Excessive Consumption of the Sugar Rich Longan Fruit Promoted the Development of Non-alcoholic Fatty Liver Disease via Mediating Gut Dysbiosis

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Research

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Abstract

Background: Controversy exists as towards the association of excessive fruits intake and certain disease risks. Longan is an edible fruit rich in high levels of fructose, glucose and sucrose. The aim of this study was to provide direct evidence on the effect of the sugar rich longan fruit on the development of non-alcoholic fatty liver disease (NAFLD). Chemical profiling of longan fruit was conducted using LC-HRMS and HPLC-ELSD.

Results: Longan extracts at the doses of 4.0 g/kg, 8.0 and 16.0 g/kg were orally administered for 4 weeks to healthy C57BL/6J mice or to C57BL/6J mice fed with a HFD diet. Fecal microbiome was analyzed by 16S rRNA sequencing. The amounts of short chain fatty acids (SCFAs) in colonic contents were determined by GC-MS. Colon and liver tissues were used for histopathological examination after H&E, Masson's trichrome, and Oil-red O staining. ELISA method was used for biochemical analysis in serum. In mice fed a normal diet, repeated longan intake for 4 weeks at excess doses (8 or 16 g/kg), but not the normal dose (4 g/kg), promoted inflammation and gut dysbiosis-like status and reduced short-chain fatty acids (SCFAs) production. In high-fat diet (HFD)-fed mice, longan intake at 4 g/kg hardly influenced the NAFLD development. In contrast, excess longan intake (8 or 16 g/kg) promoted NAFLD pathogenesis, including increased abnormality in hepatic indices, elevated inflammation, and gut permeability associated with more severe liver steatosis and fibrosis. Moreover, the exacerbated pathogenic markers were positively correlated with increased blood sugar, aggravated HFD-associated microbial dysbiosis.

Conclusions: Effects mediated by excess longan intake resembled that of equivalent free sugars supplementation, suggesting that high level of free sugars in fruits contributed to the promotion of NAFLD development as demonstrated in case of excessive longan intake.

1. Introduction

Dietary free sugars, including fructose, glucose and sucrose, have been indicated as important risk factor for overweight and non-alcoholic fatty liver disease (NAFLD).[1, 2] Free sugars were reported to disrupt the gut-liver axis *via* increasing gut permeability, altering gut microbiota, and mediating abnormal glycometabolism, which promoted *de novo* lipogenesis in the liver.[3, 4] Fruits are one of the food sources of free sugars. Although it is acknowledged that a diet rich in fruits has been associated with many health benefits, there have been inconsistent findings between dietary fruits or fruit juices and risk of certain diseases.[5, 6] Several studies showed that greater consumption of sugar-sweetened fruit juice, or even 100% fruit juice, is associated with higher risk of type 2 diabetes, weight gain, or overall cancer.[6–9] We speculate that, apart from many micronutrients of vitamins, minerals and antioxidants, specific fruits or fruit extracts are rich in free sugars that may result in harmful effect on health, particularly under excess intake. To guide precision fruit intake, it is thus of great necessity to identify fruits with potential health risks. However, to the best of our knowledge, the evidence for the high sugar fruits as a risk factor for diseases is insufficient.

Longan is a subtropical fruit of *Dimocarpus longan* Lour. (*Sapindaceae* family), which is distributed and planted in Asia-Pacific regions, such as China, Vietnam, Thailand, India, Australia, America and Africa.[10] The bioactive ingredients of longan mainly include polysaccharides and polyphenolics (flavonoids, tannins, and procyanidins), which exhibit antioxidant, anti-inflammatory, immunomodulatory, anti-fatigue and anti-tumor activities.[11–13]

However, the excessive intake of longan fruits is found in daily life frequently to cause unpleasant symptoms such as oral dryness and ulcers, gum bleeding and swelling that are likely due to induction of inflammation. Notably, the incidence of hepatitis was found to increase during the harvest seasons in places of longan production, and the excessive longan intake was suspected to be the cause.[14] However, there is no firm evidence linking the excessive intake of longan with the increased risk of liver diseases.

Of particular note, in addition to the non-caloric bioactive constituents, we determined high levels of free sugars including fructose, glucose and sucrose in longan (Table 1). We then hypothesized that long-term intake of excessive longan, combined with high free sugar intake, could induce NAFLD progression by disrupting the gut-liver homeostasis. In the present study, we assessed the risk of excessive free sugar intake accompanied with longan ingestion, and investigated whether longan intake posed a risk for development of NAFLD. Our findings would provide the first evidence for an example of high sugar fruits such as longan as risk factor for NAFLD and to advocate for rational consumption of such fruits.

Table 1
The quantitative results of fructose, glucose and sucrose in dried longans.

Batches	Fructose (%)	Glucose (%)	Sucrose (%)	Total (%)
S1	19.8	14.7	36.5	71.0
S2	21.7	22.8	23.3	67.8
S3	18.6	11.4	33.2	63.2
S4	15.8	12.4	40.3	68.5
S5	20.3	19.5	37.6	77.4
S6	15.8	10.5	40.9	67.2
S7	17.6	10.8	36.5	64.9
S8	12.1	11.2	33.4	56.7
S9	16.5	12.5	45.7	74.7
S10	17.7	10.7	44.3	72.7
Mean ± SD	17.6 ± 2.7	13.7 ± 4.2	37.2 ± 6.4	68.4 ± 6.0

2. Materials And Methods

2.1. Materials and reagents

Distilled water was prepared *via* Milli-Q system (Millipore). D-(-)-fructose, D-(+)-glucose and D-(+)-sucrose (with purity higher than 99%) were purchased from Sigma-Aldrich (USA). Different batches of dried longan arillus were collected from local retail stores in Shenzhen (Guangdong, China), Chengdu and Luzhou (Sichuan, China). Voucher specimens (No. 20160335) of longan arillus are deposited at Southwest Medical University, Luzhou, Sichuan, China.

2.2. Preparation of longan extract for LC-MS analysis and biological assays

The 1.0 kg dried longan (Kangmei Pharmaceutical Co., Ltd., Guangdong, China) was subjected to extraction using boiling distilled water (8 L) for three times (1.0 h each time) followed by lyophilization till complete dryness. The resultant longan extract (LE) was stored at -80°C for subsequent LC-MS analysis and animal studies.

2.3. Chemical profiling of longan by liquid chromatography coupled with high-resolution mass spectrometry (LC-HRMS) analysis

LC-HRMS-based identification of constituents of longan was performed on a X500R Q-TOF mass spectrometer (AB Sciex, Concord, Canada) coupled with an ExionLC™ system (AB Sciex, Concord, Canada). Chromatographic separation was conducted using a XR-ODSII C18 column (2.0*100 mm, 2.2 μm), with column temperature monitoring at 40 ^{o}C. The mobile phases consisted of distilled water (A, with 0.1% formic acid) and acetonitrile (B, with 0.1% formic acid). The gradient program for mobile phase elution at 0.25 mL/min was set as follows: 0-5 min, 2% B; 5-15 min, 2-10% B; 15-18 min 10-100% B; 18-21 min, 100% B. The injection volume was 5.0 μL and the autosampler was set at 15 ^{o}C. The TOF MS was monitored at negative electrospray ionization (ESI) mode, with spray voltage at -4500 V, source temperature at 500 ^{o}C, curtain gas at 35 psi, CAD gas at 7 psi, declustering potential (DP) at -80 V and scan range of 100-1000 Da. Data acquisition and processing were performed on SCIEX OS software.

2.4. Determination of free sugars in longan

The quantification of free sugars (fructose, glucose and sucrose) in longan arillus was conducted based on the previously developed method.[15] Briefly, using an Agilent 1100 liquid chromatograph system (Agilent Technologies, USA) coupled with an Alltech 2000 evaporative light scattering detector (ELSD; Grace, USA) was used. Chromatographic separation was achieved on an Asahipak NH_2P-504E column (4.6 mm × 250 mm, Shodex, Japan) maintained at 30°C. The mobile phases consisted of water and acetonitrile (*v*/*v*, 25:75) at a flow rate of 1 mL/min. The drift tube temperature was set at 110°C, with nitrogen flow rate at 2.5 L/min. The injection volume was 10 μL. The calibration curves were y=1.431x+5.102 (r=0.999) for fructose, y=1.510x+6.544 (r=0.999) for glucose, and y=1.41x+5.9 (r=1) for

sucrose, where y represented logarithm of peak area while x for logarithm of analyte concentration. The detection range was 1.32-6.60 μg for fructose, 1.60-8.00 μg for glucose, and 2.10-10.50 μg for sucrose.

The extraction of an aliquot of dried longan powder (0.2 g) was conducted using 100 mL solution (water and acetonitrile, 50:50 (*v*/*v*)) under sonication for 30 min. The supernatant was filtered through a 0.45-μm nylon membrane filter before analysis.

2.5. Animals

SPF-grade male4-week-old C57BL/6J mice were obtained from Beijing HFK Bio-Technology Co., Ltd. (Beijing, China) and were housed in ventilated animal cages (5 mice per cage) at the Animal Center of Southwest Medical University under controlled conditions (Temperature, 22 ± 2°C; relative humidity, 55-60%; and a regular 12/12 h light/dark cycle). Mice had free access to sterilized standard chow and tap water. The care of animals and all experimental procedures have been approved by the Committee on Use and Care of Animals of Southwest Medical University (Reference No., 2020226). All mice were subjected for adaption into the environment for at least 7 days before experiments.

2.6. Normal mice feeding experiment

All mice were fed with standard diet (#LAD0011; Trophic Animal Feed High-Tech Co., Ltd., Jiangsu, China), which were randomly grouped (n=7 in control group; n=5 in each LE-treated group). Mice in LE-L, LE-M and LE-H groups were orally administered with LE (dissolved in sterilized distilled water) at low, medium and high dosage of 4, 8 and 16 g/kg, respectively, every other day for 4 consecutive weeks. Mice in control group (Ctrl) were orally given distilled water.

Mice feces were collected after the 4-week treatment at 3:00-5:00 pm to minimize possible circadian effects. Fecal samples were immediately stored in sterilized tubes on ice and transferred into -80 ^{o}C within 2 h. Mice were anaesthetized by pentobarbital sodium solution, with blood collected via cardiac puncture. Blood was further centrifuged after coagulation at 4 ^{o}C at sequential 3000 and 12,000 rpm/min for 5 and 10 min, respectively, to obtain serum, which were subsequently stored at -80 ^{o}C. Colonic contents, colon and liver tissue specimens were further collected. Fresh colon and liver specimens were washed with ice-cold saline and stored at -80 ^{o}C.

2.7. High-fat diet (HFD) induced NAFLD

Mice were randomly allocated into 4 groups (Ctrl, HFD, HFD+LE-M and HFD+LE-H groups). To establish a NAFLD model, mice were fed with a high-fat diet (HFD, comprising 20% fat, 1% cholesterol and 0.2% bile salt; #TP28707; Trophic Animal Feed High-Tech Co., Ltd., Jiangsu, China) for 8 weeks. HFD-fed mice were orally administered with 0, 4, 8 and 16 g/kg LE (dissolved in distilled water) in HFD, HFD+LE-L, HFD+LE-M and HFD+LE-H group (n=10 per group), respectively, every other day from the beginning of the 5th week. Ctrl mice (n=10) were fed with normal diet (#LAD0011) and were orally gavaged with distilled water every other day from the beginning of the 5th week.

On the day before the end of experiment, fecal specimens of each mouse were collected as previous section described and stored at -80 ^{o}C within 2 h. After fasting overnight, a drop of blood from tail vein of each mouse was collected for measuring blood glucose level using the Accu-Chek Active Blood Glucose Meter (#10138059, Roche). Mice were anaesthetized by pentobarbital sodium solution, with blood collected via cardiac puncture for serum preparation. Colonic contents, colon and liver samples were collected, washed and stored at -80 ^{o}C.

