Chapter 1

Review of Literature

1. Introduction

1.1. Global Consumption of Sugar

Sugar, a basic constituent of diets worldwide, makes people think of pleasure and derision at the same moment (Clemens *et al*., 2016). Its physiological importance is unquestionable as it is the basic currency of energy in the bloodstream and the body. However, abnormalities of its clearance from the bloodstream and its use create problems. Sugar including fructose has been charged for many non-communicable diseases like cardiovascular disease, type 2 diabetes, and metabolic syndrome.

Sugars mainly, sucrose and fructose have been consumed by humans since years as it is present in fruits, vegetables. In the beginning of the twentieth century, the consumption of sugar increased throughout the world because of low cost and colonial trade (Standage, 2005). Urban development in high earnings countries led to increase in intake of high-fat high-sugar diets which made a significant proportion of the total increased caloric load (Drewnowski, 2003; Ogden *et al*., 2007; Canoy and Iain, 2007). Obesity has also increased with the use of high fructose corn-syrup (HFCS) instead of sucrose in sweetened beverages (Reilly *et al*., 2005; Abegunde *et al*., 2007; WHO, 2009; 2014). The 2015 Dietary Guidelines Advisory Committee's noted a positive relation between weight gain and sugar consumption thereby suggesting an upper limit of 10% of total energy from sugar, a level at which the added sugar contributed to increased risk of mortality from cardiovascular disease, especially among those with a BMI > 25 (Bray and Popkin, 2014; Yang *et al.,* 2014; DHHS/USDA 2015). This recommendation is consistent with the WHO report, which stated that the sugar consumption was associated with weight gain. Limit on sugar intake was also recommended as a strategy to manage body weight (WHO, 2015). Surprisingly, according to WHO, all regions of the world, excluding Eastern Asia and Western Africa, have already exceeded the new level of daily intake of free sugars for adults and children (Fig. 1.1).

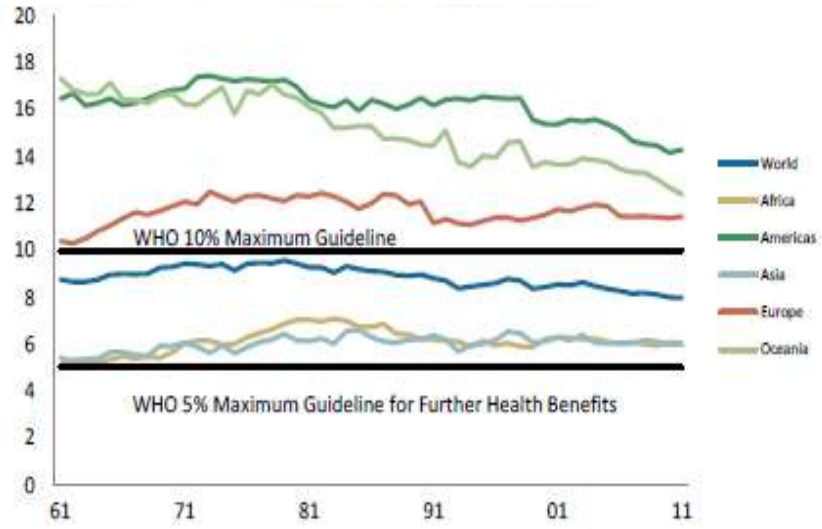

Figure 1.1: Sugar and sweeteners as percentage of total caloric intake (WHO, 2015).

1.2 Metabolism of Fructose

Dietary glucose, starch and fat are considered as 'primary dietary substrates' as they can be directly used as an energy source by all cells of the organism (Campos and Tappy, 2016). On the contrary, fructose is considered as 'subsidiary substrates' as it cannot be used directly as energy sources and it has to be processed by specialized metabolic cells which express a comprehensive set of metabolic enzymes. This two-step metabolism in which subsidiary substrates are converted into primary substrates in splanchnic organs comes at some energy cost and reduces their net energy yield.

