions, was higher in highlanders than lowlanders (P<0.01). Furthermore, this score tended to be lower in PH patients than controls among highlanders (P =0.056) but not among lowlanders (P=0.840). In addition, the gut microbiome showed a good performance in distinguishing PH patients from controls in both lowlanders and highlanders.

Conclusions: Our study reported differently altered gut microbiome profiles between highland and lowland PH patients, highlighting the distinct microbial mechanism in PH between highlanders and lowlanders.

Keyword: Pulmonary hypertension; Hypoxemia; Microbiota; Metagenomics; Living environment.

Introduction

Pulmonary hypertension (PH) is a group of malignant vascular disorders, and its pathogenesis is not well delineated [1, 2]. Recent evidence has indicated the potential role of microbial ecology in PH development [3]. Proposed mechanisms include the gut microbial-involved production of cardiovascular-related metabolites such as trimethylamine oxide (TMAO) and lactate [4], the modulation effects of certain bacterial species (e.g., *Lactobacillus*) on endothelium-dependent tissue oxygenation and eventually the remodeling of the vascular system [5]. Various studies revealed that the altered gut microbial composition and specific bacterial taxonomies (e.g., *Bifidobacterium*, *Streptococcus*, and *Acidaminococcus spp.*) were associated with hypertension and might drive pathological abnormalities in the cardiovascular system [6–9]. To our knowledge, there are limited studies reported gut dysbiosis and microbial metabolites in PH patients thus far [10].

It is known that the specific pathogenic mechanisms differ to some degree between highland and lowland PH. For example, the hypoxic stimuli induced pulmonary arterial vasoconstriction and vascular remodeling greatly contributed to the inception and progression of PH in highlanders, but this may not be the case in lowlanders [1]. Besides, different overall gut composition and relative abundances of specific bacteria groups (i.e., *Firmicutes* and *Bacteroidetes*) were observed between highlanders and lowlanders [11, 12]. However, little is known about whether the associations of the gut microbiome with PH are different between highlanders and lowlanders.

In the current study, we characterized the gut metagenome profiles of PH patients and controls enrolled from the Tibetan plateau and the plain areas. We hypothesized that distinct gut microbiome profiles are related to the different PH pathogenesis between highlanders and lowlanders.

METHODS

Study Participants

This study enrolled PH patients and controls among highlanders living on the Tibetan plateau range and lowlanders living on the plains (Shanghai) from June 2018 to June 2019. Highlanders were defined as those born, raised, and living on the Tibetan plateau range. Lowlanders were

defined as those born, raised, and living in Shanghai. Participants were excluded if they 1) self-reported use of antihypertension medication, pulmonary vasodilator therapies, probiotics, or antibiotics in the past month, 2) suffered from general bowel diseases (such as diarrhea, inflammatory bowel disease, irritable bowel syndrome, bowel removal) and overt systemic diseases (such as diabetes, chronic kidney disease, coronary artery disease, cancers) as determined by medical records and physical examination by clinicians, or 3) had PH secondary to left-sided cardiac disease, PH secondary to lung diseases, chronic thromboembolic pulmonary hypertension, and other relevant etiologies in appropriate clinical scenarios (such as portopulmonary hypertension and PH secondary to sickle cell disease) [1]. Eventually, 101 participants (68 highlanders and 33 lowlanders) were included in the current analysis.

All participants provided their written informed consent to study procedures conforming to the latest revision of the Declaration of Helsinki standards. The ethical committee of Xizang Minzu University and Zhongshan Hospital Fudan University approved this study.

Outcomes and Covariates Assessment

Pulmonary artery pressure was measured by echocardiography (GE VIVID Q) at a local clinic at an altitude of 5070 m for highlanders and at Zhongshan Hospital at an altitude of 6 m for lowlanders. PH patients were defined as participants with a mean pulmonary artery pressure >30 mmHg or a systolic pulmonary artery pressure >50 mmHg [13]. Because pure tricuspid regurgitation (TR) or right atrial enlargement (RAE) represents a physiological adaptation rather than organic impaired pulmonary circulation [14], controls were further classified into 1) TR/RAE controls if they had TR or RAE identified by echocardiography, and 2) normal controls if they did not have TR and RAE in the secondary analysis.

Data on participant characteristics, including sex, age, ever smoking or drinking status (yes/no), medication use, and medical history, were obtained from medical records and face-to-face interviews. Body mass index was defined as the weight (kilograms) divided by the square of the body height (meters).

Gut Microbiome Profiling

Stool samples were collected using sterile stool collection kits containing storage solutions during the clinic visit. Stool samples were frozen at –80°C after collection within 24 h until further processing in the central lab. Genomic DNA was extracted using the QIAamp DNA

Micro Kit (QIAGEN, Germany), and then quantified with Qubit 3.0 (Thermo Fisher Scientific, USA) and Qubit dsDNA HS Assay Kit (Thermo Fisher Scientific, USA) according to the manufacturer's guidelines. The Illumina sequencing libraries were prepared from isolated DNA and normalized to 1-ng input per sample. Metagenomic shotgun sequencing of fecal samples was carried out on the NovaSeq 6000 platform for paired-end 150-bp reads (Illumina, USA). The data size per sample of raw sequence data was ~12.9 Gbp with up to 85.9 million reads.

All sequenced metagenomes were quality controlled using KneadData v0.7.4 and were mapped to remove host-derived and rDNA sequences using Bowtie2 v2.4.4 against Genome Reference Consortium Human Build 37 (GRCh37) and SILVA database (version 128) [15]. Adapter sequences and low-quality bases were trimmed by Trimmomatic (version 0.39, with parameters ILLUMINACLIP: NexteraPE-PE.fa:2:30:10:8:TRUE SLIDINGWINDOW:4:20 MINLEN:75). The quality of processed metagenomes was examined using the FastQC toolkit v0.11.9. Taxonomic profiles of the relative abundances of all bacteria were quantified by MetaPhlan 3.0.13 [16], and a total of 287 microbial species (represented by 112 genera and by nine phyla) presented in more than 10% of total samples with a relative abundance of more than 0.01% were included in our subsequent analysis. The profiling and quantification of TMA-synthesis enzymes and their functional genes were determined by HUMAnN v3.0.0 [16] using the MetaCyc database, UniRef orthologous gene family catalog, and Enzyme Commission gene families, and only those presented in more than 10% of samples were used in the downstream analysis.

Statistical Analysis

Differences in the basic characteristics between PH patients and controls were analyzed using Wilcoxon rank-sum tests (for continuous traits) or $\chi 2$ tests (for categorical traits). Based on the relative abundance of microbial species, the microbial species richness of a participant (α-diversity) was computed using the Shannon index, and microbiome dissimilarity (β-diversity) was assessed by principal coordinate analysis (PCoA) using the Bray-Curtis dissimilarity matrix. The differences in the α-diversity and the relative abundance of taxonomic and functional features were assessed by Wilcoxon rank-sum test for comparison between two groups and by the Kruskal-Wallis test for comparisons among more than two groups. The significance of

difference in β-diversity was examined between groups by permutational multivariate analysis of variance (PERMANOVA) with 999 permutations.

In consideration of the previously reported role of TMA-producing bacteria in PH development [10], a gut microbial (GMB)-TMA score was calculated based on the presence of 23 species with functional genes encoding TMA-synthesis enzymes detected in the current study (ranged 0-23; not present in participants=0, and present in participants=1). In addition, considering the prior knowledge on the beneficial effects of the probiotic *Lactobacillus* on cardiovascular health and preventing PH [17, 18], a GMB-*Lactobacillus* score was calculated based on the detectable presence of eight *Lactobacillus* species (i.e., *Lactobacillus mucosae*, *Lactobacillus rogosae*, *Lactobacillus ruminis*, *Lactobacillus salivarius*, *Lactobacillus kefiranofaciens*, *Lactobacillus kefiri*, *Lactobacillus oris*, and *Lactobacillus sanfranciscensis*) in the current study (ranged 0-8; absent in participants=0, present in participants=1).

