Effect of Time Restricted Feeding on Metabolic Risk and Circadian Rhythm Associated with Gut Microbiome in Healthy Males

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Running Head: Preventive role of TRF against metabolic diseases

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Abstract

Time restricted feeding (TRF) confer protection against nutritional challenges that predispose obesity and metabolic risks through involvement of clock genes and gut microbiome, but the underline mechanism is not clearly understood. Therefore, this study examined the effects of TRF on metabolic markers and circadian rhythm associated with gut microbiota in healthy males. Two groups (TRF, n=56; Non-TRF, n=24) of male adults were enrolled. TRF group provided blood at Pre-TRF and Post-TRF, while Non-TRF one time after 25 days of trial. Serum lipid and liver profiles were determined. RT-PCR was applied for circadian and inflammatory genes expression. The 16S rRNA gene were sequenced at Illumina Miseq v3 platform to comprehensively catalogue the composition and abundance of bacteria in stool. We showed that TRF ameliorated the serum lipid and liver profiles of the individuals. In TRF group, gut microbial richness was significantly enhanced, with enrichment of the Prevotellaceae and Bacteroidaceae. TRF enhanced circadian genes expression probably by activation of Sirt1, which is positively associated with gut microbiome richness. TRF could be a safe remedy for the prevention of metabolic diseases related to dyslipidemia, while regulates circadian rhythm associated with gut microbiome modulation.

Keywords Circadian rhythm: Gut microbiome: Metabolic risk: Time restricted feeding: Lipid profile

Introduction

Intermittent fasting (IF) results in many health benefits ranging from prevention to enhanced treatment of many metabolic diseases\(^1\). Currently, intermittent fasting categorized in to whole day fasting (WDF), every other day fasting (EODF) and time restricted feeding (TRF). Correspondingly, TRF, refers to time restricted food consumption for a period of counted hours and allows daily fasting duration greater than 12 hr, irrespective to alter the quality and quantity of nutrients\(^2\). In context of therapeutic and sustained benefits of TRF, a translational research is necessary to develop fasting-associated interventions into accessible, effective, and inexpensive treatments with the potential to improve health span.
Circadian system is a 24 hr daily rhythms in behavior, physiology and metabolism that are continued under constant feeding/fasting, light/dark and sleep/awakening conditions\(^1\). Agitation of circadian oscillator components leads to diabetes, obesity and develop impaired glucose tolerance and signs of metabolic diseases either in tissue specific or whole body loss of function in mouse models\(^3\). Fasting cycle profoundly affect the circadian system by driving daily rhythm in the activities of nutrient homeostasis key regulators including AMPK, CREB and AKT\(^4\). AMPK degrades cryptochrome (CRY) by promoting its phosphorylation\(^5\). Moreover, fluctuation in the level or activity of sirtuins especially Sirt1 with the cellular energy state also disturb circadian system\(^6\). SIRT1 activates circadian system through recruitment to the CLOCK:BMAL1 chromatin complex and by physically associating with CLOCK, operate the circadian system\(^7\), but there is lack of data on effect of TRF on Sirt1 relating to gut microbiota. Although fasting driven other metabolic regulators lead to coordinated oscillations at the transcriptional level\(^8\), which in turn synchronize daily circadian rhythms in cell repair, division, cellular metabolism and growth in a tissue-specific manner\(^9\).

Modern humans face complex health challenges in treating metabolic diseases. IF can be a therapeutic lifestyle approach for reducing the risk of many metabolic diseases like obesity and hypertension\(^8\). After consumption of 500 kcal with relatively high protein diet for two days a week for 6 months reduced abdominal fat and blood pressure while increased insulin sensitivity in overweight individuals\(^10\). IF may also diminish inflammation, as reported that practicing two months EODF resulted in a significant reduction in inflammatory markers in asthma patients\(^11\). TRF showed significantly improved rhythms and increased thermogenesis, which leads to reduced liver steatosis, adiposity, reduced serum cholesterol, normal glucose tolerance and increased bile acid production in mice\(^8\).

