Dietary Copper-Fructose Interactions Alter Gut Microbial Activity in Male Rats

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Abstract
Dietary copper-fructose interactions contribute to the development of nonalcoholic fatty liver disease (NAFLD). Gut microbiota play critical roles in the pathogenesis of NAFLD. The aim of this study was to determine the effect of different dietary doses of copper and their interactions with high fructose on gut microbiome. Male weanling Sprague-Dawley rats were fed diets with adequate copper (6 ppm, CuA), marginal copper (1.5 ppm, CuM) (low-copper) or supplemented copper (20 ppm, CuS) (high-copper) for 4 weeks. Deionized water or deionized water containing 30% fructose (w/v) was given ad lib. Copper status, liver enzymes, gut barrier function and gut microbiome were evaluated. Both low- and high-copper diets led to liver injury in high fructose fed rats, and this was associated with gut barrier dysfunction, as shown by the markedly decreased tight junction proteins and increased gut permeability. 16S rDNA sequencing analysis revealed distinct alterations of the gut microbiome associated with dietary low- and high-copper/high-fructose feeding. The common features of the alterations of the gut microbiome were the increased abundance of Firmicutes and the depletion of Akkermansia. However, they differed mainly within the phylum, Firmicutes. Our data demonstrated that a complex interplay between host, microbes and dietary copper-fructose interaction regulates gut microbial metabolic activity, which may contribute to the development of liver injury and hepatic steatosis. The distinct alterations of gut microbial activity, which were associated with the different dietary doses of copper and fructose, imply that separate mechanism(s) may be involved.

Keywords: Copper; Fructose; Gut microbiome; Gut barrier function; NAFLD.
New & Noteworthy

1. Dietary low- and high-copper/high fructose induced liver injury are associated with distinct alterations of gut microbiome.

2. Dietary copper level plays a critical role in maintaining the gut barrier integrity, likely through acting on the intestinal tight junction proteins and the protective commensal bacteria, Akkermansia.

3. The alterations of gut microbiome induced by dietary low- and high-copper with or without fructose differ mainly within the phylum, Firmicutes.
Glossary: NAFLD, nonalcoholic fatty liver disease; NASH, nonalcoholic steatohepatitis; ALT, alanine aminotransferase; AST, aspartate aminotransferase; NEFA, non-esterified fatty acids; MCP-1, monocyte chemoattractant protein-1; LBP, Lipopolysaccharide binding protein; HOMA-IR, homeostasis model assessment of insulin resistance; LPS, lipopolysaccharide; PAS, periodic acid-Schiff; Slc31a1(Ctr1), solute carrier family 31(copper transporters) member 1; TLR, toll like receptor; SCFAs, short chain fatty acids.
Introduction

NAFLD is now the most common liver disease in the United States, with an increasing prevalence worldwide. A recent study showed the prevalence of NAFLD and nonalcoholic steatohepatitis (NASH) to be 46% and 12.2%, respectively, in middle-aged adults in the United States (59). Moreover, NASH is now the third most common indication for liver transplantation in the United States (11). Unfortunately, the etiology and progression of NAFLD are both complex and remain poorly understood. Thus far, there is no FDA-approved drug therapy. Therefore, there is an unmet need for mechanistic studies and therapeutic targets.

The rapid rise in NAFLD suggests a possible role for environmental factors in the pathogenesis of this disease. Accumulating evidence indicates that dietary fructose intake is closely associated with the prevalence of obesity and NAFLD (5, 38). Recently, low copper availability was observed in NAFLD patients, and a copper deficient diet induces fatty liver in rodents (1, 2). In fact, inadequate copper intake (27, 29, 30) and increased fructose consumption represent two important nutritional problems in the United States (52, 57). Further, dietary copper-fructose interactions worsen copper status and are associated with the metabolic syndrome (19-21). Our previous studies demonstrated that copper-fructose interactions exacerbate liver injury and accelerate hepatic fat accumulation, which may contribute to dietary fructose-induced fatty liver (46, 47). However, the underlying mechanisms by which dietary copper-fructose interactions induce hepatic steatosis remain to be determined. One proposed mechanism by which fructose may cause hepatic lipid accumulation is that fructose is converted to fatty acids at a greater rate than glucose (52). However, even with increased de novo lipogenesis, it
remains largely unknown how 10% of calorie intake from fructose for a typical American, or even excessive dietary fructose consumption of up to 18% of caloric intake for a NAFLD patient, causes fatty liver (38, 52). In fact, the intestinal absorptive capacity of fructose is much lower than for glucose (16, 17, 22, 42). In contrast, fructose leads to a significantly higher hepatic lipid accumulation than glucose, even though the total caloric intake from fructose is less than glucose (8), suggesting that the lipogenic effect of fructose may not merely depend on metabolism in the liver.

A growing body of evidence has shown that gut microbiota play a critical role in the pathogenesis of obesity, diabetes and the metabolic syndrome, as well as NAFLD (10, 23). Interactions between diet, gut microbiota and host genetics are thought to be critical in the development of obesity and NAFLD (32, 54). In fact, the role of fructose in the induction of intestinal bacteria overgrowth, gut permeability and endotoxemia is well documented (8, 50). Several lines of evidence indicate that copper might be involved in the regulation of gut microbiota and gut barrier function. First, copper has been used as an antimicrobial agent throughout the ages (26). Second, the response to copper stress varies greatly among different bacteria species (39, 45). It appears that copper homeostasis is an important factor in the pathogenesis of steatosis (1, 2, 37, 51). However, whether and how dietary copper-fructose interactions contribute to the development of steatosis through altering gut microbiota and gut barrier function remain unknown. In this study, we fed rats with different levels of copper with or without fructose to evaluate the role of dietary copper-fructose interactions in the etiology of hepatic steatosis from the perspective of gut microbiota and gut barrier function.
Materials and Methods

Animal experiments. Male weanling Sprague-Dawley rats (35-45g) from the Harlan Laboratories (Indianapolis, IN) were fed (ad lib) a purified AIN-76 diet with a defined copper content in the form of cupric carbonate. The rats received 1.6 ppm, 6.0 ppm or 20 ppm of copper as marginal, adequate or supplemental doses, respectively, for 4 weeks. Control animals were fed adequate copper with no added fructose. The animals were single housed in stainless steel cages without bedding in a temperature- and humidity-controlled room with a 12:12h light-dark cycle. Animals had free access to either deionized water or deionized water containing 30% fructose (w/v). Fructose enriched drinking water was changed twice a week. After fasting overnight, all the animals were killed under anesthesia with pentobarbital (50 mg/kg I.P. injection). Blood was collected from the inferior vena cava, and citrated plasma was stored at -80°C for further analysis. Portions of liver tissue were fixed with 10% formalin for subsequent sectioning, while others were snap-frozen with liquid nitrogen. All studies were approved by the University of Louisville Institutional Animal Care and Use Committee, which is certified by the American Association of Accreditation of Laboratory Animal Care.