Random forest-based models of 287 microbial species were constructed to classify PH patients and controls in highlanders or lowlanders separately and validated by 10-fold cross-validation testing (999 trees, balanced class weight, max features=square root of all features) using the R package randomForest v4.6-14 (https://www.stat.berkeley.edu/~breiman/RandomForests/). The set of predictable microbial species with the minimum cross-validation error and the smallest number of species was selected as the optimal set. For each test, the receiver operating characteristic (ROC) curve was constructed to evaluate the performance of the constructed models, and the area under the parametric curve (AUC) was computed using the R package pROC v1.18.0. All statistical tests were 2-sided and an alpha value of 0.05 was considered to indicate statistical significance. All the data analyses were conducted in R v4.1.1 (https://www.R-project.org/).

Results

Basic Characteristics of the study population

Among the 101 participants (mean age, 43.4±14.6 years; 50.5% were women) included in the final analysis, 13 (12.9%) were PH patients including six highlanders and seven lowlanders, and the other 88 participants were controls including 45 TR/RAE controls (97.8% were highlanders) and 43 normal controls (41.9% were highlanders). Among lowlanders, PH patients were more likely to be systemic hypertensive compared with controls (P=0.028), while other characteristics

showed no significant difference between PH patients and controls in both highlanders and lowlanders (P>0.05) (Table 1).

The gut microbial composition was associated with PH status in both lowlanders and highlanders

The α-diversity measured by the Shannon index was similar between PH patients and controls (P=0.730, Figure 1A), or among PH patients, TR/RAE controls, and normal controls (P=0.580, Figure S1A). However, a distinct microbial composition was observed in PH patients compared with controls (P=0.003, PERMANOVA, Figure 1B), and among PH patients, TR/RAE controls and normal controls (P=0.001, Figure S1B).

Lowland PH patients tended to have a lower α-diversity than either lowland controls (P=0.009, Figure 1C) or highland PH patients (P=0.001, Figure 1C). No significant difference was observed between PH patients and controls in highlanders (P=0.082, Figure 1C), or among PH patients, TR/RAE controls, and normal controls in highlanders (P=0.920 [H.Normal v.s. H.TR/RAE], P=0.090 [H.Normal v.s. H.PH], P=0.100 [H.TR/RAE v.s. H.PH], Figure S1C). The gut microbial composition tented to be different among PH patients and controls among the lowlanders (P=0.050, PERMANOVA, Figure 1D) but not among the highlanders (P=0.252, PERMANOVA, Figure 1D). At the phylum level, Bacteroidetes and Firmicutes were the most abundant phyla in our population (Figures 1E and S1D), and at the genus level, *Prevotella* was dominantly enriched in highlanders whereas *Bacteroide* was the most prevalent genus in lowlanders (Figures 1F and S1E).

TMA-related Species Were More Enriched in Lowlanders with PH

Among the analyzed microbial species, the relative abundances of 20 bacterial species (e.g., *Clostridium symbiosum*, *Bacteroides massiliensis*, *Clostridium saccharolyticum*, *Gordonibacter pamelaeae*, and *Eisenbergiella massiliensis*) were increased in PH patients, and that of four species (*Allisonella histaminiformans*, *Sellimonas intestinalis*, *Absiella dolichum*, and *Bacteroides coprocola*) were increased in controls (P<0.01, Figure 2A). Among these differential species, five species (i.e., *Citrobacter portucalensis*, *Clostridium aldenense*, *Clostridium bolteae*, *Citrobacter youngae*, and *Cl. saccharolyticum*) carried functional genes encoding TMA-synthesis enzymes (i.e., carnitine monooxygenase gene [*cntA*], choline TMA-lyase gene [*cutC*], and betaine reductase complex component B subunit beta gene [*grdH*]). Furthermore,

among all the analyzed species in the current study, 23 species captured homologous genes encoding enzymes producing TMA, including six species that contained *cntA* and *cutC* (i.e., *Escherichia coli*, *Klebsiella pneumoniae*, *Citrobacter freundii*, *Enterobacter cloacae complex*, *Klebsiella quasipneumoniae*, and *Klebsiella variicola*), and five species contained *cutC* and *grdH* (i.e., *Hungatella hathewayi*, *Dorea longicatena*, *Cl. aldenense*, *Clostridium citroniae*, and *Clostridium clostridioforme*) (Figure 2B).

Based on these 23 species containing genes encoding TMA-producing enzymes, we calculated a GMB-TMA score and assessed its association with PH. Although there was no difference in the distribution of GMB-TMA scores between PH patients and all controls (P=0.330, Figure 3A and P=0.520 [Normal v.s. PH], P=0.240 [TR/RAE v.s. PH], Figure S2A), among lowlanders, PH patients were more likely to have a higher GMB-TMA score compared with controls (11.14±3.58 in PH patients *v.s.* 8.12±2.03 in controls, P=0.022, Figure 3B). Among highlanders, no significant discrepancy was observed between PH patients and all controls (P=0.057, Figures 3B and P=0.110 [H.Normal v.s. H.PH], P=0.058 [H.TR/RAE v.s. H.PH], Figure S2B). Consistently, the genes encoding the TMA-synthesis enzymes were more likely to cluster in PH patients than controls (P<0.001, Figure 3C and P=0.210 [Normal v.s. TR/RAE], P<0.001 [Normal v.s. PH], P<0.001 [TR/RAE v.s. PH], Figure S2C), and lowland PH patients tended to have a higher relative abundance of these genes compared with lowland controls (P<0.001, Figure 3D). In highlanders, the relative abundance of these genes was similar between PH patients and all controls (P=0.790, Figure 3D and P=0.520 [H.Normal v.s. H.PH], P=0.940 [H.TR/RAE v.s. H.PH], Figure S2D).

***Lactobacillus* Was a Potential Microbial Marker of PH among Highlanders**

Out of the 26 differential gut microbial genera in highland and lowland normal controls, three genera (i.e., *Lactobacillus*, *Lactococcus*, and *Leuconostoc*) involved in the production of lactate, which regulates prolonged hypoxia responses in cardiovascular disorders [19, 20], were more abundant in highland normal controls than in lowland normal controls (P<0.01, Figure 4A). At the species level, most detectable species (*i.e.*, *L. mucosae*, *L. rogosae*, *L. ruminis*, *L. salivarius*, *L. kefiranofaciens*, *L. kefiri*, and *L. sanfranciscensis*) belonging to *Lactobacillus* genus were more abundant in highland normal controls than that in lowland normal controls (all P<0.05 expect for *L. sanfranciscensis*, Figure S3).

Lactobacillus has been demonstrated to reduce blood pressure and be beneficial in cardiovascular functions via the modulation of immune-inflammatory and oxidative stress response [17]. Thus, we calculated a GMB-Lactobacillus score at the species level based on eight detectable species belonging to the *Lactobacillus* genus, including the revealed seven species and *L. oris*. The GMB-*Lactobacillus* score was comparable between PH patients and controls in either the combined population (*P*=0.066, Figure 4B), the highlanders only (*P*=0.056, Figure 4C), or the lowlanders only (*P*=0.800, Figure 4C). However, notable discrepancies in the GMB-*Lactobacillus* score between lowlanders and highlanders were observed (0.36±0.65 in lowlanders *v.s.* 2.54±1.32 in highlanders, *P*<0.001, Figure 4D). In subgroup analysis, highland PH patients had a higher GMB-*Lactobacillus* score compared with lowland patients (1.83±0.75 in highland PH patients *v.s.* 0.43±0.79 in lowland PH patients, *P*=0.007, Figure 4C), and consistent findings were observed in controls (2.61±1.35 in highland controls *v.s.* 0.35±0.63 in lowland controls, *P*<0.001, Figure 4C). In addition, among highlanders, TR/RAE controls presented a comparable distribution of GMB-*Lactobacillus* score to normal controls (*P*=0.660), and both control groups presented a suggestive higher GMB-*Lactobacillus* score than PH patients (*P for difference in normal controls and PH patients*=0.050, and *P for difference in TR/RAE controls and PH patients*=0.076, Figure S4).