2.8. Biochemical analysis

Serum levels of TNF-α and IL-6 were determined by ELISA kits (#E-EL-M0049c and #E-EL-M0044c, Elabscience Biotechnology Co., Ltd) according to the manufacturer's protocol. The level of aspartate transaminase (AST, #C010-2), alanine transaminase (ALT, #C009-2), total cholesterol (TC, #A111-1) and triglyceride (TG, #A110-1) in serum and liver tissues were measured with kits purchased from Nanjing Jiancheng Bioengineering Institute, Jiangsu, China.

2.9. H&E Masson's trichrome and Oil-red O staining of animal tissues

Formalin-fixed paraffin-embedded sections were stained with H&E or Masson's trichrome stain as previously reported.[16] Frozen liver sections were used for oil-red O staining according to previous report.[16] H&E- and Masson-stained sections were inspected by Nikon Eclipse Ts2R+FL microscope. Images of oil-red O-stained sections were captured by a Motic microscope (Motic).

For the NAFLD mice, the NAFLD activity score (NAS) ranged from 0 to 13: steatosis (0-3), lobular inflammation (0-3), hepatocellular ballooning (0-3), and fibrosis (0-4).

2.10. DNA extraction and PCR amplification

Microbial DNA was extracted from mouse fecal specimens using the E.Z.N.A.® soil DNA Kit (Omega Bio-tek) according to the manufacturer's instruction. The DNA concentration and purification were measured by NanoDrop 2000 (Thermo Scientific), with the DNA quality examined by 1% agarose gel electrophoresis. The bacterial 16S rRNA gene (V3-V4 hypervariable regions) were amplified with primers 338F (5'-ACTCCTACGGGAGGCAGCAG-3') and 806R (5'-GGACTACHVGGGTWTCTAAT-3') by thermocycler PCR system (GeneAmp 9700) as we previously reported.[17, 18] The resultant PCR products were extracted from a 2% agarose gel and further purified using the AxyPrep DNA Gel Extraction Kit (Axygen Biosciences) and quantified using QuantiFluor™-ST (Promega).

2.11. Illumina MiSeq sequencing

Purified amplicons were pooled in equimolar and paired-end sequenced (2×300) on an Illumina MiSeq platform (Illumina) according to the standard protocols by Majorbio Bio-Pharm Technology Co. Ltd. (Shanghai, China).

2.12. Processing of sequencing data

Analysis of the fecal microbial community was performed using the free online platform of Majorbio Cloud Platform (www.majorbio.com) and Microbiomeanalyst (https://www.microbiomeanalyst.ca/).[19] Raw fastq files were demultiplexed, quality-filtered by Trimmomatic and merged by FLASH with the criteria as we previously described.[17]

Operational taxonomic units (OTUs) were clustered with 97% similarity cut off using UPARSE (version7.1) and chimeric sequences were identified and removed using UCHIME. The taxonomy of each 16S rRNA gene sequence was analyzed by RDP Classifier algorithm against the Silva (SSU123) 16S rRNA database using confidence threshold of 70%.

Rarefaction curves and α diversity were analyzed using mothur v1.30.1 and β diversity was analyzed using QIIME. Partial least squares discriminant analysis (PLS-DA) was performed in R tools using package mixOmics. Data structure was assessed by principal co-ordinates analysis (PCoA) using the Bray-Curtis dissimilarity matrices. Linear discriminant analysis (LDA) coupled with effect size (LEfSe) was achieved using LEfSe program. Canonical correlation analysis (CCA) was performed to see the correlation of microbial changes and other factors.

Based on 16S rRNA gene sequence data, the open-source R package of Tax4Fun was applied to monitor functional changes of microbial communities mapping with Kyoto Encyclopedia of Genes and Genomes (KEGG) reference database.

2.13 Short-chain fatty acids (SCFAs) determination in colon

The determination of SCFAs, including acetic acid, propionic acid, isobutyric acid, butyric acid, isovaleric acid, valeric acid and caproic acid, was performed based on the method we previously developed[15] using gas chromatography coupled with mass spectrometry (GC-MS). The method was fully validated with linearity, limit of quantification, intra-day and inter-day precision, repeatability, and recovery. For sample preparation, the colonic content (50 mg) was vortex mixed with 15% phosphoric acid (50 μL), 125 μg/mL isocaproic acid (internal standard, 100 μL), and ether (400 μL) for 1 min, which was further centrifuged at 12000 rpm for 10 min at 4 ^{o}C. The resultant supernatant (1 μL) was directly injected for GC-MS analysis.

2.14 Statistical analysis

Statistical difference was assessed by GraphPad Prism 7.0 software based on unpaired student's *t* test (for comparison between two groups) or one-way ANOVA with a *post hoc* Tukey test (for comparison among three or more groups). A *p* value less than 0.05 was considered statistically significant.

Table 2
Identified compounds from longan by LC-HRMS.

No.	RT (min)	Molecular formula ($[M-H]^-$)	$[M-H]^-$		Identified compounds
			Detected (m/z)	Error (ppm)	
1	1.01	$C_6H_{11}O_6$	179.0540	-6.783	Fructose
2	1.13	$C_6H_{13}N_4O_2$	173.1032	-0.590	Arginine
3	1.13	$C_{12}H_{21}O_{11}$	341.1048	-8.906	Sucrose
4	1.15	$C_{10}H_{12}N_5O_4$	266.0864	-7.443	Adenosine
5	1.15	$C_{15}H_{13}O_6$	289.0685	-7.488	Epicatechin
6	1.19	$C_{10}H_{13}N_2O_5$	241.0817	-0.821	Thymidine
7	1.19	$C_9H_{12}O_5N_3$	242.0759	-5.151	Cytidine
8	1.19	$C_4H_6NO_4$	132.0287	-3.289	Aspartic acid
9	1.22	$C_{10}H_9O_4$	193.0481	-7.435	Ferulic acid
10	1.22	$C_6H_{11}O_7$	195.049	-4.764	Gluconic acid
11	1.23	$C_5H_9O_6$	165.0381	-7.662	Arabinonic acid
12	1.25	$C_5H_8NO_4$	146.0447	-0.577	Glutamic acid
13	1.26	$C_6H_{11}O_6$	179.0540	-5.666	Glucose
14	1.26	$C_9H_7O_4$	179.0341	1.200	Caffeic acid
15	1.26	$C_7H_{11}O_8$	223.0432	-7.370	4-*O*-methyl-glucaric acid
16	1.3	$C_{10}H_{11}N_4O_5$	267.0704	-7.473	Inosine
17	1.34	$C_7H_{11}O_6$	191.0538	-6.357	Quinic acid
18	1.41	$C_4H_5O_5$	133.0127	-3.381	Malic acid
19	2.05	$C_5H_4N_5$	134.0456	-3.892	Adenine
20	2.13	$C_6H_7O_7$	191.0173	-6.958	Citric acid
21	2.13	$C_4H_3N_2O_2$	111.0183	-5.440	Uracil

No.	RT (min)	Molecular formula ($[M-H]^-$)	$[M-H]^-$		Identified compounds
			Detected (m/z)	Error (ppm)	
22	2.74	$C_9H_{11}O_6N_2$	243.0602	-3.960	Uridine
23	2.77	$C_4H_5O_4$	117.0175	-6.282	Succinic acid
24	4.56	$C_{10}H_{12}O_5N_5$	282.0809	-8.490	Guanosine
25	4.85	$C_7H_5O_5$	169.0119	-7.394	Gallic acid
26	4.87	$C_5H_5N_2O_2$	125.0338	-6.030	Thymine
27	12.26	$C_{15}H_9O_7$	301.0315	-9.232	Quercetin
28	17.52	$C_{27}H_{21}O_{18}$	633.0665	-9.067	Corilagin
29	17.63	$C_{15}H_9O_6$	285.0369	-8.646	Kaempferol
30	17.64	$C_{12}H_{23}O_2$	199.1679	-6.811	Lauric acid
31	17.78	$C_7H_7O_2$	123.0441	0.358	4-Methylcatechol
32	17.87	$C_{29}H_{49}O$	413.3746	-7.723	β-Sitosterol

3. Results

3.1. Determination of free sugar in longan

The contents of free sugars including glucose, fructose and sucrose were determined in different batches of dried longan using HPLC-ELSD method. The relative levels of fructose, glucose, and sucrose in longan fruits were determined at 17.6 ± 2.7, 13.7 ± 4.2, and 37.2 ± 6.4% (*w/w*), respectively (Table 1). The total contents of free sugars amounted for 68.4 ± 6.0% (*w/w*) of dried weight longan suggestive of being a sugar rich fruit as that of litchi and mangoes.

According to the guideline issued by the World Health Organization (WHO)[17], it is recommended to reduce the daily intake of free sugars to less than 10% of total energy intake (~50 g, strong recommendation) or preferably to 5% (~25 g, conditional recommendation) throughout life course for both children and adults. Considering the high level of free sugars in longan, the inappropriate intake of longan may pose a risk of excessive free sugar intake, that is, daily consumption of longan more than 36 g or 72 g (equivalent to 25 or 50 g free sugars) would probably have a potentially bad effect on health (Table 3). Notably, as documented in Chinese Pharmacopeia (2015 edition),[20] the recommended daily dose for adult is 15 g for longan in TCM, and the use in real scenarios can be as high as 30 g.

Table 3
Risk assessment of excess free sugar intake accompanied with longan consumption.

Daily free sugar intake (Total energy, %)*	Health risk	Equivalent dried longan consumption
<25 g (<5%)	Low	<36 g
25-50 g (5-10%)	Medium	36-72 g
>50 g (>10%)	High	>72 g

*According to the WHO guideline on intake of sugars for adults and children, it is recommended to reduce the intake of free sugars to less than 10% of total energy intake (Strong recommendation), with preferably reduction to 5% of total energy intake (Conditional recommendation).

3.2. Identification of bioactive components in longan fruit extract using LC-HRMS

Considering that sugars amounted for *ca*. 70% of the fruit composition, determination of secondary bioactives that would characterize longan fruit, LC-HRMS was employed using a reverse phase column that is more suited for phytonutrients characterization.[21] Considering the enrichment of fruit in phenolics,[22] negative ionization MS mode was used for chemical profiling. The LC-HRMS chromatogram for longan analysis is displayed in Fig. 1, and the results for identification of main components are shown in Table 2. A total of 31 small-molecular constituents from longan were identified based on their retention time and HRMS data, which included mostly free sugars (fructose, glucose and sucrose) amounting for the major peaks eluting earlier in the chromatogram, along with nucleosides (thymidine, adenosine, cytidine, adenine, inosine, adenine, etc.), organic acids (quinic acid, malic acid, citric acid, succinic acid). Among these, adenosine accounts for fruits anxiolytic, sedative and analgesic effects.[23] Detected polyphenols in fruits included mostly phenolic acids (ferulic acid, caffeic acid and gallic acid) and flavonoids (epicatechin, quercetin and kaempferol). Although phenolics are known to be associated with a low prevalence of metabolic diseases, including obesity, and insulin resistance concurrent with lowering the main risk factors involved in the pathogenesis of NAFLD,[24] such effects was not observed in case of longan fruit likely due to their presence at trace levels (Fig. 1).

