Chapter I

Introduction

The human gastrointestinal tract consists of very diverse and complex microbial communities, which plays a significant role in the maintenance of human health. This huge collection of the microbial communities is commonly referred as hidden metabolic 'organ' due to their enormous impact on human health, metabolism, physiology, nutrition and immune function. The human gut is mainly consisting of the four major dominant microbial phyla such as Firmicutes, Bacteroidetes, Actinobacteria and Proteobacteria. It hosts a huge number and varieties of microorganisms, including at least 10 phyla belonging to ~1,000 species **(Guinane and Cotter, 295).** The gut microbiota is dominated by bacteria; with more than 90% of the species belonging to Firmicutes and Bacteroidetes phyla with variable and diverse population, which changes from person to person. It has conserved set of core gut microbiota and the core microbiome, which are common among all the individuals and necessary for the gut functioning **(Ley et al, 1022; Dore and Corthier, S9).** Firmicutes are the largest phylum, with over 200 genera, majorly including *Mycoplasma*, *Lactobacillus*, *Clostridium* and *Bacillus* species **(Gerritsen et al, 211)**. The Bacteroidetes is composed of four major classes of Gram-negative, non spore forming, anaerobic or aerobic and rod-shaped bacteria which are broadly distributed in the gut. Actinobacteria are a Gram positive bacteria residing in the gut, which play a major role in the digestion of cellulose **(Flint et al, 297).** The gut microbiota is majorly distributed in the three areas of the gastrointestinal tract, i.e. stomach, small intestine and colon. Gut microbial species of these different locations play different functions in the human. The stomach contains a very little number of the microbial species due to its acidic environment **(Sekirov et al, 861).** The microbial

species of the small intestine play an important role in the absorption of the simple nutrient molecules such as sugars, while colonic microbial population has a main role in the fermentation process of the indigestible food molecules **(Guinane and Cotter, 296).** Diet plays a vital role in deciding the types of the microbes, which colonize the gastrointestinal tract. Diet affects the composition of the gut microbiota, which might regulate the immune and inflammatory responses. The gut microbiota composition has been observed significantly altered in the vegetarians and the westernized diet consuming human individuals. Increased proportions of the Bacteroides/Prevotella group, *Bacteroides thetaiotaomicron, Clostridium clostridioforme* and *Faecalibacterium prausnitzii* were observed in the vegetarian group. On the other side, fat consuming mice had shown increased levels of *Bilophila*, *Turicibacter*, and *Bacteroides* in their gut, while the fish oil-fed mice had contained unusually high levels of lactic acid bacteria, Verrucomicrobia and Actinobacteria. Verrucomicrobia is a group of microbes, which contains *Akkermansia* species that has been widely associated with the good metabolic health **(Caesar et al, 2).** One study focusing on the dietary intake of the obese individuals demonstrated that consumption of the low-carbohydrate diet resulted in the low concentration of butyrate in the fecal along with a low abundance of *Roseburia* and *Eubacterium rectale* species along with the other butyrate producing microbial group **(Duncan et al, 1073).**

An investigation of the fecal samples from 10 volunteers had revealed that there was a significant increase in the proportions of *R. bromii* and *E. rectale* in the volunteers group consuming the resistant starch. It also led to the increased abundance of *Bifidobacterium adolescentis* and *Parabacteroides distasonis* **(Martinez et al, 3).** The major problem of western diet, i.e. high fat and sugar (digestible saccharides) is that these nutrients are regularly absorbed in the duodenum, which leaves very few amounts of substrates for the colonic bacteria. Adequate intake of the fiber promotes the growth of the healthy microbiota that significantly lowers the predominance of inflammatory diseases mainly through the release of short chain fatty acids (SCFAs).One of the important activities of the gut microbiota is to break down the complex plant polysaccharides which, yield SCFAs according to the fiber content present in the diet through fermentation **(Binder et al, 307).** Two important groups of SCFAs producing bacteria are found within the

Firmicutes phyla, i.e. *Eubacterium rectale* and *Roseburia* species comprising of around 5–10% of the total microbiota **(Aminov et al, 6373).**

SCFAs are the final end products of fermentation of dietary fibers by the anaerobic intestinal microbiota, which have been shown to exert the number of beneficial effects on mammalian energy metabolism **(den Besten et al, 2325).** They are the saturated aliphatic organic acids, which consist of one to six carbon moieties as shown in Figure 1.1 **(Carnicom, 02).** Acetate (C2), propionate (C3), and butyrate (C4) are the most abundant SCFAs, which are present in an estimated molar ratio of 60:20:20 in the colon and stool samples **(Topping and Clifton, 1035).**

