Food & Function

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DOI: 10.1039/C6FO01467D

Kodo millet whole grain and bran supplementation prevents high-fat diet induced derangements in lipid profile, inflammatory status and gut bacteria in mice

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DOI: 10.1039/C6FO01467D

Abstract

Protective role of kodo millet whole grain and bran supplementation in diet induced obesity has not been investigated. Here we have studied the role of kodo millet supplementation in age matched Swiss albino mice that were randomly divided into groups and fed their respective diets for 16 weeks. High fat diet increased weight gain, reduced glucose tolerance, increased serum lipids, altered hepatic and adipocyte gene expression and caused dysbiosis in the intestinal beneficial bacteria. Kodo millet supplementation did not affect weight gain but it improved glucose tolerance and prevented increase in serum cholesterol and lipid parameters ($P \leq 0.05$), modulated adipogenesis related gene expression, decreased serum IL-6 and LPS levels ($P \leq 0.05$), promoted selected beneficial gut bacterial abundances (*Lactobacillus* sp., Bifidobacteria, *Akkermansia* and *Roseburia* spp.) and improved total short chain fatty acid production ($P \leq 0.05$) and acetate levels ($P \leq 0.05$) in cecal contents. This study provides an evidence that kodo millet supplementation alleviates high-fat diet induced changes and hence can be incorporated as a functional ingredient for the management of obesity.

Keywords: Obesity, gut bacteria, lipopolysaccharide, inflammation, kodo millet, short-chain fatty acids

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List of Abbreviations

ACC, acetyl-CoA carboxylase;

AUC, area under curve;

C/EBPα, CCAAT/enhancer-binding protein alpha;

F4/80, epidermal growth factor-like module-containing mucin-like hormone receptor (EMR) 1;

FASN, fatty acid synthase;

GAPDH, glyceraldehyde-3-phosphate dehydrogenase;

HOMA-IR, homeostasis model assessment of insulin resistance

HSL, hormone-sensitive lipase;

KM, kodo millet;

LPS, lipopolysaccharide;

MCP-1, monocyte chemoattractant protein 1;

NEFA, non-esterified fatty acids;

NFκB, nuclear factor kappa light chain enhancer of activated B cells;

OGTT, oral glucose tolerance test;

PPARγ, peroxisome proliferator-activated receptor gamma;

SYBR, NN-dimethyl-N-[4-[(3-methyl-1,3-benzothialzol-2-ylidene)methyl]-1-phenylquinolin-1-ium-2-yl]-N-propylpropane-1,3-diamine;

TAG, triacylglycerol;

WAT, white adipose tissue;

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DOI: 10.1039/C6FO01467D

1. Introduction

Obesity is a metabolic disorder that has become a major health concern worldwide.[1] Gut microbial dysbiosis in obesity has been associated with increased energy harvest and elevated serum proinflammatory lipopolysaccharide (LPS) causing associated comorbidities.[2] Anti-obesity medications pose side effects.[3] Hence dietary approaches for the management of obesity together with enhanced physical activity is deemed important. Amongst dietary approaches, whole grain consumption has been suggested as a safe strategy to regulate the weight gain.[4] Ferulic acid present in cereals especially from wheat bran has been shown to ameliorate obesity in rats.[5] Millets are small cereals and their nutritive potentials are comparable to the major cereals, rice and wheat.[6] Interventions with proso millet, sorghum, oats, buckwheat and cereal grains have been explored for their protective effects in diet induced obesity.[7-9] Previously we have shown the beneficial effects of finger millet whole grain/bran as a nutraceutical ingredient in the prevention of diet-induced obesity.[10] Kodo millet (KM) (*Paspalum scrobiculatum*), comes under whole grain category, is grown in various parts of Asia and West Africa. In India KM is grown in Andhra Pradesh, Tamil Nadu, Orissa, Bihar, Madhya Pradesh, Maharashtra and Gujarat. It is extremely drought and salt tolerant and grains are very coarse with a horny seed coat. The grains are cooked and consumed similar to rice. KM has higher dietary fibre, polyphenols and mineral content than staple rice or wheat. Importantly, it lacks gluten and this makes it an alternate crop to wheat and can be explored for preparing healthy foods. KM ethanolic extract at 500 mg kg^{-1} body weight showed hypoglycemic and hyperinsulimic effects in alloxan induced diabetic rats.[11] Polyphenol rich extract of KM has been shown to exert antagonistic activity against *Staphylococcus aureus*, *Leuconostoc mesenteroides*, *Bacillus*

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cereus and *Enterococcus faecalis*.[12] KM whole grain supplementation has been shown to alleviate diabetic complications in rats through improvement in oxidative stress parameters and glucose tolerance.[13] However, the protective effect of KM whole grain (KM-WG) and bran (KM-BR) supplementation on rodent models of high-fat diet induced alterations and gut microbial derangements have not been explored and that forms the basis for the present study.

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2. Material and Methods

2.1 Preparation of Kodo millet whole grain flour and bran

Kodo millet, variety: JK-65 (KM) grain was surface cleaned with water, dried and ground to a fine flour to prepare KM whole grain (KM-WG). KM Bran (KM-BR) was collected from the whole grain flour by repeated washing of the starchy endosperm with water, dried completely and stored in airtight container at 4 ºC until further use. Moisture, protein and ash content of KM-WG and BR were determined using standard DGHS Lab Manual 03 protocols; dietary fibre was determined as per PBTI/SOP/27/TP-17 protocol and carbohydrate, fat and energy content was determined as per PBTI/SOP/18/TP-11 protocols. All these tests were got done by Punjab Biotechnology Incubator, Mohali, Punjab (India), accredited by National Accreditation Board for Testing and Calibration Laboratories, India.

