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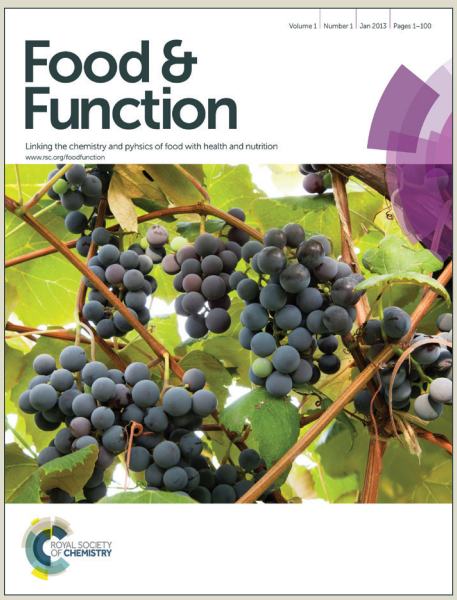

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Title: Influence of milk fermented by *Lactobacillus rhamnosus* NCDC 17 alone and in combination with herbal ingredients on diet induced adiposity and related gene expression in C57BL/6J mice

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Abstract

Obesity has become a major health problem in developed countries and is rapidly catching up in the developing world due to change in the life style. Dietary incorporation of functional foods, including probiotic fermented milk and herbal ingredients is being tried to ameliorate the metabolic disorders. In the present study, the effects of dietary supplementation of a probiotic (*Lactobacillus rhamnosus* NCDC 17) fermented milk alone or either of herbal preparations (*Aloe vera*/*Gymnema sylvestre* powders, 1% w/w) on progression of obesity has been studied in high fat diet fed C57BL/6J mice for 12 weeks. At the end of experimental period, oral administration of *L. rhamnosus* and herbs showed a significant decrease in the body weight, epididymal fat mass, fasting blood glucose and serum insulin levels. Supplementation of probiotic *L. rhamnosus* NCDC 17 alone and in combination with herbs showed a significant decrease in the adipocyte cell size and increase in the number. Finally, obesity related adipokines levels were maintained to normal by the treatment groups. Thus, dietary intervention of milk fermented with probiotic *L. rhamnosus* alone or in combination with any of the herbal preparations seems to exhibit anti-obesity and anti-inflammatory properties.

Introduction

Obesity is associated with multi-factorial disorders, in which excessive fat is accumulated in the adipose tissue results in increased energy consumption and decreased energy expenditure.[1] The accumulation of ample fat inside the body leads to many pathological diseases such as insulin resistance, type 2 diabetes mellitus (T2DM), ischaemic heart disease, retinopathy, neuropathy and cancer, which can lead to failure of several organs and termed as metabolic syndrome or syndrome X.[2] Current pharmacological treatments are not successful in cutting down the weight gain and its related disorders.[3] Therefore, alternative therapies are emerging the aforesaid diseases.

The gut microbiota are supposed to play an important role towards obesity, however, this is still controversial. More than 10^{12} microorganisms are inhabited in the gastrointestinal (GI) tract.[4] The major phyla includes *firmicutes* and *bacteroides*[5] and alteration of above phyla occurs in the GI tract of obese and lean subjects. Probiotics are defined as live microorganisms when administered in an adequate amounts confer a health benefit on the host.[6] Now-a-days, researchers have keen interest in using probiotic organisms like lactobacilli and bifidobacteria species for their anti-obesity effects.[7] Several probiotics showed different effects on gut microbiota and obesity.[8] Intra-gastric gavage administration of *Lactobacillus rhamnosus* PL 60 fed to high fat diet mice showed a decrease in the body weight gain and epididymal fat mass.[9] In another study, *Lactobacillus rhamnosus* GG showed a significant reduction of epididymal fat mass and obesity related biomarkers in the liver (*viz.*, acetyl-CoA carboxylase, fatty acid synthase and stearoyl CoA desaturase-I) of obese mice.[10]

Now-a-days, plant extracts are having great attention as a therapeutic weapons for the treatment of obesity and its associated diseases. Both *Aloe vera* (AV) and *Gymnema sylvestre* (GS) extracts have been widely used for their anti-obesity and anti-diabetic effects since

ancient periods.[11,12] Shin and co-workers[13] reported that dietary aloe formulas showed reduction in the anti-inflammatory markers in white adipose tissue of obese mice. Administration of the dried AV gel powder showed decrease of body weight, visceral fat weight, body fat and serum lipids in obese rats.[14] In another report, *Aloe vera* phytosterols administration displayed decreased in the random and fasting blood glucose levels and reduced visceral fat weights in Zucker Diabetic Fatty (ZDF) rats.[15,16] Similarly, GS supplementation also showed decrease body mass index, visceral fat pad weight, serum lipids in high fat diet (HFD) fed rodents.[17-19] The active components (gymnemic acid IV fraction and dihydroxy gymnemic triacetate) isolated from *Gymnema sylvestre* displayed hypoglycemic and hypolipidemic effects in diabetic rats.[20,21]

The bioactive components (acemannan, aloin & gymnemic acids) present in the AV and GS may be responsible for the growth and metabolic activity of lactobacilli strains during fermentation. Further, it can excite to examine the effects of administration of herbal ingredients together with the probiotics. There is a paucity of information on anti-obesity of indigenous strains of lactobacilli. Moreover, even contradictory findings have also been available in the literature regarding probiotic lactobacilli species. In our previous study, *Lactobacillus rhamnosus* NCDC 17 (LR17) strain was selected from four different lactobacilli strains and exhibited good probiotic attributes *viz*., such as acid tolerance, bile tolerance and surface hydrophobicity under *in vitro* conditions.[22] In addition, LR17 exhibited angiotensin-converting enzyme inhibitory and extent of proteolysis activities which are linked with anti-hypertensive effect.[23] Accordingly, the present investigation was carried out to study anti-obesity effects of milk fermented by LR17 alone or in combination with herbal extracts (AV/GS) in HFD fed C57BL/6J mice.

