Chapter II

Review of Literature

2.1: Gut microbiota

Mammals are co-evolved with the microorganisms of the surrounding environment and each individual consists of very unique set of the microorganisms, known as the microbiota **(Ley et al, 1025).** The human body contains an immense number of fungi, archaea, viruses and bacteria, where they live in the peaceful coexistence with their hosts **(Savage, 120; Neish, 72).** The composition and functions of the microbial communities have been extensively studied in the recent era of the time. However the role of viruses and archaea are still unclear. It is predictable that the human microbiota contains 10^{14} bacterial cells, which is almost 10 times higher than the number of human cells present in our bodies **(Hsiao et al, 857).** Microorganisms initially started to colonize at each surface of the body, which is later exposed to the external environment. Microorganisms flourish on the skin and in the genitourinary, gastrointestinal as well as respiratory tracts **(Verstraelen et al, 3; Chiller et al, 171).** The gastrointestinal tract is the most profoundly colonized organs. Colon alone is estimated to contain more than 70% of all the microorganisms in the human body **(Savage et al, 121).**

The gut microbiota is mainly composed of the strict anaerobes, which dominate the facultative anaerobes and aerobes by two to three orders of magnitude **(Gordon and Dubos, 252).** It has been reported that more than 50 different bacterial phyla reside in the gut. However, the human gut microbiota is mainly dominated by Bacteroidetes and Firmicutes phyla. Other phyla, such as Proteobacteria, Verrucomicrobia, Fusobacteria, Actinobacteria and Cyanobacteria are found to be present in minor proportions **(Schloss et al, 687; Eckburg et al, 1636).** Recent research involving large number of human subjects has suggested that, the group of human microbiota is composed of more than 35,000 bacterial species **(Frank et al, 13782).** The human stomach contains 10^1 to 10^3 bacteria per gram of content, while small intestine contains 10^4 to 10^7 bacteria per gram, which progresses to 10^{11} to 10^{12} bacterial cells per gram in the colon **(Serikov et al, 860)** (Figure 2.1). It was suggested that the enrichment of the different bacterial groups had differed at different sites when comparing the biopsy samples of the small intestine and colon from the healthy people. The bacilli class of the Firmicutes and Actinobacteria was found to be enriched in the small intestine, whereas, Bacteroidetes and the Lachnospiraceae family of the Firmicutes were found to be more prevalent in the colonic samples **(Frank et al, 13781).**

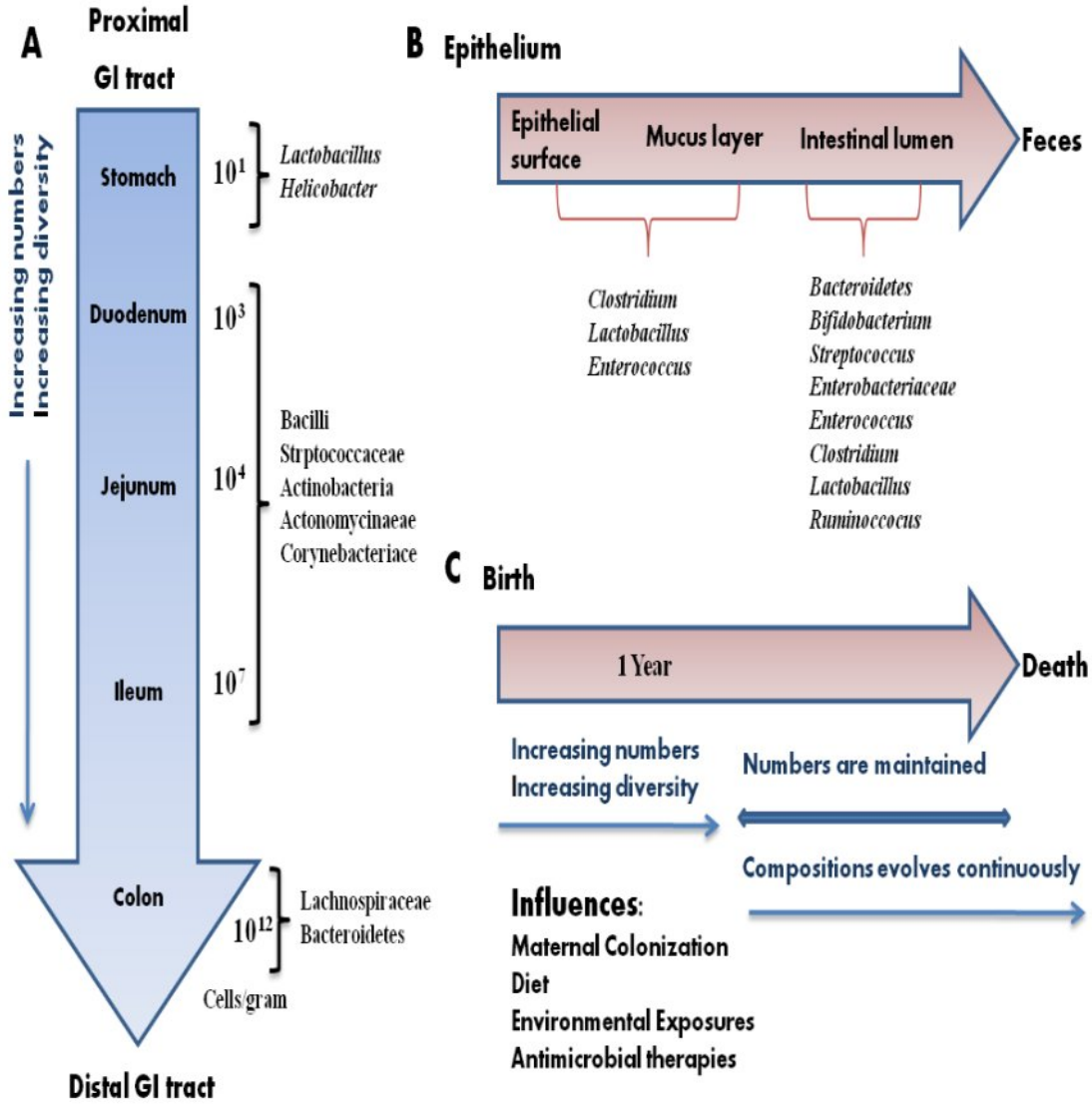

Figure 2.1: Diversity and distribution of the gastrointestinal microflora. (A) Distribution of the microflora from stomach to colon (B) Distribution of the microflora on the gastrointestinal epithelial layers (C) Effects of the various external factors on the gut dominant microflora **(Serikov et al, 860)**

The gut microbiota plays various pivotal functions in the human body, such as vitamin synthesis, intestinal motility, bile acid transformation and enterohepatic cycling and cholesterol metabolism. Many essential vitamins produced by the gut microbiota, such as folate and biotin are involved in the epigenetic regulation and maintenance **(Morowitz et al, 773).** The gut microbiota influences

gut motor functions through various mechanisms, such as by releasing the bacterial fermentation end products through mediators released by gut immune systems as well as by gut neuroendocrine factors **(Quigley, 142).** The number of reports state that intestinal microbiota may control the host cholesterol metabolism through mechanisms, such as bile acid modifications which affect enterohepatic circulation, *de novo* synthesis of bile acids, cholesterol absorption and inhibition of lipoprotein lipase enzymes **(Chiang, 1194).** However, the most important function of the gut microbiota is their role in the metabolism of the non-digestible complex dietary components. The important metabolites, i.e. SCFAs have been produced by the anaerobes within the through the fermentation of endogenous (e.g., epithelial-derived mucus) and exogenous (i.e., dietary) substrates. Metabolism of the major SCFAs, such as acetate, propionate, butyrate and lactate has been carried out by the host tissues, including the liver, muscle and brain. However, half of the energy produced by these organs is taken up only by the colonocytes as a fuel. These metabolites not only provide energy and nutrients for the bacterial growth, but also recover the energy for the host. One report suggested that, germfree mice had exhibited a reduced uptake of glucose in the intestine and require greater caloric intake to sustain normal body weight as compared with colonized controls **(Backhed et al, 15718).**

