

Influence of Gut Microbiota on Inflammation and Pathogenesis of Sugar Rich Diet Induced Diabetes

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Abstract

Type 2 diabetes is characterized by peripheral insulin resistance. Besides immune and inflammatory mechanisms, other pathways involve interaction between gut microbiota and metabolic syndrome. The present study was designed to understand gut microbiota alteration following High Sugar Diet (HSD) and its effect on physiology and gastrointestinal immunology. Male wistar rats were fed with high fructose and HSD for 60 days. Composition of fecal microbiota by DGGE and proinflammatory cytokines in serum was investigated. Expressions of genes such as TLR2, TLR4 and NF- κ B in various tissues were also studied. The bacteria coliforms and clostridium level were higher and Lactobacillus was lower in both sugar rich diet fed rats. Highly diverse and densely populated bands were observed in HSD group by DGGE fingerprint. The band profiles of sugar fed group have clustered together. Elevated mRNA expression of TLR2, TLR4, and NF- κ B were observed in HSD groups. Increased inflammation was confirmed by blood and tissue biochemical assay and enhanced serum pro-inflammatory cytokines in HSD diet groups. Gut microbiota strongly influenced the metabolic profiling of individuals fed with high calorie intake. The diverse microbial population and increased coliforms and clostridium may affect host gene expression. Targeting TLRs and microbiota could be promising therapeutic approach.

Key words:

Sugar rich diet; Gut microbiota; DGGE; TLRs; NF- κ B; Inflammation

Introduction

Diabetes mellitus has emerged as a foremost health care problem globally. According to International Diabetes Federation, currently there are 371 million people alive with diabetes mellitus worldwide and this number is anticipated to increase to 552 million by 2030 i.e. a 51% raise [1]. Insulin resistance and progressive pancreatic beta cell failure are key pathological mechanisms in natural history of T2D [2]. Several cross-sectional studies have shown that the inflammatory markers i.e. C-reactive protein (CRP), interleukin-6 (IL-6) and TNF- α are positively correlated with features of insulin resistance syndrome [3].

Consumption of high calorie diet and sedentary lifestyles disorders is rapidly becoming the most important health issue in most of the developed countries. Potential pathogenic mechanisms in diabetes include hyperglycemia, IR, oxidative stress, and inflammation that could culminate in the increased susceptibility to complications [4]. Recent insights into the activation of the innate immune system and inflammation via TLR (Toll-like receptor) activation in diabetes has led to significant interest in the key signalling mechanisms as novel therapeutic targets for a range of inflammatory and immune diseases [5].

It was found that gut microbiome contributes to metabolic disorders by triggering systemic inflammation [6]. The immune system co-evolves with the microbiota during after birth and unlocks the host

and microbiota to coexist in a mutually beneficial relationship [7]. The innate immune system has appeared as a key regulator of the gut microbiota and recognition of microbe-associated molecular patterns, is executed by families of pattern-recognition molecules with a special role for Toll-like receptors (TLRs) [8]. Recent findings specify that TLRs, which are over expressed in the affected tissue of most inflammatory disorders, can involve crosstalk between the immune systems and whole body metabolism [8]. It has been documented that TLR4, a ligand for lipopolysaccharides on Gram-negative bacteria, is involved in the stimulation of proinflammatory cytokine expression in macrophages, adipocytes, and liver [9].

Gut microbiota is being gradually more recognized as an important factor connecting genes, environment, and immune system [10]. The microbial population of the gut can also have an influence on metabolic processes, such as energy harvest from food, and should be considered an environmental factor that can cause insulin resistance, obesity, diabetes and other metabolic diseases [11]. Due to complex interaction among multiple susceptibility genes and between genetic and environmental factors, genetic examination of diabetes is complex and poorly understood.

The human gut "microbiome" is a complex consortium of trillions of microbes, and their genomes (gut microbiome) contain at least 100 times as many genes as human eukaryote genome [10]. This microbiome provides the host with enhanced metabolic capabilities, protection against pathogens, modulation of gastrointestinal development and protected immune system [11]. Understanding of the gut microbiota may improve transform therapeutic strategies for many important diseases and also progress the productivity of the

pharmaceutical industry. The significant involvement of the gut microbiota in human health and disease suggests that modulation of commensal microbial population through antibiotics, probiotics, and prebiotics or in combination could be a novel therapeutic approach.

The objective of this work was to understand the influence of sugar rich diet such as fructose and sucrose on gut microbiota imbalance leads to inflammation and observation of crosstalk among diet, microbiota, host gene expression and inflammation.

Materials and Methods

Chemicals and reagents

Synthesized oligonucleotides (IDT, USA), PCR master mix (Takara, South Korea), cDNA synthesis kit (Thermo Scientific, USA) and QIAamp Stool DNA Kit (Qiagen, Germany) were purchased. MRS, MacConkey and Yeast and mold agar were from Himedia (India). FreeStyle Optium H Blood Glucose Monitor from Abbott (UK) and all electrophoresis reagents (Biorad, USA) were used in this study.

Test animals and sample collection

All the procedures conducted in the present study were approved by the Institutional Animal Care and Use Committee, Nirma University, Ahmedabad under the CPCSEA guidelines of Ministry of Environment and Forest, New Delhi (Protocol No. IS/BT/FAC-12-1010). Male wistar rats of 8-10 weeks were obtained from Cadila Pharmaceuticals limited (Ahmedabad, India). The animals were housed in standard cages, and three rats in each cage. During the study the temperature was maintained at $22 \pm 1^\circ\text{C}$ and relative humidity at $55 \pm 5\%$, air was changed 8-10 times per hour. Diets and water (adjusted to pH 3.05 by citric acid to prevent growth of microorganisms) were provided ad libitum.

Diets and experimental design

The animals were randomized into three different dietary groups with 6 animals per group. Two groups were fed with fructose and sucrose rich diet. Third group was fed with diets contained normal rodent diets (Amrut agro foods, Mumbai) and considered as control group. Animals were fed their respective diets for 60 days. Body weight and food consumption were recorded weekly. Faecal samples excreted during the last 24 hours of the experiment were collected from the cages of the rats, each sample thus representing the three animals, which were caged together. All specimens were weighed and homogenized gently by hand. Faecal aliquots for molecular analysis were stored at -80°C before DNA extraction. Blood was collected from the experimental animals for biochemical analysis.

Blood and tissue sample collection

All animals were observed for their normal condition, clinical signs and mortality. The blood samples (approximately 1 ml) were collected from the retro orbital plexus under mild anesthesia into the microfuge tubes for collection of serum on day 0 (for baseline values) and the last day of the experiment. The plasma was used to determine levels of blood glucose, total cholesterol, triacylglycerol, high-density lipoprotein cholesterol (HDL-C), Total protein and urea. The pathophysiology evaluations were carried out by biomarkers by ALT (Glutamate pyruvate transaminase, SGPT) and AST (Serum glutamate oxaloacetate transaminase, SGOT) were estimated from rat serum by

Accucare diagnostic kit (India). Hepatic triglyceride and cholesterol were analyzed using Accucare diagnostic kit (India) while liver glycogen content was estimated by method described earlier [12].