Glucose and fructose elicit differential central and peripheral responses (Fig. 1.2). Fructose is sweeter than glucose with intensity profile that reaches a larger peak and diminishes more quickly than glucose (Hanover and White, 1993).Glucose is absorbed by a sodium coupled co-transporter and is the main sugar circulating in bloodstream, acting as fuel source in brain and arrives at the liver via portal circulation (Havel, 2005; Tappy and Kim, 2010). In contrast, fructose is absorbed by enterocytes through a specific fructose transporter, GLUT5 via a non-sodium-dependent process. In liver cells, citrate and ATP allosterically inhibit phosphofructokinase thereby limiting hepatic extraction of glucose whereas fructose metabolism is unregulated because fructose bypasses the main regulatory step in glycolysis, catalyzed by phosphofructokinase. Glucose is taken up by pancreatic β-cells through GLUT2 transporters where it stimulates insulin secretion, a hormone

that increases central satiety signaling and reduces hedonic feeding behavior whereas fructose is unable to directly stimulate insulin secretion because of low expression of fructose specific transporters on β-cells (Curry, 1989; Woods *et al*., 1996). Consumption of fructose leads to reduced post-prandial leptin secretion, a hormone produced by adipocytes that serves as a key signal to the brain to decrease appetite and to increase energy expenditure from adipose tissues when compared to glucose (Teff *et al*., 2009). When compared to ingestion of glucose, fructose ingestion results in smaller increase in the appetite suppressing hormone, glucagon-like-1 polypeptide (GLP-1) in the small intestine and a smaller reduction in the hunger-stimulating hormone, ghrelin (Kong *et al*., 1999; Lindqvist *et al*., 2008; Page *et al*., 2013).

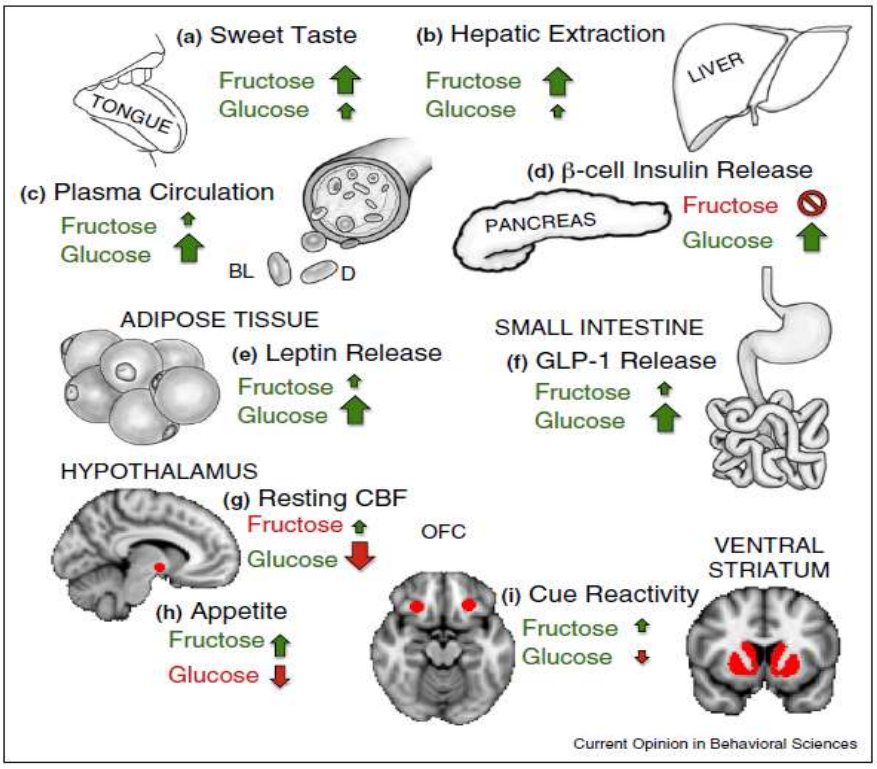

Figure 1.2: Differential central and peripheral response to glucose and fructose (Page *et al*., 2016).

Metabolism of different sugars mainly occurs in liver, kidney and small intestine (Coffee *et al*., 2009). Fructose that enters the liver can have variety of primary fates including catabolism to pyruvate followed by further utilization for energy; the production of lactate; the conversion to glucose, which can be further metabolized within the liver or exported to the general circulation; the storage of the

energy as glycogen, which accounts for a relatively high fraction of absorbed fructose or can lead to secondary fates including lipogenesis and export of fructose to extrahepatic tissues (Tappy and Kim, 2010; Laughlin *et al.*, 2014). In liver, fructokinase converts fructose to fructose-1-phosphate which is a substrate for aldolase that suppresses the glycolytic pathway (Fig.1.3) (Collison *et al.*, 2009). This pathway ultimately bypasses the control step for entering into glycolysis and results in the production of higher levels of ATP, citrate and fatty acids. Thus, most of the consumed fructose is converted to triglycerides in de novo lipogenesis (Parks *et al.*, 2008). These triglycerides are packed with apoB100 as VLDL or may accumulate in the liver and initiate nonalcoholic fatty liver disease (NAFLD).

