FOOD & FUNCTION

Fuzhuan tea consumption imparts hepatoprotective effects and alters intestinal microbiota in high saturated fat diet-fed rats

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Received: August 17, 2015
Revised: November 30, 2015
Accepted: January 18, 2016

Scope: Nonalcoholic fatty liver disease is an obesity-related disorder characterized by lipid infiltration of the liver. Management is limited to lifestyle modifications, highlighting the need for alternative therapeutic options. The objective of this study was to examine if fermented Fuzhuan tea prevents metabolic impairments associated with development of hepatic steatosis.

Methods and results: Rats consumed control (CON) or high saturated fat (SAT) diets with or without Fuzhuan tea for 8 weeks. Outcomes included enzymatic and gene expression measures of metabolic dysregulation in liver and adipose tissue. Pyrosequencing was used to assess intestinal microbiota adaptations. Fuzhuan tea prevented diet-induced inflammation in the liver. Liver triglycerides of ~18 mg/g were observed in SAT-fed animals, but remained similar to CON diet levels (~12 mg/g) when supplemented with Fuzhuan tea. In adipose tissue, tea treatment prevented SAT-induced inflammation and reduced plasma leptin approximately twofold. Fuzhuan tea also altered intestinal function and was associated with a threefold increase in two *Lactobacillus spp*.

Conclusions: These data suggest that Fuzhuan tea protects against liver and adipose tissue stress induced by a high SAT diet and positively influences intestinal function. Further investigation of the molecular targets of Fuzhuan tea is warranted.

Keywords:
Fuzhuan tea / Inflammation / Lactobacillus / Nonalcoholic fatty liver disease / Saturated fat

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1 Introduction

Nonalcoholic fatty liver disease (NAFLD) is an emerging obesity-related disorder characterized by lipid infiltration (steatosis) of the liver in the absence of chronic alcohol consumption. In some individuals, steatosis progresses to nonalcoholic steatohepatitis, which is characterized by steatosis, inflammation, apoptosis, and fibrosis [1]. NAFLD is now recognized as the most common cause of chronic liver enzyme elevations and cryptogenic cirrhosis [2]. The estimates of NAFLD prevalence in a US population, based on determination of hepatic steatosis by ultrasonography or biopsy, suggest that 19–46% of tested individuals had NAFLD, which may translate to as many as 28.8 million adults afflicted with this condition [3].

Although the underlying pathogenesis of NAFLD is not known, several mechanisms for the development of steatosis and disease progression have been proposed. One suggested mechanism for steatosis development is a combination of diet, de novo lipogenesis, and increased free fatty acid delivery. The latter may account for as much as 60% of stored lipids within the liver of patients with NAFLD [4] suggesting that the regulation of adipose tissue lipid stores and adipokine levels are critical factors in NAFLD development. Another suggested mechanism is disruption of ER homeostasis, often referred to as ER stress, which has been observed in

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Abbreviations: ALT, alanine aminotransferase; **NAFLD**, nonalcoholic fatty liver disease; **SAT**, saturated fat

liver and adipose tissue of humans with NAFLD and/or obesity [5–8]. The signaling pathway activated by disruption of ER homeostasis, the unfolded protein response, has been linked to lipid biosynthesis, insulin resistance, inflammation, and apoptosis characteristic of nonalcoholic steatohepatitis [5, 9, 10]. Finally, recent studies have suggested that development of NAFLD may be linked to gastrointestinal health and the composition of the gut microbiota. Translocation of bacterial LPS with dietary fats or through leaky epithelial cell junctions has been demonstrated to mediate systemic inflammatory responses through Toll-like receptors and other pathogen-recognition receptors [11]. In addition, there is some evidence that specific members of the gut microbial community result in endogenous ethanol production and elevated blood alcohol levels in the absence of alcohol consumption [12].