The byproducts of microbial metabolism, which are generated through the conversion of carbohydrates to organic acids provide up to half of the energy requirement of colonocytes **(Lopez et al, 157).** Gut microbial interactions are mainly complex and fluid, which are capable of adjusting to various physiological perturbations that are encountered on a regular basis. However, the host pathobiology, alterations of diet, medications, and other environmental factors can trigger the major and selective shifts in the gut microbiota, which can upset critical inter-microbe as well as host-microbe relationships in turn to initiate the pathophysiological processes and diseases **(Sun et al, 134).** According to several reports, dysbiosis of the gut microbiota can be associated with the pathogenesis of both intestinal as well as extra-intestinal disorders. Intestinal disorders include IBD, irritable bowel syndrome (IBS), colorectal cancer and celiac disease. On the other hand, extra-intestinal disorders include allergy, asthma, metabolic syndrome, cardiovascular disease, obesity and T2D **(Carding et al, 03).**

2.2: Gut microbiota and energy harvest

The role of the gut microbiota in the energy harvest and storage of the ingested nutrients was supported by the studies conducted in germfree mice. One research demonstrated that, when germfree mice were introduced with the gut microbiota of the conventionally raised mice receiving normal chow diet had resulted in a 60% increase in the body fat and developed IR within 2 weeks of the duration. They also increased the chow diet consumption by 29% and decreased the insulin activity by 27% as compared with germfree mice **(Turnbaugh et al, 1028).** It was consequently observed that germfree mice had acquired the obese phenotype when the intestinal microbiota from the conventionally raised obese mice was transferred into them **(Turnbaugh et al, 1029).** These observations support the hypothesis that the composition of the gut microbiota affects the amount of energy extracted from the diet. An increased diet intake and body fat was accompanied by adipocyte hypertrophy, IR and increased levels of circulating leptin as well as glucose. An increased fecal energy harvest was found to be observed by the obese mice compared to their lean counterparts. Various mechanisms have been identified, which link the gut microbiota with increased fatty acid metabolism and storage of the fat. These include: (I) intestinal absorption of monosaccharide following the fermentation of indigestible polysaccharides to produce SCFAs, along with increased hepatic lipogenesis via carbohydrate and sterol response-element binding proteins (II) suppression of fasting-induced adipocyte factor (FIAF), a circulating inhibitor of lipoprotein lipase, which catalyzes the release of fatty acids from triacylglycerol which are then taken up by skeletal muscle and adipose tissue (III) suppression of adenosine monophosphate-activated protein kinase (AMPK), a cellular fuel which is activated in response to the metabolic stresses such as exercise, hypoxia, and glucose deprivation (IV) the interaction between products of polysaccharide fermentation (i.e., SCFAs) and GPR41 expressed by enteroendocrine cells in the gut epithelium reduces the expression of neuropeptides YY(PYY), an enteroendocrine cell–derived hormone which inhibits gut motility, thereby increasing intestinal transit rate and, possibly reducing nutrient contact time **(Backhed et al, 979; Forbes et al, 3764).**

2.3: Diabetes and inflammation

According to the IDF survey, diabetes is the chronic endocrine disorder, which has affected more than 415 million people in 2015 and further it is estimated to increase upto 640 million by 2040 all around the world **(IDF, 2015)**. Obesity and T2D are commonly associated with low-grade systematic inflammation, which is considered by the activation of the immune system. This increases the release of the adipocytes derived bioactive metabolites like FFAs, lipids and proinflammatory cytokines **(Shi et al, 3017; Jung et al, 6186).** During the early 1950s, there were some evidences, which suggested a strong correlation between inflammation and IR. However, the exact mechanism was not defined. Since past some years, researchers have found that, IL-1β, TNF-α, IL-6 and CRP are the major acute phase responsive inflammatory proteins, which are the strongest predictors for the development of T2D **(Gani et al, 69).** The inflammation originates from the adipose tissue through the secretion of several bioactive molecules, such as cytokines, fatty acids and chemokines, which activates the macrophage infiltration. Various proinflammatory cytokines were found to be overexpressed in the adipose tissues of rodent models in obesity **(Jung et al, 6185; Makki et al, 02).** TNF-α was found to be overexpressed in the adipose tissue as well as the skeletal muscles of the obese human subjects **(Uysal et al, 610).** The body weight of the obese people was found to be significantly reduced by decreasing serum TNF-α levels and improved insulin sensitivity **(Uysal et al, 611)**. Administration of recombinant TNF-α into cultured cells or in whole animals has been found to produce an impaired insulin signaling pathway. An obese mouse model lacking a functional TNF-α receptor or its fragment has shown an increase in the insulin sensitivity as compared to their wild-type counterparts. Thus, it could be concluded that over expression of TNF-α and other inflammatory cytokines from the adipose tissue is a significant marker for the obesity, which in turn lead to the progression of IR **(Uysal et al, 4833).** An increased expression of inflammatory cytokines might change the structural conformation of the insulin receptor through phosphorylation of the serine or threonine moiety, thus insulin cannot bind to the receptor, which downregulate the IRS pathway and develop IR **(Savage, 830).**

IL-6 is secreted by various cell types, such as monocytes, fibroblasts, endothelial cells and macrophages. Adipocytes isolated from obese people showed an increased expression of IL-6, which can be associated with the extent of IR **(Weisberg et al, 1798).** Adipose tissue IL-6 mRNA expression has been shown to be increased in diabetic human, which can be correlated with decreased rate of insulin stimulated glucose uptake **(Kristiansen et al, S115).** IL-6 plays an important role in driving the acute inflammatory response and orchestrates the production of acute-phase proteins. Circulating IL-6 levels have been correlated with IR and a marker of endothelial dysfunction **(Tabit et al, 62).** However, moderate increase in the plasma levels of the bacterial LPS, an inflammatory agent elevated through a fat or sugar-enriched diet creates metabolic endotoxemia. One group has reported that LPS, a major component of the outer membrane was found to be responsible for the onset of metabolic diseases **(Cani et al, 1762).** The highly insulin resistant Asian individuals have shown an increased circulatory LPS levels, LPS activity and tight junction proteins with positive correlation with inflammatory markers and poor glycemic/lipid control **(Jayashree et al, 203).**

2.4: Gut microflora and diabetes

Gut microbiota have been recently identified as an environmental factor involving in the energy homeostasis and progression of the disease conditions. A healthy human gut contains complex association of various microorganisms, which plays a major role in the regulation of metabolism and inflammation. Gut microbiota participates in the development of the obesity and diabetes, thus manipulation of it can be an important therapeutic tool in diabetes care **(Everard and cani, 75).** According to various metagenomic studies, gut microbiota composition was found to be altered between healthy and diabetic subjects. T2D is associated with the alteration in certain gut dominant microbial bacteria, such as *Bifidobacterium* and *Lactobacillus.* Moreover, Firmicutes to Bacteroidetes ratio was also found to be altered between healthy and diabetic subjects. Diabetic children have shown significantly reduced quantity of the bacteria, which are essential for regulating the gut integrity as compared to healthy control **(Murri et al, 01).** The

qPCR analysis has shown significant alteration in three strains, i.e. *Bacteroides vulgatus*, *Bifidobacterial* genus and *Clostridium leptum* subgroup at different degrees in the diabetic group of animals, whereas copy number of *Bifidobacteria* was significantly reduced **(Wu et al, 69)**. Patients with T2D were characterized by moderate gut microbial imbalance with reduction in butyrate-producing bacteria, an increase in opportunistic pathogens and enhancement of the bacterial functions, such as spot reduction as well as oxidative stress resistance **(Qin et al, 55)**. One research investigation has shown the reduced amount of the Firmicutes phyla and Clostridia class in a T2D animal group compared with control, whereas β-proteobacteria was found to be highly enriched in the diabetic group **(Larsen et al, 05)**. Gut metagenomic composition study between T2D and normal subjects have found the increased number of *Lactobacillus* species, while the decreased amount of *Clostridium* species in the diabetic group **(Karlsson et al, 96).**