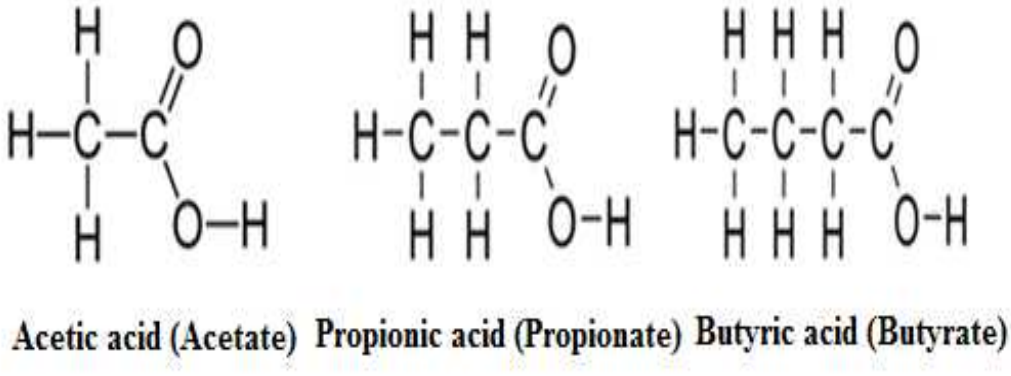

Figure 1.1: Chemical structure of the major short chain fatty acids, i.e acetate, propionate and butyrate **(Carnicom, 02).**

The amount of the SCFAs in the gut can be varied according to the dietary pattern of the individuals. SCFAs levels were strongly linked to the quantity of vegetables, fruits, legumes and fiber consumption irrespective of the type of diet normally eaten. Moreover, higher levels of the SCFAs were found in vegans, vegetarians and those who consistently followed a Mediterranean diet **(Darzi et al, 121)**. SCFAs produced by the microbial species of the cecum and colon are also found in the hepatic, portal and peripheral blood **(Cummings et al, 1221).** They influence on the lipid, glucose and cholesterol metabolism in the various tissues **(Bergman et al, 54)**. SCFAs are transported from the lumen of the host intestine into the blood circulation, which is then taken up

by the various organs, where they can act as substrates or signaling molecules. SCFAs play an important role in the carbohydrate, lipid and protein metabolism through binding with the G-protein coupled receptors (GPCRs). SCFAs can regulate gene expression by binding to the two main GPCRs, such as GPR41 (also known as FFAR3) and GPR43 (also known as FFAR2). Signaling through GPCRs affects several different functions depending upon the cell type. For example, SCFAs suppress inflammation through GPR43 signaling pathway in the immune cells such as neutrophils **(Maslowski et al, 1284; Zaibi et al, 2381)** and modulate the secretion of Glucagon like peptide-1 (GLP-1) hormone by enteroendocrine L-cells in the distal small intestine and colon to improve the insulin secretion and antidiabetic effects **(Tolhurst et al, 366).** In addition, SCFAs produced by the gut microbiota induce neuropeptides YY (PYY) expression by L-cells through a GPR43-dependent mechanism. GPR43-deficient conventionally raised mice have shown a reduction in the adiposity compared with conventional wild-type mice, whereas germfree wild-type and GPR43-deficient mice had shown similar adiposity **(Samuel et al, 16767).** This indicates that the gut microbiota may affect the fat deposition is the body thorough acting on the SCFA receptors. The SCFAs transport mechanism has been mostly studied in the colonocytes, which are the first host cells that take up SCFAs and depend largely on the butyrate for their energy requirement. Propionate and butyrate activate the intestinal gluconeogenesis via gut-brain neural circuit; thereby promote the metabolic benefits on body weight and glucose control **(De Vadder et al, 87)**. Acetate reduces the appetite by changing the expression profiles of neuropeptides secreted by hypothalamus through the activation of TCA cycle **(Frost et al, 5)**. SCFAs have been shown to increase the anti- inflammatory properties through the induction of T regulatory (T_{reg}) cells **(Smith et al, 569).**

The gut microbiota plays role in various important physiological functions, such as digestion, metabolism, nutrient extraction, vitamin synthesis, prevention against colonization by pathogens and immune modulation. All the mammals co-exist in the beneficial symmetry with all the gut resided microorganisms. However, the disruption of this mutualistic relationship can noticeable itself in the progression of several inflammatory diseases, such as inflammatory bowel disease (IBD), colon cancer, ulcerative colitis (UC), crohn's disease (CD), obesity and type 2 diabetes (T2D). The disruption or dysbiosis of the gut microbiota can be caused by various external

environmental factors, such as diet, medication, diseases, stress, hormones and toxins **(Carding et al, 3; Zhang et al, 01).** However, diet and medication are the most prominent modulators of the human gut microbiota.

