

## Decaffeinated coffee consumption induces expression of tight junction proteins in high fat diet fed rats

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### ABSTRACT

**Background:** Recent evidence indicates that gut microbiota plays a key role in the development of NAFLD through the gut-liver axis. An altered gut permeability induced by alterations of tight junction (TJ) proteins allows the passage of bacteria and substances leading to liver inflammation, hepatocyte damage and fibrosis. This study aims to evaluate the influence of decaffeinated coffee on gut permeability in a rat model of fat liver damage induced by a high fat diet (HFD).

**Methods:** Twelve male Wistar rats were assigned to 3 groups. The first group received HFD for 5 months and drank water. The second group received HFD for 5 months and drank water added with 1.2mL decaffeinated coffee/day starting from the 4th month. The third group received standard diet (SD) and drank water. Protein and mRNA expression levels of Toll-Like Receptor-4 (TLR-4), Occludin and Zonula occludens-1 (ZO-1) were assessed in rat intestines.

**Results:** A significant reduction of Occludin and ZO-1 was observed in HFD fed rats ( $0.97 \pm 0.05$  vs  $0.15 \pm 0.08$   $p < 0.01$ , and  $0.97 \pm 0.05$  vs  $0.57 \pm 0.14$   $p < 0.001$  respectively). This reduction was reverted in HFD+COFFEE rats ( $0.15 \pm 0.08$  vs  $0.83 \pm 0.27$   $p < 0.01$  and  $0.57 \pm 0.14$  vs  $0.85 \pm 0.12$   $p < 0.01$  respectively). The TLR-4 expression up-regulated by HFD was partially reduced by coffee administration.

**Conclusions:** HFD impairs the intestinal TJ barrier integrity. Coffee increases the expression of TJ proteins, reverting the altered gut permeability and reducing TLR-4 expression.

**Key Words:** HFD, coffee, Occludin, Zonulin-1, rat model, TLR-4.

**BACKGROUND**

Coffee is perhaps the most famous beverage worldwide, having an important role in human socialization for at least 1200 years. Coffee is appreciated for its distinct aroma and flavour [1], with its widespread use being mainly due to its psychoactive and invigorating properties [2]. Caffeine is the coffee component responsible for these effects and is the most studied component of coffee. However, coffee is a complex mixture of about 1000 compounds, including several bioactive compounds such as chlorogenic acid, melanoidins and diterpenes [3, 4]. Coffee is considered one of the major contributors of antioxidant phytochemicals in the diet [5]. Polyphenols are among the most abundant bioactive compounds in coffee and have been associated with numerous health properties including antioxidant, anti-inflammatory, and prebiotic properties [6]. A growing body of evidence indicates that coffee consumption is associated with improved hepatic outcomes, the benefits spanning from positive effects on liver enzymes, improvement of hepatic steatosis and fibrosis, and reduced risk of cirrhosis and hepatocellular carcinoma [7, 8, 9, 10]. The evidence comes from epidemiological and clinical observations as well as experiments performed in animal models of liver injury [11, 12]. However, the molecular mechanisms underpinning the effects on the liver are not completely elucidated. In a previous study from our research group coffee consumption was demonstrated to protect the liver from the damage caused by high fat diet (HFD), through reduction of hepatic fat accumulation via increased fatty acid  $\beta$ -oxidation, reduction of liver inflammation via modulation of genes and expression of inflammatory cytokines, and amelioration of systemic and liver oxidative status through the glutathione system [13]. It is well documented that a high fat, high calories solid diet [14] affects the gut microbiota composition and gut permeability, resulting in bacterial translocation and hepatic inflammation. All these steps contribute to realizing a close connection between gut alteration and liver disease [15, 16]. The exposure to bacterial product of intestinal origins can lead to alteration of tight junction (TJ) proteins and impaired gut epithelial integrity causing bacterial translocation [17, 18]. The passage of luminal antigens initiates inflammatory process via Toll-like receptor (TLR) signalling. It is worthwhile mentioning that within the gut mucosa there is an intricate system of immune receptors which recognize the structurally conserved molecules of microbial products. The luminal antigens and pathogens in the intestinal epithelium facilitate the response of the innate immune system by exaggerating the extent of TLR signalling, ultimately leading to the development of intestinal inflammation and damage with associated changes in junctional localization of TJ proteins [occludin and Zonula occludens protein 1 (ZO-1)]. Primary classes of such receptors include the TLR and nod-like receptors (NLR) which recognize a variety of bacterial products including lipopolysaccharides (LPS), flagellin, peptidoglycan, and bacterial DNA. The primary consequence of TLR/NLR activation is to broadly induce the expression of host-defence genes acting against several pathogens and driving the host tissue damage by activating the cascade of pro-inflammatory cytokines [19]. In a recent study, Guo et al. demonstrated for the first time that TLR-4 signal transduction pathway plays an important role in intestinal barrier regulation [20]. Additionally, animal studies suggest that chronic intake of a HFD lead to individual adaptation to intestinal alterations with a loss of TJ proteins in the upper parts of the small intestine, elevated