A growing body of evidence has demonstrated that saturated fatty acids, derived from adipose tissue or the diet, provoke ER stress, inflammation, and liver injury to a greater extent than unsaturated fatty acids [13, 14]. Management of NAFLD includes recommendations to reduce body weight, limit consumption of saturated fat and simple sugars, and increase physical activity [15]; however, alternative pharmaceutical and/or nutraceutical treatments need to be explored. Fuzhuan tea, a Chinese brick-style tea that undergoes controlled fermentation by the fungus *Eurotium cristatum*, may be a suitable candidate. Previous investigations have shown that Fuzhuan tea has a distinct chemical profile from green teas, novel phytochemical compounds [16–19], and can lower plasma lipid levels in humans with elevated LDL cholesterol [20]. In high fat diet-fed rats, Fuzhuan tea reduced body weight and hepatic steatosis, and suppressed development of hypolipidemia [21]. Another study demonstrated that rats consuming Fuzhuan tea with HFD showed protein expression consistent with reduced lipogenesis and enhanced β-oxidation, tricarboxylic acid cycle, and electron transport when compared with HFD-fed rats not receiving the tea [22]. Finally, several studies have demonstrated inhibitory effects of Fuzhuan tea on enteric pathogens and one study showed stimulation of specific commensal organisms, including *Lactobacilli* [23]. Based on this body of literature, we hypothesized that Fuzhuan tea consuming rats on a high saturated fat diet (SAT) would show reduced metabolic impairment and a more favorable intestinal microbial profile relative to SAT-consuming animals not supplemented with tea. To test this hypothesis, we examined the ability of Fuzhuan tea to prevent liver and adipose tissue inflammation and explored intestinal microbiota adaptations to a high SAT diet and Fuzhuan tea.

2 Methods

2.1 Animal research

Adult male Wistar rats (Charles River, Wilmington, MA, USA) weighing ~200–250 g were individually housed under controlled conditions. During the 8 wk experiment, rats had free access to either a control diet (CON; $n = 13$) or a diet high in saturated fat (SAT; $n = 13$) (Supporting Information Table 1). A subset of five animals from each group was supplemented with Fuzhuan tea (Naturalin Bio-Resources, Hunan, China). Tea was provided as a powdered extract to the drinking water. Extract concentration was adjusted each week based on the average animal weight and the amount of beverage consumed in order to maintain a target dosage of 1400 mg/kg/wk, which was previously shown to reduce plasma lipids [24]. Body mass, food intake, and tea consumption were recorded weekly. All rats were fasted for 4 h prior to termination. After being weighed and anesthetized with isoflurane, blood was collected by cardiac puncture. A portion of the right lobe of the liver was frozen in liquid nitrogen. Inguinal (IWAT), epididymal (EWAT), perirenal (PWAT), visceral white adipose tissue (VWAT; mesenteric and omental), and interscapular brown adipose (BAT), were dissected and weighed. IWAT and VWAT were frozen in liquid nitrogen. Cecal contents and terminal ileum were also collected and frozen in liquid nitrogen. All samples were stored at −80°C. Procedures were conducted under protocol number 14–5067A, which was reviewed and approved by the Colorado State University Institutional Animal Care and Use Committee.

2.2 Glucose tolerance test (GTT)

One week before experiment termination, rats were fasted overnight and blood glucose was determined from tail vein blood. Rats received an intraperitoneal injection of 1.5 g/kg dextrose and blood glucose was assessed at baseline, 15, 30, 45, 60, and 120 min postinjection. In addition, ~150 μL of blood was collected at baseline, 15, 60 and 120 min for measurement of plasma insulin (Linco Research, St. Charles, MO, USA).

2.3 Plasma alanine aminotransferase

Alanine aminotransferase (ALT) concentration was determined on plasma samples according to manufacturer instructions (Cayman Chemical, Ann Arbor, MI, USA).

2.4 Plasma adipokines

Plasma insulin, leptin, monocyte chemotactic protein-1 (MCP-1), IL-1β, IL-6, tumor necrosis factor α (TNF-α), and plasminogen activator inhibitor-1 concentrations were determined using commercial multiplex kits (EMD Millipore Corporation, Billerica, MA, USA) and analyzed on a Luminex instrument (LX200; Millipore, Austin, TX, USA).

2.5 Liver and adipose tissue RNA isolation, cDNA synthesis, and real-time PCR

Liver RNA was isolated with Trizol (Life Technologies, Grand Island, NY, USA) according to the manufacturer's instructions and adipose tissue RNA was isolated with lipid-specific RNAeasy mini-kit columns (Qiagen, Valencia, CA). iScript (Bio-Rad, Hercules, CA, USA) was used to synthesize cDNA from 0.25 μg total RNA. Primer sequences are shown in Supporting Information Table 2. Primers were optimized as previously described [25]. Samples were run in triplicate using an iCycler and iQ SYBR Green Supermix (Bio-Rad). Expression patterns of genes of interest were normalized to constitutively expressed β2 microglobulin and relative expression was quantified as previously described [25].

