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Ferulic acid increases intestinal Lactobacillus and improves cardiac function in TAC mice

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ABSTRACT

Ferulic acid, a main ingredient of Ligusticum, exhibits anti-oxidant and anti-inflammation effects in heart diseases. Some studies indicate that gut microbiome is associated with the generation of ferulic acid. Whether the beneficial effect of ferulic is raised by the alteration of gut microbiota is still unknown. This study examined the effect of sodium ferulate on gut microbiome and cardiac function in TAC mice. Cell Counting Kit-8 (CCK8) assay verified that ferulic acid has low toxicity in vitro and that ferulic acid inhibited the up-regulation of β-MHC and ANP induced by Angiotensin II. In addition, daily supplement of 50 mg/kg sodium ferulate improved the ejection fraction and changed the gut microbiota composition of TAC mice. Relative abundance of *Lactobacillus* and *Parabacteroides* are increased in TAC mice gavaged with sodium ferulate. In addition, *Lactobacillus* is negatively correlated with HW/BW and LW/BW ratio. These results suggest that the beneficial effect of ferulic in TAC mice is probably through the regulation of gut microbiota.

1. Introduction

Cardiac hypertrophy is a compensated stage of heart failure, which is generally accompanied by oxidative stress. Ferulic acid, a main ingredient from ligusticum, could exert beneficial effects in various chronic diseases. Previous studies suggested that ferulic acid inhibits lipid peroxidation and exhibits DNA protective effect [1]. In addition, Ferulic acid inhibits renal tubulointerstitial fibrosis [2] and improves cognition and neurodegeneration in Alzheimer's disease [3]. Some studies also indicated its roles in cardio-protection and anti-myocardial hypertrophy [4].These universal functions might be due to its anti-oxidant and anti-inflammatory effects.

Recent evidences have showed that the gut microbiota influences the generation of ferulic acid [5,6] whilst ferulic acid itself can inversely modulate the gut microbiota [7]. Gut microbiota plays an important role in the regulation of health and various diseases [8–12]. For example, Trimethylamine N-oxide (TMAO), a metabolic product influenced by gut microbiota, promotes the genesis and development of various cardiovascular diseases (CVDs) [13–15]. It is also known that diet or drugs might interact with the host body via changing the intestinal bacterial flora. Therefore, the alteration of gut microbiota composition would probably affect prognosis and progress of CVDs.

Thus, we hypothesized that the beneficial effects of ferulic acid would probably be achieved through the gut microbiota. In this study, we examined the effects of sodium ferulate on gut microbiome and cardiac function in transverse aortic constriction (TAC) mice.

2. Methods

2.1. Experimental animals

All C57BL/6 J mice and Neonatal Sprague-Dawley rats were obtained from Laboratory Animal center of Southern Medical University. The mice were fed normal chow and water ad libitum under a 12 -h

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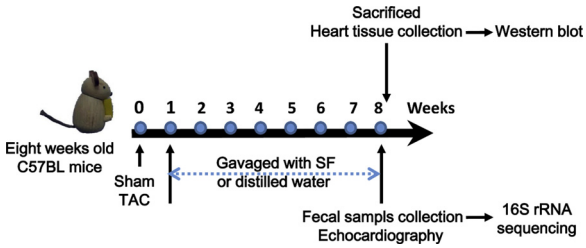

Fig. 1. Time-line diagram of TAC mice.

light/dark cycle. Ten-week-old male mice were subjected to TAC or Sham operation and gavaged with 50 mg/kg Sodium Ferulate (SF) or distilled water for 7 weeks, while neonatal rats were sacrificed for cells extraction. The study was performed in accordance with the recommendations of the Guide for the Care and Use of Laboratory Animals (NIH, 8th Edition, 2011). The study was approved by the ethics review board of Southern Medical University. The time-line diagram of TAC mice is shown in Fig. 1.

2.2. Isolation and culture of neonatal rat ventricular cardiomyocytes (NRVMs)

Neonatal Sprague-Dawley rats were sacrificed via 2% isoflurane inhalation and subsequent cervical dislocation. Hearts were removed, dissected and enzymatically digested with 0.2% pancreatin and collagenase II (1 mg/ml) as previously described [16]. After collection, centrifugation and re-suspension, cells were pre-plated for 90 min of differential adhesion in DMEM containing penicillin and streptomycin (100:1) (Thermo Fisher) and supplemented with 10% FBS (Fetal calf serum) to reduce non-myocyte contamination and then plated in culture dishes and incubated at 37 °C in 5% CO_2 incubator. Following 48 h at 37 °C, NRVMs were treated with various concentrations of ferulic acid (Selleck) and stimulated with 10^{-5} M Ang II (Abcam) for 24 h in DMEM without FBS.