The animals were sacrificed at the end of the study (after 60 day of high fructose and sucrose diet fed) by deep dose of anaesthesia. The liver, distal ileum and proximal colon were collected. Tissue samples were blotted with paper towel to remove blood, rinsed in saline, blotted to remove excess fluid for and stored at -70°C . A small portion of liver, distal ileum and proximal colon was excised from animals of each group fixed in 10 % v/v formalin saline and processed for standard histopathological procedures. Paraffin embedded specimens were cut into 5 μm sections (Yorco Sales Pvt. Ltd., New Delhi) and stained with hematoxylin and eosin for histopathological evaluations. The histopathological tissues sections were viewed and digitally photographed using a Cat-Cam 3.0 MP Trinocular microscope with an attached digital 3XM picture camera (Catalyst Biotech, Mumbai, India).

Oral glucose tolerance test

One day before the termination of the experiment, animals were subjected to an oral glucose tolerance test. Oral glucose tolerance tests were performed between 8.0 and 10.0 h at weekly intervals. Briefly, after overnight fasting, animals received a glucose load (2 g/kg) orally. Blood samples were collected from the tail vein at 0 min (before glucose administration), 15,30,60,90 and 120 min after glucose administration. Glucose concentration was determined with a FreeStyle Optium H Blood Glucose Monitor (Abbott, UK). Area under the curve for glucose (AUC glucose) was determined using the trapezoidal rule.

Selective cultivation of bacteria from faecal samples

Anaerobic and aerobic cultivable bacteria in fresh faecal samples were enumerated on yeast and mold agar (Himedia, India) for yeast and mold, Lactobacillus spp. (and others) on MRS (Himedia), coliforms on MacConkey agar (Himedia). Samples were homogenized and appropriate dilutions were spread plated on the selective agars, incubated for 48 h at 37°C , and resultant colonies were counted in CFU/gm of faeces.

RNA isolation and Reverse transcription-polymerase chain reaction (RT-PCR)

RNA was isolated from fresh tissue samples (distal ileum, proximal colon, liver, kidney and brain) using TRI reagent (Sigma-Aldrich), according to the manufacturer's protocols. RNA concentration was quantified by determining optical density at 260 and 280 nm (UV-2450, Spectrophotometer, Shimadzu (Japan)). The expressions of TLR2 and TLR4 (Distal ileum, proximal colon, liver, kidney and brain) while NF- κB mRNA (Distal ileum, proximal colon) was assessed by RT-PCR. Reverse transcription (RT) was performed using a First strand cDNA synthesis Kit (Thermo Scientific, USA). β -actin was served as a normalization control. The primer sequences were shown in Table S1.

Polymerase chain reaction (PCR) was performed with 50 μL reaction mixture of 3 μL of RT product, 25 μL of PCR master mix (Emerald master mix, Clontech, Korea) containing 1.5 mmol/L MgCl_2 , 2.5 U Taq DNA polymerase, 100 $\mu\text{mol/L}$ dNTP, 0.1 $\mu\text{mol/L}$ primer, and 1 \times Taq DNA polymerase magnesium-free buffer. PCR products were electrophoresed on a 1.5% ethidium bromide-stained

agarose gel and saved as digital images. Relative quantities of different mRNA expressions were analyzed by TotalLab 1.0 software (Magnitec Ltd., Israel), normalized with β -actin expression.

For DNA extraction, frozen faecal samples were diluted 1:10 (w/v) in PBS and thawed at 4°C. DNA was extracted from 2 ml of the 10-1 dilution by a method previously described [13], purified using the QIAamp DNA Stool Mini Kit (Qiagen, Hilden, Germany) and stored in 30 μ l autoclaved water at -20°C until use. Extract quality was determined by electrophoresis on 1% (w/v) agarose gels containing 10 μ g/ml ethidium bromide, and visualized under UV-Transilluminator.

Molecular fingerprints of the rat fecal microbiota by DGGE

The V3 region of the 16S rRNA gene from each DNA sample was amplified with universal primers P3 (5'-CGCCCGCCGCGCGCGGGCGGGGCGGGGGCACGGGGG-3') and P2 (5'-ATTACCGCGGCTGCTGG-3'), by following the protocol described before [14] for denaturing gradient gel electrophoresis (DGGE) analysis. Each 25 μ l PCR reaction mixture contained 1.5U of rTaq DNA polymerase, 2 mM MgCl₂, 2.5 μ l of the corresponding 10x buffer (Takara, South Korea), 200 μ M each dNTP, 12.5 pmol of each primer and 20 ng of total DNA template, and was conducted in a Thermocycler PCR system (Thermal cycler, Eppendorf, Germany). DGGE was carried out as previously described using a DCode™ Universal Mutation Detection System and gradient former model 475 according to the manufacturer's instructions (Bio-Rad Labs, Hercules, California).

PCR products (200 ng) were separated on 9% acrylamide (acrylamide-bis 37.5:1) stock solutions (Bio-Rad) in 1x TAE (20 mM Tris, 10 mM acetate, 0.5 M EDTA, pH 7.4). The gels were made with denaturing gradients ranging from 25 to 65%. The 100% denaturant solution contained 40% formamide and 7 M urea. The PCR product was mixed with 3 μ l loading dye before loading. Gels were run in 1x TAE at 60°C for 16 hr at 36 V, 28 mA, stained with the fluorescent dye for 45 min, and viewed by UV transillumination. The TotalLab software, version 1.00 was used for normalization of band patterns from DGGE gels and for neighbor joining and UPGMA Dendrogram. PCA analysis was performed by using XLSTAT software.

Measurement of inflammatory response

Levels of inflammatory cytokines in sera collected from various animal groups were estimated using BDTM Multiplex CBA Mouse

Inflammation kit (BD Biosciences, San Diego, CA, USA). Serum was subjected for quantifying the levels of cytokines IL-6, IL-10, MCP-1, IFN- γ , TNF, IL-12 p70. Briefly, different capture bead populations were mixed, incubated with recombinant protein standards or test samples, and subsequently incubated with PE-conjugated detection antibodies (measured in FL2) to form sandwich complexes. The standard and test samples were analyzed using BDTM CBA software on a flow cytometric platform.

Statistical analysis

All the values are expressed as mean \pm SD. Statistics was applied using graph pad prism software version 5. One way ANOVA followed by Tukey's multiple comparison test was used to determine the statistical significance between various groups. Differences were considered to be statistically significant when p<0.05. The significant value on comparison with control group were indicated with asterisk (*) while that compared with HFD group was indicated with hash (#).

Results

Weight gain and blood biochemical parameters

The rat supplemented with high fructose (HFD) and high sucrose diet (HSD) had a significant increase in body weight (BW) compared with rat on a normal diet (CD) with p values <0.001 and p<0.01, respectively. There were no significant changes in HDL-C and total protein level among the three groups (Table 1).

HFD and HSD rats also had significantly higher plasma triglyceride concentrations (167.88% and 154.22% respectively (p<0.001) and hepatic triglyceride (TG) levels (229.61% and 178.78% respectively, (p<0.001) compared to the controls. However, the hepatic TG levels of HSD rats were significantly lower (22.13%, p<0.01) than HFD rats (Table 1). In case of plasma and hepatic cholesterol, the HFD and HSD rats were significantly higher (p<0.001) than CD group. The free fatty acid levels were significantly higher in both the sugar diet fed group (p<0.001) than CD group. The liver injury markers such as SGPT (p<0.001, p<0.01) and SGOT (p<0.001) of serum were found significant increased levels in HFD and HSD group (Table 1). These results showed that long-term high fructose and high sucrose diet induced damage to liver cells as compared to control group.