2.2 Animal Diets

Experimental diets were prepared by mixing kodo millet whole grain (KM-WG) and bran (KM-BR) with normal diet (ND) or high fat diet (HFD) (TestDiets, No. 58125). Normal diet (TestDiet No. 58124) and ND supplemented with 20% (dry weight) with KM-WG or KM-BR. Normal diets derived 10% energy from fat and were isocaloric *viz.*, ND (3.79 Kcal g^{-1}), ND+20%WG (3.76 Kcal g^{-1}) and ND+20%BR (3.76 Kcal g^{-1}). HFD and HFD supplemented with 10% or 20% KM-WG or KM-BR derived ~45% energy from fat and were isocaloric *viz.*, HFD (4.66 Kcal g^{-1}), HFD+10%WG (4.65 Kcal g^{-1}), HFD+20%WG (4.63 Kcal g^{-1}), HFD-10%BR (4.64 Kcal g^{-1}) and HFD-20%BR (4.63 Kcal g^{-1}). Normal diets and HFD diets were not isocaloric. Detailed diet composition and their ingredients have been explained in **ESI 1.**

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2.3 Animal Study Design

Swiss albino mice (8 week old, weighing 22 ± 2 g) were housed in the Central Animal Facility (CAF) of National Institute of Pharmaceutical Education and Research (NIPER), SAS Nagar, Punjab, India. The experimental procedures were approved by Institutional Animal Ethical Committee (IAEC), National Institute of Pharmaceutical Education and Research (NIPER) and were conducted as per the guidelines of the Committee for the Purpose of Control and Supervision on Experiments on Animals (CPCSEA) and Indian National Science Academy (INSA) for the use and care of experimental animals.

Standard laboratory conditions (Temperature 22 ± 2 °C; humidity 55 ± 5%), with 12 h light-dark cycles were maintained all throughout the experiment. Access to food and water was provided *ad libitum.* Mice were acclimatized to standard experimental conditions for a week. Age matched mice were randomly divided into the following eight experimental groups (n=8) (i) Control (Ctrl) group fed with a normal diet (ND) (ii) High-fat diet fed group (HFD), (iii) HFD + 10% KM WG (HFD-10WG), (iv) HFD + 10% KM BR (HFD-10BR), (v) HFD + 20% KM WG (HFD-20WG), (vi) HFD + 20% KM BR (HFD-20BR), (vii) A *per se* group fed with ND + 20% WG (ND-20WG) and (viii) A *per se* group fed ND + 20% BR (ND-20BR). Mice were fed with corresponding diets for 16 weeks and body weights of mice were measured for every alternate week.

2.4 Oral glucose tolerance test (OGTT)

Mice were fasted (6 h) following which OGTT was performed on the 16th week, prior sacrifice as described elsewhere.[10] Briefly, fasting glucose levels were measured from the blood that was collected by tail snip method prior to glucose administration (T=0) using Arkray Glucocard 01 Sensor (Arkray Factory, Inc. Shiga, Japan). Mice

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were then gavaged with 2 g kg^{-1} glucose and blood glucose concentration was measured at 15, 30, 60 and 90 min. The area under the curve (AUC) was determined for each group.

2.5 Serum biochemical analysis

Mice were continued to feed on their respective diets after OGTT for another 24 h. Fresh blood collected was allowed to stand on ice and coagulate for 20 min and centrifuged at 1700 x g for 15 min to collect the serum. Using the basal blood glucose level (mM L^{-1}) and fasted serum insulin level, Homeostasis Model Assessment of insulin resistance index (HOMA-IR) was calculated. Other insulin sensitivity indices, quantitative insulin sensitivity check index (QUICKI) [QUICKI = 1/(log glucose in mg dL^{-1} + log insulin in μIU mL^{-1})], Reversed-QUICKI [Reversed-QUICKI = 1/(log glucose in mg dL^{-1}+ log insulin in μIU mL^{-1} + log NEFA in nM μL^{-1})] and McAuley's index in terms of fat-free mass (Mffm/I) for insulin resistance [Mffm/I = exp (2.63-0.28 ln(Insulin in μIU/mL) - 0.31 ln (TAGs mM/L)] using fasted blood glucose and serum insulin and serum TAG were calculated.[14] Triacylglycerols (TAG), total cholesterol (TC), HDL-cholesterol (HDL-c), LDL-c/VLDL-cholesterol (LDL-c/VLDL-c) (Accurex Biochemical Pvt. Ltd., Mumbai, India), non-esterified fatty acids (NEFA; Sigma-Aldrich, St. Louis, USA), serum cytokines (Raybiotech Inc., Norcross, GA, USA), serum LPS (CUSA Biotech, Hubei, China) were determined using commercially available kits as per manufacturer's instructions.

2.6 Tissue collections

At the end of the experiment (24 h after OGTT), mice were sacrificed by cervical dislocation. Subcutaneous white adipose tissue (sWAT), visceral white adipose tissue (vWAT), liver, pancreas, skeletal muscle and cecum contents were collected; snap frozen and stored at -80ºC till further analysis.

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2.6.1 Liver triacylglycerol analysis. The method described by Folch *et. al.* was used with slight modifications to extract lipids from liver homogenate.[15] Briefly, 100 -150 mg of liver tissue was excised and washed with 0.1M PBS (pH 7.4) to remove extraneous blood cells. Tissue was then freeze-dried and about 5-10 mg was weighed and crushed in a 2.0 mL centrifuge tube and 1.5 mL of chloroform-methanol 2:1 (v/v) was added. To this 400 µL of H_2O was added for clear separation of layers of the two solvents. The mixture was vortexed for 1 min and centrifuged at 3000 x g for 10 min at room temperature. Chloroform phase containing lipid fraction was collected in a fresh tube and completely dried under nitrogen gas. The dried fraction containing triacylglycerols (TAG) was quantified using commercially available kit as per the manufacturer's instructions.

2.6.2 Gene expression analysis in liver and vWAT. Total RNA was extracted from liver and vWAT using RNA ISO Plus (Takara Bio Inc., Otsu, Shiga, Japan) based on the method described elsewhere.[16] Briefly, the tissues were homogenized using RNA ISO Plus solution. Chloroform was added to the homogenate and mixed well. Contents were centrifuged and the supernatant containing RNA was pipetted out into a fresh tube. Isopropanol was added to extract the total RNA. Precipitated RNA was suspended in buffer and quantification of RNA was performed using Infinite M200 PRO NanoQuant (Tecan, Männedorf, Switzerland) and integrity was checked using 1.2 % agarose gel. Pure and intact total RNA samples (2 µg) were reverse-transcribed to complementary DNA using the RT^2 First Strand Synthesis Kit according to the manufacturer's instructions (RT^2 first strand cDNA synthesis kit, Qiagen, Venlow, Netherlands).