Experimental

This section describes the experimental details of the study conducted using obesity prone C57BL/6J mice. The effects of probiotic (LR17) and two types of herbal preparations *viz.*, AV and GS were investigated in mice fed HFD for 12 weeks duration.

Probiotic culture and preparation of fermented milk

Probiotic culture (LR17) was obtained from the National Collection of Dairy Cultures (NCDC, Dairy Microbiology Division) of the institute. Skim milk (SM) was obtained from experimental dairy of the institute, heated to 90 °C for 30 min and cooled to room temperature without exposing to air. The activated culture of LR17 was inoculated at 1% (v/v) level and incubated at 37 °C for 18 h. After that, the prepared fermented milk was stored at refrigeration temperature (4 °C) and fed to the respective groups. The viable counts were determined on MRS agar. An appropriate quantity of fermented milk was diluted with 0.85 % saline and mixed uniformly with a vortex mixer. Serial dilutions were prepared and proper dilutions were plated on MRS agar by pour plating method and colony counting done in a routine manner. Milk fermented with LR17 contained viable counts was in the range of 8.0 to 8.5 log cfu mL^{-1}.

Herbal ingredients

Aloe vera gel powder (200x) containing 8.8% aloin was procured from M/s Indichem, Mumbai while *Gymnema sylvestre* powder containing 75% gymnemic acids was a product of M/s Ambe Phytoextracts Pvt. Ltd, New Delhi. The herbal powder (AV/GS) was added to HFD at 1% w/w level.

Animals and treatment

Thirty male C57BL/6J mice (25-28 g) were obtained from National Institute of Nutrition, Hyderabad (India). The animals were acclimatized on normal chow for 1 week under 12 h light/dark conditions at 22 ± 1 °C and 50 ± 10 % relative humidity and were fed *ad libitum* on

respective diets. The experiment was carried out in accordance with the guidelines of the institutional animal ethics committee.

After an acclimatization period, the mice were randomly divided into six groups (n=6/group, 3 mice/cage) and fed with control + SM, HFD + SM (60 kcal% fat), HFD + LR17, HFD + AV + LR17 and HFD + GS + LR17 for 12 weeks. These were designated as CTRL, HFD, HFD-LR, HFD-AVLR and HFD-GSLR, respectively. The HFD or HFD containing the herbal ingredient was presented to animals during the dark phase (5:00 pm-9:30 am), while skim or fermented milk was fed in light phase (9:30 am-5:00 pm). The solid diet was removed from the cages during light phase to ensure that the animals could consume their respective liquid diets. The compositions of diets are given in Table 1. Body weights were taken at weekly intervals. At the final stage of the experimental period, mice were sacrificed by cervical dislocation under anaesthesia using diethyl ether and blood was collected by cardiac puncture to determine serum insulin and lipid profile. Liver, spleen, kidney and epididymal fat (E.fat) were collected and weighted. A portion of epididymal adipose tissue was stored in buffered formalin (10%) for determination of adipocyte size and number. The rest of the portion of E.fat was stored in RNAlater at -20 °C for gene expression study.

Analysis of fasting blood glucose level

The fasting blood glucose levels were determined at 0, 6 and 12 weeks by using a glucometer (Accu-Chek® active, Roche Diagnostics, Germany) and comparisons were made between the different groups.

Oral glucose tolerance test (OGTT)

Before the end of experiment, mice from different treatment groups were fasted for 12 h and then administered glucose solution by intragastric gavage (1.0 g kg^{-1} b.wt, 20% glucose solution). Blood samples were drawn by puncturing the tail vein with needle gun and glucose

levels were measured at 0, 30, 60 and 120 min after glucose administration, using a glucometer.

Determination of serum and liver lipids

Serum total cholesterol (TC), triglycerides (TG) and HDL-cholesterol levels were estimated enzymatically using the kit according to manufacturer's instructions (Span Diagnostics Ltd, Surat, India). A Friedewald's equation was used for the calculation of VLDL and LDL-cholesterol levels. To determine liver lipids, tissue (100 mg) was homogenized in one mL of isopropanol and shaken for 45 min. The samples were centrifuged at 3000 *g* for 10 min to collect the supernatant and TG & TC levels were analysed using commercial enzymatic kits.[16]

Measurement of insulin and HOMA index

Serum insulin levels were measured by sandwich ELISA (Crystal chem. Inc, USA). Insulin resistance was assessed by homeostasis model assessment (HOMA-IR). It was calculated by multiplying fasting serum insulin ($\mu U\ L^{-1}$) with fasting blood glucose ($mM\ L^{-1}$) divided by 22.5.[24]

Histological analysis

After sacrificing mice, epididymal fat tissue was taken from three animals in each group (3 replicates/sample). These were fixed at 10 % (v/v) neutral-buffered formalin and embedded in paraffin, sectioned (4 μm thickness) and mounted on glass slides. Hematoxylin-eosin stained slides were examined (3 fields/spot) under magnification x 200 (Nikon, Eclipse Ti-S, Japan). Images were taken and cell size & number were determined using Image J software (National Institutes of Health, Bethesda, MD, USA).