Diabetes is the most general chronic endocrine disorder, which has affected around 415 million people in 2015, that is predictable to increase up to 642 million by 2040 across the globe **(IDF, 2015).** Insulin resistance (IR) is a condition with the decreased efficiency of the insulin for the glucose utilization by the liver, adipose tissue and skeletal muscles as well as increased hepatic lipid accumulation. Consumption of sugar or fat rich diet and sedentary lifestyle are becoming the major root for diabetes onset and progression in various developing and developed countries **(Cani et al, 1473).** Genetic and environmental factors, along with age progression are responsible for the progression of obesity and T2D, which lead to the development of the cardiovascular diseases **(Shoelson et al, 2175; Hu et al, 696)**. These diseases are associated with low-grade inflammation, which is triggered by the activation of the innate immune system. Emerging evidence from latest epidemiological and biochemical studies, suggests that the high dietary intake of the fructose has rapidly become an important causative factor for the development of the metabolic syndrome. Daily consumption of 85–100 grams of the fructose leads to the rapid stimulation of the lipogenesis in the liver, which accumulates the triglycerides. It further reduces the insulin sensitivity and progress to the metabolic disorders. These diseases also progress by the increased release of the adipocyte derived bioactive metabolites and proinflammatory cytokines **(Shoelson et al, 2175; Olefsky and Glass, 222).**

Higher calorie intake has been associated with a variety of diet-induced complications such as metabolic syndrome, cardiovascular diseases and non-alcoholic fatty liver disease (NAFLD) **(Tauriainen et al, 01).** Supplementation of the carbohydrate, sugar and fat containing dietary components has been widely known to induce the symptoms of the human metabolic syndrome in the rodent models **(Panchal and Brown, 03).** Consumption of sucrose and high fructose corn syrup (HFCS) in the form of the processed foods, such as beverages, jams, jellies, baked goods and dairy products has been drastically increased amongst the majority of the population. HFCS is an inexpensive and sweeter than the sucrose, which enhances its palatability and hence is

rapidly replacing sucrose in the processed foods. An increased intake of the refined carbohydrates, such as HFCS is associated with the development of hypertension, obesity, T2D, kidney and cardiovascular diseases, both in humans and rodents **(Raatz et al, 2262; Kuzma et al, 1375; Stanhope, 52).** Detrimental effects of the fructose on health can be accredited to its metabolic pathway. After dietary intake, fructose is transported via the portal circulation to the liver following absorption in the gastrointestinal tract, where it enters into hepatocytes via the glucose transporter GLUT5 independent of the insulin and rapidly metabolized **(Sato et al, 640, Jegatheesan et al, 193).** The intermediate metabolites of the fructose metabolism are used as a substrate for the very low density lipoprotein (VLDL) synthesis. When sprague dawley rats were fed with 65% of the fructose diet for continues 10 weeks, it induced hypertriglyceridemia and hyperinsulinemia **(Nakagawa et al, F625)**. Jena and co-researchers had reported that administration of the high fructose diet in the wistar rats had significantly increased the blood lipid parameters and developed the IR within 45 days **(Jena et al, 3810).**