2.6 Bacterial sequencing and qPCR enumeration

DNA was extracted from fecal pellets using the MoBio Ultraclean Soil DNA isolation kit (MoBio, Carlsbad, CA, USA). DNA was quantified using PicoGreen (Quanti-it Picogreen, Life Technologies) and diluted to 10 ng/μL. Sequencing libraries were constructed using primers for the V3-V5 region of the 16s rRNA gene and pyrosequencing was conducted at Research and Testing Laboratory (Lubbock, TX, USA) on a Roche GS FLX+ using Titanium chemistry. Sequence data were aligned to the bacterial-subset SILVA alignment and classified using the Green Genes database and NCBI BLAST.

Quantitative PCR reactions using *Lactobacillus* genus primers (forward: 5′-AGCAGTAGGGAATCTTCCA-3′, reverse: 5′-CACCGCTACACATGGAG-3′) [26] were prepared with SsoAdvanced SyberGreen and run on a CFX96 thermal cycler. Optimized cycling conditions were as follows: 95°C for 3 min and then 40 cycles of 95°C 15s, 66°C 15s, 72°C 10s, and 85°C 5s. Sample quantification was determined by comparing to a 5-point standard curve of *Lactobacillus plantarum* amplicons The16s copy number was calculated as follows [27]:

$$\begin{aligned}\text{Gene copies} &= \left(\text{DNA concentration}\left(\text{ng}/\mu\text{L}\right)\right) \\ &\times \left(\frac{1\text{ g}}{1000^3\text{ ng}}\right) \times \left(\frac{1\text{mole bp DNA}}{660\text{ g DNA}}\right) \\ &\times (6.023 \times 10^{23}\text{bp/mol bp}) \\ &\times \left(\frac{1\text{ copy}}{\text{genome or plasmid size (bp)}}\right) \\ &\times (\text{volume of template } (\mu\text{L}))\end{aligned}$$

2.7 Endotoxin quantification

Plasma was diluted 1:5 in endotoxin-free water and heated at 70°C for 10 min. The Limulus Amebocyte Lysate chromogenic assay (Lonza, Basil, Switzerland) was used according to the manufacturer's instructions and a 5-point standard curve of defined endotoxin concentrations was used for quantification.

2.8 Intestinal alkaline phosphatase

Ileal tissue was weighed, homogenized, and assayed for activity of alkaline phosphatase using the SensoLyte pNPP Alkaline Phosphatase Assay Kit (Anaspec Inc., Fremont, CA, USA) following manufacturer's instructions.

2.9 Statistical analyses

Data are expressed as mean ± SEM. Outliers were identified and removed using a modified Thompson Tau test for systemic endotoxin and intestinal alkaline phosphatase (IAP) assays [28]. Body and adipose mass, food intake, GTT and insulin AUC, triglyceride concentration, gene expression, liver ALT concentration, fatty acid composition, adipo/cytokines, systemic endotoxin, IAP, and *Lactobacillus* gene copy were analyzed using two-way ANOVA with a diet × group (2 × 2) design. Post hoc analysis of groups were made using Tukey's tests, except for sequence data where post hoc analysis was done with Bonferroni corrected *p*-values (SPSS for Windows, release 11.5.0; SPSS, Chicago, IL, USA and XLSTAT-Pro, Addinsoft Inc. Paris, France). For all experiments, differences among groups were considered statistically significant if $p \leq 0.05$.

3 Results

3.1 Physiologic responses to Fuzhuan tea treatment

Based on previously published studies [24], we chose a Fuzhuan tea dose of 1400 mg/kg body weight per week. Actual achieved doses ranged from 1200 to 1800 mg/kg/wk per animal (Supporting Information Fig. 1). Animals consuming the SAT diet had higher overall energy intake compared to CON, regardless of Fuzhuan tea treatment (Table 1). Fuzhuan tea treated animals on the SAT diet had significantly lower inguinal and total adipose tissue than CON animals without tea and significantly lower perirenal adipose tissue than both SAT and CON no-tea rats. Despite reductions in specific adipose tissue depots, there was no significant difference in total body weight change between SAT and CON without tea and Fuzhuan tea treated SAT animals. Fuzhuan tea treated animals on the CON diet gained significantly less weight than the other groups. The glucose and insulin responses to a glucose injection were not significantly different among groups (Table 1).