List of primers used in the present study is given in supplementary **ESI 2**. Expression of *ACC* and *HSL* in liver and *NFκB, MCP-1*, *F4/8, C/EBPα* and *PPARγ* in vWAT was

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determined by qRT-PCR (Applied Biosystems 7500 Fast Real-Time PCR, Massachusetts, USA) using SYBR green (RT^2 ROX qPCR, Qiagen, Venlow, Netherlands) under the following conditions: held at 95 ºC for 10 min, followed by forty cycles at 95 ºC and 60 ºC for 1 min each. Data were analyzed using the $\Delta\Delta C_T$ method; values are expressed as fold change (FC) relative to the Ctrl group. *β-actin* and *GAPDH* were used as reference genes. ΔC_T was calculated using *β-actin*.

2.6.3 Relative abundances of selected gut bacteria in cecal contents.

DNA was isolated from 100 mg of cecal content collected from each mouse using QIAamp DNA Stool Mini Kit. Briefly, cecal contents were incubated at 70 °C using lysis buffer followed by addition of InhibitEX matrix to remove PCR inhibitors. Contents were centrifuged and DNA in the supernatant was purified on QIAamp Mini spin columns and the purified DNA was eluted from the column using a low-salt buffer. DNA was quantified using Infinite M200 PRO NanoQuant (Tecan, Männedorf, Switzerland). SYBR green based real-time quantification of bacteria *was* performed using specific primers (**ESI 2**). Total bacterial DNA was normalized and results were expressed as relative fold change (FC) of bacterial DNA abundance compared with the ND fed mice.

2.6.4 Quantification of short chain fatty acids (SCFA) in cecal content.

Approximately, 50mg of cecum content was extracted with 500 µL of acidified water (pH 1-2) by rigorous vortexing. The samples were incubated at room temperature for 10 min followed by centrifugation at 3850 x g for 20 min at 4 ºC and filtered through 0.2 µm nylon membrane filter (Millipore Millex-GN).[11]

An Agilent HPLC system equipped with controller pump (model no. G1311C), auto sampler unit (model no. G1329B) and a diode array detector (DAD; model no. G1315D) set at 210 nm (peak width 2.5 Hz, band width 2 nm) was used for SCFA

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quantification. Separation of acetate, propionate and butyrate was achieved chromatographically at 60 ºC using an anion exchange column (Zorbax Hi-Plex H, 300 × 7.7 mm; 8 µm particle size, Agilent technologies, CA, USA) along with a guard column (Hi-Plex H cartridge, 3 X 5 mm internal diameter) made of monodisperse, sulfonated styrenedivinylbenzene copolymer. The mobile phase used was a mixture of 0.1% formic acid prepared in Milli-Q water (Merck Millipore, 0.22 µm filtered, resistivity 18.1-18.3 MΩ cm). The column was made to attain equilibrium and eluted with isocratic flow rate of 0.6 mL min^{-1} at 60 ºC for 30 min. Injection volume was 20 µL and three cecal extracts from each group was injected in duplicates. Data acquired was processed with EZchrom elite software. Peak areas of acetate, propionate and butyrate were calculated using various known concentrations and drawing a calibration curve of the same.[16] Adipic acid was used as internal standard, while analyzing cecal samples as well as SCFA standards. Data was represented as µM concentration of SCFAs per mg of cecal content.

Statistical analysis: Values are expressed as mean ± SEM. Intergroup variation was analyzed using One-way ANOVA followed by Tukey's *post hoc* test. Serum lipid parameters were analyzed using Two-way ANOVA followed by Bonferroni *post hoc* test using GraphPad Prism 5 software (GraphPad Software, Inc., CA, USA). $P \leq 0{\cdot}05$ was considered as significant in all tests.

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3.0 RESULTS

3.1 Proximate nutritional composition of Kodo Millet

KM whole grain flour and bran was rich in total carbohydrate content while bran had higher dietary fiber and ash content relative to whole grain. The total energy content of whole grain and bran per 100 g was similar **Table 1**.

3.2 Body weight gain and OGTT

HFD fed mice showed a higher body weight gain at the end of 16 weeks relative to ND fed mice **(Table 2)**. HFD-WG and HFD-BR supplementation at 10 and 20% doses did not prevent the body weight gain **(Table 2)**. Average food intake was same among all the experimental groups **(Table 2)**. Fasting blood glucose levels were similar in all the diet groups **(Table 2)**. However in OGTT, HFD fed mice showed higher AUC than ND fed mice. HFD-BR fed mice at 10 and 20% doses and HFD-20WG showed decreased AUC in OGTT relative to HFD fed mice **(figure 1a)**. Glucose clearance was remarkably quick at the 15 min time point for HFD-10WG and overall AUC was much reduced in HFD-10BR & HFD-20BR relative to HFD fed mice **(figure 1a and 1b)**. No difference in the vWAT weights was observed among the experimental groups (data not shown). Hepatic triglycerides (TAGs) was increased in HFD mice, while its level was decreased in HFD-WG and HFD-BR fed mice at both doses with better normalization of TAGs at higher (20%) dose **(figure 1c)**. TAG levels of ND-20WG and BR fed mice were similar to ND fed mice **(figure 1c)**.

3.3 Serum lipid parameters, insulin, resistin and leptin levels

HFD-fed mice had higher serum TAG, TC, HDL-c, LDL-c, VLDL-c and NEFA than ND-fed mice (**Table 2**). HFD-WG and HFD-BR at 10 and 20% doses decreased their levels relative to HFD fed mice. ND-WG and ND-BR fed mice showed reduction in

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LDL-c while showing an increase in HDL-c relative to ND fed mice whereas TAG, TC, VLDL-c and NEFA remained same as that of ND fed mice. Insulin sensitivity indices (HOMA IR, QUICKI, Reversed-QUICKI, and McAuley's) suggested insulin resistant state caused by HFD while HFD-10BR was seen most effective in preventing the condition (**Table 2**). There was a decreasing trend in serum insulin levels while leptin was not altered significantly among the experimental groups (**figure 2a & 2b**).

3.4 Systemic IL-6 and LPS levels

HFD-fed mice had increased levels of IL-6 to 117.8 pg ml^{-1} and LPS to 3.36 ng ml^{-1} relative to ND fed mice while HFD-WG supplementation at 10 and 20% doses reduced the IL-6 to 37.2 & 49 pg ml^{-1} and LPS levels to 0.95 & 1.05 ng ml^{-1}. HFD-BR at 10 and 20% doses reduced IL-6 to 35.5 & 28.2 pg ml^{-1} and LPS to 1.3 & 0.65 ng ml^{-1} relative to HFD alone fed mice. ND-WG and ND-BR feeding decreased the LPS levels to 0.39 & 0.3 ng ml^{-1} relative to ND alone fed mice (**figure 2c and 2d**). There was no change in TNFα levels among the experimental groups (data not shown).