translocation of endotoxin, and an induction of TLRs expression in the liver [21, 22]. An increased intestinal mucosa permeability typical of genetically obese mice [23] and found in HFD fed mice [24] leads to portal endotoxemia. Gut-derived bacterial endotoxins pass into the liver through the portal circulation, thereby activating Kupffer cells, the resident hepatic macrophages by the TLR-4 complex on the cell surface. This background suggests that the TLR-4 receptor for bacterial endotoxin plays a key role in the gut-liver axis [25].

The aim of the present study was to evaluate the effect of decaffeinated coffee beverage on gut permeability in a model of HFD fed rats. This goal was pursued by monitoring the gene and protein expression of the main intestinal integrity-related proteins, i.e. Occludin and ZO-1.

## METHODS

### Preparation of coffee-based beverage

The coffee used in this study was prepared by filtering the grounds on a paper filter with a mix of boiling water and decaffeinated coffee powder (4:1 v:w) (Illy Caffè®, Trieste, Italy). Filtered decaffeinated coffee was portioned and stored at -20 °C until used. A volume of 1.2 mL of coffee was added to the drinking water of rats belonging to the HFD+COFFEE group. This daily amount of coffee corresponded to 6 cups of espresso coffee or 2 cups of filtered coffee for a person weighing 70 kg [13].

### Animals and treatments

Twelve male Wistar rats weighing 180-220g were randomly housed in wire-bottomed cages. Animals were obtained from Harlan Italy (Harlan, San Pietro al Natisone UD, Italy) and maintained under controlled temperature conditions of  $22 \pm 2$  °C, at a relative humidity of  $55 \pm 5\%$  with a 12-hour light/dark cycle and free access to water.

Rats were randomly divided into three groups (each group  $n = 4$  rats) and maintained for 20 weeks with free access to food and beverage. The first group: *HFD*, received a fat-rich diet (58% of energy derived from fat, 18% from protein, and 24% from carbohydrates, 5.6 kcal/g), and drank water. The second group: *HFD+COFFEE*, received the same fat-rich diet and drank the coffee-based beverage from the 4<sup>th</sup> month. The third group: *SD*, represented the healthy control group and received a standard rat feed (5% of energy derived from fat, 18% from proteins, and 77% from carbohydrates, 3.3 kcal/g) and drank water.

Food intake and body weight were recorded twice weekly. During the experiment, the rats in the 3 groups grew up gradually and no unexpected death occurred. All animals were sacrificed after five months of treatment.

Animals were euthanized under deep anaesthesia, and the proximal intestine comprising the duodenum was excised and flushed with physiological saline. The intestine was cut longitudinally and the mucosa was scraped, snap frozen, and stored at -80°C until use.

All animals received humane care according to the criteria outlined in the National Institutes of Health Guide for the Care and Use of Laboratory Animals (National Institutes of Health Publication 86-23; revised 1985). The experimental protocol was approved by the Ethic Committee of University of Naples “Federico II”.

### Protein extraction and western blot analysis

Frozen proximal duodenum samples were homogenized in RIPA lysis buffer 0.1% sodium dodecyl sulphate (SDS), 0.5% deoxycholate, 1% Nonidet, 100mM NaCl, 10mM Tris-HCl-pH 7.4) containing a protease inhibitor cocktail (Sigma, St Louis, Missouri, USA). After 30 min at 4 °C, tissue lysates were clarified by centrifugation at 14,000 rpm for 10 min at 4 °C. The cleared tissue lysates were collected, the protein concentration of each sample was determined by the Bradford assay (Bio-Rad, Melville, NY, USA) and stored at -80°C.

