Animal Microbiome

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Cardiac function of colorectal cancer mice is remotely controlled by gut microbiota: regulating serum metabolites and myocardial cytokines

Zhan-Kui Gao[1,2,3], Chao-Yuan Fan[1,2,3], Bo-Wen Zhang[1,2,3], Jia-Xin Geng[1,2,3], Xing Han[1,2,3], Dan-Qi Xu[1,2,3], Muhammad Arshad[1,2,3], Hao-Xuan Sun[1], Jiong-Yi Li[1], Xiangyuan Jin[5*] and Xiao-Qin Mu[1,2,3,4*]

Abstract

Several studies have indicated that the dysregulation of microbial metabolites and the inflammatory environment resulting from microbial dysbiosis may contribute to the occurrence and progression of cardiovascular diseases. Therefore, restoring the disordered gut microbiota in patients with colorectal cancer by fecal microbiota transplantation (FMT) has the potential to reduce the incidence of cardiac disease. In this study, we identified cardiac dysfunction in azomethane and dextran sodium sulfate-induced colorectal cancer mice. Intestinal microbes from healthy mice were transferred to colorectal cancer mice, which vastly reversed the disorder of the gut microbiota and effectively alleviated cardiac dysfunction. Moreover, FMT regulated the expression of serum metabolites such as uridine triphosphate (UTP), tiamulin, andrographolide, and N-Acetyl-D-glucosamine, as well as cytokines like TGF-β, IRF5, and β-MHC in the heart. These findings uncover that the disturbed gut microbiota causes cardiac dysfunction in colorectal cancer mice by modulating the expression of serum metabolites and cytokines, which could be alleviated by treatment with FMT.

Keywords Colorectal cancer, Cardiac injury, Intestinal microbiota, Fecal microbiota transplantation

*Correspondence:
Xiangyuan Jin
jinxiangyuan@hotmail.com
Xiao-Qin Mu
muxiaoqin@ems.hrbmu.edu.cn
[1]Genomics Research Center (Key Laboratory of Gut Microbiota and Pharmacogenomics of Heilongjiang Province), College of Pharmacy, Harbin Medical University, Harbin 150081, China
[2]National Key Laboratory of Frigid Zone Cardiovascular Diseases, Harbin Medical University, Harbin 150081, China
[3]HMU-UCCSM Centre for Infection and Genomics, Harbin Medical University, Harbin 150081, China
[4]Translational Medicine Research and Cooperation Center of Northern China, Heilongjiang Academy of Medical Sciences, Harbin 150081, China
[5]Department of Thoracic Surgery, Harbin Medical University Cancer Hospital, Harbin, Heilongjiang, China

Introduction

Cardiovascular disease caused by cancer has become the second leading cause of death in cancer patients [1], with a 42% higher risk compared to individuals without cancer [2]. Cardiac dysfunction in patients with colorectal cancer generally manifests as heart failure, arrhythmia, valvular stenosis, left ventricular dysfunction, and pericardial effusion [3]. The 10-year cumulative incidence of new cardiovascular disease in elderly patients with colorectal cancer is 57.4%, which is 2–4 times higher than that in patients without a cancer history [4].

The disorder of microbial metabolites induced by the dysbiosis in the gut microbiota is common in patients

with colorectal cancer, which may create a cradle for cancer-derived cardiac damage [5]. Trimethylamine N-oxide, a metabolite produced by gut bacteria such as *Acinetobacter calcoaceticus*, *Acinetobacter baumannii*, *Pelobacter acetylenicus*, and *Pelobacter carbinolicus*, aggravates heart failure by causing cardiac hypertrophy and fibrosis, exacerbating mitochondrial dysfunction and remodeling of myocardial T-tubules [6]. N, N,N-trimethyl-5-aminovaleric acid, a metabolite produced by *Enterococcus faecalis* and *Pseudomonas aeruginosa*, not only results in excess accumulation of the lipid in the hearts, which exerts lipotoxicity, but also suppresses the biosynthesis and uptake of carnitine associated with fatty acid oxidation and then impairs myocardial energy homeostasis [7]. Conversely, short-chain fatty acids, the metabolites of the gut microbes such as *Bacteroides*, *Bifidobacterium*, and *Faecalibacterium* [8, 9], exert anti-atherosclerotic action mediated by the activation of cognate G-protein coupled receptors GPR41 and GPR43, inhibiting the production of proinflammatory cytokines [10]. The diet of high short-chain fatty acids such as acetate reduces the thickening of the left ventricle wall and cardiac fibrosis and inhibits the dilatation of the left ventricular chamber in the deoxycorticosterone acetate-salt hypertensive mice [11]. Therefore, the intestinal microbiota can be regarded as a bridge between colorectal cancer and cardiac injury.

It is also impossible to ignore the significance of the inflammatory microenvironment caused by the dysbiosis of the gut microbiota, which could potentially induce cardiac dysfunction. TNF-α is found in certain animal models of cancer cachexia and is thought to be the main inflammatory mediators of cancer cachexia [12]. IL-6, IL-6 receptor, and macrophage marker F4/80 are all elevated in the hearts of tumor mouse, indicating increased local inflammation [13]. The overexpression of TNF-α and IL-6 up-regulates the ubiquitin-dependent degradation of the sarcomere by activating the NF-κB pathway and then induces significant myocardial atrophy [14]. Elevated IL-6 may trigger the expression of β-MHC. The shift of α-MHC to β-MHC was obviously observed in cancer mice with impaired heart function, which was characterized by increased fibrosis, disruptive myocardial structure [13]. However, in mice with antibiotic-associated diarrhea, gastric perfusion of *Lactiplantibacillus plantarum* 2–33 can significantly lower levels of proinflammatory factors TNF-α and IFN-γ and raise levels of anti-inflammatory factors IL-4 and IL-10 in serum [15]. *Lactobacillus reuteri* I5007 inhibits the elevated mRNA expression of TNF-α and IL-6 in porcine jejunal epithelial cells induced by lipopolysaccharide in vitro [16]. In the mesenteric lymph nodes of piglets treated with *Lactobacillus reuteri* I5007, the relative abundance of mRNA for TGF-β was higher and that for IFN-γ was lower [17]. Therefore, it may be possible to prevent tumor-induced cardiac dysfunction by managing the gut microbiota to regulate the expression of inflammatory cytokines inside the tumor microenvironment.

Maintaining the balance of the intestinal microbiota is of great significance for the prevention and alleviation of cardiac dysfunction. The approximately 100 trillion microorganisms that colonize the human gut are the most important active components of the intestinal microecosystem [18]. The interaction of different intestinal microbes plays important roles in maintaining the balance of the intestinal microecosystem, the stability of metabolites, and regulating the inflammatory response [19]. In this study, fecal microbiota transplantation (FMT) was adopted for the treatment of cardiac dysfunction induced by colorectal cancer. FMT reversed the disorder of the intestinal microbiota and subsequently inhibited the abnormality of microbial metabolites, such as uridine triphosphate (UTP), tiamulin, andrographolide, and N-Acetyl-D-glucosamine, and cytokines like TGF-β, IRF5, and β-MHC in the heart. This study may provide promising strategies for the prevention of cardiovascular diseases in cancer patients.

