EVALUATING THE ROLE OF SCFAs IN COMBINATION TO REVERSE THE DIABETIC CHARACTERISTICS

A Dissertation Thesis

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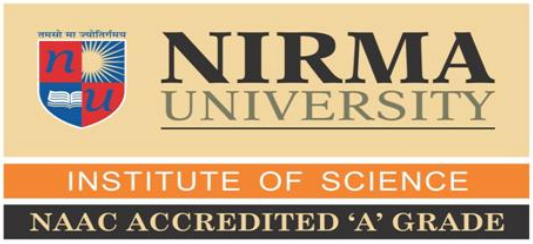

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DECLARATION

We declare that the thesis entitled **"Evaluating the role of SCFAs in the combination to reverse the diabetic characteristics"** has been prepared by us under the guidance of Dr. Sriram Seshadri, Assistant Professor, Institute of Science, Nirma University. No part of this thesis has formed the basis for the award of any degree or fellowship previously.

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ABBREVATIONS

- **GI** - Gastro intestinal
- **SCFAs** – Short chain fatty acids
- **HDACs** – Histone deacetylases
- **GPCRs** – G protein coupled receptors
- **FFARs** – Free fatty acid receptors
- **GLP 1** – Glucagon like peptide 1
- **PYY** – Peptide tyrosine tyrosine
- **GIP** – Gastric inhibitory poly peptide
- **T2D** – Type 2 diabetes
- **LPS** – Lipopolysaccharides
- **IR** – Insulin resistance
- **TLR** – Toll like receptor
- **TG** – Triglyceride
- **HFD** – High fat diet
- **NFκB** - Nuclear factor kappa-light-chain-enhancer of activated B cells
- **TNFα** – Tumor necrosis factor α
- **LRR**- C-terminal-Leucine rich repeats
- **PAMPs** – Pathogen associated molecular patterns
- **TIR** – Toll/IL 1 receptor
- **HSP** – Heat shock protein
- **NOD** – Nucleotide oligomerizing domain
- **NLRs** – Nod like receptor
- **CARD -** Caspase recruitment domain
- **PYD -** Pyrin domain
- **BIR -** Baculovirus inhibitor receptor
- **IFNγ** –Interferon γ
- **TNF-β** – Tumor necrosis factor β

- **L-PK** – Liver pyruvate kinase
- **FAS** – Fatty acid synthase
- **ACC** – Acetyl Co A carboxylase
- **SCD-1** – Stearoyl Co A desaturase
- **SREBP** – Sterol response element binding protein
- **ChREBP** – Carbohydrate responsive element binding protein
- **SRE -** Sterol response element
- **LXR** –Liver X receptor
- **ChoRE** – Carbohydrate responsive element
- **TR** – Thyroid hormone receptor
- **PPAR -** Peroxisome proliferator-activated receptors
- **PXR –** Pregnane X Receptor
- **FXR –** Farnesoid X receptor
- **CAR –** Constitutive androstane receptor
- **ER α –**Estrogen α
- **OGTT** – Oral glucose tolerance test
- **A** – Acetate
- **P** – Propionate
- **B** – Butyrate
- **SGOT** – Serum glutamate oxaloacetate transaminase
- **SGPT** – Serum glutamate pyruvate transaminase
- **ELISA** – Enzyme linked immune sorbent assay
- **HPLC** – High performance liquid chromatography
- **ANOVA** –Analysis of variance
- **SD** – Standard deviation
- **DNA -** Deoxyribonucleic acid
- **HSD** – High Sugar diet

ABSTRACT

Short chain fatty acid (SCFAs) are gut bacterial fermentation product. The most predominant SCFAs found in gut are Acetate, propionate and butyrate, present normally in the ratio of 60:25:15 respectively. In many studies, SCFAs are found to have anti-inflammatory effect. In the previous studies from our institute, microflora alteration was seen in diabetic group along with the SCFAs alteration which was restored by use of single SCFA. On that basis, we have hypothesized that HFD+HSD fed mice are treated with SCFAs combination for activation of FFARs and checked for reversal of inflammation, reversion to insulin sensitivity and restoration of gut flora. This research aimed to investigate the anti-diabetic characteristics of all the combined SCFAs group, to perform SCFA profile and to check for restoration of gut flora. The C57/BL6 mice were divided into three different groups: Normal control group (control diet), Diabetic or HSD+HFD group and treatment group with SCFAs combination (AP, AB and BP). After 60 days of induction and 60 days of treatment study, significant changes in different biochemical parameters such as body weight, OGTT, fasting glucose, SGOT, cholesterol and triglyceride level have been observed. Also, gene expression of liver and histopathological analysis of pancreas was done and considerable decrease in inflammation was found in combination treatment mice.

INTRODUCTION

Combination therapy is currently one of the more preferred strategy for various treatment studies. In combination therapy, two and more chemical agents or biologically active molecules are used and administered in fixed dose combination. Physicians have prescribed it to manage and treat a variety of disease conditions. Combinational therapy is generally used or switched up when monotherapy is not responding as expected. The adverse effect of single agent in monotherapy can be minimized by using the same in combination with a compound which reduces its adverse effect to make a combinational therapy. The combinational partner as well as their inter se ratio needs to be appropriately calculated to obtain the desired biological response. Combined agent can work in many ways first in which both the chemicals act together and has role in reduction of disease condition or another way in which one chemical is toxic and potential while other has role to reduce its toxicity.

Different studies have demonstrated the beneficial effects of combinational approach for treatment of chronic conditions like diabetes mellitus, neurological conditions, rheumatoid arthritis, pulmonary diseases, Alzheimer's and other diseases.

For diabetes treatment, combinational therapy is often used because with two chemicals which act at two different site for a better glycemic control. Single chemical need high concentration doses for recovery and can have side effects while using chemicals in combination require lower concentration doses and thus has lesser side effect. Anti-diabetic drug individually, may not be sufficient to solve the diabetic complications. Metformin along with sulfonylureas provides synergistic effect, decreases side effects and mortality. Metformin was also combined with insulin which supports insulin release and reduces the weight gain (Kupsal, Mudigonda, VBK Sai, Neelala, & Rani Hanumanth, 2016).

Recent studies suggested that metformin mediate gut microbiota shift and may be involved in the alteration of a variety of short chain fatty acid producing bacteria of gut origin (De La Cuesta-Zuluaga et al., 2017).

Metformin has been reported to increase butyrate producing microbes in gut. Synergism is also seen in molecular mechanism of action of butyrate and metformin (Maniar et al., 2017).

Microorganisms in huge number that residues in the gut, are collectivity referred to as microbiota. The ratio of anaerobes to aerobes bacteria in gut is 1000:1. Most of the anaerobes are localized in colon. The gut contains 10^{12} organism/gram of colonic content, which contains some of the most prevalent species such as Bifidobacterium, Bacteroides, fusobacterium and Eubacteria.

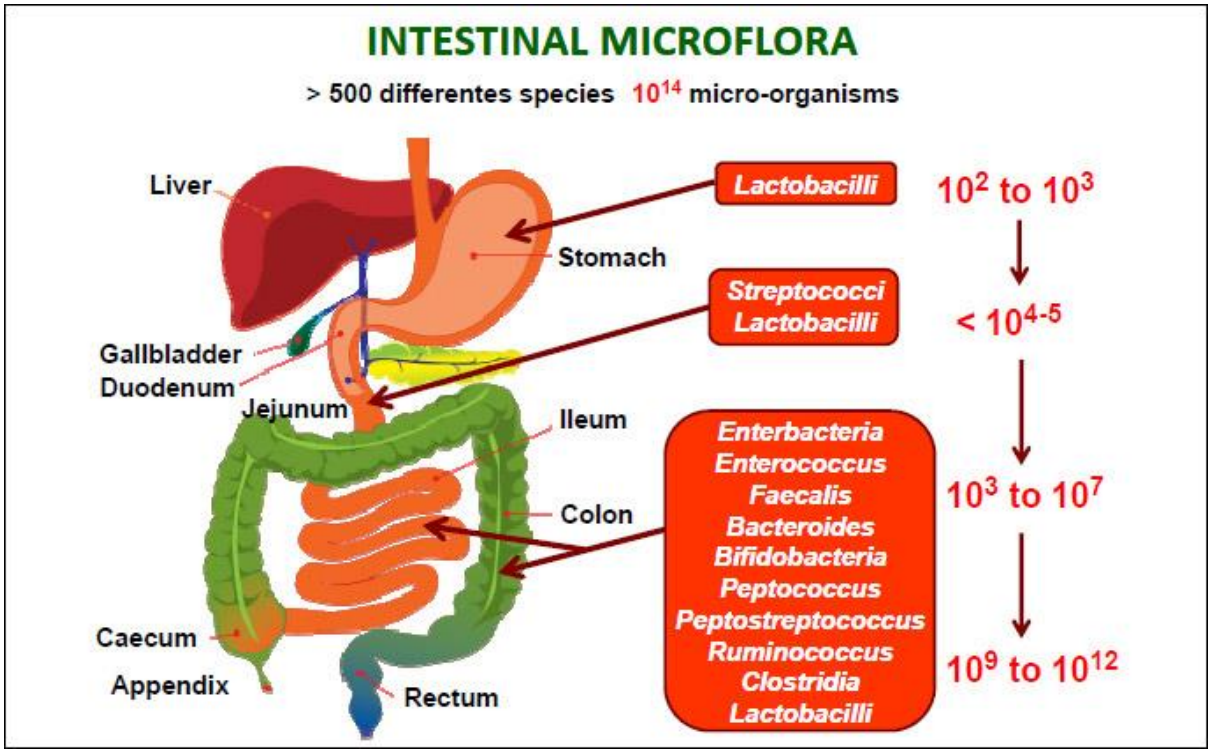


Figure 1: Microbial composition and numbers across GI tract. The gut microbiota of human contains 10^{14} bacterial cells, which is 10 times more than the human body cells. The bacterial cells shows the continuity in numbers, starting from 10^2 to 10^3 bacteria per gram of stomach and duodenum content, followed by 10^4 to 10^7 cells per gram in jejunum and ileum and 10^9 to 10^{12} bacteria per gram of colon content (Konturek, Haziri, Brzozowski, Hess, & Heyman, 2015).