Total protein extracts (50 µg) were boiled in Laemmli buffer (0.125 M Tris-HCl (pH 6-8), 4% SDS, 20% glycerol, 10% 2-mercaptoethanol, 0.002% bromophenol blue) for 5 min before electrophoresis. The samples were subjected to SDS-PAGE (10 or 7 % polyacrylamide as required) under reducing conditions. After electrophoresis, proteins were transferred to nitrocellulose membrane (Pure Nitrocellulose Membrane, 0.45 µm, Bio-Rad Laboratories, Inc., Hercules, CA, USA); complete transfer was assessed using pre-stained protein standards (Invitrogen, Carlsbad, CA, USA). To block non-specific binding sites the membranes were treated for 1 h with blocking solution of 20% FBS in TNT (10 mM-Tris (pH 8), 150 mM NaCl and 0.05% Tween-20), and then were incubated overnight at 4 °C with the required primary antibody: Rabbit polyclonal IgG anti-Zo-1 (1:500) (Invitrogen™; Paisley, UK); Mouse monoclonal IgG anti-Occludin (1:200) (Invitrogen™; Paisley, UK); Mouse monoclonal IgG anti β Actin (1:2000) (Cell Signaling Technology). The antibodies were opportunely diluted in 20% FBS in TNT (0.5% Tween-20). The membranes were washed three times in changes of Tween-Tris-buffered saline (TTBS; 20 mM-Tris-HCl (pH 7.6), 150 mM-NaCl, 5% Tween-20) for 30 min. After washing with TBS, membranes were incubated for 2 h (at room temperature) with the appropriate HRP secondary antibody and immunoreactive proteins were detected by development with ECL according to the manufacturer's instructions.

### RNA extraction and Real Time PCR analysis

Total RNA was isolated from frozen duodenum tissue, using the RNeasy Plus Mini Kit (Qiagen Inc., Milan, Italy) following the manufacturer's instructions. Purity and concentration were determined by Eppendorf BioPhotometer. Single strand cDNA fragment was obtained according to the instructions of the High Capacity cDNA Reverse Transcription (Applied Biosystems®); ZO-1, Occludin, TLR-4 and GAPDH (endogenous control) gene expression levels were analyzed by Taq- Man\_Gene Expression Assays (Applied Biosystems). Quantitative Real-Time PCR was carried in triplicate using pre-optimized primer/probe mixture (Rn00569848\_m1, Rn02116071\_s1, Rn00580064\_m1, Rn01775763\_g1) and TaqMan universal PCR master mix (Applied Biosystems) on a StepOne™ Real-Time PCR System (Applied Biosystems). We analyzed the relative gene expression data using the  $\Delta\Delta C_t$  method. The sample values represent X-fold differences from a control sample (given a designated value of 1) within the same experiment.