Gut Microbial Signatures Could Distinguish PH Patients from Normal Controls

With the above-observed differences in gut microbial features between lowlanders and highlanders, we explored the potential of gut microbiota markers for the classification of PH patients in lowlanders and highlanders separately. Because there was only one lowland TR/RAE control in our study (Table 1), we only included normal controls and PH patients to make random forest modeling in highlanders and lowlanders comparable. A model among lowlanders including 25 microbial species achieved an accuracy of 93.8% (Figure 5A), and a model among highlanders of 15 species presented a performance of the accuracy of 85.7% (Figure 5B). Of note, among the 25 microbial features in the lowland model, five species, i.e., *Flavonifractor plautii*, *H. hathewayi*, *Cl. bolteae*, *K. quasipneumoniae*, and *D. longicatena*, carried functional genes encoding TMA-synthesis enzymes, and the first three species exhibited higher relative abundances in PH patients compared with controls (all *P*<0.05, Figure S5). Among these 15 species in the highland model, the top three predictable species for PH patients, i.e., *Roseburia sp*

CAG:182, *Eubacterium sp CAG:38*, and *Roseburia sp CAG:471*, were more abundant in PH patients compared with controls (all $P<0.05$, Figure S6).

Discussion

In this study of the gut metagenome in PH involving highlanders and lowlanders, we evaluated their gut microbiota profiles associated with PH pathogenesis. We observed distinct gut microbial composition in PH patients compared to controls. In addition, compared with controls, lowland PH patients carried higher abundances of bacterial species involved in the production of TMA, while highland PH patients tended to carry a decreased abundance of *Lactobacillus*. These divergent patterns of gut microbiota suggested the distinct potential microbial etiology in PH between highlanders and lowlanders.

PH is a complex disease involving abnormal alterations in lung, brain, and multiple other organs [21]. Data on population studies of the gut microbiota with PH are limited. Consistent with the results from the only two studies of shotgun metagenomics in PH [10, 22], we observed that the altered gut microbiota composition was associated with PH. In the current study, the further disparity of the gut microbial profile among PH patients, TR/RAE controls, and normal controls suggested that the gut microbial communities possibly changed in physiological adaptations of chronically hypoxic stimulus. Moreover, we observed a lower richness of gut microbiota in lowland PH patients than in highland PH patients, which might be partially attributed to the distinct gut microbiota composition in individuals with different altitude residences [23]. In line with the previous observations [11], one of the dominant phyla of microbiota was Prevotella in the plateau-living Tibetan individuals, and the plain-living residents exhibited high enrichment of microbes from the phylum Bacteroidetes in this study.

TMA is synthesized by gut microbes and then in liver is converted to TMAO, which promotes vascular inflammation and oxidative stress [24]. Previous studies have revealed that a higher circulating level of TMAO is a risk factor for various cardiovascular events such as hypertension, heart failure, and atherosclerosis [25-27]. Alterations in the bacterial communities associated with TMAO metabolism have been linked to the progression of PH among lowlanders [18, 19]. For example, TMA/TMAO-producing bacteria (e.g., *Clostridium*, *Citrobacter*, and *Desulfovibrio*) were enriched in PH patients [10], and circulating TMAO was related with severe PH progression [28]. In the current study, bacteria species with homologous genes encoding

enzymes involved in the production of TMA were enriched in PH patients compared with controls among lowlanders, but not among highlanders. This is consistent with previous observation that no significant difference in the abundance of TMA-producing bacteria and TMAO circulating levels between high-land patients with coronary heart disease and their healthy counterparts [29]. In view of this, we suggest that the microbiota-dependent TMAO mechanism might be more involved in the development of PH in lowlanders, but not among highlanders.

Chronic hypoxic exposure gradually induces pulmonary artery pressure elevation, the structural remodeling of pulmonary vessels, and persistent vasoconstriction for permanent high altitude populations [30]. The hypoxic environment can accelerate the growth of strict anaerobes such as *Lactobacillus* due to the limited oxygen concentration in the intestinal microenvironment [31, 32]. This might explain that highlanders intrinsically carried the more abundant gut *Lactobacillus* than lowlanders in the current study. In hypoxia rat models, the relative abundance of *Lactobacillus* was lower in the PH group than in the control group [33]. The abundance of this bacteria was not obviously different between the combined PH patients and controls in our study, but showed a greater disparity between PH patients and controls among highlanders. Recent sutdies have reported the role of *Lactobacillus* supplementation in maintaining healthy cardiovascular functions, partially through the hypoxia-induced *Lactobacillus*-derived metabolites (e.g., lactate) to reduce inflammation and oxidant damage [4, 17]. In addition, in the Yorkshire swine fed with *Lactobacillus plantarum*, a decreased expression was observed in the ischemic myocardial tissue of hypoxia-inducible factor 1α, a crucial regulator of the proliferative and inflammatory responses to hypoxia [34], and an increased expression was found of endothelial nitric oxide synthase [5], which promotes the production of nitric oxide, a potent vasodilator of the pulmonary circulation [35]. Collectively, these observations support *Lactobacillus* is a potential microbial marker of PH in highlanders, while its mechanism remains to be further clarified.

This study design is unique in that the highlanders were recruited from those who permanently lived on the Tibetan plateau at an altitude of 5070 m, the highest altitude reported in relevant studies, allowing us to reveal the distinct potential microbial etiologies in PH in different altitude environments. Our study has several limitations. First, PH patients in this study were

defined by transthoracic echocardiography, not the gold standard, i.e., right heart catheterization, which is invasive and not available in local highland clinics. As such, PH patients due to heart failure with preserved ejection fraction might be included in the PH patients, especially among an old population with a high prevalence of hypertension. Second, we cannot refer to the causal inference in the alterations in gut microbiota and PH in this observational study. Third, though, to the best of our knowledge, it is thus far the largest study of the gut metagenomes in PH with different altitude residences, our sample size might be too small to reveal the influences of the demographic factors (e.g., sex, age, and body mass index) on the differences in PH gut microbiome pathogenesis and could not support to perform multiple comparisons. Meanwhile, the ROC analysis with only a few cases tended to be overtrained, and therefore affected its real performance in predicting PH. Future population studies with large sample sizes as well as mechanism experiments are warranted to validate our findings.

In summary, this study has reported the altered gut microbiota in PH patients using whole-genome shotgun sequencing data from participants in different altitude living environments. Our findings imply the gut microbial species related to TMA may participate in PH development among lowlanders, and gut microbial *Lactobacillus is* involved in the etiology of PH among highlanders. These potentially microbiota-dependent mechanisms remain to be further elucidated. Our findings may provide gut microbial targets for the early prevention of PH in lowlanders and highlanders.

Acknowledgments

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Affiliations

Key Laboratory for Molecular Genetic Mechanisms and Intervention Research on High Altitude Disease of Tibet Autonomous Region, School of Medicine, Xizang Minzu University, Shannxi,

China (L.L. K., W.X. D.， L.F. M., X. Y.， Z.Y. Z., J. L.). State Key Laboratory of Genetic Engineering, School of Life Sciences and Human Phenome Institute, Fudan University, Shanghai, China (Y. Z., Q.M. H, Z.H. S., Z.D. M., M.M. K., J.J. Z.). Department of Cardiology, Shanghai Institute of Cardiovascular Disease, ZhongShan Hospital, Shanghai, China (Y. Z., D.D. C., D.X. Z., Y.X. D.).

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Disclosures

None.