Besides diet, gut microbiota also play a significant role in the body weight gain and glucose homeostasis through energy harvest, but the insight mechanism of induction is still not clear. Growing evidences suggested that obese and insulin resistant people were characterized by an altered gut microbiota composition along with the major shift in two phyla, i.e. Firmicutes and Bacteroidetes **(Lynch et al, 142).** The scientists have compared the gut metagenomic composition between T2D and healthy animal groups and observed a significant increase in *Lactobacillus* species and decrease in *Clostridium* species in the former group **(Karlsson et al, 100).** The relative proportions of Firmicutes and Clostridia phyla were found to be significantly decreased in T2D patients compared to control in one quantitative PCR (qPCR) investigation. However, β-Proteobacteria class was found to be highly enriched in the diabetic group as compared to the non diabetic control **(Larsen et al, 02).** The amounts of the bacteria, which are important in the maintenance of the gut epithelial integrity, were found to be decreased in the children with diabetes compared to the healthy ones **(Murri et al, 03).** Gut microbiota regulate insulin sensitivity through different factors, but gut derived lipopolysaccharide (LPS) and SCFAs are the major ones. Obesity and T2D are associated with an increase in the Gram negative microbial population in the gut. High fat or sugar diet considerably increases the Gram-negative

microflora population in the gut. The LPS present in the outer membrane of the Gram-negative microbiota produce the metabolic endotoxemia in the gut, which modulate the intestinal permeability and develop inflammation and IR.

Inflammation induced IR is the key feature of T2D and obesity, which is governed by both genetic as well as environmental factors. Obesity creates the low grade inflammation in adipose tissue, which has a key role in metabolic dysfunction and T2D **(Esser et al, 144).** Adipose tissue acts as an origin of inflammation and involves in the secretion of various bioactive molecules such as cytokines, fatty acids and chemokines which further activates the macrophage infiltration and develops inflammation. Inflammatory markers, such as C-reactive proteins (CRP), interleukin-6 (IL-6) and TNF-α are efficiently correlated with the characteristics of IR according to the existing reports **(Wieser et al, 119**; **Lecube et al, 1098)**. Adipose tissue is well known to secrete a large number of the proteins, which can be termed as adipokines. These adipokines act in an autocrine, paracrine, or endocrine fashion to control various metabolic functions. More than fifty adipokines have been recognized with different functional roles. Adiponectin and leptin are the most studied adipokine, which have a role in the pathogenesis of obesity and T2D **(Kwon and Pessin, 01).**

Adiponectin hormone is also known as adipocyte complement-related protein which is highly expressed in adipose tissue only. This hormone enhances the insulin sensitivity in muscle and liver. It also increases the free fatty acids (FFAs) oxidation in the several tissues, including muscle fibers **(Yamauchi et al, 941).** It also decreases serum FFAs, glucose, and triacylglycerol concentrations in the body. Leptin is the first adipocyte hormone, which mainly influences the food intake through the direct action on the hypothalamus **(Halaas et al, 544; Lee et al, 633).** Plasma leptin concentrations are highly correlated with body mass index (BMI) in human and rodent models **(Maffei et al, 1155).** Mice lacking the gene coding for leptin (*ob/ob* mice) were found to be with obese and diabetic characteristics. Administration of the leptin through regular injections in *ob/ob* mice was found to reduce the food intake and weight gain with an increase in the metabolic rate **(Smith et al, 433).** Mice and rats with a genetic mutation affecting the leptin receptor in the hypothalamus exhibited a similar phenotype to the *ob/ob* mice **(Chua et al, 997).**

The immune system is one of the important systems to defend against pathogens. Vertebrates are constantly endangered by the attack of harmful microorganisms and have evolved immune arms to eliminate the pathogens from the body. The vertebrate immune system is majorly consists of two arms: innate and acquired immunity. The innate immune system is known as germ encoded and nonspecific host defense system against pathogens. It is mediated by phagocytes, macrophages and other immune cells. In contrast, acquired immunity is mainly involved in the specific elimination of pathogens in the delayed phase of infection with the generation of immunological memory. The recognition of the microbial pathogens is the crucial step for the initiation of innate immune responses, such as inflammation, which is mainly mediated by germ line-encoded varieties of extracellular or intracellular pattern recognition receptors (PRRs). PRRs recognize the varieties of molecular structures shared by the pathogenic microorganisms, known as pathogen-associated molecular patterns (PAMPs). PRRs initiate the series of the signaling cascades upon PAMPs recognition to perform the first line host defensive response necessary for killing the infectious microorganisms. Four different classes of PRR families have been identified. These families consist of transmembrane proteins, mainly like toll-like receptors (TLRs), NOD-like receptors (NLRs) and c-type lectin receptors (CLRs) **(Akira et al, 784; Osorio et al, 652).** In spite of their great contribution in the human defense system, they are known to be involved in the pathogenesis of obesity and T2D. The TLRs and NLRs are the most extensively studied PRRs in the pathogenesis of the inflammation mediated IR.