There is an additional link of nutrition and metabolic diseases with gut microbiome. Diet is a critical determinant of the gut microbial composition. Gut commensal bacteria and their metabolites can be both beneficial and harmful effects on host metabolism. Bacteria derived ammonia, hydrogen sulfide and N-nitroso compounds, from dietary protein can activate inflammatory pathways, induce DNA damage and reactive oxygen species (ROS)\(^12\). In addition, the end product of dietary choline produced in the gut called Trimethylamine-N-oxide (TMAO), correlates with CVD, stroke and promote arteriosclerosis\(^13\). IF confers protection in multiple...
sclerosis by increasing gut microbiome richness, changing their composition and metabolic pathway\textsuperscript{(14)}. It is expected that TRF may trigger the fasting physiology within the time restriction paradigm. In turn, this process coordinates with robust circadian rhythm and enhance normal host metabolism in relation to gut microbiota.

Recently IF is one of the attractive lifestyle intervention therapies for many metabolic diseases although the underline mechanism by which IF regulates circadian rhythm by activation of Sirt1 and modulate gut microbiota is unclear. Therefore, this study for the very first time demonstrated that TRF modulate the circadian system through stimulation of Sirt1 and increase gut microbial diversity in human individuals. We found that microbiome richness and abundance are positively or negatively associated with circadian rhythm, lipid and liver profiles respectively, indicating an important role of the microbiome as an integrator of the effects of TRF.

Methods

Participants and Study Design

In April, 2018 we initiated a multi-center study to examine the feasibility and effects of TRF on biomarkers and genetic predisposition of metabolic disease risks in young male healthy adults. This trial was conducted according to the guidelines laid down in the Declaration of Helsinki and approved by the ethical review committee of Nanjing Medical University, Jiangsu China under approval number (NJMUIRB (2018)004). International male students were recruited from three universities of Nanjing city i-e Nanjing Medical University, Nanjing Agriculture University and Nanjing University of Science and Technology China. All participants provided a written consent form. Inclusion criteria for enrollment in study were: 1) only male adults; 2) young aged; 3) absence of ongoing any chronic and infectious diseases; 4) currently not use any supplementation. Individuals were excluded if followed any strict diet plan or food for weight loss or gain. Females were excluded due to reproductive periods like menses as it influence the internal body biochemistry for short time. Participants were divided in to Non-TRF group (n=24), in which participants received their regular diet on daily basis with no time restriction and TRF group (n=56), who underwent daily fasting of 16 hours for 25 days. Study participants, assistant dietitian and the study principal investigator knew the group assignment...
after randomization. The laboratory assistants and nurses who analyzed and collected the samples were blinded to the group assignment.

Study Procedures and Sample Collection

After enrollment, an assistant dietitian instructed the TRF group to strictly follow the study protocol. In TRF group, participants were allowed to consume their normal diet with no food restriction for only 8 hours per 24 hrs i-e from 7:30 pm to 3:30 am for 25 days. Assistant dietitian kept phone and email contact with the participants to provide additional information as required and encouragement between the 2 visits. Non-TRF group continued their regular diet and were not given any specific instructions or time restriction. In TRF group, the participants provided blood at Pre-TRF (baseline) and Post-TRF (after 25 days) and stool samples at one point while from Non-TRF, blood and stool samples were collected only one time after 25 days of trial at Sir Run Run Hospital affiliated with Nanjing Medical University. After completion of trial period, both groups (TRF and Non-TRF) were informed through Wechat at the same time that not take any food from 3:00 am to 15:00 pm. The blood was drawn in same time period between 15:00 pm to 18:00 pm from both groups. At each visit the blood was drawn in the afternoon from both groups after a 12 hours fast. Socio-demographic and body composition including body weight, BMI, %fat, WHR and BMR were also measured as shown in Table S1.