Several evidences have discovered that T2D patients demonstrated an altered intestinal microbiota, which was characterized by the reduction of Bacteroidetes/Firmicutes ratio and some useful bacteria (e.g. *Bifidobacteria*) with an increased number of various endotoxin-producing Gram-negative bacteria **(Schwiertz et al, 190; Hildebrandt et al, 1716; Wu et al, 71).** Germfree mice having similar age and genetic profile as conventional mice had shown 40% lower weight gain upon feeding with normal chow diet, whereas germ-free mice colonized with the gut microbiota derived from the conventional mice increased their fat mass and developed IR within 2 weeks. It has been shown that body weight gain and fat accumulation is suppressed in high fat diet consuming germfree mice, leading to the lowered glycemia and insulinemia **(Turnbaugh et al, 1028).** Obese mice were characterized by the 50% reduction in the population of Bacteroidetes and an increased population of Firmicutes as compared with lean mice. However, diabetic mice have shown an opposite trend with an increased abundance of Bacteroidetes and decrease in Firmicutes **(Ley et al, 11072).** Similarly, obese human subjects have shown less bacterial diversity, reduced Bacteroidetes/Firmicutes ratio along with higher abundance of the pro-inflammatory Proteobacteria population **(Million et al, 100).**

2.5: Diet and gut microflora

The mammalian gut microbiota composition is strongly influenced by a variety of factors, including, birth acquired microbial species, host genetics, immunological factors, antibiotic usage and dietary effects **(O'Mahony et al, 263; Turnbaugh et al, 02)**. Diet can have a major and distinct impact on the gut environment, including gut transit time and pH of the lumen. Moreover, differential intake of the three main macronutrients (carbohydrate, protein and fat) can significantly affects the gut microbiota composition. Changes in the dietary pattern have shown a significant impact on the composition of the intestinal microbiota. In fact, dietary changes could reveal around 57% of the total structural difference in the gut microbiota, whereas changes in the genetics accounted for not more than 12% **(Zhang et al, 232).** Metagenomic studies focusing on the fecal microbiota composition of the vegetarian people versus omnivorous diets revealed that the omnivorous were enriched with the bacteria belonging to the *Clostridium* cluster X, which represent the major butyrate-producing microorganisms. In contrast, the gut microbiota of vegetarian people was observed to be enriched with Bacteroides and *Faecalibacterium prausnitzii* species **(Kabeerdoss et al**, **953**; **Dominguez-Bello et al, 11973).**

Western diet is defined by a very little presence of the complex fibers and a high amount of fat and refined sugars. This kind of diet has been observed to promote the scarcity of major fiber-degrading bacteria, such as *Prevotella*, *Succinivibrio*, *Treponema* and *Bifidobacteria.* In contrast, whole grain products not only exerts a bifidogenic effect with the enhancement of *Bifidobacteria,* but also assisted to increase the population of *Collinsella*, *Atopobium* and *Clostridium* cluster IV **(Jonnalagadda et al, 1012S**; **Ou et al, 114; Schnorr et al, 02).** The gut microbiota composition comparison between two human cohorts receiving either a plant or meat-based diet had highlighted the distinct profiles of the microbiota. Notably, the meat-based diet was shown to increase the abundance of bile-tolerant bacteria, such as *Alistipes*, *Bilophila* and *Bacteroides*, with a concomitant decrease of Firmicutes **(David et al, 559).** Long-term of diet intake containing high fruit and legume fibre, which is typically consumed by a rural agrarian community, is found to be associated with greater diversity and noticeable changes in the fecal microbiota

composition. A 'Western', high-animal fat/high-sugar diet (also typically low in fruit and vegetable fibre) had potentially decreased the amount of beneficial Firmicutes (such as the *Roseburia*/*Eubacterium* group and *Faecalibacterium* species, which ferment dietary plant polysaccharides to beneficial SCFAs) and promoted the growth of bacteria belonging to the Proteobacteria phylum including mucosa-associated enteric gut pathogens and pathobionts, such as adherent, invasive *E. coli* as shown in Figure 2.2. According to a research, the developments of the obesity-related inflammation depend on the effect on the gut microbiota composition through feeding of the lipids in various rodent models including mice **(Caesar et al, 658).** Mice fed with a high-lard diet for 11 weeks had developed the symptoms of metabolic disorders, while the mice fed with fish oil found to be remained healthy. When the scientists transplanted the gut microbiota of fish oil-fed mice to the antibiotic-treated mice and consequently given the lard diet. The mice were found to be protected from the typical detrimental effects of the saturated fat. The lard-fed mice had shown increased levels of *Bilophila*, *Turicibacter*, and *Bacteroides* in their guts, while the fish oil-fed mice had observed strangely with high levels of the lactic acid bacteria group, Actinobacteria, and Verrucomicrobia—a group containing *Akkermansia*, which has previously been associated with good metabolic health **(Simpson and Campbell, 158).**

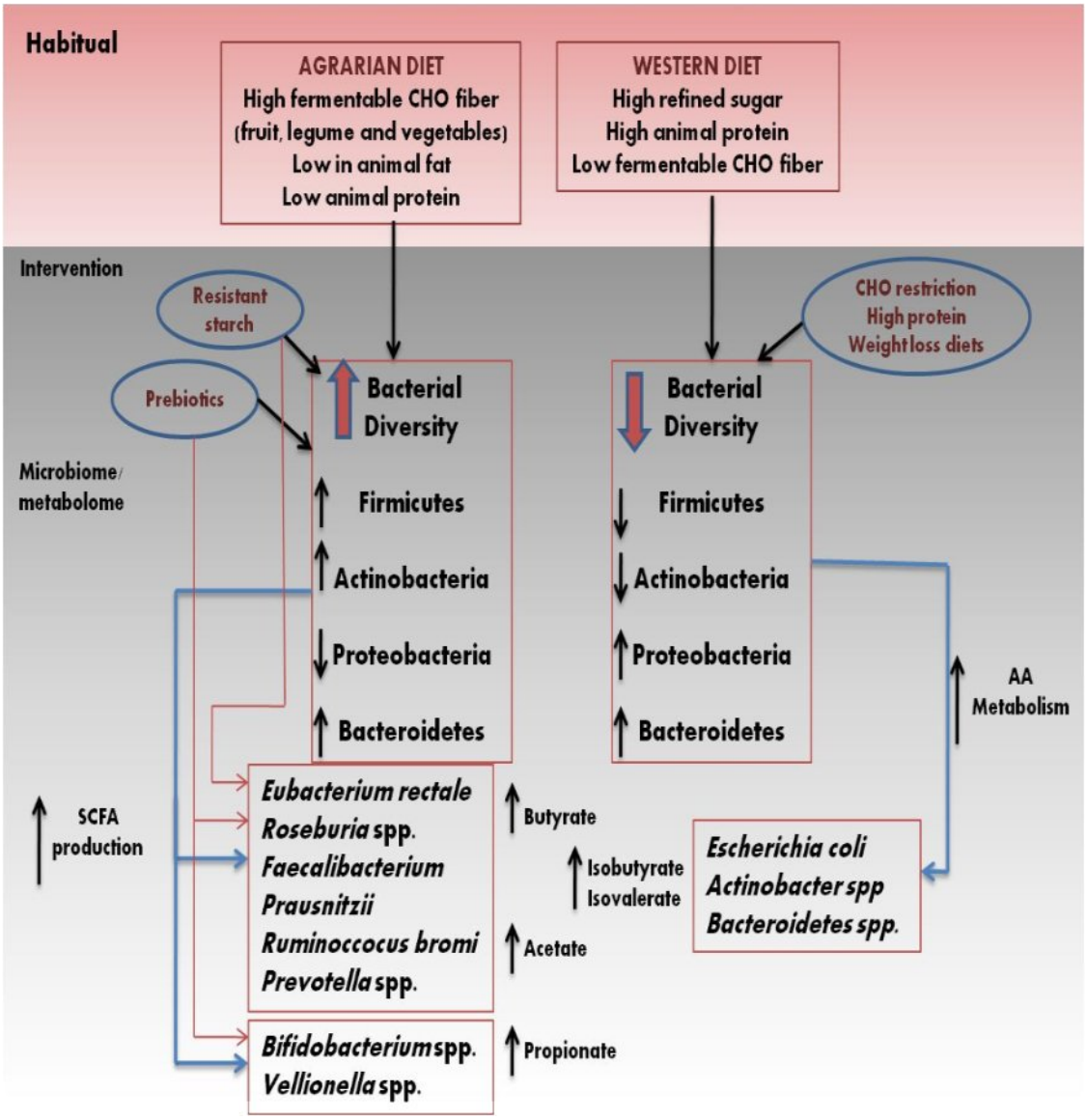

Figure 2.2: Overview of the long-term and short-term impact of dietary fiber on the intestinal microbiome and metabolome. AA, amino acid; AIEC, adherent, invasive *E. coli*; CHO, carbohydrate; FODMAP, fermentable oligo, di and monosaccharide and polyols; SCFA, short-chain fatty acids; spp., species **(Simpson and Campbell, 173).**