3.5 Gene expression analysis in Liver and vWAT

Hepatic *ACC* gene expression was decreased upon HFD supplementation relative to ND fed mice (**figure 1d**). Supplementation with HFD-WG at 10 and 20% levels promoted its expression, whereas HFD-BR feeding did not show increase relative to HFD alone fed mice (**figure 1d**). No change in the expression levels of *HSL* between HFD, ND and HFD-WG 10% fed mice was observed. However, supplementation with HFD-20WG, HFD-10BR and HFD-20BR enhanced its expression significantly (**figure 1e**). ND-20BR feeding also increased the expression of *HSL* (**figure 1e**).
In vWAT, HFD feeding promoted the expression of adipocyte markers *C/EBPα* and *PPARγ* as well as inflammatory markers*, NFκB; MCP-1* and *F4/80* relative to ND fed

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mice (**figure 1f-1j**). Feeding HFD-10WG and HFD-10BR decreased the expression levels of the adiposity markers and *NFκB* relative to HFD alone fed mice (**figure 1f – 1h**) whereas feeding HFD-20WG and HFD-20BR decreased the expression of *MCP-1* and *F4/80* relative to HFD and ND fed mice (**figure 1i & 1j**). Feeding ND-20WG decreased the expression of *NFκB* whereas expression of *MCP-1* and *F4/80* genes was reduced with ND-20WG and ND-20BR feeding while showing significant change in adiposity markers.

3.6 Abundances of selected gut bacteria

HFD feeding decreased the abundances of Bifidobacteria and *A. muciniphila,* whereas no effect on *Lactobacillus sp.* was seen relative to ND fed mice (**figure 3a, 3b and 3d**). Feeding mice with HFD-WG and HFD-BR at 10 and 20% doses enhanced the cecal abundances of *Lactobacillus sp.* and *Roseburia spp.* whereas Bifidobacteria and Firmicutes was enhanced with HFD-20WG and HFD-20BR relative to HFD and ND alone fed mice. *A. muciniphila* abundance was enhanced with HFD-10WG and HFD-10BR whereas HFD-20WG and HFD-20BR feeding did not show any effect relative to HFD alone fed mice (**figure 3d**). Bacteroidetes did not show any significant difference among all the experimental groups (**figure 3f**). ND-20WG and ND-20BR feeding enhanced the abundances of *Lactobacillus sp.,* Bifidobacteria, *Roseburia spp.* and Firmicutes relative to ND alone fed mice, whereas *A. muciniphila* showed a significant decrease (**figure 3b, 3c and 3e**).

3.7 SCFA analysis

Total SCFA, acetate, propionate and butyrate levels in cecal content was reduced in HFD fed mice relative to ND fed mice (**figure 3g – 3j**). HFD-WG and HFD-BR feeding at 10 and 20% doses increased the total SCFA and acetate levels relative to HFD fed mice (**figure 3g and 3h**) although propionate and butyrate levels showed

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an increased trend relative to HFD fed mice (**figure 3I and 3J**). No difference in SCFA production was observed between ND, ND-20WG and ND-20BR fed mice (**figure 3g-j**).

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4. DISCUSSION

In the present study the potential of kodo millet whole grain and bran supplementation in counteracting HFD induced biochemical and gene level alterations in mice has been evaluated, as KM is rich in dietary fibres and bioactive phytochemicals. Although KM-WG and BR supplementation did not affect weight gain, which is in alignment with the report on other millets,[17] it prevented HFD-induced lipid derangements in mice. Triacylglycerols, total cholesterol, HDL-c, LDL-c and VLDL-c were decreased whereas NEFA levels remained same relative to HFD fed mice. It is to be noted that *per se* supplementation with KM-WG and KM-BR did not increase any lipid parameters as compared to the NPD fed mice. Also, there was no change in fasting blood glucose upon KM-WG and BR supplementation. But interestingly, KM-BR improved the glucose tolerance at both doses and at lower dose prevented the rise in insulin resistance indices suggesting benefits of KM supplementation. This is in alignment with our earlier study finger millet.[10] KM-WG and BR supplementation did not improve oxidative stress parameters in liver, vWAT, sWAT, muscle or pancreas (Data not shown). This is in contrast to earlier reports where KM whole grain and ethanolic extracts have been shown to improve the oxidative stress parameters *in vivo*.[11,13]

Further, KM supplementation prevented alterations in gene expression caused by HFD in liver and vWAT. In liver, the expression of *HSL* was enhanced with higher dose of KM-WG and at both doses of KM-BR causing hydrolysis of triacylglycerols. As a result lower accumulation of triglycerols in liver was observed in mice fed with high dose KM-WG and both doses of KM-BR relative to HFD alone fed mice. Expression of *FASN* and *ACOX* was enhanced (non-significantly) upon KM supplementation relative to HFD fed mice. This is due to feedback inhibition of *FASN*

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as HFD is a rich source of lipids.[18] Some recent reports suggest that zinc-α2-glycoprotein involvement in body fat loss, which leads to decreased *FASN* expression while increasing *HSL* expression.[19]

In vWAT, *PPARγ* (an adipogenic gene) and *ACOX1* (a fatty acid oxidation gene) were down regulated with lower dose of KM-WG and BR supplementation. No change in their expression was observed with higher dose of KM-WG and BR relative to HFD alone fed mice. PPARs act as sensing proteins for fatty acids and their derivatives and thus play an important role in energy regulation pathway.[20] Reduction in expression of *PPARγ* and *ACOX1* in the mice with lower dose of KM-WG and BR supplementation and *C/EBPα* at both doses is suggestive of decreased adipogenesis in vWAT.[20,21] Higher dose of KM-WG supplementation enhanced the expression of *PGC1α,* an upstream transcription factor to various adipogenesis and mitochondrial biogenesis related genes such as *PPARγ* and *UCP-1*.[22,23] Decreased expression of lipid synthesis and increased hepatic lipase genes, along with decreased liver and serum lipids suggested that bioactives from millets especially dietary fibers and polyphenols might have prevented the storage of TAGs and cholesterol.[24-26]