Materials and methods

The colorectal cancer mouse model

Eight-week-old male Balb/c mice were obtained from Beijing Vital River Lab (Beijing, China) and adaptively housed at the SPF Laboratory Animal Center for one week. In the first week of establishing the colorectal cancer model, the mice were intraperitoneally injected with the carcinogen azoxymethane (AOM, Sigma) at a dose of 10 mg/kg. In the second week, mice were given drinking water containing 2.5% dextran sulfate sodium (DSS, MP Biomedicals). During the third and fourth weeks, the mice normally drink distilled water without any reagents. The treatment from week 2 to week 4 was repeated twice to complete the establishment of the colorectal cancer model. AOM/DSS-induced inflammatory colorectal cancer model in male Balb/c mice is a traditional technique with a high carcinogenic rate [20]. Mice were randomly divided into three experimental groups based on body weight, with four mice per cage. The study was approved by the Ethics Committee of Harbin Medical University (IRB1040723).

Fecal microbiota transplantation

Fresh stool ensured the acquisition of active gut microbiota. Before transplantation, fresh fecal samples were collected from mice in the control group and mixed in PBS (200 mg/ml) by 10 s vortex. The suspension was centrifuged at 1500 rpm for 1 min to remove food debris. The supernatant was finally collected for subsequent transplantation. Each recipient mouse in FMT group received

1 mL of the microbial suspension via enema every 3 days for 9 weeks. Equivalent enemas with diluent PBS were not given to the normal control mice or the colorectal cancer mice since PBS flushing altered the diversity of the gut bacteria [5].

16S rDNA high-throughput sequencing

The mice were fed without any treatment for one week after FMT to avoid the adverse conditions that the transplanted microbial solution left in their intestines during the analysis of the intestinal microbiota. Subsequently, the mice were transferred to clean cages to collect fresh fecal samples, with two tubes of feces collected per cage. Six fecal samples from different cages within the same experimental group were used for high-throughput sequencing of the gut microbiota (Biomarker, Beijing, China). Based on the Illumina HiSeq platform, sequencing data for six replicates in each experimental group was obtained and submitted to NCBI (PRJNA1060903). Microbial diversity was analyzed based on a representative OTU sequence with 97% similarity.

Untargeted metabolomics assay of serum

For serum metabolomics, 100 μL serum and 500 μL extracted liquid containing internal standards (volume ratio of methanol and acetonitrile = 1:1, 20 mg/L internal standard) were mixed and ultrasounded on ice for 10 min. The solution was then centrifuged at 12,000 rpm and 4℃ for 15 min after standing at -20℃ for 1 h. The concentrated and dried extracts of the supernatant were redissolved in 160 μL of extraction solvent. Following centrifugation, 120 μL of supernatant was injected into a liquid chromatography tandem mass spectrometry (LC-MS/MS) instrument. The LC-MS/MS system is composed of WatersTM Acquity I-Class PLUS ultra-high-performance liquid chromatography and a Waters Xevo G2-XS QTOF high- resolution mass spectrometer in series. Chromatographic separation was achieved using the Waters Acquity UPLC HSS T3 chromatography column (1.8 μm, 2.1 mm*100 mm) with gradient elution of mobile phases A (0.1% formic acid aqueous solution) and B (0.1% formic acid acetonitrile) at a flow rate of 400 μL/min. The scanning range was 50–1200 m/z. The drying gas flow rate and temperature were maintained at 800 L/h and 500℃, respectively. The capillary voltage was 2000 V (the positive ion mode) or -2000 V (the negative ion mode). Metabolomic raw data collected by MassLynx V 4.2 was processed for peak extraction and peak alignment using Progenesis QI software. The fold change > 2 or < 0.5, the variable importance in projection ≥ 1, and the P value < 0.05 were used as the selection criteria for differential metabolites. The analysis of metabolomic data was performed on the BMKCloud platform.

Electrocardiographic record

Mice were anesthetized with avertin (Sigma-Aldrich, USA) and then fixed in the supine position. The electrode needle was subcutaneously inserted into their limbs, with the positive electrode connected to the left lower limb and the negative electrode connected to the right upper limb. The Lead II surface electrocardiogram was continuously monitored for 10 min using a pair of electrodes connected to a BL420s multichannel recorder (TME Technology, China).

Echocardiographic measurements

Mice were anesthetized with avertin, and an ultrasound machine Vevo 2100 (Visualsonics, Ontario, Canada) equipped with a 10-MHz phased-array transducer was used to detect the left ventricular function. The parameters applied to the analysis included the left ventricular internal dimension at end-diastole (LVIDd), the left ventricular internal dimension at systole (LVIDs), the ejection fraction (EF%), and fractional shortening (FS%). EF was determined automatically by the machine, while FS was calculated according to the following equation: [(LVIDd-LVIDs)/LVIDd]×100%.

HE staining

The paraffin sections of heart tissue were dewaxed and rehydrated with xylene and ethanol before being stained with hematoxylin for 2 min and eosin for 1 min. The tissues were dehydrated and transparent with ethanol and xylene, then sealed with neutral resin and visualized under a microscope (Zeiss).

Immunohistochemical detection

The paraffin sections of heart tissue were dewaxed with xylene and rehydrated with ethanol. The endogenous peroxidase and biotin were inactivated with 3% H_2O_2 for 10 min. Antigen retrieval was performed in a microwave oven with sodium citrate buffer for 2 min at high power and then for 10 min at low power. After cooling to room temperature, the tissue section was blocked with 50% goat serum for 1 h and then incubated with the primary antibody cleaved caspase-3 (1:1000, 9661, Cell Signaling Technology) at 4 °C over night. The next day, the tissue was incubated with horseradish peroxidase-labeled secondary antibody HRP goat anti-rabbit IgG (H + L) (1:500, AS014, ABclonal) at 37℃ for 1 h. The DAB peroxidase substrate kit (ZSGB-BIO) was used for color development. The sections were dehydrated with ethanol and xylene before being sealed with the neutral resin. Image J was utilized to analyze the intensity of positive markers by transforming the image signal into a digital signal.