Assessment of copper status. Plasma ceruloplasmin was measured on the basis of its oxidase activity (44). Plasma copper and liver copper were measured by Varian SpectrAA 880/GTA-100 graphite furnace atomic absorption spectrometer (AAS) (Worcester Polytechnic Institute, Worcester, MA).

Liver enzyme and plasma biochemical assays. Liver enzymes and plasma biochemical assays were performed with commercially available kits: alanine aminotransferase (ALT), aspartate aminotransferase (AST), glucose, cholesterol,
triglyceride (Infinity, Thermo Electron, Melbourne, Australia); non-esterified fatty acids (NEFA) (Wako Chemicals, Richmond, VA); insulin (Lino Research, St. Charles, MO); and monocyte chemoattractant protein-1 (MCP-1) (Invitrogen Corporation, Camarillo, CA).

**Determination of gut permeability, plasma endotoxin and lipopolysaccharide binding protein (LBP).** For *ex vivo* detection of intestinal permeability, a freshly isolated 10 cm section of ileum was rinsed with modified Krebs-Henseleit bicarbonate buffer (KHBB, pH 7.4). 100 µl FITC-dextran (molecular weight 4,000, FD-4, 40 mg/ml) was injected into the lumen before the gut was ligated to form a sac, as described previously (64). The gut sac was then placed in KHBB and incubated at 37°C for 20 min. The FD-4 that penetrated from the lumen into the incubation buffer was measured spectrofluorometrically with an excitation wavelength of 485 nm and an emission wavelength of 530 nm. The FD-4 permeability was expressed as micrograms per centimeter per minute. Plasma samples were diluted 2-fold in pyrogen-free water and heated at 75°C for 10 min and cooled down to room temperature (64). Endotoxin (lipopolysaccharide, LPS) was measured using the limulus ameobocyte lysate (LAL) kit (Lonza Walkersville Inc., Walkersville, MD) according to the manufacturer’s instructions. The control standard endotoxin (CSE) is from bacterial strain E.coli O111:B4. Samples were not spiked. Lipopolysaccharide binding protein (LBP) was determined using a commercially available ELISA kit (Cell Sciences, Canton, MA).

**Histology.** For goblet cell analysis, paraffin-embedded ileum sections were stained with periodic acid-Schiff (PAS). The goblet cells were stained pink and the number of goblet cells were quantified as per villus (24).
Isolation of RNA and real time RT-PCR. Total RNA was extracted from the liver, and the mucosa of the duodenum and ileum using TRIZOL (Invitrogen, Carlsbad, CA). Primers were designed using Primer-BLAST (http://www.ncbi.nlm.nih.gov/tools/primer-blast/index.cgi?LINK_LOC=BlastHome) or from published papers (supporting information). Real-time PCR was performed with an ABI prism 7500 sequence detection system and SYBR green I dye reagents. The relative gene expression was analyzed using the 2-ΔΔCt method.

Western blot. Equal amounts of protein extracted from the mucosa of ileum homogenate were loaded and resolved on 4%-15% SDS-polyacrylamide gels, and transferred to PVDF membrane (Millipore, Bedford, MA). The membrane was blocked and probed with primary antibody (1:1000 dilution) for Claudin-1 (catalog number: 374900), Occludin (catalog number: 33-1500) (ThermoFisher Scientific Inc., Waltham, MA), and Reg3B (Catalog number: MAB1996) (R&D Systems Inc., Minneapolis, MN), overnight at 4°C, and incubated with the corresponding horseradish peroxidase-conjugated secondary antibody. Protein signals were visualized using the enhanced chemiluminescence system (Amersham Biosciences, Little Chalfont, UK). Band intensities were quantified using Image J software (http://rsb.info.nih.gov/ij/).

16S rDNA Gene Library Preparation and Sequencing on the Illumina MiSeq. Fecal pellets were collected into sterile tubes at the end of the experiment and stored at -80°C. Microbial genomic DNA was extracted from frozen fecal samples using PowerSoil DNA isolation kit (MO BIO Laboratories, Inc., Carlsbad, CA) according to the manufacturer’s instruction. The composition of fecal microbiota was analyzed using Illumina MiSeq technology targeting the variable V3 and V4 regions of 16S ribosomal
RNA. Primers that are complementary upstream and downstream of the region of interest were designed with overhang adapters, and were used to amplify templates from genomic DNA (Amplicon PCR). A subsequent limited-cycle amplification step was performed to add multiplexing indices and Illumina sequencing adapters (Index PCR). Each step was followed by the PCR clean-up, using AMPure XP beads to obtain a purified library. After libraries were normalized, pooled, and denatured, sequencing was done on the MiSeq system using paired 300bp reads and v3 reagents.

**Sequencing Data Analysis.** Quality control of raw sequence files was performed using FastQC and further analyzed using QIIME 1.9. Chimeric sequences were removed by using both reference and *de novo* approaches. The non-chimeric sequences were assigned to operational taxonomy units (OUTs) at 97% similarity to the clustered Greengenes database (15, 36). Rarefaction curve using the PD_whole_tree provided by QIIME was used as a metric of α-diversity (31). Principal coordinate analysis (PCoA) were performed to compare microbial community structure between samples (β-diversity) using weighted UniFrac (34).