Gut flora plays a crucial role in regulating gut function and conserving host health*(Tsukumo, Carvalho, Carvalho-Filho, & Saad, 2009)*. Normally, microbial population of gastro intestine provides protection against many pathogen, and the shift in gut microbiota is highly regulated by diet, which is the key factor to host health *(Fujimura, Slusher, Cabana, & Lynch, 2010).*

Gut microbiota alteration leads to change in microbial products such as SCFAs, LPS and metabolites that affect insulin resistance. Type 2 diabetes is also associated with microbiota dysbiosis *(Wen & Duffy, 2017).*

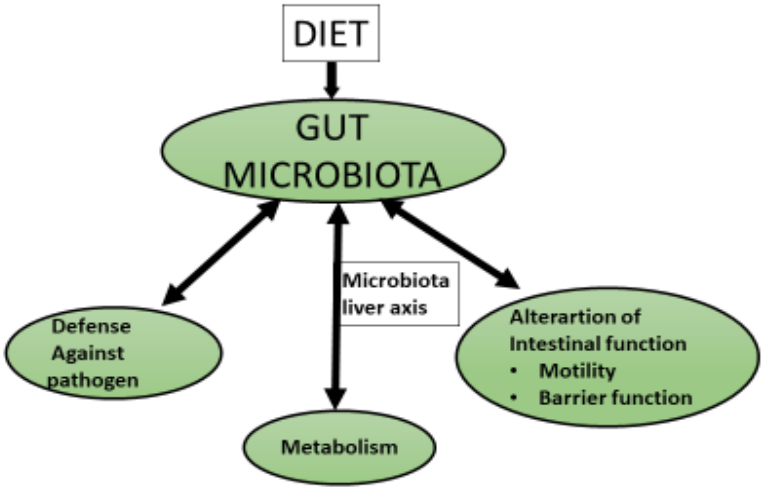


Figure 2: **Diet influenced physiological role of microbiota:** It has bidirectional role: 1. Defence against pathogens, 2. Effect on Liver metabolism, 3. Alteration of intestinal function including Motility and Intestinal permeability (Konturek, Haziri, Brzozowski, Hess, & Heyman, 2015).

SCFAs are fermentation products of gut bacteria produced when fibrous food including, dietary fiber, undigested proteins, resistant starch, and other endogenous substance reaches colon where fibers are metabolized by anaerobic gut bacteria giving rise to it's end products (Puddu, Sanguineti, Montecucco, & Viviani, 2014).

Short Chain Fatty Acids are fatty acids comprising up to 6 carbon atoms having aliphatic conformation. It includes C1-formic acid, C2-acetic acid, C3-propionic acid, C4-butyric acid, C5-valaric acid. In colon 90-95% of SCFAs content comprises of acetate, propionate, butyrate and their individual make up is about A-60%, P-25%, B-15% (Puddu, Sanguineti, Montecucco, & Viviani, 2014).

Types of substrate influence the SCFAs production, the consumption of increased dietary fiber elevated the SCFAs concentration whereas its concentration decreases in high fat diet intake (Puddu, Sanguineti, Montecucco, & Viviani, 2014).

The regulatory role of SCFAs is exerted through numerous mechanism including histone deacetylases (HDACs) inhibition, metabolic regulation and G-Protein coupled receptor (GPCRs) activation (Kim, Park, & Kim, 2014).

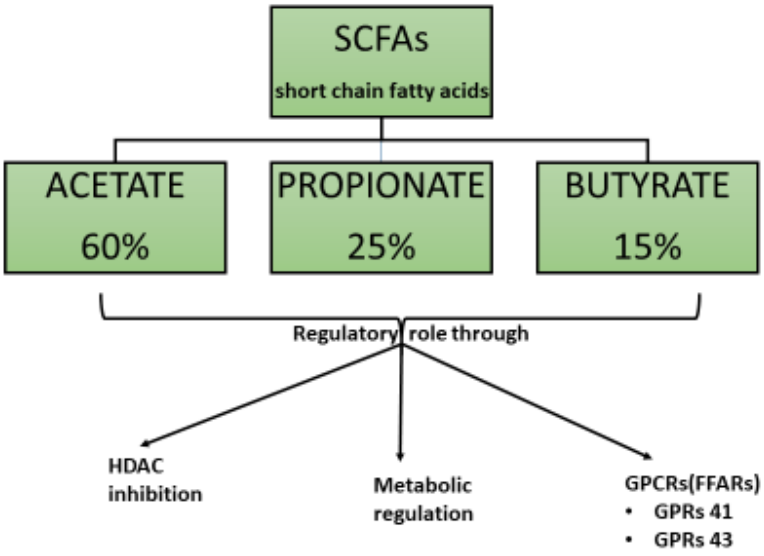


Figure 3: Major SCFAs and their regulatory function. 3 major SCFAs produced in gut are Acetate, Propionate and Butyrate in the ratio of 60:25:15 respectively. SCFAs exert their regulatory role through 1. HDAC inhibition, 2. Metabolic regulation, 3. GPCRs activation (Kim, Park, & Kim, 2014).

SCFA bound to GPCRs causes receptor activation and mediate cell signaling cascade. Two major SCFAs receptor includes, FFAR2 (GPR43) and FFAR3 (GPR41). FFAR2 and FFAR3 both has 40% similarity but they differ in their specificity for ligand. These receptor present in the number of cells like mucosal mast cells, enteroendocrine L cells, adipose tissues, monocytes and neutrophils. All the three major SCFAs can bind to both FFAR2 and FFAR3 while butyrate/propionate specifically binds to FFAR3. FFAR2 has role in GLP1 secretion and immune modulation while FFAR3 has a role in leptin production stimulated by SCFAs *(Puddu, Sanguineti, Montecucco, & Viviani, 2014).*

Table 1: Expression of SCFAs receptors on cell surface and their major functions(Kim, Park, & Kim, 2014).

Receptor	Cell type	Function
(1)GPR41/FFAR3 (butyrate)	Enteroendocrine Adipocytes Pancreas Sympathetic ganglia	• Leptin production • Gut hormone regulation • Innate immunity • Sympathetic activation
(2)GPR43/FFAR2 (acetate and propionate)	α cells Mucosal mast cell Leukocytes Gut epithelial cell Adipocytes	• Secretion of PYY &GLP-1 • Anti inflammatory effect • Anti tumor activity • Treg differentiation

SCFAs have metabolic regulation directly through HDAC inhibitors which further promote β-cells of pancreas to develop, proliferate and differentiate or indirectly through increased secretion of GLP1 from enteroendocrine L cells further promoting insulin secretion. SCFAs mediate the decrease in release of pro inflammatory cytokines from adipose tissue. This way SCFAs exert anti-inflammatory effects by improving glucose uptake and insulin resistance *(Puddu, Sanguineti, Montecucco, & Viviani, 2014)* (Fig.4).

Sodium butyrate administration significantly increase GLP-1 and GIP in plasma and moderately increase PYY, 10 minutes after dosing, while Sodium propionate significantly increased insulin, GIP and amylin, it did not show any increase in PYY and GLP-1 level*(Kim, Park, & Kim, 2014*).

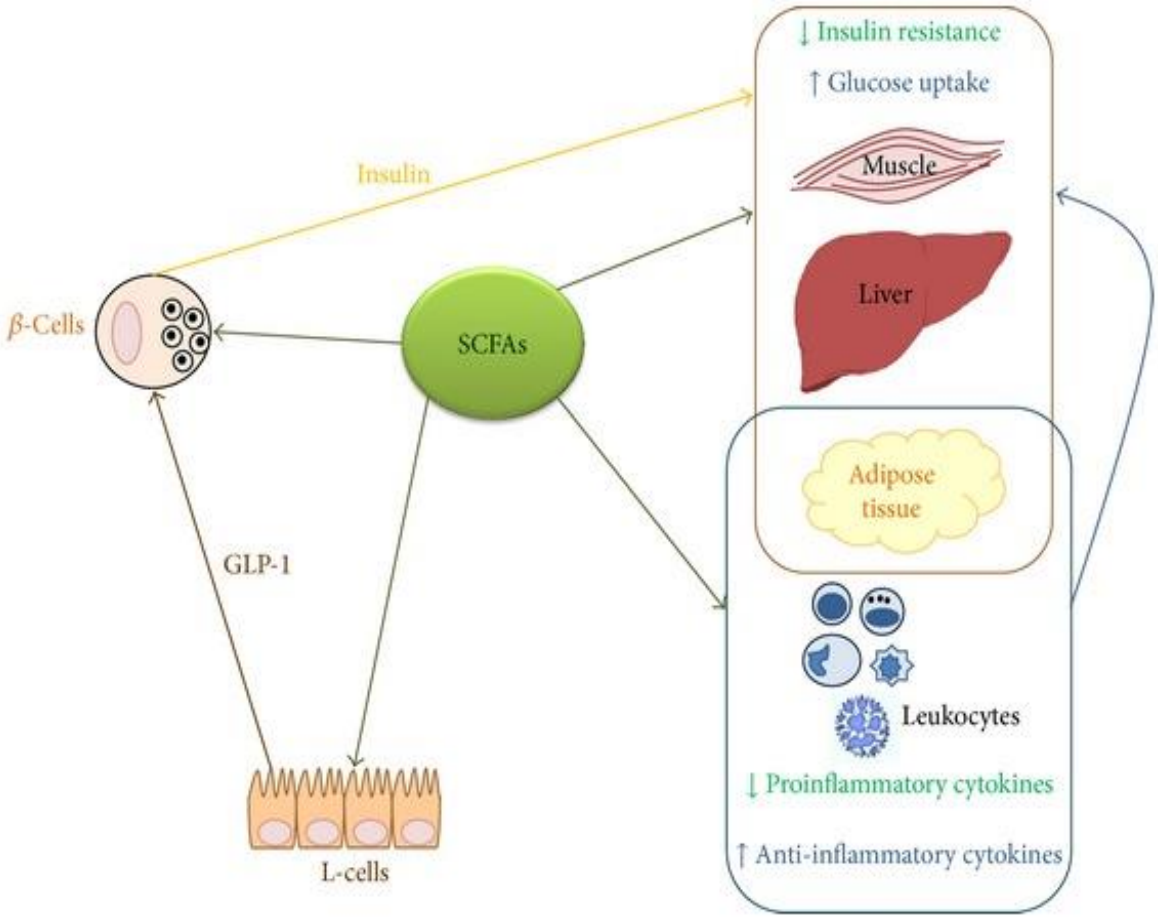


Figure 4 : **SCFAs improves metabolic function in T2D** (Puddu, Sanguineti, Montecucco, & Viviani, 2014).

Dietary changes affect the gut microbiota composition and its metabolic products. In high fat diet SCFAs production decrease and lipopolysaccharides (LPS) increases because of the shift in gut microbiota towards more gram negative bacteria *(Puddu, Sanguineti, Montecucco, & Viviani, 2014).* Studies have shown intestinal flora as the main contributor of T2D development. T2D arose from the reduction of *clostridiales* bacteria, which are butyrate producers.

LPS was associated with the increased permeability in the colon epithelium induced due to secretion of more pro-inflammatory molecules. This is referred as "metabolic endotoxemia". Rise in LPS is directly linked to IR. Enterocytes absorb LPS, conjugate with chylomicrons and transferred to plasma. This way it leads to a correlation between dietary fats and LPS absorption which can also be associated with gut microbiota changes .Binding of LPS with TLR4 leads to production of pro-inflammatory cytokines and thus impaired β cells of pancreas *(Puddu, Sanguineti, Montecucco, & Viviani, 2014).* A positive corelationship has been shown in mice between plasma concentration of LPS, insulin resistance, triglyceride (TG) accumulation and body weight.

LPS, component of gram negative bacteria are involved in the development of inflammation and T2D. This suggest that LPS concentration is higher in diabetic patients and involved in pathogenesis of T2D (Fig.5).