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Table 1. Basic Characteristics of the Participants

Variables	PH (N=13)	Controls (N=88)		*P* value
		Normal (N=43)	TR/RAE (N=45)	
Highlanders	6	18	44	
Male (%)	2(33.33)	7(38.88)	26(59.09)	0.228
Age (mean (SD))	42.67 (15.10)	39.00 (14.74)	41.82 (12.41)	0.719
BMI (mean (SD))	20.50 (2.21)	21.13 (5.79)	21.43 (3.83)	0.874
Smoking (%)	1 (16.67)	3 (16.67)	6 (13.64)	0.945
Drinking (%)	2 (33.33)	4 (22.22)	9 (20.45)	0.775
Systemic hypertension (%)	1 (16.67)	2 (11.11)	6 (13.64)	0.933
Lowlanders	7	25	1	
Male (%)	6 (85.70)	10 (40.00)	0 (0.00)	0.062
Age (mean (SD))	64.43 (10.11)	44.20 (15.28)	61.00 (-)	-
BMI (mean (SD))	24.98 (1.95)	21.89 (2.87)	22.86 (-)	-
Smoking (%)	3 (42.86)	2 (8.00)	0 (0.00)	0.069

Systemic hypertension (%)	5 (71.43)	6 (24.00)	1 (100.00)	0.028

-, denotes not applicable; TR, Tricuspid Regurgitation; RAE, Right Atrial Enlargement; PH, Pulmonary Hypertension; BMI, Body Mass Index. Statistical methods: Wilcoxon rank-sum tests for continuous traits and $\chi 2$ tests for categorical traits.

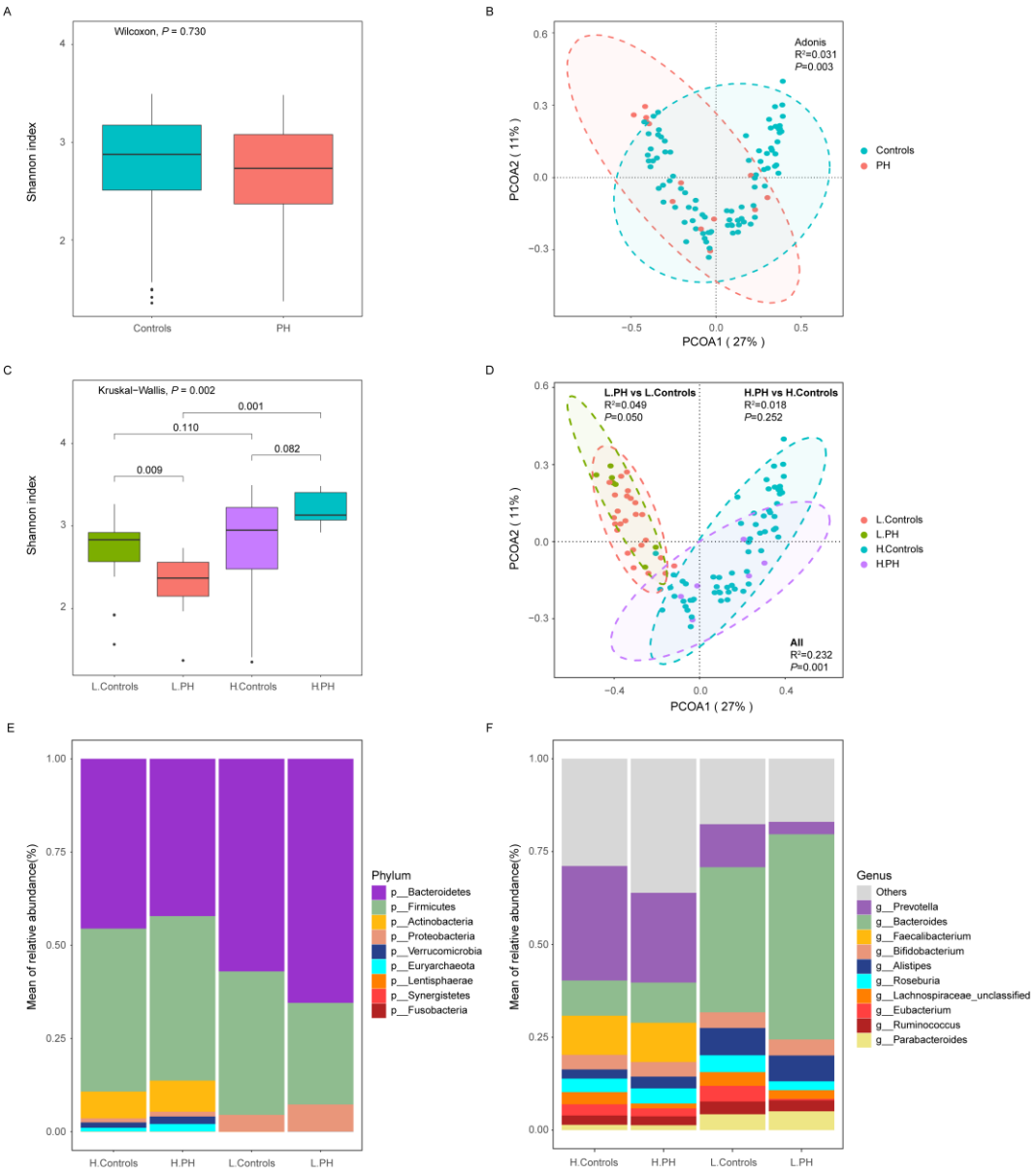
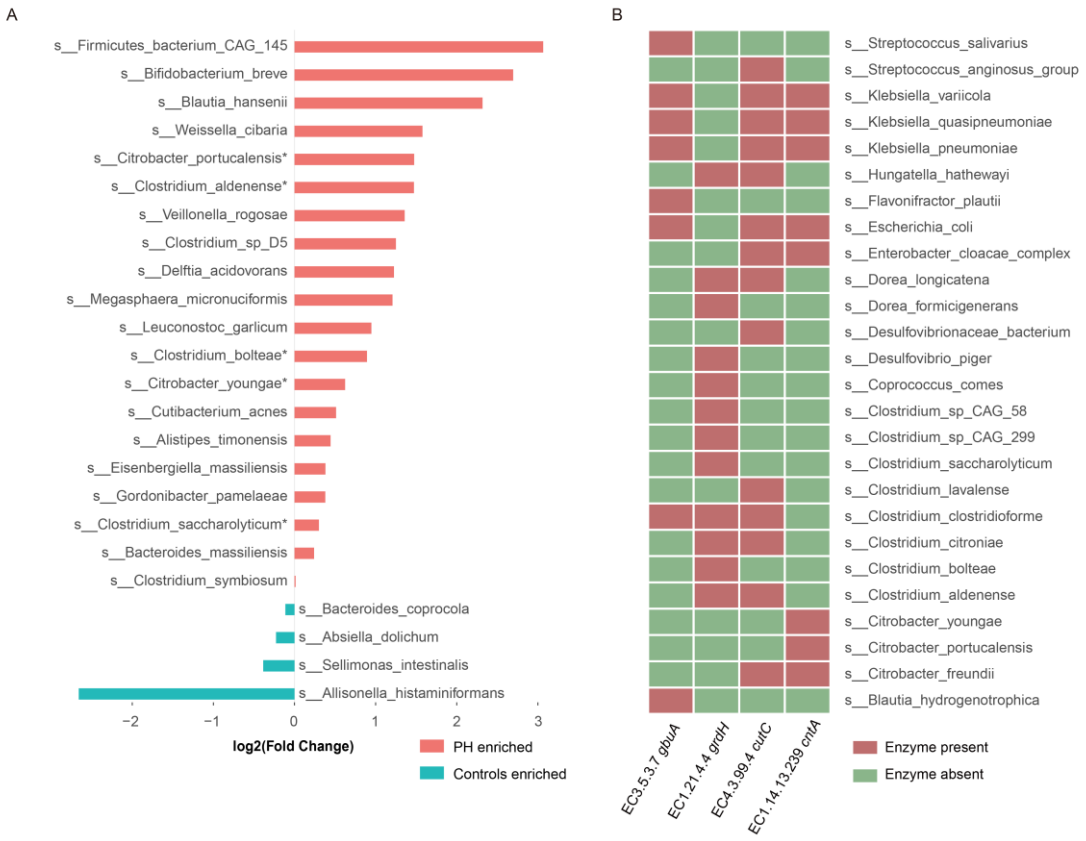