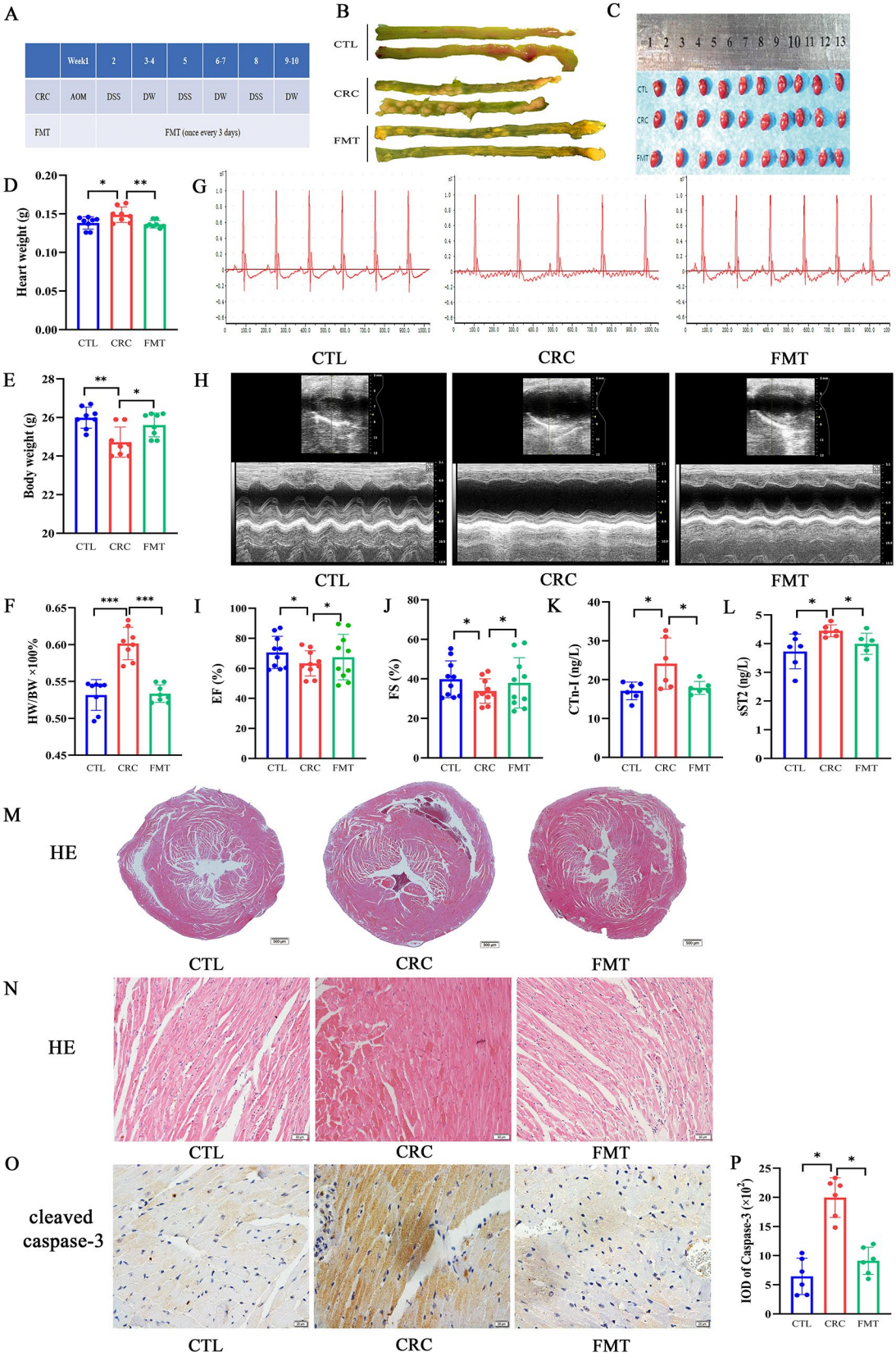

Fig. 1 (See legend on next page.)

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Fig. 1 FMT alleviated cardiac injury in colorectal cancer mice. (**A**) Protocol for the mouse model of colorectal cancer (CRC) induced by azoxymethane (AOM) and dextran sulfate sodium (DSS) with or without treatment of fecal microbiota transplantation (FMT). DW, distilled water. (**B**-**C**) Pictures of intestinal tissue and heart from each experimental group, including normal control mice (CTL), colorectal cancer mice (CRC), and colorectal cancer mice treated with fecal microbiota transplantation (FMT). (**D**-**E**) Heart weight and body weight from each experimental group, $n = 8$. (**F**) The ratio of heart weight and body weight (HW/BW), $^{**}P < 0.01$, $n = 8$. (**G**) Representative raw traces of the electrocardiogram. (**H**) Representative images of echocardiographs. (**I**-**J**) Ejection fraction (EF%) and fractional shortening (FS%) of hearts, $^{*}P < 0.05$ (CTL, $n = 10$; CRC, $n = 10$; FMT, $n = 10$). (**K-L**) Levels of CTn-I and sST2 in serum analyzed by ELISA, $^{*}P < 0.05$, $n = 6$. (**M**-**N**) HE staining of heart tissues. (**O**-**P**) Immunohistochemical staining of cleaved caspase-3 in heart tissues, $^{*}P < 0.05$, $n = 6$

ELISA

The levels of cardiac troponin I (CTn-I) and soluble growth stimulator gene 2 protein (sST2) were measured by using the mouse CTn-I kit (MM-0791M2, MEIMIAN) and the mouse sST2 kit (MM-1070M2, MEIMIAN) according to the manufacturer's instructions. In order to obtain the supernatant, the blood samples were centrifuged at 3000 rpm for 20 min at 4℃ after naturally coagulating for 10 min at room temperature. Diluted standard solutions with different concentrations (8 μg/L, 4 μg/L, 2 μg/L, 1 μg/L, 0.5 μg/L) were added to the standard wells. Sample dilution and 10 μL of diluted serum were added to the testing sample wells, respectively. The plate was then incubated at 37℃ for 30 min. After washing five times, HRP-conjugate reagent was added. The plate was then incubated and washed as previously described. Chromogen solution A and chromogen solution B were added and then preserved for 10 min at 37℃ in the dark. The OD value was detected at 450 nm after adding 50 μL of the stop solution.

Western blot

Tissue proteins of the heart were denatured for 5 min at 100℃ in 5×SDS-PAGE loading buffer and then electrophoresed on 6% or 10% tris-glysine gels, depending on the different molecular weights. The intact PVDF membrane and electrophoresed tris-glycine gels were preserved during the initial use of the new antibody to accurately locate the protein bands. In subsequent tests, only those membranes containing target protein bands were retained for further use. The separated protein was transferred to the PVDF membrane and then blocked with 5% non-fat milk (P0216-300 g, Beyotime) at room temperature for 2 h. The membrane was incubated with primary antibodies for TGF-β (1:1000, A18692, Abclonal), β-MHC (1:1000, A7564, Abclonal), IRF-5 (1:1000, A16388, Abclonal), GAPDH (1:2000, A19056, Abclonal), and Tubulin (1:1000, AP0064, Bioworld) at 4℃ over night. Subsequently, the membrane was incubated with horseradish peroxidase labeled secondary antibody HRP goat anti-rabbit IgG (H + L) (1:5000, AS014, ABclonal) at room temperature for 2 h. Tanon-5200 and Tanon MP were used to measure the band intensity.