It is well established that chronic low grade inflammation prevails in the adipose tissue due to high circulatory levels of LPS originating from the gut leading to obesity associated comorbidities.[27] Stromal vascular fraction in adipose tissue and adipocytes *per se* produce proinflammatory cytokines in chronic obesity due to M1 macrophage infiltration (and higher expression of *MCP-1*, a marker for macrophage infiltration).[28] In the present study, low dose of KM-WG and BR supplementation reduced the expression of *NFκB*, an upstream transcription factor for inflammatory markers such as *TNFα* and *iNOS.* In vWAT, the expression of *MCP-1* and *F4/80* was

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decreased with both doses of KM-WG and BR supplementation suggesting reversal from proinflammatory stress as a result of HFD feeding. IL-6 and LPS levels were low in the mice fed with KM-WG and BR at both the doses relative to HFD fed mice suggesting physiological improvement through enhanced gut barrier function as a result of beneficial modulation of gut bacteria.[25,29,30] Higher IL-6 level has been reported in obese subjects relative to their lean counterparts.[31] Role of IL-6 in obesity is ambiguous as some studies suggests elevated IL-6 imparts anti-inflammatory activity while others reported that low IL-6 level is beneficial in counteracting obesity.[32] Overall this study shows that kodo millet supplementation could counteract HFD induced proinflammatory stress.

HFD feeding caused reduction in beneficial gut bacteria such as Bifidobacteria and *A. muciniphila* as reported by others.[10] As a result LPS might have translocated from intestine due to dampened intestinal barrier function. This is the root cause for chronic low grade inflammation in adipose tissue leading to metabolic endotoxaemia, insulin resistance and type 2 diabetes.[33] KM-WG and BR supplementation increased the abundance of *Lactobacillus sp.*, Bifidobacteria, *Roseburia* spp. and *A. muciniphila* ("prebiotic effect"). Increased *A. muciniphila* abundance might have helped in the mucus formation, which otherwise has been depleted in obese mice.[34] Enhanced bifidobacteria, *A. muciniphila* and *Roseburia* might have helped in restoring the intestinal barrier function. This was augmented by our observation that low systemic LPS levels in mice supplemented with KM. Promoting beneficial bacterial abundances, especially *A. muciniphila,* rather than exogenous administration by dietary regimen such as kodo millet supplementation is not only economical but safe as reported by other researchers.[35,36]

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Enhanced beneficial gut bacteria due to KM supplementation resulted in improved SCFA profile with high acetate levels in the cecal contents. Recent studies suggest that acetate influences central nervous system and can reduce appetite.[37] We speculate a mechanism involving fermentable carbohydrates along with polyphenols in KM, which not only binds to excess lipids in HFD but promotes beneficial gut bacteria that restore host health through LPS, IL-6 and short chain fatty acid mechanism. Further, comprehensive studies on metagenomics, gene expression and consequences of high acetate levels upon KM supplementation in the gut might give more insights on the protective efficacy of KM feeding.

CONCLUSIONS:

Our results suggest that kodo millet whole grain and bran supplementation shifted the physiology of mice from obese to non-obese conditions. Although KM did not help in weight reduction but it improved serum cholesterol, lipid parameters exerted prebiotic effect on selected gut bacteria and improved SCFA production especially acetate and prevented the increase in IL6 and LPS levels in serum suggesting improved gut barrier function and pro-inflammatory state. The consequence of KM supplementation in over weight and obese humans needs to be evaluated.

Acknowledgments This research was supported by grants from Department of Biotechnology (grant no. BT/PR6273/FNS/20/622/2012) and Department of Science and Technology (grant no. SB/FT/LS-224/2012), Government of India. Authors would like to thank DBT-eLibrary Consortium (DeICON) for providing access to online Journals.

Author's contribution: KKK, MB and KKB designed the experiments. PK, SMS, SJ conducted the animal studies; RK Boparai, SMS, PK, RK Baboota and SJ contributed to animal dissections; SMS, PK, DPS, RK Baboota performed the

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experiments. Podili K helped in oxidative stress experiments. SMS, KKK and MB analysed the data and wrote the manuscript. JK, Podili. K and RK Boparai contributed in editing and improving the manuscript. All authors read and approved the manuscript.

Conflict of interest

The authors declare no conflicts of interest.

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Table Legends

Table 1: Proximate nutritional composition of Kodo Millet

Table 2: Effect of kodo millet whole grain and bran supplementation on body weight gain, average food intake, fasting glucose levels, insulin resistance indices and serum lipid parameters.

Foot note: * Represents significance vs ND fed group, † represents significance vs HFD fed group. One-Way ANOVA with Tukey's *post hoc* test was used for body weight, indexes and associated tests ($P \leq 0.05$). Two-Way ANOVA with Bonferroni *post hoc* test was used for serum lipid parameters ($P \leq 0.05$).

Figure Legends

figure 1 Effect of kodo millet whole grain and bran supplementation on (a) oral glucose tolerance (n=5), (b) area under the curve in OGTT (n=5), (c) liver triglycerides (n=5); Hepatic gene expression of (d) *ACC*, (e) *HSL*. Visceral white adipose tissue expression of adiposity genes (n=3) (f) *C/EBPα* (g) *PPARγ* and inflammatory genes (h) *NFκB,* (i) *MCP-1* (j) *F4/80*. Serum protein levels of. C_T values were normalized against β-actin housekeeping gene. Expression is expressed as positive fold change with respect to control group (n=3). Values are expressed as means ± SEM and in representation of gene expression as fold changes ± SEM equivalent of ΔC_T value. ***** Represents significance vs ND, † represents significance vs HFD, ‡ represents significance vs KM-10WG, **#** represents significance vs KM-10BR. One-way ANOVA statistical test with Tukey's *post hoc* analysis was used ($P \leq 0.05$).

figure 2 Effect of kodo millet whole grain and bran on serum protein levels of (a) insulin, (b) leptin (c) interleukin 6 (d) lipopolysaccharides (n=5). Values expressed as means ± SEM. ***** Represents significance vs ND, † represents significance vs HFD. One-way-ANOVA statistical test with Tukey's *post hoc* analysis was used ($P \leq 0.05$).