3.3. Excessive intake of longan induces inflammation in mice

It has been recognized that there is certain association between sugary drink consumption and inflammation.[25] To see whether longan induces inflammation, the impact of longan intake at different doses was further investigated on mice. The doses of longan used for mice were set as 4, 8 and 16 g/kg (approximately equivalent to human doses of 20-28, 40-56 and 80-112 g dried longan daily, respectively), which potentially poses a low, medium and high risk of excessive free sugar intake. The 4 g/kg longan in

mice (LE-L group) was approximately at the maximum recommended dose, while the dosages at 8 g/kg (LE-M group) and 16 g/kg (LE-H group) were regarded as excess longan intake.

After a 4-week oral administration of water extract of longan, mice in LE-M group (4 out of 6 mice) and LE-H group (3 out of 6 mice) displayed increased infiltration of inflammatory cells in colon (Fig. 2A) and liver (Fig. 2B) tissues. In comparison, mice in Ctrl and LE-L group had no sign of inflammatory cell infiltration in either colon or liver samples after 4 weeks of low-dose longan (4 g/kg) treatment (Fig. 2A and 2B). Besides, compared to Ctrl mice, levels of the proinflammatory factors of TNF-α and IL-6 in serum were significantly elevated in the LE-H group ($p < 0.05$) (Fig. 2C and 2D). The TNF-α level was also increased in LE-M group ($p < 0.05$) (Fig. 2C). The LE-L group showed unchanged serum levels of TNF-α and IL-6 (Fig. 2C and 2D). The results indicate that excess longan (8 or 16 g/kg), for a 4-week supplementation, could mediate a pro-inflammatory status in mouse colons and livers. Nevertheless, it should be noted that administration of longan fruit extract at much lower doses at a dose of 100-400 mg/kg in mice showed an anti-inflammatory action as manifested by reduction in inhibited lipopolysaccharide (LPS)-induced nitric oxide (NO) levels in macrophages and inhibition on paw edema development following carrageenan treatment.[26]

3.4. Excess intake of longan changes intestinal microbial structure in mice

Considering longan inflammation induction in the colon, we further examined whether such action was mediated in part *via* affecting colonic microbiota composition. As displayed in PLS-DA plot (based on genus level), samples from different groups of Ctrl, LE-L, LE-M and LE-H were clearly separated (Fig. 2E), suggesting that longan markedly mediated structural changes of colonic microbiota in mice. The loading plot for PLS-DA analysis showed the significance of bacterial genera contributing to discriminating different groups (Fig. 2F).

To compare difference in colonic microbiota among groups, one-way ANOVA test was conducted for different taxon levels. At phylum level, the abundance of Firmicutes was gradually increased while the abundance of Bacteroidetes was decreased following longan intake (Fig. 2G). As a decreased Bacteroidetes/Firmicutes (B/F) ratio has been previously implicated as an indicator of certain pathological conditions,[27] here we showed that the B/F ratio was reduced along with increased longan doses, with significant difference observed in LE-H group (Fig. 2H). At genus level, heat map dendrogram of the most significant ones (highlighted with purple color, with Comp 1 or Comp 2 values > 0.125, or < -0.125) suggested that there were remarkably different patterns of generic abundance across different groups (Fig. 3A), which could effectively distinguish longan supplemented groups with Ctrl group. with increasing longan doses, *norank_f__Lachnospiraceae*, *unclassified_f__Lachnospiraceae*, *Lachnospiraceae_NK4A136_group*, *Desulfovibrio*, and *Alistipes* were increased, while the most abundant genus *norank_f__Muribaculaceae* was dramatically decreased (Fig. 3B).

In order to distinguish the predominant taxon, LEfSe analysis was further performed. LEfSe was applied to generate a cladogram to display the specific bacteria associated with longan intake. After 4 weeks of longan administration, *Desulfovibrio*, *unclassified_f__Lachnospiraceae*, *Lachnospiraceae_NK4A136_group*, *norank_f__Lachnospiraceae*, *[Eubacterium]_brachy_group*, *Family_XIII_UCG-001* and *Ruminococcaceae* family were enriched in LE-M and LE-H groups, while *norank_f__Muribaculaceae* was enriched in Ctrl (Fig. 3C).

Furthermore, functional prediction by Tax4Fun as a result of longan intake demonstrated that the 4 weeks of longan treatment, particularly with excess longan intake (LE-M and LE-H groups), significantly enriched the annotated KEGG pathways related to signal transduction and energy metabolism (Fig. 3D). On the other hand, several metabolic pathways regarding the glycan biosynthesis and metabolism, carbohydrate metabolism, lipid metabolism and metabolism of other amino acids were significantly decreased in LE-M and/or LE-H group (Fig. 3D). It is suggested that LE-fed mice specifically displayed changed metabolic pathways.

3.5. Excess intake of longan reduces SCFA production in mice

SCFAs are the end products of microbial fermentation in gut and have been suggested as important mediators of host health.[28] To see the alteration of SCFA production resulting from microbial changes, we further analyzed SCFA levels (i.e., acetic acid, propionic acid, isobutyric acid, butyric acid, isovaleric acid, valeric acid and caproic acid) in colon (Fig. 3E-L). After a 4-week administration of longan, the levels of acetic acid and isobutyric acid were significantly decreased in LE-H group (Fig. 3E and 3I), while caproic acid was increased in all longan-treated groups (Fig. 3K). The other SCFAs were not changed. As a result, the total production of SCFAs in LE-H group was significantly decreased (Fig. 3L), suggesting that the gut homeostasis was impacted particularly in LE-H group.

Collectively, the results indicate that repeated longan intake (particularly for the excess doses in LE-M and LE-H groups) induces a pro-inflammatory status and disrupts gut homeostasis *via* altering intestinal microbial communities and related metabolism.

3.6. Excessive longan intake promotes the development of NAFLD in mice

Further, the effect of longan intake at different doses on the pathogenesis of NAFLD was determined for liver markers. Low-, medium- and high-dose longan (from the 5th to 8th week) was supplemented together with dietary HFD, and the development of NAFLD after 8 weeks was assessed (Fig. 4 and Fig. 5).

Intriguingly, the results showed that longan extract at a normal dose of 4 g/kg did not significantly influence the HFD-induced NAFLD development in mice (Fig. 4). Particularly, the HFD+LE-L group did not show aggravated histological changes, when compared to HFD group (Fig. 4C and 4D). Regarding the serum levels of AST (Fig. 4E), ALT (Fig. 4F) and TC (Fig. 4G), there was no significant difference between

HFD and HFD+LE-L group. However, it also demonstrated that the serum IL-6 and TG levels were reduced in HFD+LE-L group, compared to HFD group (Fig. 4B and 4H). The result suggests that the low-dose LE may have certain beneficial effect on NAFLD, although major liver markers have no improvement.

The effect of excessive dose LE on NAFLD was further analyzed (Fig. 5A). The HFD-induced NAFLD mouse model was observed with elevated levels of AST and ALT in liver and serum, increased hepatic TC and TG and unchanged blood sugar level (Fig. 5B-H). The histological observation showed typical NAFLD manifestations including increased infiltration of inflammatory cells and ballooned hepatocytes (H&E stain) (Fig. 5I), more lipid droplets in hepatocytes (Oil-red stain) (Fig. 5J), and mild liver fibrosis (Masson's trichrome stain) (Fig. 5K), with increased histological score (Fig. 5L). The serum levels of proinflammatory IL-6 and TNF-α were increased (Fig. 5M and 5N) and the serum LPS level was elevated (Fig. 5O), suggesting the increased inflammation and gut permeability. Compared with HFD-fed alone mice, the combined longan-treated and HFD-fed mice (HFD+LE-M and HFD+LE-H groups) demonstrated significantly increased levels of AST, ALT, TC and TG in liver or serum (Fig. 5B-G), higher histopathological score (more obvious hepatocytes ballooning, fat accumulation and fibrosis) (Fig. 5I-L), more inflammation and serum LPS (Fig. 5M-N), together with elevated blood glucose level (Fig. 5H). The results suggest that excess longan intake exacerbated the NAFLD symptoms in mice regarding the enhanced *de novo* lipogenesis and increased liver steatosis and fibrosis.

Notably, the supplementation of high free sugars (with equivalent dose to 16 g/kg longan) had a similar effect with the high-dose longan on promotion of NAFLD, as seen with elevated levels of AST, ALT, TC, TG, IL-6 and TNF-α in liver or serum and increased histological score that were generally comparable with HFD+LE-H group (Fig. 5B-O). The results demonstrate that free sugars might contribute to the longan-induced NAFLD promotion.

3.7. Progression of NAFLD by excess longan supplementation is associated with enhanced gut dysbiosis and reduced SCFAs production

We then investigated the effect of combined longan intake and HFD on gut microbiota in NAFLD mice. The results showed that HFD alone decreased Sobs index (Fig. 6A) and Shannon index (Fig. 6B) of gut microbial communities, but with no statistical difference. Whereas, mice treated with combined longan or free sugars with HFD (HFD+LE-M, HFD+LE-H and/or HFD+FS groups) demonstrated significantly decreased Sobs index and Shannon index (Fig. 6A and 6B). Therefore, HFD had a considerable impact on both microbial richness and diversity, and that the combined longan or free sugar treatment could further decrease HFD-induced reduction of microbial richness and diversity in mice.

PCoA analysis on OUT level (Fig. 6C and 6D) showed that the NAFLD mice (including the HFD, HFD+LE-M, HFD+LE-H and HFD+FS groups) showed structural difference in microbial communities from that in Ctrl mice. The samples from HFD+LE-M, HFD+LE-H and HFD+FS groups were gradually separated out

from that of HFD group. It is suggested that HFD-induced microbial changes are enhanced by excess longan or free sugar supplementation.

Compared to Ctrl mice, HFD-fed mice at the phylum level had remarkable changes (Fig. 6E). *Firmicutes* and *Bacteroidota* were the predominant phyla in the gut. *Firmicutes* was increased from 46.22% (Ctrl) to 50.50% (HFD), 63.71% (HFD+LE-M), 65.74% (HFD+LE-H) and 54% (HFD+FS), while the *Bacteroidetes* (*Bacteroidota*) was decreased as: 33.1% (Ctrl), 9.9% (HFD), 5.1% (HFD+LE-M), 5.6% (HFD+LE-H) and 5.5% (HFD+FS) (Fig. 6F), along with significantly reduced B/F ratio in HFD, HFD+LE-M, HFD+LE-H and HFD+FS groups (Fig. 6G). HFD diet promoted the growth of *Desulfobacterota*, however, the longan or free sugar supplementation reversed the change (Fig. 6F). In addition, *Actinobacteriota* was significantly elevated after intake of free sugar but not longan (Fig. 6F).

At genus level, HFD alone induced a significant increase of 6 genera, including *Desulfovibrio*, *Faecalibaculum*, *Streptococcus*, and *Lactococcus*, among others, and a significant decrease of 15 genera, including *norank_f__Muribaculaceae*, *norank_f__norank_o__Clostridia_UCG-014*, *Candidatus_Saccharimonas* and *norank_f__Ruminococcaceae*, among others (Fig. 7). In order to identify key altered gut microbial communities among groups, LEfse was performed (Fig. 8A and 8B). *Desulfovibrio*, *Turicibacter*, *norank_f__Muribaculaceae*, *Lactobacillus*, *Faecalibaculum*, *Akkermansia*, *Dubosiella*, *Prevotellaceae_UCG_001*, *norank_f_UCG_001* and *norank_f__Lachnospiraceae* were considerably enriched in different groups which had LDA scores larger than 3.5 (Fig. 8B).