Statistical analysis

The Student-Newman-Keuls-q test was used for statistical analysis by Graphpad Prism v8. All data were reported as mean ± SD. A two-tailed value of $p < 0.05$ was taken to indicate statistical significance.

Results

FMT alleviated cardiac injury in colorectal cancer mice

Figure 1A showed the procedures for using azoxymethane (AOM) and dextran sulfate sodium (DSS) to induce the mouse model of colorectal cancer as well as the frequency of FMT. Figure 1B-C displayed pictures of the intestinal tissues and hearts. The AOM/DSS-induced colorectal cancer mice's body weight dramatically reduced and their heart weight significantly rose when compared to the normal control group, both of which led to the changes in the HW/BW index (Fig. 1D-F). Electrocardiograms recorded decelerated heart rate (11/12) and ST-segment elevation (9/12) in colorectal cancer mice. In FMT-treated mice, the incidences of decelerated heart rate (2/10) and ST-segment elevation (1/10) were significantly reduced (Fig. 1G). Compared with normal mice, the ejection fraction (EF%) and fractional shortening (FS%) were decreased by 10.3% and 15.1% in colorectal cancer mice, respectively, which were significantly reversed by the treatment of FMT (Fig. 1H-J). Furthermore, the increased production of cardiac troponin I (CTn-I) and soluble growth stimulator gene 2 protein (sST2) in serum, which indicates aggravated myocardial injury and fibrosis in colorectal cancer mice, was inhibited by the treatment of FMT (Fig. 1K-L). HE staining revealed that the cardiomyocytes were spindle-shaped and orderly in arrangement in normal mice. In contrast, obvious alterations appeared in the hearts of colorectal cancer mice, which were mainly manifested as thickened myocardium, larger and disordered cardiomyocytes. FMT significantly reversed the alteration of cardiac morphology in colorectal cancer mice (Fig. 1M-N). IHC staining showed that the expression of cleaved caspase-3 in the heart was elevated in colorectal cancer mice compared with normal mice, which was effectively inhibited by treatment with FMT (Fig. 1O-P). The regulation of gut microbiota achieved by FMT alleviated the cardiac injury in colorectal cancer mice, indicating that gut microbiota may play an important role in maintaining cardiac functions.

FMT reversed the dysbiosis of the intestinal microbiota in colorectal cancer mice with cardiac injury

In total, 1,682,612 clean reads were obtained after quality filter control and assembly. There were 17 phyla, 29 classes, 57 orders, 94 families, 183 genera, and 199 species in the gut microbiota. The abundance of intestinal microbiota in colorectal cancer mice with cardiac injury was different from that in normal control mice at the levels of phylum, family, and genus (Fig. 2A-C).

In the gut of normal mice, the following genus-level dominants were found: *Alistipes* (11.177%), Prevotellaceae_UCG-001 (5.060%), Ruminococcaceae_UCG-014

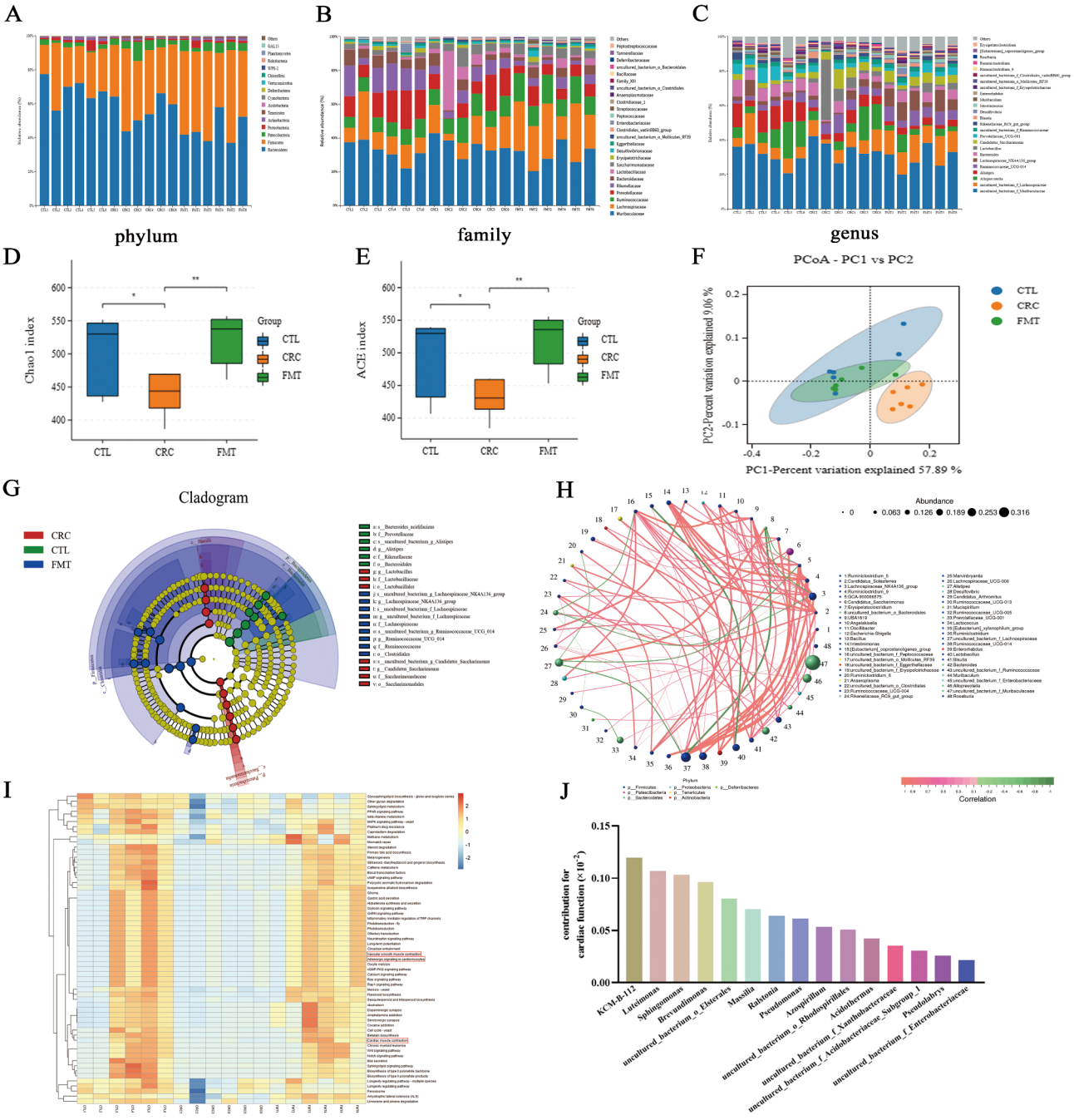

Fig. 2 FMT reversed the microbial dysbiosis of colorectal cancer mice with cardiac injury. (**A**-**C**) Relative abundance of the intestinal microbiota at levels of phylum (**A**), family (**B**), and genus (**C**). (**D** and **E**) Alpha diversity analysis reflects the richness and diversity of intestinal microbiota by Chao 1 and ACE indexes, $^{*}P < 0.05$, $^{**}P < 0.01$, $n = 6$. (**F**) Beta diversity analysis reflects the microbial community structure by principal coordinates analysis. (**G**) Representative bacteria identified by LefSe analysis. (**H**) Microbial network based on Spearman rank correlation analysis (corr > 0.1 and $p < 0.05$). (**I**) Metabolic pathways annotated in the KEGG database. (**J**) Gut microbiota involved in cardiac function revealed by PICRUSt2 analysis