2.6: SCFAs receptors

The main function of the gut microbiota is to produce the metabolites i.e SCFAs through the fermentation of non-digestible complex carbohydrates. Acetate, propionate, and butyrate are the most abundant SCFAs **(den Besten et al, 2325).** All these SCFAs are quickly assimilated into host carbohydrates and lipid derivatives to provide ~10% of our daily energy supplies. However, there are marked differences in the way through which they are metabolized. Butyrate is the major intestinal fuel, which supplies ~60–70 % of the energy needs of the isolated colonocytes. Moreover, acetate, propionate and butyrate stimulate the epithelial cell proliferation and its differentiation in rats. SCFAs might affect the gut and host metabolism via the activation of two main GPCRs, such as GPR41, also known as free fatty acid receptor 3 (FFAR3) and GPR43, also known as free fatty acid receptor 2 (FFAR2). Propionate mainly acts as a precursor for gluconeogenesis, whereas acetate and butyrate are rather incorporated into fatty acids and cholesterol metabolism **(Le poul et al, 25481)**. SCFAs regulate metabolism by inhibiting the histone deacetylases and chain length–dependent activation of the endogenous GPR41 and GPCR43 along with serving as an energy source **(Samuel et al, 16767; Vinolo et al, 859).** The long chain butyrate is more selective for binding with GPR41, while the short length acetate is more selective for binding with GPR43 and propionate binds to both receptors **(Brown et al, 11312).** GPR41 is primarily activated by propionate followed by butyrate and acetate. GPR43 is activated by all three SCFAs at a similar rate. GPR41 and GPR43 proteins and mRNA have been found in human colonic tissue. Notably, both receptors are also expressed in human white adipose tissue, skeletal muscle and liver, which indicate that SCFAs might also affect substrate and energy metabolism directly in peripheral tissues. Furthermore, GPR109a is also known as hydroxycarboxylic acid receptor 2, which is found to be expressed in, adipocytes, immune cells and gut epithelial cells. It has been found to be activated by the butyrate, but not acetate or propionate **(Thangaraju et al, 2826; Jobin, 08).**

2.7: Regulation of fatty acid metabolism by SCFAs

SCFAs regulate homeostasis amongst fatty acid synthesis, fatty acid oxidation, and lipolysis in the body. Fatty acid oxidation is activated by SCFAs, while *de novo* synthesis and lipolysis are inhibited by them. This process is resulted in a reduction of FFAs concentration in the plasma and a decrease in body weight **(Heimann et al, 81).** SCFAs have been shown to increase the AMPK activity in liver and muscle tissues **(Gao et al, 1511; Yamashita et al, 1236).** An activation of AMPK triggers peroxisome proliferator-activated receptor gamma co activator (PGC)-1α expression, which is known to control the transcriptional activity of several transcription factors such as peroxisome proliferator-activated receptors (PPARs), i.e. PPARα, PPARδ, PPARγ, liver X receptor (LXR), and farnesoid X receptor (FXR), all important in the regulation of cholesterol, lipid, and glucose metabolism **(Lin et al, 361; Jager et al, 12017)**. Hepatic fatty acid lipolysis seems to be unaffected by SCFAs, but lipolysis in adipose tissue is strongly reduced by SCFAs **(Ge et al, 4519).** In isolated adipocytes, acetate and propionate were found to inhibit lipolysis via ffr2 activation as shown in Figure 2.3 **(Yamashita et al, 1236).** The decreased rate of the lipolysis was in consistent with the reports from the human studies, which suggested that intravenous administration of acetate and propionate reduced plasma FFAs and glycerol. An inhibition of the lipolysis mediated by FFAR2 is most likely through the inactivation of the hormone-sensitive lipase (HSL), which hydrolyzes triglycerides and is one of the key molecules controlling lipolysis in adipose tissue **(Carmen and Victor, 402).**

2.8: Regulation of glucose metabolism by SCFAs

The results from dietary intervention studies in humans indicate that, SCFAs might be used to regulate energy intake and body weight. In an acute crossover trial with healthy volunteers, an intake of 10 gm of inulin propionate ester increased satiety and reduced appetite, as measured via

visual analogue scales for hunger and satiety, compared with inulin alone **(Al-Lahham et al, 357).** In several *in vitro* studies using intestinal cell lines from rodents and humans, SCFAs stimulated the secretion of PYY and GLP-1 from L-cells in a GPR41 and GPR43 dependent manner. Moreover, the use of rodent knockout animal models has highlighted the importance of the SCFA receptor GPR43 in GLP-1 and PYY secretion. Knock out of GPR43 in mice lowers *in vivo* basal levels of active GLP-1 by ~43% (P<0.01) and blunts GLP-1 levels after glucose gavage by ~47% (P<0.01), compared with wild-type littermates **(Chambers et al, 1745).** SCFA might also beneficially affect the control of body weight by influencing energy expenditure. In obese mice, oral administration of sodium butyrate, leads to the loss of body weight, via an increased energy expenditure and fat oxidation. Intracolonic infusions of SCFAs in rats and pigs increased blood concentrations of PYY, but unfortunately no data on glucose metabolism was reported **(Cherbut et al, G1417).** GLP-1 indirectly regulates blood glucose levels by increasing the secretion of insulin and decreasing the secretion of glucagon by the pancreas as shown in Figure 2.3 **(Canfora et al, 581)**. Intracolonic infusions of SCFAs and intake of fibers increased the plasma GLP-1 concentrations and glucose uptake by the adipose tissue **(Zhou et al, E1160)**. In addition, mice lacking FFAR2 or FFAR3 exhibited a reduced SCFA-triggered GLP-1 secretion *in vitro* and *in vivo*, and a parallel impairment of glucose tolerance **(Tolhurst et al, 364).**