Table 1. Food intake and physiologic and metabolic parameters after 8 wk

	No Tea		Tea	
	CON	SAT	CON	SAT
Cumulative FI (kcal)	4611 ± 146.5[a]	5704 ± 204.9[b]	4500 ± 75.1[a]	5666 ± 124.5[b]
Normalized IWAT adipose mass (mg/g of body mass)	20 ± 2[a]	18 ± 2[a,b]	16 ± 2[a,b]	14 ± 1[b]
Normalized EWAT adipose mass (mg/g of body mass)	21 ± 2	21 ± 2	15 ± 1	16 ± 2
Normalized RWAT adipose mass (mg/g of body mass)	24 ± 2[a]	23 ± 3[a]	17 ± 3[a,b]	15 ± 1[b]
Normalized VWAT adipose mass (mg/g of body mass)	17 ± 2	17 ± 2	14 ± 1	15 ± 1
Normalized total adipose mass (mg/g of body mass)	83 ± 6[a]	74 ± 8[a,b]	62 ± 4[a,b]	59 ± 4[b]
Body weight change (g)	280.6 ± 12.2[a]	279.5 ± 11.1[a]	248.8 ± 11.1[b]	289 ± 7.38[a]
GTT AUC × 10^4 (mg/dL/120 min)	1.57 ± 0.07	1.60 ± 0.19	1.82 ± 0.16	1.53 ± 0.12
Insulin AUC (pg/mL/120 min)	47.13 ± 9.1	64.5 ± 11	30.13 ± 12	48.30 ± 8.02
Liver triglycerides (mg/g)	11.8 ± 0.56[a]	17.87 ± 1.17[b]	13.57 ± 0.76[a]	12.54 ± 1.73[a]
Alanine aminotransferase (U/L)	32.5 ± 6.5[a]	76.9 ± 3.5[b]	47.1 ± 2.9[a]	45.6 ± 1.5[a]

Different letters indicate significant differences ($p < 0.05$).

3.2 Liver triglycerides and indices of injury and ER stress

Fuzhuan tea prevented increases in liver triglycerides and plasma ALT that were observed in the SAT group relative to CON (Table 1). Fuzhuan tea also prevented SAT-induced increases in gene markers of liver fibrosis such as α-smooth muscle actin (*Sma/Acta2*), transforming growth factor-β (*Tgfb1*), and collagen-α1 (*Col1a1*) mRNA, inflammation: Caspase-1 (*Casp1*), and ER stress: spliced X-box binding protein-1 (*Xbp1s*), glucose-regulated protein 78 (*Grp78*), C/EBP homologous protein (*Chop*) and growth arrest, and DNA damage inducible protein 34 (*Gadd34*) (Fig. 1A–H).

3.3 Adipose tissue gene expression, insulin, and adipo/cytokine concentration

Because Fuzhuan tea treatment prevented many negative effects of the SAT diet on liver injury and inflammation, we sought to determine whether this was tissue specific or if tea treatment could also decrease gene markers of inflammation in subcutaneous and visceral adipose tissue.

The increased mRNA expression of monocyte chemoattractant protein 1 (*Mcp-1*) (a protein which contributes to macrophage infiltration) observed in the SAT group was prevented by Fuzhuan tea treatment in both subcutaneous and visceral adipose tissue (Fig. 2A and B). Fuzhuan tea also prevented the increase in expression of the inflammatory cytokine tumor necrosis factor α (*TNF*-α mRNA that was observed in the SAT animals without tea (Fig. 2C and D). Tea treatment did not alter expression of the above genes in the CON diet group. Fuzhuan tea treatment also significantly decreased systemic leptin and plasminogen activator inhibitor concentrations in both the CON and SAT groups (Fig. 2E and F).

3.4 Systemic endotoxemia and IAP

Fuzhuan tea treatment significantly reduced plasma endotoxin in both diet groups; however, there was no significant difference between Fuzhuan tea supplemented SAT and CON (Fig. 3A). IAP, an enzyme that plays a role in the neutralization of luminal endotoxin, was higher in Fuzhuan tea treated SAT animals compared with SAT animals without tea (Fig. 3B).