In particular, the abundance of *norank_f__Muribaculaceae* (Fig. 8C) and *Lactobacillus* (Fig. 8D) in HFD group were found decreased with no statistical significance, but in HFD+LE-M, HFD+LE-H and HFD+FS groups they were significantly reduced when compared to Ctrl group. The abundance of *Desulfovibrio* (Fig. 8E) was significantly increased in HFD group, but was further decreased after LE or free sugar supplementation. *Faecalibaculum* (Fig. 8F) and *Turicibacter* (Fig. 8G) showed a trend of increase in HFD-fed mice, with the highest enrichment in mice supplemented with excessive longan or free sugars. *Dubosiella* had a similar change with *Faecalibaculum*, with highest enrichment in HFD+LE-M, HFD+LE-H and HFD+FS groups (Fig. 8H). The abundance of *Lachnospiraceae_NK4A136_group*, *unclassified_f__Lachnospiraceae*, *norank_f__Lachnospiraceae*, and *Prevotellaceae_UCG-001* were remarkably decreased in HFD-fed mice with or without intake of longan or free sugars (Fig. 8I-J).

As a result of microbial alterations, the production of SCFAs was altered (Fig. 9A-H). The contents of acetic acid and propionic acid were significantly reduced in HFD+LE-M and/or HFD+LE-H groups (Fig. 9A and 9B). Caproic acid also experienced a remarkable decrease after longan intake in HFD-fed mice (Fig. 9G). The other SCFAs were not changed (Fig. 9C-F). The total SCFA levels in HFD+LE-M and HFD+LE-H groups was at the lowest level (Fig. 9H).

The above results suggest that excess longan or free sugar intake similarly aggravates HFD-mediated disruption of gut homeostasis.

3.8. Correlation analysis of association of key microbial changes and pathological abnormities among groups

We subsequently performed CCA analysis to reveal the association of key microbial and metabolic changes with pathological abnormities within gut-liver axis including inflammation, liver injury, hyperglycemia and SCFA production. The CCA analysis (Fig. 9I) showed that the composition of microbial communities was altered at the genus level as the introduction of HFD diet and further supplementation of longan or free sugars. In NAFLD mice, *Dubosiella* and *Faecalibaculum*, together with elevated blood glucose level, increased histological scores, inflammation, liver TC/TG/AST/ALT levels and decreased SCFAs, were positively correlated with HFD feeding, particularly for the HFD+LE-M, HFD+LE-H and HFD+FS groups, while *norank_f__Muribaculaceae* and *Lactobacillus* were found negatively correlated.

Collectively, the results demonstrate that excess longan or free sugars could exacerbate gut dysbiosis and microbial metabolism, which is significantly correlated with progression of NAFLD in mice.

4. Discussion

A diet rich in fat or free sugars has been suggested to promote the development of NAFLD. A recent study showed that 8 weeks of a diet low in free sugars compared with usual diet led to a significant improvement in hepatic steatosis in adolescent boys with NAFLD.[29] Although dietary fruits are important source of free sugars, several studies implicated that higher intake of fruits or fruit juices enhanced antioxidant status in healthy men,[30] and resulted in favorable changes in anthropometry and insulin levels overweight and obese subjects,[31] which may be due to the presence of antioxidants and other micronutrients in fruits. However, there are also studies demonstrating an adverse effect of fruit juice on type 2 diabetes or cancers.[5, 9] It is speculated that not all fruits or fruit extracts are beneficial, particularly under excess intake. As a popular fruit, although many reports showed health benefits of longan in human or animals,[26] some researchers linked excessive intake of longan to increased incidence of liver disease,[14] but with no firm evidence. Therefore, the aim of this study was to investigate the potential association of excessive intake of longan with the progression of NAFLD based on the homeostasis of the gut-liver axis. The effect of equivalent free sugars was further examined on NAFLD mice for comparison to healthy animals.

Here, we demonstrated for the first time that excessive intake of longan (at 8 and 16 g/kg) significantly promoted the progression of NAFLD in mice as evidenced by liver injury indices as well as histological observations. Compared with HFD-fed mice, the longan supplemented with HFD (HFD+LE-M and HFD+LE-H groups) showed increased abnormality in hepatic indices (higher liver TC and TG levels and elevated liver or serum AST and ALT levels), enhanced inflammation (higher serum levels of IL-6 and TNF-α and increased inflammatory cell infiltration in liver tissue), and more severe steatosis and liver fibrosis. At the meantime, supplementation of free sugars (10.88 g/kg, equivalent to 16 g/kg longan) showed

similar impact on promotion of NAFLD compared with that using 16 g/kg longan. The results indicate that longan or free sugar intake at excess pose a risk for NAFLD.

Underlying chemicals mediating for these abnormalities was mostly revealed from the fruit richness in free sugars (fructose, 17.6%; glucose, 13.7%; and sucrose, 37.2%), and further in longan supplementation d with excessive free sugar intake. Sucrose, a disaccharide, is hydrolyzed inside the gut into glucose and fructose prior to absorption. Excess dietary free sugars can be metabolized into fatty acids for further lipogenesis. Fructose has a more lipogenic ability than glucose,[32] due to peripheral consumption of glucose is higher than that of fructose;[33] and thatfructose bypasses key steps in glycolysis and is a more efficient substrate for lipogenesis. The excess doses for longan used in mice are set as 8 g/kg (daily 40-56 g human equivalents) and 16 g/kg (daily 80-112 g human equivalents), which have exceeded the daily-intake of free sugars recommended by WHO (< 25-50 g; equal to 36-72 g longan) for children and adults. After a repeated consumption of longan at 8 or 16 g/kg for 4 weeks in HFD-fed mice, the blood glucose level was significantly increased compared to those not supplemented with longan. It is speculated that excess free sugars, accompanied with longan supplementation, have led to impaired glycometabolism and enhanced *de novo* lipogenesis in HFD-fed mice, which resulted in increased fat accumulation in hepatocytes and subsequent vesicular steatosis. Moreover, the longan-induced gut dysbiosis was quite similar to that mediated by fructose in reports (discussed in next paragraph). Notably, both excess longan and free sugars could promote NAFLD development in HFD-fed mice, displaying comparable impacts. It is suggested that high free sugar level in longan should be the main factor resulting in the progression of NAFLD along with other unknown chemicals. In addition, it is also found in the present study that the low-dose LE may have certain beneficial effect on NAFLD via decreasing serum level of TG and IL-6. This was to some extent in accordance with previous finding that longan at low dose protected from inflammation.[26] The benefit of longan may largely owe to its phenolics and potentially polysaccharides.[25, 34] It is speculated that under excessive dose the detrimental effect of longan may override its benefits.

Furthermore, excess longan intake promotes the development of NAFLD *via* disrupting intestinal homeostasis in mice. The complex reciprocal interaction within gut-liver axis has been shown to play an essential role in the development of liver diseases in response to dietary, genetic and environmental factors.[35] Disruption in gut homeostasis at several interconnected levels, including the gut microbiome, the microbial metabolites such as SCFAs and endotoxins, and mucus and epithelial barriers, has a profound impact on the pathogenesis of NAFLD.[35] It has long been acknowledged that patients or animals with NAFLD exhibit dysbiosis with significantly altered gut microbial communities at the phylum, genus and species levels.[36] Dysbiosis led to increased gut permeability, microbial translocation and absorption of microbial products, which increased inflammation and cell injury and altered metabolism in liver.[35] Previous study has shown that gut microbiota determined the development of NAFLD independently of obesity.[37] In the present study, we firstly demonstrated that excess longan (8 or 16 g/kg) but not the low-dose longan (4 g/kg) supplemented for 4 weeks increased systemic inflammation in normal mice, occurred with structurally changed intestinal microbiome. The altered gut microbiota was

characterized by decreased B/F ratio and changed specific microbial communities mainly including the decrease of the nonpathogenic *norank_f__Muribaculaceae*, and the increase of *Desulfovibrio* and several genera in *Lachnospiraceae* family including the *unclassified_f__Lachnospiraceae*, *Lachnospiraceae_NK4A136_group* and *norank_f__Lachnospiraceae*, among others. An increased abundance of the obese microbiota *Firmicutes* concurrent with decreased abundance of the lean microbiota *Bacteroidetes*, with a decreased B/F ratio, were reportedly associated with obesity and NAFLD in both human and animals.[27] The *norank_f__Muribaculaceae* has been reported to be potentially beneficial *via* relieving inflammation, suppressing harmful bacteria and/or promoting anticancer immunity.[38–40] *Desulfovibrio* can generate the potentially toxic hydrogen sulfide, contributing to gut inflammation and being associated with IBD progression.[41, 42] *Lachnospiraceae* bacteria are generally nonpathogenic and are suggested to produce SCFAs.[43] However, several previous reports have showed that the high-fructose diet led to increased abundance of *Desulfovibrio* and the *Lachnospiraceae* family in mice,[44, 45] which was in consistent with current findings by excess longan intake containing high level of sugars. Additionally, previous studies have demonstrated that certain SCFAs such as acetic acid and butyric acid were able to inhibit inflammation, while the caproic acid tended to promote TH1 and TH17 differentiation and support inflammation.[46] The results in the current study confirmed that longan treatment (particularly for the LE-H) reduced acetic acid and isobutyric acid, and increased the caproic acid, which might lead to increased inflammation in mice. Thus, our results apparently indicated that the excess longan intake for 4 weeks induced inflammation and dysbiosis-like status in mice, which may increase disease susceptibility.

Further evidence for the disturbance of intestinal homeostasis by excess longan was obtained on HFD-fed mice. In NAFLD mice, the 8-week HFD feeding mediated significant changes in gut microbiota, featuring dysbiosis as follows: i) reduced microbial richness and diversity; ii) decreased B/F ratio; and iii) substantial alteration of microbial compositions characterized by increased abundance of potentially pathogenic bacteria of *Desulfovibrio*, *Faecalibaculum* and *Streptococcus*, and decreased the beneficial *norank_f__Muribaculaceae* and *Lactococcus*, among others. Notably, longan supplemented with HFD promoted most of these changes, as seen with the lower Sobs and Shannon indices and B/F ratio in HFD+LE-M and/or HFD+LE-H group. Although longan reduced the HFD-induced increase of LPS-producing *Desulfovibrio* [47] in mice, longan intake enhanced the HFD-associated alterations including the decrease of *norank_f__Muribaculaceae* and *Lactobacillus* and the increase of *Dubosiella, Turicibacter* and *Faecalibaculum*. The decrease of *norank_f__Muribaculaceae* abundance has been previously implicated in HFD-fed mice.[39, 48] *Lactobacillus* can protect against NAFLD by attenuating inflammation in mice.[49] Although both *Turicibacter* and *Faecalibaculum* are producers of SCFAs such as butyrate or lactic acid, they were previously reported to be increased in mice fed an HFD.[50, 51] Although *Lachnospiraceae* family was found increased in normal mice after longan supplementation, its abundance was lower in HFD+LE-M and HFD+LE-H groups compared to HFD group. Its role in longan-treated mice requires further investigation. Furthermore, in the present study, the acetic acid, propionic acid, caproic acid, as well as the total SCFA level was found mostly decreased in HFD+LE-M and HFD+LE-

H groups, compared to Ctrl. Although there no significant difference between HFD and HFD+LE-M or HFD+LE-H, it seems that HFD+LE-M or HFD+LE-H had the lowest level of acetic acid, propionic acid, and total SCFAs. Acetic acid as one of the most abundant SCFA was also found decreased in normal mice treated with LE-H, which is consistent with the finding in NAFLD mice. However, the results for caproic acid were not consistent in two mouse models. This might be due to the effect of LE-H on regulating caproic acid was compromised by HFD feeding, since HFD diet significantly reduced the caproic acid production. Notably, the alterations of HFD-associated microbial communities and decreased SCFA production mediated by excess longan were strongly associated with the NAFLD progression. It is thus suggested that excess longan promoted dysbiosis, thereby promoting the NAFLD pathogenesis in mice, and with free sugars to demonstrate a similar effect to that of longan in HFD-fed mice.