Parameters	Baseline Levels	Control	HFD	HSD
Body weight (gm)	210 \pm 15	245 \pm 15	285 \pm 10**	273 \pm 15
FBG (mg/dL)	86.38 \pm 4.2	87.92 \pm 3.28	173.2 \pm 13.21***	164 \pm 12.67***
Triglycerides (mg/dL)	92.42 \pm 2.32	94.02 \pm 5.63	157.85 \pm 4.36***	145.65 \pm 7.24***
Total Cholesterol (mg/dL)	72.96 \pm 1.46	73.7 \pm 7.5	97.8 \pm 4.7***	93.21 \pm 2.7***
HDL-C (mg/dL)	40.62 \pm 2.01	43.5 \pm 7.52	38.8 \pm 4.7	42.32 \pm 3.25
SGPT (ALT) IU/L	31.18 \pm 2.01	33.26 \pm 1.8	45.07 \pm 3.2***	39.73 \pm 2.57***##
SGOT (AST) IU/L	28.09 \pm 1.89	29.39 \pm 2.4	45.03 \pm 2.71***	37.86 \pm 3.21***##
Total Protein (g/dl)	7.98 \pm 0.72	8.14 \pm 1.12	7.04 \pm 1.23	7.42 \pm 1.27

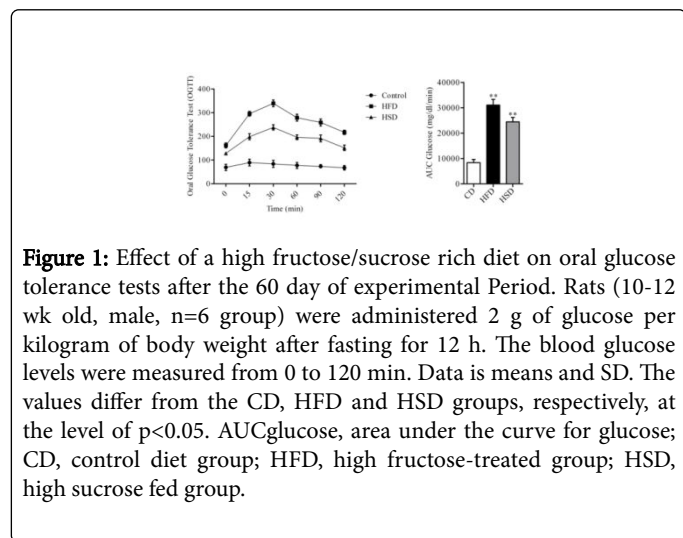
Total Urea (mg/dl)	50.67 ± 1.76	52.49 ± 2.49	47.44 ± 1.25***	48.07 ± 1.35**
Liver weight (gm)	-	4.2 ± 0.3	5.6 ± 0.3***	5.1 ± 0.1****##
Adipose tissue weight (gm)	-	4.90 ± 0.80	7.32 ± 1.3**	6.8 ± 1.2*
Free Fatty Acids	22.32 ± 2.51	24.69 ± 3.2	45.37 ± 5.8***	43.28 ± 4.6***

Table 1: Body weight and Serum profile in Control, HFD and HSD group rats at baseline after 60 days HFD: High fructose diet, HSD: High sucrose diet fed group, HDL-C: high-density lipoprotein cholesterol. Values are presented as mean ± SD drawn from pooled spillage of three cages of the same group and calculated as six animals per group. Values with different superscript letters are significantly different. *compared with control group; #compared with HFD group except CD group; ***p<0.001, **p<0.01, *p<0.05, ###p<0.001, ##p<0.01, #p<0.05.

Oral glucose tolerance test (OGTT) in high-fructose and sucrose rich diet rats

The incremental changes in plasma glucose concentrations of rats following an oral glucose intake were observed (Figure 1). After animals received a glucose load orally, an increment in plasma glucose and insulin concentrations was peaked at 15 min. The incremental glucose concentrations of the HFD and HSD group were significantly higher than those of CD group at 15, 30 and 60 min. There were no significant differences in the incremental glucose concentrations at 60, 90 and 120 min between the HFD and HSD group.

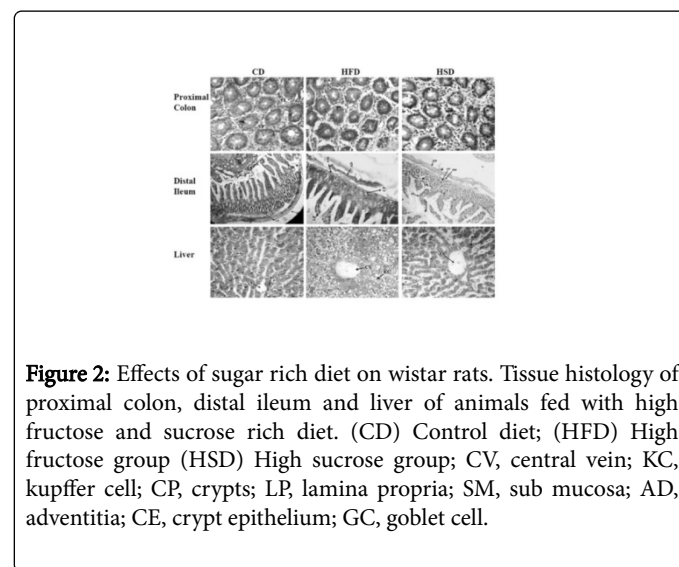
As shown in Figure 1, the incremental AUCs (area under the curves) of plasma glucose (AUC_{glucose}) concentration during OGTT of the HFD and HSD group was elevated significantly (p<0.01) approximately 270% and 191% compared to CD group respectively while HSD rats having 22% lower AUC glucose concentration than HFD group.



Effect of Sugar rich diet of morphology of liver, ileum and colon

The livers of the HFD and HSD group were larger compared with those in the CD group and became beige. The hepatic cells with clear cytoplasm, nucleus, nucleolus and central vein in the CD group

showed normal histology (Figure 2). The liver sections of the HFD and HSD rats exhibited massive fatty changes and severe steatosis with cytoplasmic vacuoles confirmed by histopathological examination. Intestinal segments revealed villi edema, lymphocytes infiltration and goblet cells hyperplasia in HFD and HSD fed rats. The crypts of colon contain higher goblet cells in HFD and HSD rats than CD group. The epithelial layer was largely destroyed in the ileum and less damaged in the colon. Significant destruction of enterocytes was noted in the ileum but was minimal in the ileum. Villus structure was also severely affected in HFD rats compared to HSD. The diabetic animals (HFD and HSD fed) were found height in villus or width or crypt depth and necrosis was also observed in samples and number of goblet cells as compared to the CD group.