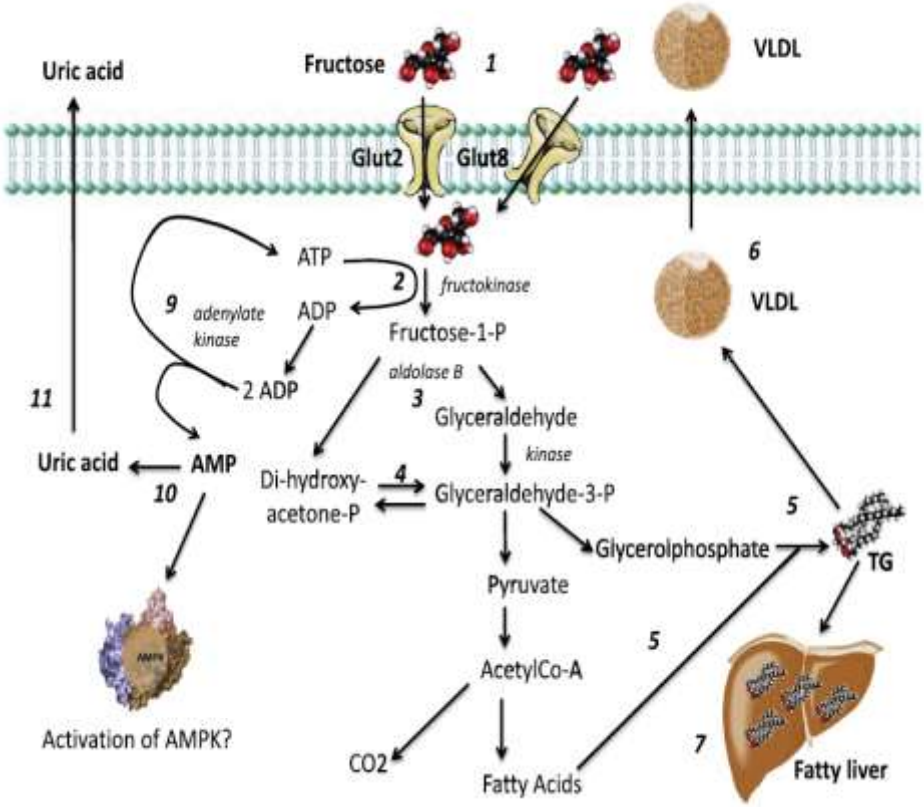

Figure 1.3: Fructose metabolism in the liver (Gugliucci., 2016).

1.3 Metabolic Effects of Fructose

Animal studies have shown a high-fructose diet induces metabolic syndrome resulting in hyperinsulinemia, insulin resistance, glucose tolerance reduction, hypertension, hypertriglyceridemia, and decreased high-density lipoprotein cholesterol (Yokozawa *et al.*, 2008; Chan *et al.*, 2013). In healthy male subjects,

overconsumption of fructose causes visceral adiposity, tight junction disruption, hepatic insulin resistance, reduced lipid oxidation, and increased triglycerides (Fig.1.4) (Abdel-Sayed *et al*., 2008; Couchepin *et al*., 2008). Additionally, the hyperlipidemic effects of excessive fructose consumption leads to increased production of inflammatory cytokines, which may enhance hepatic secretion and/or delay clearance of very-low-density lipoprotein, thereby increasing serum triglyceride and cholesterol concentrations in adult rats (Kelley *et al*., 2004).