### Statistical analysis

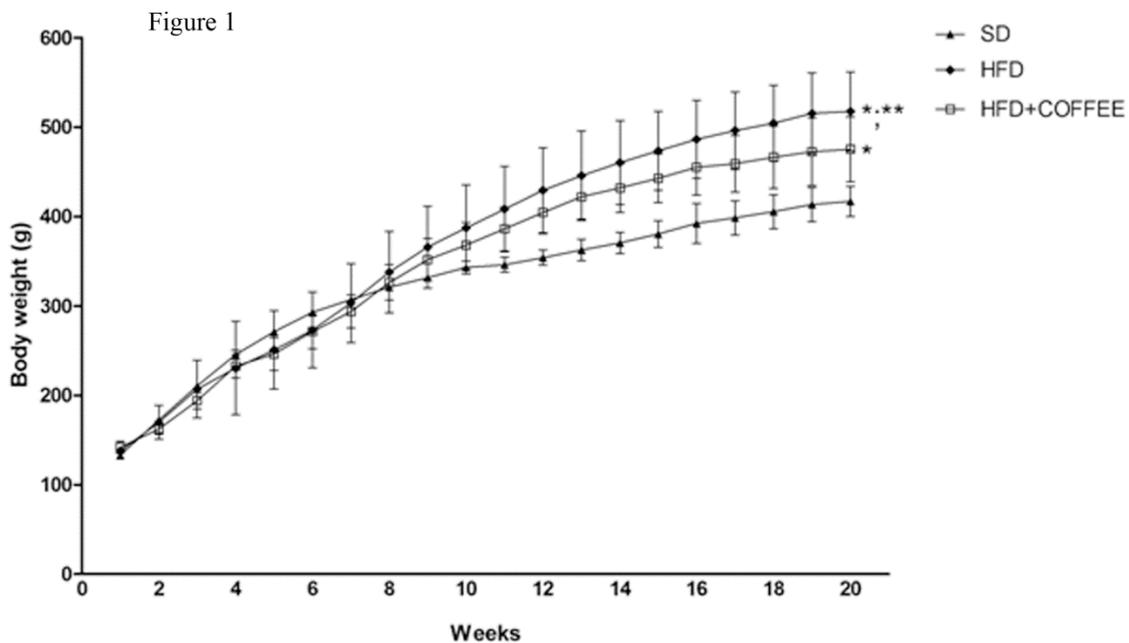
All values were expressed as the mean  $\pm$  standard deviation. Appropriately, the results were analyzed by students *t*-test and one-way analysis of variance (ANOVA), followed by Tukey's

post test, using Prism GraphPad 5 software (GraphPad Software Inc., San Diego, CA, USA). A  $p$  value  $< 0.05$  was considered as statistically significant.

## RESULTS

### Adherence to diet

The average weekly energy intake for HFD groups was significantly higher compared to SD group ( $601.5 \pm 78.66$  kcal/weeks vs  $497.6 \pm 60.50$  kcal/weeks,  $p < 0.00001$ ). Whereas energy intake between HFD and HFD+COFFEE was similar ( $601.5 \pm 78.66$  kcal/weeks vs  $554.9 \pm 61.46$  kcal/weeks,  $p = ns$ ). As expected, the body weight of rats (Figure 1) in SD group was significantly lower than HFD and HFD+COFFEE rats ( $323.4 \pm 81.01$  g vs  $370.0 \pm 123.4$  g,  $p < 0.001$  and  $323.4 \pm 81.01$  g vs  $350.2 \pm 109.9$  g,  $p < 0.001$ , respectively). Interestingly, body weight of HFD rats was significantly higher compared to HFD+COFFEE rats ( $370.0 \pm 123.4$  g vs  $350.2 \pm 109.9$  g,  $p < 0.01$ ).

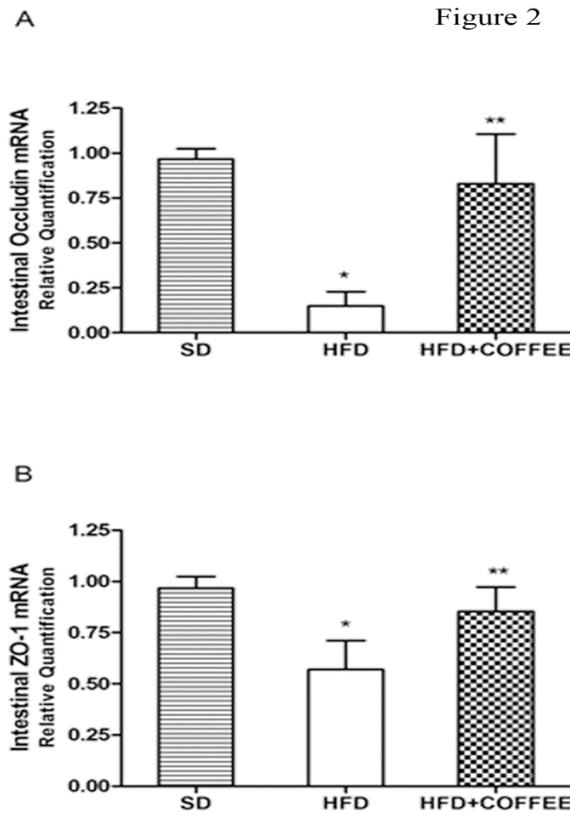


**Figure 1.** Body weight (g) of rats during the study period. Values are expressed as mean  $\pm$  SD (\* $p < 0.001$  vs SD; \*\* $p < 0.01$  vs HFD).