Blood Serum Analysis

Venous blood samples were centrifuged at 4°C for 15 min at 4000 rpm. After collection, serum was portioned and sealed tubes stored at −80°C until analysis. Serum concentrations of total cholesterol (TC), LDL-C and HDL-C, triglycerides (TG), Alanine aminotransferase (ALP), aspartate aminotransferase (AST) and GGT activities were measured at laboratory of School of Public Health, Nanjing Medical University using a commercial reagent (Nanjing Jiancheng Bioengineering Institute, China) with the Roche/Hitachi Cobas c311 analyser (Roche diagnostics, Mannheim, Germany) followed by previously described procedure\(^{15,16}\).

Enzyme-Linked Immunosorbent Assay (ELISA)

ELISA kit (Youxuan Biotechnologies, Shanghai) was used to detect the serum level of IL-1β and TNF-α. Human serum samples were seeded in the microplates and then incubated with
HRP-conjugate reagent for 1 h at 37°C. Chromogen solution A and B were added for another 15 minutes at 37°C. Optical density values were measured at 450 nm within 15 minutes.

**RNA Extraction and RT-PCR**

Total RNA was isolated from 200 µl serum samples using RNAiso Plus (TaKaRaBio Technology, Dalian, China), and was reverse-transcribed to cDNA using the Prime Script™ RT Master Mix. The quantification of RNA was analyzed by qPCR using SYBR Premix Ex Taq II (TaKaRaBio Technology, Dalian, China). The primer sequences are presented in Table 1. Clock, Bmal1, Sirt1, IL-1β, and TNF-α primers and human U6 primers as an internal reference were obtained from RiboBio (Guangzhou, China). All the PCR analyses were conducted using the $2^{-\Delta\Delta CT}$ method.

**Table. 1: qPCR primers**

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<tr>
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<th>Forward primers</th>
<th>Reverse primers</th>
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<tr>
<td><strong>Clock</strong></td>
<td>AAATACTCTCTACTCATCTGCTGG</td>
<td>ATGGCTCCTTTTGTTGCTATTG</td>
</tr>
<tr>
<td><strong>Bmal1</strong></td>
<td>CTGGCTAGAGGTGTATACGGTTTGG</td>
<td>GGTCACCTCAAAGCGATT TTC</td>
</tr>
<tr>
<td><strong>Actin</strong></td>
<td>TCCACCTTCAGCAGATGTG</td>
<td>GCATTTGCGGTGGACGAT</td>
</tr>
<tr>
<td><strong>Sirt1</strong></td>
<td>CTTCAGGTCAAGGGATGGTAT</td>
<td>GCGTGTCATGTCTTCTGGGTAT</td>
</tr>
<tr>
<td><strong>IL-1β</strong></td>
<td>AGAAGCTTCCACCAATACTC</td>
<td>AGCACCTAGTTGTAAGGAAG</td>
</tr>
<tr>
<td><strong>TNF-α</strong></td>
<td>ATGTGCTCCTACCCACACC</td>
<td>GTCGGTCACCCCTTCTCCAGCT</td>
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**Gut Microbiota Analysis**

After samples collection from both groups, stools were immediately frozen at -80°C until DNA extraction. Metagenomic DNA was extracted using the Power Soil DNA Isolation Kit (Mo Bio Laboratories, Carlsbad, CA, USA) according to manufacturer’s instruction. The V1-V3 region of the 16S rRNA gene were sequenced at Illumina Miseq v3 platform (2x300bp paired-end reads) to comprehensively catalogue composition and abundance of the bacteria in the stool samples. 16S gene sequences were subjected to primer trimming, assembly by FLASH with default parameters(17), and taxonomic assignment by RDP classifier(18). Diversity and richness in gut microbiota was measured as described(19).
Quantification and Statistical Analysis