Figure 1. Altered gut microbiota composition in PH patients. A, The boxplots show the Shannon diversity of the intestinal microflora was comparable between PH patients and controls. **B**, The PCoA plots show a distinct Bray- Curtis distance of dissimilarity in the intestinal microbiota between PH patients and controls. **C**, The Shannon diversity of PH patients decreased compared with controls among lowlanders but not among highlanders. **D**, The PCoA plots show a distinct Bray- Curtis distance of dissimilarity in the intestinal microbiota between PH patients and controls in lowlanders and highlanders. **E** and **F**, The relative abundance of the intestinal microbiome was plotted in taxa bars based on the phylum level (**E**) and genus level (**F**). L, lowlanders; H, highlanders; PH, pulmonary



hypertension.

Figure 2. The gut microbiota of PH patients contained a higher abundance of TMA-producing microbiota. **A**, The bars showed species with different relative abundences between PH patients and controls ($P < 0.01$, Wilcoxon rank-sum test). The species carrying functional genes encoding TMA-synthesis enzymes were marked with a *black star*. **B**, The heat map showed the prevalence of TMA-produced enzymes in the genomes of the intestinal microbiome. PH, pulmonary hypertension.

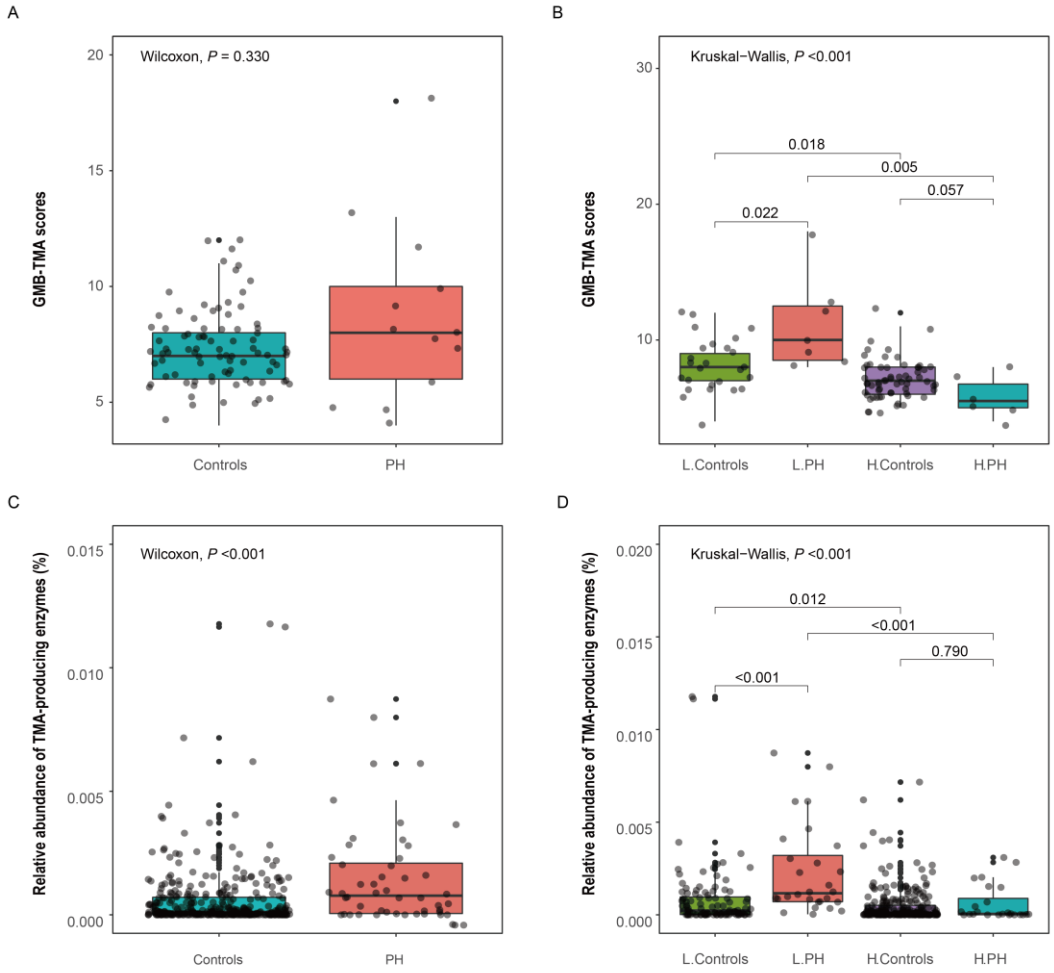


Figure 3. Comparison of TMA-producing microbiota in the gut microbiota between PH patients and controls in lowlanders and highlanders. A and **B**, The boxplots show the discrepancy of the GMB-TMA score between PH patients and controls (**A**), and between them in lowlanders or highlanders separately (**B**). **C** and **D**, The boxplots show the discrepancy of relative abundance of TMA-produced enzymes between PH patients and controls (**C**), and between them in lowlanders or highlanders separately (**D**). L, lowlanders; H, highlanders; PH, pulmonary hypertension.