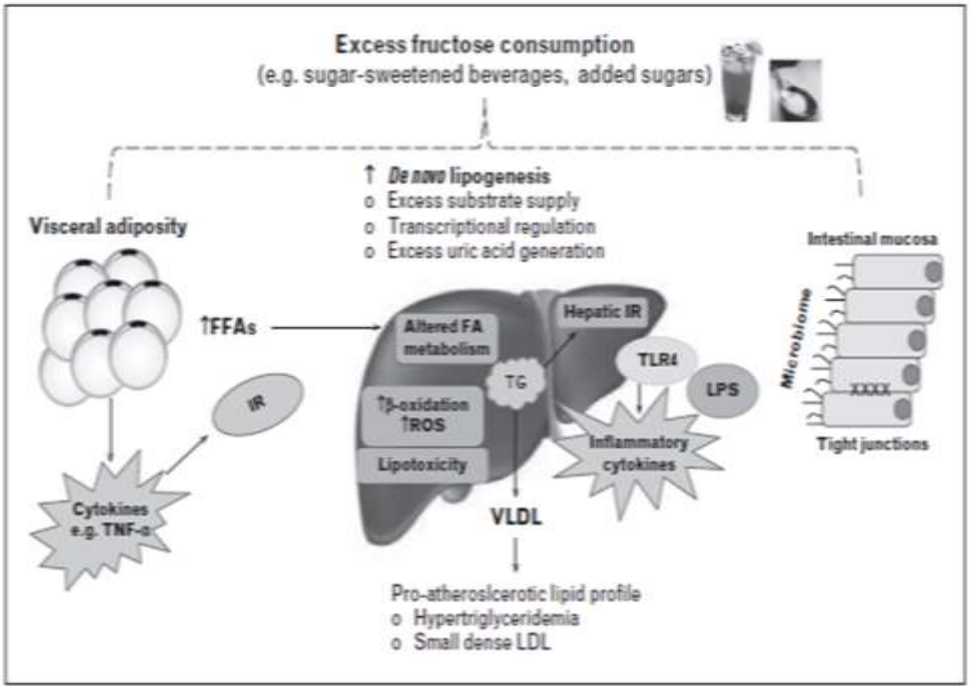

Figure 1.4: Metabolic effects of excess fructose consumption (Jin *et al*., 2015).

1.3.1 Dyslipidemia

Overconsumption of fructose leads to hepatic overproduction of large VLDL particles along with delayed clearance by lipoprotein lipase, increasing gastrointestinal tract plasma triglycerides and formation of small, dense LDL particles, thereby creating a proatherogenic environment leading to metabolic dyslipidemia (Stanhope *et al.*, 2010). Additionally, fructose consumption lowers postheparin lipoprotein lipase activity. More than 90% of fructose that is metabolized by the liver may directly upregulate transcriptional factors regulating de novo lipogenesis or indirectly by inducing ER stress, insulin resistance, and decreased

mitochondrial metabolism producing uric acid and reactive oxygen species leading to de novo lipogenesis (Fig.1.5). In healthy adults, consumption of fructose or HFCS-sweetened beverages but not glucose for 2 weeks increased postprandial triglycerides, small dense LDL particles, oxidized LDL, and remnant-like particle lipoprotein triglycerides (Stanhope *et al*., 2011).

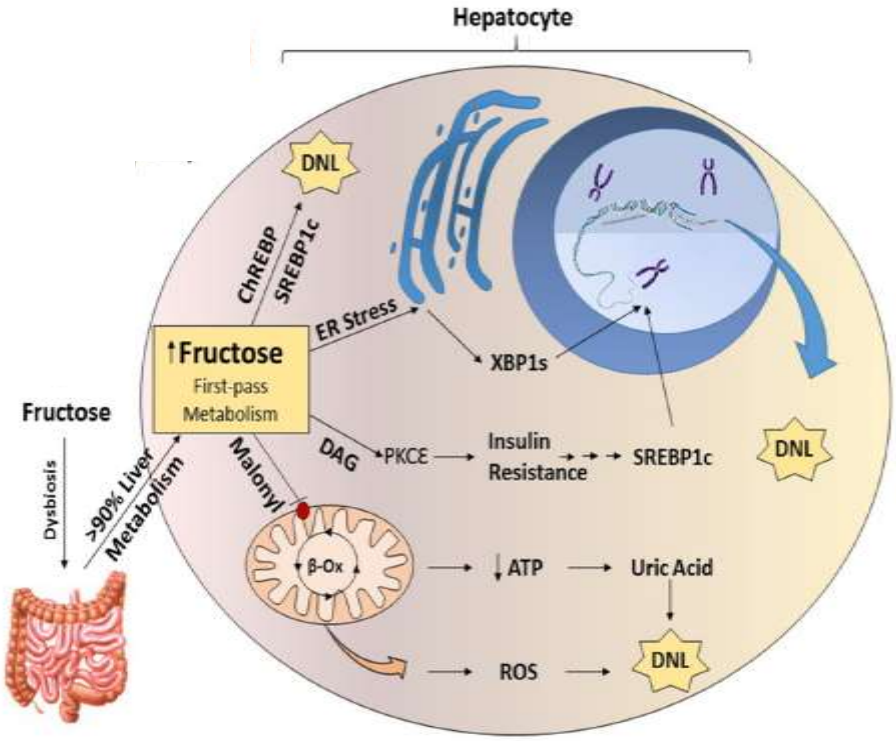

Figure 1.5: Lipogenic potential of fructose (Softic *et al*., 2016)

1.3.2 Visceral adiposity

In both adults and children, overconsumption of fructose is associated with increased visceral adiposity (Stanhope *et al.*, 2009; Pollock *et al.*, 2012). High fructose leads to development of lipid vesicles significantly earlier in human adipocytes cultured in growth media containing high fructose (Robubi *et al.*, 2014). Inflamed visceral adipose tissue is metabolically active and produces numerous inflammatory cytokines that leads to insulin resistance whereby failing to suppress the hormone sensitive lipase leading to elevated free fatty acids being delivered to the liver (Lim *et al.*, 2010).