Nutrient intake of the participants of both groups (TRF and Non-TRF) are presented (as mean ± SD) in Table S2. Differences in these characteristics between the two groups were compared using t-test or the Mann-Whitney test, as appropriate. The treatment effect for laboratory measures were evaluated using an analysis of covariance (ANCOVA). The ANCOVA model is a recommended approach for analyzing baseline and follow-up measurements. Analyses were performed using Graph Pad Prism 8.0. The abundance of a taxon in a stool sample was indicated as the relative abundance, which was calculated by dividing the number of reads for a taxon by the total read counts of the sample. Principle component analysis (PCoA) is an ordination technique that aims to discover the data pattern in N-dimensional spaces. It represents the major variation among objects in a reduced dimensional space. It is used to explore the microbiome data structure on the basis of dissimilarity measurement (Bray-Curtis dissimilarity) among samples. Permutational multivariate ANOVA (PERMANOVA) was used for formal statistical testing to investigate the whole microbial community difference between groups. ANCOVA was conducted only for those taxa with average relative abundance >0.05%. Pearson or Spearman correlation was conducted to correlate log transformed relative abundance of gut microbiome. The statistical analysis was performed using R. Differential analysis of the gut microbiome composition between two groups was performed using LEfSe, a software to support high-dimensional class comparisons for microbiome data. A FDR q value was considered significant in all of the microbiome data analysis.

Results

Regulation of lipid profile by TRF

Dyslipidemia accounts one of the major risk factors of obesity, atherosclerosis and metabolic disorders. TRF reverse and inhibit diet induced obesity and associated metabolic diseases in mice without changes in nutritional intake by altering the lipid levels. For the first time, we investigated the antihyperlipidemic effect of TRF in humans. Serum TC (p<0.0001) and TG (p=0.0003 and p=0.0052) levels reduced significantly in post-TRF compared to pre and non-TRF respectively (Fig. 1A and 1B), while LDL-c was remain constant in all groups (Fig. 1C).
After TRF intervention, there was significant (p<0.0001) increment in HDL-c level compared to non-TRF (Fig. 1D).

**TRF improves liver function by regulating its enzymes**

Elevated liver enzymes especially ALT are associated with obesity-induced liver steatosis in mice fed a high fat diet\(^7\). Frequent and high caloric intake throughout the day and night exclusively attributed to obesity and ultimately disturb the liver enzymes and albumin level. In our results, AKP/GGT level was significantly (p<0.0009) decreased in post-TRF than non-TRF (Fig. 2A). Serum AST (p=0.0390 & p=0.0268) and ALT (p=0.0003 & p=0.0174) were also reduced significantly in post-TRF compared to pre and non-TRF respectively (Fig. 2B & 2C). TRF has also a profound effect on serum albumin, which recorded significantly (p<0.0001) lower compared to pre-TRF (Fig. 2D). This indicates that TRF can regulate normal liver metabolism by modulating hepatic enzymes.

**TRF reduced production of pro-inflammatory cytokines**

Excess nutrients intake induces inflammatory response, which has been causally linked to dysregulation of glucose and lipid metabolism\(^{24}\). Dyslipidemia and obesity-associated inflammation predispose to the pathogenesis of atherosclerosis, which is a clinical manifestation of vascular inflammation in metabolic diseases\(^{25}\). Regarding the role of inflammation in atherosclerosis, IL-1\(\beta\) is increased in atherosclerosis and associated with disease severity\(^{26}\). TNF-\(\alpha\) and IL-1\(\beta\) are the most attributable pro-inflammatory cytokines of metabolic dysregulation secreted by adipose tissue\(^{27}\). Therefore, we studied the effect of TRF on IL-1\(\beta\) and TNF-\(\alpha\). However, the mRNA and serum levels of both cytokines downregulated in post-TRF group compared to pre and non-TRF groups but did not reach statistical significance (Fig. 3A, 3B, 3C & 3D).