Effect of sugar rich diet on the faecal microflora species in sugar rich diet rats

Lactobacilli and Bifidobacteria have been used as beneficial microflora and GRAS species, whereas some species like Clostridium would be harmful as a result of their metabolic activities. The number of lactobacilli, Yeast and moulds, Coliforms, Bifidobacteria and Clostridia in control group were regarded as 100% for comparison. these results (Figure 3) indicated that the number of Lactobacilli, Yeast and moulds, Coliforms, Bifidobacteria and Clostridia in HFD group were 55.10 ± 2.4%, 105.25 ± 2.8%, 180.06 ± 5.2%, 93.47 ± 3.2%, 130.61 ± 4.2%, respectively; whereas, in HSD group were 59.51 ± 2.3%, 89.05

$\pm 3.4\%$, $157.65 \pm 4.9\%$, $94.89 \pm 4.1\%$, $124.48 \pm 2.8\%$, respectively. These results revealed that no significant change on the number of yeast, moulds, and Bifidobacteria in both HFD and HSD group was noticed as compared to control group. However, the number of coliforms and Clostridia in both HFD and HSD group was significantly higher ($p < 0.05$) than that of control group.

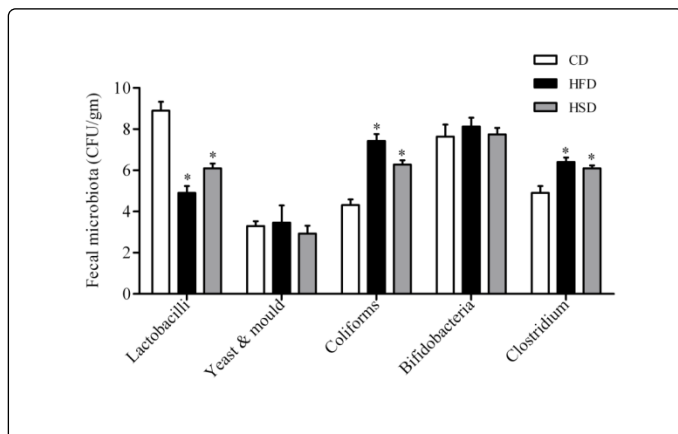


Figure 3: Enumeration of microbial profiling from the faecal samples of rats after 60 days of the respective diets. Averages and standard deviations of six samples, each representing 6 animals, are presented. Values with different superscript letters are significantly different. *compared with control group; #compared with HFD group except CD group; * $p > 0.05$.

Effects on DGGE fingerprints of faecal microbiota

DGGE allows the separation of genomic DNA fragments of the same length that differ in their nucleotide sequence, due to differences in duplex denaturing characteristics. Fragment separation is improved by the addition of a GC clamp to one end of the duplex DNA. In this study DGGE analysis of PCR amplified fragments of the V2-V3 16S rDNA region with total bacterial DNA extracted from faecal samples of different fed groups' shows that each individual harbors a characteristic bacterial profile that remains stable during the study period. The average number of bands obtained on genomic DNA-based, V3 region of DGGE profiles was 21 ± 3 , while the numbers of bands obtained differ significantly in high fructose (36 ± 3) and high sucrose (32 ± 2) dietary groups (Figure 4). The genomic DNA and V3 variable region of 16S rDNA was enzymatically amplified in the PCR with primer to conserved regions of 16S- rRNA genes from experimental group.

By comparing the DNA based DGGE profiles obtained from the faecal samples (Figure 4) of different group, extensive diversity of bands and densely populated bands were observed in HFD and HSD diet group than in the control diet. The microbial population of rats fed on control diet and Fructose/sucrose rich diet displayed an entirely different profile.

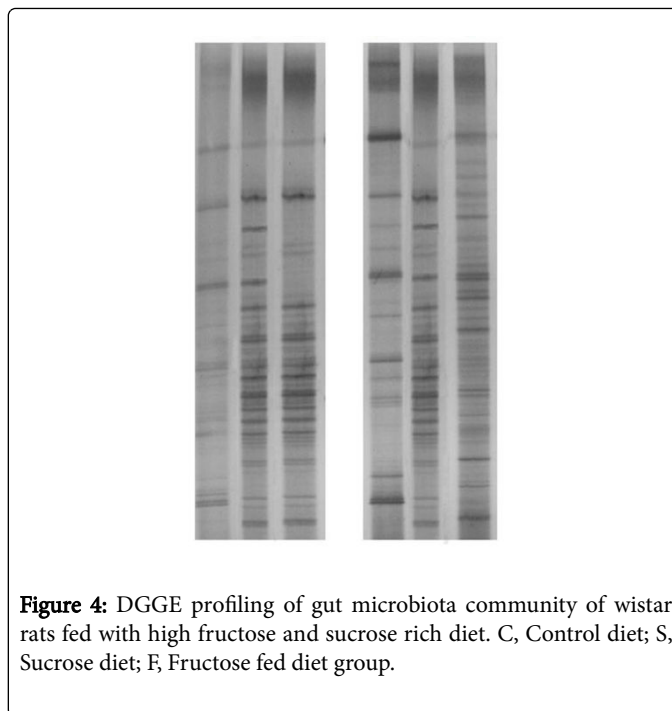


Figure 4: DGGE profiling of gut microbiota community of wistar rats fed with high fructose and sucrose rich diet. C, Control diet; S, Sucrose diet; F, Fructose fed diet group.

The neighbor joining (Figure S1) and UPGMA Dendrogram (Figure S2) of analyzed DGGE gel was also showed different profile of microbial community present in CD group compared with sugar fed dietary groups. Principal Component Analysis of obtained DNA-based DGGE profiles revealed that samples from animals belonging to the sugar fed dietary group clustered together, but control diet groups produced different profiles (Figure 5).

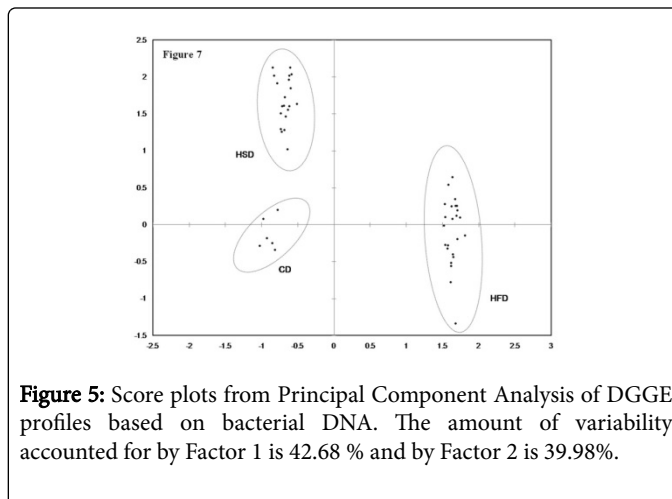


Figure 5: Score plots from Principal Component Analysis of DGGE profiles based on bacterial DNA. The amount of variability accounted for by Factor 1 is 42.68 % and by Factor 2 is 39.98%.

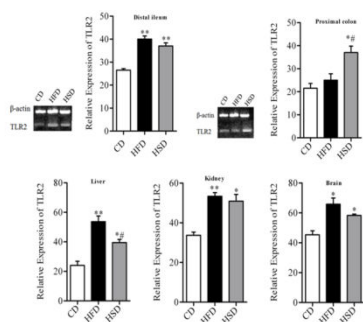


Figure 6: The expression of TLR2 mRNA of distal ileum, proximal colon, liver, kidney and brain of rats of CD, HFD and HSD group. *Differences are compared with control group and # compared with HSD group except CD group; ($p < 0.05$).