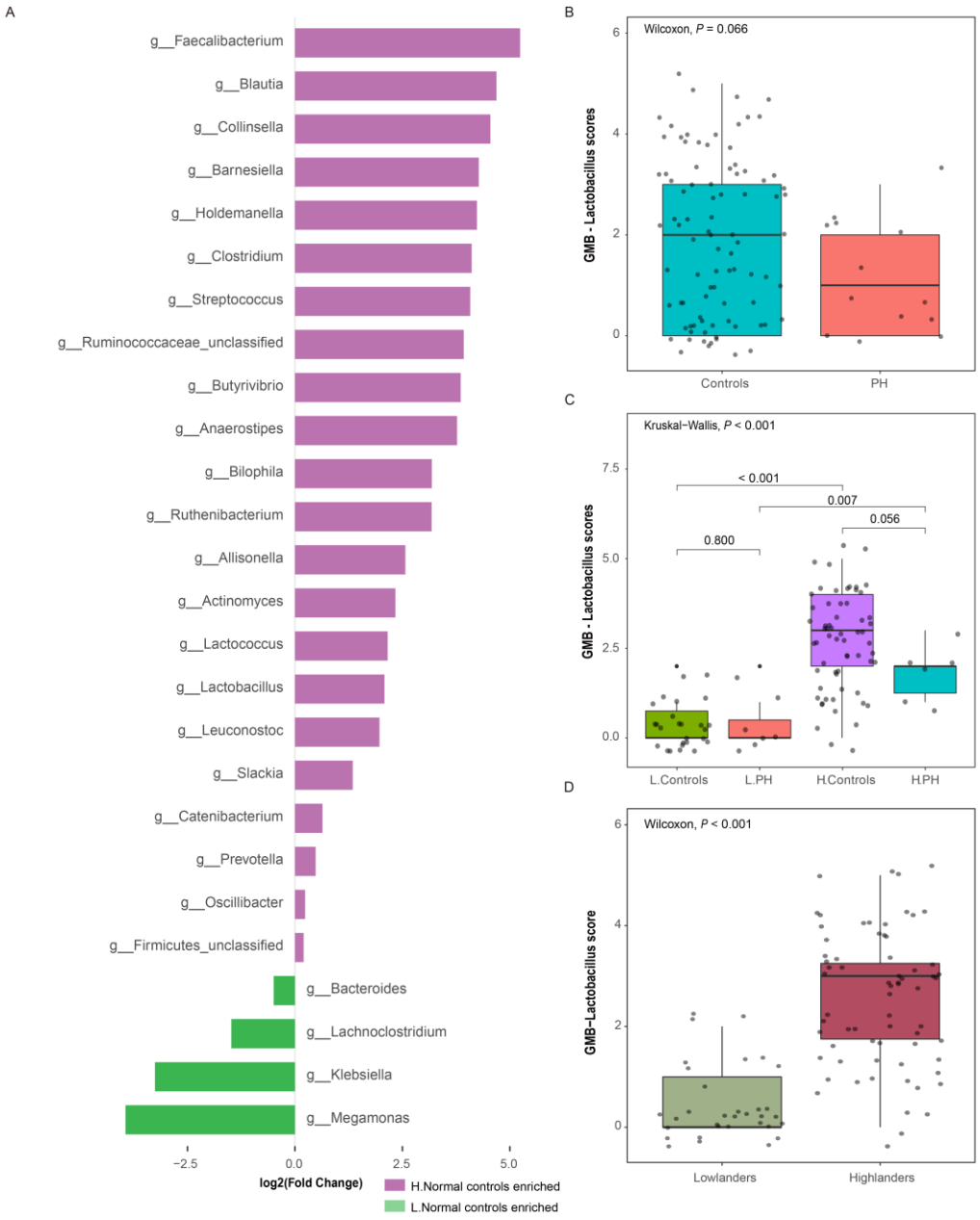


Figure 4. The GMB-Lactobacillus scores varied between highlanders and lowlanders. A, The bars represented genus with differential relative abundances between normal controls in highlanders (H.Normal controls) and normal controls in lowlanders (L.Normal controls) (P < 0.01, Wilcoxon rank-sum test). **B**, **C**, and **D**, The boxplots show the discrepancy of GMB-Lactobacillus score between PH patients and controls (**B**), between them in lowlanders and highlanders, separately (**C**), and between all lowlanders and all highlanders (**D**). L, lowlanders; H, highlanders; PH, pulmonary hypertension.

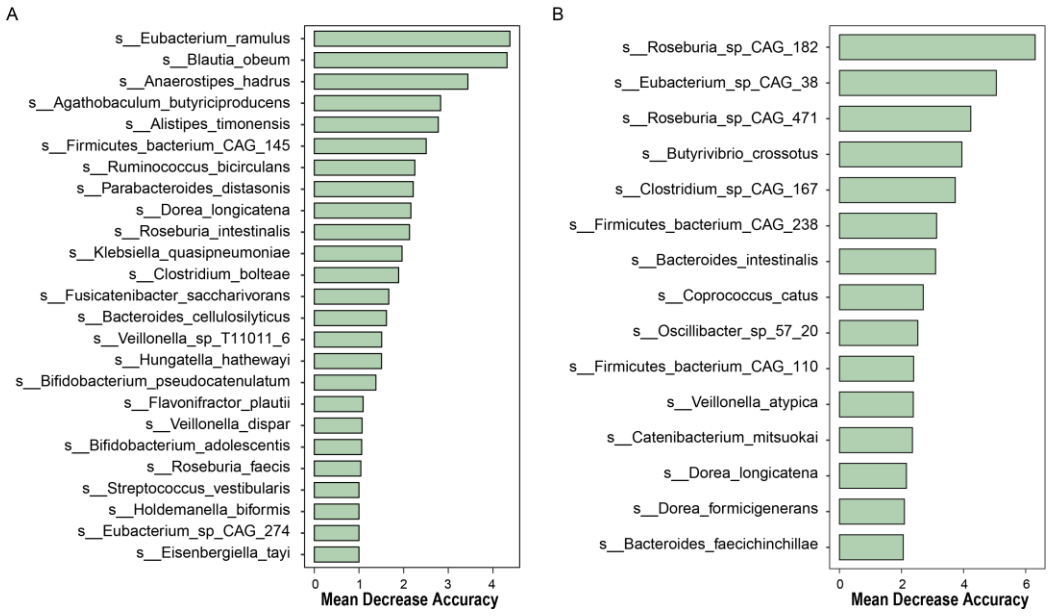


Figure 5. Random forest modeling identified a subset of taxa distinguishing PH patients from normal controls in lowlanders (A) or highlanders (B). The random forest algorithm was used to train the 25 and 15 most important species, in lowlanders or highlanders, to distinguish PH patients from normal controls. The species in the optimal set are ranked from top to bottom by decreasing mean decrease accuracy. Since mean decrease accuracy quantifies the strength of each respective predictor, the best predictors of PH are at the top of the plot.

Supplementary Data

Additional file 1: Figure S1. Distinct gut microbiota diversity among PH patients, TR/RAE controls, and normal controls. **Figure S2.** The relative abundances of TMA-producing gut microbiota were abundant in highland PH patients. **Figure S3.** The relative abundance of species belonging to *Lactobacillus* genus was compared between normal highland controls and normal lowland controls. **Figure S4.** The gut GMB-Lactobacillus score in highland PH patients was lower than that in normal controls. **Figure S5.** The relative abundance of species found in Random Forest modeling to predict PH was compared between PH patients and normal controls in lowlanders. **Figure S6.** The relative abundance of species found in Random Forest modeling to predict PH was compared between PH patients and normal controls in highlanders.