3.5 Fuzhuan tea alters intestinal bacteria populations

Cecal contents from CON and SAT without tea and Fuzhuan tea treated SAT animals were analyzed for microbial composition by pyrosequencing, and a total of seven different bacterial phyla observed. Firmicutes, Bacteroidetes, and Verrucomicrobia represented ~97% of total sequences. The CON animals had a significantly higher amount of Bacteroidetes and Verrucomicrobia and significantly lower Firmicutes than SAT animals, regardless of Fuzhuan tea treatment (Supporting Information Fig. 2). Actinobacteria was only present in the Fuzhuan tea treated animals, although it only accounted for ~0.1% of the total community composition.

Bacterial sequences that most closely matched *Lactobacillus johnsonii* and a *Lactobacillus sp*. were significantly higher in Fuzhuan tea treated SAT animals than in CON or SAT groups without tea treatment (Fig. 3C). Quantitative PCR using *Lactobacillus*-specific primers confirmed that there were fewer *Lactobacillus* in SAT without tea treatment than in the other groups (Fig. 3D).

4 Discussion

NAFLD is a growing public health concern and the current interventions for this condition are limited to diet and

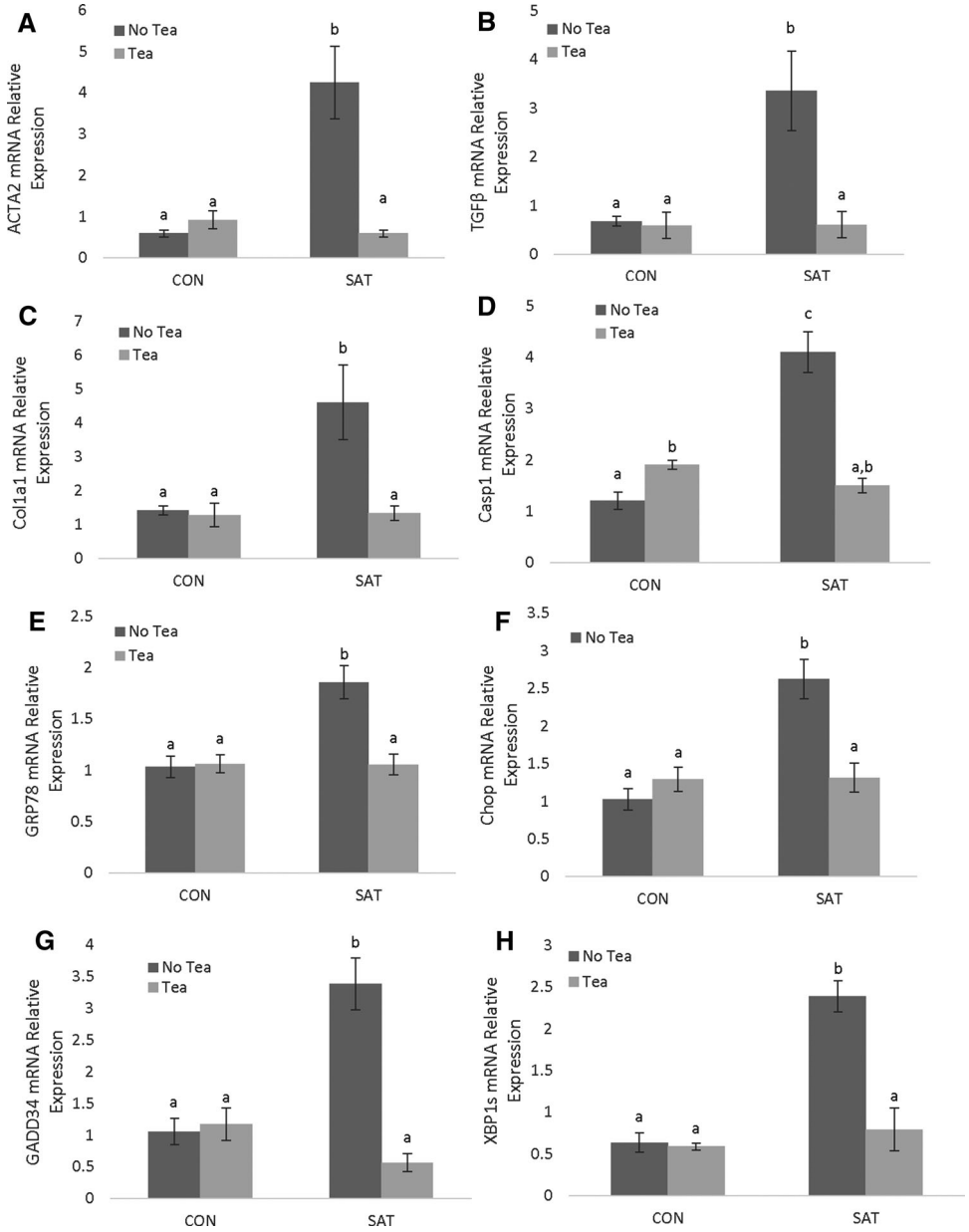

Figure 1. Gene markers of liver fibrosis and ER stress—Fuzhuan tea prevented SAT-associated increases in gene markers of liver fibrosis: (A) α-smooth muscle actin (*Sma/Acta2*), (B) transforming growth factor β (*Tgfb1*), (C) collagen-α1 (*Col1a1*); inflammation: (D) Caspase-1 (*C*asp1); as well as those involved in ER stress: (E) glucose-regulated protein 78 (Grp78), (F) C/EBP homologous protein (Chop), (G) growth arrest and DNA damage inducible protein 34 (*Gadd34*), and (H) spliced X-box-binding protein-1 (*Xbp1s*) ($p \leq 0.05$; unlike letters indicates statistical significance).