Gene expression

TLR2 and TLR4 mRNA expressions were analyzed by RT-PCR from distal ileum and proximal colon, liver, kidney and brain tissue whereas NF-kB mRNA expressions were analyzed from distal ileum and proximal colon.

The TLR2 mRNA expression in ileum, liver and kidney of HFD rats were significantly increased ($p < 0.01$), while in HSD group TLR2 mRNA level in proximal colon, liver, kidney and brain was elevated significantly ($p < 0.05$) than CD group (Figure 6). No difference was observed in TLR2 mRNA in proximal colon of CD and HFD group.

The TLR4 mRNA level in proximal colon of both HFD and HSD rats were significantly increased ($p < 0.001$) in liver and ileum ($p < 0.01$), kidney ($p < 0.05$) than CD group (Figure 7).

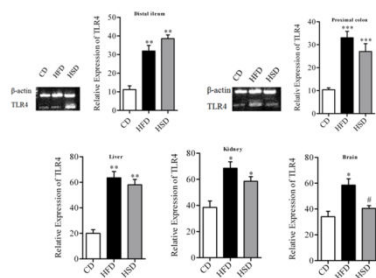


Figure 7: The expression of TLR4 mRNA of distal ileum, proximal colon, liver, kidney and brain of rats of CD, HFD and HSD group. *Differences are compared with control group and # compared with HSD group except CD group; ($p < 0.05$).

No difference of TLR4 mRNA level was observed in brain among CD and HSD group but the brain of HFD rats had increased significantly ($p < 0.05$). The NF-kB expression in distal ileum and

proximal colon were significantly elevated in both HFD ($p < 0.01$) and HSD group ($p < 0.05$) than that of CD group (Figure 8).

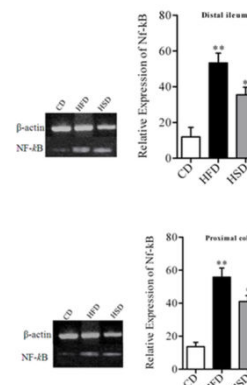


Figure 8: The expression of NF-kB mRNA of distal ileum and proximal colon, of rats of CD, HFD and HSD group. *Differences are compared with control group and # compared with HSD group except CD group; ($p < 0.05$).

Effect of sugar rich diet in serum inflammatory cytokines

Six important insulin resistance-related inflammatory cytokines namely IL-6, IL-10, TNF- α , INF- γ , IL-12p70 were evaluated (Figure 9). The concentrations of IL-6, TNF- α and INF- γ in HFD and HSD group were significantly increased ($p < 0.001$) as compared to control group, and IL-12 level also elevated significantly ($p < 0.05$) in HFD rats but no difference was observed in HSD group when compared to CD group. The MCP-1 and IL-12p70 levels in both the diet group was significantly higher ($p < 0.01$) than control rats. No significant difference was observed in serum IL-10 level of different diet groups.

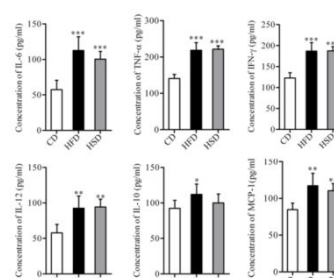


Figure 9: Effect of Sugar rich diet in progress of inflammation in wistar rat. Cytokines (IL-6, TNF- α , INF- γ , MCP-1, IL-12 and IL-10) level in serum of high-fructose and high sucrose diet rats at the end of the experimental period (60 days) were determined. The data were expressed as mean \pm SD, ($n = 6$). TNF- α , tumor necrosis factor α ; IL-6, interleukin 6; MCP-1, monocyte chemoattractant protein-1; * $p < 0.05$ compared with Control group; *** $p < 0.001$, ** $p < 0.01$ and * $p < 0.05$.

Discussion

The qualitative and quantitative changes of diet intake influence the gut microbiota. The bacteria of the large intestine respond to changes in diet, especially to the type and quantity of dietary carbohydrate. The consequence of increased carbohydrate intake leads to decreased in pH of the gut lumen and significantly alteration in bacterial metabolism and commensal prevalence [15].

The increased occurrence of obesity and type II diabetes cannot be ascribed only to changes in the human genome, nutritional habits, or reduction of physical activity in our daily lives. There is one most important new environmental factor, namely gut microbiota [16]. The significant involvement of the gut microbiota in human health and disease suggests that manipulation of commensal microbial composition through probiotics could be a novel therapeutic approach [17].

Consumption of high carbohydrate and fat diet leads rodent model to mimic the human metabolic syndrome and testing for potential therapeutic interventions [18]. In the metabolic pathway, fructose and sucrose rapidly induces hepatic de novo lipogenesis, increased intestinal permeability associated with high fructose and sucrose feeding may lead to endotoxemia and increase the secretion of inflammatory cytokines, which, may lead to insulin resistance and diabetes [19].

While increased body weight alone does not necessarily represent obesity, there is few other parameters such as increased body-fat and increased TG levels, may be leads to obese status. The present study demonstrated that feeding of the high fructose (HFD) and high sucrose (HSD) diet caused gains in body and white adipose tissue (WAT) weight and hepatic steatosis in 8 weeks, which is consistent with other studies [20]. This might be an effect of concentration of sucrose, as others have shown that at higher concentrations (65%) of sucrose and fructose rats can become overweight. Although there was no difference in food intake between animals given the control or HFD or HSD diet. Thus, rapid onset of visceral obesity and fatty liver may occur with intake of a high sugar diet that is high in fructose and sucrose. Diet-induced diabetes is largely caused by disorders of fat metabolism, resulting in an excess deposition of fat in various tissues. Diet is considered to be one of the important environmental factors

influencing the composition of the gut microbiota within a host and affecting their functional relationships [21,22].

Impaired glucose tolerance testing is an important diagnostic indicator for type 2 diabetes [12]. Our results from OGTT indicated that an increase in blood glucose level was observed after high-fructose as well as sucrose-diet feeding.

The present data suggests that rats with high sugar diet (Fructose and Sucrose) do not maintain a normal body weight. In a related study, female rats with access to high fructose corn syrup (HFCS) showed enhanced body weight and abdominal fat pad compared to controls after 8 weeks of dietary status. However, in the current study, no significant difference in body weight was observed between HFD and HSD rats, although a more rapid weight gain was noted in HFCS-consuming animals compared to fructose- and sucrose-consuming animals.

Fructose and sucrose consumption by young rats has been revealed to induce lower glucose tolerance and reduced insulin sensitivity as well as increased TG, cholesterol, and body fat [22]. The elevated TG levels are commonly associated with a cluster of metabolic risk factors known as the metabolic syndrome [23]. Further, adverse effects precipitated by increased fructose intake include negative effects on cardiovascular and kidney functions. Earlier studies reported that high sugar diet can lead to insulin resistance, while defects in the insulin-signaling pathway may be due to high fat diet [24]. In this study, HFD and HSD rats reproduced the changes in blood glucose, plasma triglyceride and cholesterol levels which may leads to insulin resistance and impaired glucose tolerance. These altered markers suggest a multiple organ function compromise, such as the adipose tissue (increased FFA levels) and the liver (high cholesterol and triglyceride levels) (Table 2). It has also been reported that FFAs regulate gene expression, especially those involved in lipid and carbohydrate metabolism. Our results are in agreement with the earlier reports on significant elevation of FFAs in both HFD and HSD diet as compared to control fed. Chronically elevated FFAs may also impair insulin secretory function through toxic effects on pancreatic beta cells of metabolic changes seen in the adipose tissue, skeletal muscle and liver of insulin-resistant animals and subjects [25].