**Statistical analysis.** Data were expressed as mean ± SD (Standard Deviation) and analyzed using two-way ANOVA testing factors of copper, fructose, and their interactions (copper × fructose), followed by Newman Keuls Multiple Comparison Test. PCoA comparing the mean distance between two groups using a two-sided Student’s t-test and the nonparametric p-values were calculated using 999 Monte Carlo permutations. Differences at P ≤ 0.05 were considered to be statistically significant.

**Results**
Effects of different dietary doses of copper and high fructose feeding on body weight, liver weight, epididymal fat weight, and blood metabolites

As shown in Table 1, no significant differences in body weight and body weight gain were observed between CuA and CuM rats with or without fructose feeding. However, high fructose feeding led to a significant reduction in body weight and body weight gain in CuS rats. In addition, high fructose feeding led to a significantly elevated liver/body weight ratio in both CuM and CuS rats. Liver weight was elevated in rats fed with marginal copper plus fructose (CuMF) compared to CuM rats. No obvious changes in epididymal fat or epididymal fat/body weight ratio were observed between groups.

Plasma cholesterol levels were lower in rats fed with adequate copper plus fructose (CuAF) than in CuA rats. Moreover, cholesterol was elevated in CuMF rats compared to the CuM rats. Plasma triglyceride levels did not differ between groups. High fructose feeding led to a marked reduction in plasma NEFA levels in both CuM and CuS rats. Similar effects were observed in plasma glucose levels and HOMA-IR. (For the purpose of minimizing the numbers of animal used to accomplish the research, some data was reused in Table 1 (CuMF) and part of Figs. 1-3 (CuA and CuMF) (48) to make an explicit comparison that would be much harder for the reader to make otherwise. We declare that the experiments were performed at the same time and same location).

Copper Status in response to dietary low- or high-copper high-fructose feeding

Plasma ceruloplasmin activity and plasma copper, as well as liver copper were significantly lower in CuM rats compared to CuA rats. High fructose feeding led to slightly decreased plasma copper and liver copper levels in both CuAF and CuMF rats compared to controls, but differences did not reach statistical significance. Plasma
ceruloplasmin activity and liver copper were significantly lower in CuS rats compared to CuA rats without high fructose feeding. On the contrary, high fructose feeding (CuSF) led to significantly higher plasma ceruloplasmin activity and higher plasma copper levels compared to CuS rats (with no added fructose) (Fig.1A, B, C). The mRNA expression of duodenal Slc31a1 (copper transporter-1, Ctr-1) was significantly downregulated by high fructose feeding in both CuM rats and CuS rats. Hepatic mRNA expression of Slc31a1 was significantly downregulated by high fructose feeding in CuAF rats compared to control. In addition, Slc31a1 was also downregulated in both CuM and CuS rats, but levels were not affected by high fructose feeding (Fig.1D and E).

**Both Dietary low- and high-copper induce hepatic steatosis and liver injury in high-fructose fed rats**

Both low copper and high copper diets led to markedly elevated plasma AST in those rats also fed high fructose compared to controls (Fig. 2B). High-copper/high-fructose diet led to significantly elevated plasma ALT compared to controls, whereas low-copper/high-fructose diet led to slightly elevated plasma ALT levels, which did not reach statistical significance (Fig. 2A). Plasma MCP-1 was modestly but significantly elevated in CuMF rats and CuS rats as well as in CuSF rats compared to controls (Fig. 2C). Both low- and high-copper/high fructose diet induced hepatic steatosis, which has been shown in our previous work (data not shown here) (58).

**Effects of different dietary doses of copper and high fructose feeding on plasma endotoxin, LBP and gut permeability**

Plasma endotoxin levels were significantly elevated in CuM rats compared to controls, and they were further elevated by high fructose feeding (CuMF). Plasma endotoxin levels
were not elevated in CuS rats compared to CuA rats. They were slightly, but significantly higher in CuSF rats than in CuA rats. However, plasma endotoxin levels in CuS rats were significantly lower than in CuM rats (Fig. 3A). Plasma LBP levels were significantly elevated by high fructose feeding, irrespective of dietary copper content (Fig. 3B). However, gut permeability, as evaluated by \textit{ex vivo} measurement of ileum permeability to FD-4, was significantly increased in CuSF rats (fed with high fructose) compared to controls. Gut permeability was also slightly increased in CuM rats. However, the difference did not reach the statistical significance (Fig. 3C).

\textit{Tight junction protein and Reg3B expression as well as goblet cell numbers in ileum epithelium in response to different dietary doses of copper and high fructose}

Claudin-1 and Occludin protein expression in ileum epithelium as shown by western blot was significantly downregulated in both low- and high-copper diet fed rats compared to controls, and Claudin-1 expression was further inhibited by dietary fructose (Fig. 4A). However, Reg3b protein expression in ileum epithelium was significantly upregulated in both low- and high-copper diet fed rats, and to a lesser extent in low copper diet and a greater extent in high copper diet fed rats. Two-way ANOVA revealed that Reg3b expression was related to dietary copper level, but not to fructose (Fig. 4A). In line with this finding, IL-22 mRNA expression, which is required for the induction of Reg3B (63), showed a trend of upregulation in the ileum of CuMF rat and markedly increase in CuSF rats (data not shown). Antimicrobial peptides expression at the mRNA level showed similar trends with protein expression. However, the mRNA expression of tight junction proteins was not consistent with protein expression, suggesting different levels of regulation (supporting information). The number of goblet cells as shown by PAS
staining was significantly reduced in the ileum of both CuMF and CuSF rats (Fig. 4B and 4C). Taken together, decreased expression of tight junction proteins and goblet cell numbers in the ileum of CuMF and CuSF rats suggested disrupted gut barrier function. The increased antimicrobial peptides expression is likely a response to increased gut pathobionts.