lifestyle modifications. In the present study, we demonstrate that a microbially fermented tea, Fuzhuan tea, prevents the negative effects of a high SAT diet on the intestines, liver, and adipose tissue in a Wistar rat model. Numerous studies have shown beneficial effects of green tea components, particularly catechins and other polyphenols on weight regulation, and glucose and lipid metabolism in human and animal models [29]. Tea compounds that have demonstrated beneficial effects on hepatic lipid metabolism, including epigallocatechin-3-gallate [30] and caffeine [31,32] are also found in varying levels in Fuzhuan tea. Unlike most teas, Fuzhuan tea is also rich in organic acids and contains several novel compounds, such a newly reported isoprenoid, and strictin, astragalin, and isovitexin [19] that may contribute to the effects reported here. NAFLD is characterized by hepatic steatosis, ER stress, inflammation, and liver injury [5, 9, 10]. The high SAT diet used in the present study resulted in a liver phenotype that included all of the above adaptations. The presence of Fuzhuan tea over an 8 wk period prevented diet-induced hepatic steatosis and indices of ER stress, inflammation, and liver injury.

Interestingly, the observed alterations in various organs in response to the SAT occurred in the absence of significant weight gain. The lack of SAT-induced increases in body/adiposity mass may be due to our selection of dietary fats. Control diets consisted of 12% fat from safflower and corn oil while SAT had 45% fat from cocoa butter. The simplest explanation for the lack of significant changes in body