Analysis of mRNA expression of genes in epididymal fat

Total RNA was isolated from epididymal fat by using TRIzol (Sigmal-Aldrich, USA). The purity was determined by spectrophotometerically on the basis of A_{260}/A_{280} ratio, while integrity was checked by agarose gel electrophoresis. The cDNA template was synthesised by reverse transcription of 500 ng of total RNA using first strand cDNA synthesis kit (Thermo scientific, USA). SYBR Green was used for real-time PCR detection. The primers (adiponectin, leptin, resistin, PPARγ, UCP2, TNF-α and IL-6) used for qRT-PCR are listed in Table 2. β-actin was used as a reference gene. All samples were analysed in duplicate in a 96-well reaction plate and the data was analysed according to the $2^{-\Delta\Delta CT}$ method.

Statistical analysis

All data were expressed as mean ± SEM. Statistical analysis was done with one or two way ANOVA using Tukey's tests (GraphPad Software, version 5.01) for body and organ weights, fasting blood glucose, serum & liver lipids, insulin, OGTT, HOMA-IR and gene expression.

Results

Body weight and organ weights

As shown in Table 3, high fat diet fed mice showed an increase in the body weight gain over 12 weeks experimental period (27.29 ± 0.71g, mean ± SEM). Administration of fermented milk containing LR (25.18 ± 0.55g, $p<0.05$) alone and in combination with herbs showed lowering of the body weight. However, a significant difference was observed in the presence of HFD-GSLR (25.20 ± 0.41g, $p<0.05$) fed group.

High caloric intake results accumulation of energy in the form of body fat, i.e. epididymal fat, was significantly higher in HFD fed (1.10 ± 0.09 g) mice. The weight of epididymal fat pad was significantly ($p<0.05$) decreased in all treatments and the values were found to be 0.55 ± 0.11, 0.68 ± 0.07 and 0.64 ± 0.09 g for HFD-LR, HFD-AVLR and HFD-

GSLR fed groups, respectively. No significant difference was observed in the weight of liver and kidney among the different dietary interventions.

Fasting blood glucose level

In Fig 2, fasting blood glucose levels were determined at 0, 6 and 12 weeks, respectively. High fat diet fed obese mice showed a significant increase in the glucose levels at 12 weeks (152.20 ± 6.96 mg dL^{-1}, $p<0.05$). Oral administration of probiotic LR (100.20 ± 7.58 mg dL^{-1}, $p<0.05$) and a combination of herbs showed decreases in the circulatory glucose levels whereas, GS-LR fed group (118.20 ± 7.61 mg dL^{-1}) alone made a significant contribution.

Glucose tolerance test

In OGTT experiment, HFD fed mice exhibited a significant increase in the blood glucose levels upon glucose loading compared to control group. As shown in (Fig. 1), supplementation with LR alone or in combination with GSLR in HFD fed mice showed a remarkable lowering of glucose levels during 30 and 60 min while, similar results were also observed in the case of area under the curve (AUC).

Serum and liver lipids

The serum and liver lipids were analysed for all treatment groups (Table 3). Serum TG levels (131.20 ± 6.87 mg dL^{-1}) were found to be significantly higher in HFD group. Supplementation of probiotic LR (90.95 ± 7.73 mg dL^{-1}) alone and in combination with herbal extracts AV/GS (87.64 ± 6.85/84.45 ± 8.69 mg dL^{-1}) displays a significantly decreased in the TG levels. No effect was observed with the remaining serum lipids. In other hand, a significant decreased in hepatic TG levels were observed with LR alone (11.93 ± 1.01) either of AV/GS (9.90 ± 1.42/13.46 ± 0.60) compared to HFD (18.45 ± 0.52 mg g^{-1} tissue) fed group. However, no significant difference was observed with liver TC levels in all dietary interventions.

Serum insulin and HOMA-IR index

In Table 3, serum insulin and IR index values were presented. Serum insulin concentrations were significantly higher in HFD fed group and observed to be 172.90 ± 24.28 pmol L^{-1}. These insulin levels were significantly reverted back to normal with probiotic LR (76.19 ± 9.54 pmol L^{-1}) alone and in combination with herbs AV/GS with the values of 98.14 ± 8.07 and 86.51 ± 5.89 pmol L^{-1}, respectively. Similar results were found with the calculated HOMA-IR index values.

Adipocyte size and number

Histological analysis of epididymal fat showed that the cell size was significantly increased and the number was decreased significantly in HFD fed group. Oral administration of LR alone or in combination with herbal ingredients (AVLR/GSLR) showed a significant positive effect in obese mice (Fig. 3).