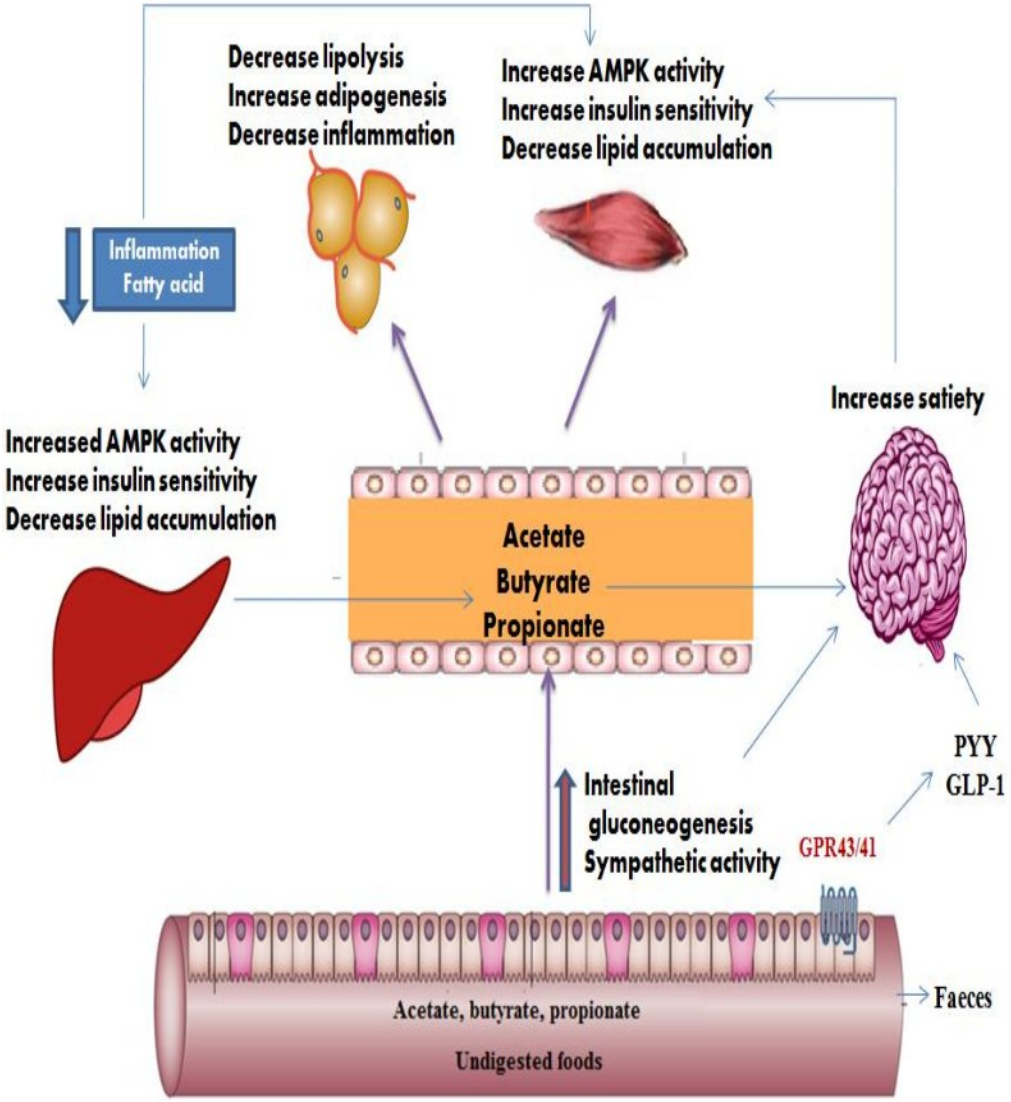

Figure 2.3: SCFA and inter organ crosstalk. Fermentation of indigestible foods in the distal intestine results in the production of SCFA **(Canfora et al, 581).**

2.9: Pattern recognition receptors (PRRs)

The human immune system is one of the leading properties that nature has bestowed on us to fight against various pathogens. Vertebrates are frequently endangered by the attack of dangerous microorganisms and have evolved immune arms to remove the pathogens from the body. The vertebrate immune system mainly made up of two arms: innate and acquired immunity **(Bayne, 297).** The innate immune system is a germ encoded, non specific host defense system against foreign particles, which is mediated by phagocytes, macrophages and other immune cells. Acquired immunity mainly involves the specific elimination of the pathogens with the generation of immunological memory **(Akira, 784; Kurtz, 190).** Detection of microbial pathogens is a fundamental factor for the initiation of the innate immune responses such as inflammation, which is mediated by various germ-lines encoded extracellular or intracellular PRRs. PRRs identify certain molecular structures, which are mostly shared by the pathogenic microorganisms known as PAMPs and Danger associated molecular patterns (DAMPs). DAMPs are secreted by the stressed cells undergoing necrosis, can act as danger signals for promotion of inflammatory response. Upon recognition of PAMPs and DAMPs, PRRs initiate a series of signaling events which perform the first line of host defensive responses, essential for killing infectious microorganisms **(Kawai and Akira, 319).** Four different classes of PRRs families, such as TLRs, NLRs, c-type lectin receptors (CLRs) and cytoplasmic proteins, such as retinoic acid-inducible gene (RIG)-I-like receptors (RLRs) have been identified **(Boller and Felix, 381).** TLRs and NLRs are the most extensively studied PRRs acknowledged to be involved in inflammation induced IR.

2.9.1: Toll like Receptors (TLRs)

The mammalian and mouse TLRs comprise of a large family consisting of 11 and 13 members respectively. TLRs are divided into major two groups depending upon their cellular localization; a group which is expressed extensively on cell surfaces, includes TLR1, TLR2, TLR4, TLR5, TLR6, TLR11 and another group is comprised of TLR3, TLR7, TLR8 and TLR9, which is expressed solely on intracellular vesicles like endoplasmic reticulum, endosomes, lysosomes and

endolysosomes. Different types of TLRs sense different types of ligands and attach to the various types of adaptor proteins to start signaling events, which lead to the activation of the inflammatory process (Figure 2.4) **(Kawai and Akira, 376).** TLRs are mainly characterized by an extracellular leucine-rich repeat (LRR) domain, which is involved in the recognition PAMPs through a cytoplasmic Toll/IL-1 (TIR) domain that activates downstream signaling adaptor protein molecules, including MyD88, IRAKs and TRAF6 **(Akira et al, 677).** TLR2 recognizes a variety of microorganism derived products like lipoproteins from several pathogens, peptidoglycan moiety from both Gram positive and Gram negative microorganisms, zymosan from some fungi and lipoteichoic acid from certain Gram positive microorganisms **(Smith et al, 35)**. It also recognizes LPS of certain non enterobacteria, such as *Leptospira interrogans*, *Porphyromonas gingivalis* and *Helicobacter pylori* species **(Takeuchi et al, 935).** TLR4 is a major receptor found in the recognition of a variety of ligands derived from the Gram-negative bacteria. LPS is an important integral component of the outer membrane of Gram-negative bacteria which acts as a main ligand for TLR4. It has been shown to be involved in the recognition of a variety of endogenous ligand derived from cellular stress or necrosis, which includes DAMPs like heat shock proteins (HSPs), mainly HSP60 and HSP70, the extra domain of fibronectins proteins, oligosaccharides moiety of hyaluronic acid, heparin sulfate and fibrinogen at some lesser extent.

A relationship between gut microbiota and inflammation in the pathogenesis metabolic disorders has been identified in the recent era of time. Many dietary factors such as lipids, FFAs and glucose along with gut microbiota alteration trigger the progression of metabolic disorders by activating the TLRs and NLRs. Significant increase in TLR2 and TLR4 expression along with their ligands was found at both mRNA and proteins levels people with T2D **(Dasu et al, 861).** High fat diet induced inflammation was found to be reduced in TLR2 deficient mice, which resulted in increased insulin-stimulated glucose transport in cultured adipocytes **(Davis et al, 138).** The TLR2 knockout mice were protected from the harmful effects of high fat diet compared with TLR2 positive controls. It improved glucose tolerance and insulin sensitivity with decreased macrophage infiltration and inflammatory cytokine expression after 20 weeks

of high fat diet feeding. This suggests the molecular link between enhanced dietary lipid intake and maintenance of glucose homeostasis **(Himes and Smith, 733).**

Short-term inhibition of TLR2 mRNA expression by TLR2 oligonucleotides antisense in diet-induced obese mice leads to a downregulated signaling pathway and increased insulin sensitivity **(Caricilli et al, 399).** Other studies have reported that TLR2 knockout mice exhibited a decreased body weight and adiposity along with protection against IR, weight gain and co-morbidities on high fat diet than control mice **(Song et al, 739).** Murakami and colleagues analyzed the over-expression of TLR2 and TNF-α in isolated adipocytes from obese mice using flow cytometry, which was significantly associated with IR **(Murakami et al, 727).** Reyna and colleagues have shown that FFA concentration was directly associated with an increase in TLR4 gene and protein expression, which can be correlated with the IR condition in obese and diabetic subjects **(Reyna et al, 2595).** Furthermore, researchers have shown that the TLR4 deficient mouse strain fed on a diet rich in saturated fat were protected from systemic inflammation **(Davis et al, 1248).** Another data reported that saturated fatty acids like palmitate stimulates both TLR2 and TLR4 signals in insulin (INS-1) cells, showing the involvement of TLR2 and TLR4 in fatty acid induced IR. An activation of the stress-related JNK pathway through TLR4 stimulation was involved in palmitate-induced INS-1 cell death, which suggests that activation of an innate immunity signal may be involved in fatty acid induced lipotoxicity in cells **(Lee et al, 321).** An increase in the palmitate levels among all the other circulating FFA species would be the key to clearly demonstrate the activation of inflammation via the TLR4/myD88 pathway which results in cellular dysfunction **(Eguchi et al, 518).**

