Response to Reviewers' comments

Reviewer#1

1- The authors have not been well explained if the participants were clearly informed of all the study procedures before signing the consent form, and whether the subjects have completed the Study Questionnaires so as to provide personal details and information about health, diet, smoking activity and lifestyle at the time of sample collection.

A: Thanks for the reviewer's comments. Yes, all the participants were clearly informed of all the study procedures before signing the consent form, and the subjects have completed the Study Questionnaires to provide personal details and information about health, diet, smoking activity and lifestyle at the time of sample collection.

2- The authors have not been clarified whether this study was approved by the Ethics Committee of the University and what is the number of approvals.

A: Thanks for the reviewer's comments. The information has been provided in the title page in the original manuscript. This has also been added to the Materials and Methods section in the revision.

"The study was approved by the ethics committee of the sixth affiliated hospital of Kunming Medical University (approval no. 2023-kmykdx6f-66). All participants provided written informed consent."

3- There are many QC tests that can be used to evaluate performance, precision, and accuracy throughout the study.

A: We agree with the reviewer's comments that we need to evaluate performance, precision, and accuracy throughout the study. First, the quantity and quality of extracted DNAs were measured using a NanoDrop NC2000 spectrophotometer and agarose gel electrophoresis, respectively. Then, raw sequence data were demultiplexed using the demux plugin following by primers cutting with

cutadapt plugin. Sequences were also quality filtered, denoised, merged and chimera removed. Non-singleton amplicon sequence variants (ASVs) were aligned with mafft and used to construct a phylogeny with fasttree2.

4- The data seemed of good quality and had been validated using appropriate quality control methods. However, only very basic statistics were performed. More sophisticated techniques such as Analysis of Variance (ANOVA) and Principal Components Analysis (PCA) are required to elucidate the interactions in the data.

A: Thanks for the reviewer's comments. PCA and PERMANOVA were performed and the results have been added to the results section in the revision. We also performed principle coordiante analysis (PCoA) for the gut microbiota data and the results were shown in Figures 2 and 3.

5- The authors have not explained the effect of gender, therefore the effect of other factors such as gender, food, and smoking activity requires investigations.

A: We agree with the reviewer's comments that the effect of gender, food, and smoking activity need to be investigated. However, the sample size is relatively small and therefore, we did not perform the subgroup (e.g., male vs. female) comparison. This is a limitation of this study and this has been added to the revision.

6- I suggest the title of article: Comparative study of type 2 diabetes-associated gut microbiota between the Dai and Han populations

A: Thanks, the title has been changed to "Comparative study of type 2 diabetes-associated gut microbiota between the Dai and Han populations" according to the reviewer's suggestion.

Reviewer#2
SPECIFIC COMMENTS TO AUTHORS
The topic of manuscript is very interesting and obtained results are relevant.

However, authors should address the following points:

Isn't type 2 diabetes clinically characterized mainly by hyperglycemia? Please, clarify if the enrolled individuals with type 2 diabetes were newly diagnosed and drug-naïve.

A: According to the guideline for the prevention and treatment of type 2 diabetes mellitus in China (2020 edition), the type 2 diabetes were characterized by fasting blood glucose (FBG) ⩾ 7.0 mmol/L, or hemoglobin A1c (HbA1c) ⩾ 6.5%. The enrolled individuals with type 2 diabetes were newly diagnosed and drug-naïve. This information has been added to the revision.

Why adult control and type 2 diabetes individuals were not matched by age? If participants with type 2 diabetes were treated with antidiabetic drugs this should be reported as well, especially knowing these drugs may alter gut microbiota.

A: Thanks for the reviewer's comments. Although the mean age of adult control and type 2 diabetes individuals were not match, there's no significant difference between them. Also, we included healthy children and healthy adults to see the effect of age on gut microbiota.

Participants with type 2 diabetes were newly diagnosed and drug-naïve.

Discussion section consists mostly of a repetition of the obtained results; authors should put their results in the context of a previous knowledge in the studied area

Some of the references are duplicated in the Reference list (e.g. 26 and 32).

A: Thanks for the reviewer's comments. We have revised the Discussion section in the revision. The references have been updated in the revision.

Once the abbreviations are defined, they should be used consistently throughout the text.

A: Thanks, we have checked the abbreviations to make sure they were used consistently throughout the text.