(3.008%), Candidatus_Saccharimonas (2.198%), *Muribaculum* (1.388%), uncultured_bacterium_f_Enterobacteriaceae (0.727%), *Blautia* (0.512%), *[Eubacterium]_coprostanoligenes_group* (0.509%), uncultured_bacterium_o_Mollicutes_RF39 (0.403%), uncultured_bacterium_f_Peptococcaceae (0.281%), Lachnospiraceae_UCG-006 (0.211%), *Angelakisella* (0.169%), Ruminococcaceae_UCG-013 (0.158%), *Bacillus* (0.130%), *Oscillibacter* (0.117%), Ruminococcaceae_UCG-005 (0.114%), *Marvinbryantia* (0.110%), Escherichia-Shigella (0.071%), and Ruminiclostridium_6 (0.015%). However, the abundance of some bacteria was significantly decreased in colorectal cancer mice with cardiac injury, such as *Alistipes* (4.901%), Prevotellaceae_UCG-001 (2.181%), *Muribaculum* (0.621%), Lachnospiraceae_UCG-006 (0.137%), *Angelakisella* (0.080%), *[Eubacterium]_coprostanoligenes_group* (0.040%), *Marvinbryantia* (0.044%), Escherichia-Shigella (0.025%), and *Bacillus* (0.002%). In addition, several bacteria, such as *Blautia* (1.282%), uncultured_bacterium_o_Mollicutes_RF39 (0.854%), Ruminococcaceae_UCG-013 (0.340%), *Oscillibacter* (0.242%), and *Ruminiclostridium_6* (0.103%), were shown to be predominately abundant in the gut of mice with heart injuries. FMT significantly reversed the abnormal levels of these gut bacteria, such as Prevotellaceae_UCG-001 (3.118%), *Muribaculum* (0.698%), uncultured_bacterium_o_Mollicutes_RF39 (0.635%), *[Eubacterium]_coprostanoligenes_group* (0.590%), Lachnospiraceae_UCG-006 (0.304%), *Oscillibacter* (0.207%), Ruminococcaceae_UCG-013 (0.205%), *Angelakisella* (0.196%), *Bacillus* (0.090%), *Marvinbryantia* (0.074%), and Escherichia-Shigella (0.056%). Statistically, as indicated by the ACE and Chao1 indexes, which reflect the intestinal bacterial richness and diversity, the dysbiosis of the intestinal microbiota was significantly reversed by FMT (Fig. 2D and E).

Principal coordinates analysis (PCoA) was used to extract the most important elements from multi-dimensional data, which can classify multiple samples and further show the differences in genus diversity among samples. A distinct segregation between normal mice and heart-injured mice was observed by the PCoA based on the unweighted unifrac algorithm. The treatment of FMT reduced the segregation in the microbiota between them (Fig. 2F). During microbiota transplantation, certain gut bacteria, such as *Ruminococcaceae_UCG-014* and *Lachnospiraceae_NK4A136_group*, were more likely colonize the gut, as determined by Lefsey analysis. Others, for example, *Alistipes*, were less likely colonize, requiring individualized transplantation by in vitro culture as a supplement (Fig. 2G). The complex interactions between the gut microbiota were important for maintaining microbial homeostasis. In this study, 48 genera of bacteria exhibited close correlations, of which 86 red lines showed positive correlations and 14 green lines showed negative correlations (Fig. 2H). PICRUSt2 was utilized to investigate the function of the microbial community. Metabolic pathways such as vascular smooth muscle contraction, adrenergic signaling in cardiomyocytes, and cardiac muscle contraction were annotated in the KEGG database (Fig. 2I). The following gut bacteria were found to be potentially contributors to these three pathways: KCM-B-112, *Luteimonas*, *Sphingomonas*, *Brevundimonas*, uncultured_bacterium_o_Elsterales, *Massilia*, *Ralstonia*, *Pseudomonas*, *Azospirillum*, uncultured_bacterium_o_Rhodospirillales, *Acidothermus*, uncultured_bacterium_f_Xanthobacteraceae, uncultured_bacterium_f_Acidobacteriaceae_Subgroup_1, *Pseudolabrys*, and uncultured_bacterium_f_Enterobacteriaceae (Fig. 2J).

FMT regulated the serum metabolites in colorectal cancer mice with cardiac injury

A total of 11,412 peaks were detected based on the LC-QTOF platform, with 2,595 metabolites being annotated. We identified 335 significantly different metabolites between the normal mice and colorectal cancer mice, of which 165 were up-regulated and 170 were down-regulated in the serum samples (Fig. 3A). FMT inhibited the upregulation of eight metabolites, including 2,4-dihydroxy-6-pentylbenzoate, UTP, L,L-Cyclo (prolylalanyl), 2-hydroxy-6-ketononatrienedioate, P1,P4-Bis(5'-uridyl) tetraphosphate, CPA (18:1(11Z)/0:0), tiamulin, and Asn Ile Lys Lys (Fig. 3B and D). These metabolites may contribute to cardiac dysfunction in colorectal cancer mice. Meanwhile, FMT inhibited the downregulation of 62 metabolites, such as stigmast-4-ene-3,6-dione, gibberellin A112, 3beta,5beta-Ketodiol, nosantine, 10Z-nonadecenoic acid, 5-(10,13-nonadecadienyl)-1,3-benzenediol, CAY10514, N-acetyl-D-glucosamine, 11beta,21-dihydroxy-3,20-oxo-5beta-pregnan-18-al, angiotensin (5–8), and andrographolide (Fig. 3B and E). These metabolites might have beneficial effects on heart health. There were complex correlations among these metabolites, most of which were positive (Fig. 3C).