2.3. Measurements of cell viability

Cell viability under different concentrations of ferulic acid was determined using the Cell Counting Kit-8 (Dojindo, Tokyo, Japan) according to manufacturer's guideline.

2.4. Western blotting

Proteins were extracted from the cultured cardiomyocytes and heart tissue by RIPA buffer (Beyotime biotechnology, Shanghai, China) which were mixed with protease inhibitor (Sigma, U.S.A) (1:100) and quantified by BCA assay (Thermo fisher). Primary antibodies used included Anti-β-MHC antibody (1:1000, Abcam, U.S.A), Anti-ANP antibody (1:500, Abcam, U.S.A) and anti-GAPDH antibody (1:3000, Proteintech, U.S.A). Second antibody used was goal anti-rabbit IgG-HRP (1:3000,

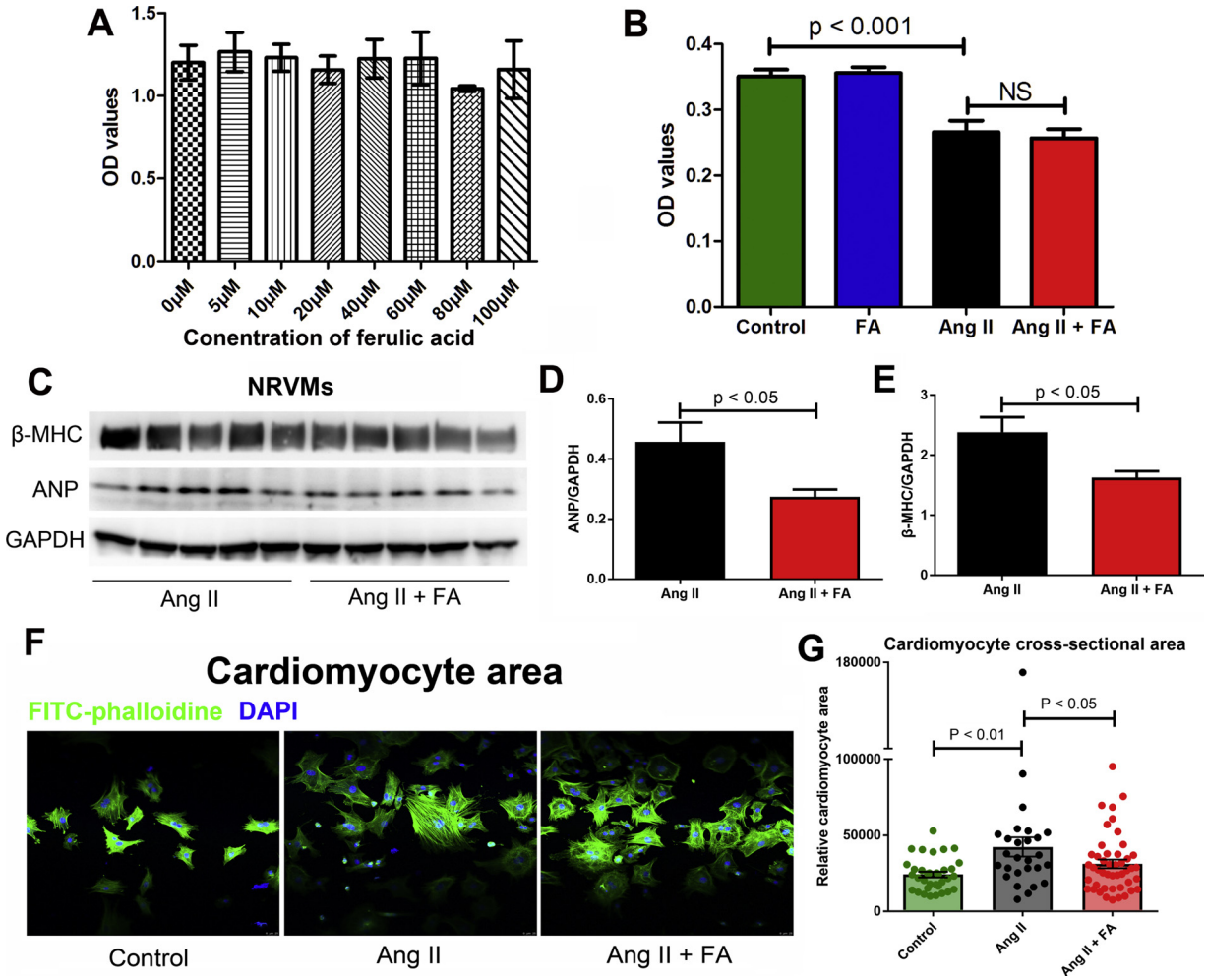

Fig. 2. (A) CCK8 assay to test the cell viability of NRVMs in different concentrations of ferulic acid (n = 4–6 per group). (B) Cell viability of NRVMs challenged with angiotension II (Ang II). (C–E) Western bloting of ANP and β-MHC between Ang II and Ang II + FA groups (n = 5 per group). (F–G) NRVMs were exposed to Ang II and ferulic acid, and their cross-sectional areas were visualized by Phalloidine staining (n = 34, 27 and 46 in control, Ang II and Ang II + FA groups, respectively).