**TRF regulates circadian rhythm and its stimulator**

Feeding and fasting driven metabolic regulators, associated extensively with circadian oscillation that coordinated in a number of metabolic pathways\(^7\). However, under nutritional challenges such as one posed by high-fat diet, circadian oscillations are necessary and sufficient for preventing metabolic disorders\(^{28}\). Therefore, we have identified in humans, that TRF improves the circadian oscillation for normal metabolic regulation. The mRNA level of Bmal1
(p= 0.0020) and Clock (p=0.0302) genes were significantly upregulated by TRF intervention compared to pre and non-TRF group respectively (Fig. 4A & 4B). Sirt1 activation modulate the circadian physiology in mice\(^{(29)}\). In this study, we showed that activation of Sirt1 can regulate the circadian rhythm. As in post-TRF group the mRNA level of Sirt1 significantly (p= 0.0068 & p=0.0300) upregulated compared to pre and non-TRF groups respectively (Fig. 4C).

**TRF increases Gut microbiome diversity and has profound association with Sirt1 expression and HDL level**

Recently, animal and human studies have been discovered that reduced microbiome diversity, altered gut microbial activities and scattered microbiome relative abundance especially of two phyla i-e Bacteroidetes and Firmicutes are associated with obesity development\(^{(30,31)}\). Using the relative abundance of the differential abundant microbial taxa, we separated the post-TRF microbiota from non-TRF group. However there is no substantial overlap between the two groups as illustrates by applying PCoA analysis (Fig. 5A). Microbial richness is a measure of alpha diversity for gut microbiota\(^{(32)}\), and reflects complexity of a microbial community with higher diversity being associated with more healthy gut microbiome\(^{(33,34)}\). TRF significantly increased microbial diversity compared to non-TRF group (Fig. 5B, p< 0.005). Gut microbiota might modulate systemic metabolic responses\(^{(35)}\), and also we observed that TRF modulate gut microbiota, which exhibit regulation of genetic pathway. Indeed, the Sirt1 expression was positively correlated with gut microbiome richness (r=0.45, p<0.0201, Pearson correlation; Fig. 5C). Serum HDL level showed a trend towards positive association (r=0.42, p<0.0289, pearson correlation; Fig. 5D), while serum albumin has significant negative correlation with gut microbiome richness (r=-0.37, p<0.0495; Fig. 5E). This indicates that TRF reduces the burden of metabolic risk through regulation of Sirt1 expression, serum HDL and albumin levels induced by gut microbiome modulation.

**TRF results in significantly different microbial composition in gut microbiome**

We applied linear discriminant analysis (LDA) combined effect size measurements to explore the significant changes and relative richness of bacterial community in both groups. At genus level, 34 and 18 bacteria were enriched in TRF and non-TRF group respectively. Prevotellaceae (prevotella_9 and prevotella_2) and Bacteroidetes were the most abundant in TRF
group with greater than 2.5 LDA score, while in non-TRF, Escherichia Shigella and Peptostreptococcus were abundant at genus level (Fig. 6A). Further, we found an association of LDL-c and TG with Bacteriodia belongs to phylum Bacteroidetes that maintain a complex and generally beneficial relationship with the host when retained in the gut\(^{36}\). The relative richness of Bacteroidia was significantly negative association with LDL-c \((r=-0.46, p=0.0186)\) and TG \((r=-0.40, 0.0364)\) (Fig. 6B and 6C). This indicates that the reduced serum level of LDL-c and TG are probably related with increased Bacteroidia diversity.

**Association of healthy gut microbiome with circadian genes**

After determination of combined microbial richness, we further analyzed whether there is any association of specific microbiome with circadian genes. We found that some of the beneficial bacteria including Prevotellaceae \((r=0.67, p=0.0003)\), prevotella_9 \((r=0.70, p=0.0002)\) and Bacteriodia \((r=0.40, p=0.0370)\) were significantly positive association with Bmal gene (Fig. 7A, 7B, & 7C). Previous studies suggested that Sirt1 functioning as a molecular link between circadian rhythms and metabolic control and also an important modulator and activator of clock genes expression\(^{37,38}\). Interestingly, we reported that expression of Clock and Bmal1 probably upregulated with increase in Sirt1 expression. Here the relative abundance of Prevotellaceae \((r=0.70, p=0.0002)\), Prevotella_9 \((r=0.73, p<0.0001)\), Bacteriodia \((r=0.45, p=0.0199)\), Dialisster \((r=0.50, p=0.0105)\) and Prevotella_2 \((r=0.69, p=0.0002)\) were significantly positive associated with Sirt1 expression (Fig. 7D, 7E, 7F, 7G, 7H).