TLR4 can recognize the varieties of the microbial structures, including LPS, peptidoglycan and high mobility group proteins. After binding with its ligands, it leads to the activation of the two downstream pathways: mitogen activated protein kinase (MAPK) and nuclear factor-kappa B (NF-κB) pathway. TLR4 plays a significant role in the intestinal innate immune system as it is the first line for the recognition of the gastrointestinal tract bacteria. TLR2 recognize the peptidoglycan moiety, which is present in the outer membrane of the microbial species. Activation of the TLR2 and TLR4 by their respective ligands activates the downstream signaling pathways through binding with various adaptor proteins. The activation of the TLRs signaling pathway through bacterial stimuli activates the NF-κB pathway by degrading the Iκβ protein

complex. Degradation of Iκβ protein complex allows the NF-κB to translocate into the nucleus where it induces the expression of the proinflammatory cytokines **(Habelhah, 736).**

The cytosol located NLRs are known as nucleotide-binding oligomerization domain (NOD). NOD containing receptors are the definite group of intracellular proteins, which correspond to the 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) and acidic transactivating domain, or baculovirus inhibitor repeat (BIR), (b) a central NOD domain which mediates self oligomerization during activation and (c) a C-terminal leucine-rich repeat (LRR) that detects and binds with PAMPs **(Chen et al, 367; Shaw et al, 624).** NLR1 and NLR2 are the earliest identified and best characterized NLRs **(Kobayashi et al, 195; Optiz et al, 485).** NOD1 also known as CARD4, which detects the various substructure of the peptidoglycan named as iE-DAP (-D-glutamyldiaminopimelic acid). It is found to be present in both Gram-negative and Gram-positive bacteria, but it selectively identifies the Gram negative bacteria. NOD2 also known as CARD15, which detects the muramyl dipeptide (MDP), largest active component of peptidoglycan motif, which is present in both Gram-negative and Gram-positive bacteria. Upon binding with their respective ligands NLR1 and NLR2 undergo self-oligomerization to recruit and activate the adaptor protein receptor-interacting serine/threonine-protein kinase 2 (RIPK2) which activates NF-κB and the MAPKs pathways **(Park et al, 2382; Abbott et al, 2217).**

Activation of the NF-κB through its nuclear translocation by degrading the various cytoplasmic complexes induces the transcription of proinflammatory genes, which plays a central role in the progression of the inflammation **(De Martin et al, E85).** NF-κB is found to be highly activated at the sites of inflammation in various diseases. Besides proinflammatory cytokines, it can also induce the transcription of various chemokines, adhesion molecules as well as inducible nitric oxide (iNOS). The over expression and activation of the NF-κB induces the recruitment of inflammatory cells and production of proinflammatory mediators like IL-1β, IL-6, IL-8, and TNF-α. TNF-α is a potent proinflammatory cytokine primarily secreted from myeloid cells via activation of MAPK and NF-κB signaling pathways resulting in the release of other inflammatory

cytokines, such as IL-1β and IL-6 as shown in Figure 1.2 **(Makki et al, 02).** It is the first white adipose tissue derived inflammatory cytokine reported to be implicated in the initiation and progression of the IR **(Cawthorn and Sethi, 119).** The TNF-α level is found to be positively correlated with other markers of IR. An increased level of the TNF-α phosphorylate the serine or threonine moiety of the insulin receptor and downregulates the insulin receptor signaling (IRS) signaling pathway to progress the IR **(Luft et al, 03).** Beside its negative interference with the IRS pathway, it indirectly mediates IR also by altering the adipocyte differentiation and lipid metabolism. It is also known to induce the lipolysis and secretion of FFAs, which contribute to an increase hepatic glucose production **(Green et al, 78; Ryden et al, 168).** The elevated levels of the various adipokines and proinflammatory cytokines were observed in a recent case cohort study involving the middle-aged obese individuals with diabetes, which suggests the significant role of the inflammatory cytokines in the adult onset of diabetes in obese individuals **(Luft et al, 05)**. IL-6 is produced by the several cell types, such as endothelial cells, monocytes, fibroblasts and macrophages. Adipocyte isolated from the obese individuals has shown the upregulation of the IL-6 expression, which can be further correlated with the progression of IR **(Sjoholm et al, 06; Yudkin et al, 210).**