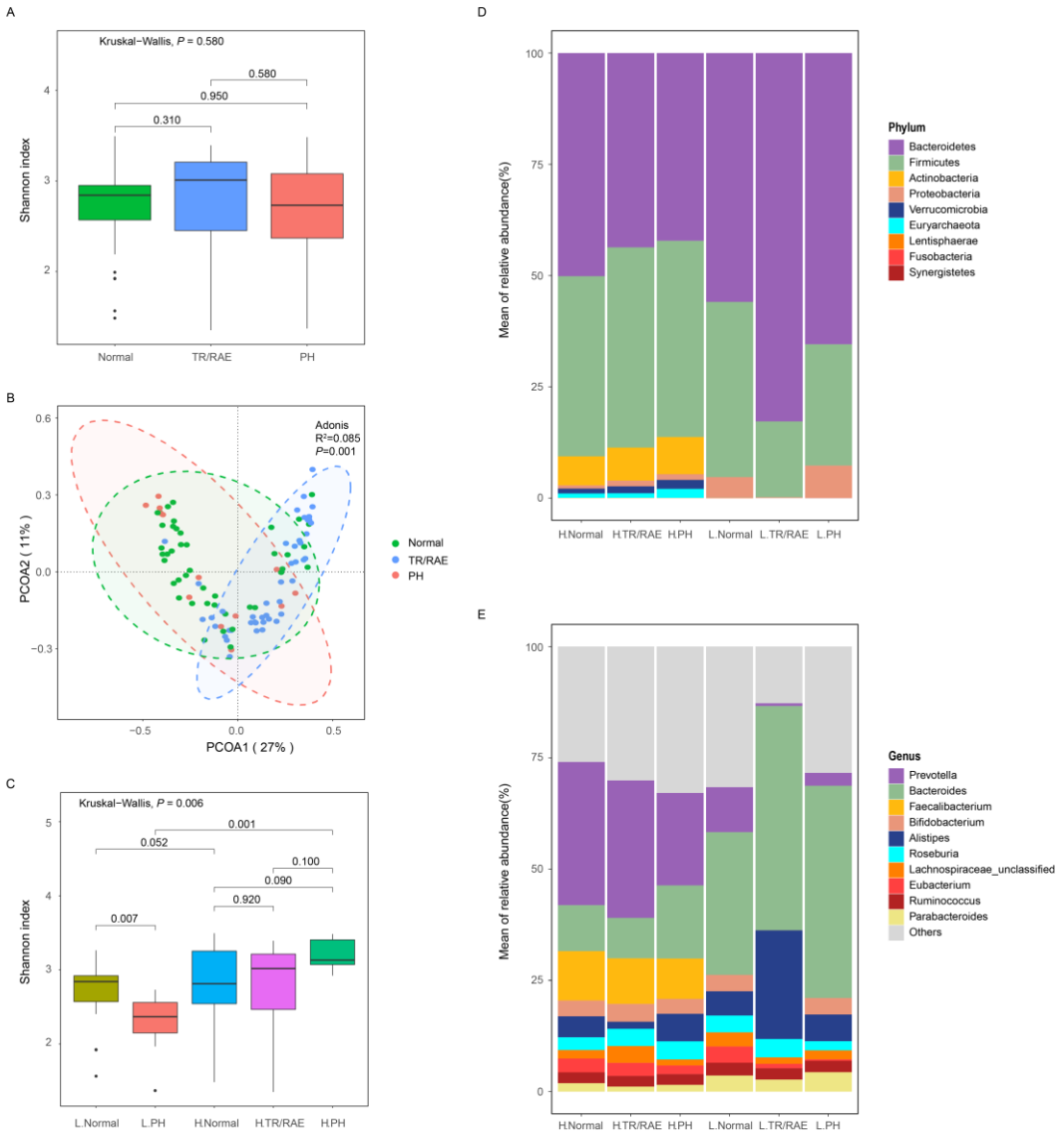


Figure S1. Distinct gut microbiota diversity among PH patients, TR/RAE controls, and normal controls. A, The boxplots show the Shannon diversity of the intestinal microflora among PH patients, TR/RAE controls, and normal controls. **B**, The PCoA plots show Bray- Curtis distance of dissimilarity in the intestinal microbiota among PH patients, TR/RAE controls, and normal controls. **C**, The boxplots show the Shannon diversity analysis of the intestinal microflora among PH patients, TR/RAE controls, and normal controls in highlanders or lowlanders separately. **D** and **E**, The relative abundance of the intestinal microbiome of PH patients, TR/RAE controls, and normal controls in lowlanders and highlanders was plotted in taxa bars based on the phylum level (**D**) and genus level (**E**). L, lowlanders; H, highlanders; PH, pulmonary hypertension; TR, tricuspid regurgitation; RAE, right atrial enlargement.

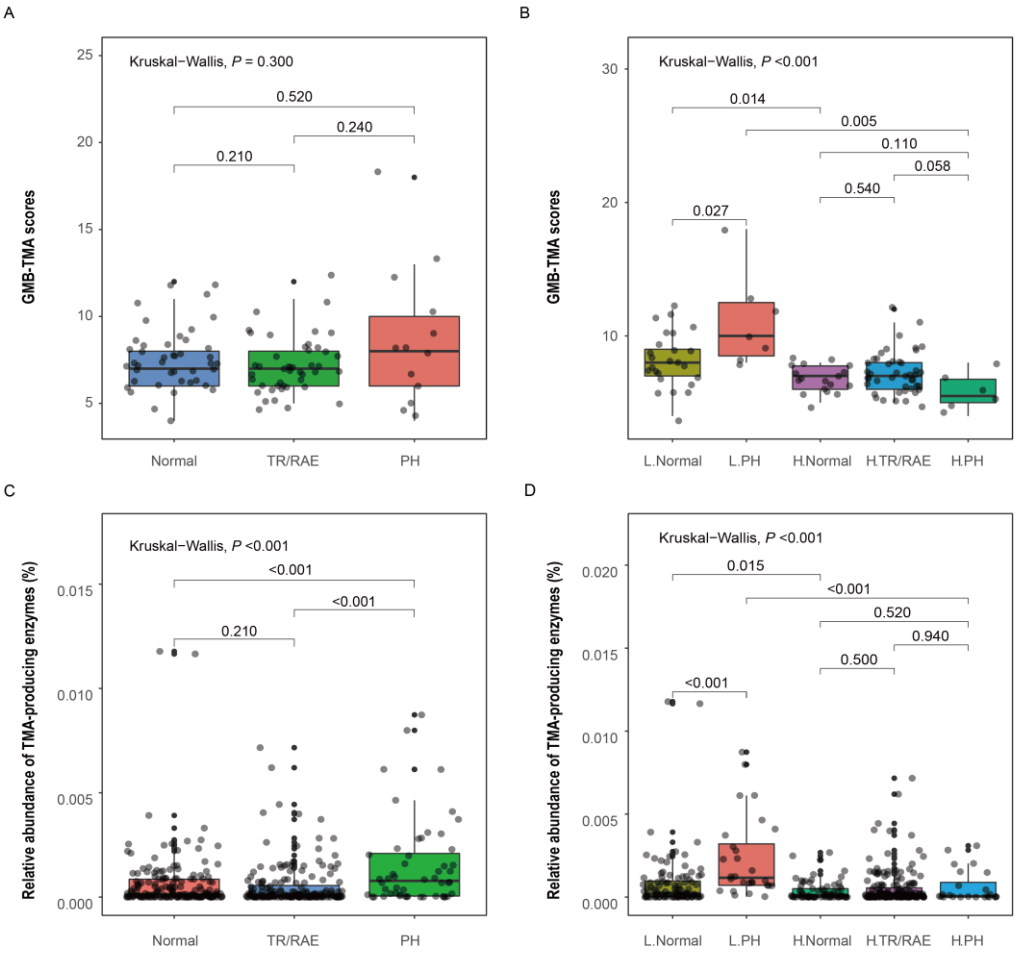


Figure S2. The relative abundances of TMA-producing gut microbiota were abundant in highland PH patients. A and **B**, The boxplots show the discrepancy of GMB-TMA score among PH patients, TR/RAE controls, and normal controls (**A**), and among them in lowlanders or highlanders separately (**B**). **C** and **D**, The boxplots show the discrepancy of relative abundance of TMA-produced enzymes among PH patients, TR/RAE controls, and normal controls (**C**), and among them in lowlanders or highlanders separately (**D**). L, lowlanders; H, highlanders; PH, pulmonary hypertension; TR, tricuspid regurgitation; RAE, right atrial enlargement.

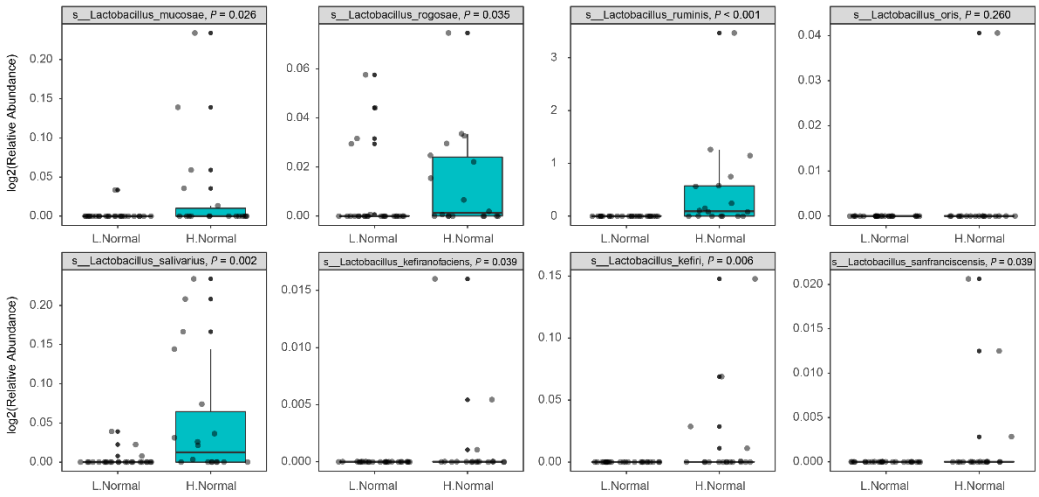


Figure S3. The relative abundance of species belonging to *Lactobacillus* genus was compared between normal highland controls and normal lowland controls. L, lowlanders; H, highlanders.