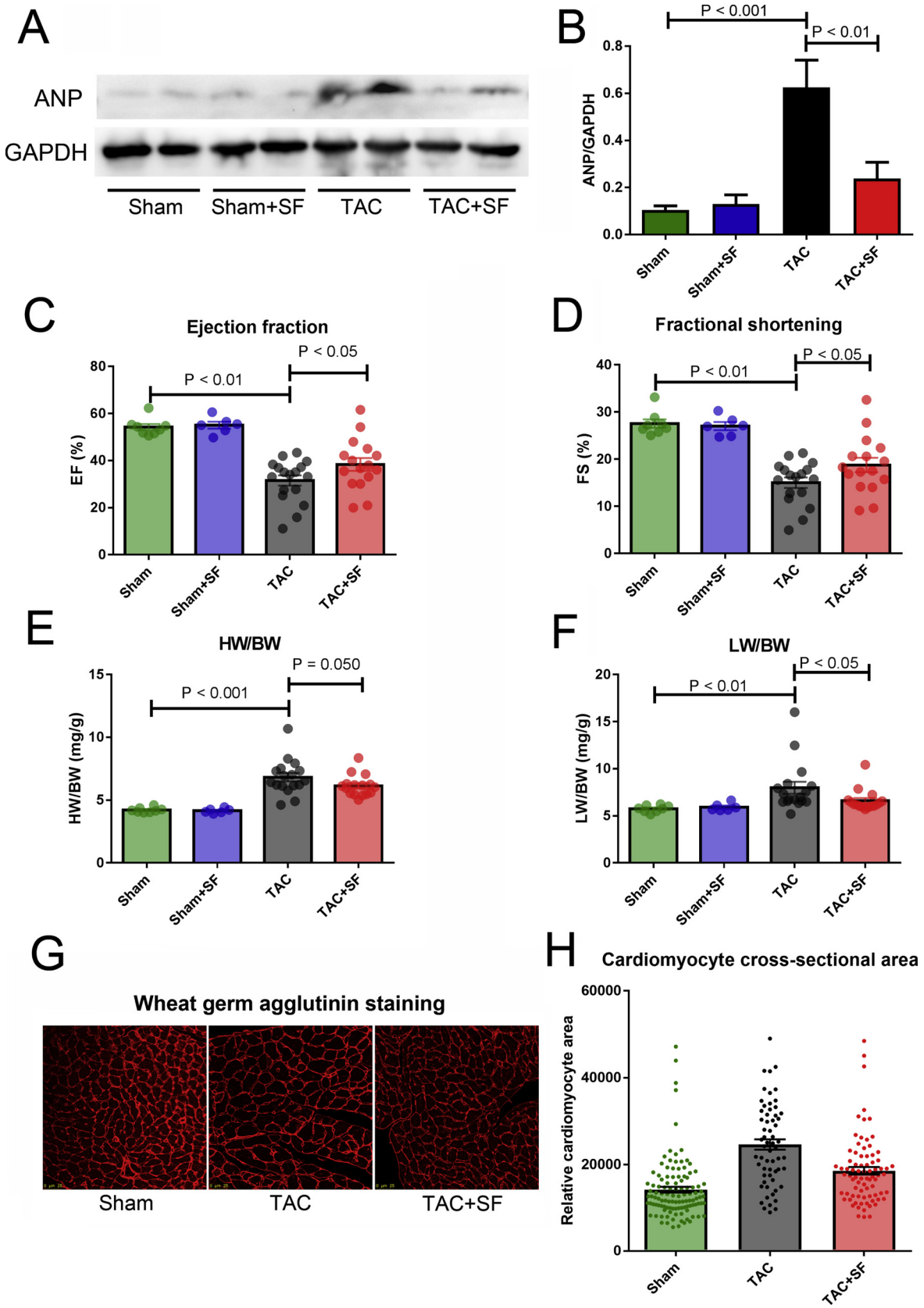

Fig. 3. (A–B) Relative expression of ANP in heart tissue among Sham, Sham + SF, TAC and TAC + SF groups (n = 4 per group). Cardiac function reflected by (C) Ejection Fraction (EF%) and (D) Fractional Shortening (FS%) as well as (E) the ratio of heart weight to body weight (HW/BW), (F) the ratio of lung weight to body weight (LW/BW) (n = 8, 6, 17 and 16 in Sham, Sham + SF, TAC and TAC + SF groups, respectively). (G) the representative picture of heart tissue sections stained with WGA in each group and (H) their relative cardiomyocyte cross-sectional areas in these regions of interest (n = 112, 61 and 79 in Sham, TAC and TAC + SF mice, respectively).