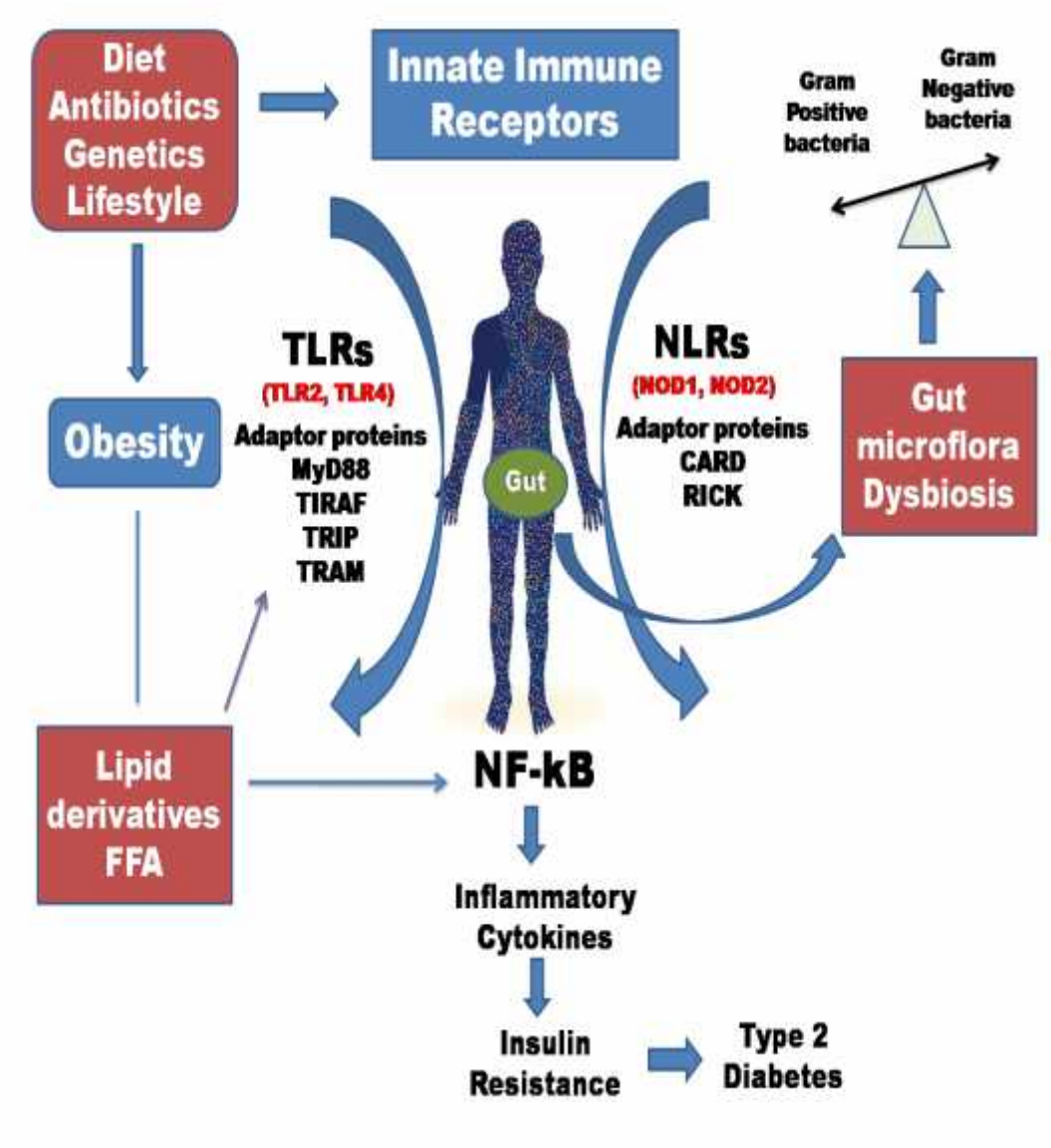

Figure 1.2: TLR and NLR cross talk in pathogenesis of Type 2 Diabetes. Innate immune receptors like TLRs (TLR2, TLR4) and NLRs (NLR1, NLR2) play an important role in pathogenesis of the obesity mediated insulin resistance.