High fat diet increase gut permeability and inflammation via NFκB phosphorylation and TNF α up regulation in the ileum. This is counteracted by anti inflammatory effect of SCFAs, which probably increases due to intake of dietary fibers *(Puddu, Sanguineti, Montecucco, & Viviani, 2014).*

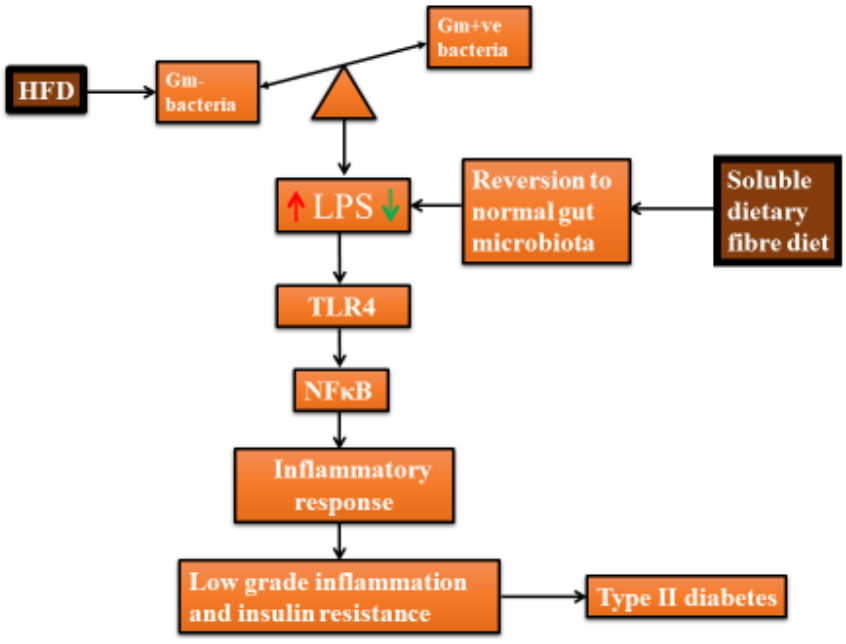


Figure 5: HFD induces gut microbiota dysbiosis by altering LPS concentration leading to NFkB phoshorylation followed by inflammation and T2D.

Type 2 diabetes is associated with low grade inflammation because of increased pro-inflammatory cytokine production and gut microbiota alteration. TLRs and NLRs are pathogen recognition receptor which contributes to innate immunity. TLRs and NLRs are known to activate NFκB and releases pro-inflammatory cytokines.Depending upon its cellular location, TLRs are grouped into two, those present on cell surface i.e, TLR1,TLR2,TLR4,TLR5,TLR6,TLR11 and the second present intracellularly i.e, TLR3,TLR7,TLR8,TLR9. The extra cellular region of TLRs is leucine rich repeat (LRR) domain which recognizes pathogen associated molecular patterns (PAMPs) and its cytoplasmic domain Toll/IL-1(TIR) activates downstream signaling (Prajapati, Jena, Rajput, Purandhar, & Seshadri, 2014) (Fig.6).

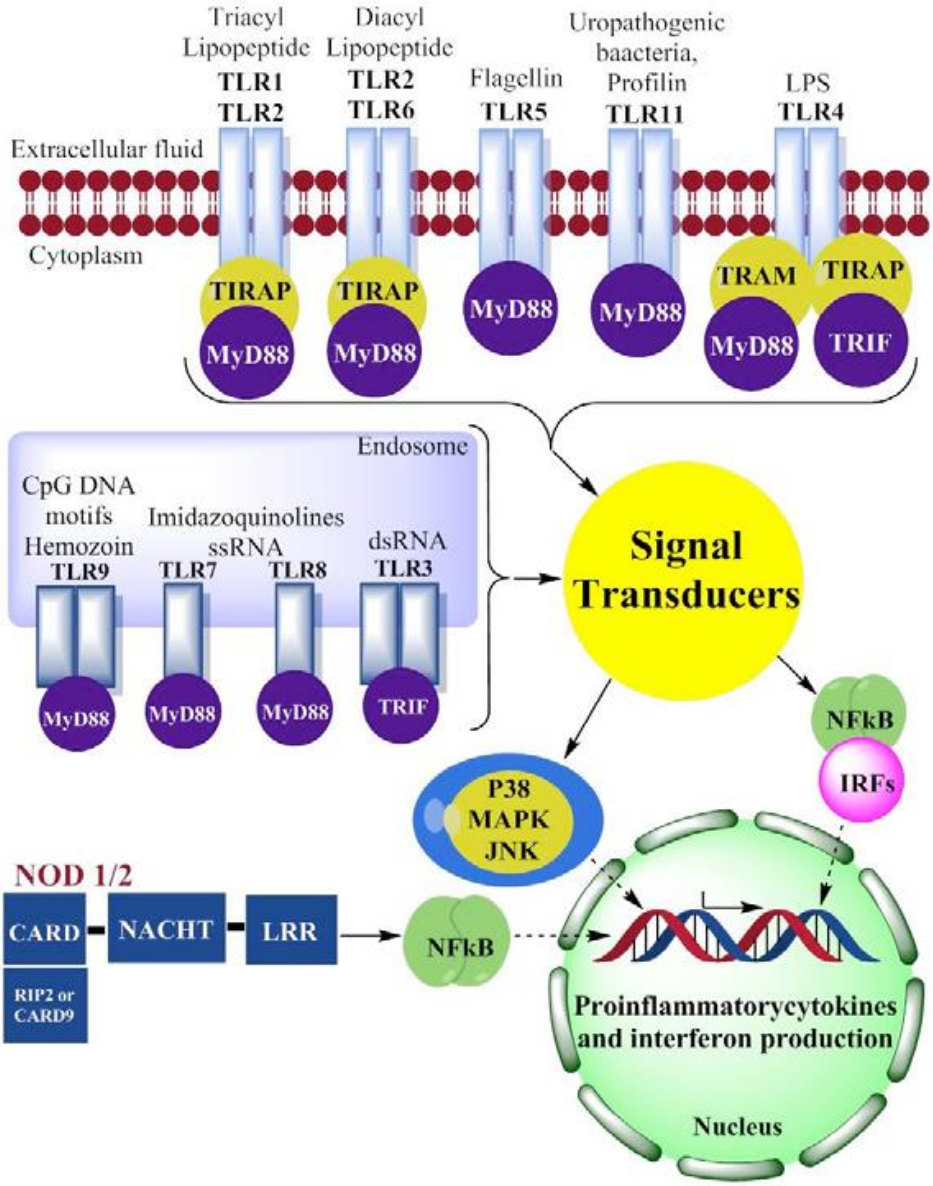


Figure 6: TLRs present on the plasma membrane and endosome for recognition of microbial components (Prajapati, Jena, Rajput, Purandhar, & Seshadri, 2014).

TLR2 recognizes peptidoglycans and lipopeptide from gram positive and gram negative bacteria. It recognizes and differentiate Diacyl and Triacyl lipopeptide with the help of TLR1 and TLR6.TLR4 ligands are derived from gram negative bacteria. LPS is one of the component and act as ligand for TLR4. Other endogenous ligands are also recognized by TLR4 which includes Heat Shock Protein (HSP), i.e., HSP60, fibronectins and hyaluronic acid (Prajapati, Jena, Rajput, Purandhar, & Seshadri, 2014).

NOD (nucleotide oligomerizing domain) is cytoplasmic NLRs (NOD like receptor) present intracellularly and involved in innate immunity. NLR family consists of the following i.e., CARD (Caspase recruitment domain), PYD (Pyrin domain), BIR domain (Baculovirus inhibitor receptor); Central domain, NOD and LRR (c-terminal-Leucine rich repeats), C-terminal domain that detects PAMPs. NOD1 (CARD4) recognizes peptidoglycan present in gram positive and gram negative bacteria but majorly from gram negative bacteria. NOD2 (CARD15) recognizes peptidoglycan component muramyl dipeptide, from both gram positive and gram negative bacteria.

NLR and TLRs crosstalk induces NFκB and has role in insulin resistance and inflammation (Prajapati, Jena, Rajput, Purandhar, & Seshadri, 2014)(Fig. 7).

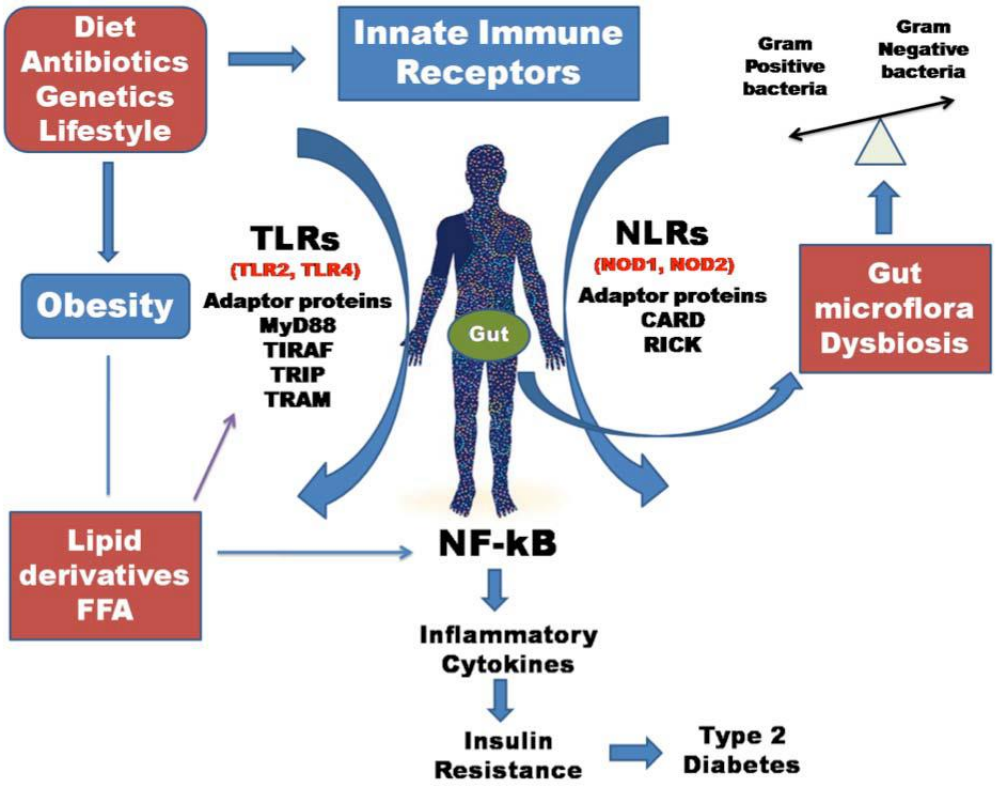


Figure 7: Innate immune receptors like TLRs (TLR2, TLR4) and NLRs (NOD1, NOD2) crosstalk in pathogenesis of T2D and insulin resistance. Increased LPS mediate TLR and NLR pathways in association with other adapter proteins. TLRs and NLRs mediate activation of NFkB central pathway which leads to inflammatory cytokines and IR (Prajapati, Jena, Rajput, Purandhar, & Seshadri, 2014).

In T2D, local and systemic inflammation and promoting insulin resistance has been confirmed through a critical role of adaptive immune system. It has been shown that CD4+ Tcell play an important role in obesity induced insulin resistance. CD4+ Tcell can be divided into pro-inflammatory Th1, Th17 and anti-inflammatory Th2 and FoxP3+ Treg cells based on cytokine production and functionality. CD4+ Tcell trigger cell mediated immunity and phagocyte dependent inflammation. Th1 cells could produce IFNγ, IL2, TNFβ ; wherein Th2 cells regulate antibody response produces IL4, IL5, IL6, IL9, IL10, IL13. Th1 and Th2 cells play important role in regulating inflammatory process, although they are activated later than macrophage in inflammation (*Kim, Park, & Kim, 2014*).