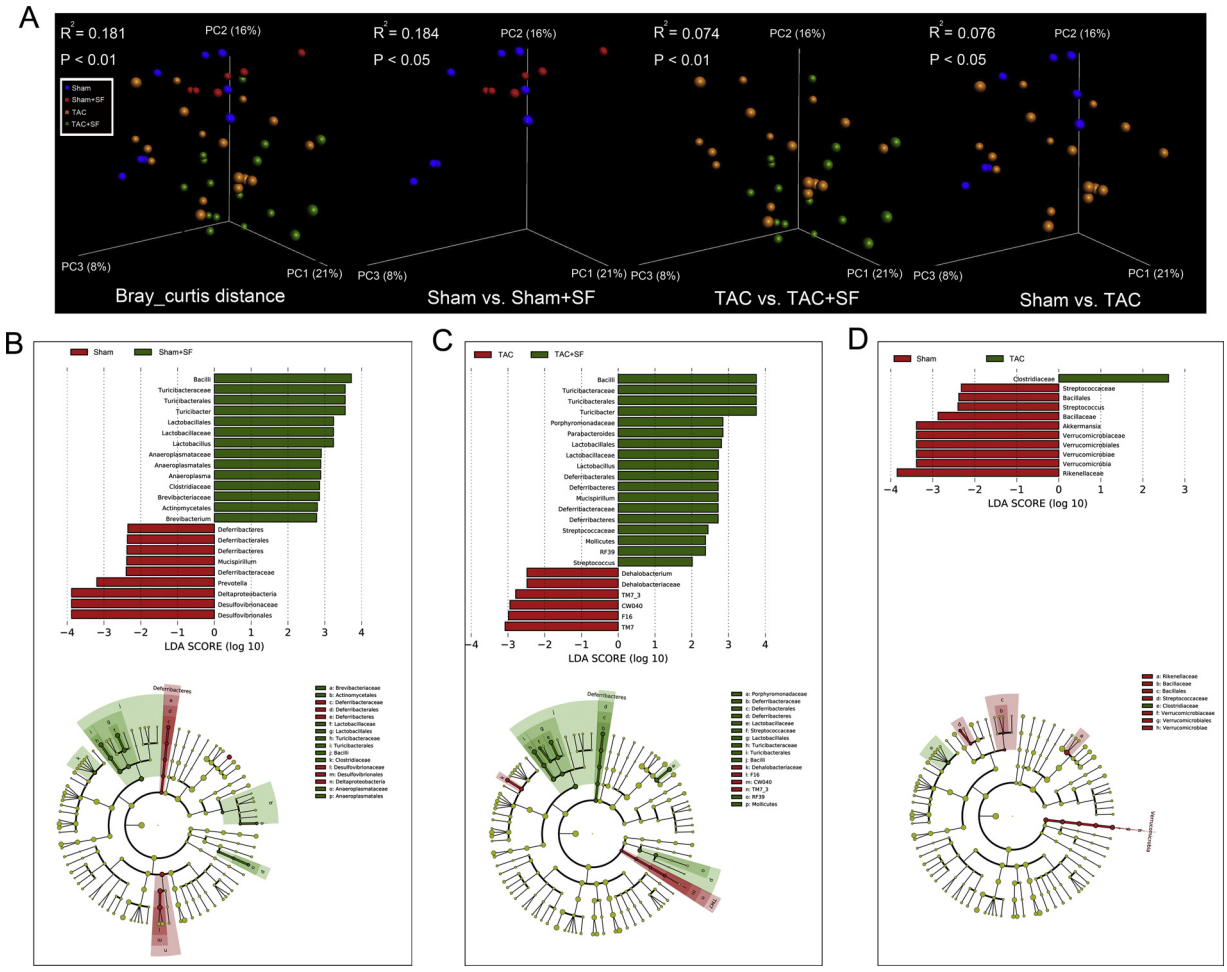

Fig. 4. (A) Comparison of the microbial community among Sham, Sham + SF, TAC and TAC + SF groups based on Bray_curtis distance obtained in a PCoA analysis. (B–D) LEfSE in the bacterial community structure between these groups (n = 8, 6, 17 and 16 in Sham, Sham + SF, TAC and TAC + SF groups, respectively).