**Alterations of gut microbiome in response to different dietary doses of copper and high-fructose as analyzed by 16S rDNA pyrosequencing**

A total of 1,328,075 reads were obtained from a total of 38 fecal samples with a mean of 34,949 reads per sample. Eleven bacteria phyla, 62 families and 110 genera were identified in the gut microbiomes. Alpha-diversity reflects the diversity within the samples. The CuA group showed a large difference from the other five groups, which have a similar distribution. However, there is no statistically significant difference between groups (Fig. 5A). Beta-diversity was evaluated by UniFrac analysis. Unweighted UniFrac analysis demonstrated that CuS and CuSF groups are clustered together but different from other groups (Fig. 5B). The mean distance between groups CuS and CuA, CuSF and CuA, and CuMF and CuSF, are significantly different (nonparametric p-value < 0.05) (supporting information), suggesting CuS and CuSF rats have a similar bacteria community which is different from CuA and CuMF rats. Weighted UniFrac analysis revealed that the diversity between CuSF and CuA, and CuMF and CuSF are significantly different (Fig. 5B) (nonparametric p-value < 0.05) (supporting information). At the phylum level, while Firmicutes was significantly increased in CuMF, CuS and CuSF rats, Bacteroidetes was decreased in CuMF rats, which led to a robustly increased Firmicutes/Bacteroidetes ratio in CuMF rats compared to CuA rats (19.87 Versus 6.46,
CuMF versus CuA). Proteobacteria showed a trend of increase by dietary fructose or low/high copper level, and two-way ANOVA analysis suggested a role for fructose in boosting Proteobacteria. In addition, Verrucomicrobia were significantly decreased in CuMF, CuS and CuSF rats. It appears that significant alterations in gut microbiome composition at the phylum level occurred in both CuMF and CuSF rats, with CuMF rats being characterized with a more pronounced obesity phenotype, as shown by a robustly increased Firmicutes/Bacteroidetes ratio (33, 53). Further, significant changes were also observed at the family and genus levels. While Bifidobacteriaceae (family) and Bifidobacterium (genus), under the phylum-Actinobacteria, were significantly decreased in CuSF rat, Bacteroidaceae and Bacteroides under the phylum-Bacteroidetes, were markedly decreased in CuMF rats. Lactobacillaceae and Lactobacillus, under the phylum-Firmicutes, were significantly increased in both CuS and CuSF rats to a similar extent. Peptostreptococcaceae was significantly increased only in CuMF rats. While Lachnospiraceae showed a trend of increase in both CuMF and CuSF rats, Ruminococcaceae was markedly reduced in both CuMF and CuSF rats to a similar extent. In addition, a significant increase in Erysipelotrichaceae was only found in CuS and CuSF rats. Enterobacteriaceae, although in low abundance, was markedly increased in CuSF rats. Notably, Akkermansia was markedly reduced in CuMF, CuS and CuSF rats, which showed same trend as that in phylum level, suggesting that either low or high copper may reduce the abundance of Akkermansia, and Akkermansia depletion is a common feature in both CuMF and CuSF rats (Table 2). Accumulated evidence showed that Akkermansia plays a critical role in maintaining gut barrier function and has beneficial effects on the metabolic syndrome (13, 14, 18). Overall, it appears that dietary
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Copper levels have profound effects on the composition of gut bacteria and dietary low or high copper with or without fructose may shape gut microbiota differently.

**Real-time PCR using 16S rRNA gene primers for specific bacteria.**

To gain insights into the altered gut permeability and varied plasma endotoxin levels between groups, fecal gut microbiota were analyzed by real-time PCR using 16S rRNA gene primers for specific gram-negative bacteria. The alterations of the abundance of Enterobacteriaceae, γ-Proteobacteria and Bacteroidetes were further validated by real-time PCR and showed similar trends as did the results from the 16s rDNA sequencing (Fig. 6). In addition, the abundance of *Samonella spp.*, a gram-negative bacterial genus belonging to the Enterobacteriaceae family, was also significantly increased in high-copper/high-fructose fed rats (CuSF). To examine whether bacterial products other than LPS might contribute to the development of high-copper high-fructose induced steatosis, we found that the H7 antigen expression of *E. coli*, which relates to bacterial flagella growth, was robustly increased in CuS rats (Fig. 6).

**Discussion**

The principal finding of the current study was that both dietary low copper-fructose and high copper-fructose interactions induced liver injury and impaired gut barrier function, and were associated with distinct alterations of gut microbiome. The novel insight obtained from this study is that different doses of dietary copper and/or fructose may shape the gut microbiome in distinct ways, which may contribute to the development of NAFLD through differential mechanisms.