Parameters	Control	HFD	HSD
Liver Triglyceride (mg/gm)	10.84 ± 1.76	24.89 ± 3.23 ^{***}	19.38 ± 2.57 ^{***##}
Liver Cholesterol (mg/gm)	1.57 ± 0.24	1.13 ± 0.27 ^{**}	0.98 ± 0.13 ^{***}
Liver Glycogen (mg/gm)	8.14 ± 0.8	6.91 ± 1.08	7.04 ± 0.74

Table 2: Tissue biochemical analysis HFD: High fructose diet, HSD: High sucrose diet fed group, Values are presented as mean ± SD drawn from pooled spillage of three cages of the same group and calculated for six animals per group. Values with different superscript letters are significantly different. *compared with control group; #compared with HFD group except CD group; ^{***}p<0.001, ^{**}p<0.01, ^{##}p<0.01.

Serum AST is comprised of two distinct isoenzymes, one located in cytoplasm, and the other in mitochondria. In the liver, 60-80% of the total AST activity is of mitochondrial origin. In this study, the serum levels of AST and ALT, two critical markers of liver injury, were increased in HFD and HSD fed rats. This elevation of serum AST and ALT may leads to liver injury and this has been observed in our study.

Insulin resistance plays a decisive role in hepatic steatosis, which is closely associated with obesity [26]. Our results from histological

analysis demonstrated that a prominent hepatic steatosis with cytoplasmic vacuoles was observed in HFD and HSD rats. The liver uses fatty acids hydrolyzed from fat to synthesize cholesterol and TG [21]. Excess fructose and sucrose intake was not able to equilibrate lipid metabolism, which in turn accumulate of TG in liver and an increased liver lipid profile, and hepatic steatosis takes place.

Indigenous bacteria proliferate into the intervillous spaces in the small intestine, thus intestinal villi become shorter in sites. In this

study, ileum have unusual prominent deep surface convolutions of the villi developed without increase in height of the villi.

Intestinal flora influences the human health such as immunomodulation, improved digestion and absorption, vitamin synthesis, inhibition of the growth of potential pathogens, cholesterol reduction, colonic fermentation and lowering of gas distension [27]. Regular intake of high calorie/sugar (fructose and sucrose) rich diet influenced gut microbiota composition. The study data showed that HFD and HSD diet influences the gut microbial community with significant decrease of lactobacilli (commensal bacteria) and increase in coliforms and clostridium population.

Scientists have reported that upon feeding of high fat enriched diet, the gut microbiota was involved in the progression of metabolic endotoxemia, adipose tissue inflammation and metabolic disorder may be due to increase in gut permeability and LPS (lipopolysaccharide) absorption. Earlier reports suggest that metabolic disorders were clearly having a relation with LPS [16]. Clostridium species could trigger the inflammatory process in human inflammatory bowel diseases and increase the incidence and growth rate of colonic tumors induced in animals, whereas other genera such as Lactobacillus and Bifidobacteria prevent tumorigenesis [28]. However, there were no significant change in the number of Bifidobacteria as well as yeast and moulds in all three diet groups. Pathogenic gut Microbiota is reported to stimulate LPS production and secretion from intestinal epithelial cells, which can bind to cytokine receptors on liver cells and adipocytes triggering pro-inflammatory cytokine release [29].

Analysis of extracted genomic DNA in faeces from animals fed with high fructose, high sucrose and control diets by DGGE resulted in different profiles from animals belonging to each of the three dietary groups. The average number of bands obtained by DGGE was approximately 21 (CD), 32 (HFD) and 36 (HSD) bands observed in the dietary groups, indicating that the overall complexity of the microbiota was altered by the ingested sugar rich diets. The DDGE profile showed the fructose, sucrose fed diet and the control diet produced different phylogenetic bacterial fingerprints (Figure 4). DGGE image defining that change in the diet affected the phylogenetic composition of the fecal microbiota (Figures 5 and 6). The DGGE band patterns also indicate the effect of sugar rich diet in large intestine with diverse microbial community. Indeed the starch, fructose and sucrose were normally digested in the small intestine [30].

Inflammation could be triggered by the detection of highly conserved molecules, termed 'pathogen associated molecular patterns' (PAMPs), which are expressed by microbes but not host cells and that are usually recognized by pattern recognition receptors (PPRs), such as Toll-like receptors (TLRs) and the inhibition of the NF- κ B inflammatory pathway. TLRs are responsible for cellular responses against bacterial infections, initiation of inflammation, production of antimicrobial peptides, activation of cellular repair and survival pathways. TLR2 and TLR4 are most important sensors of pathogenic bacteria, for maintaining bacterial homeostasis [31].

When sensitivity to the hormone insulin was weaker or stopped by the cells or tissues of an organism is called as insulin resistance in which the ability of the host to lower blood sugar levels can become impaired in a process that can eventually lead to the development of type II diabetes. Recent evidence suggests that both of these conditions may be promoted by the stimulation of TLR-signalling [32].

Gut microbiota and energy metabolism are regulated by a complex gut environment and microbial complexes which influence signaling

processes. Thus, in this study investigated mRNA expression of key pathogen recognition receptors and inflammatory mediators such as TLR2, TLR4 and NF- κ B. The interactions among inflammation, hyperglycemia, insulin resistance, and type 2 diabetes have clear implications for metabolic disorder via innate immune system. The pathways lead to the production of inflammatory cytokines that include interleukin-6 (IL-6), IL-12, IL-18, IFN- γ , and TNF- α . It has been reported that activation of TLR2 and TLR4 promotes expression of inflammatory cytokines [33].

In this study, mRNA expression levels of TLR2, TLR4, and NF- κ B were elevated in the distal ileum, proximal colon, liver and adipose tissues of rats fed with high fructose and sucrose compared to control group rats. Type 2 diabetes and insulin resistance is recognized by enhanced inflammatory cytokines and infiltration of adipose tissue with macrophages. From most of the inflammatory pathways, the NF- κ B was associated with macrophage infiltration and stimulation of inflammatory cytokines and chemokines like TNF and MCP-1 [34].

TLR2 mRNA expression level was higher in HFD and HSD rats showed that elevated inflammation activated in response to inflammatory cytokines and free fatty acids. Furthermore, increased free fatty acids and TG in our HFD and HSD animals can be important factors for causing insulin resistance in our study. High glucose concentration in blood induces inflammatory cytokines, chemokines, p38 MAPK, NF- κ B activity, and TLR2 expression. Additionally, TLR2 stimulation with peptidoglycan was associated with increased levels of TNF- α and IL-6. Our data implied that TLR2 can contribute to the development of insulin resistance in HFD and HSD rats which is in agreement with other reports [35].