CST, U.S.A). Immunoreactive bands were detected by ECL Substrate (FDbio, Hangzhou, China) and visualized by Gene Gnome Imaging System (Syngene Bio Imaging) and quantified by densitometry with Image J software (NIH).

2.5. Phalloidin staining

NRVMs were treated with Ang II (10^{-6} M) or ferulic acid (40 μM) for 24 h. After cells were washed three times by phosphate buffer (PBS), they were fixed with 4% paraformaldehyde for 10 min and washed by phosphate buffered saline containing 0.1% Triton X-100, and then stained with FITC-phalloidin (Actin-tracker green) (Beyotime, China) and DAPI (Beyotime, China) for 60 min and 5 min, respectively. And the results were observed by confocal microscopy.

2.6. Model of transverse aortic constriction (TAC)

The mice subjected to operation in the study were anesthetized using an intraperitoneal injection of a mixture of xylazine (5 mg/kg) and ketamine (100 mg/kg). After anesthesia, the mice underwent Transverse Aortic Constriction (TAC). The method was described previously [17]. Briefly, after an endotracheal tube was inserted, the mice were supported by a respiratory machine (ventilator). The thorax of the mice was opened and underwent banding of arcus aortae with 26 gauge needle. The needle was withdrawn after tight banding while on the other hand, sham operated mice were subjected to the same treatment without banding. Eight weeks after operation, the mice were sacrificed by overdose of anesthesia with intra-peritoneal injection of pentobarbital sodium and cervical dislocation. Weight of the heart and lungs as well as the length of the tibia were measured subsequently.

2.7. Echocardiography

Echocardiography was performed in anesthetized (2% isoflurane) mice using a VEVO2100 system (Visual Sonic, North American). The long axis and short axis were acquired in B-Mode, while the measurements were performed under M-Mode.

2.8. Wheat germ agglutinin (WGA) staining

Heart tissue cardiomyocyte cross-sectional areas were visualized by WGA staining Briefly, after heart tissues were embedded and cleanly sliced, tissue slices were dewaxed and the antigen was restored by sodium citrate followed by incubation with WGA (Wheat Germ Agglutinin, Texas Red-X Conjugate, Thermal Fisher) (1:1000, 1 mg/ml) for 48 h.

Excessive dye was removed and washed 3 times by PBS for 5 min. WGA staining was examined and photographed using a Leica SP8 microscope.

2.9. DNA extraction, 16S rRNA gene amplification, bioinformatics and statistical analysis

Fecal samples were collected in a sterility tube and then processed

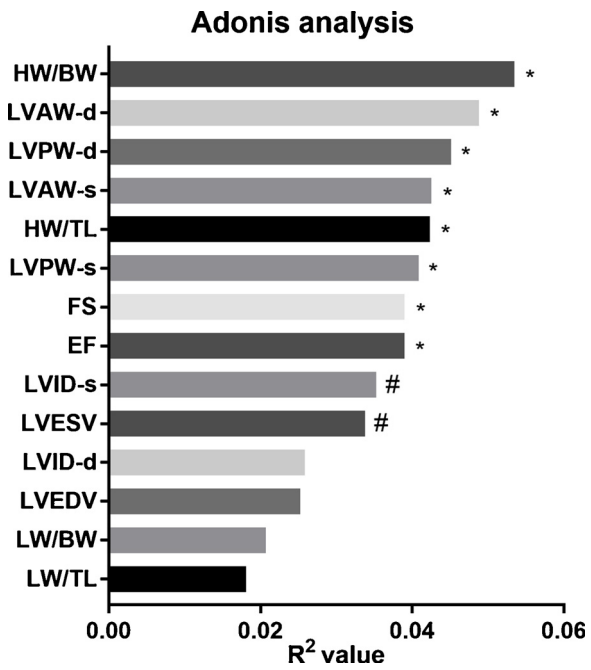

Fig. 5. Beta diversity of the gut microbial community among cardiac indices. HW/BW, the ratio of heart weight/body weight; LW/BW, the ratio of lung weight/body weight; LW/TL, the ratio of lung weight to tibial length; HW/TL, the ratio of heart weight to tibial length. LVAW-d, diastolic left anterior wall thickness; LVAW-s, systolic left anterior wall thickness; LVPW, left ventricular posterior wall thickness; LVID, left ventricular internal diameter; FS, fractional shortening; EF, ejection fraction; LVESD, left ventricular end-systolic diameter; The R2 value represents the results of Adonis in Bray-curtis distance.*P < 0.05, #P < 0.1.

with a Fecal Total DNA EXTRACT kit (Qiagen, Germany) for the extraction of bacterial genomic DNA. The process was strictly performed according to the manufacturer's protocol. The 16S rRNA V4 hypervariable region was amplified and sequenced by Illumina HiSeq PE150.