**Discussion**

Disturbed dietary/feeding pattern bothers metabolic pathways entrained by both feeding and circadian rhythms. This chronological misalignment in cellular metabolism in combination with nutrient quality and gut microbiota predisposes obesity and metabolic syndrome\(^1\). Many studies have observed that IF exerts a strong anit-obesity, anti-diabetic, anti-inflammatory and cardoprotective effects in animal models\(^3\). Currently the recommended lifestyle modification focused on alteration of individual nutrition and gut microbiome that are interlinked with metabolic risk. This is the first report found that the mRNA expressions of Sirt1 and circadian genes (Bmal1 and Clock) were higher and the microbiota were improved after TRF, the beneficial effect of TRF probably related with the Sirt1 activation. Therefore, we introduced
TRF, a lifestyle intervention that can prevent metabolic risk through modification of circadian rhythm, gut microbiome by preserving natural feeding rhythms.

Lipid homeostasis maintained by interaction of circadian components with metabolic regulators. Herein we show that TRF reduced the lipid level including TC, TG and increased HDL in the circulation. Similarly in a previous study, intermittent energy restriction induced a decrease in TC and TG\textsuperscript{10}. TRF reduced fatty acids synthesis and increased fat oxidation, which in turn leads to reduction in adiposity\textsuperscript{23}, and it is demonstrated that TRF is a lifestyle therapy against metabolic diseases by inhibiting dyslipidemia\textsuperscript{8}.

Liver metabolism and homeostasis maintained by mutual coordination of metabolic regulatory enzymes and circadian oscillation. Altered liver metabolism induced by increased level of hepatic enzymes could be a risk for development of metabolic diseases. We showed that TRF reduced the serum level of liver enzymes including AKP, AST, ALT and albumin, as reported previously in mice that TRF prevents hepatomegaly and liver failure by reducing the serum ALT\textsuperscript{8}.

In animal models, IF has shown potential role against inflammation\textsuperscript{39}. Therefore, we studied the effect of TRF on IL-1β and TNF-α in human. The reduction in these two cytokines were non-significant. Conversely, fasting mimicking-diet (FMD) improved inflammation by returning C-reactive protein, a marker of inflammation in cardiovascular disease in a very limited sample of seven subjects\textsuperscript{40}. IF also reduced inflammation through less production of IL-17 by altering gut microbiota in mice with multiple sclerosis\textsuperscript{14}. However, EODF significantly reduced TNF-alpha in patients suffering from asthma after two months\textsuperscript{17}. This discrepancies may be due to sample size or mode of fasting, inflammation markers and even study subjects.

Food restriction not only increase the rhythmic transcripts but also improves amplitude, synchronizes the phases of oscillations and reduces food intake. Feeding affects the clock through nutrient sensing pathway by anabolic targets. The mTOR pathway phosphorylates casein kinase 1 which further phosphorylate the clock component called PER, as a result changing its stability\textsuperscript{41}. Besides, erratic dietary pattern including disturbed feeding, eating attitude and poor nutrition can cause chronic disruption in circadian rhythm that increasing the metabolic risk. As previously reported that after ten days of misalignment of circadian system, individuals developed insulin resistance, elevated post-prandial glucose and increased arterial pressure\textsuperscript{42}. 
Improvement in circadian rhythm helps in prevention or reverse metabolic diseases. This is in line with a study reported that Sirt1 gene expression was increased after 48 h of fasting in 10 healthy lean men\(^{(43)}\). In disease condition, the circadian genes usually downregulated as reported previously, that Bmal1 and Sirt1 expression decreased significantly and resulted in aggravation of osteoarthritis-like gene expression changes under IL-1\(\beta\) stimulation. Overexpression of Bmal1 through stimulation of Sirt1 relieved the metabolic imbalance induced by IL-1\(\beta\) in osteoarthritis\(^{(43)}\). Therefore, we can suggest that Sirt1 probably acts as a stimulator for circadian genes.