5. Conclusions

In conclusion, we provided the first evidence that the excess longan supplementation (8 and 16 g/kg) significantly promoted the development of NAFLD in mice, which was tightly associated with excess free sugar intake and the disruption of intestinal homeostasis (Fig. 10). Such effect can be resembled by the intake of free sugars. The results provide a scientific basis for excess longan or free sugar mediated NAFLD via disrupting gut-liver axis. Our findings warrant rational consumption of longan fruit or other fruits containing high free sugars such as litchi as a dietary supplement among the general population and advocate a precision fruit intake by NAFLD patients. Determination of these effects in cases of other fruits of rich sugar composition such as litchi, grapes and cherries should confirm our hypothesis and extend to examine other etiologies or diseases in the future ideally in clinical studies to be conclusive.

Declarations

Ethical Approval and Consent to participate

The care of animals and all experimental procedures have been approved by the Committee on Use and Care of Animals of Southwest Medical University (Reference No., 2020226).

Consent for publication

All authors in this manuscript have agree to publication on this journal.

Competing interests

The authors reported no declarations of interest.

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Author contributions

Xu Wu, Jianbo Xiao and Yisheng He conceived and supervised the project, interpreted the results and finalized the manuscript. Huimin Huang and Mingxing Li contributed to experimental designs, performed experiments, interpreted the results, generated figures and wrote the manuscript. Yi Wang, Xiaoxiao Wu, Qin Wang and Shengpeng Wang performed experiments, interpreted the results, and revised manuscript. Jing Shen, Zhangang Xiao, Yueshui Zhao, Huijiao Ji, Fukuan Du, Jing Li and Yu Chen performed experiments, and interpreted the results. Yifei Yang, Lin Liu, Zhigui Wu and Chi Hin Cho interpreted the results and revised manuscript. All authors discussed the results and revision of the manuscript, and approved the manuscript.

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Figures

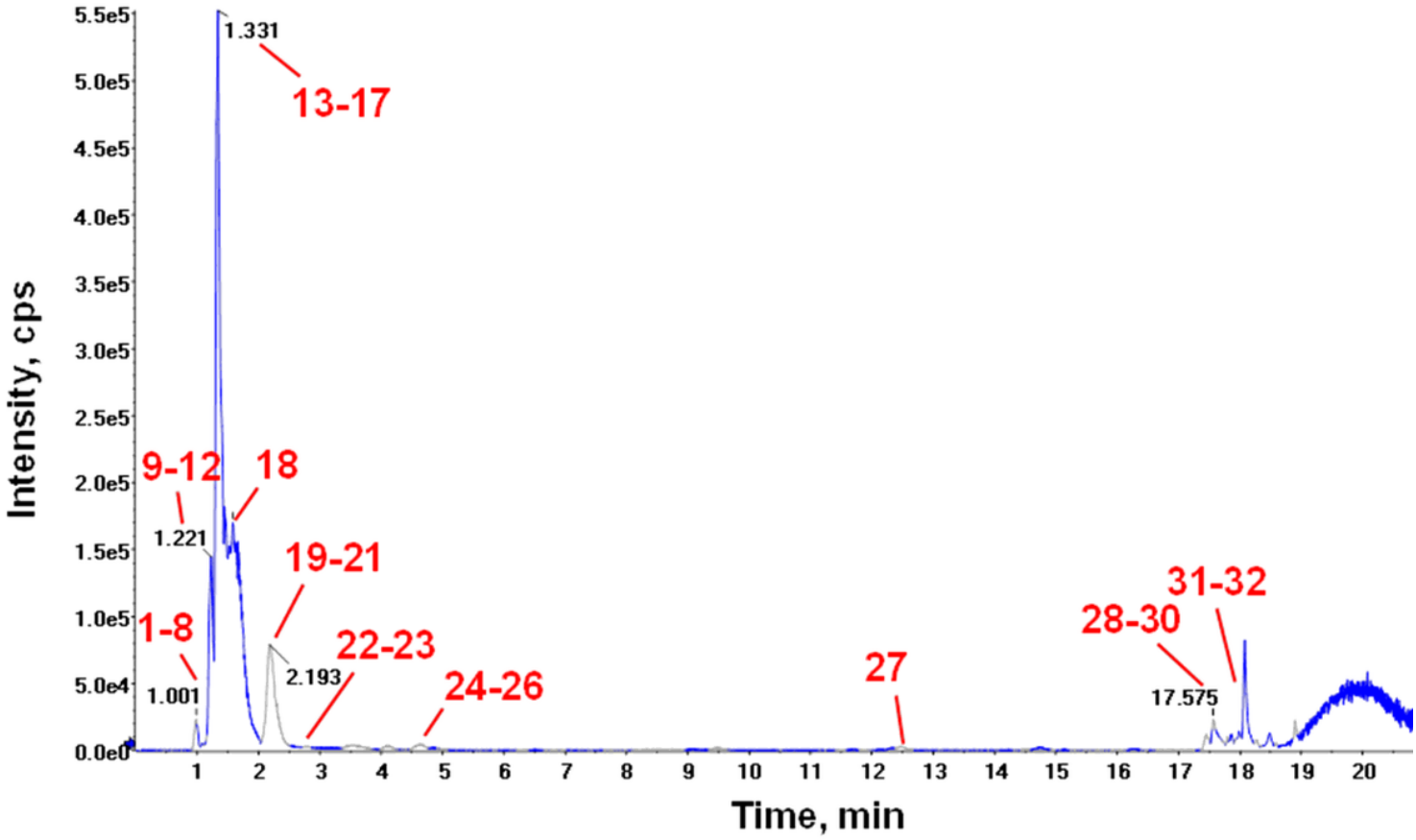

Figure 1

LC-HRMS chromatogram of longan extract. The number labelled with red color shows identified constituents in Table 2.

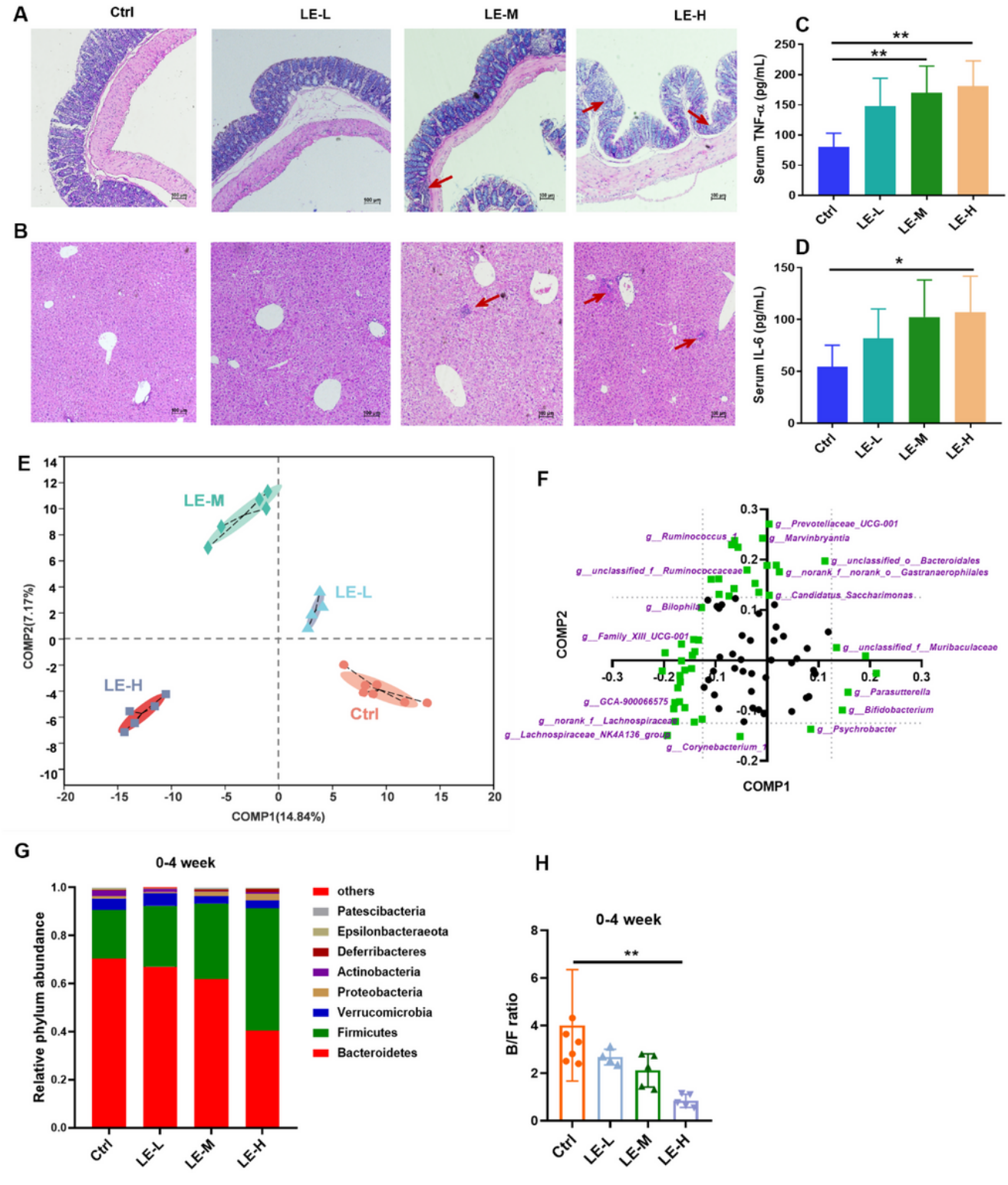

Figure 2

Excessive longan intake promotes inflammation in mice, induces structural changes of intestinal microbiota. (A and B) H&E staining of colon (A) and liver sections (B) after 4 weeks of longan consumption. The oral dosage of longan for Ctrl, LE-L, LE-M and LE-H group was at 0, 4, 8 and 16 g/kg, respectively. Red arrow shows inflammatory cell infiltration. (C and D) Serum levels of TNF-α and IL-6 in mice after 4 weeks of longan intake. (E) PLS-DA plot based on microbial OUT level after 4 weeks of

longan administration. (F) Loading plot of bacterial genus contributing to PLS-DA grouping of samples. To distinguish the most significant contributors (indicated by purple color), a threshold (>0.125 or <-0.125) was set for component 1 (Comp 1) and component 2 (Comp 2). (G) Phylum level differences among groups. (H) Ratio of Bacteroidetes/Firmicutes (B/F). Data are presented as mean ± SD (N=4-6). * p< 0.05, **p < 0.01, ***p < 0.001, compared to Ctrl.