We used the QIIME [18] to process the raw sequences, cluster operational taxonomic unit (OTU) and for further classification. The details in processing sequences have been described previously [9]. Briefly, PCoA analysis was performed based on the Bray-Curtis distance and Adonis was used to estimate the amount of dissimilarity in microbial compositions between groups. We used LEfSE (linear discriminant analysis effect size) to identify features that differed between the groups. The threshold on the logarithmic LDA score for discriminative features was set to 2.0.

Quantitative data were displayed as mean ± SEM (standard error of mean). Normal distribution test was performed to determine parameter or non-parameter tests. Comparison between two experimental groups was based on a two-tailed t-test, while comparisons of parameters among more than 3 groups were analyzed by ANOVA followed by LSD or Dunnett's T3 for post hoc multiple comparisons. All statistical analyses were performed using SPSS 20.0 and R version 3.5.1.

3. Results

3.1. Cyto-toxicity test of ferulic acid in NRVMs by CCK8

In order to verify the toxicity of ferulic acid, CCK8 assays were performed. No significant difference was detected after the treatment of various concentration of ferulic acid from 0 μM to 100 μM for 24 h (n = 4–6 per group) (Fig. 2A).

3.2. Effect of ferulic acid on NRVMs treated by Angiotension II

Then, we explored the effect of ferulic acid in NRVMs challenged with Angiotension II (10^{-5} M). We found that Ang II treatment reduced the cell viability compared with control ($P < 0.001$), while 40 μM ferulic acid treatment did not reverse this impairment (Fig. 2B). However, the expression of ANP ($P < 0.05$) and β-MHC ($P < 0.05$) in Ang II treated cells were down-regulated by ferulic acid (n = 5 per group) (Fig. 2C E).

3.3. Cardiomyocyte cross-sectional area visualized by Phalloidin staining

Next, NRVMs were visualized by Phalloidin staining and measured by image pro plus software. We found that ferulic acid decreased the cross-sectional area of hypertrophy NRVMs ($P < 0.05$) (n = 34, 27 and 46 in control, Ang II and Ang II + FA groups, respectively) (Fig. 2F–G)

3.4. Sodium ferulate supplementation improves cardiac function of TAC mice

The cardiac beneficial effects of sodium ferulate have been proved in previous studies [19]. Our results are in accordance with these findings. TAC mice gavaged with 50 mg/kg soudium ferulate (SF) expressed decreased levels of ANP in heart tissue compared with TAC mice ($P < 0.01$) (Fig. 3A–B). Although the ratio of HW/BW in TAC + SF mice seemed to have a downward trend when compared with TAC group (Fig. 3E, $P = 0.050$), mice gavaged with SF have higher levels of ejection fraction (Fig. 3C), Fractional Shortening (Fig. 3D) and lower levels of LW/BW (Fig. 3F) ratio when compared with their corresponding TAC groups (n = 8, 6, 17 and 16 in Sham, Sham + SF, TAC and TAC + SF groups, respectively). We also visualized the cardiomyocytes sizes in heart tissue by WGA staining and measure their relative cardiomyocyte cross-sectional areas in regions of interest (Fig. 3G–H).

3.5. Sodium Ferulate supplementation changes the gut microbiota and increases intestinal abundance of Lactobacillus

Then, we performed PCoA in QIIME to identify differences in the composition of the bacterial community structure between Sham and Sham + SF, Sham and TAC as well as TAC and TAC + SF groups (Fig. 4A). Fecal samples were clustered into four distinct sets in these groups, indicating their different bacterial community structures. These differences were further confirmed by the results of the Bray-curtis distance values in the Adonis analysis which were significantly different in these groups ($R^2 = 0.184$, $P < 0.05$, Sham *vs.* Sham + SF groups) ($R^2 = 0.076$, $P < 0.05$, Sham *vs* TAC groups) ($R^2 = 0.074$, $P < 0.01$, TAC *vs.* TAC + SF groups) (Fig. 4A). These results, altogether indicate that the gut microbiome between Sham and TAC are different. In addition ferulic admission alters gut microbiota of Sham as well as TAC mice.