The surface expression of TLR2 and TLR4 was significantly increased in monocytes in type 1 diabetes (T1D) patients and *db/db* mice at mRNA levels via palmitate and LPS induction, while decreasing by pioglitazone treatment. Other findings also demonstrated that expression and activity of the functional receptors of TLR2 and TLR4 were increased in the monocytes of patients with metabolic syndrome, which could also be contributed to the increased risk for metabolic disorders **(Fabre et al, 01).** NOD proteins are important innate immune components

which are involved in diet-induced inflammation and IR. Acute activation of NOD proteins by mimetics of bacterial peptidoglycan (PGN) causes whole-body inflammatory IR *in vivo* by altering both glucose tolerance and glucose production. NOD1/2 or double knockout mice are more insulin tolerant compared with wild type control mice following 16 weeks of high fat diet, which was evidenced by decreased insulin tolerance. After the high fat diet, all these mice showed lower gonadal adipose and liver masses compared with wild type control mice with reduced adipose size. Thus, it could be concluded that mice were protected from the high fat-induced IR, lipid accumulation and inflammation in adipose tissue, skeletal muscle and liver **(Schertzer and Klip, E585).** NOD1 activator like bacterial PGN motifs caused acute systemic IR in mice, which further suppressed insulin action in the liver and isolated hepatocytes as well as decreased insulin-mediated glucose uptake in adipocytes **(Tamrakar et al, 5624).**

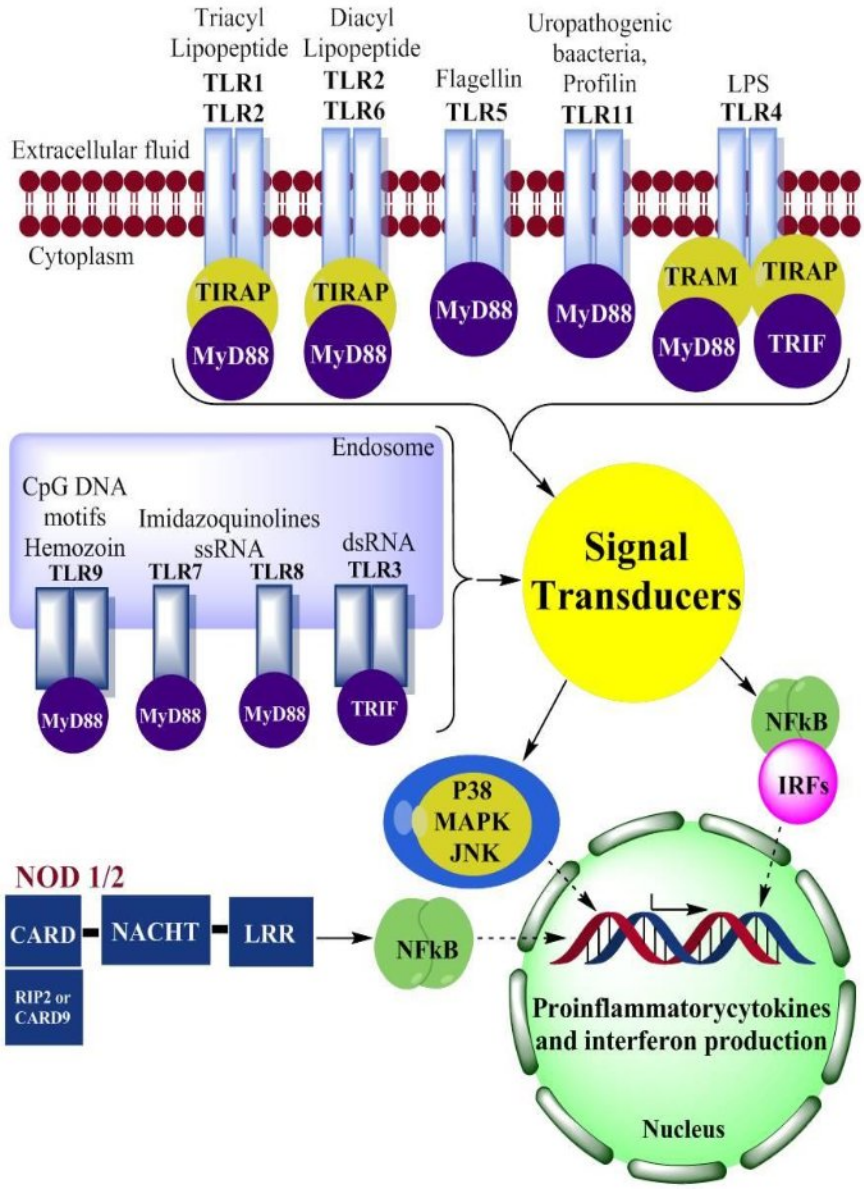

Figure 2.4: TLR and NLR signaling pathway: TLR1-11 have been identified in human for recognition of various microbial components and localized on a plasma membrane and endosomes **(Akira et al, 678)**

2.9.2: NOD like Receptors (NLRs)

The cytosolic NLRs known as nucleotide-binding oligomerization domain (NOD) containing receptors are a specialized group of intracellular proteins, which represent a main component of the host innate immune system. This family of proteins is defined by a tripartite structure mainly consisting of (a) variable N-terminal protein-protein interaction domain, known as caspase recruitment domain (CARD), pyrin domain (PYD), acidic transactivating domain, or baculovirus inhibitor repeat (BIR), (b) a central NOD domain, which mediates oligomerization with own that occurs during activation and (c) a C-terminal leucine-rich repeat (LRR) that detects PAMPs **(Chen et al, 365; Ting et al, 288).** NOD1 is also known as CARD4, which recognizes various substructure of the peptidoglycan, namely iE-DAP (-D-glutamyldiaminopimelic acid),which is found to be present in both Gram-negative and Gram-positive bacteria but specifically, senses the Gram negative bacteria. NOD2 is also known as CARD15, which recognizes muramyl dipeptide (MDP), largest active component of peptidoglycan motif, which is present in both Gram-negative and Gram-positive bacteria. Upon recognition of their respective ligands, both NOD1 and NOD2 self-oligomerize to recruit and activate the adaptor protein RIP2, which is necessary for the activation of both NF-κB and the MAPKs pathways (Figure 2.4) **(Hasegawa et al, 376; Abbott et al, 2219; Hayden and Ghosh, 2198).**

Peptidoglycan motifs that acts on NOD2 induces muscle cell- autonomous IR suggests that NOD2 alone is capable of acutely inducing IR within muscle cells, possibly through the activation of endogenous inflammatory signals and/or through cytokine production, restricting upstream insulin signals. Therefore, NOD1 ligand-mediated IR seems to involve cross talk between cells from the various tissues, likely adipose and hepatic, with indirect manifestation in skeletal muscle **(Tamrakar et al, 5626).** Amar and his co-workers have observed the bacterial translocation towards adipose tissue and blood only after one week of high-fat diet mediated inflammation induction. This translocation is prevented in mice lacking the microbial recognition receptors NOD1 or CD14, suggesting that these receptors have important roles in the development of the low-grade inflammatory state that characterizes IR **(Amar et al, 562).** Administering PGN-based NOD1 agonists to adipocytes of wild type mice leads to the activation of inflammatory cytokines, impairing insulin signaling and decreasing insulin-stimulated glucose uptake, but such effect was found to be absent in NOD1 knockout mice

(Zhao et al, E589). Along with NOD1 and NOD2, the list of innate immune components involved in IR is growing and also includes NOD-like receptors such as NLRP3 **(Vandanmagsar et al, 181).** It is a daunting but important task to understand how components of the immune system coordinate inflammation resulting in IR.