Quantitative real time PCR

Further, we measured the gene expression analysis in epididymal fat tissue (Fig. 4). Adipokines which are involved in the energy metabolism, i.e. adiponectin (AdiopQ) and UCP2 expression levels were down regulated in HFD group. Milk fermented by probiotic LR alone and in combination with herbs showed a significant up regulation of both AdipoQ and UCP2 mRNA expression. However, leptin and resistin gene expression was up regulated in HFD fed group, these expression levels were significantly reverted back to normal upon probiotic LR alone and in combination with GS supplementation. In contrast, no significant difference was observed with PPARγ expression in all different dietary interventions. Pro-inflammatory cytokines (TNF-α and IL-6) were significantly increased in HFD fed group. Administration of milk fermented by LR alone and in combination with both AV/GS significantly decreased inflammatory marker genes.

Discussion

The feeding of milk fermented *Lactobacillus rhamnosus* NCDC 17 showed a significant reduction in body weight gain in HFD fed group. Few reports are available on the anti-obesity effect of LR probiotic culture. Our results were consistently similar to Lee and co-workers[9] observed that *L. rhamnosus* PL60 administration to HFD fed mice showed a significant reduction in the body weight and no effect on feed intake. In contrary, Ji *et al.*[10] studied that oral administration of *Lactobacillus rhamnosus* GG (LGG) did not show any significant effect in the reduction of body weight and feed intake compared to normal chow fed mice. In a recent report by Kumar *et al.*[25] also observed that no significant effect on body weight gain and feed intake in hypercholesterolemic rats by LGG strain. Oral administration of milk fermented by LR culture in combination with AV had no effect on reduction in the body weight. Similar results were obtained by Kumar and co-workers[25] upon administration of *Aloe vera* gel extract and in combination with LGG in hypercholesterolemic rats. Moreover, GSLR showed reduction in the body weight gain. To the best of our knowledge, no reports are available with probiotic in combination with GS extract. In this study, reducing body weight gain and epididymal fat mass in the HFD mice was due to increasing energy expenditure. The increase in the energy expenditure is associated with less fat accumulation via mRNA expression of uncoupling protein (UCP2). On the other hand, conjugated linoleic acid (trans-10, cis-12-CLA) produced by the lactobacilli might be responsible to decrease body weight gain with unknown mechanism.[9,26] The contradictory results were observed due to lactobacilli strain specific and route of administration and treatment length.

Insulin plays an important role in the regulation of glucose homeostasis and lipid metabolism. Body weight gain and fat deposition were responsible for insulin resistance which results failure of target organs (adipose tissue and muscle) for the action of insulin and associated with metabolic abnormalities such as glucose intolerance, hyperlipidaemia, hepatic

steatosis, and hypertension as occurs.[27-30] The high fat diet fed obese mice showed a significant increase in glucose and insulin levels after 12 weeks experimental period. These were decreased by lactobacilli (LAB) culture alone and in combination with *Aloe vera* and *Gymnema sylvestre* extracts. The results on blood glucose levels in the present study suggest that the effects of probiotic fermented milk may be due to the improvement of insulin sensitivity. This is also supported by the insulin levels measured by ELISA in probiotic fed groups. Hypoglycemic effect of *Aloe vera* might be associated with pancreatic insulin synthesis and its secretion responsible for the lowering of circulatory glucose levels[31] whereas, gymnemic acids present in the *Gymnema sylvestre* binds to the glucose receptors to prevent the intestinal glucose absorption.[12]

A high-fat intake results in increased levels of free fatty acids in the circulation.[31,32] Administration of NCDC lactobacilli culture alone and in combination with herbs displayed a significant reduction in the serum triglycerides levels in DIO mice. Similar to our results, Lee *et al*.[9] observed that *L. rhamnosus* PL60 showed no effect on serum TC and LDL-C levels in diet induced obese mice. Recently, Kumar *et al*.[25] stated that *Lactobacillus rhamnosus* GG administration to hypercholesterolemic rats showed a decrease in the serum lipids (TC, TG, VLDL-C, LDL-C) levels. Next, we measured the hepatocyte lipid (TG and TC) levels after 12 weeks. The liver is responsible for the utilization of fatty acids from the HFD diet to synthesize cholesterol and triglycerides. High fat intake of mice results alteration in the lipid metabolism, resulting in accumulation of fat in the hepatocyte.[33] Administration of lactic acid bacteria (LAB) culture and in combination with both herbs showed a significant reduction in the lowering of liver TG and no effect on TC levels. However, phytosterols present in the herbs are don't extensively absorbed from the intestine, but it can bind to the lipids and prevent its absorption to show a hypolipidaemic effect.[34]. Further, we determined the adipose tissue cell size and number. Administration of LAB culture alone and in combination with

both herbs showed a significant decrease in the cell size, whereas a significant increase in the number in HFD fed group. Our results were similar with other several LAB cultures showed reduction in the adipose tissue size in high fat diet mice.[4,35] To our contradictory results Lee *et al*.[9] reported that *L. rhamnosus* PL60 from human origin supplementation, the size of epididymal adipocytes was not reduced in DIO mice. The results suggest an accumulation of fat due to increase intake of energy by mice receiving high fat diet. Seemingly, the hypertrophic adipocytes produce abnormal adipokines and cytokines, including the inflammatory markers.