Metagenomics study revealed the common features shared by low- and high-copper with fructose in shaping gut microbiota. These included significantly increased
Firmicutes, a trend of increase in Proteobacteria, and depletion of Verrucomicrobiaceae (Akkermansia), as well as markedly reduced Bifidobacteriaceae and Bifidobacterium in CuSF rats and to a lesser extent in CuMF rats. Two-way ANOVA analysis deciphered the significant role of copper in shaping Firmicutes (Lactobacillaceae, Lactobacillus, Erysipelotrichaceae, etc.) and Verrucomicrobiaceae (Akkermansia), and the significant role of dietary fructose in boosting Proteobacteria and both copper and fructose in reducing Ruminococcaceae (Table 2). Both Akkermansia and Bifidobacterium are beneficial to the host and play critical roles in maintaining gut barrier function (9, 18), highlighting the essential role of copper homeostasis in gut barrier function likely through maintaining gut microbiota eubiosis. Although both low- and high-copper increased Firmicutes to a similar extent, the Firmicutes/Bacteroidetes ratio was higher in CuMF rats (19.87) than in CuSF rats (8.28), due to lower Bacteroidetes in CuMF rats. Moreover, copper levels shaped the family and genus within Firmicutes differently. While increased Firmicutes in low copper diet fed rats was predominantly due to the increases in Lachnospiraceae and Peptostreptococcaceae, the increased Firmicutes in high copper fed rats was due to the increase in Lactobacillaceae (Lactobacillus), Lachnospiraceae and Erysipelotrichaceae. The increased abundance of Peptostreptococcaceae and Erysipelotrichaceae was associated with high caloric diet-induced hepatic steatosis in mice, which might contribute to disease progression (35). A recent study showed that NAFLD patients had increased Lactobacillus, Lachnospiraceae and decreased Ruminococcaceae in their fecal microbiome (40). Moreover, reduced abundance of Lachnospiraceae and Ruminococcaceae were observed in human obese and NASH patients (65). In addition, mice fed a high-fat diet exhibited decreased Bacteroidetes and
increased Firmicutes and Proteobacteria as well as increased \textit{Lactobacillus} (25, 62). Further, Proteobacteria was significantly elevated in NASH patients as well as in the mice fed with high fat diet (25, 65). The differences between these studies might be due to the different disease stages and variability of experimental design. In our study, both low-copper/high-fructose and high-copper/high-fructose-induced hepatic steatosis recapitulated, at least partially, the gut microbiome profiles of obese, NAFLD and NASH. They shared common features, such as increased Firmicutes and Proteobacteria, decreased Ruminococcaceae and Verrucomicrobia in the fecal microbiome, but with distinct profiles. Low-copper/high-fructose fed rats were characterized with more robustly elevated Firmicutes/Bacteroidetes ratio and higher Peptostreptococcaceae, whereas significantly elevated \textit{Lactobacillus} and Erysipelotrichaceae, as well as markedly decreased \textit{Bifidobacterium}, were seen in high-copper/high-fructose fed rats, suggesting differential mechanisms might be involved. In line with this, fecal metabolomics showed that distinct alterations of fecal metabolites are associated with dietary copper level and fructose (58), further supporting the concept that differential mechanisms through host-microbes interaction may contribute to development of steatosis. However, how the altered gut microbiome and metabolites contribute to the pathogenesis of steatosis remains largely unknown.

Although the beneficial role of \textit{Lactobacillus} as a probiotic has been shown by previous study (60), an increased abundance of \textit{Lactobacillus} species has been reported in diabetes and obesity as well as in NAFLD (3, 4, 28, 40). Therefore, whether \textit{Lactobacillus} contributes to the disease development or is a consequence of the disease remain unclear. Lower abundance of Lachnospiraceae and greater abundance of
Erysipelotrichaceae were observed in a colitis mouse model, whereas administration of Lachnospiraceae limited colitis, in part, by suppressing the expansion of Erysipelotrichaceae (12). In contrast with previous studies, our data showed a trend of an increase in Lachnospiraceae in both CuMF and CuSF rats. Lachnospiraceae and Ruminococcaceae are two dominant families of butyrate generating bacteria (56). However, the opposite trend of alterations of these two families in our experimental groups in the current study, together with the decreased fecal short chain fatty acids (SCFAs) in CuMF and CuSF rats in our previous study (58), indicates that future studies on segmental alterations of intestinal (cecal) microbiome and metabolites are needed to determine whether decreased fecal SCFAs is due to increased absorption.

In addition to the alteration of gut microbiome, our data showed that both dietary low copper and high copper with or without fructose play critical roles in disrupting gut barrier function, as supported by markedly downregulated intestinal tight junction proteins and decreased goblet cell numbers. Moreover, the disrupted gut barrier function was associated with gut microbiota dysbiosis, characterized by Akkermansia depletion, which has been well documented to be critical for maintaining gut barrier function (13, 14, 18). To our knowledge, this the first study to show specific effects of copper levels on Akkermansia. Further study is needed to examine whether copper homeostasis directly acts on the integrity of the gut barrier and/or acts via inducing gut microbiome dysbiosis.

Another interesting finding is that endotoxemia is present in only CuMF rats, but not in CuSF rats. Moreover, the increased abundance of the gut Gram-negative bacteria, Proteobacteria (Enterobacteriaceae), did not parallel plasma endotoxin levels, despite the fact that both of these regimens resulted in increased gut permeability, adding further
support to the concept that different mechanisms might be involved in the development of steatosis in these two states. The high copper diet did not lead to excessive hepatic copper accumulation, as distinct from Wilson’s disease, which is characterized by higher liver copper and low plasma ceruloplasmin (43). Our data showed that plasma ceruloplasmin activity and liver copper were slightly lower in rats fed a high-copper diet than in rats fed an adequate copper diet, likely due to the body’s compensatory mechanisms to reduce copper absorption in response to a high-copper diet. Indeed, previous work has demonstrated that decreased copper absorption and increased intestinal copper retention in rats fed with increased dietary copper is due to changes in copper transporters (7). Collectively, our data do not support a role for liver copper toxicity in the induction of steatosis in CuSF rats.

The inconsistency between plasma endotoxin levels and increased gut permeability as well as increased abundance of gram-negative bacteria indicate that bacterial products other than LPS might contribute to the development of steatosis. We found that H7 antigen expression of *E. coli*, which related to bacteria flagella growth (49), was robustly increased in CuS and CuSF rats. Flagellin, the major component of flagella, is a toll like receptor (TLR) 5 ligand. Whether gut bacteria-derived flagellin contributes to steatosis remains to be determined. Elevated plasma LBP levels seen in CuMF and CuSF rats suggested the possibility of elevated plasma LPS. A recent elegant study showed that LPSs derived from different bacteria have different structure and may play differential roles in immune responses (55). Currently, the most commonly used method to measure LPS is the LAL assay, with standard endotoxin being from the bacterial strain, *E.coli*. Therefore, different types of LPS may not be equally measured by this LAL assay. In line
with our results, a recent study showed that the majority of obese NASH patients did not have elevated serum endotoxin levels (61). One proposed mechanism underlying the pathogenesis of NASH is endogenous ethanol production, and this concept is supported by the findings of elevated blood ethanol levels and upregulation of alcohol-metabolizing enzymes in the livers of NAFLD and NASH patients (6, 65, 66). Moreover, the ethanol producing bacteria, *Escherichia*, were significantly increased in NASH patients (65). However, additional studies are needed to validate this concept.