In addition, TRF increased gut microbiome richness and abundance at different levels. Importantly, we found that Sirt1 activation and increased level of HDL-c were significantly positive associated with an increase in microbial richness. These data strongly suggest that TRF-induced alteration in gut microbiome richness can intervene a systemic anti-obesity response and protection against metabolic risk, which is in agreement with previous study\(^{(14)}\). It is well known that nutrition plays an important role in gut microbiome modulation\(^{(44)}\). Gut microbiota dysbiosis mediate the pathogenesis of several human diseases\(^{(45)}\). In this study, we found that TRF increased microbial diversity and was positive correlated with HDL and Sirt1. These results are in consistent with previous studies reported that gut microbial community structure was significantly influenced and more diverse after long term intervention of CR than people eating western diets\(^{(46)}\) while IF also significantly increased the microbiome richness\(^{(14)}\). TRF had a striking effect on microbiome composition. Interestingly, we also showed that Bacteroidia inversely correlated with LDL-c and TG level that exhibited anti-obesity response. This is in line with a study suggested that increased abundance of Bacterioidetes members is directly associated with weight loss in mice\(^{(47)}\). In our study, TRF exhibited a significant increase in the abundance of Bacteriodia and Bacterioidetes, which is in agreement with previous reports in animal models\(^{(48, 49)}\). Low abundance of Bacterioidetes enhanced the development of inflammatory conditions including obesity, atherosclerosis, neurodegenerative diseases and diabetes\(^{(50)}\).

Dysbiosis and disturbed interaction of host-microbe may cause circadian misalignment leading to the pathogenesis of metabolic disorders. Overexpression of Bmal1 decreases hyperlipidemia and atherosclerosis through modulation of lipoprotein production and biliary cholesterol excretion in ApoE\(^{-/-}\) mice\(^{(51)}\). Besides food intake and daylight cycle, microbiota are
also involved in control of circadian system that regulate intestinal physiology and systemic metabolism\(^{(52)}\). Gut microbiome has circadian rhythmicity and helps the host circadian clock function. Here, we found a significant positive correlation of Bmal1 with Prevotella and Bacteroidia and Sirt1 with Prevotellaceae, Bacteroidia and Dialisster. Prevotella derived SCFAs produced by the fermentation of non-digestible fiber facilitate peripheral clock adjustment in mouse tissues\(^{(53)}\). Bmal1 expression disturbs during gut microbiome ablation resulting in prediabetic phenotype and ileal corticosterone overproduction. In addition, lacking of healthy gut microbiota leading to a trends in downregulation of clock control genes expression, especially involved in metabolic regulation\(^{(54)}\).

In conclusion, the current study showed that TRF reduced serum lipid and liver profiles, while induced downregulation of inflammatory genes. In addition, TRF increased the microbial richness and abundance, which probably associated with circadian rhythm and lipid levels. Therefore, TRF could be a safe therapeutic remedy for the prevention of metabolic diseases related to dyslipidemia and elevated liver function, while regulates circadian rhythm associated with gut microbiome modulation.

**Conflict of Interest**

All authors declare no conflict of interest regarding this paper.