The whole manuscript requires light language polishing

A: Thanks, the manuscript has been edited by a professional English language editing company, Filipodia publishing. The certificate has also been submitted along with the revised manuscript.

Table 1 is very busy and hard to follow; please, check the accuracy of data (e.g. total cholesterol among type 2 diabetes individuals in the Han population)

A: Thanks for the reviewer's comments. We have double checked the data and it is correct as one patient had abnormally high TC level (289 mmol/L).

Response to Reviewer #03490943 comments

Specific comments to authors

The topic of manuscript is very interesting and obtained results are relevant. However, authors should address the following points:

Isn't type 2 diabetes clinically characterized mainly by hyperglycemia? Please, clarify if the enrolled individuals with type 2 diabetes were newly diagnosed and drug-naïve.

A: Thank you for your comments. We have mentioned in the METHOD section a clear diagnostic approach to diabetic disease and enrollment criteria: "Patients with T2DM met the following diagnostic criteria: (1) Fasting blood glucose ≥ 7.0 mmol/L; or (2) Hemoglobin A1c ≥ 6.5%. The enrolled individuals with T2DM were newly diagnosed and drug-naïve. Subjects who had been treated with antibiotics in the previous 3 mo, were pregnant or lactating, or had inflammatory bowel disease were excluded from the study. The patients and healthy adults in each population were age-matched ($p>0.05$). ". Based on our criteria, all patients grouped as T2DM fulfilled the following conditions: T2DM were characterized by fasting blood glucose (FBG) ≥ 7.0 mmol/L, or Hemoglobin A1c (HbA1c) ≥ 6.5%, according to the guideline for the prevention and treatment of type 2 diabetes mellitus in China (2020 edition). In line with our enrollment criteria, patients with T2DM were enrolled who were newly diagnosed and unmedicated.

Why adult control and type 2 diabetes individuals were not matched by age? If participants with type 2 diabetes were treated with antidiabetic drugs this should be reported as well, especially knowing these drugs may alter gut microbiota.

We thank the reviewers for the comments. We apologize that our previous statement was somewhat confusing to you. In fact, before inclusion in the analyses, we had performed age matching between the diseased and healthy adult groups, and as you can see, the results of the statistical tests for their two age groups were

not significant ($p>0.05$). In addition, we included healthy children and healthy adults to look specifically at the effect of age on the gut microbiota.

To address the age impact, we selected eight samples with better-matched ages for repeated analysis, and the results were consistent with the main text. We found that: there were no observable differences in the gut microbiota between healthy adults and T2DM patients in the Dai population in the PCoA analysis (Figure R1). As you have pointed out, the large mean difference in age between the Dai healthy adult and T2D groups is a shortcoming of our study. We have explained this point additionally in the DISCUSSION section: “Despite being matched for age (significance less than 0.05), there was a large deviation from the mean value of the age of healthy adults with diabetes in Dai who were actually enrolled in the analysis. We also selected some of these samples with matched age means for subset analysis, and the results were consistent with the existing result.” .

We have also mentioned the enrollment criteria in the METHOD section for detail: "Patients with T2DM met the following diagnostic criteria: (1) Fasting blood glucose ≥ 7.0 mmol/L; or (2) Hemoglobin A1c ≥ 6.5%. The enrolled T2DM patients were newly diagnosed and drug-naïve. Subjects who had been treated with antibiotics in the previous 3 mo, were pregnant or lactating, or had inflammatory bowel disease were excluded from the study. The patients and healthy adults in each population were age-matched ($p>0.05$).".