There are several limitations of the current study. A purified AIN-76-based diet containing nearly 40% sucrose (w/w) was used. Therefore, the sucrose in the diet could be a potential confounding factor which may raise the baseline levels for some control groups (such as the gut microbiota composition) and/or increase the variations for some indices, and therefore may blunt the differences between groups. However, we repeatedly observed the significant differences in most of the major endpoints when rats were given excessive fructose from drinking water. Moreover, most manifestations of the copper deficiency were prominent in the experiment involving dietary copper-fructose interaction when AIN-93 diet was substituted for the AIN-76. AIN-93 diet contains only 10% sucrose (41). To move the science forward, we will replace the AIN-76 diet with AIN-93 diet in subsequent studies. Another limitation was that we did not collect fecal pellets at day one and at the middle of the experiment, which could have provided information on longitudinal alterations of gut microbiome within each group. Future study on the segmental alterations of the gut microbiome between the small and the large intestine, as well as therapeutic interventions with antibiotics or probiotics would provide a better understanding for the role of gut microbiota in gut barrier function and energy
harvesting. Sex differences with regard to the alteration of the gut microbiome induced by dietary fructose-copper interaction also warrant investigation.

In summary, our current study provides novel insights into the pathogenesis of NAFLD with regard to copper-fructose interactions at the intestine level. The studies indicate that copper homeostasis is critical in maintaining gut microbiota eubiosis and gut barrier integrity, which are critical in the prevention of NAFLD.
Figure Legends

Fig. 1. Effects of different dietary doses of copper and high fructose feeding on copper status and mRNA expression of copper transporter 1 (Ctr1 or Slc31a1). (A) Plasma ceruloplasmin. (B) Plasma copper. (C) Liver copper. (D) Duodenum Slc31a1 mRNA. (E) Liver Slc31a1 mRNA. Data represent means ± SD (n=5-8). Statistical significance was set to p≤0.05. P values displayed for factors copper (Cu), Fructose (F) and interaction (Cu×F) by Two-way ANOVA followed with Neurman-Keuls post hoc test. CuA, adequate copper diet; CuM, marginal copper diet; CuS, copper supplementation diet; Slc31a1 (Ctr1), solute carrier family 31(copper transporters) member 1.

Fig. 2. Effects of dietary different doses of copper and high fructose feeding on plasma ALT, AST and MCP-1. (A) ALT. (B) AST. (C) MCP-1. Data represent means ± SD (n=5-8). Statistical significance was set to p≤0.05. P values displayed for factors copper (Cu), Fructose (F) and interaction (Cu×F) by Two-way ANOVA followed with Neurman-Keuls post hoc test. CuA, adequate copper diet; CuM, marginal copper diet; CuS, copper supplementation diet.

Fig. 3. Effects of different dietary doses of copper and high fructose feeding on plasma endotoxin, LPS binding protein (LBP), and gut permeability. (A) Plasma endotoxin. (B) Plasma LBP. (C) Gut permeability. The penetration of intraluminal FD-4 to the incubation buffer was determined after incubation of ileum sac for 20 min. Data represent means ± SD (n=5-7). Statistical significance was set to p≤0.05. P values displayed for factors copper (Cu), Fructose (F) and interaction (Cu×F) by Two-way
ANOVA followed with Neurman-Keuls post hoc test. **CuA**, adequate copper diet; **CuM**, marginal copper diet; **CuS**, copper supplementation diet.

**Fig. 4. Effects of different dietary doses of copper and high fructose on tight junction protein expression and goblet cell number in ileum.** (A) Western Blot of Claudin-1, Occludin and Reg3B Expression. Data represent means ± SD (n=4). (B) Representative photomicrographs of PAS staining (100×). (C) Quantification of goblet cell numbers per villi. Data represent means ± SD (n=5-8). Statistical significance was set to p≤0.05. P values displayed for factors copper (Cu), Fructose (F) and interaction (Cu×F) by Two-way ANOVA followed with Neurman-Keuls post hoc test. **CuA**, adequate copper diet; **CuM**, marginal copper diet; **CuS**, copper supplementation diet; **CuAF**, adequate copper diet +30% fructose drinking; **CuMF**, marginal copper deficient diet +30% fructose drinking; **CuSF**, copper supplementation diet +30% fructose drinking.

**Fig. 5. Effects of different dietary doses of copper and high fructose on bacterial diversity and abundance.** (A) alpha diversity. (B) beta diversity. (C) Taxonomic composition of the gut microbiota at the phylum level. **CuA**, adequate copper diet; **CuM**, marginal copper diet; **CuS**, copper supplementation diet; **CuAF**, adequate copper diet +30% fructose drinking; **CuMF**, marginal copper deficient diet +30% fructose drinking; **CuSF**, copper supplementation diet +30% fructose drinking.

**Fig. 6. Alterations of gut microbiome in response to dietary different doses of copper and high fructose feeding.** (A) Enterobacteriaceae. (B) γ-Proteobacteria. (C) Bacteroidetes. (D) InA (S. Typhimurium). (E) RGfliCh7 (E. coli O157:H7). Data represent means ± SD (n=5-8). Statistical significance was set to p≤0.05. P values displayed for factors copper (Cu), Fructose (F) and interaction (Cu×F) by Two-way
ANOVA followed with Neurman-Keuls *post hoc* test. **CuA**, adequate copper diet; **CuM**, marginal copper diet; **CuS**, copper supplementation diet.
Acknowledgment: We thank Ned Smith, Sharon A. Gordon and Dr. David Barker for technical assistance. We thank Sabine Waigel for the help with 16S rDNA sequencing. We thank Renwei Wang for assistance in statistical analysis. We thank Marion McClain for careful reading of this manuscript. Sequencing was performed with assistance of the UofL Genomics Facility and Bioinformatics, which are supported by NIH/NIGMS Phase III COBRE P30 GM106396 (Donald Miller), NIH/NIGMS KY-INBRE P20GM103436 (Nigel Cooper), the James Graham Brown Foundation, and user fees.