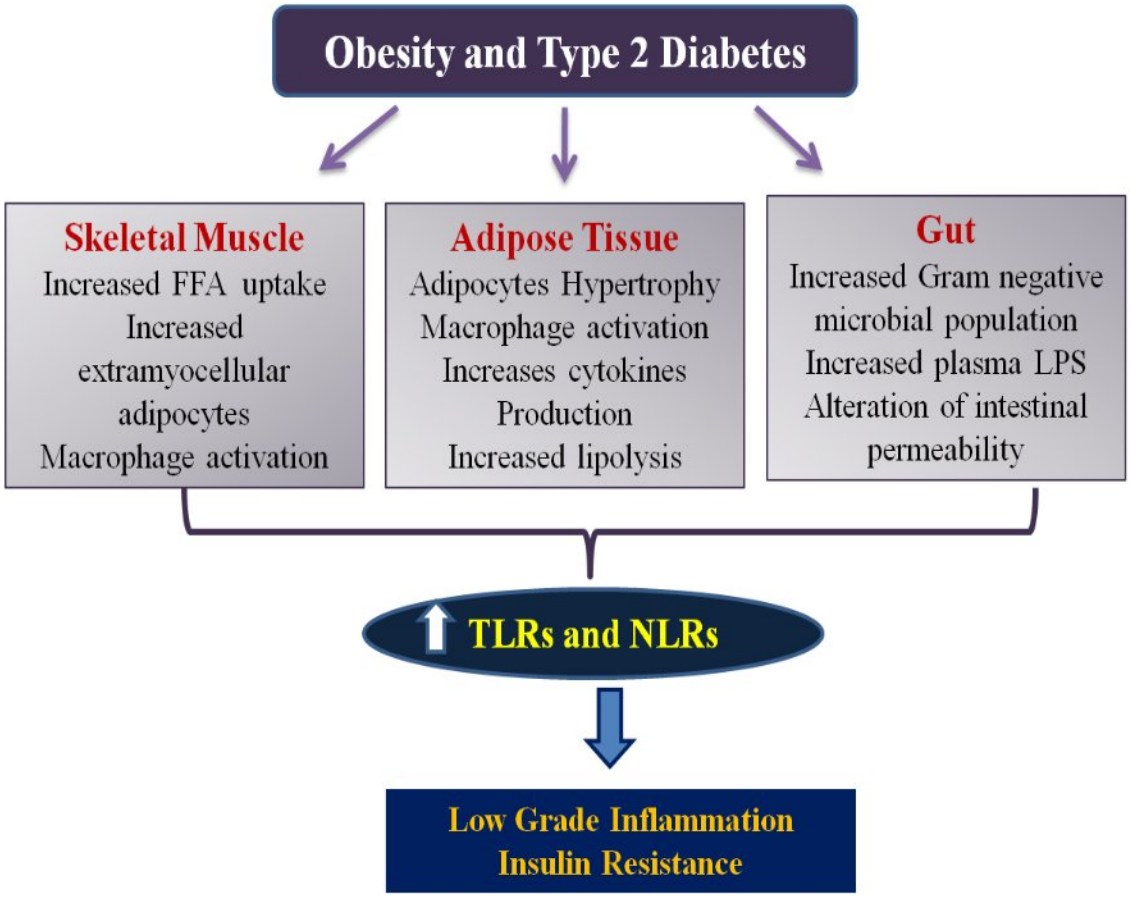

Figure 2.5: Obesity mediated development of inflammation and insulin resistance. Obesity induced alteration in the skeletal muscles and adipose tissue, increase the plasma FFAs. Obesity is also involved with changing in gut microbiota composition towards an increase in gram negative microbial population. Both FFAs and LPS triggers the up-regulation of the TLRs and NLRs mediated inflammatory signaling pathway, which further activates NF-κB and leads to the development of inflammation mediated IR.

2.10: Gut microbiota modulation for management of metabolic disorders

Various evidences from the recent researches have linked the dysbiosis of the healthy gut microbiota composition for the development of metabolic diseases. Thus, various approaches have been proposed for restoring the gut microbiota, which can be developed as an emerging and fascinating therapy **(Cammarota et al, 365).**

2.10.1: Modulation with probiotics

Probiotics administration is the one of the approaches for the modulation of the gut microbiota for the management of various disorders. Live microorganisms, which exert beneficial health effects on the host after entering the gut by improving the intestinal microbial balance, can be termed as probiotics **(Forsythe, 901).** The *Lactobacilli* and *Bifidobacteria* are most commonly used probiotics strains. They are considered safe for human ingestion with very few or no cases of the adverse effects. Numerous reports from animal and human studies suggest that alteration of gut microbiota through ingestion of probiotics can be an efficient strategy to treat metabolic diseases **(Haukioja, 350).** Application of genetically engineered strains of *Escherichia coli* in Caco-2 cell line had resulted in the secretion of insulin and insulinotropic protein such as GLP-1 **(Duan et al, 7437).** One study found that, administration of a probiotic containing *Lactobacillus reuteri* significantly reduced the elevated levels of serum insulin, leptin, glucose, c-peptide, liver injury markers, glycated hemoglobin and lipid parameters in high fructose-fed rats **(Hsieh et al, 2).** An administration of *L. plantarum*, *L. acidophilus* and *L. casei* in high fat diet induced obese mice and alloxan-induced diabetic rats, resulted in the suppression of body weight, hyperglycemia, insulin and lipase activity, reduction in non-esterified fatty acids and drop in adipocyte derived IL-1β mRNA expression **(Arasu et al, S7; Sakai et al, 144; Park et al, 145).** Probiotic

supplementation modulated gut microbiota composition with improved glucose tolerance, increased GLUT4, PPAR-gamma and lipogenic genes mRNA expression as well as a reduced expression of pro-inflammatory markers such as IL-6 and TNF-α in fructose-fed rats **(Zhang et al, 4).**

2.10.2: Modulation with prebiotics

In addition to probiotics, gut microbiota can also be modulated by administration of prebiotics. Prebiotic is a food ingredient or carbohydrates resistant to the digestion, absorption and degradation in the upper digestive tract that promotes to the growth of intestinal beneficial microorganisms **(Wollowski et al, 451S).** Prebiotic administration in obese mice noticeably lowered fasting glucose, improved glucose tolerance and leptin sensitivity, enhanced levels of satiety hormones, reduced low-grade inflammation and muscle fat content. These effects were linked with an altered gut microbiota composition, i.e. decreased abundance of Firmicutes and increased number of Bacteroidetes **(Parnell et al, 709)**. Honey, natural product enriched in oligosaccharides has been shown to increase the glycemic control and improve the metabolic disturbances in diabetic rats **(Kajiwara et al, 214).** Inulin has shown to increase bacterial deconjugation of bile acids, increase the villus height in the colon with increased cecal weight **(Kuo et al, e60270).** Laminarin or fucoidan supplementation decreased the population of *Enterobacteriaceae* and the abundance of attaching *Escherichia coli*, which improved the gut intestinal barrier functions in pigs **(Walsh et al, 1630).** These prebiotics had also significantly downregulated the colonic mRNA expression of proinflammatory cytokines **(Walsh et al, 4121).** Baer and colleagues had reported that soluble fiber feeding had increased the weight gain in high fat induced obese mice, while insoluble fiber feeding had reduced the body weight **(Baer et al, 579).** These findings seem to indicate that in addition to the non-digestible carbohydrates, other constituents of prebiotics play a role in altering gut microbiota composition and subsequently improving the metabolic disorders.