Furthermore, we analyzed mRNA expression of adipokines such as adiponectin (AdipoQ), leptin, resistin, PPARγ, UCP2 and pro-inflammatory genes (TNF-α and IL-6) in epididymal fat tissue by qRT-PCR method against β-actin used as a reference gene. The adiponectin secreted from the adipocytes played an important role in regulating energy homeostasis. It is responsible for the inactivation of AcetylCoA carboxylase via AMP activated protein kinase (AMPK) which ultimate activation of β-oxidation of fatty acids to decrease energy conservation. Our results demonstrated that, high fat diet fed mice exhibited a significant decrease and increase in the adiponectin and leptin mRNA expression. The adipoQ and leptin levels were significantly increased and decreased in all treatment groups (except AVLR) in obese mice. In other hand, no significant effect was observed in case of PPARγ expression. Kondo *et al*.[36] demonstrated that *bifidobacterium breve* strain B-3 supplementation to HFD fed mice showed up regulation of adiponectin levels, whereas no effect on leptin and PPARγ expression. In addition, obese mice showed, mRNA expression of resistin was down regulated by different treatment groups. Major active principle compounds (anthraquinones, polyphenols and acemannan etc) present in the *Aloe vera* are becoming a major dietary energy source for the bacterial growth and metabolised in the large intestine. These compounds can't be degraded in the human gastrointestinal (GI) tract and converted

into short chain fatty acids (SCFAs) such as acetate, butyrate and propionate by the gut microbiota under anaerobic conditions. The SCFAs are bind to the G protein-coupled receptors/free fatty acid receptors (expressed in the intestine) to activate AMPK pathway for the β-oxidation of fatty acids to decrease adiposity.[37] SCFAs are stimulates anorexigenic gut hormones such as Glucagon like peptide-1 (GLP-1) and Peptide YY (PYY) which acts as a satiety effect by reducing energy utilization from the diet.

An increase in the energy expenditure results in a significant reduction of fat accumulation in the adipose tissue.[38] Genes play an important role involved in the increase of thermogenesis in adipose tissue. In our study, UCP2 mRNA expression levels were significantly increased after feeding a potential probiotic alone and in combination with herbs. Obesity and insulin resistance are associated with the excess accumulation of fat in the adipose tissue, leads to less vascularisation results hypoxia (less oxygen) and further infiltration of macrophages and inflammatory cytokines.[28,30] In the present study, both pro-inflammatory cytokines (TNF- α and IL-6) were significantly down regulated by all dietary interventions. Probiotics are playing an important possible mechanism involved in down regulation of pro-inflammatory gene expression in the adipose tissue by improving the intestinal tight junction proteins (ZO1 and occludin) to prevent the lipopolysaccharide (LPS which is secreted from the harmful bacteria) binding to cytokine receptors present in the adipose tissue and liver. An increase in the circulator LPS levels results in metabolic endotoxemia, which further leads to inflammation and metabolic disorders.[39,40] The polysaccharide (acemannan) of *Aloe vera* is responsible for the activation of macrophages to secrete cytokines viz. TNF-α, IL-1, IL-6 and interferon-γ or INF-γ. The active components (anthraquinones, polyphenols and acemannan etc) present in the *Aloe vera* showed an anti-inflammatory activity by inhibiting the cyclooxygenase in the arachidonic acid pathway.[41]

Conclusions

Due to potential health benefits of probiotic lactobacilli species, they are widely used as food ingredients in the form of fermented milk products. The present results indicate that the milk fermented by probiotic LR alone and in combination of AVLR/GSLR reduced body weight gain and epididymal fat mass. This is the first study where probiotic (LR) in combination with herbal ingredients were used. Further, lactobacilli and combination with both herbs showed decrease in epididymal fat cell size, fasting blood glucose, serum insulin and lipid levels. Relative mRNA expression analysis of obesity related genes in epididymal tissue suggested that a positive effect by all treatment groups. Though, this is an interesting active area of research to delineate the mechanistic aspects clearly as to how the probiotic live cells or their metabolic products change the gut environment. Fractionation of preparations from herbs like *A. vera* and *G. sylvestre* to study the effects of individual components may also be an important field of research to investigate their effectiveness in amelioration of diet induced obesity and insulin resistance.

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List of Figure legends

Fig 1 – Effect of different treatments on a) oral glucose tolerance test (OGTT) b) AUC in mice fed with high fat diet. Values are expressed as mean ± SEM. (n=6, [a-b]Mean values with unlike superscript letters were significantly different ($p<0.05$ and $p<0.01$).

Fig 2 – Effect of different treatments on fasting blood glucose levels in mice fed with high fat diet. [a-b]Mean values with different superscripts differ significantly ($p<0.05$).

Fig 3 – Effect of different treatments on photomicrographs of a) hematoxylin and eosin of adipose tissue (200x) (b) Adipocyte mean area (μm^2) and cell number in mice fed with high fat diet. Values are expressed as mean ± SEM (n=3, $p<0.001$).

Fig 4 – Effect of different treatments on mRNA expression levels of different genes in mice fed with high fat diet. Values are expressed as mean ± SEM (n=3). $^{a-b}$Mean values with different superscripts differ significantly ($p<0.05$).