Dysbiosis of the stable gut microbiota through various environmental factors can lead to the development of the various diseases including T2D. Therefore, modulation and restoration of the disrupted gut microbiota can be a potential and an emerging therapy to prevent the progression of

diseases. Gut microbiota can be modulated using prebiotics, prebiotics and antibiotics for the prevention of metabolic disorders. Probiotics are live microorganisms, which exert beneficial health effects on the host after its oral administration to improve the intestinal microbial balance **(Goldenberg et al, 04).** The most commonly used probiotics strains are *Lactobacilli, Bifidobacteria* and some species of the *Escherichia.* Prebiotics are mainly the indigestible food materials, which promote the growth of intestinal beneficial microorganisms. They are most frequently used to stimulate the growth of either *Lactobacilli* or *Bifidobacteria* population. Inulin and oligofructose or fructo-oligosaccharides are the most commonly used prebiotics. One research study had observed that supplementation of chow diet along with the SCFAs or fructooligosaccharides caused a shift in the gut microbiota composition in the mice, which strongly correlated with beneficial effects on the body weight, adiposity and glycemic index. These physiological changes were brought via butyrate and propionate mediated activation of the intestinal gluconeogenesis **(Kumar et al, 01).**

Gut microbiota modulation using antimicrobial agents is an emerging and attractive therapeutic strategy to prevent the inflammatory diseases. The success of using this approach is ultimately depends on the target specificity of the antimicrobial agents as the detrimental consequences by the overuse of the broad-spectrum antibiotics have become more apparent in the recent years. Broad-spectrum antibiotics have been commonly used by the clinicians for the treatment of a wide range of infections. However, the spread of the antibiotic resistance is now posing a serious problem in health care settings due to their frequent use **(Korotkevich, 07).** In addition, antibiotic therapies not only affect the target microorganism but can also disturb the microbial communities of the gut. The extent of this damage has been recently become more apparent through the application of high throughput DNA-based sequencing technologies to assess the composition of gut microbial population **(Cotter et al, 193).** The recent reports suggested that gut microbiota dysbiosis can be restored by using various types of the antibiotics alone or in the combination. The effect of the antibiotic administration for the prevention of the obesity and T2D mainly depends upon their antimicrobial spectrum **(Mbakwa et al, 107).**

The targeted drug delivery system is also known as a smart drug delivery system. The system involves delivering the drug or medication to the patient in such a manner that it increases the concentration of the drug in a desired region of the body. The targeted drug delivery can overcome certain limitations of the conventional delivery system to achieve the higher therapeutic efficacy with least toxicity. Various types of the formulations, such as nanoparticles, liposomes, microspheres, dendrimenrs etc. have been developed for delivering the drug at the target site in a controlled fashion **(Wilczewska et al, 1021; Philip and Philip, 81).** Delivering the drug using microspheres is one of the most advanced approaches in the recent era of the time. The microspheres are typically a free flowing powder, which may be made up of proteins or synthetic polymers that is biodegradable in the nature and having a particle size less than 200 µM. The eudragit series of the polymers are widely used in the drug delivery system for its pH sensitive drug release behavior. Eudragit S100 (ES) and Eudragit L100 (EL) are composed of the methacrylic acid and methyl methacrylate. ES and EL are known as pH sensitive polymer due to its unique dissolution at pH 7.0 and pH 5.5 respectively. Thus, they are widely used in various applications such as, enteric coating and drug delivery. Eudragit can also be used in combination with several other polymers, such as hydroxypropyl methyl cellulose (HPMC) and talc for the controlled drug delivery system **(Raffin et al, 361; Yoo et al, 263; Gursoy and cevik, 567)**.

Chitosan polymer is a biodegradable in the nature and inhibits the growth of the harmful bacteria in the gut. Therefore, it acts as a prebiotics to promote the growth of the probiotics to exert better digestion and immunomodulation. Chitosan is a polymer of glucosamine, which is obtained from the deacetylation of chitin. It is widely distributed in the nature mainly in the crustaceans, insects and fungal cell walls **(Kean and Thanou, 6; Martins et al, 20800).** It exhibits various unique properties, such as biodegradability, mucoadhesiveness, easy availability, biocompatibility and non-toxicity **(Jarmila and Vavrikova, 3598).** It is very well known and frequently used in the pharmaceutical and biotechnology industries for the drug delivery purposes. Various biological activities of the chitosan, such as antitumor, wound healing and broad spectrum antimicrobial against Gram negative, Gram positive bacteria and fungi have been presented by various studies

(Lee et al, 320; Rou et al, 1146). The chitosan has been reported for its hypocholesterolemic and hypolipidemic activities in human and rodent models **(Tai et al, 1703).**