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figure 3 Effect of kodo millet whole grain and bran supplementation on relative bacterial abundances of (a) *Lactobacillus* sp. (b) Bifidobacteria, (c) *Roseburia* spp., (d) *Akkermansia muciniphila* (e) Firmicutes (f) Bacteroidetes, (g) total short chain fatty acids (h) acetate (i) propionate (j) butyrate. C_T values were normalized against genus specific total bacteria sequence primer. Expression is expressed as positive fold change with respect to control group. For relative abundances of selected bacteria the data expressed as mean fold changes ± SEM equivalent of ΔC_T values (n=5). For SCFA analysis, values expressed as mean concentration/mg cecal content ± SEM (n=4). ***** represents significance vs ND, † represents significance vs HFD, ‡ represents significance vs KM-10WG (low dose WG), # represents significance vs KM-10BR (low dose BR). One-way ANOVA statistical test with Tukey's *post hoc* analysis was used ($P \leq 0.05$).

figure 4 Schematic diagram depicting overall effects of kodo millet whole grain and bran supplementation in HFD fed mice. KM supplementation improved glucose tolerance while reducing serum lipids, systemic LPS and IL-6 levels. Hepatic TAGs were reduced in the liver along with reduced expression of lipid metabolism genes. Inflammation related genes in visceral WAT were down regulated upon KM-WG/BR supplementation; while at 10% KM WG/BR supplementation down regulated adipogenesis genes. Total SCFAs were increased upon KM-WG/BR supplementation with increase in acetate levels in the cecum with increased *Lactobacillus* and *Roseburia* spp. abundances while at 20% dose of KM WG/BR Bifidobacteria abundance was increased whereas 10% KM-BR supplementation increased the abundance of *Akkermansia muciniphila*.

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Table 1

Component	KM Whole Grain (%)	KM Bran (%)
Protein	5.35	4.92
Carbohydrate	80.74	79.84
Fat	2.53	2.83
Total dietary fiber	26.40	48.42
Ash	2.97	5.33
Moisture	8.41	7.07
Energy	367.1 Kcal $100g^{-1}$	364.5 Kcal $100g^{-1}$

Table 2: Effect of kodo millet whole grain and bran supplementation on body weight gain, average food intake, fasting glucose levels, insulin resistance indices and serum lipid parameters.

	Dietary groups							
	ND	**HFD**	**HFD-10WG**	**HFD-20WG**	**HFD-10BR**	**HFD-20BR**	**ND-WG**	**ND-BR**
Weight gain (g)	10.6 ± 0.5	16.8 ± 0.3[a]	13.3 ± 0.7	13.5 ± 0.6	13.2 ± 1	11.6 ± 0.8	10.2 ± 2.3	11 ± 1.6
Average food intake (g day^{-1} per animal)	3.8 ± 0.4	4.1 ± 0.3	3.8 ± 0.4	3.9 ± 0.2	3.6 ± 0.3	3.8 ± 0.1	4.1 ± 0.3	3.9 ± 0.1
Fasting glucose (mg dL^{-1})	119.4 ± 18.9	151.1 ± 10.3	127.4 ± 12.2	120.4 ± 15.9	117.6 ± 13	123.8 ± 11.2	120 ± 10.3	121 ± 15
HOMA-IR	1.0 ± 0.3	9.8 ± 0.8[a]	5.4 ± 0.7[b]	5 ± 0.6[b]	3.6 ± 1.4[b]	4.1 ± 0.5[b]	2.9 ± 0.9[a]	4.8 ± 1.6[a]
QUICKI Index	0.36 ± 0.01	0.27 ± 0.003[a]	0.30 ± 0.006	0.30 ± 0.004	0.33 ± 0.02[b]	0.31 ± 0.006	0.33 ± 0.01	0.31 ± 0.01
Reversed-Quickie	0.24 ± 0.008	0.17 ± 0.002[a]	0.18 ± 0.004	0.19 ± 0.003	0.20 ± 0.008	0.19 ± 0.001	0.19 ± 0.001	0.21 ± 0.006†
McAulay Index	2.11 ± 0.08	1.18 ± 0.03[a]	1.4 ± 0.06	1.49 ± 0.08	1.93 ± 0.24[b]	1.66 ± 0.05	1.87 ± 0.23	1.68 ± 0.11
TAG (mg dL^{-1})	97.4 ± 3.7	154 ± 8.5[a]	129.8 ± 6.7[b]	104.9 ± 5.01[b]	84.9 ± 3[b]	88.3 ± 2.9[b]	101.9 ± 4.3	86.6 ± 3.6[a]
Total cholesterol (mg dL^{-1})	117.4 ± 15.3	219.2 ± 17.8[a]	171.4 ± 3.6[b]	138.4 ± 6.2[b]	146.7 ± 10[b]	128.7 ± 3.2[b]	121.6 ± 6.5	113.4 ± 3.9[a]
HDL-c (mg dL^{-1})	56.3 ± 13.4	140.8 ± 11.7[a]	119.8 ± 2[b]	89.6 ± 5.3[b]	101.3 ± 11.1[b]	87.2 ± 2.1[b]	82.4 ± 4.3[a]	82.7 ± 5.3
LDL-c (mg dL^{-1})	27 ± 4.4	67.9 ± 13.6[a]	26.3 ± 5.5[b]	17.6 ± 1.9[b]	18.9 ± 2.6[b]	16.9 ± 3.5[b]	11.9 ± 1.6[a]	9.9 ± 3.7[a]
VLDL-c (mg dL^{-1})	23.5 ± 3	43.8 ± 3.6[a]	34.3 ± 0.7[b]	27.7 ± 1.2[b]	29.3 ± 2[b]	25.8 ± 0.6[b]	22.67 ± 0.8	24.3 ± 1.3
NEFA (nM μL^{-1})	26.8 ± 3.9	123.1 ± 20.9[a]	119.3 ± 18	89 ± 11.5[b]	87.8 ± 6[b]	75.2 ± 7.7[b]	29 ± 0.8	27.3 ± 2.6

a Represents significance vs ND fed group, b represents significance vs HFD fed group. One-Way ANOVA with Tukey's post hoc test was used for body weight, indexes and associated tests ($P \leq 0.05$). Two-Way ANOVA with Bonferroni post hoc test was used for serum lipid parameters ($P \leq 0.05$).