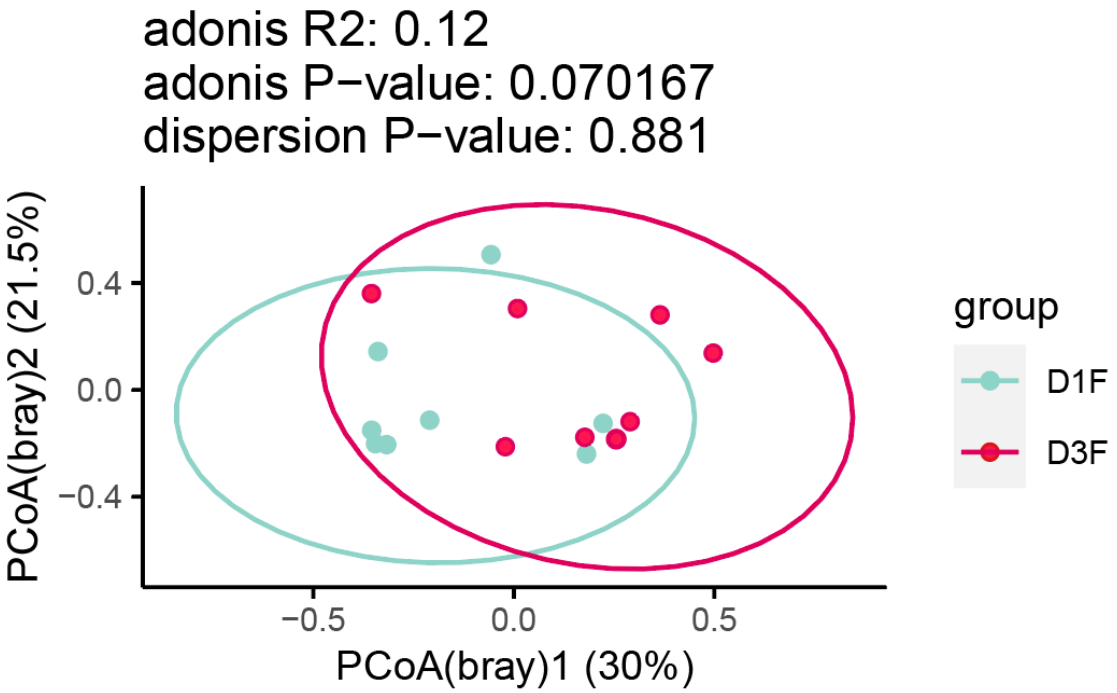

Figure R1: Clustering of gut microbiota composition between the healthy adults and T2DM patients in the Dai population (n=8 pre group, 49.5±13.9 and 51.1±4.0 yr). There were no significant differences between two groups (P > 0.05), which is consistent to our original result.

Discussion section consists mostly of a repetition of the obtained results; authors should put their results in the context of a previous knowledge in the studied area Some of the references are duplicated in the Reference list (e.g. 26 and 32).

A: Thanks for the reviewer's comments. We have revised the Discussion section in the revision. The references have been updated in the revision. Please see below.

"The relationship between gut microbiota and T2DM is becoming increasingly important. In the past decade, studies have supported the role of gut microbiota in the pathogenesis of T2DM[24-28]. Some researchers have reported that there are discriminating microbiota compositions between the Han and the Tibetans populations[16-18] and also different among the different ethnicities: Han, Zang, Bai, Hani, Dai, and Miao (including both healthy urban and rural residents of each ethnicity)[29]. However, the underlying differences of the gut microbiota between

the Han and Dai populations have not been elucidated. Here, we performed a comparative analysis of the gut microbiota in subjects with and without T2DM from the Dai and Han populations in Yunnan Province, China. To the best of our knowledge, this is the first time to compare the T2DM-associated gut microbiota between Han and Dai populations.

The alpha diversity of the gut microbiota, which reflects the abundance, evenness, and richness[30], might vary between ethnic groups in part due to the varied prevalence of T2DM among ethnic groups[31]. Interestingly, our study showed that there was no significant difference in alpha diversity between the Han and Dai populations, suggesting that the abundance, evenness, and richness of the gut microbiota were not significantly different between the Han and Dai populations. However, the diversity of gut microbiota was decreased in T2DM patients compared to healthy adults in both the Han and Dai populations (Figure 1), which is consistent with the previous results in different populations of the world, including other populations in China[32-34].

The gut microbiota is associated with the age of host[35]. Despite being matched for age (significance less than 0.05), there was a large deviation from the mean value of the age of healthy adults with diabetes in Dai who were actually enrolled in the analysis. We also selected some of these samples with matched age means for subset analysis, and the results were consistent with the existing result. To determine the influence of age on gut microbiota, we also conducted a comparison of gut microbiota between healthy children and healthy adults in the Han and Dai populations. These findings suggested that the difference in the gut microbiota between healthy children and healthy adults was greater in the Han population than the Dai population. The observed higher relative abundances of genus *Bacteroides* in children and higher relative abundances of genus *Blautia* in adults were consistent with the previous studies[36].