Table 1 Composition of different diets

Components	Control Diet (%)	HFD (%)
Starch	53	25
Casein	20	20
Sucrose	10	10
Soyabean oil	7	7
Lard	0	28
Cellulose	5	5

Vitamin mixture (AOAC, 1990)	1	1
Mineral mixture (AOAC, 1990)	3.5	3.5
Choline chloride	0.2	0.2
Methionine	0.3	0.3

Table 2 Sequence of primers used for quantitative real time PCR

Gene	Primer sequence (5'-3')	Annealing Temperature (°C)	Size amplification product (bp)
Adiponectin	F - GGATGCTACTGTTGCAAG R - CATGTACACCGTGATGTG	60	152
Leptin	F - TGACACCAAAACCCTCATCA R - TCATTGGCTATCTGCAGCAC	60	213
Resistin	F - TTCCTTGTCCCTGAACTGCT R - CAAGACTGCTGTGCCTTCTG	60	186
PPARγ	F - CTGGCCTCCCTGATGAATAA R - GGCGGTCTCCACTGAGAATA	60	205
UCP2	F - GCCACTTCACTTCTGCCTTC R - GAAGGCATGAACCCCTTGTA	60	181
IL-6	F - AGTTGCCTTCTTGGGACTGA R - CAGAATTGCCATTGCACAAC	60	191
TNF-α	F - GTCGTAGCAAACCACCAAG R - AGAGAACCTGGGAGTAGATAAG	60	145
β-Actin	F - TGTTACCAACTGGGACGACA R - GGGGTGTTGAAGGTCTCAAA	60	165

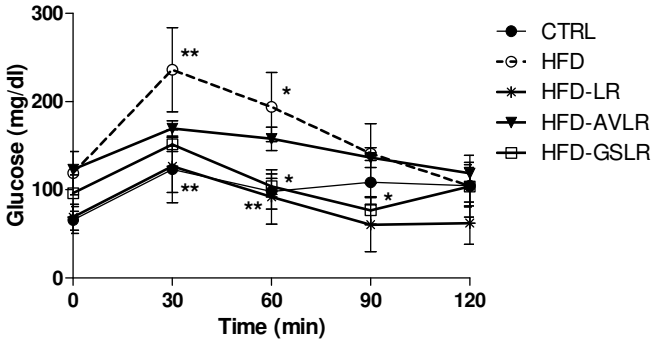
Table 3 Body weight, organ weights, insulin, IR index, serum and liver lipids

Parameters	CTRL	HFD	HFD + LR17	HFD + AV + LR17	HFD + GS + LR + 17
Body weight (g)					
Initial	22.27 ± 0.80	22.45 ± 1.10	22.60 ± 0.99	22.52 ± 1.20	22.70 ± 1.32
Final	25.40 ± 0.56[a]	27.29 ± 0.71[a]	25.18 ± 0.55[b]	25.81 ± 0.24[a]	25.20 ± 0.41[b]
Epdidymal fat (g)	0.56 ± 0.05[a]	1.10 ± 0.09[b]	0.55 ± 0.11[a]	0.68 ± 0.07[a]	0.64 ± 0.09[a]
Liver (g)	0.98 ± 0.07	0.96 ± 0.05	0.95 ± 0.02	0.88 ± 0.07	0.83 ± 0.08
Kidney (g)	0.36 ± 0.04	0.40 ± 0.05	0.42 ± 0.02	0.41 ± 0.01	0.33 ± 0.03
Insulin (pmol/l)	80.58 ± 5.74[a]	172.9 ± 9.28[b]	76.19 ± 9.54[a]	98.14 ± 8.07[a]	86.51 ± 5.89[a]
HOMA-IR index values	3.03 ± 0.40[a]	9.35 ± 1.31[b]	2.53 ± 0.27[a]	4.16 ± 0.41[a]	3.45 ± 0.28[a]
TG (mg/dl)	80.58 ± 5.46[a]	131.2 ± 6.87[b]	90.95 ± 7.73[a]	87.64 ± 6.85[a]	84.45 ± 8.69[a]
TC (mg/dl)	97.81 ± 4.54	137.8 ± 5.21	90.95 ± 2.74	96.58 ± 3.86	111.4 ± 5.58
HDL-C (mg/dl)	73.80 ± 2.29	78.42 ± 6.06	74.5 ± 6.21	67.18 ± 9.16	89.35 ± 4.94
LDL-C (mg/dl)	12.27 ± 1.22	16.68 ± 1.82	17.38 ± 1.64	16.96 ± 2.60	18.41 ± 2.70
VLDL-C (mg/dl)	16.12 ± 1.09	21.24 ± 1.85	18.19 ± 1.54	17.06 ± 1.15	16.29 ± 1.37
TG (mg/g liver tissue)	11.94 ± 1.00[a]	18.45 ± 0.52[b]	11.93 ± 1.01[a]	9.90 ± 1.42[a]	13.46 ± 0.60[a]
TC (mg/g liver tissue)	1.59 ± 0.19	2.83 ± 0.23	1.45 ± 0.20	2.06 ± 0.21	1.90 ± 0.21

SM=skim milk, HFD=high fat diet and IR=insulin resistance

[a-b]Mean values within a row with unlike superscript letters were significantly different ($p<0.05$, Tukey's test)

a)



b)