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Disclosures

No conflicts of interest, financial or otherwise, are declared by the authors.
References


41. Reeves PG, Ralston NV, Idso JP, and Lukaski HC. Contrasting and cooperative effects of copper and iron deficiencies in male rats fed different


Table 1. Effects of dietary different doses of copper and high fructose feeding on body weight, liver/body weight ratio, epididymal fat weight, and plasma Indices

<table>
<thead>
<tr>
<th>Variable</th>
<th>CuA</th>
<th>CuAF</th>
<th>CuM</th>
<th>CuMF</th>
<th>CuS</th>
<th>CuSF</th>
<th>Two-way ANOVA</th>
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<tr>
<td>BW (g)</td>
<td>252.4±17.3</td>
<td>242.6±20.1</td>
<td>235.3±7.3</td>
<td>242.3±15.8</td>
<td>257.9±26.1</td>
<td>237.3±13.3</td>
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<tr>
<td>BW gain (g)</td>
<td>200.6±15.0</td>
<td>190.9±18.9</td>
<td>185.1±7.9</td>
<td>190.0±14.5</td>
<td>208.2±21.2</td>
<td>188.8±12.0</td>
<td>Cu×F, p=0.0051</td>
</tr>
<tr>
<td>LW (g)</td>
<td>11.06±1.06</td>
<td>10.03±1.02</td>
<td>8.63±0.59</td>
<td>10.24±1.31</td>
<td>10.11±0.74</td>
<td>10.53±1.22</td>
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<tr>
<td>LW/BW (%)</td>
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<td>4.45±0.56</td>
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</tr>
<tr>
<td>EFW (g)</td>
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<td>2.77±0.54</td>
<td>3.00±0.51</td>
<td>2.79±0.48</td>
<td>2.81±0.64</td>
<td>Cu, p=0.0002</td>
</tr>
<tr>
<td>EFW/BW (%)</td>
<td>1.24±0.21</td>
<td>1.09±0.11</td>
<td>1.18±0.21</td>
<td>1.23±0.14</td>
<td>1.08±0.12</td>
<td>1.18±0.25</td>
<td>Cu, p=0.0025</td>
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<tr>
<td>Plasma Indices</td>
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<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>TG (mg/dl)</td>
<td>46.7±13.6</td>
<td>42.2±10.3</td>
<td>33.6±8.5</td>
<td>43.2±9.3</td>
<td>35.3±8.1</td>
<td>39.9±12.4</td>
<td>Cu, p=0.0004</td>
</tr>
<tr>
<td>CHO (mg/dl)</td>
<td>58.4±8.6</td>
<td>45.6±9.9</td>
<td>32.3±8.0</td>
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<td>38.8±9.2</td>
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<td>NEFA (μM)</td>
<td>398.1±121.5</td>
<td>331.0±119.7</td>
<td>347.2±74.7</td>
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<td>495.6±113.4</td>
<td>390.8±86.0</td>
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<tr>
<td>Glucose (mg/dl)</td>
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<td>104.8±7.0</td>
<td>90.6±11.0</td>
<td>110±10.9</td>
<td>89.6±14.1</td>
<td>112.9±7.1</td>
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</tr>
<tr>
<td>Insulin (ng/ml)</td>
<td>0.45±0.06</td>
<td>0.50±0.07</td>
<td>0.45±0.05</td>
<td>0.52±0.13</td>
<td>0.43±0.04</td>
<td>0.44±0.08</td>
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</tr>
<tr>
<td>HOMA-IR</td>
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<td>3.18±0.41</td>
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<td>2.39±0.43</td>
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<td>Cu×F, p=0.0017</td>
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Data are expressed as means ± SD (n=5-8). Statistical significance was set to p≤0.05. P values displayed for copper (Cu), Fructose (F) and interaction (Cu×F) by Two-way ANOVA followed with Neurman-Keuls post hoc test. * versus CuA; # versus CuAF; $ versus CuM; † versus CuMF; ‡ versus CuSF. CuA, adequate copper diet; CuM, marginal copper diet; CuS, copper supplementation diet. CuAF, adequate copper diet +30% fructose drinking; CuMF, marginal copper deficient diet +30% fructose drinking; CuSF, copper supplementation diet +30% fructose drinking; BW, Body weight; FC, food consumption; LW, liver weight; EPW, Epididymal fat weight; TG, tryglyceride; CHO, cholesterol; NEFA, non-esterified fatty acid. HOMA-IR, homeostasis model assessment of insulin resistance. International formula for HOMA-IR calculator: [fasting glucose (mmol/L) x fasting Insulin (mU/L)/22.5].
### Table 2. Mean Abundance of Gut Microbiome Taxa.

<table>
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<tr>
<th>Bacteria</th>
<th>CuA</th>
<th>CuAF</th>
<th>CuM</th>
<th>CuMF</th>
<th>CuS</th>
<th>CuF</th>
<th>CuXF</th>
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<td>9.43</td>
<td>11.76</td>
<td>11.48</td>
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<td>10.36</td>
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<td>0.17</td>
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<td>6.95</td>
<td>3.21</td>
</tr>
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<td>5.55</td>
<td>6.95</td>
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<td><strong>7.87</strong></td>
<td><strong>4.14</strong></td>
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<td>4.12</td>
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<td>N</td>
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<td>4.87</td>
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**P value of factors (two-way ANOVA)**
COPPER-FRUCTOSE INTERACTIONS AND NAFLD

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<th>Phyla</th>
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<td>0.24</td>
<td>1.21</td>
<td>0.61</td>
<td>1.97</td>
<td>0.58</td>
<td>1.37</td>
<td>N</td>
<td>N</td>
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<tr>
<td>g__Sutterella</td>
<td>0.24</td>
<td>1.21</td>
<td>0.61</td>
<td>1.97</td>
<td>0.58</td>
<td>1.37</td>
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<td>f__Enterobacteriaceae</td>
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<td>0.06</td>
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<td>0.22</td>
<td>0.12</td>
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<td>g__unknown*</td>
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<td>0.05</td>
<td>0.12</td>
<td>0.22</td>
<td>0.12</td>
<td>0.82</td>
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<td>p__Verrucomicrobia</td>
<td>10.41</td>
<td>6.16</td>
<td>3.85</td>
<td>0.88</td>
<td>0.48</td>
<td>0.9</td>
<td>Y</td>
<td>N</td>
<td>N</td>
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<tr>
<td>f__Verrucomicrobiaceae</td>
<td>10.41</td>
<td>6.16</td>
<td>3.85</td>
<td>0.88</td>
<td>0.48</td>
<td>0.9</td>
<td>Y</td>
<td>N</td>
<td>N</td>
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<tr>
<td>g__Akkermansia</td>
<td>10.41</td>
<td>6.16</td>
<td>3.85</td>
<td>0.88</td>
<td>0.48</td>
<td>0.9</td>
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<td>Unassigned;Other</td>
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<td>1.93</td>
<td>1.32</td>
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</table>