Many researchers have reported that the gut microbiota diversity is affected by T2DM[26,37]. After comparing the gut microbiota between healthy adults and T2DM

patients in the Han population, we observed a significant difference in the gut microbiota. However, there was no clear distinction between healthy adults and T2DM patients in the Dai population. The underlying reason behind this warrant further investigation. Moreover, our data showed that the T2DM patients of the Dai population possessed a distinctive microbiota composition characterized by a high abundance of Proteobacteria, which is consistent with the previous results[38]. Recent evidence has also shown that Proteobacteria in gut microbial dysbiosis is essential for metabolic disorders[39]. Interestingly, we found that T2DM patients from the Han population had an increase in *Bacteroidetes*, *Bacteroidales*, *Megamonas* and *Bacteroidia* within the phylum Bacteroidetes. This discovery conflicted with the results of some earlier studies [32,40]. The possible reason might be that the proportion of Bacteroidales abundance can be altered by high-calorie diets[41], which is also a possible cause of T2DM.

Although we have identified that both age and T2DM influence the gut microbiota, it is unknown which has a greater effect. We explored the differences in bacteria between healthy children to healthy adults and healthy adults to T2DM in both ethnic groups. The results showed that the differences of healthy children between the Han and Dai population were still significant in healthy adults. However, these changes in T2DM patients were not statistically significant. These results demonstrated that the differences were influenced more by age than T2DM during the transition from healthy children to healthy adults and T2DM patients in both the Han and Dai populations.

Several limitations of this study should be taken into account. First, the sample size was relatively small, which limits the generalizability of the findings. It should be confirmed in a larger scale of samples in the future. Second, the effect of gender, food, and smoking activity were not investigated in the study. Third, the metabolic profile requires further investigation to confirm the relationship between the imbalance of metabolism and gut microbiota alterations."

Once the abbreviations are defined, they should be used consistently throughout the text.

A: Thanks, we have checked the abbreviations to make sure they were used consistently throughout the text.

The whole manuscript requires light language polishing

A: Thanks, the manuscript has been edited by a professional English language editing company, Filipodia publishing. The certificate has also been submitted along with the revised manuscript.

Table 1 is very busy and hard to follow; please, check the accuracy of data (e.g. total cholesterol among type 2 diabetes individuals in the Han population)

A: Table1 we are describing in the order of basic information, blood glucose indicators, lipid indicators, serum biochemical index, liver function, etc., we have gone through a rearrangement, see below. After double-checking all the raw data, we believe that the value you mentioned is an abnormal value due to a writing error. We have updated the TC values in the table. Thank you for your comments.

Table 1 Subject demographics and biochemical blood indices

Parameter	Han population			Dai population		
	Healthy adults, n = 8	T2DM patients, n = 12	Healthy children, n = 15	Healthy adults, n = 10	T2DM patients, n = 12	Healthy children, n = 10
Sex as male/female	3/5	8/4	7/8	5/5	9/3	4/6
Age in yr	50.63 ± 5.85	55.27 ± 11.76	5.00 ± 2.10[1]	45.70 ± 14.66[2]	55.92 ± 9.07	5.50 ± 1.18[3]
FBG in mmol/L	4.85 ± 0.38	9.98 ± 5.89[1]	3.23 ± 0.99	5.62 ± 1.36	7.74 ± 2.39[3]	4.52 ± 0.29
HbA1c as %	5.55 ± 0.24	8.55 ± 2.59[1]	5.23 ± 0.15	5.58 ± 0.19	7.24 ± 0.77[3,4]	5.19 ± 0.35
TG in mmol/L	1.90 ± 0.63	2.22 ± 1.54	0.97 ± 0.24[1]	3.90 ± 4.10[2]	2.48 ± 1.91	1.12 ± 0.45[3]
TC in mmol/L	5.15 ± 0.61	3.76 ± 1.45[1]	4.01 ± 0.88	5.51 ± 1.04	4.85 ± 1.26[4]	3.91 ± 0.57[3]
HDL-C in mmol/L	1.28 ± 0.28	0.97 ± 0.22[1]	1.48 ± 0.34	1.23 ± 0.28	1.26 ± 0.40	1.39 ± 0.42
LDL-C in mmol/L	3.12 ± 0.64	1.82 ± 0.71[1]	2.16 ± 0.63[1]	2.98 ± 1.23	2.75 ± 1.05[4]	2.16 ± 0.38
APO-A1 in g/L	1.75 ± 0.27	1.30 ± 0.30[1]	1.75 ± 0.36	1.83 ± 0.23	1.74 ± 0.37[4]	1.61 ± 0.36
APO-B in g/L	1.04 ± 0.15	0.68 ± 0.18[1]	0.72 ± 0.19[1]	0.98 ± 0.24	0.94 ± 0.29	0.66 ± 0.09[3]
WBC as 10^9/L	6.06 ± 1.44	6.41 ± 1.87	6.95 ± 1.11	6.79 ± 1.43	7.07 ± 2.09	8.32 ± 2.38