To further confirm the effect of ferulic in the gut microbiome of mice, we performed a LEfSE analysis to determine which bacterial taxa were distinct within these groups. Sham + SF group have higher level of *Lactobacillus* and *Turicibacter* (Fig. 4B). *Turicibacter*, *Parabacteroides*, *Lactobacillus*, *Mucispirillum*, *Streptococcus* were significantly increased in TAC + SF group, while *Dehalobacterium* was significantly more abundant in TAC mice (Fig. 4C). Comparing Sham and TAC groups, we found that *Akkermansia*, a newly discovered probiotics which could be increased by physical exercise [20], were decreased in TAC mice (Fig. 4D) (n = 8, 6, 17 and 16 in Sham, Sham + SF, TAC and TAC + SF groups, respectively).

3.6. Association between gut microbial community and cardiac indices

Next, we explored the relationship between cardiac indices and beta

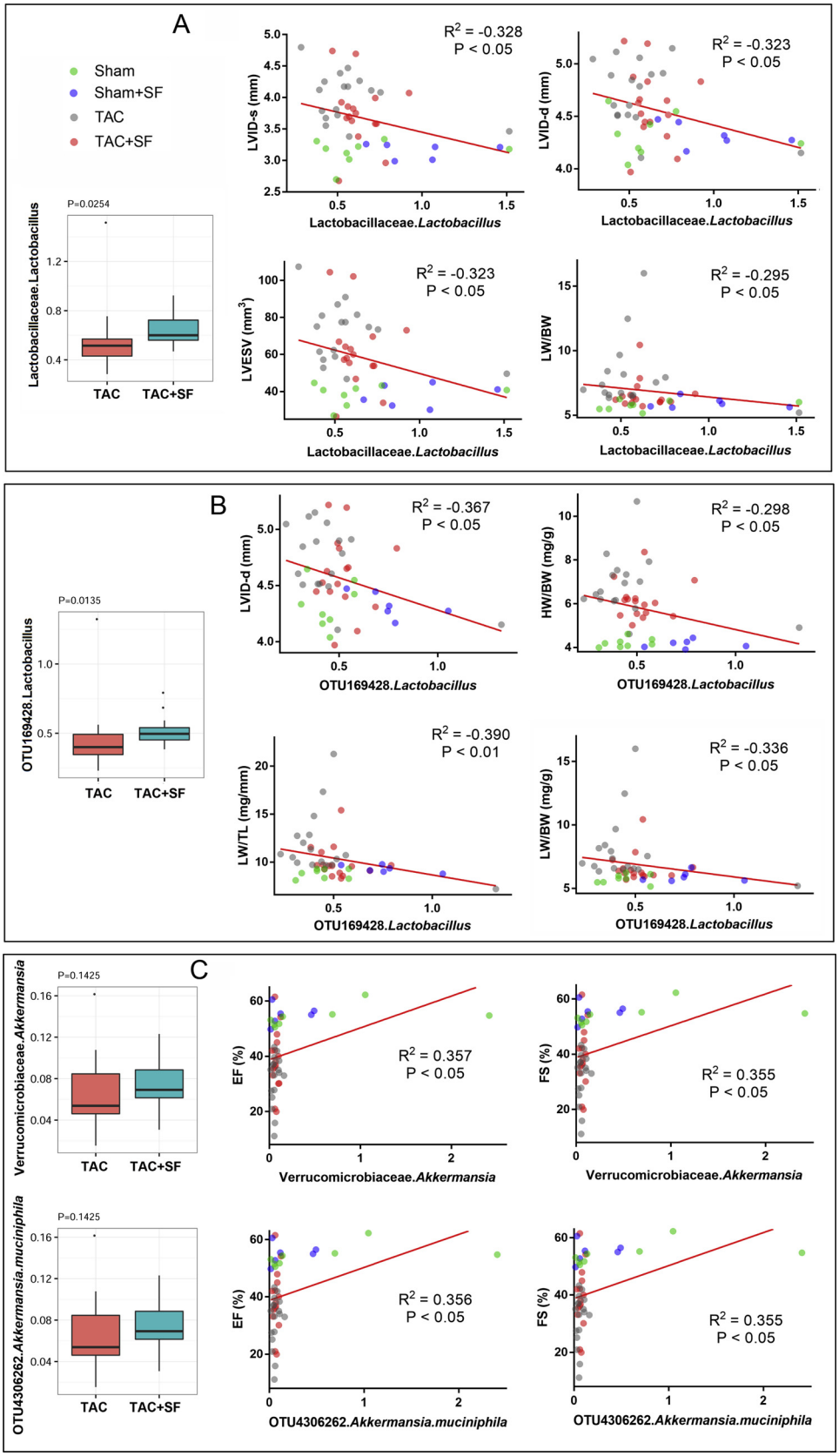

Fig. 6. (A) The relative abundance of Lactobacillaceae *Lactobacillus* between TAC and TAC + SF groups, while the scatter plots show its correlations with cardiac function. (B) The relative abundance of OTU169428 *Lactobacillus* between TAC and TAC + SF groups, and the scatter plots show its correlations with cardiac function (C) The relative abundance of *Akkermansia* and OTU4306262 *Akkermansia* between TAC and TAC + SF groups, and their correlations with cardiac indices (n = 8, 6, 17 and 16 in Sham, Sham + SF, TAC and TAC + SF groups, respectively).

diversity in the gut microbial community. The results showed that HW/BW, LVAW-d, LVPW-d, LVAW-s, HW/TL, LVPW-s, FS and EF were significantly correlated with the gut microbiota in Adonis analysis (Bray-curtis distance, $P < 0.05$) (Fig. 5).