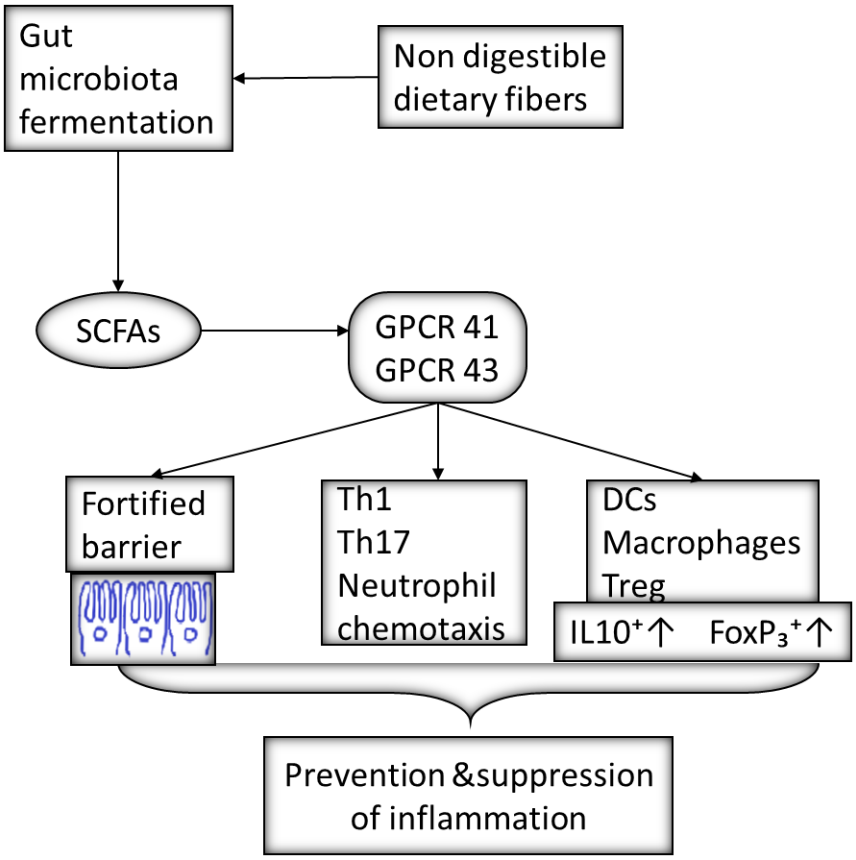


Figure 8:SCFAs regulates tissue inflammation through their effect on multiple cells. SCFAs regulates intestinal epithelial cells by improving gut barrier functions. The other cells which gets affected are antigen presenting cells (APCs). SCFAs acts on dendritic cells (DCs) and leads to the generation of tolerogenic T cells because of the decreased expression of MHC II molecules, co-stimulatory molecules, CCR7 and cytokines. In this way, SCFAs lowers inflammatory T cells. SCFAs act directly on naïve T cells and increases its differentiation in effector and IL10 producing T cells. Together it enhances the gut barrier function, neutrophil recruitment, T cell function for prevention and suppression of inflammation *(Kim, Park, & Kim, 2014*).

According to recent studies, it has been found that Tcells can be regulated by SCFAs. SCFAs either positively or negatively regulate FoxP3+ cells depending on Tcell activation. SCFAs on T cells, enhances the generation of Th1 and Th17 cells in Tcell polarization condition. Mechanism like, metabolic regulation, HDAC inhibition and GPCR activation signals are bound together to regulate directly or indirectly Tcell differentiation. SCFAs promote Tcell production of IL10 which prevent inflammatory responses. Propionate and butyrate directly suppress HDACs inhibition and Treg generation was increased by SCFAs as a result of inhibition HDAC by SCFAs (*Kim, Park, & Kim, 2014*).

In T2D, impairment of both insulin secretion and insulin action takes place. Normally, Insulin promotes glucose uptake and its utilization. Insulin maintains glucose homeostasis by increasing peripheral glucose uptake and by decreasing hepatic glucose production but in insulin resistant state, glucose clearance decreases in response to the insulin *(Bouché, Serdy, Kahn, & Goldfine, 2004).*

The homeostasis of whole body glucose is majorly mediated by Liver. Liver stores a large proportion of glucose from ingested meal simultaneously release it back into circulation. It suggests that fasting hyperglycemia is a result of increased hepatic glucose production. Elevated fasting blood glucose level are reflection to β cells destruction *(De La Cuesta-Zuluaga et al., 2017).*

The liver has major role in controlling different pathways of glucose metabolism like Glycolysis, Gluconeogenesis, glycogenesis and glycogenolysis. For functioning of these system both acute and chronic regulation of enzymes are required. The acute control constitutes post translational modification of these enzymes and for chronic control expression of genes for these enzymes is necessary.

Liver perform de novo lipogenesis in which triglyceride is formed from excess carbohydrate. Increase in lipogenesis is resulting from the transcriptional gene activation of lipogenic and glycolytic enzymes; (L-PK) liver pyruvate kinase, fatty acid synthase (FAS), acetyl CoA carboxylase (ACC) and stearoyl CoA desaturase (SCD1).

Insulin mediate its transcriptional effect through SREBP-1c. SREBP-1c has capacity to attach sterol response element (SRE), in target genes. SREBP-1c is regulated by itself as well as LXR (Liver X receptor). Control regulator of lipogenic pathway is insulin along with that glucose acts as signaling molecule for denovo lipogenesis, and shows synergy with insulin.

Glucose controls the expression of genes through Glucose-signaling transcription factor, (Carbohydrate Responsive Element Binding Protein (ChREBP). Glucose regulates the activation and entry of ChREBP from cytoplasm to the nucleus and promote the binding of ChREBP to ChoRE (carbohydrate responsive element) in the region of ACC and FAS lipogenic genes and L-PK glycolytic gene. Inhibition of ChREBP in liver improve hepatic steatosis and also insulin resistance in obese mice. The expression of ChREBP is regulated by LXR and thyroid hormone receptor (TR).

Recent studies suggested that other nuclear receptor agonist like PPAR γ, PXR or antagonists like FXR, CAR, and ER α interact with ChREBP and promote lipid storage in hepatocytes. ChREBP gene is directly responsible for glucose repression of PPAR α gene which is present in β cells of pancreas, it suggests that ChREBP is important for suppression of β oxidation in pancreas *(Poupeau & Postic, 2011).*

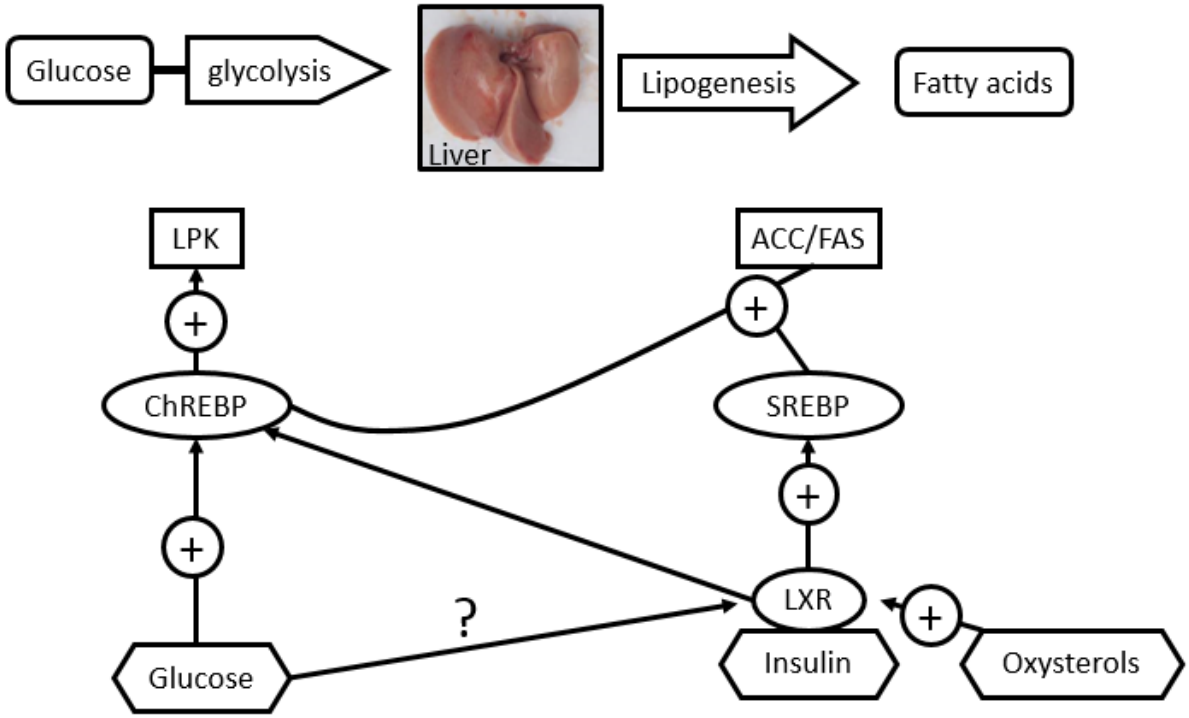


Figure 9: Role of ChREBP, SREBP-1C and LXR in type 2 diabetes.

The conversion of glucose into fatty acids through de novo lipogenesis is regulated by both glucose and insulin signaling pathway in response to dietary carbohydrates. Glucose activates ChREBP by stimulating its gene expression by promoting its binding to carbohydrate responsive element in its promoter region by

regulating its entry through cytosol to nucleus. For induction of L-PK, ChREBP is required which is dependent on glucose. Combined action of FAS and ACC genes is under ChREBP and SREBP-1C is under glucose and insulin combined action. Whereas, LXR are central for the insulin- mediated activation of SREBP-1C (*Ferre, 2014*).

In type 2 diabetes PPARs play an efficient role in decreasing the glucose induced insulin secretion and lessen the efficiency in insulin action. Activation of PPARα in HFD mice of insulin resistance, distinctly improve insulin sensitivity. Intracellular fatty acids and their byproducts interfere with insulin-stimulated glucose metabolism either via metabolism competition or through insulin signaling pathway.

PPARs are the nuclear receptor which bind to peroxisome proliferator response element on the DNA. PPARα and PPARγ are the most implicated in lipid metabolism and insulin sensitivity.

PPAR α activation in terms of insulin sensitivity, it has been shown that mice are protected from high fat diet induced insulin resistance.

PPARγ present in adipose tissue i.e. insulin sensitive tissue. Thus to decrease in plasma concentration, the activation of PPARγ leads to transport of fatty acids into adipose tissue. PPARγ on adipocyte hormone shown to modulate insulin sensitivity.