Numbers listed under study groups are percentages (n=5-8/group). Statistical significance was set to \( p \leq 0.05 \). P values displayed for factors copper (Cu), Fructose (F) and interaction (Cu×F) by Two-way ANOVA. Y, \( p \) value of factor \( \leq 0.05 \). N, \( p \) value of factor >0.05. CuA, adequate copper diet; CuM, marginal copper diet; CuS, copper supplementation diet. CuAF, adequate copper diet +30% fructose drinking; CuMF, marginal copper deficient diet +30% fructose drinking; CuSF, copper supplementation diet +30% fructose drinking.

P. Phyla with average abundance greater than 1% in any of the groups are listed.
f. Families with average abundance greater than 0.5% in any of the groups are listed.
g. Genera with average abundance greater than 0.5% in any of the groups are listed.
*16S rRNA sequence distinct from any known genera in this family/genus.
Fig. 1

A. Plasma Ceruloplasmin (U/L)

B. Plasma Copper (µg/dL)

C. Liver Copper (µg/g dry weight)

D. Duodenum Slc31a1 mRNA (fold change)

E. Liver Slc31a1 mRNA (fold change)

- * versus CuA fructose (-)
- # versus CuA fructose (+)
- $ versus CuM fructose (-)
- † versus CuM fructose (+)
- ‡ versus CuS fructose (-)

Cu, p<0.0001
Cu, p<0.0001 Cu×F, p=0.0165
Cu, p<0.0001
F, p=0.0167
F, p=0.0053 Cu×F, p=0.0018
Fig. 2

A

Cu, p=0.0008

Fructose(-) Fructose(+)

* versus CuA fructose (-)
# versus CuA fructose (+)
$ versus CuM fructose (-)
† versus CuM fructose (+)
‡ versus CuS fructose (-)

B

Cu, p<0.0001
F, p<0.0001
Cu×F, p=0.0101

C

Cu, p=0.0014

PLASMA MCP-1 (pg/ml)
Fig. 3

A

Plasma Endotoxin (EU/ml)

Cu, p<0.0001
F, p=0.0152

CuA  CuM  CuS

F, p=0.0019

B

Plasma LBP (ng/ml)

Fructose(-)
Fructose(+)

C

FD-4 Permeability (µg/cm/min)

Cu, p=0.0201

* versus CuA fructose (-)
# versus CuA fructose (+)
$ versus CuM fructose (-)
† versus CuM fructose (+)
### Fig. 4

#### A

<table>
<thead>
<tr>
<th>Clade 1</th>
<th>CuA</th>
<th>CuAF</th>
<th>CuM</th>
<th>CuMF</th>
<th>CuS</th>
<th>CuSF</th>
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<tr>
<td><strong>β-actin</strong></td>
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<tr>
<td><strong>Claudin-1/β-actin Ratio</strong></td>
<td>1.00±0.44</td>
<td>1.31±0.22</td>
<td>0.65±0.22*#</td>
<td>0.26±0.13**$</td>
<td>0.67±0.12#†</td>
<td>0.42±0.05**#</td>
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<td>(Cu, p&lt;0.0001; Cu×F, p=0.0172)</td>
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</table>

<table>
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<th>Occludin</th>
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<th>CuAF</th>
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<th>CuMF</th>
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<tr>
<td><strong>β-actin</strong></td>
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<tr>
<td><strong>Occludin/β-actin Ratio</strong></td>
<td>1.00±0.28</td>
<td>0.77±0.32</td>
<td>0.64±0.11*</td>
<td>0.41±0.08*#</td>
<td>0.43±0.15**#</td>
<td>0.35±0.04*#$</td>
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<tr>
<td>(Cu, p=0.0002; F, p=0.0341)</td>
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<table>
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<th>Reg3B</th>
<th>CuA</th>
<th>CuAF</th>
<th>CuM</th>
<th>CuMF</th>
<th>CuS</th>
<th>CuSF</th>
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<tbody>
<tr>
<td><strong>β-actin</strong></td>
<td></td>
<td></td>
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<td></td>
<td></td>
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<tr>
<td><strong>Reg3B/β-actin Ratio</strong></td>
<td>1.00±0.32</td>
<td>1.04±0.35</td>
<td>1.50±0.41</td>
<td>1.81±0.11**#</td>
<td>2.62±0.75**$†</td>
<td>2.25±0.38**$ $</td>
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<tr>
<td>(Cu, p=0.0001)</td>
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</table>

* versus CuA fructose (-)
# versus CuA fructose (+)
$ versus CuM fructose (-)
† versus CuM fructose (+)
Fig. 4

B

Ileum PAS X 100

Fructose (-)

Fructose (+)

C

Goblet Cells/villi

CuA           CuM           CuS

Fructose(-)    Fructose(+)    *

* versus CuA fructose (-)
Fig. 5

A  \(\alpha\) diversity

B  \(\beta\) diversity

Unweighted UniFrac

Weighted UniFrac
**Fig. 6**

A. Enterobacteriaceae (fold change) vs. Cu, p=0.0189 vs. CuA fructose (-) # vs. CuM fructose (-) $ vs. CuS fructose (-) † vs. CuM fructose (+) ‡ vs. CuS fructose (+)

B. γ-Proteobacteria (fold change)

C. Bacteroidetes (fold change) * vs. CuA fructose (-) † vs. CuM fructose (+)

D. InvA (Salmonella spp.) (fold change)

E. RGfliC7 (O157:H7) (fold change)