3.7. The correlation of Lactobacillus, Akkermansia and cardiac indices

It is possible that the advantage of sodium ferulate in the heart is achieved by influencing intestinal bacteria. Thus, we performed correlation analysis between cardiac indices and *Lactobacillus*, a probiotics increased in TAC mice gavaged with soudium ferulate (Fig. 6A–B). A genus from *Lactobacillus* is also negatively correlated with LVID-s, LVID-d, LVESV and LW/BW (Fig. 6A), while an OTU from Lactobacillus is negatively correlated with LVID-d, HW/BW, HW/TL and LW/TL (Fig. 6B). These findings indicated that *Lactobacillus* is associated with cardiac function. In addition, *Akkermansia* might also be a cardiovascular disease therapeutic target for its positive correlation with EF and FS (Fig. 6C), though it showed no significance between TAC and TAC + SF groups (n = 8, 6, 17 and 16 in Sham, Sham + SF, TAC and TAC + SF groups, respectively).

4. Discussion

Ferulic acid, a potential anti-oxidant reagent, is derived from Chinese herbs, but it can also be generated by intestinal bacteria [21]. A recent study indicated that the ingestion of plant fibers increases the amount of ferulic acid through affecting gut microbiota [21]. Although accumulating evidence has suggested that ferulic acid is beneficial in CVDs [22,23], no study has investigated the interactions between ferulic acid and gut microbiota in heart diseases. In this study, we find that sodium ferulate, a soluble form of ferulic acid, ameliorates cardiac failure possibly through regulating the gut microbiota.

Gut microbota have been shown to be closely linked with cardiovascular diseases [9,15] and the harbors of probiotics would alleviate the inflammation of host body and even slow the progress of diseases. In this study, we found that mice gavaged with ferulic harbored a greater amount of *Lactobacillus*. In addition, *Lactobacillus* is negatively correlated with cardiac-hypertrophy indices. These results suggest that the benefits of ferulic acid might be realized by increasing intestinal *Lactobacillus*. *Lactobacillus* is a recognized probiotics which has been proposed to offer protection from heart diseases [24]. *Lactobacillus murinus* can also counter hypertension, while high salt intake reduces intestinal *Lactobacillus* and increases blood pressure [25]. TAC which is a model of pressure overload is accompanied by excessive activation of the neuroendocrine system, resulting in an increase in plasma angiotensin II and norepinephrine [26]. This pathophysiological process, to some extent, is similar to that of hypertension. The reduction of *Lactobacillus* might be due to the challenge of angiotensin II, which severely constricts intestinal blood vessels. Therefore, it was not unexpected to find that *Lactobacillus was* negatively correlated with the ratio of HW/BW, LW/BW as well as left ventricular internal dimension (LVID) in our study.

In addition, *Parabacteroides*, a symbiotic bacteria, is also increased in TAC + SF group. *Parabacteroides,* which plays crucial role in anti-obesity, is associated with enhanced intestinal integrity and reduced levels of inflammation and insulin resistance [27,28]. In addition, *Parabacteroides* is associated with reduced atherosclerosis [29]. These effects of *Parabacteroides* would probably be favorable in CVDs.

Taken altogether, the beneficial effect of ferulic is possible through the regulation of gut microbiota composition. Although sodium ferulate increases ejection fraction and the relative abundance of *Lactobacillus* in TAC mice, its anti-hypertrophy effect in vivo still needs to be verified. This consequence might be due to the intake of sodium salt which might partly abolish the beneficial effect of ferulic acid. Hypernatremia would increase serum osmotic pressure which would contribute to hypertension and this pathologic change would increase cardiac afterload and reduce the effect of ferulic acid in anti-cardiac hypertrophy. Therefore, it is not surprising to find that *streptococcus* is harbored in TAC + SF group. Though, beyond the scope of this study, the intake of sodium salt still should be cautious in CVDs.

In conclusion, the increase in heart function by sodium ferulate might partly be explained by the alteration of the gut microbiota.

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Declaration of Competing Interest

None.

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