Intestinal microbiota alleviated cardiac injury by downregulating TGF-β, IRF5, and β-MHC

As an immune cytokine stimulated by *Bacteroides fragilis* [21], *Bacteroides uniformis*, and *Clostridium ramosum*, TGF-β aided in the hypertrophic expansion of cardiomyocytes as well as the proliferation of cardiac fibroblasts and their phenotypic transition into myofibroblasts [22]. Patients with viral myocarditis have elevated levels of IRF5, which binds to cis-regulatory sites in the nucleus and increases the production of inflammatory proteins including TNF-α and IL-6, hence aggravating cardiac damage [23]. β-MHC, a major component of the cardiac

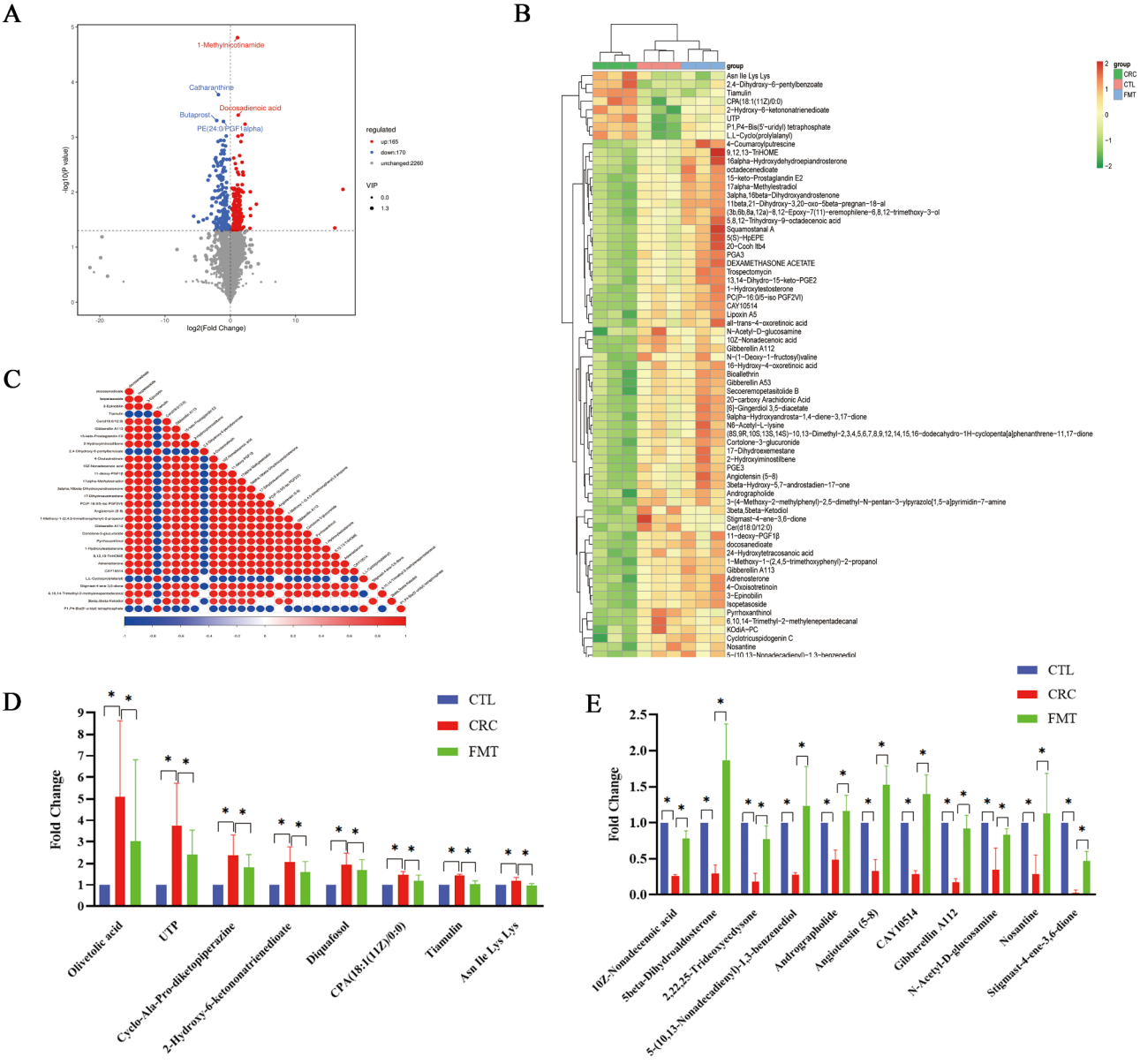

Fig. 3 FMT restored the balance of gut microbiota metabolites to alleviate cardiac injury in colorectal cancer mice. (**A**)The altered metabolites between the CTL and CRC groups indicated by the volcano plot. (**B**) Pearson cluster analysis of 70 metabolites in response to FMT intervention. (**C**) Correlation analysis for the top 30 of the 70 metabolites. (**D** and **E**) The metabolites with the most different expression, $^{*}P < 0.05$, $n = 3$

contractile apparatus, is also involved in the regulation of myocardial fibrosis and cardiac hypertrophy [24]. This study found that the cytokines TGF-β, IRF5, and β-MHC were significantly overexpressed in the myocardium of colorectal cancer mice (Fig. 4A), suggesting that the cardiac dysfunction caused by colorectal cancer may be mediated by these cytokines. The expression of these three cytokines was sensitive to the alternation of the gut microbiota. The balance of gut microbiota created with FMT could dramatically restrict their overexpression, thereby preventing heart damage.

Spearman correlation analysis was employed to investigate the correlation among cytokines, gut microbiota, and serum metabolites. Of interest, some gut bacteria with higher abundance in healthy mice, such as *[Eubacterium]_coprostanoligenes_group*, uncultured_bacterium_f_Enterobacteriaceae, *Angelakisella*, *Bacillus*, and *Marvinbryantia*, showed a negative correlation with the expression of TGF-β, IRF5, and β-MHC (Fig. 4B), indicating that they may have a strong inhibitory effect on the three cytokines. Meanwhile, the majority of serum metabolites, including (8 S,9R,10 S,13 S,14 S)-10,13-Dimethyl-2,3,4,5,6,7,8,9,12,14,15,16-dodeca-