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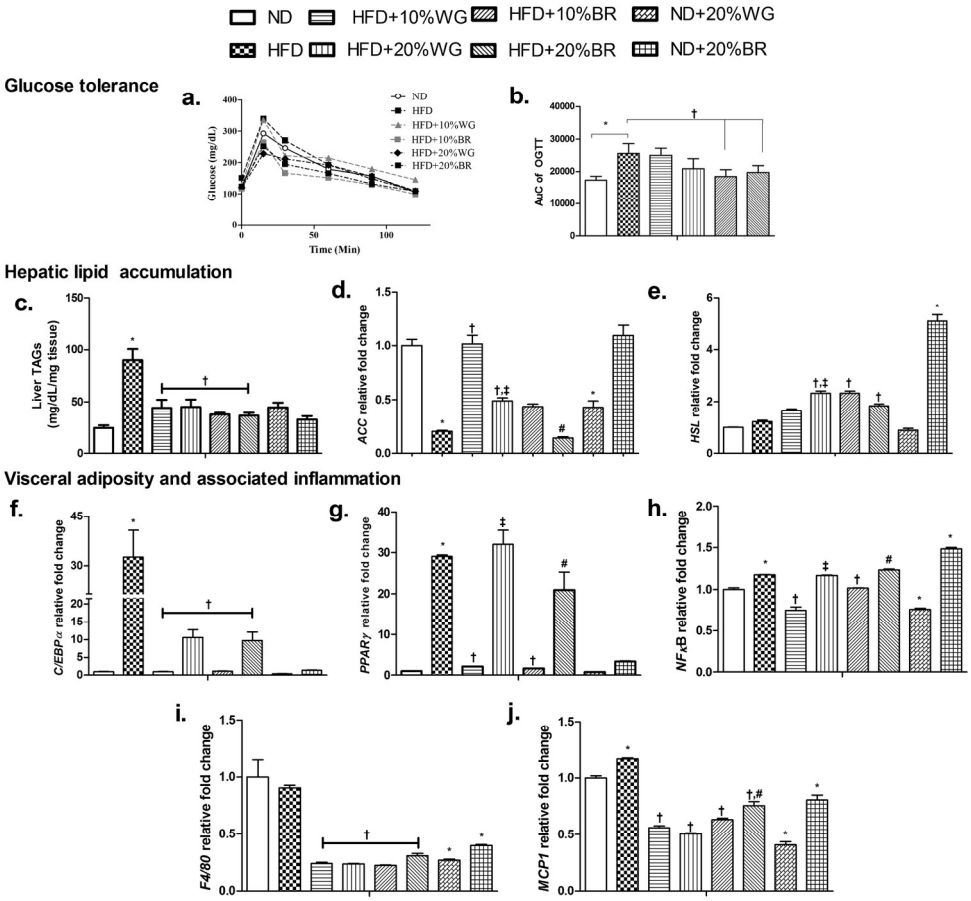

Effect of kodo millet whole grain and bran supplementation on (a) oral glucose tolerance (n=5), (b) area under the curve in OGTT (n=5), (c) liver triglycerides (n=5); Hepatic gene expression of (d) ACC, (e) HSL. Visceral white adipose tissue expression of adiposity genes (n=3) (f) C/EBPα (g) PPARγ and inflammatory genes (h) NFκB, (i) MCP-1 (j) F4/80. Serum protein levels of. CT values were normalized against β-actin housekeeping gene. Expression is expressed as positive fold change with respect to control group (n=3). Values are expressed as means ± SEM and in representation of gene expression as fold changes ± SEM equivalent of ΔCT value. * Represents significance vs ND, † represents significance vs HFD, ‡ represents significance vs KM-10WG, # represents significance vs KM-10BR. One-way ANOVA statistical test with Tukey's post hoc analysis was used ($P \leq 0.05$).

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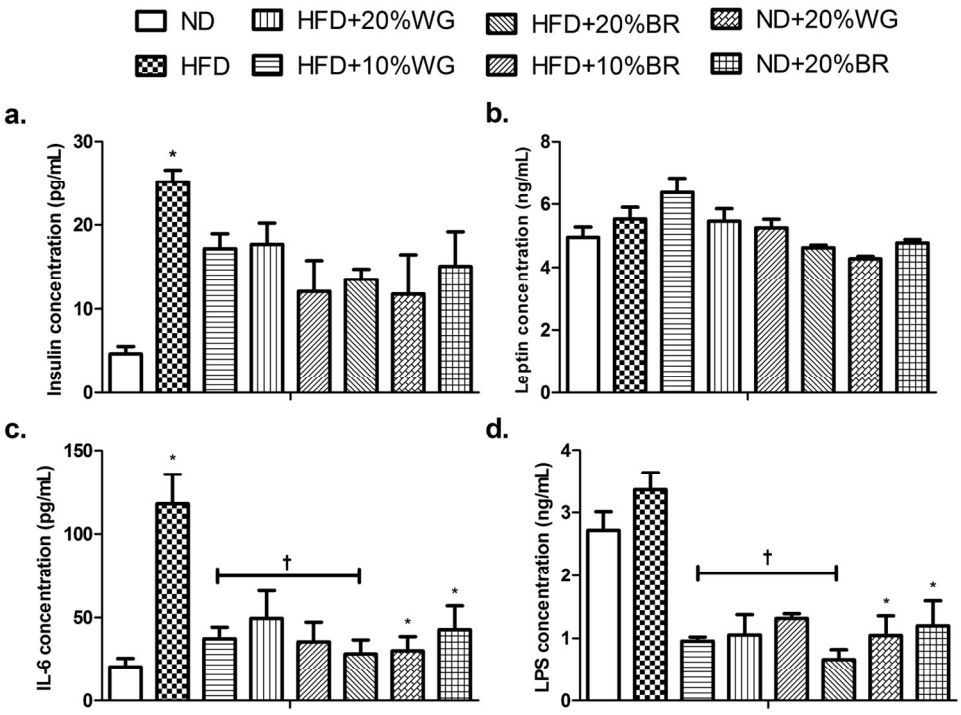

Effect of kodo millet whole grain and bran on serum protein levels of (a) insulin, (b) leptin (c) interleukin 6 (d) lipopolysaccharides (n=5). Values expressed as means ± SEM. * Represents significance vs ND, † represents significance vs HFD. One-way ANOVA statistical test with Tukey's post hoc analysis was used ($P \leq 0.05$).