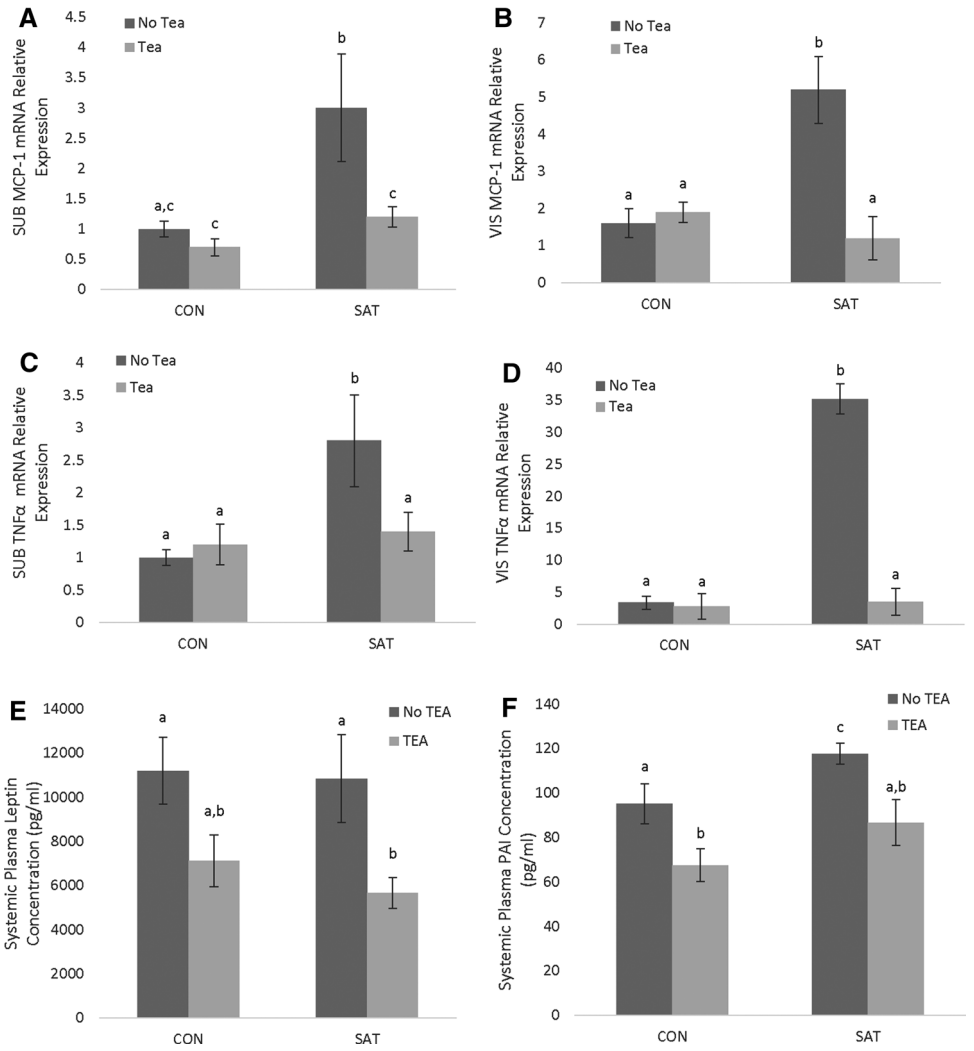

Figure 2. Markers of adipose tissue inflammation and adipokines—increased expression of monocyte chemoattractant protein 1 (*Mcp-1*) observed in the SAT group was prevented by Fuzhuan tea treatment in both (A) subcutaneous and (B) visceral adipose tissue. Fuzhuan tea also prevented the increase in tumor necrosis factor α *(TNF-α)* expression that occurred in the SAT animals in both (C) subcutaneous and (D) visceral adipose tissue ($p \leq 0.05$; unlike letters indicates statistical significance). Fuzhuan tea treatment decreased systemic (E) leptin and (F) plasminogen activator inhibitor concentrations in both the CON and SAT groups ($p \leq 0.05$; unlike letters indicates statistical significance).

weight is the variable digestibility of these fats. Previous studies have demonstrated that bioavailability of cocoa butter in rats is relatively low when compared with other fats such as corn oil [33]. This decreased digestibility would account for the significant increase in food intake of SAT rats, without the resulting increase in body mass compared with control. Despite the lack of difference in body weight and fat mass between SAT and CON, the SAT diet resulted in impaired regulation of adipose tissue (e.g. increased expression of AT inflammatory pathways), which was prevented in Fuzhuan tea fed animals.

Systemic endotoxin, which interacts with Toll-like receptor 4 and other receptors to mediate an inflammatory response, is also emerging as an important factor in NAFLD and liver inflammation [34]. In the present study, endotoxin levels were reduced and IAP activity was increased by Fuzhuan tea treatment. These data suggest that Fuzhuan tea may influence intestinal barrier integrity and alter endotoxin activity. Among its functions, IAP dephosphorylates bacterial endotoxin rendering it inactive, regulates lipid absorption in enterocytes, and limits transepithelial movement of bacteria [35]. In vitro studies have shown increased transepithelial electrical resistance in cell monolayers treated with tea polyphenols [36]. Therefore, combined reduction in endotoxin activity and reduction in endotoxin translocation to the plasma may account for some of the Fuzhuan tea mediated effects observed. We also demonstrate an increase in cecal *Lactobacilli* in Fuzhuan tea treated rats compared with the respective nontreated groups. *Lactobacillus spp*. chemically and competitively exclude pathogens, stimulate mucin secretion from intestinal L-cells, and modulate inflammatory responses through stimulation of Treg cells [37]. One of the *Lactobacillus* species that distinguished Fuzhuan tea treated animals from nontreated SAT and CON groups was identified as *L. johnsonii*. When given as a probiotic to outbred ICR mice on a high-fat diet, *L. johnsonii* prevented steatosis and reduced oxidative stress in the liver, and induced changes in the composition of the commensal biota [38].