PPARα and γ are presently tested and seem to show combine positive effects on insulin sensitivity and on lipid parameters (*Denechaud, Dentin, Girard & Postic, 2008*)

PPARs belongs to superfamily of nuclear receptor which acts on DNA response as heterodimer. PPARs consists of 2 isoforms (α and γ). PPARα primarily expressed in liver with high fatty acid oxidation, Gluconeogensis, and decrease, glycolysis in liver. PPARγ is highly expressed in adipose tissue which mediates oxidation and Lypolysis and insulin sensitivity and decrease the inflammation

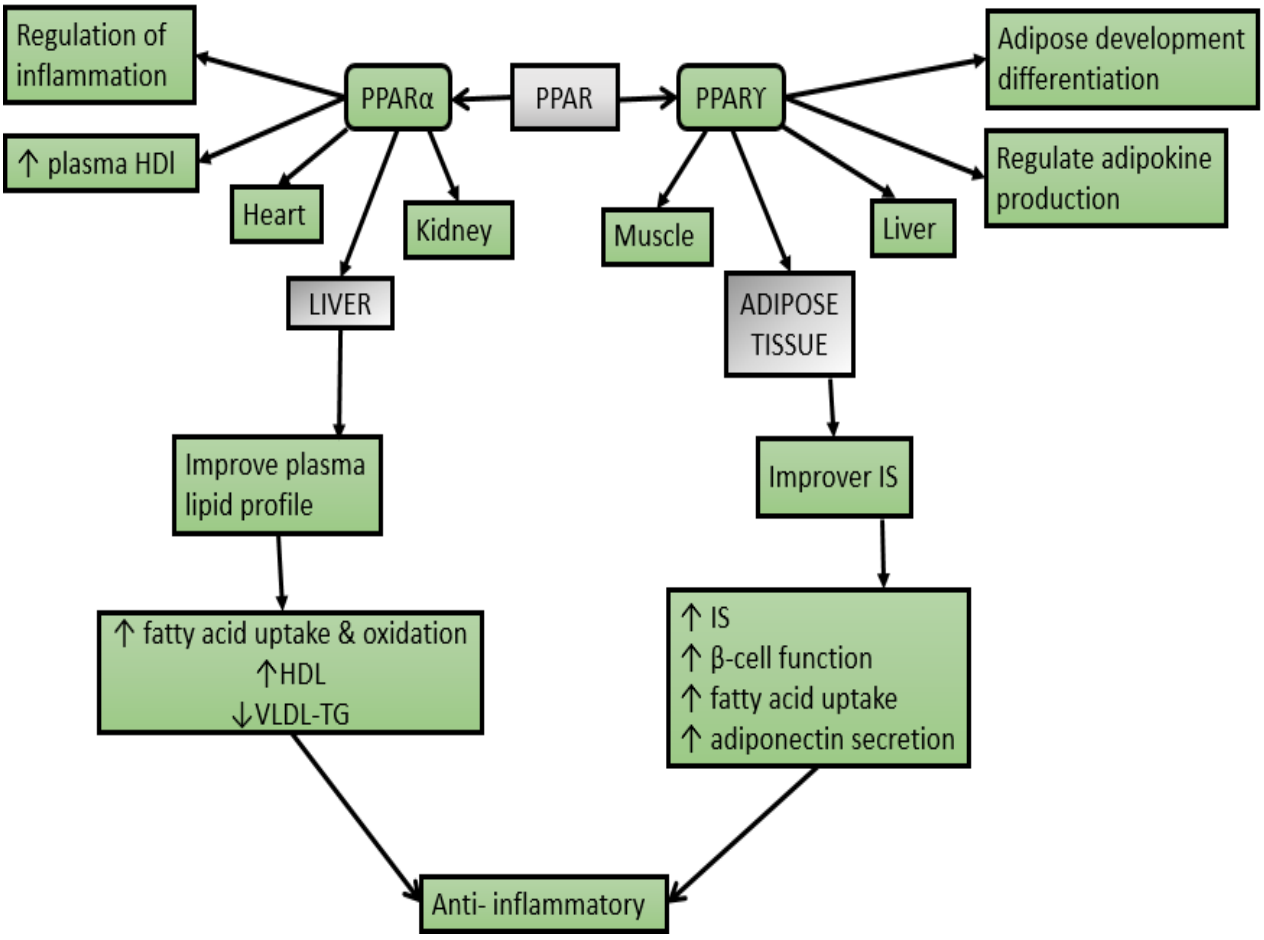


Figure 10: Role of PPARs in type 2 diabetes.

HYPOTHESIS

SCFAs has pro inflammatory effect. Thus, targeting SCFA receptor i.e. GPRs by different combinations of SCFAs for reduction of inflammatory characteristic, reversion to insulin sensitivity and modulation of gut flora for possible treatment of T2D.

OBJECTIVE

1. To evaluate the response of various diabetic characteristic in all the three combination groups.
2. To perform SCFAs profile in treated animals.
3. To restore the altered gut flora.

MATERIAL AND METHOD

EXPERIMENTAL ANIMALS

C57/BL6 male mice, 6-8 weeks old of 15-20 gms weight were acquired from Zydus pharmaceuticals, Ahmedabad. Mice were maintained and taken care under controlled condition at central animal facility, Institute of pharmacy, Nirma university. Diet and water were provided to mice everyday according to their grouping. The animals were subjected to studies in accordance with the ethical guidelines for care and use of laboratory animals of institutional animal care and use committee, Nirma university, Ahmedabad (The Protocol has been approved under IAEC proposal no. IS/PHD/18/025).

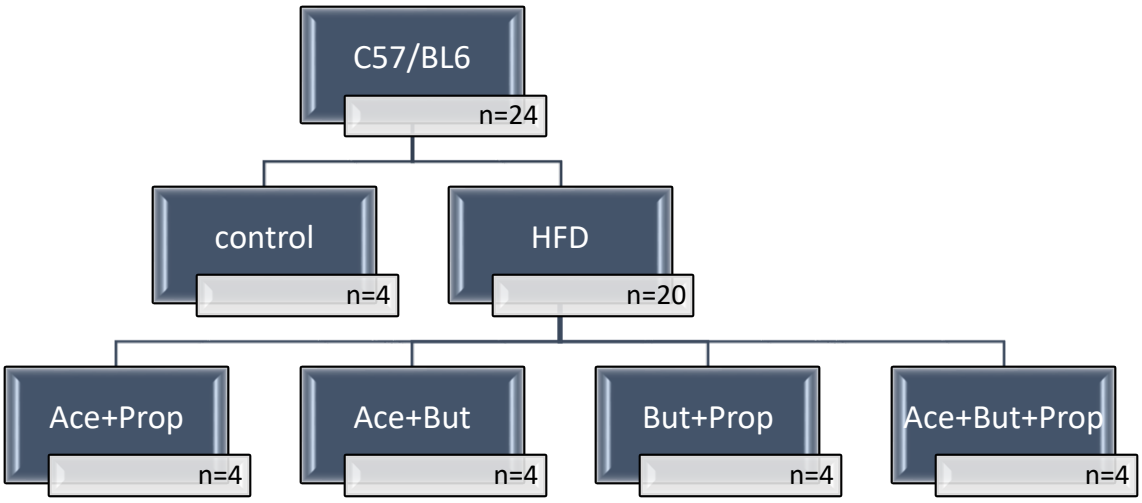


Figure11: Grouping of Experimental Animals.

ANIMAL DIET

Animals were subjected to different diet based on their groups. Animals were always given sufficient diet and water except 12 hours prior performing of blood collection and oral glucose tolerance test (OGTT).

The diet composition for normal control and high fat diet (HFD) mice is as follows:

Table 2: Animal Diet table.

Ingredients	**100 gm (control)**	**100 gm (HFD+HSD)**	**100 gm (Control+SCFAs)**
Corn starch	65 gm	-	65 gm
Wheat bran	5 gm	5 gm	5 gm
Groundnut oil	5 ml	5 ml	5 ml
Casein	20 gm	20 gm	20 gm
Methionine	0.3 gm	0.3 gm	0.3 gm
Salt mix	3.5 gm	3.5 gm	3.5 gm
Vitamin mix	1.0 gm	1.0 gm	1.0 gm
Choline chloride	0.2 gm	0.2 gm	0.2 gm
Fructose/Lard	-	45 gm/15 gm	-
Sucrose /fructose	-	30 gm/30gm	-
SCFAs			SCFAs will be added in diet in different ratio of combination for treatment.

INDUCTION STRATEGY

Mice were fed high fat diet and high sugar diet comprising Fructose or Lard and Fructose or Sucrose respectively for 60 days to allow them to develop diabetes and simultaneously OGTT were performed at regular intervals in order to confirm the induction of diabetes.

TREATMENT STRATEGY

The diabetes induced mice due to HFD diet were treated with acetate + butyrate (5%+10% w/w), butyrate + propionate (10% +10% w/w), acetate + propionate (5%+10% w/w), acetate + butyrate +propionate (5%+10%+10% w/w). All SCFAs were mixed with the control diet and was given to individual groups for 60 days. As within a short span of time all mice of A+B+P group were died thus, we were not able to continue with further analysis.

AUTOPSY SCHEDULE

Regular oral glucose tolerance test (OGTT) tests were performed of experimental animals to check glucose tolerance and insulin resistance along with triglyceride (TG) and cholesterol assays to check diabetic induction and upon induction also they were further performed at frequent intervals to ascertain the reversal of diabetes.

Following induction, 4 animals out of HFD treated animals were sacrificed and subjected to analyze different parameters and rest diabetic animals were treated with different SCFAs combination diet.

After 60 days treatment with SCFAs, animals were subjected to autopsy and a day prior to autopsy, OGTT and fresh fecal samples were collected from animals and analyzed.

Mice were sacrificed by euthanasia under dose of thiopental, followed by autopsy. Liver, pancreas, small intestine, large intestine, adipose tissues, muscle, lymph node, spleen were collected from each animal and stored at -20˚c blood was collected by retro-orbital plexus and serum was separated from it after centrifugation at 10,000 rpm for 15 minutes at 37˚c.

PARAMETERS

OGTT (oral glucose tolerance test)

OGTT was performed at regular intervals to check occurrence of diabetes in mice. 12 hours prior to performing OGTT, diet was removed. First estimation of fasting glucose levels were done and then oral dosing of glucose was given to animals as per their weights (29 kgs). Glucose estimation was done using glucose strips and calculated with free optimum H blood glucose monitor (Abbott, UK) at different time intervals of 0, 30, 60 and 120 minutes after oral dosing.

Blood collection:

Collection of blood started from the initial day of the experiment till the last day of the autopsy 90th day. 250µl blood was collected in the microfuge (without anticoagulant) tube from retro orbital plexus by giving mild anesthesia using diethyl ether followed by centrifugation at 10,000rpm for 10min at 4°C and further serum was separated and transferred to microfuge tubes for biochemical assays.

Serum Analysis:

Serum was used for the estimation of glucose, TG (Triglycerides), Cholesterol, SGOT (Serum glutamate oxaloacetate transaminase), SGPT (Serum glutamate pyruvate transaminase) using diagnostic kit (Accucare reagent kit) and following procedure as per the manufacturer's protocol.

Cytokines were analyzed by ELISA kit based techniques (Accucare reagent kit).