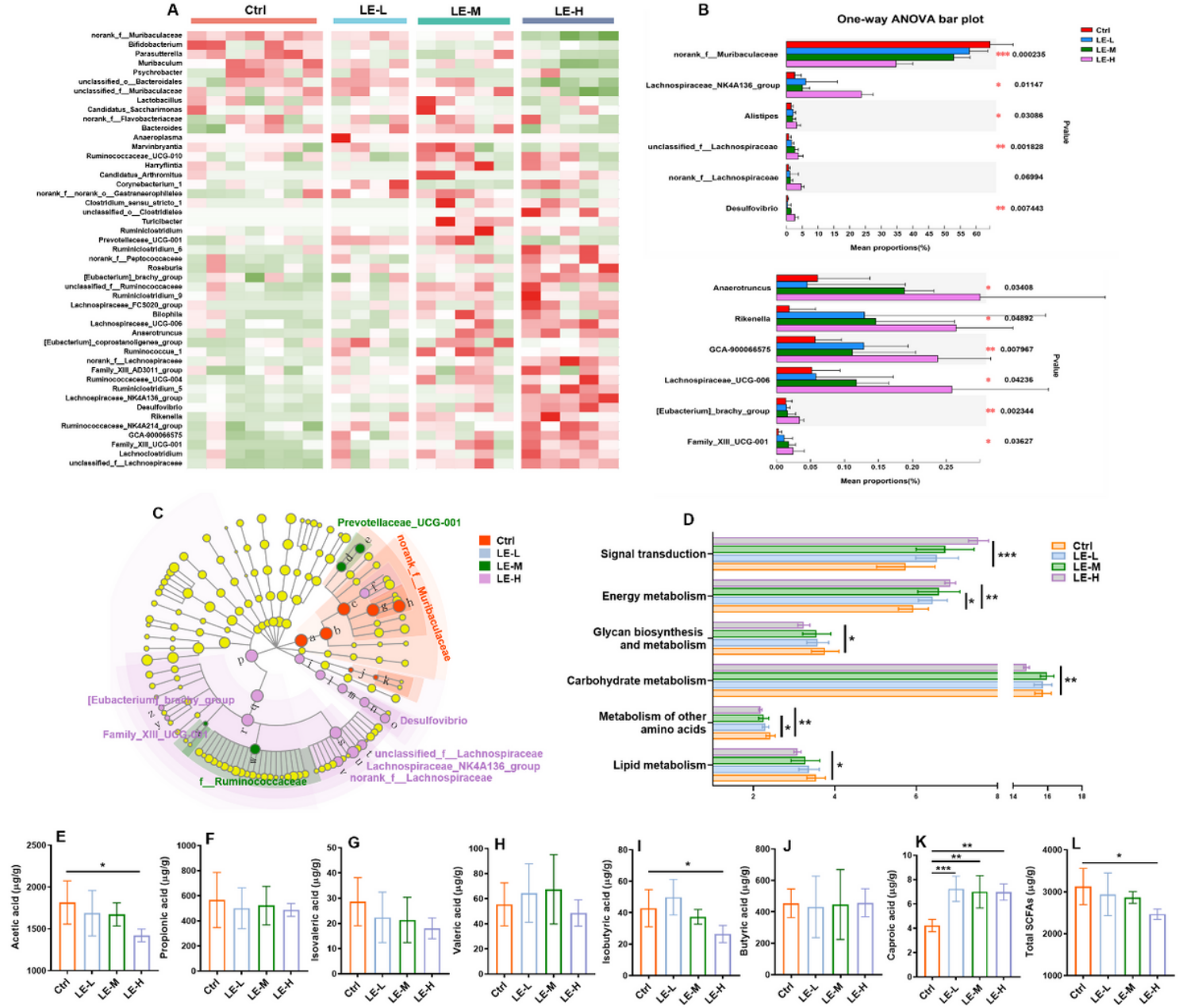

Figure 3

Excessive longan intake specifically alters gut microbiome in normal mice and changes short-chain fatty acids (SCFAs) production. (A) Heatmap analysis based on identified microbe contributors. The most significant contributors were selected by setting a threshold (>0.125 or <-0.125) for the first two components in the loading plot of PLS-DA grouping (Figure 2F). (B) Generic difference among groups after 4 weeks of longan administration in normal mice. (C) Cladogram for Linear discriminant analysis

(LDA) score and LDA effect size (LEfSe) analysis showing enriched bacterial genera in each group. (D) Functional features of the resulting bacterial communities with longan intake predicted by Tax4Fun. Contents of acetic acid (E), propionic acid (F), isovaleric acid (G), valeric acid (H), isobutyric acid (I), butyric acid (J), and caproic acid (K) in colonic lumen. (L) Total contents of SCFAs. Data are presented as mean ± SD (N=4-6). *p < 0.05, **p < 0.01, ***p < 0.001, by one-way ANOVA followed by post hoc Tukey test.

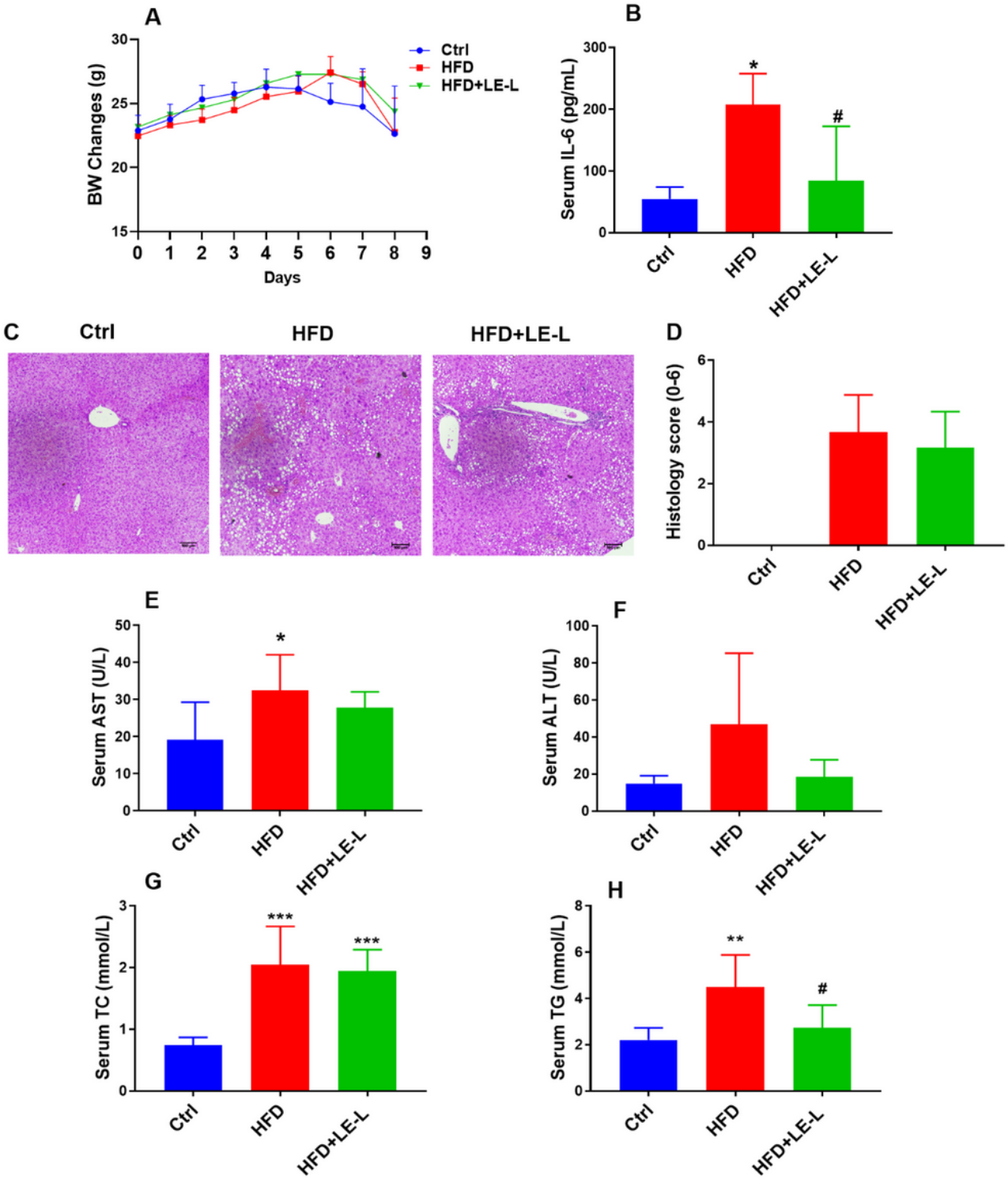

Figure 4

Longan extract at a normal dose of 4 g/kg did not exaggerate the NAFLD pathogenesis in mice. (A) Body weight of mice. (B) Serum level of IL-6. (C) H&E-stained sections of liver tissue. (D) Histological score based on H&E staining. (E) Serum level of AST. (F) Serum level of ALT. (G) Serum level of TC. (F) Serum level of TG. Ctrl, normal diet-fed mice; HFD, HFD-fed mice; HFD+LE-L, HFD-fed mice supplemented with low-dose longan (4 g/kg). Data are presented as mean ± SD (N=6). *p < 0.05, **p < 0.01, ***p < 0.001, compared with Ctrl; #p < 0.05, compared with HFD, by one-way ANOVA followed by post hoc Tukey test.

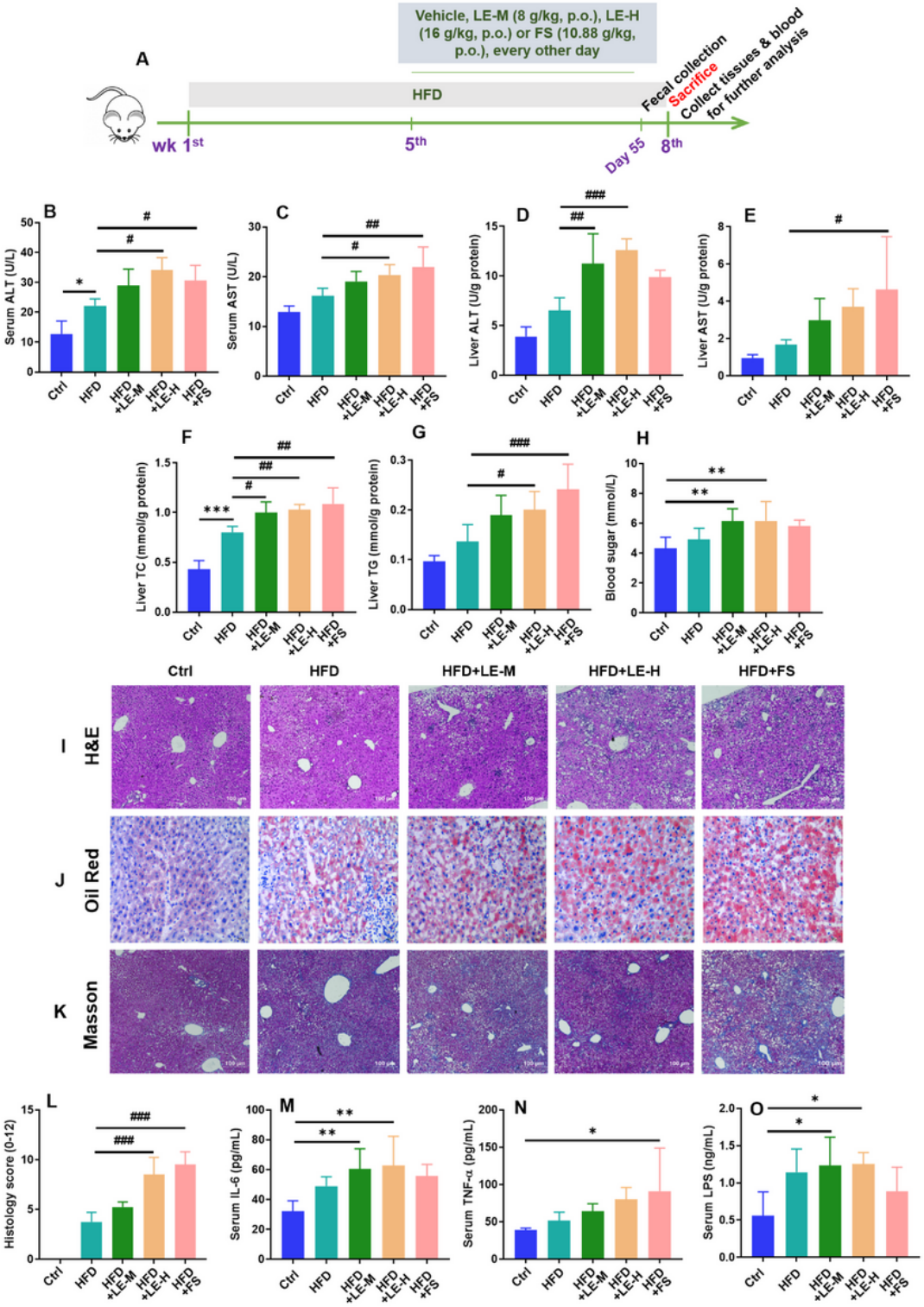

Figure 5

Excessive intake of longan and free sugars aggravates HFD-induced NAFLD. (A) Experimental design of mouse study. (B) Serum ALT level. (C) Serum AST level. (D) Liver ALT level. (E) Liver AST level. (F) Liver TC level. (G) Liver TG level. (H) Blood sugar level. (I) H&E staining of liver sections. (J) Oil-red staining of liver sections. (K) Masson's trichrome staining of liver sections. (L) Histological score based on H&E, Masson's trichrome and Oil-red stains. (M) Serum level of IL-6. (N) Serum level of TNF-α. (O) Serum LPS level. Ctrl, normal diet-fed mice; HFD, HFD-fed mice; HFD+LE-M, HFD-fed mice supplemented with medium-dose longan (8 g/kg); HFD+LE-H, HFD-fed mice supplemented with high-dose longan (16 g/kg); HFD+FS, HFD-fed mice supplemented with free sugars (10.88 g/kg, equivalent to that contained in high-dose longan). Data are presented as mean ± SD. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, compared to Ctrl; #$p < 0.05$, ##$p < 0.01$, ###$p < 0.001$, compared to HFD group, by one-way ANOVA followed by post hoc Tukey test.