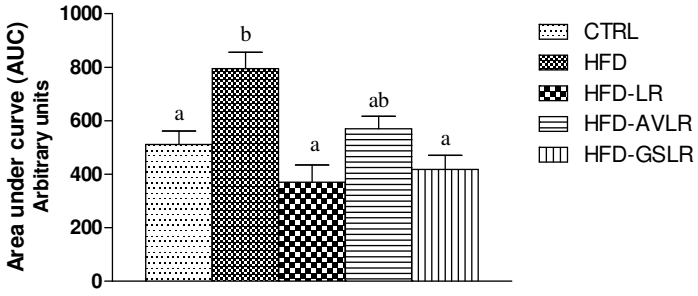


Fig. 1

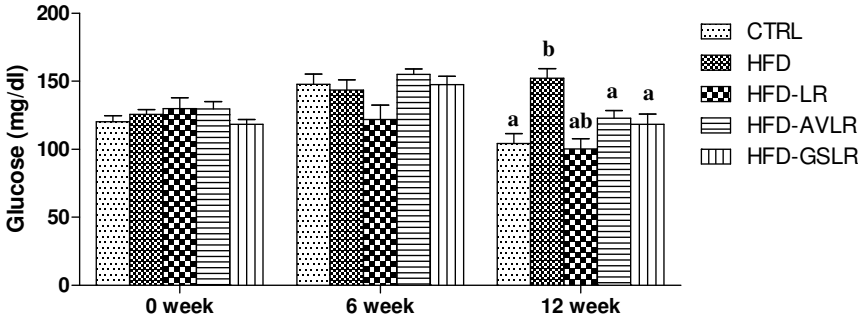
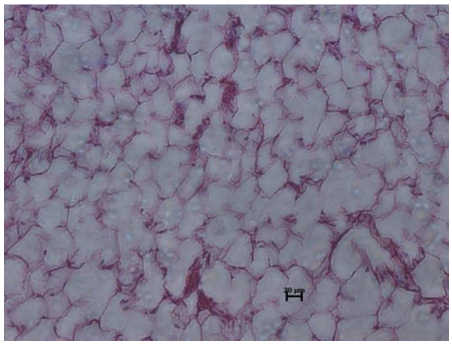


Fig. 2



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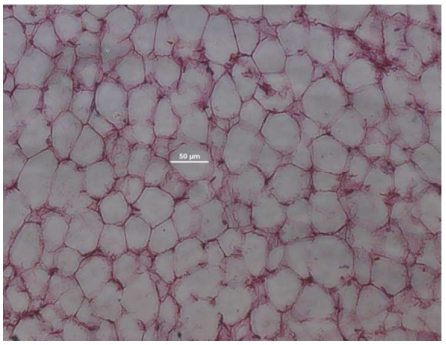
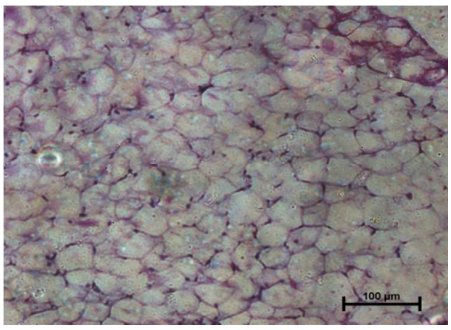
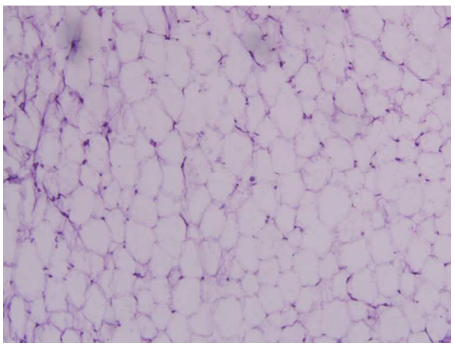


HFD



HFD-LR



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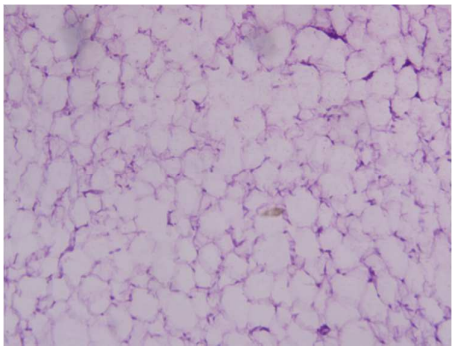
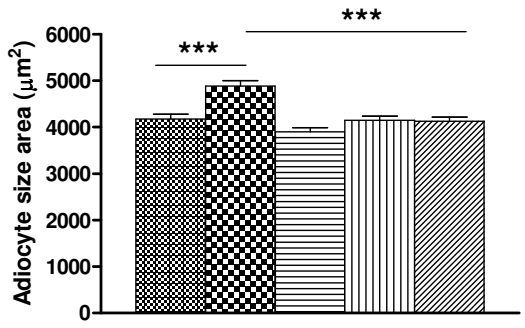
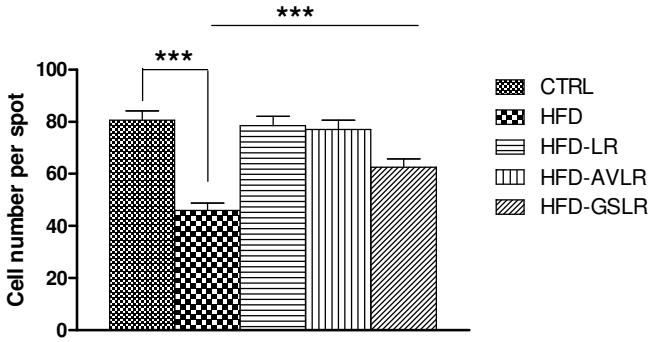