Colonic fecal collection:

Fresh colonic fecal samples were collected from the all combination groups on 90th day that is the scheduled autopsy time after 60 days of treatment and stored at -20°c for further microbial gut microflora quantification

FECAL GENE EXPRESSION ANALYSIS

DNA Isolation:

Colonic samples which were stored at -20°c, were taken for the DNA isolation using QIAamp DNA stool Mini kit, Qiagen, Germany as per the instruction mentioned in the manufacturer's protocol for all combination groups. Characteristics of isolated DNA was determined by electrophoresis on 2% agarose gel along with ethidium bromide and imaged in Gel documentation system with absorbance taken at 260nm and DNA concentration was documented.

TISSUE SPECIFIC GENE EXPRESSSION

RNA Isolation:

RNA were isolated from liver tissue samples of subjects following the SAMBROOK-RUSELL (2001) protocol for RNA isolation and were further subjected to gene expression studies.

Primer designing for gene expression study:

Using Integrated DNA technologies, primers of different combination genes were designed. The nucleotide sequence for each gene was blasted with primer sequence to validate the complete alignment of primer with mRNA sequence.

HISTOPATHOLOGICAL STUDIES

Liver tissues were dissected out from experimental animals at the time of autopsy and were fixed in formaldehyde to prepare paraffin blocks and slides and then slides were viewed and digitally photographed using Catcam 3.0 MP Trinocular microscope with an attached digital 3XM picture camera (Catalyst Biotech, Mumbai, India).

SCFAs PROFILE

In order to quantify SCFAs proportion in subjects, High Performance Liquid Chromatography (HPLC) for fecal and serum samples were performed using 4.6×10mm column with 3.5 micron sieve size in Agilent HPLC instrument. For HPLC, standard protocol was followed.

For Sample preparation, excreted fecal was weighed 100 mg and added to 1ml of H_3PO_4 followed by being crushed and vortexed vigorously and further centrifuged at 10,000 rpm for 15 minutes at 4˚C. Later supernatant was collected separately. For filtration, 500µl of H_3PO_4 was added to it. 20 µl of this filtered sample was used for SCFA profiling.

Standards used for acetate, propionate and butyrate profiling were in the sodium acetate, sodium propionate, and sodium butyrate form respectively and they were acquired from SIGMA-ALDRICH, USA.

10mg/ml of stock was prepared for acetate, propionate and butyrate which was further diluted to 1mg/ml, 0.1mg/ml, 0.01mg/ml and 0.001mg/ml followed by filtration then standard run in HPLC column.

STATISTICAL ANALYSIS

In the form of mean ±SD, results were expressed. One way analysis of variance (ANOVA) was used for analysis of statistical difference between the means of various groups. T-test was performed using Graphpad Prism software (V 5.0) for the evaluation of statistical significance.

Table 3: Primers for Gene expression.

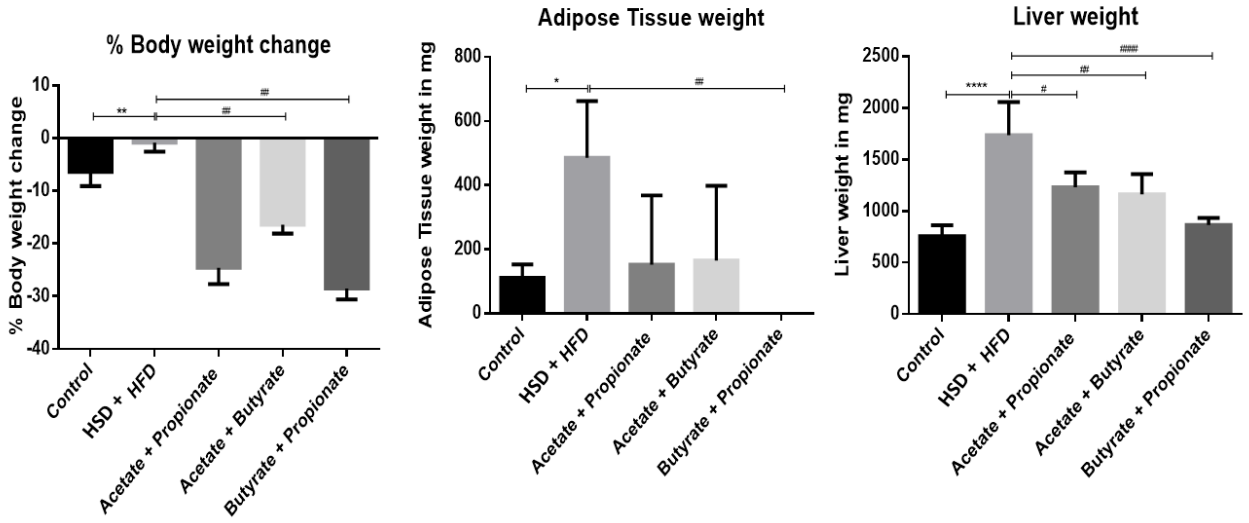
GENES	FORWARD PRIMER **(FP)**
	REVERSE PRIMER **(RP)**
LXR	**FP:-** GTTTCACCGTGTCCTTTGTG
	RP:- CCTGTTGACTCTCCCTTAATGC
SREBP-1C	**FP:-** GCAGCCACCATCTAGCCTG
	RP:- CAGCAGTGAGTCTGCCTTGAT
ChREBP	**FP:-** CCTGAAGACCCTAAGACCAAG
	PR:- TCTAAGCCATGCACCTTGAC
GPR41	**FP:-** TCCAAGTTCCAAGCCGAC
	RP:- TGCGGTCCACTCTTTTCTC
GPR43	**FP:-** AGGTTTGCTACTGATCCGC
	RP:- GTACCCCTTCTGCTTGACTTC

RESULTS

Body weight and tissue weight

There is a significant increase in the body weight of HSD+HFD group mice in comparison to control which is correlated with the increase in the adipose tissue weight and the liver weight in HSD+HFD mice, i.e, these mice are undergoing lipedemia.

After treatment, significant decrease in body weight is seen in AB and AP combination along with the significant decrease in adipose tissue and liver tissue weight. Therefore, it suggests that change in lipid balance has impact on reducing steatosis.



Graph 1: Body weight and tissue weight

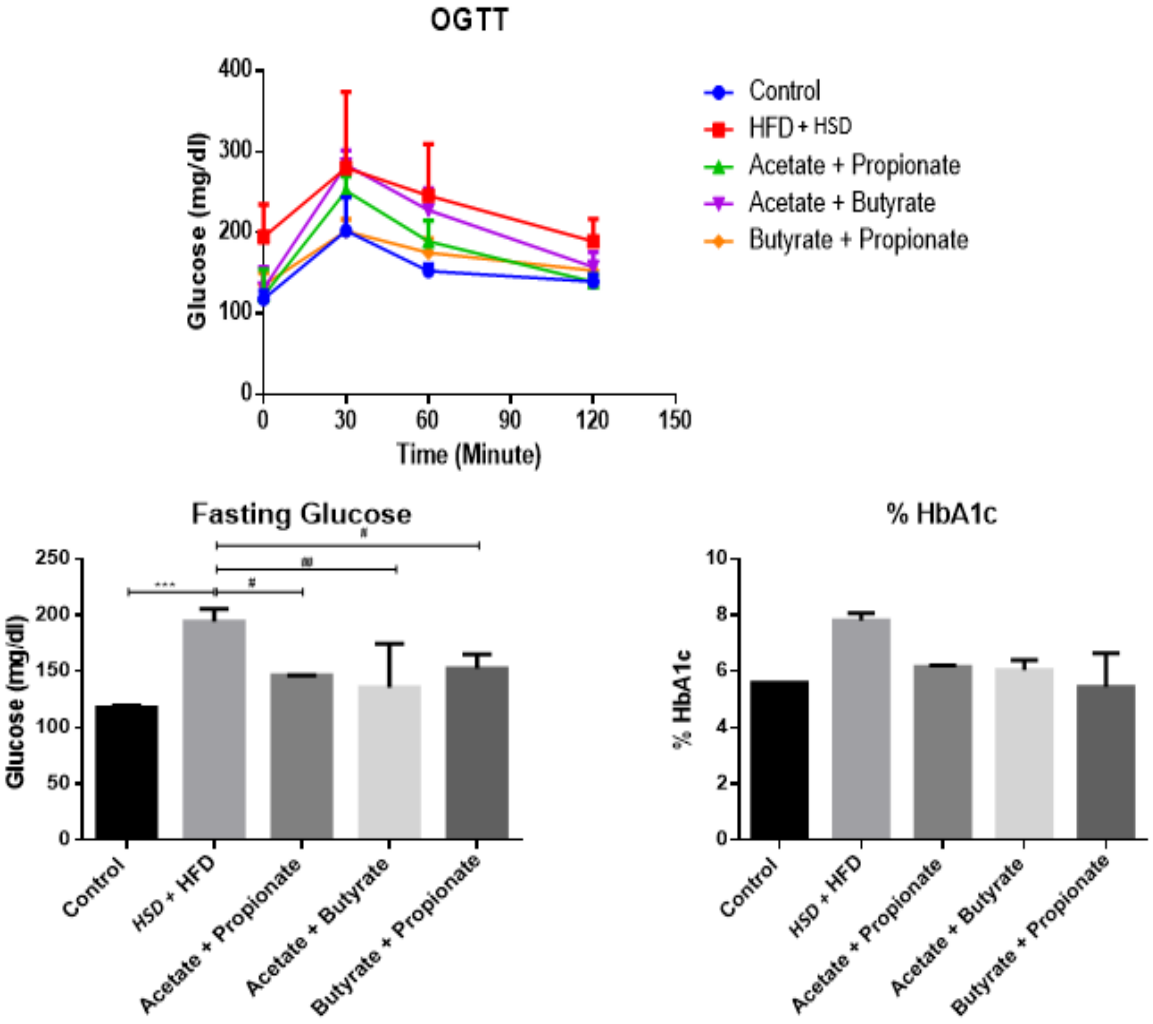
Biochemical parameters

In mice, after an oral glucose intake, the increased changes in plasma glucose concentration were seen. An increased peak of plasma glucose was seen at 30min after oral administration of glucose. The 30min peak was higher in HFD in comparison to control also, after 2 hours the glucose levels are higher than 140mg/dl that confirms the hyperglycemic condition in HFD+HSD group. Also, fasting plasma glucose is above 126mg/dl which is diagnostic of diabetes.

After treatment, 30min peak were largely seen to be decreased in BP followed by AP and lastly AB. 2hours OGTT values of all treated group is below 140mg/dl that confirms the reversal from hyperglycemia to normal but the difference is larger in BP group.

Fasting glucose were seen to be equal in all 3 treated group and it is under normal values i.e. < 126mg/dl.

Our HbA1c results doesn't show much significant changes but treated group had shown decrease % HbA1c than HFD+HSD group.