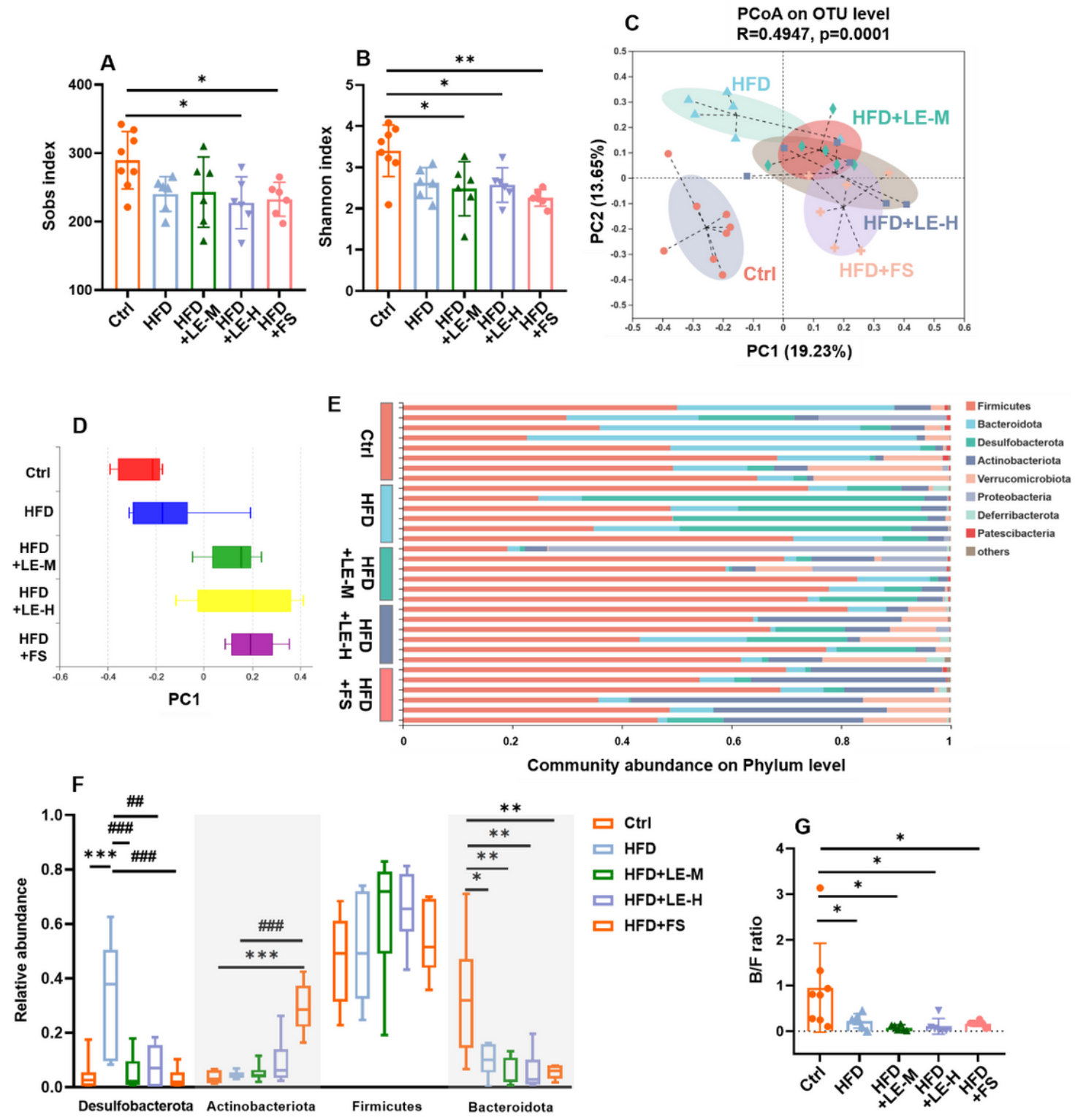

Figure 6

Excessive intake of longan and free sugars promotes HFD-induced dysbiosis. (A) Sobs index for microbial richness. (B) Shannon index for microbial diversity. (C) PCoA analysis based on OUT level and (D) the discrete degree of PC1. (E) Community abundance on phylum level. (F) Abundance differences of the main phyla among groups. (G) Ratio of Bacteroidetes/Firmicutes (B/F). Data are presented as mean

± SD (N=6-7). *p < 0.05, **p < 0.01, ***p < 0.001, compared to Ctrl; #p < 0.05, ##p < 0.01, ###p < 0.001, compared to HFD group, by one-way ANOVA followed by post hoc Tukey test.

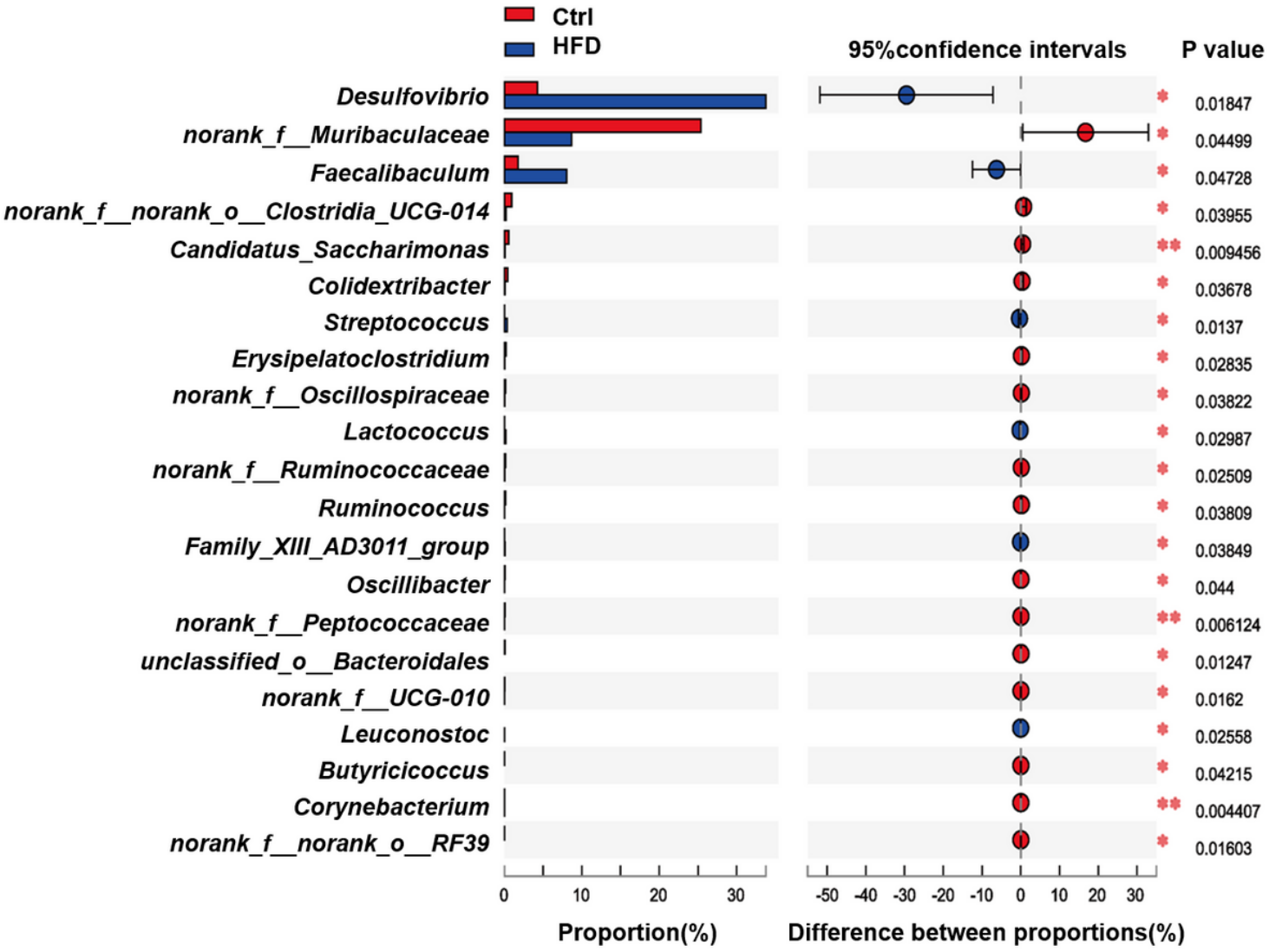

Figure 7

Generic difference between HFD and Ctrl group. *p < 0.05, **p < 0.01, ***p < 0.001, compared to Ctrl, by student t test (N=6-7).