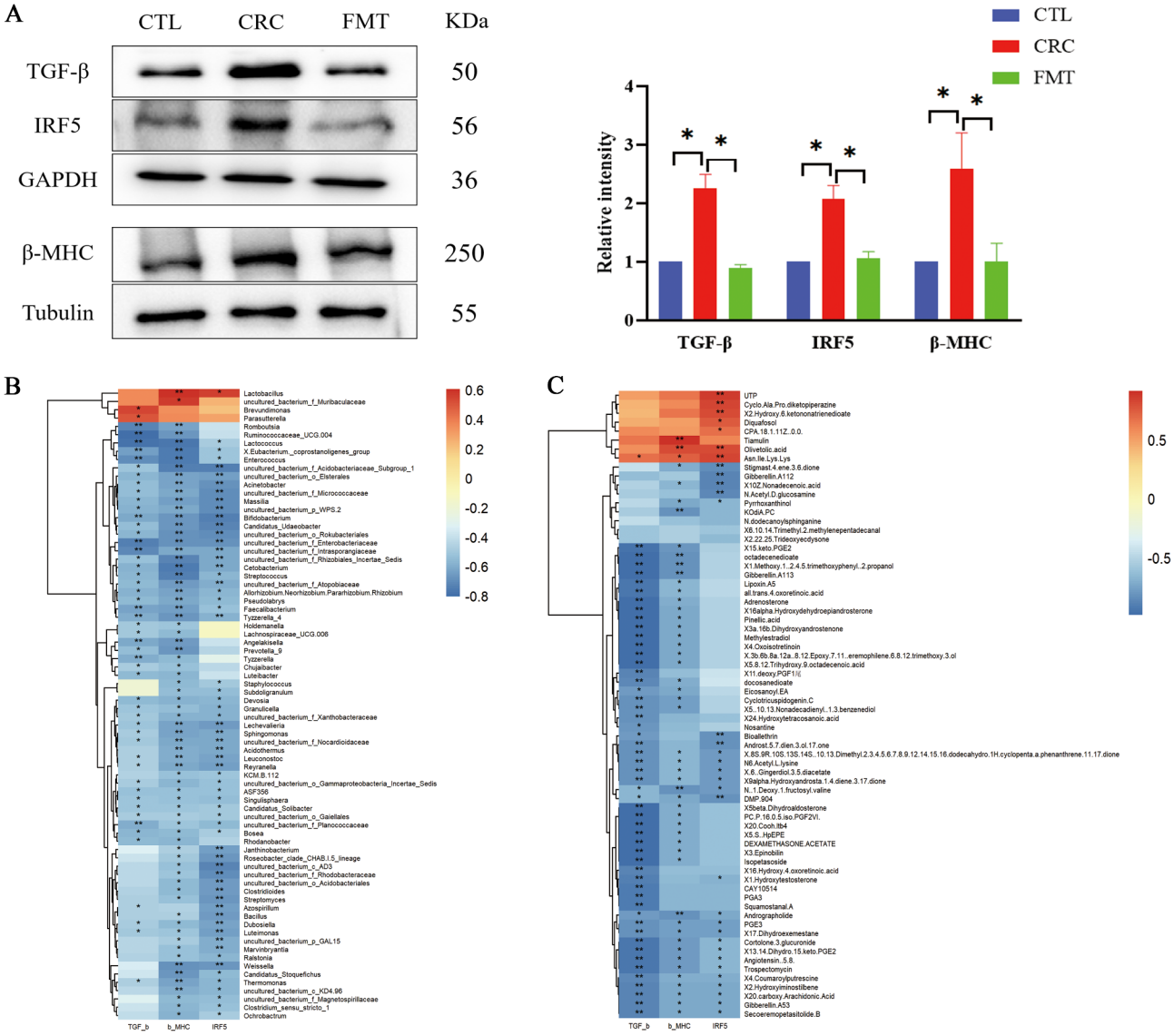

Fig. 4 FMT downregulated the expression of TGF-β, IRF5, and β-MHC in the heart to alleviate cardiac injury in colorectal cancer mice. (**A**) Western blot analysis for TGF-β, IRF5, and β-MHC from heart tissue. *$p < 0.05$, $n = 3$. (**B**) Heatmap of Spearman's correlations between the intestinal microbiota and TGF-β, IRF5, and β-MHC. *$p < 0.05$, **$p < 0.01$. (**C**) Heatmap of Spearman's correlations between serum metabolites and TGF-β, IRF5, and β-MHC. *$p < 0.05$, **$p < 0.01$

hydro-1 H-cyclopenta[a]phenanthrene-11,17-dione, N6-Acetyl-L-lysine, [6]-Gingerdiol 3,5-diacetate, 9alpha-Hydroxyandrosta-1,4-diene-3,17-dione, N-(1-Deoxy-1-fructosyl)valine, DMP-904, Andrographolide, PGE3, 17-Dihydroexemestane, Cortolone-3-glucuronide, 13,14-Dihydro-15-keto-PGE2, Angiotensin (5–8), Trospectomycin, 4-Coumaroylputrescine, 2-Hydroxyiminostilbene, 20-carboxy Arachidonic Acid, Gibberellin A53, Secoeremopetasitolide B, have been found to exhibit negative relationships with the three cytokines, suggesting a possible inhibitory influence on cytokine expression (Fig. 4C). The results indicated that cardiac dysfunction may develop from the overexpression of TGF-β, IRF5, and β-MHC in the heart, which is promoted by microial dysbiosis and the abnormally generated metabolites that follow. This is significantly lessened by the equilibrium of serum metabolites and gut bacteria that FMT produces.

Discussion

In this study, we found that colorectal cancer mice with disordered intestinal microbiota suffer from cardiac dysfunction, mainly manifested as decreased heart rate, elevated ST-segment, decreased indexes of EF% and FS%, and enlarged and disordered cardiomyocytes. After regulating the intestinal microbiota by FMT, the cardiac dysfunction was alleviated, suggesting that the intestinal microbiota may remotely control cardiac function. The alleviation was achieved mainly by regulating the

production of serum metabolites such as UTP, tiamulin, andrographolide, and N-Acetyl-D-glucosamine, as well as the expression of cytokines TGF-β, IRF5, and β-MHC in the heart.

As the gut-heart axis has been proposed recently [25], the link between gut microbiota and cardiovascular disease is receiving more attention. Enhancing the abundance of *Alistipes* alleviates atherosclerosis symptoms by suppressing the inflammatory response, which lowers the production of NLRP3, IL-6, IL-1β, and TNF-α while increasing the level of IL-10 in mice fed a high-fat diet [26]. However, Clostridiacea produces TMA, which is the precursor of TMAO, a pro-atherogenic chemical [27]. A bidirectional Mendelian randomization reported that *Blautia* may raise the risk of endocarditis [28]. *Blautia* increases the atherosclerotic burden in high-fat diet mice by elevating the expression of total cholesterol, low-density lipoprotein cholesterol, alanine transaminase, and aspartate transaminase in serum [29]. In this study, we observed less abundance of *Alistipes* (11.177–4.901%) and more abundance of *Blautia* (0.512–1.282%) and uncultured_bacterium_o_Clostridiales (0.018–0.032%) in colorectal cancer mice. Additionally, *Bacillus subtilis* DE111 improves endothelial function and decreases the risk of cardiovascular disease in adults by modulating blood lipids, particularly by lowering the levels of total cholesterol and non-high-density lipoprotein-cholesterol in serum [30]. *Bacillus subtilis* sp. efficiently lessens the degenerative alterations in the myocardial and aortic tissues by releasing polysaccharide to scavenge free radicals, reduces oxidative stress, and ultimately shields the heart from harm brought on by diabetes [31]. *Prevotella* levels were lower in 218 patients with atherosclerotic cardiovascular disease than in 187 healthy controls, according to a metagenome-wide association analysis [32]. In our investigation, the abundance of *Bacillus* (0.130–0.002%) *and Prevotella_9* (0.011–0.005%) in colorectal cancer mice was considerably lower, and the FMT intervention raised their levels (*Bacillus*, 0.002–0.090%; *Prevotella_9*, 0.005–0.015%). These studies establish a connection between two seemingly unrelated entities: the intestinal microbiota and the heart. In this research, FMT alleviated cardiac dysfunction by reversing disordered gut microbiota in colorectal cancer mice, suggesting that the intestinal microbiota indeed remotely regulates cardiac function.