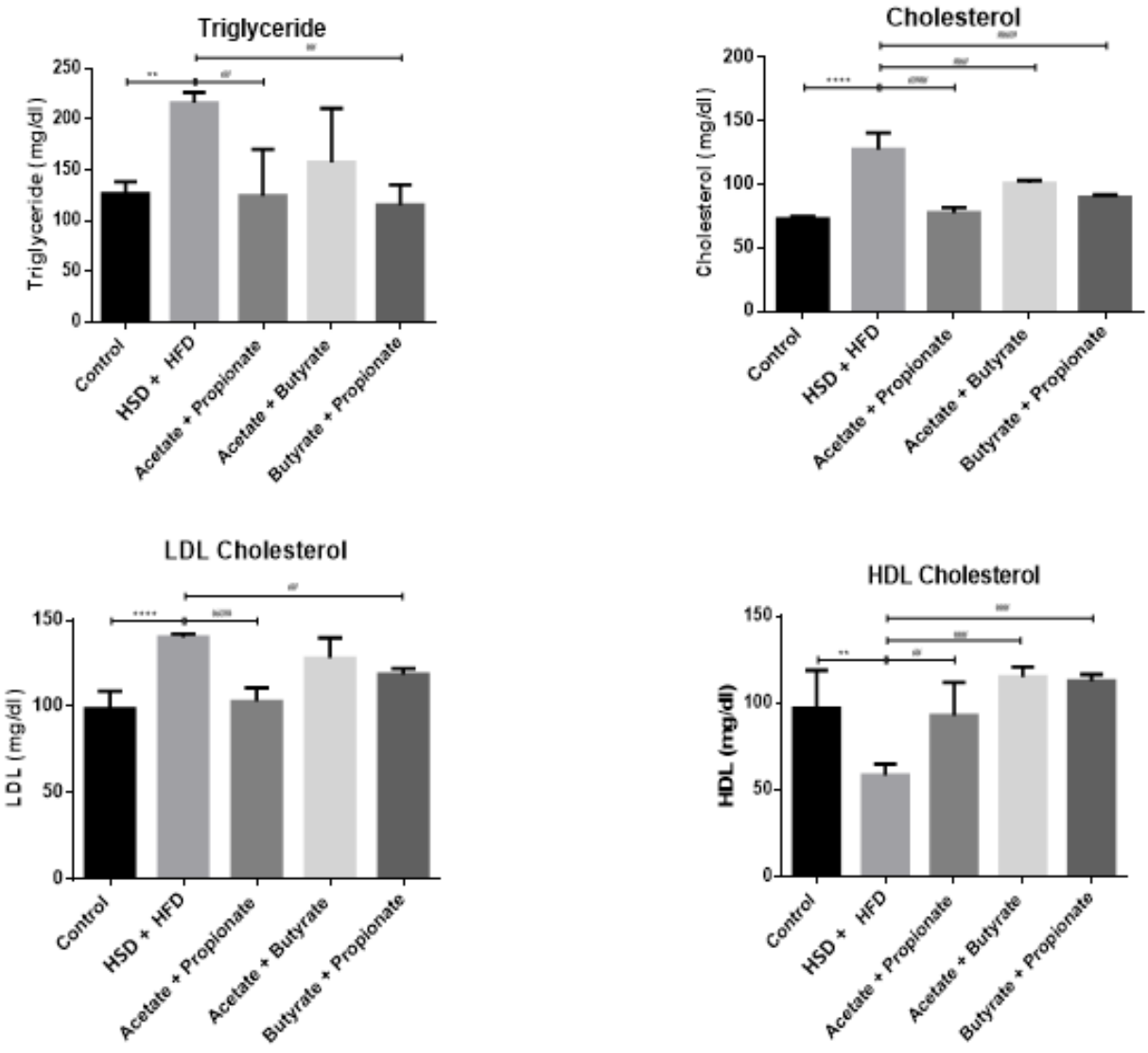


Graph 2: Biochemical perameters

Lipid profile

Elevated TG, Cholesterol and LDL Cholesterol levels in HFD+HSD group is associated with hyperlipidemia and insulin resistance, decreased HDL cholesterol confirms it.

After treatment, all the treated group shows decrease in TG , Cholesterol and LDL but more of the siginficance was seen in BP and AP group. HDL (good cholesterol) was also elevated in all group but it is highly significiant in AB and BP and this helps lowers LDL in these group.

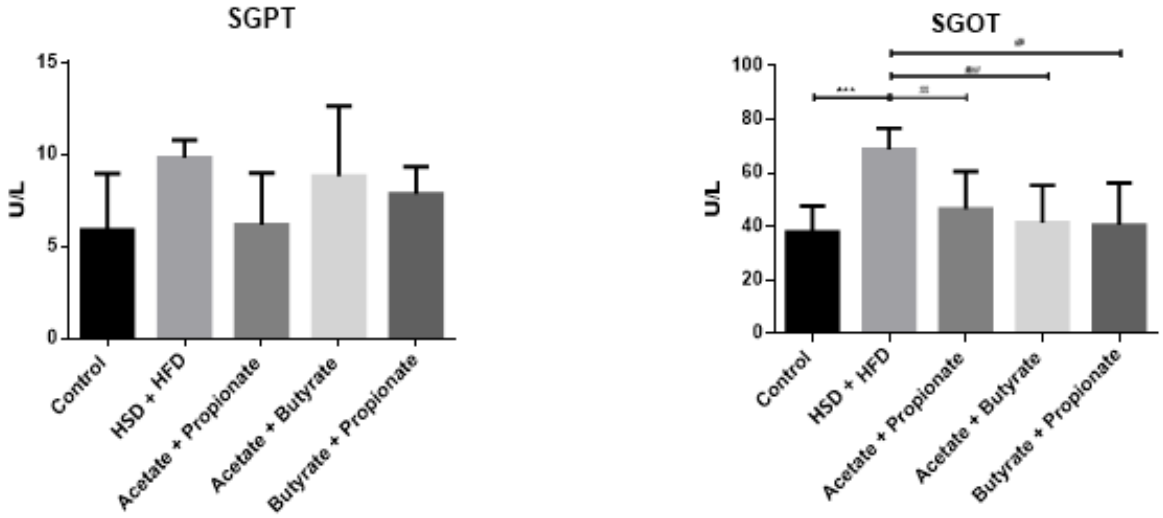


Graph 3: Lipid profile

Serum Biochemistry

SGPT (Serum glutamate pyruvate transaminase) is an enzyme which is normally present in liver and released into the blood when liver cells get damaged. In HFD+HSD group, there is poor blood glucose control, fat gets deposited in liver, leading to rise in SGPT/SGOT. In SGPT, there is no significant result seen.

SGOT (Serum Glutamate Oxaloacetate Transaminase) shows significant elevation in HFD+HSD group which is correlated with liver inflammation or liver disease. Significant decrease was seen in treated group which reaches normal range 5-40 units/lit specifically in AB and BP group.



Graph 4: Serum Biochemistry.

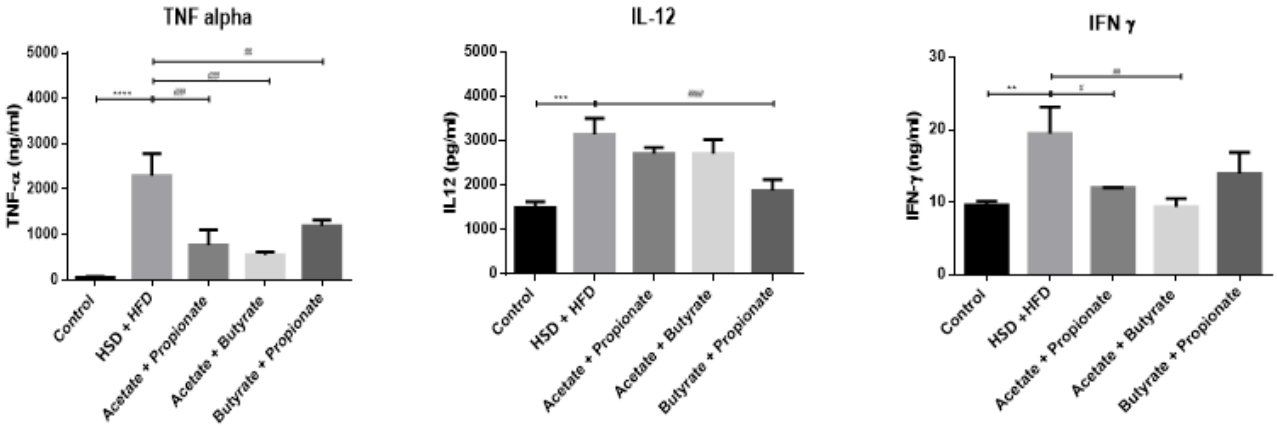
Cytokines Profile

TNF- α is one of the important pro-inflammatory mediator. There is significant increase in the TNF- α in the HFD+HSD group, and it is directly linked with IR and pathogenesis of T2D.After SCFAs combination treatment, significant decrease in TNF-α was seen in all of the group but mainly in AB group that suggests decreased pro-inflammatory response.

IL-2 is pro-inflammatory cytokine involved in differentiation of T-cells into Th1 cells. IL12 stimulates the production of TNF- αand TNF-γ. In HFD+HSD group, high significance was seen

in increased IL-12. But all the groups does not show significant reduction in IL-12, only BP has significant reduction ($p<0.001$).

INF-γ along with TNF-α gets elevated in HFD+HSD group, as it is pro- inflammatory cytokines. The decrease was seen in AB group after treatment.