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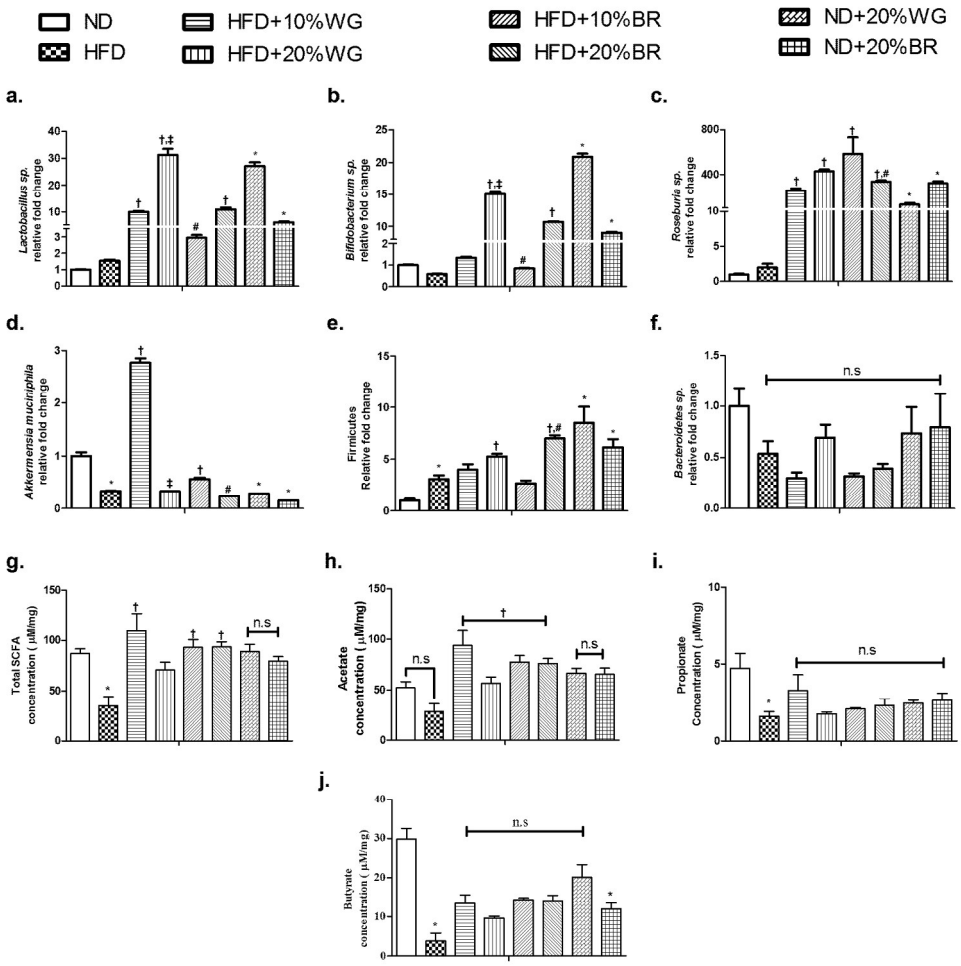

Effect of kodo millet whole grain and bran supplementation on relative bacterial abundances of (a) Lactobacillus sp. (b) Bifidobacteria, (c) Roseburia spp., (d) Akkermansia muciniphila (e) Firmicutes (f) Bacteroidetes, (g) total short chain fatty acids (h) acetate (i) propionate (j) butyrate. CT values were normalized against genus specific total bacteria sequence primer. Expression is expressed as positive fold change with respect to control group. For relative abundances of selected bacteria the data expressed as mean fold changes ± SEM equivalent of ΔCT values (n=5). For SCFA analysis, values expressed as mean concentration/mg cecal content ± SEM (n=4). * represents significance vs ND, † represents significance vs HFD, ‡ represents significance vs KM-10WG (low dose WG), # represents significance vs KM-10BR (low dose BR). One-way ANOVA statistical test with Tukey's post hoc analysis was used ($P \leq 0.05$).

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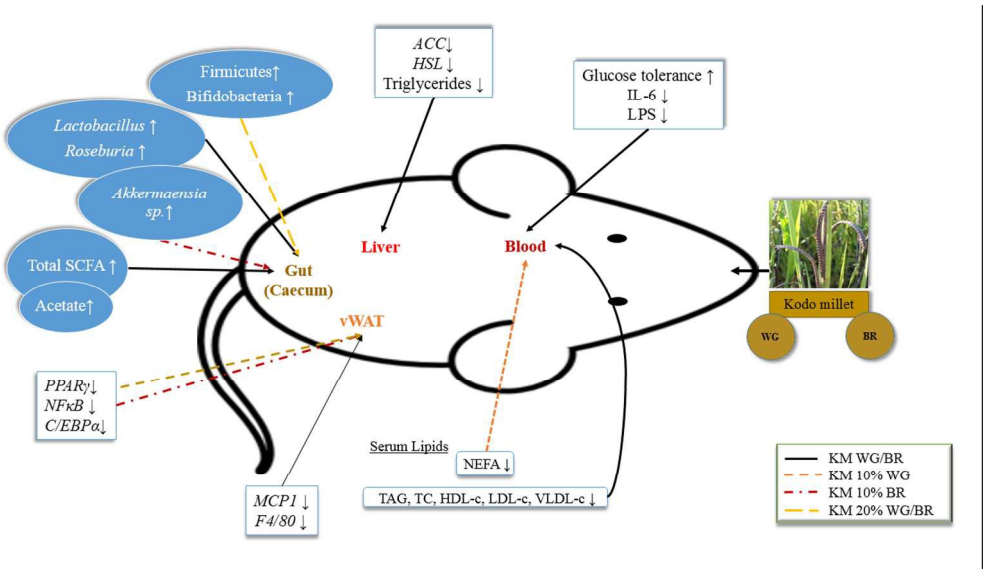

Schematic diagram depicting overall effects of kodo millet whole grain and bran supplementation in HFD fed mice. KM supplementation improved glucose tolerance while reducing serum lipids, systemic LPS and IL-6 levels. Hepatic TAGs were reduced in the liver along with reduced expression of lipid metabolism genes. Inflammation related genes in visceral WAT were down regulated upon KM-WG/BR supplementation; while at 10% KM WG/BR supplementation down regulated adipogenesis genes. Total SCFAs were increased upon KM-WG/BR supplementation with increase in acetate levels in the cecum with increased Lactobacillus and Roseburia spp. abundances while at 20% dose of KM WG/BR Bifidobacteria abundance was increased whereas 10% KM-BR supplementation increased the abundance of Akkermansia muciniphila.

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