HFD-GSLR

a)





b)

Fig. 3

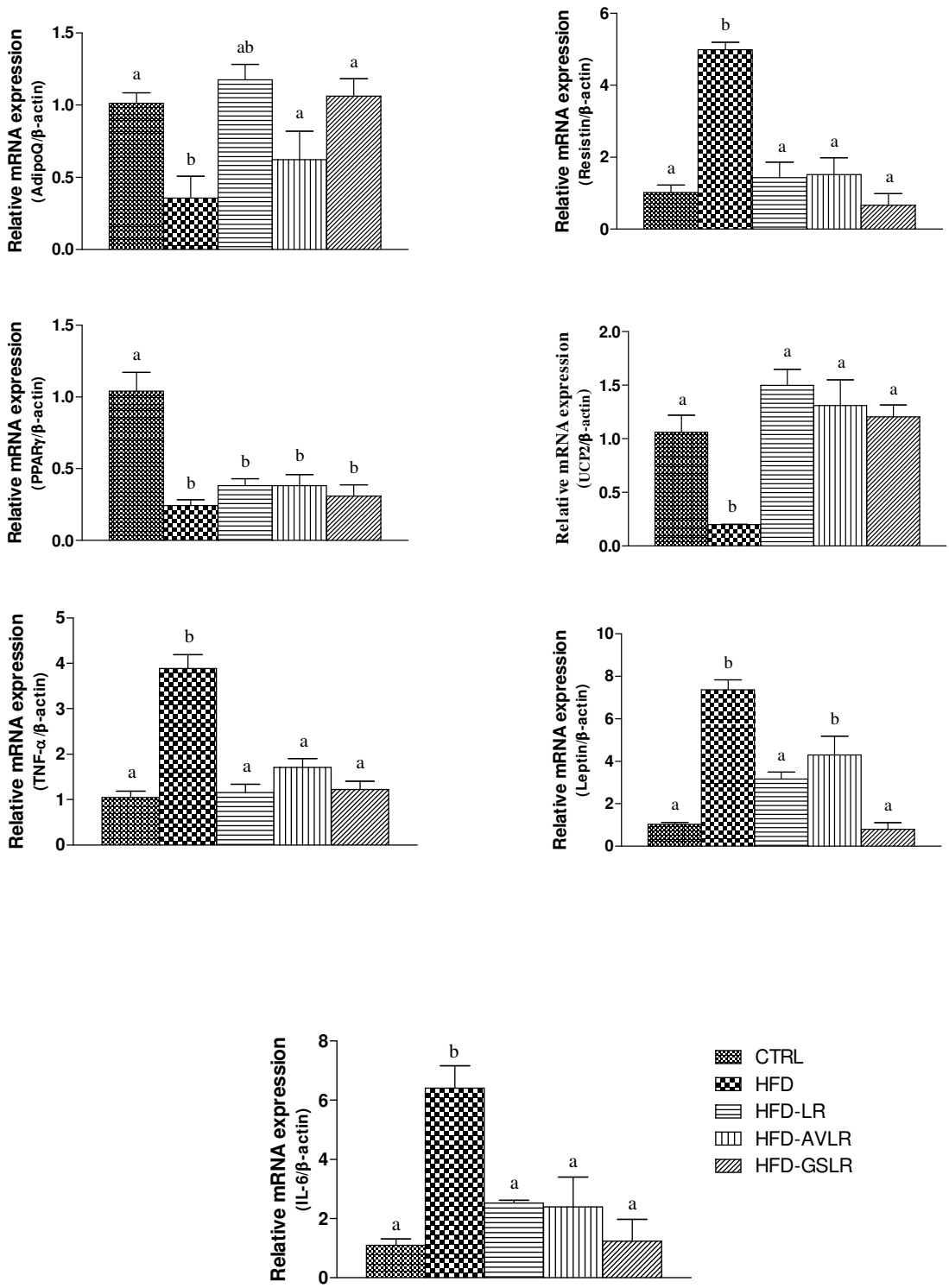
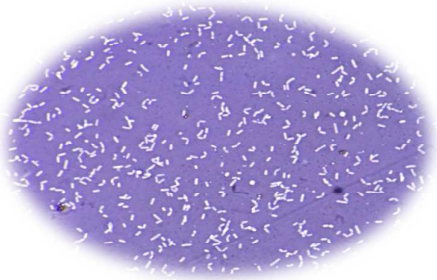


Fig. 4

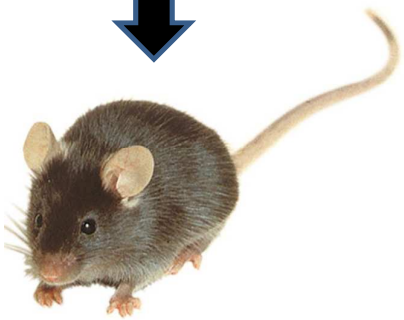
High fat diet (HFD)



Lactobacillus rhamnosus

Aloe vera

Gymnema sylvestre



C57BL/6J mice

After 12 weeks

Body weight ↓
FBG ↓
Epididymal fat mass ↓
Serum TG ↓
Liver TG & TC ↓
Serum Insulin ↓
Adipocyte size ↓
Adiponectin ↑
Leptin ↓
UCP2↑
TNF-α↓