Graph 5: Cytokine Profile

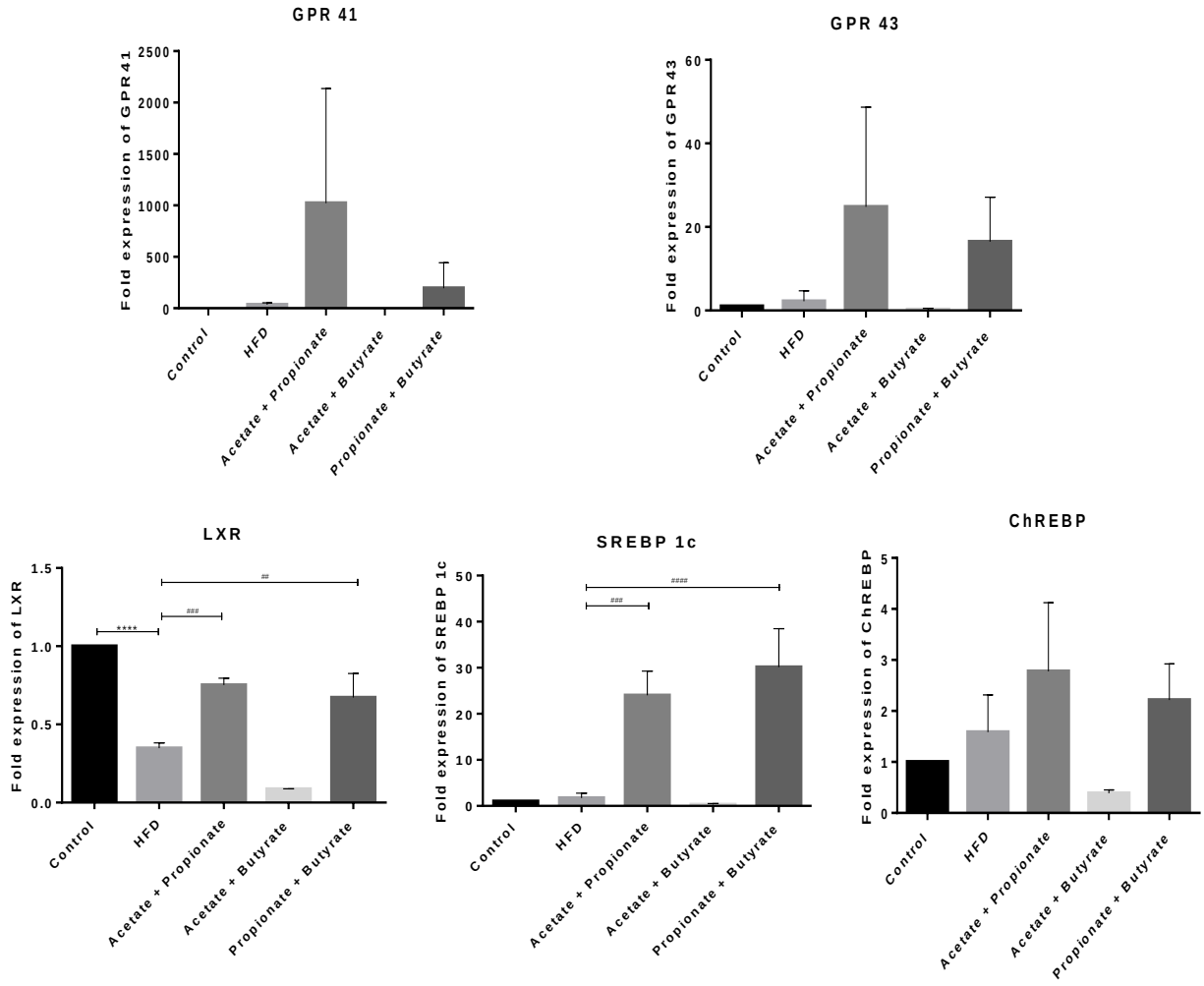
Gene Expression

Gene expression of SCFAs receptor GPR41 and GPR43 shows elevation in AP and BP group although it is not significant. (Graph 6)

SREBP1c and ChREBP are transcription factors that regulate glycolytic and lipogenic genes. Expression of ChREBP shows elevation in HFD+HSD group because its diet contains high fructose and sucrose which leads to hyperglycemic condition while reversal is seen only in AB group although it is not significant.

SREBP1c is regulated by insulin, in HFD+HSD group hyperinsulinemia takes place which leads to increased expression in SREBP1c although it is not significant. Reversion is gene expression of SREBP1c is seen in AB group.

LXR doesn't show increase in HFD+HSD group which is not supporting reported papers.

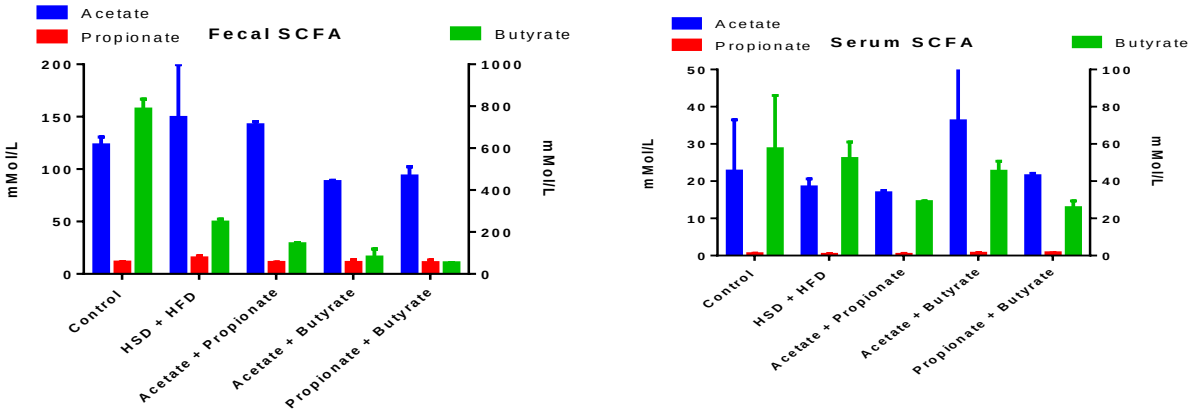


Graph 6: Gene Expression

SCFA profile

As shown in Graph 7 control has higher butyrate concentration than HFD+HSD while HFD+HSD has increased acetate which is further reduced in treatment group that gives the signal of restoration of altered gut flora.

In the serum, both decrease in acetate and butyrate were found in HFD+HSD group in compared to control while acetate is shown to be increase in AB group along with the butyrate. This is not supporting the liver SCFAs receptor expression.



Graph 7: SCFA Profile

HISTOPATHOLOGY

Histopathological analysis of pancreas was performed to know the effect of HFD+HSD and responding change due to SCFAs. In case of HFD+HSD, increase in size of islet cells and Acini cells integrity was seen compared to control.

In treatment group, results were found in AB and BP where Islet cells again gets compact and small integrity of acini cells gets normalized in comparision to HFD+HSD. But in AB, necrosis was seen where islet cells gets spread.

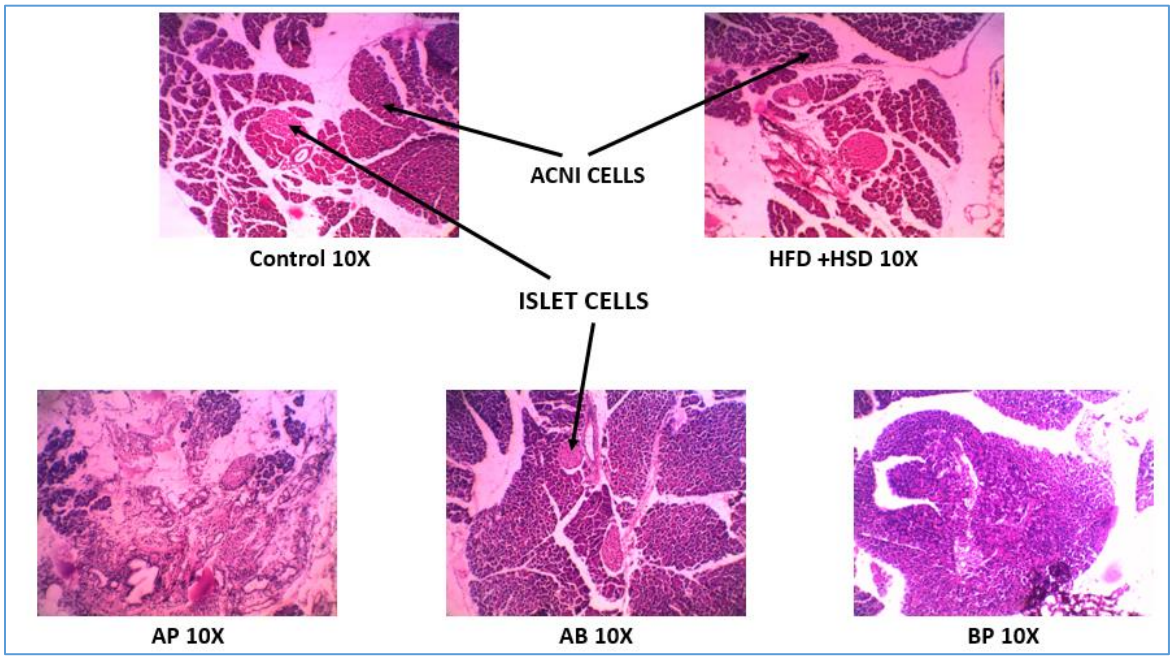


Figure 12: Histopathological slides

DISCUSSION:

In T2D, Hyperglycemic and Hyperlipedemic condition takes place where there is elevation in blood glucose level along with the fat accumulation in tissues occurs, thus increased body weight is seen. In our results, blood glucose level were reduced in all combination along with the reduction in body weight and liver and adipose tissue weight, which is highly significant in BP group.

TG and cholesterol and LDL values which gets elevated in diabetic condition gets reduced when treated with these three combinations along with which HDL values increases to lower LDL.

Liver inflammatory markers SGPT and SGOT is reported to be increased in diabetic condition and it is directly linked with liver steatosis. When fed with treatment diet, the values reaches to normal in AP and BP group that confirms reduction in inflammation.

SCFAs, gut microbial product has role in conserving host health thus we are trying to study the expression of its receptors i.e. FFAR2 and FFAR3 which is responsible for improving insulin sensitivity. Other genes regulating glucose and lipid metabolic pathways are ChREBP, SREBP and LXR. These genes are reported to be increased in diabetic condition. After treatment ChREBP and SREBP was seem to be reduced back in case of AB while LXR results doesn't follow the trend which is reported earlier.

The gut microbiota dysbiosis leads to inflammatory pathway. TNFα, pro inflammatory cytokines along with phosphorylated NFkB is pathogenesis of T2D. Elevated TNFα seems to be reduced in all treated group but highly significant in AP. IFNγ reduction was also seen in AP.

DNA isolation bands were seen but there was no further samples to carry out further analysis.

CONCLUSION:

The data from our present study indicates that combination of SCFAs can be promising for treatment of type 2 diabetes. Out of all the treatment strategy, BP and AB could improve insulin resistance and reduce inflammation. All the biochemical parameters shows significant changes highly in BP followed by AP. Cytokines profile then confirms the reduction in inflammation in AP.

SCFAs can itself be grouped together for treatment studies or for further studies, single SCFA can be grouped with hetero anti-diabetic compound having different targets for the good combination treatment for improving type 2 diabetes.

SUMMARY

Type 2 diabetes (T2D) is a multi-factorial disease prevailing around the globe. The more preferred strategy for treatment of type 2 diabetes is combinational therapy.T2D and gut flora plays an important role in regulation of gut function and changes in gut microbiota. Alteration of gut flora leads to change in microbial products such as metabolites, lipopolysaccharides (LPS), short chain fatty acids (SCFAs) that affect inflammation, insulin resistance (IR) and progressive deterioration of beta cell function. In the present study, T2D was induced using a mixed diet i.e. HFD+HSD.

Treatment or reversal of the diabetic characteristic was attempted with three different combination diet i.e. the effect on various biochemical parameters, gut microbiota and its signaling pathways were studied in both induced and treatment group. After diabetic induction, SCFAs were given as following combinations AB(ace5%+but10%), AP(ace5%+prop10%), BP(but10%+prop10%).

After 60 days of treatment using SCFAs combination, biochemical parameters, ELISA for cytokines, gene expression studies were performed to confirmed the reversal of T2D. There is significant decrease in body weight % change, fasting glucose and HbA1C in AB and BP treated group when compared with HFD. OGTT reaches to the normal values in BP group.TG, cholesterol, LDL is decreasing in all the treated group while HDL increases, in comparison to HFD. Decrease in SGPT and SGOT values in AB and BP group confirms reduced liver inflammation. AB and BP shows significant reduction in cytokine expression of TNF α and IL12 and IFNϒ. After gene expression studies it was found that BP group has higher SCFAs receptor expression. The expression of ChREBP, SREBP1c and LXR were found to be lower in AB group which confirmed reduced IR.

From the above results, we can conclude that SCFAs in combination can be preferred for treatment of T2D, out of which AB and BP are most promising combination and also one of the dissertation group found that acetate and butyrate can be used as a mono therapy as well. SCFAs itself can be used as a compound or SCFA can be grouped with another anti diabetic compound for better treatment of T2D.

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