TLR4 can recognize a variety of microbial and host structures including lipopolysaccharide, peptidoglycan, high mobility group proteins, etc. After binding to its ligands, TLR4 lead to activation of two downstream pathways: mitogen activated protein kinase (MAPK) and NF- κ B pathway. TLR4 plays an important role in intestinal innate immune system as the first line for the recognition of the intestinal tract bacteria. TLR4 is a cell surface PAMPs recognition inducing inflammation that generates innate immune responses to pathogens by inducing signaling cascades of kinase and transcription factor activation. The healthy liver contains less mRNA expression levels of TLR4 suggesting the high tolerance of the liver to LPS from the intestinal microbiota to which the liver is constantly exposed [36]. Because of the relation between the liver and intestines, Kupffer cells (KC) are the first cell to encounter gut-derived toxins including LPS which express TLR4 and are responsive to LPS [36]. Upon triggering, TLR4 signaling drives Kupffer cells to produce TNF- α , IL-1b, IL-6, IL-12, IL-18, and anti-inflammatory cytokine IL-10 [37]. In this study HFD and HSD fed rats have higher TLR4 expression in liver, distal ileum and proximal colon. TLR4 in the kidney of rats in the HFD and HSD group was higher than the control group since after the 60 day of experimental period. It indicates the expression of TLR4 in the renal tubular epithelium mediates the tubular substance leukocyte infiltration, tubular injury and activates the immunocyte and renal parenchymal cell in glomerulus. It excretes abundant inflammatory mediator and cytokine and causes the continuous inflammatory reaction that promotes the tubular interstitial fibrosis and glomerular sclerosis [38].

Tumor necrosis factor-alpha (TNF-alpha), and IL-6, considered as the main regulators of inflammation and may have a crucial role in the development of insulin resistance in type 2 diabetes. The present study revealed that feeding HFD and HSD for 60 days increased levels of

IL-6, IL-12, TNF- γ MCP-1 and IFN- γ and no significant difference in IL-10, compared to healthy controls. Increased levels of inflammatory cytokines in our study were in agreement with previous findings that an increased level of IL-6 in serum is associated with insulin resistance [39].

The high levels of inflammatory cytokines appear in type 2 diabetes and capable of thinning insulin sensitivity. The TNF- α and IL-6 found in sera are likely produced by various tissues, including activated leukocytes, adipocytes, and endothelial cells and associated with enlargement of adipose tissue and increases the number of adipose tissues macrophages. These macrophages are responsible for TNF- α expressions and mediators of inflammation, which may play a critical role in insulin resistance and the pathogenesis of type 2 diabetes [40]. Early release of macrophage-derived, proinflammatory cytokines, such as TNF, IL-1, and IL-6, are the most powerful pathological cytokines.

MCP-1 is a chemokine that recruits immune cells such as monocytes and lymphocytes (83) and a member of the chemokine family and studied for number of pathological conditions characterized by monocyte infiltration. Our data showed the possible involvement of MCP-1 in initiating and sustaining these abnormalities with type 2 diabetes. Interleukin (IL)-10 is a centrally operating anti-inflammatory cytokine that plays a crucial role in the regulation of the innate immune system. It has strong deactivating properties on the inflammatory host response mediated by macrophages and lymphocytes, and potentially inhibits the production of pro-inflammatory cytokines such as IL-6 and TNF- α [41]. In our results, no significance difference was observed among the dietary group to IL-10 when high fructose and sucrose diet was given for 60 days.

There is increasing evidence suggesting that both in the animal models as well as in human, higher intake of fructose in diet is a nutritional factor in the development of metabolic syndrome and its associated complications. Fructose is a good power source. Fructose is found in seminal fluid and sperm cells use for energy. High intake of fructose results in increased formation of triacylglycerol and transferred from the intestine to the liver for metabolism. Consumption of fructose is increasing day by day and is somehow responsible for body weight gain. Earlier evidence and the results of the present study showed that fructose and sucrose increases incidence of dyslipidemia and insulin resistance. No metabolic differences between fructose and sucrose have been noted [42]. Metabolism of fructose takes place in the liver and leads to accumulation of triglycerides in the liver (hepatic steatosis) results in impairment of lipid metabolism and enhances the expression of proinflammatory cytokine. Epidemiological studies showed that obesity, metabolic and cardiovascular disorders are also due to consumption of sweetened beverages (containing either sucrose or a mixture of glucose and fructose).

Conclusion

Diet plays a major role in determining the types of microbes that colonize the gut. Diet affects the composition of the microbiota, and the microbiota regulates immune and inflammatory responses, then diet should have easily quantifiable effects on immune responses. From our results, not much difference was found in the physiology and biochemical alteration of rats following fed with fructose and sucrose diet. High fructose and high sucrose consumption influences the gut microbial community as well as elevated expression of microbe associated molecular patterns (TLR2 and TLR4). The enhanced pro-

inflammatory signals (IL-6, TNF- α , INF- γ , IL-12) and NF- κ B expression was also observed. In this context, the coliform microbial community may elevate the gram negative bacteria with excess LPS release. These LPS is an endotoxic agents reported to increase inflammation. Both fructose and Sucrose are digestible and are not expected to reach the large intestine. The DGGE band patterns obtained indicated that these carbohydrates indeed affected the composition of bacteria in the large gut showing different molecular fingerprints. Further, metagenomic analysis is required to monitor the exact involvement of gut microbial community on the progression of diet diabetes. The probiotic intervention with increase Lactobacilli and bifidobacteria community in the GI tract may be a beneficial approach to monitor the role of colonic bacteria.

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Conflict of Interest

There is no conflict of interest among the authors.

References

1. IDF (2014) International Diabetes Federation. Diabetes atlas, 5th ed., Brussels: International Diabetes Federation.
2. Cernea S, Dobreanu M (2013) Diabetes and beta cell function: from mechanisms to evaluation and clinical implications. *Biochem Med (Zagreb)* 23: 266-280.
3. Sattar N, Wannamethee SG, Forouhi NG (2008) Novel biochemical risk factors for type 2 diabetes: pathogenic insights or prediction possibilities? *Diabetologia* 51: 926-940.
4. Olefsky JM, Glass CK (2010) Macrophages, inflammation, and insulin resistance. *Annu Rev Physiol* 72: 219-246.
5. Wong FS, Wen L (2008) Toll-like receptors and diabetes. *Ann N Y Acad Sci* 1150: 123-132.
6. Macdonald TT, Monteleone G (2005) Immunity, inflammation, and allergy in the gut. *Science* 307: 1920-1925.
7. Round JL, Mazmanian SK (2009) The gut microbiota shapes intestinal immune responses during health and disease. *Nat Rev Immunol* 9: 313-323.
8. Takeda K, Kaisho T, Akira S (2003) Toll-like receptors. *Annu Rev Immunol* 21: 335-376.
9. Creely SJ, McTernan PG, Kusminski CM, Fisher FM, Da Silva NF, et al (2007) Lipopolysaccharide activates an innate immune system response in human adipose tissue in obesity and type 2 diabetes. *Am J Physiol Endocrinol Metab* 292: E740-747.
10. Rajpal DK, Brown JR (2013) The Microbiome as a therapeutic target for metabolic disorder. *Drug Dev Res* 74: 376-384.
11. Tremaroli V, Bäckhed F (2012) Functional interactions between the gut microbiota and host metabolism. *Nature* 489: 242-249.
12. Yadav H, Jain S, Sinha PR (2007) Antidiabetic effect of probiotic dahi containing *Lactobacillus acidophilus* and *Lactobacillus casei* in high fructose fed rats. *Nutrition* 23: 62-68.
13. Leser TD, Lindcroma RH, Jensen TK, Jensen BB, Møller K (2000) Changes in bacterial community structures in the colon of pigs fed experimental diets and after infection with *Brachyspira hyodysenteriae*. *Appl Environ Microbiol* 66: 3290-3296.
14. Knapp BA, Seeber J, Podmirseg SM, Rief A, Meyer E, et al. (2009) Molecular fingerprinting analysis of the gut microbiota of *Cylindroiulus fulviceps* (Diplopoda). *Pedobiologia* 52: 325-336.
15. Underwood MA, Salzman NH, Bennett SH, Barman M, Mills DA, et al. (2009) A randomized placebo-controlled comparison of 2 probiotic/