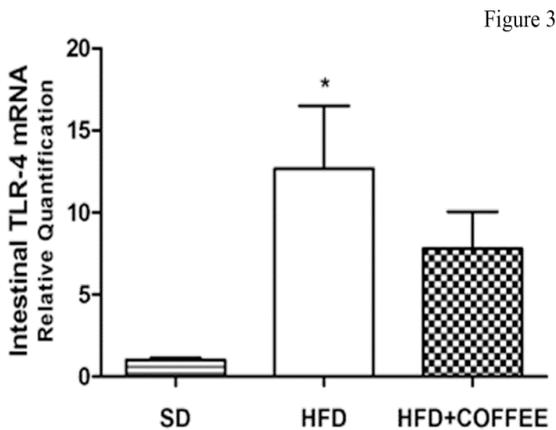
### Gene and protein expression of TJ and TLR

#### PCR analysis

The gene expression of ZO-1 and Occludin in duodenum of rats is reported in Figure 2. A significant reduction of Occludin and ZO-1 was observed in HFD rats ( $0.97 \pm 0.05$  vs  $0.15 \pm 0.08$   $p < 0.01$ , and  $0.97 \pm 0.05$  vs  $0.57 \pm 0.14$   $p < 0.001$ , respectively). This reduction was reverted by administration of coffee in HFD+COFFEE rats ( $0.15 \pm 0.08$  vs  $0.83 \pm 0.27$   $p < 0.01$ , and  $0.57 \pm 0.14$  vs  $0.85 \pm 0.12$   $p < 0.01$ , respectively). The TLR-4 expression was up-regulated by HFD ( $12.7 \pm 3.8$  vs  $1.02 \pm 0.12$   $p < 0.0001$ ) and was reduced by coffee administration even if it did not reach the statistical significance ( $7.80 \pm 2.26$  vs  $12.7 \pm 3.8$   $p = ns$ ) (Figure 3).



**Figure 2.** Occludin and ZO-1 gene expression in duodenal tissue of control rats and HFD-fed rats treated with coffee. Values are expressed as mean ± SD. (A) Occludin (\* $p < 0.01$  vs SD; \*\* $p < 0.001$  vs HFD) and (B) ZO-1 (\* $p < 0.01$  vs SD; \*\* $p < 0.01$  vs HFD).

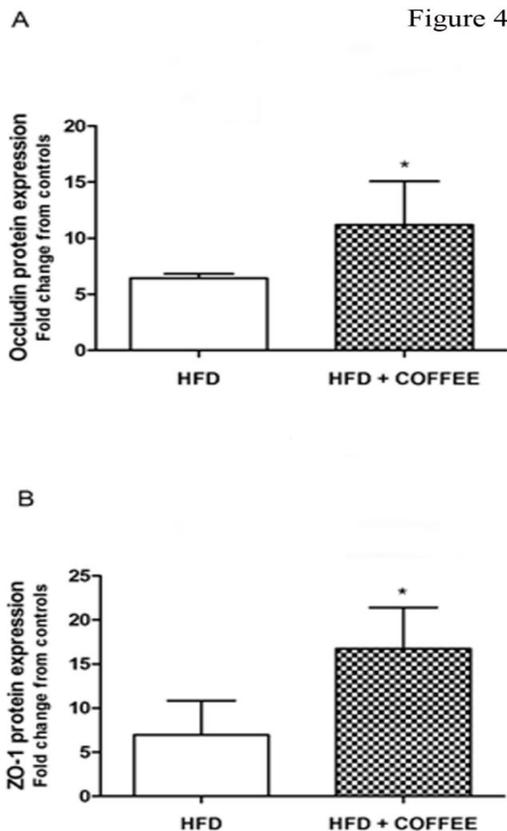


**Figure 3.** TLR-4 gene expression in duodenal tissue of control rats and HFD-fed rats treated with coffee. Values are expressed as mean ± SD, (\* $p < 0.0001$  vs SD).