**Funding/Acknowledgements**

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Figure legends

Fig. 1 TRF reduced the risk of metabolic diseases through regulation of lipid profile

(A) Serum total cholesterol (TC), (B) Triglycerides (TG), (C) low density lipoprotein-cholesterol (LDL-C) and (D) high density lipoprotein-cholesterol (HDL-C) in pre/post-TRF (n=56) and non-TRF (n=24). One-way ANOVA followed by Bonferroni post hoc tests was used, ****p<0.0000, ***p<0.0001, **p<0.001 Post-TRF vs Pre-TRF and Non-TRF.
Fig. 2 TRF improves liver function by regulating its enzymes

(A) Serum alkaline phosphatase (AKP), (B) aspartate aminotransferase (AST), (C) alanine aminotransferase (ALT) and (D) Albumin in pre/post-TRF (n=56) and non-TRF (n=24). One-way ANOVA followed by Bonferroni post hoc tests was used, ****p<0.0000, ***p<0.0001, *p<0.01 Post-TRF vs Pre-TRF and Non-TRF.
Fig. 3 TRF reduced production of pro-inflammatory cytokines

(A) Interleukin-1 beta (IL-1β) and (B) Tumor necrosis factor alpha (TNF-α) gene expression at mRNA and (C & D) Serum level in pre-TRF, post-TRF (n=56) and non-TRF (n=24). One-way ANOVA followed by Bonferroni post hoc tests was used.
Fig. 4 TRF regulates circadian rhythm and its stimulator

(A) Brain and Muscle ARNT-Like 1 (Bmal1), (B) Circadian locomoter output cycles protein kaput (Clock), (C) Sirtuin-1 (SIRT1) expression at mRNA level. One-way ANOVA followed by Bonferroni post hoc tests was used, ****p<0.0000, ***p<0.0005, **p<0.005, *p<0.05 Post-TRF vs Pre-TRF and Non-TRF.
Fig. 5 TRF increases Gut microbiome diversity and has profound association with Sirt1 expression and HDL level

Stool samples were collected from the post-TRF (after 25 days of trial n=14) and non-TRF (n=18). (A) Principle component analysis (PCoA) illustrate microbiome similarity in post-TRF and non-TRF groups at OUT level (each dot is one sample; x axis and y axis are first and second dimension of microbiome data). Samples from two groups clustered separately, indicating two distinct microbiome communities (p<0.05, PERMANOVA test). (B) Microbial richness increased significantly in post-TRF but not in non-TRF (linear regression **p<0.005). (C) Sirt1 mRNA expression was significantly positive correlated with microbial richness (r=0.45; p=0.0201, Pearson correlation). (D) Serum albumin was significantly negative correlated with microbial richness (r=−0.37, p=0.0495). (E) Serum high density lipoprotein (HDL) level was significantly positive correlated with bacterial richness (r=0.42, p=0.0289).
16 sRNA sequencing was performed for stool collected from post-TRF (n=18) and non-TRF groups (n=14). (A) A linear discriminate analysis (LDA) was conducted to identify differentially represented microbial communities abundance in two groups. Microbial community abundance with >2.5 LDA score (x axis) and p<0.05 are shown. The right side of the figure represents microbial community whose abundance was significantly higher in post-TRF group, while the left side in non-TRF. The absolute LDA value is the effect size between two groups for a particular microbial community. (B, C) Serum low density lipoprotein (LDL-c) and triglycerides (TG) were significantly negative correlation with Bacteroidia.
Fig. 7 Association of healthy gut microbiome with circadian genes

(A, B, C) Prevotellaceae (family), Prevotella_9 (genus), Bacteroidia (class) abundances were significantly positive correlated with Bmal1 gene expression ($r=0.67$, $p=0.0003$; $r=0.70$, $p=0.0002$; $r=0.40$, $p=0.0370$ respectively, Pearson correlation). (D, E, F, G, H) Prevotellaceae (family), Prevotella_9 (genus), Bacteroidia (class), Dialisster (genus), Prevotella_2 (genus) abundance was significantly positive association with Sirt1 gene expression ($r=0.70$, $p=0.0002$; $r=0.73$, $p<0.0001$; $r=0.45$, $p=0.0199$; $r=0.50$, $p=0.0105$ and $r=0.69$, $p=0.0002$ respectively).