With the development and application of metabolomics, microbial metabolites of the circulation system have been gradually revealed as biomarkers of heart disease and cancer. N-Acetyl-D-glucosamine (GlcNAc) is a metabolite of *Clostridium* sp [33]. O-GlcNAcylation activates cardiac glucose-6-phosphate dehydrogenase and improves redox homeostasis, which counteracts ischemia-reperfusion injury in cardiomyocytes [34]. Andrographolide contributes to the attenuation of cardiac hypertrophy in mice by suppressing MAPKs signaling and endoplasmic reticulum stress [35, 36]. However, uridine triphosphate is a pyrimidine derivative increased in patients with myocardial infarction, exerts positive inotropic and profibrotic effects via the P2Y2 receptor on cardiomyocytes [37, 38]. Tiamulin suppresses atrioventricular conductivity, reduces contractile tension of cardiac muscle, and relaxes coronary arteries through the Ca^{2+}-entry blocking action [39]. Cyclo-ala-pro-diketopiperazine, a type of cyclic dipeptide explored from the bioactive culture broth extract of *Pseudomonas sp.*, is significantly increased in colorectal cancer mice [40]. In this experiment, these metabolites were all aberrantly expressed in colorectal cancer mice with cardiac dysfunction. FMT treatment significantly reversed the drop in GlcNAc and andrographolide and the increase in UTP, tiamulin, and cyclo-Ala-Pro-diketopiperazine. We also observed less abundance of *Clostridium_sensu_stricto*_1 (0.058–0.001%) and more abundance of *Pseudomonas* (0.013–0.015%) in colorectal cancer mice with cardiac injury, and the FMT intervention raised *Clostridium_sensu_stricto*_1 level (0.001–0.009%). This suggests that alterations in gut microbiota have a significant impact on cardiac function, which is meditated by metabolites. Additionally, in our research, certain serum metabolites, such as olivetolic acid, 2-hydroxy-6-ketononatrienedioate, diquafosol, CPA (18:1(11Z)/0:0), Asn Ile Lys Lys, 10z-nonadecenoic acid, 5beta-dihydroaldosterone, 2,22,25-trideoxyecdysone, 5-(10,13-nonadecadienyl)-1,3-benzenediol, angiotensin (5–8), CAY10514, gibberellin a112, nosantine, and stigmast-4-ene-3,6-dione, exhibited great variations in expression between the mice that received or did not receive FMT. These metabolites may also act as mediators in regulating heart dysfunction.

Furthermore, we found that cardiac dysfunction was also alleviated by downregulating the expression of TGF-β, IRF5 and β-MHC in the heart. Strong correlations were observed between the expression of TGF-β, IRF5, β-MHC and the abundance of specific gut microbiota. Transferring fecal microbiota from the healthy donor with abundant *Lactobacillus* to chicks notably increased the expression of TGF-β [41]. The supplementation of *Lactobacillus rhamnosus* JY02 promoted the production of muscle-enhancement markers including MHC Iβ, MHC IIα, and Myo-D in mice [42]. Disorders of the intestinal microbiota in infants with congenital heart disease, as evidenced by lower level of *Bifidobacterium* and higher levels of *Enterococcus*, *Subdoligranulum*, and *Shigella*, may induce the progression of heart failure, which is caused by downregulating retinol metabolism [43]. Meanwhile, treatment of rotavirus-infected HT-29 cells with *Bifidobacterium longum* significantly reduced the relative expression of IRF5 and enhanced the cellular

antiviral immune response [44]. In this study, colorectal cancer mice with cardiac dysfunction had high levels of *Lactobacillus* and low levels of *Bifidobacterium*, and FMT restored these abnormalities. These findings imply that FMT alleviates cardiac dysfunction by controlling the abundance of intestinal flora and the expression of related cytokines, with the gut microbiota playing a significant role in this enhancement.

Serum metabolites also regulate the expression of TGF-β, β-MHC, and IRF5 in the myocardium. Angiotensin decreases the TGF-β/Smad signaling pathway, which in turn lowers expression of TGF-β and β-MHC and ultimately reduces inflammatory cell infiltration and interstitial fibrosis in the hearts [45]. We also found a negative correlation between the serum metabolite angiotensin and the expression of TGF-β, β-MHC, and IRF5. These findings highlight the complex relationship among gut microbiota, serum metabolites, and cytokines in the myocardium and suggest a potential therapeutic strategy for the treatment of colorectal cancer-induced cardiac dysfunction. Further research is necessary to determine whether bacteria and metabolites that differ significantly can be employed as indicators for the diagnosis and treatment of cardiac disorders, as well as the relationship between these bacteria and metabolites.

Conclusion

Here, we revealed that FMT reduces cardiac dysfunction in colorectal cancer mice. The underlying mechanisms involve modulation of the composition and abundance of the intestinal microbiota, as well as the expression of serum metabolites and cytokines. Our study suggests a novel strategy for cardiovascular disease, emphasizing the need to fully consider the role of the gut microbiota.

Supplementary Information

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Supplementary Material 1

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Author contributions

Zhan-Kui Gao, Writing - original draft, Visualization, Methodology, Investigation, Data curation| Chao-Yuan Fan, Validation, Formal analysis| Bo-Wen Zhang: Validation, Formal analysis| Jia-Xin Geng, Validation, Formal analysis| Xing Han, Validation, Methodology| Dan-Qi Xu, Validation, Methodology| Muhammad Arshad, Validation, Methodology| Hao-Xuan Sun, Validation, Methodology| Jiong-Yi Li, Validation, Methodology| Xiangyuan Jin: Writing - review & editing, Supervision, Conceptualization| Xiao-Qin Mu, Writing - review & editing, Supervision, Project administration, Funding acquisition, Conceptualization, Resources. All authors reviewed the manuscript.

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Data availability

Raw sequence data are available and deposited at the National Center for Biotechnology Information Sequence Read Archive with the BioProject accession code PRJNA1060903. Any requests for data, resources, and reagents should be directed to and will be fulfilled by corresponding author Xiao-Qin Mu.

Declarations

Ethics approval

The experimental procedures used in this study were approved by the Ethics Committee of Harbin Medical University (IRB1040723).

Competing interests

The authors declare no competing interests.

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