### Western Blot analysis

The protein expression of Occludin and ZO-1 is reported in Figure 4. The relative band intensity semi-quantified with Image J free software (Department of Health and Human Services, National Institutes of Health in the United State) revealed a significant reduction of both proteins in rats

fed with HFD. Coffee administration induced Occludin and ZO-1 protein expression ( $11.20 \pm 3.88$  vs  $6.45 \pm 0.37$   $p=0.02$ ;  $16.73 \pm 4.67$  vs  $6.97 \pm 3.86$   $p=0.02$ , respectively).



**Figure 4.** Occludin and ZO-1 protein expression in duodenal tissue of HFD-fed rats and HFD-fed rats treated with coffee. Values are expressed as mean  $\pm$  SD. (A) Occludin ( $*p=0.02$  vs HFD) and (B) ZO-1 ( $*p=0.02$  vs HFD).

## DISCUSSION

A growing body of evidence indicate that HFD increases gut permeability and alters gut microbiota composition [15]. Changes in intestinal barrier function and increased translocation of bacterial endotoxin were implied to be associated with the development of metabolic syndrome and obesity in animal models and in humans [22]. In particular, a significant reduction of ZO-1 gene expression was previously reported [15].

TJ are currently established as playing causative and regulatory roles in various types of disease. Disruption of TJ proteins may lead to increased permeability of the intestinal barrier to the passage of luminal antigens, thereby initiating a deep inflammatory process via TLRs signalling.

In the present study for the first time decaffeinated coffee consumption was demonstrated to be able to revert the HFD-induced alteration of gene and protein expression of Occludin and ZO-1 in rats. These findings suggest that decaffeinated coffee can reduce the damage caused by hyperlipidic diet on intestinal epithelium, thereby reducing the gut leakiness. Consequently, the induction of ZO-1 and Occludin expression by decaffeinated coffee may provide a novel molecular mechanism underpinning the protective effects of coffee against fatty liver. This

hypothesis was supported by the finding that in the HFD rat model, coffee consumption also reduced TLR-4 expression, which thereby suggests a reduction of inflammation. This is in agreement with several studies demonstrating that bioactive coffee fractions such as polyphenols and melanoidins can majorly reach the colon, thereby possibly benefitting the mucosa through a direct antioxidant and anti-inflammatory protection along the intestine or indirect action through modification of gut microbiota [26]. Accordingly, different studies showed that coffee dietary fiber could modulate gut microbiota composition towards an increase of Bacteroides-Prevotella strains which should be carefully considered as it may be associated with a protective effect on human health [27-29]. A recent study demonstrated that increased Bacteroides-Prevotella strains underpinned the preventive effects of citrulline on Western diet-induced NAFLD in rats being associated with decreased lipid deposition, increased insulin sensitivity, lower inflammatory process, and preserved antioxidant status [30]. This study strongly supports the hypothesis that also coffee might reduce liver damage through modification of gut microbiota thanks to its melanoidins and oligosaccharide fractions that behave *in vivo* as dietary fiber.

However, in the present study neither the amount of polyphenols or melanoidins in the intestine nor the microbiota composition were analyzed; thus the mechanisms behind the evidence of TJ and TLR-4 changed expression upon HFD+COFFEE vs HFD could be only hypothesized.

## CONCLUSIONS

In conclusion, our findings suggest that HFD induces disruption of intestinal TJ and increase of gut leakiness. Decaffeinated coffee is able to reduce this phenomenon. Thus, modulation of intestinal permeability may be assumed as a further mechanism underpinning the ability of coffee to reduce HFD-induced fatty liver.

**Competing Interests:** The authors declare that they have no competing interests.

**Author's Contributions:** The author's contributions are as follows – Mazzone G., Lembo V., Vitaglione P. performed the majority of experiments and analyzed the data; Mazzone G., Lembo V., Rossi A., Guarino M., performed the molecular investigations; D'Argenio G., Mazzone G., Lembo V. participated equally in treatment of animals; Morisco F., Caporaso N., Mazzone G. designed and coordinated the research; Caporaso N. Mazzone G. Morisco F., wrote the paper.

**Abbreviations:** TJ: tight junction; TLR: Toll-like receptor; ZO-1: Zonula occludens-1; HFD: high fat diet; SD: standard diet; NLR: nod-like receptors; LPS: lipopolysaccharides; SDS: sodium dodecyl sulphate.

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