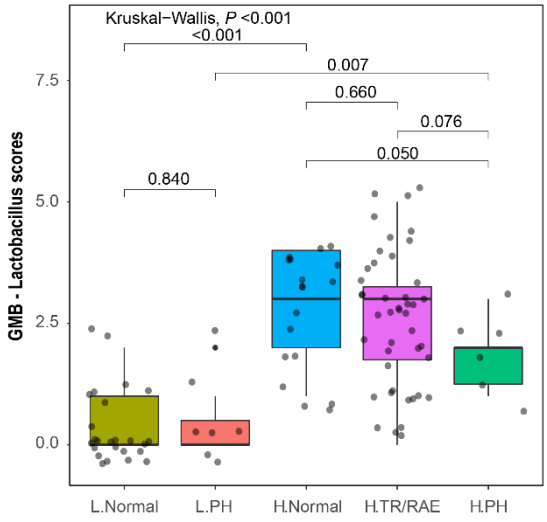


Figure S4. The gut GMB-Lactobacillus score in highland PH patients was lower than that in normal controls. The boxplots show the discrepancy of GMB-Lactobacillus scores among PH patients, TR/RAE controls, and normal controls in lowlanders or highlanders. L, lowlanders; H, highlanders; PH, pulmonary hypertension; TR, tricuspid regurgitation; RAE, right atrial enlargement.

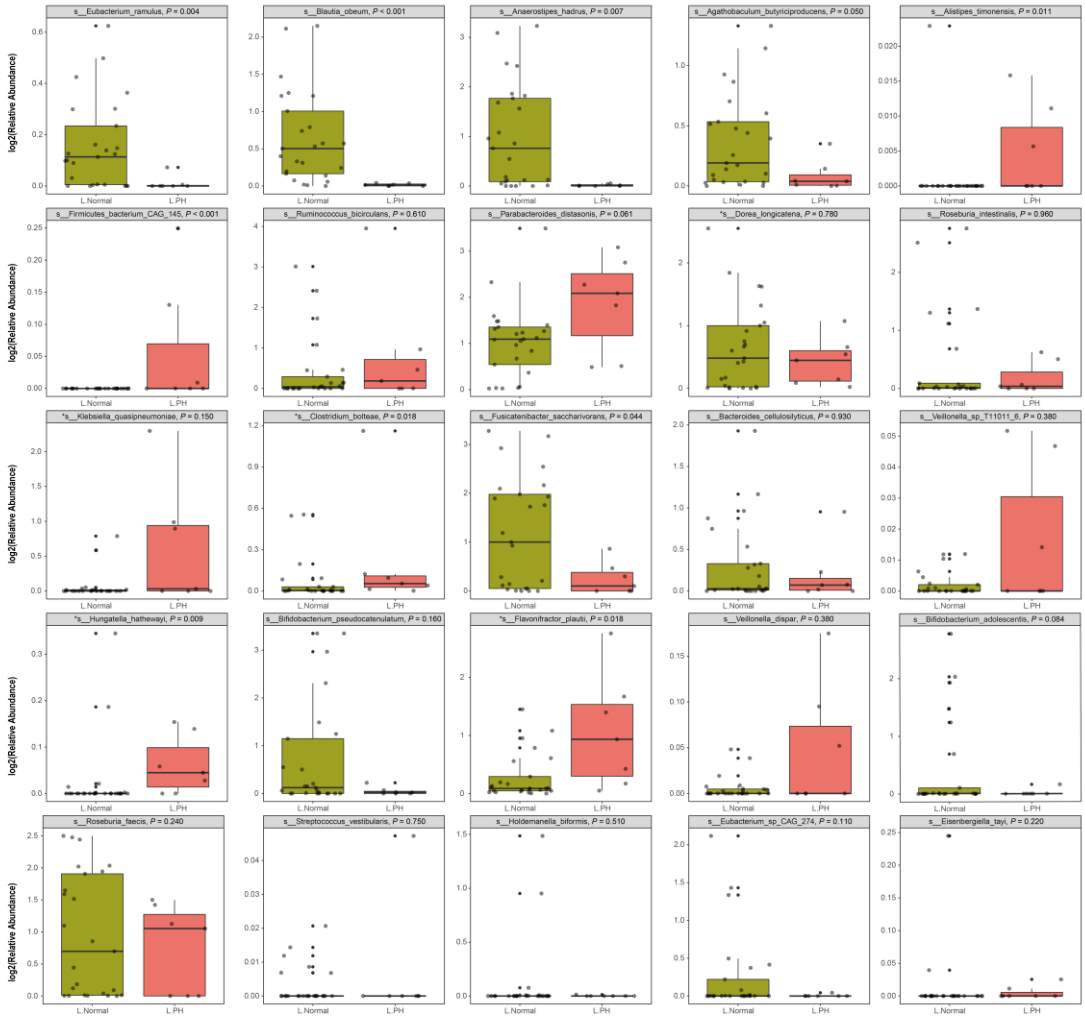


Figure S5. The relative abundance of species found in Random Forest modeling to predict PH was compared between PH patients and normal controls in lowlanders. The species carry functional genes encoding TMA-synthesis enzymes was marked with *black star*. L, lowlanders; H, highlanders; PH, pulmonary hypertension.

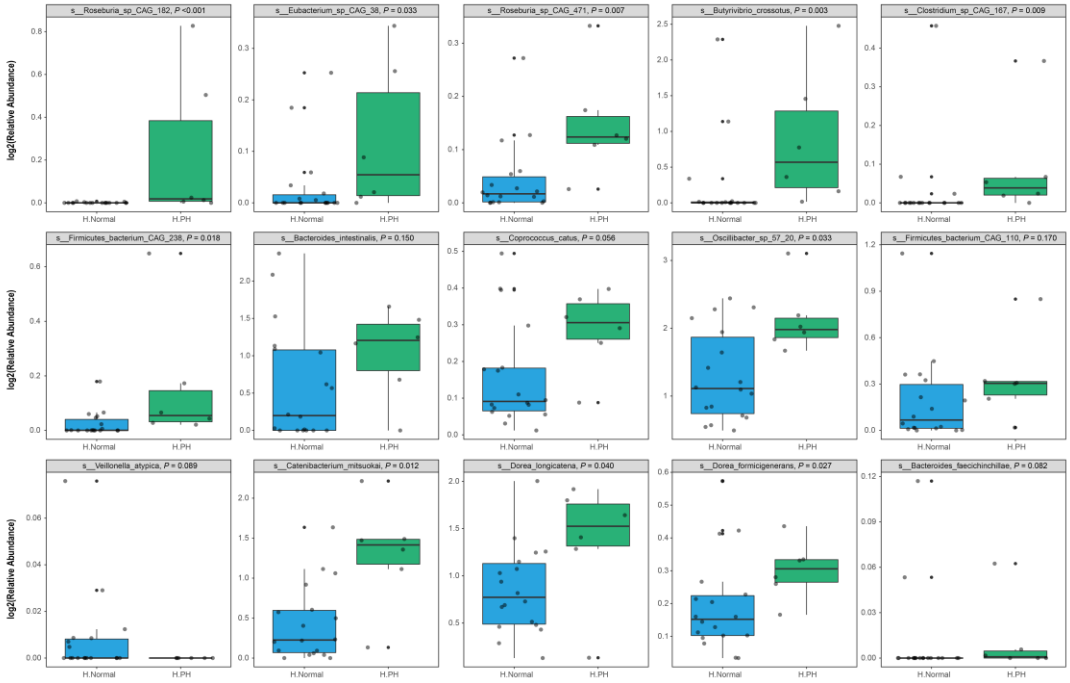


Figure S6. The relative abundance of species found in Random Forest modeling to predict PH was compared between PH patients and normal controls in highlanders. L, lowlanders; H, highlanders; PH, pulmonary hypertension.