RBC as 10^{12}/L	5.19 ± 0.66	4.82 ± 0.57	4.89 ± 0.27	5.09 ± 0.65	5.05 ± 0.57	4.90 ± 0.42
TBil in μmol/L	6.84 ± 1.59	14.19 ± 9.35[1]	6.07 ± 4.48	8.71 ± 5.08[2]	6.19 ± 1.98[4]	5.74 ± 2.38
DBil in μmol/L	3.84 ± 0.79	6.31 ± 2.92[1]	2.92 ± 2.34	3.96 ± 2.05	3.35 ± 1.02[4]	3.13 ± 1.40
IBil in μmol/L	3.00 ± 0.85	7.88 ± 6.51[1]	3.15 ± 2.28	4.75 ± 3.05[2]	2.84 ± 1.34[4]	2.61 ± 1.41[3]
ALT in U/L	22.50 ± 11.33	31.36 ± 26.34	15.80 ± 14.85	25.50 ± 15.68	26.10 ± 17.22	13.70 ± 14.96
AST in U/L	21.13 ± 4.58	27.36 ± 25.61	31.50 ± 7.18[1]	23.26 ± 8.71	23.14 ± 8.64	27.40 ± 6.45
GGT in U/L	34.75 ± 24.40	51.82 ± 90.65	9.67 ± 2.94[1]	70.40 ± 66.34[2]	38.67 ± 22.80[4]	13.50 ± 8.15[3]
BUN in mmol/L	5.63 ± 1.40	4.59 ± 1.70	3.68 ± 0.82[1]	5.12 ± 1.51	5.23 ± 2.18	3.75 ± 0.91
Cr in μmol/L	70.38 ± 8.75	69.55 ± 20.92	32.17 ± 7.60[1]	68.20 ± 17.69	85.50 ± 30.21	32.00 ± 4.00[3]
Hcy in μmol/L	15.63 ± 3.45	12.35 ± 1.55[1]	14.20 ± 3.12	13.59 ± 3.57	16.69 ± 3.47[4]	11.55 ± 1.53[5]
hs-CRP in mg/L	1.85 ± 3.35	3.46 ± 5.23	2.84 ± 6.66[5]	1.84 ± 1.57	1.83 ± 1.47[4]	0.47 ± 0.50[3]

[1]$P < 0.05$ *vs* healthy adults in the Han population; [2]$P < 0.05$, healthy adults in the Han population *vs* healthy adults in the Dai population; [3]$P < 0.05$ *vs* healthy adults in the Dai population; [4]$P < 0.05$, adult type 2 diabetes mellitus patients in the Han population *vs* adult type 2 diabetes mellitus patients in the Dai population; [5]$P < 0.05$, healthy children in the Han population *vs* healthy children in the Dai population. ALT: Alanine transaminase; APO: Apolipoprotein; AST: Aspartate aminotransferase; BUN: Blood urea nitrogen; Cr: Creatinine; DBil: Direct bilirubin;

FBG: Fasting blood glucose; GGT: Gamma glutamyl transferase; HbA1c: Hemoglobin A1c; Hcy: Homocysteine; HDL-C: High density lipoprotein cholesterol; hs-CRP: Hypersensitive C-reactive protein; IBil: Indirect bilirubin; LDL-C: Low density lipoprotein cholesterol; RBC: Red blood cell; T2DM: Type 2 diabetes mellitus; TBil: Total bilirubin; TC: Total cholesterol; TG: Triglyceride; WBC: White blood cell.