- probiotic combinations in preterm infants: impact on weight gain, intestinal microbiota, and fecal short-chain fatty acids. *J Pediatr Gastroenterol Nutr* 48: 216-225.
16. Cani PD, Amar J, Iglesias MA, Poggi M, Knauf C, et al. (2007) Metabolic endotoxemia initiates obesity and insulin resistance. *Diabetes* 56: 1761-1772.
 17. Jia W, Li H, Zhao L, Nicholson JK (2008) Gut microbiota: a potential new territory for drug targeting. *Nat Rev Drug Discov* 7: 123-129.
 18. Panchal SK, Brown L (2011) Rodent models for metabolic syndrome research. *J Biomed Biotechnol* 2011: 351982.
 19. Ribeiro RT, Lutt WW, Legare DJ, Macedo MP (2005) Insulin resistance induced by sucrose feeding in rats is due to an impairment of the hepatic parasympathetic nerves. *Diabetologia* 48: 976-983.
 20. Mélançon S, Bachelard H, Badeau M, Bourgoin F, Pitre M, et al. (2006) Effects of high-sucrose feeding on insulin resistance and hemodynamic responses to insulin in spontaneously hypertensive rats. *Am J Physiol Heart Circ Physiol* 290: H2571-2581.
 21. Wang LX, Liu K, Gao DW, Hao JK (2013) Protective effects of two *Lactobacillus plantarum* strains in hyperlipidemic mice. *World J Gastroenterol* 19: 3150-3156.
 22. de Moura RF, Ribeiro C, de Oliveira JA, Stevanato E, de Mello MA (2009) Metabolic syndrome signs in Wistar rats submitted to different high-fructose ingestion protocols. *Br J Nutr* 101: 1178-1184.
 23. Bocarsly ME, Powell ES, Avena NM, Hoebel BG (2010) High-fructose corn syrup causes characteristics of obesity in rats: increased body weight, body fat and triglyceride levels. *Pharmacol Biochem Behav* 97: 101-106.
 24. Alghamdi F, Guo M, Abdulkhalek S, Crawford N, Amith SR, et al. (2014) A novel insulin receptor-signaling platform and its link to insulin resistance and type 2 diabetes. *Cell Signal* 26: 1355-1368.
 25. Zlobine I, Gopal K, Ussher JR (2016) Lipotoxicity in obesity and diabetes-related cardiac dysfunction. *Biochim Biophys Acta* .
 26. Wallace TC, Guarner F, Madsen K, Cabana MD, Gibson G, et al. (2011) Human gut microbiota and its relationship to health and disease. *Nutr Rev* 69: 392-403.
 27. Jackson EE, Ruedy ER, Smith BJ, Lacombe VA (2015) Loss of Toll-Like Receptor 4 function partially protects against peripheral and cardiac glucose metabolic derangements during a long-term high-fat diet. *PLoS one* 10(11): e0142077.
 28. McOrist AL, Miller RB, Bird AR, Keogh JB, Noakes M, et al. (2011) Fecal butyrate levels vary widely among individuals but are usually increased by a diet high in resistant starch. *J Nutr* 141: 883-889.
 29. Musso G, Gambino R, Cassader M (2011) Interactions between gut microbiota and host metabolism predisposing to obesity and diabetes. *Annu Rev Med* 62: 361-380.
 30. DiBaise JK, Zhang H, Crowell MD, Krajmalnik-Brown R, Decker GA, et al. (2008) Gut microbiota and its possible relationship with obesity. *Mayo Clin Proc* 83: 460-469.
 31. Neish AS, Gewirtz AT, Zeng H, Young AN, Hobert ME, et al. (2000) Prokaryotic regulation of epithelial responses by inhibition of IkappaB-alpha ubiquitination. *Science* 289: 1560-1563.
 32. Erridge C (2011) Diet, commensals and the intestine as sources of pathogen-associated molecular patterns in atherosclerosis, type 2 diabetes and non-alcoholic fatty liver disease. *Atherosclerosis* 216: 1-6.
 33. Devaraj S, Tobias P, Jialal I (2011) Knockout of toll-like receptor-4 attenuates the pro-inflammatory state of diabetes. *Cytokine* 55: 441-445.
 34. Chen L, Chen R, Wang H, Liang F (2015) Mechanisms Linking Inflammation to Insulin Resistance. *Int J Endocrinol* 2015: 508409.
 35. Wang SW, Lin TM, Wang CH, Liu HH, Hwang JY (2012) Increased toll-like receptor 2 expression in peptidoglycan-treated blood monocytes is associated with insulin resistance in patients with non-diabetic rheumatoid arthritis. *Mediat Inflamm* 2012: 690525.
 36. Su GL, Klein RD, Aminlari A, Zhang HY, Steintraesser L, et al. (2000) Kupffer cell activation by lipopolysaccharide in rats: role for lipopolysaccharide binding protein and toll-like receptor 4. *Hepatology* 31: 932-936.
 37. Prajapati B, Jena PK, Rajput P, Purandhar K, Seshadri S (2014) Understanding and modulating the Toll like Receptors (TLRs) and NOD like Receptors (NLRs) cross talk in type 2 diabetes. *Curr Diabetes Rev* 10: 190-200.
 38. Liu SY, Nie XZ, Zhou WY, Chen J (2013) Expression and effect of TLR4 in rats with diabetic nephropathy. *Asian Pac J Trop Med* 6: 635-639.
 39. Mauer J, Chaurasia B, Goldau J, Vogt MC, Ruud J, et al. (2014) Signaling by IL-6 promotes alternative activation of macrophages to limit endotoxemia and obesity-associated resistance to insulin. *Nat Immunol* 15: 423-430.
 40. Marques-Vidal P, Bastardot F, von Kanel R, Paccaud F, Preisig M, et al. (2013). Association between circulating cytokine levels, diabetes and insulin resistance in a population-based sample (CoLaus study). *Clin. Endocrinol.* 78: 232-241.
 41. Pierson W, Liston A (2010) A new role for interleukin-10 in immune regulation. *Immunol Cell Biol* 88: 769-770.
 42. Erejuwa OO, Sulaiman SA, Wahab MS (2012) Fructose might contribute to the hypoglycemic effect of honey. *Molecules* 17: 1900-1915.