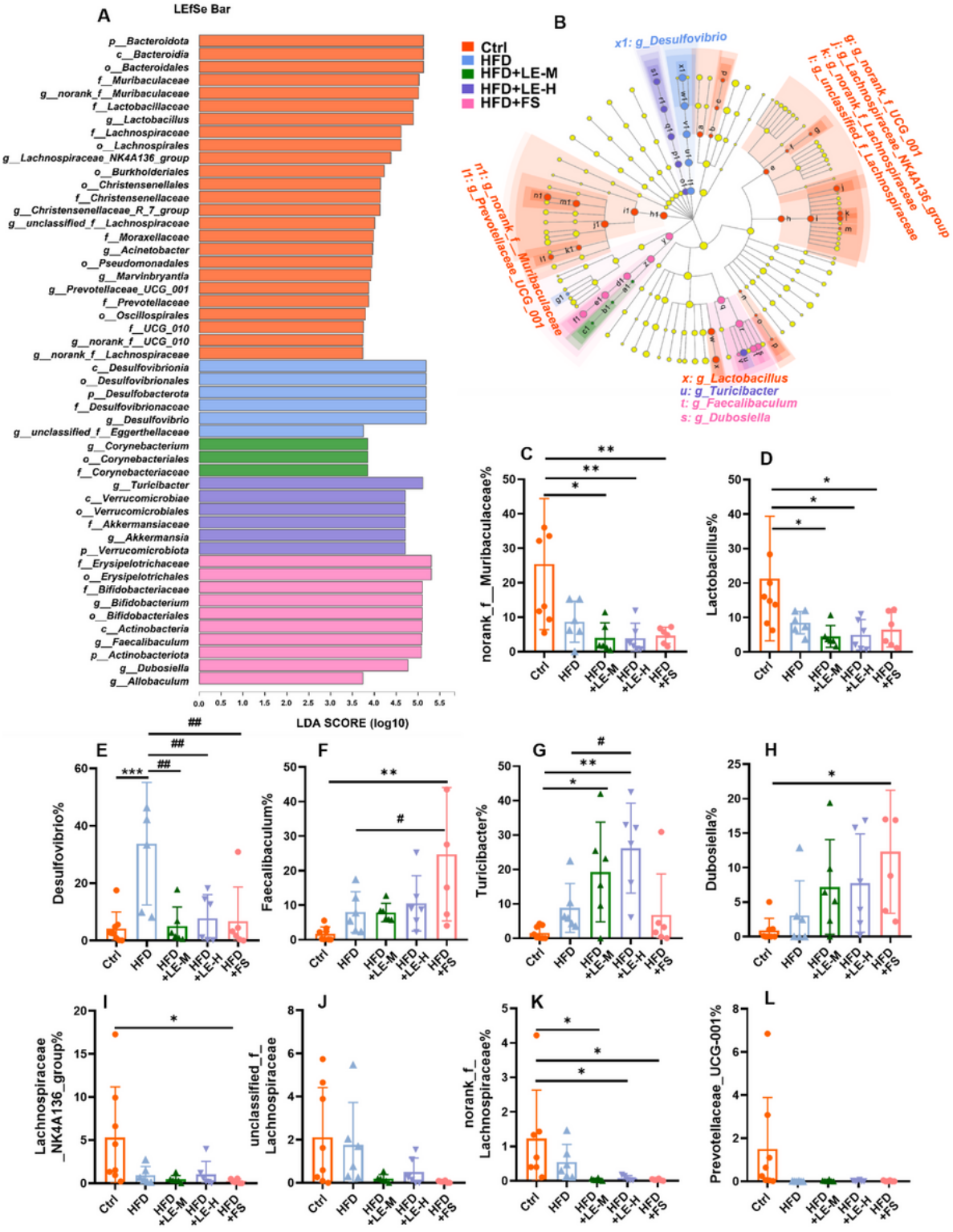

Figure 8

Identification of key microbial changes at genus level resulted from excess longan and free sugar intake in NAFLD mice. (A) Linear discriminant analysis (LDA) score and LDA effect size (LEfSe) analysis with LDA score > 3.5. (B) Cladogram for LEfSe analysis. Generic difference among groups: (C) norank_f__Muribaculaceae; (D) Lactobacillus; (E) Desulfovibrio; (F) Faecalibaculum; (G) Turicibacter; (H) Dubosiella; (I) Lachnospiraceae_NK4A136_group; (J) unclassified_f__Lachnospiraceae; (K)

norank_f__Lachnospiraceae; (L) Prevotellaceae_UCG-001. Data are presented as mean ± SD (N=6-7). *p < 0.05, **p < 0.01, ***p < 0.001, compared to Ctrl; #p < 0.05, ##p < 0.01, ###p < 0.001, compared to HFD group, by one-way ANOVA followed by post hoc Tukey test.

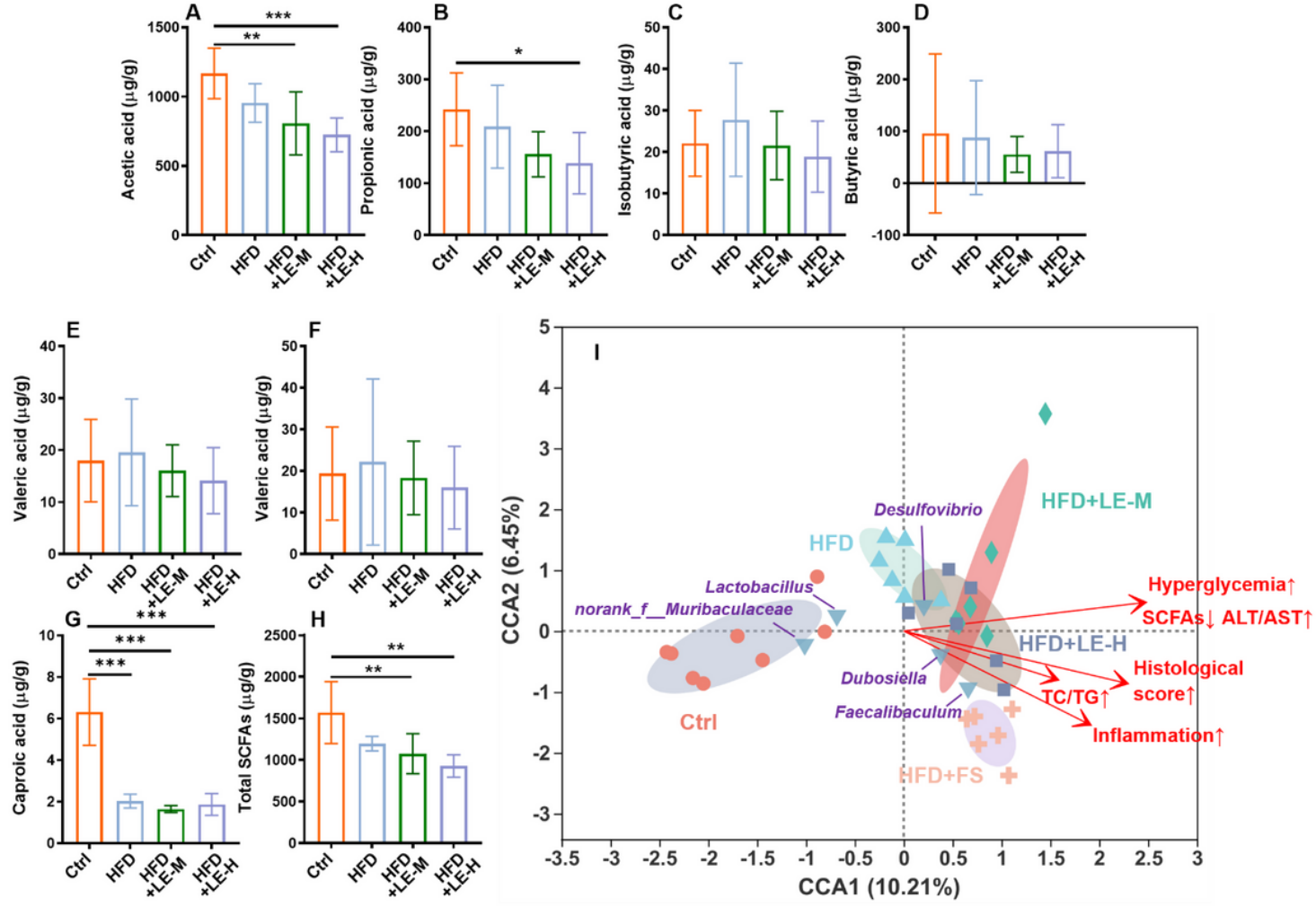

Figure 9

Excess longan intake reduces SCFAs production: Contents of acetic acid (A), propionic acid (B), isovaleric acid (C), valeric acid (D), isobutyric acid (E), butyric acid (F), caproic acid (G) and total SCFAs (H) in colonic contents. (I) Canonical correlation analysis (CCA) analysis on association of key microbial changes, SCFA production and main pathological abnormities cross different groups. Data are presented as mean ± SD (N=6-7). *p < 0.05, **p < 0.01, ***p < 0.001, compared to Ctrl; #p < 0.05, compared to HFD group, by one-way ANOVA followed by post hoc Tukey test.

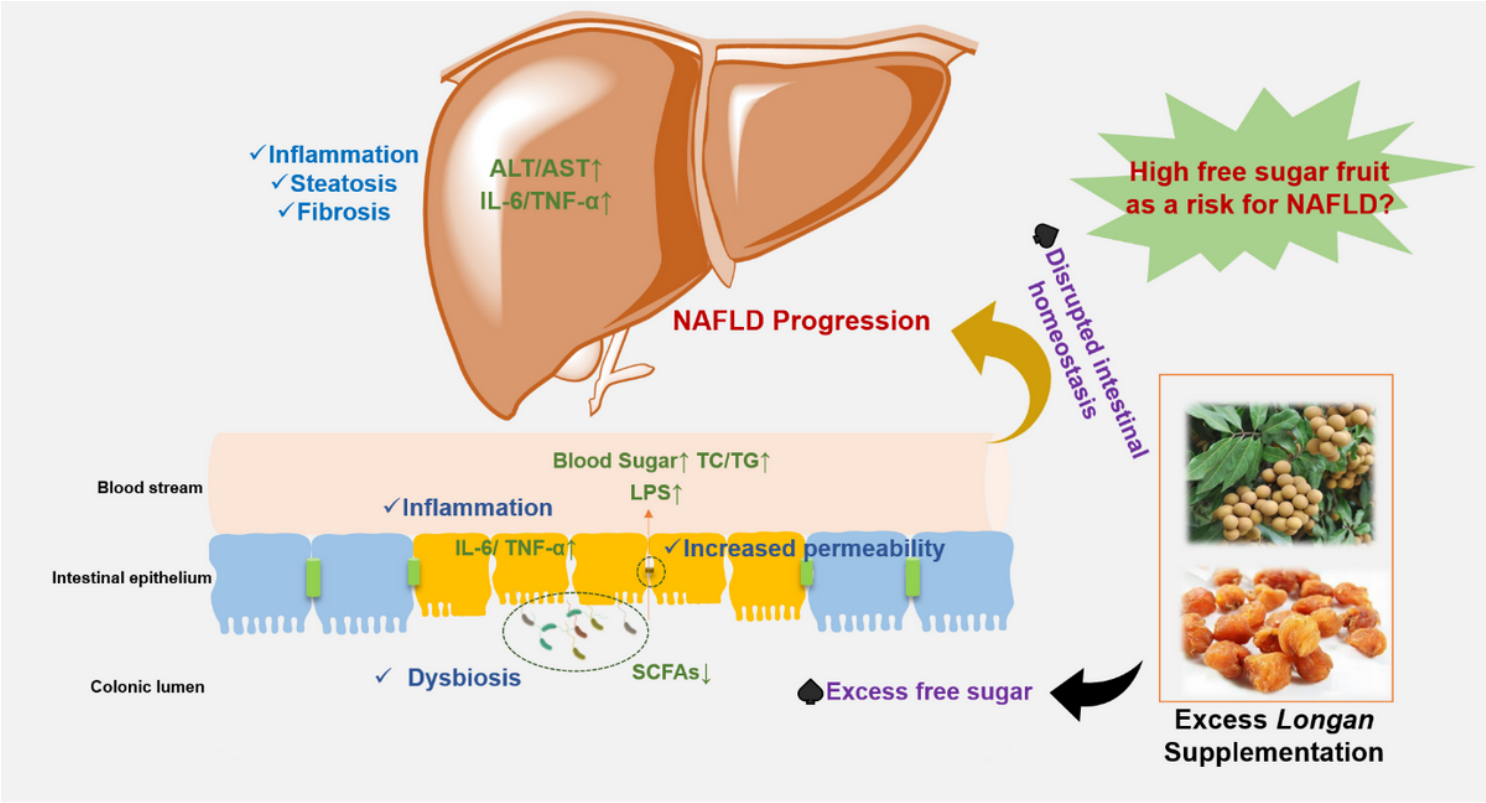

Figure 10

Schematic illustration of the progression of NAFLD development by excessive longan intake and its association with excess free sugar intake and disrupted intestinal homeostasis.