In conclusion, scientific evidence to support the traditional medicinal claims surrounding Fuzhuan tea is emerging [21].

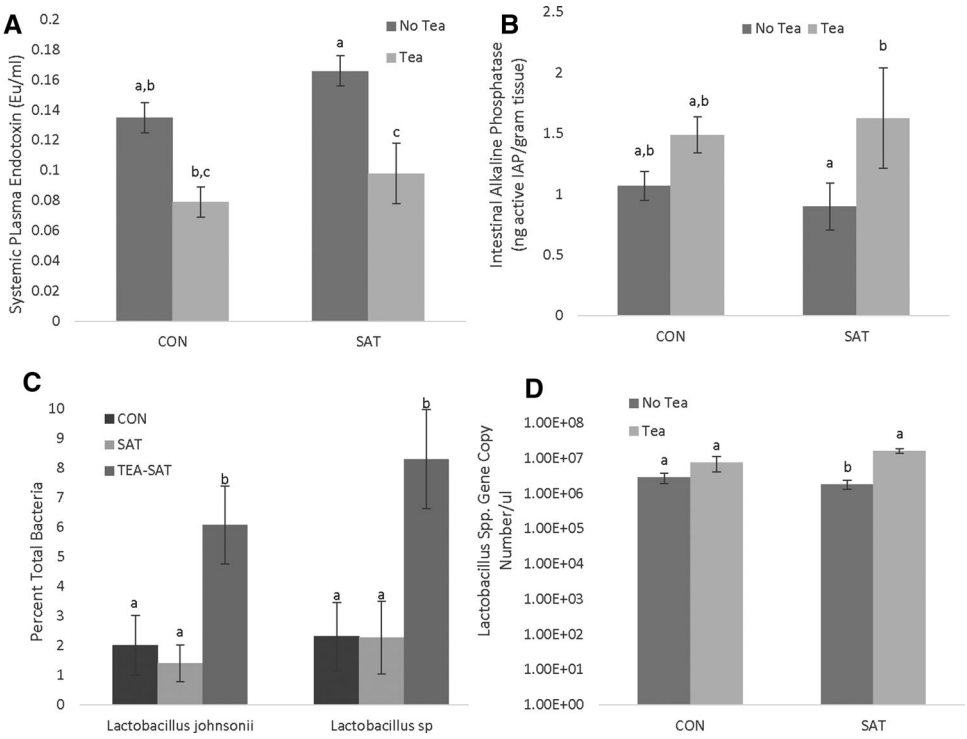

Figure 3. Markers of intestinal health and gut microbiota—(A) Fuzhuan tea treatment lowered plasma endotoxin in both diet groups. (B) IAP was higher in Fuzhuan tea treated SAT animals compared with SAT animals without tea. (C) *Lactobacillus johnsonii* and an unclassified *Lactobacillus sp.* were significantly higher in Fuzhuan tea treated SAT animals than in CON or SAT groups without tea treatment. (D) *Lactobacillus* expression was lower in SAT without tea treatment than in the other groups ($p \leq 0.05$; unlike letters indicates statistical significance).

In the present study, we show protection against markers of liver and adipose tissue stress in animals consuming a high-SAT diet. We also see potentially beneficial effects on intestinal function and gut bacteria that are independent of diet. Whether benefits seen in the liver and adipose tissue are associated with improvements in intestinal health or driven by independent mechanisms remains to be elucidated and will be the focus of future investigations.

We would like to thank Gretchen Moran and Brittany Barnett for assistance with animal care and terminations. Funding for this work was provided by NIH 7K01DK 087816 (M.T.F), and CSU College of Health and Human Science Minigrant (T.L.W.).

Experiments were planned and manuscript was written by M.T.F., C.L.G., K.C.Y., M.J.P., and T.L.W. Animal experiments were conducted by M.T.F., C.L.G., K.C.Y., A.E., L.R., M.J.P., and T.L.W. Sample analysis was conducted by M.T.F., C.L.G., K.Y.C., Y.W., D.W., M.J.P., T.M., and T.L.W. All authors read and approved the final manuscript.

The authors have declared no conflict of interest.

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