Chitosan is a polymer of glucosamine, which is obtained from deacetylation of chitin. Chitin is widely distributed in nature, mainly in the crustaceans, insects and fungal cell walls. It is widely used in pharmaceutical industries for drug delivery purposes due to its unique properties such as biodegradability, mucoadhesiveness, easy availability, biocompatibility and non-toxicity **(Ahmed and Aljaeid, 483).** It exhibits various biological activities, such as antitumor, wound healing and broad spectrum antimicrobial activity against Gram negative, Gram positive bacteria and fungi **(Ueno et al, 105; Xu et al, 5103).** It also exerts prebiotic properties by reducing the harmful bacteria in gut to promote good digestion and immunomodulation **(Lee et al, 319).** Chitosan has been reported for its hypocholesterolemic and hypolipidemic activities in rats (Zhang et al, 489). Chitosan nanopowder prepared from high and low molecular weight chitosan has shown significant hypolipidemic activities in rats **(Zhang et al, 487).** Its administration has been reported to reduce gluconeogenesis and increased the glucose uptake by skeletal muscle in streptozotocin-induced diabetic rats. It also accelerated the proliferation or neogenesis of ß cells and increased the insulin secretion in the pancreas **(Liu et al, 5795; Chiu et al, 2980).** One recent report studied that, supplementation of chitosan alleviates high fat diet induced lipogenesis in rats via adenosine monophosphate (AMP) activated protein kinase activation and inhibition of lipogenesis-associated genes **(Chiu et al, 2979).** However; the effect of chitosan on gut microflora mediated signaling pathway in diet induced diabetes is needed to be understood.

2.10.3: Modulation with antimicrobial agents

The supplementation of antimicrobial agents including broad-spectrum antibiotics is a different possible alternative to modulate the gut microbiota, which causes a diminution of microbial biodiversity and maintenance of the gut colonization by microbes. Administration of two antibiotics i.e ampicillin and norfloxacin in a combination had significantly improved the fasting glucose, glucose tolerance and IR **(Membrez et al, 2421).** These improved metabolic parameters were linked with the altered gut microbiota, which reduced the plasma endotoxin, liver triglyceride and increased hepatic glycogen storage **(Membrez et al, 2420).** Antibiotic treatment

had reduced the fasting insulin, LPS, hepatic lipid and inflammatory markers such as TNF-α in a mice fed with high fat or sugar diet **(Carvalho et al, 2824; Bergheim et al, 986).** The antibiotic administrated mice had increased colonic total SCFAs levels and gastric inhibitory polypeptide (GIP). Other recent studies have also demonstrated the beneficial effects of antibiotics on the metabolic abnormalities in obese mice, which were positively associated with the reduced diversity of gut microbiota **(Bech-Nielsen et al, 503; Murphy et al, 223).** The vancomycin administration had decreased the major Gram-positive and Gram-negative microbial genera in NOD mice, but increased the abundance of *Akkermansia muciniphila* **(Hansen et al, 2286).** Gut microbiota modulation with Cefdinir Microspheres had significantly improved IR, glucose intolerance, triglycerides, and hepatic damages in rats fed with a high sugar diet. These findings not only provided a firm theoretical basis regarding how antibiotics demonstrated their effectiveness for treating the diabetes, but also shed light on the modulation of selective gut microbiota as a potential therapeutic approach for the management of T2D **(Jena et al, 3820).**

2.11: Classification of antimicrobial agents

Antibiotics are designed to interrupt the growth of or kill bacteria. They are drugs that are derived from or chemically produced by microorganisms like bugs, fungi or bacteria. They are classified in a variety of classes, but the most effective classification is one derived from the chemical composition. Antibiotics are classified in major three classes based on the chemical composition and antibacterial spectrum **(Kuhn et al, 3).**

2.11.1: Fluroquinolones

Fluroquinolones are a class of synthetic broad spectrum antimicrobial agents which kills almost all microorganism including gram negative and gram positive bacteria. The effects of ciprofloxacin **(Brismar et al, 714),** sparfloxacin **(Ritz et al, 455),** moxifloxacin **(Samonis et al, 02)** and levofloxacin **(Ianniello et al, 168)** have been analyzed on intestinal microflora. Moxifloxacin is an oral broad spectrum quinolone antibacterial agent used in the treatment of bacterial infections caused by *S. pneumoniae, H. influenza*, *S. aureus*, *S. pyrogenes and K. pneumoniae.* It acts by inhibiting the DNA replication by acting on DNA gyrase and topoisomerase IV, which are essential for bacterial growth **(Drug bank, DB00218).**

2.11.2: Oxazolidinones

The oxazolidinones represent a novel chemical class of synthetic antimicrobial agents. They exhibit a unique mechanism of protein synthesis inhibition and display bacteriostatic activity against many important human pathogens, including methicillin-resistant *S. aureus*, vancomycin-resistant *enterococci*, and penicillin- resistant *S. pneumoniae* **(Diekema and Jones, 09).** Linezolid is totally synthetic narrow spectrum compound mainly against gram positive microorganisms. It has complete oral bioavaibility, favorable pharmacokinetics and toxicity profiles **(Drug bank, DB00601).**

2.11.3: Cephalosporins

Cephalosporins are class of beta lactam antibiotics originally derived from fungus, *cephalosporium*. Cephalosporins have bactericidal activity and it inhibits the peptidoglycan cell wall synthesis by inhibiting the final transpeptidation needed for cross-linking. According to their mechanism of action, they have classified into five generation drugs **(Yotsuji et al, 1849).** Cefdinir is a third generation cephalosporin with a broad spectrum activity against enteric gram

negative rods. It is rapidly absorbed from the gastrointestinal tract and almost entirely eliminated via renal clearance **(Drug bank, DB00535).**

2.12: Targeted drug delivery

A well designed controlled drug delivery system can overcome several limitations of conventional systems and enhance the therapeutic efficacy. To enhance the maximum therapeutic efficacy, it becomes necessary to deliver the drug at a target site with the appropriate dose showing least toxicity **(Singh and Lillard, 215).** Various formulations, such as nanoparticles, liposomes etc. have been developed for delivering a drug at a target site in a controlled fashion. One of the advanced approaches is using microspheres as a drug carrier. The microspheres are free flowing powder, consisting of proteins or synthetic polymers; which are biodegradable in the nature and ideally have a particle size less than 200 μM **(Saxena et al, 701; Nayak et al, 215)**. There are several advantages of the microspheres for the targeted drug delivery purposes as mentioned below **(Liggins et al, 1961):**

- Microspheres provide constant and prolonged therapeutic effect.
- They reduce the therapeutic drug dosing frequency to improve the patient compliance.
- The injection of the microspheres into the body is comfortable due to their spherical shape and comparatively smaller size.
- Better drug utilization improves the bioavailability and reduces the intensity of adverse effects.
- Microsphere morphology allows a controllable variability in degradation and drug release.

Many pharmaceutical dosage forms irritate the stomach due to their chemical properties. Others undergo chemical changes in gastric acid and through the action of enzymes **(Shen et al, 202)** Specific eudragit® acrylic polymers have been developed for peroral dosage forms with step-

wise release of active ingredients in the digestive tract. Eudragit have been used as pH-sensitive polymers in various applications including enteric coating materials and drug delivery vehicles and exhibited plastic deformation and significant speed sensitivity **(Tao et al, 4175).** Eudragit S-100 (ES) and Eudragit L-100 (EL) composed of methacrylic acid and methyl methacrylate (1:2, Mw= approx. 135,000), are pH-sensitive polymers owing to their unique dissolution behavior at pH 7.0 and 5.5 respectively. Eudragit in combinations with other polymers, such as hydroxypropyl methyl-cellulose, dextran and starch can also be used for the targeted drug delivery purposes. Gupta and colleagues have shown that enteric-coated epichlorohydrin cross-linked dextran microspheres had successfully delivered the 5-Fluorouracil (5-FU) at the colon milieu without any significant loss in the upper gastrointestinal tract **(Gupta et al, 240).** Rahman and colleagues had prepared sodium para amino salicylate pellets were coated with EL using fluidized bed processor and evaluated for *in vitro* dissolution behavior in 0.1 N HCl for two hours and then the media was changed to phosphate buffer pH 5.5. A 60% w/w coating level of EL has produced the most acceptable results against the gastric attack **(Rahman et al, 477).**

2.13: Objectives

1. To evaluate and determine the efficacy of various spectrum specific antibiotics on diet induced diabetes.

2. To prepare the target specific microspheres using the most effective spectrum specific antibiotics.

3. To evaluate and determine the efficacy of target specific antibiotics microspheres on diet induced diabetic animal models.