1.3.3 Insulin resistance

Fructose metabolism increases hepatic lipid load contributing to hepatic insulin resistance, through increased intrahepatic level of diacylglycerol which activates protein kinase C and inhibits the phosphorylation of insulin receptor substrate proteins, impairing downstream insulin signalling (Fig.1.6) (Birkenfeld and Gerald, 2014; Byrne and Giovanni, 2014). Fructose intake, even in a relatively small amount and over a shorter duration, leads to hepatic insulin sensitivity which may be due to a stimulation of glucogenesis and increased glycogen stores, or hepatic lipotoxicity (Stanhope *et al.*, 2009; Aeberli *et al.*, 2013). Diet-induced maternal insulin resistance was responsible for initiating dysregulation of hepatic immune system and development of de novo lipogenic pathways in offspring (Thorn *et al.*, 2014). Supporting this fact, another study noted higher prevalence of hepatic steatosis in fetus delivered by the diabetic mother suggesting that NAFLD risk is intricately linked to our early life environment where maternal, fetal, and childhood factors may predict its risk later in life (Patel *et al.*, 2015).

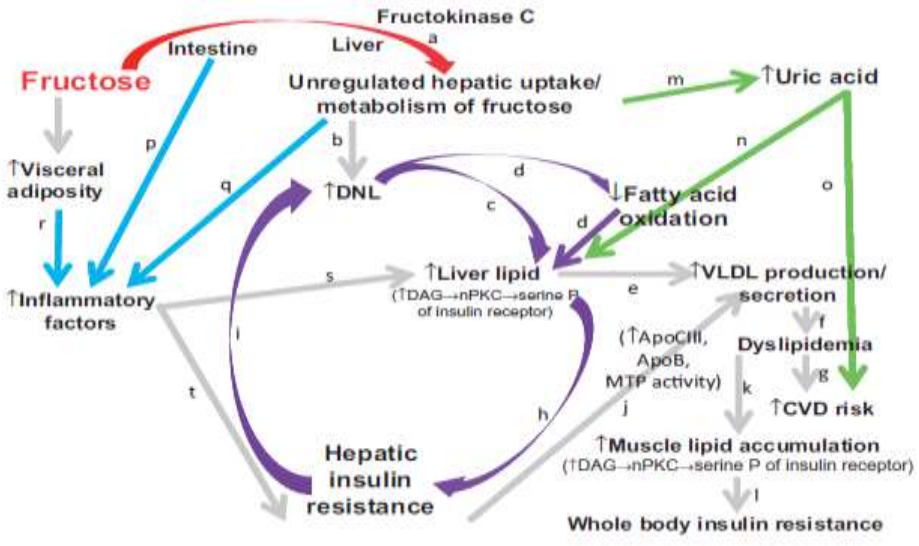

Figure 1.6: Potential mechanism by which fructose lead to hepatic insulin sensitivity (Stanhope *et al.*, 2016)

1.3.4 Fructose and oxidative stress

The ring form of fructose, a 5-membered furan with axial hydroxymethyl groups because of its unique stereochemistry is under an ionic strain, favouring the linear form of the molecule, exposing the reactive 2-keto group that can readily engage in the nonenzymatic fructosylation of exposed amino moieties of proteins via the Maillard reaction (Bremer *et al*., 2012). The Maillard reaction generates reactive oxygen species (ROS), which must be quenched by an antioxidant for reducing cellular damage. Fructose generates excessive ROS, which can lead to cellular damage and promotes the unfolded protein response (UPR), leading to metabolic syndrome (Fig.1.7). In the lipid inflamed hepatocytes, the flow of electrons in the respiratory chain is partially blocked by lipid peroxidation, peroxynitrite and ROS products increasing mitochondrial ROS and peroxynitrite formation (Pessayre *et al.*, 2007). Oxidative stress increases the release of lipid peroxidation products and cytokines contributing to the development of nonalcoholic steatohepatitis (NASH). Nonenzymatic reactions of fructose and higher production of reactive carbonyls (RCS) and oxygen species compared with glucose are believed to be mediating negative effects of fructose (Semchyshyn, 2014).

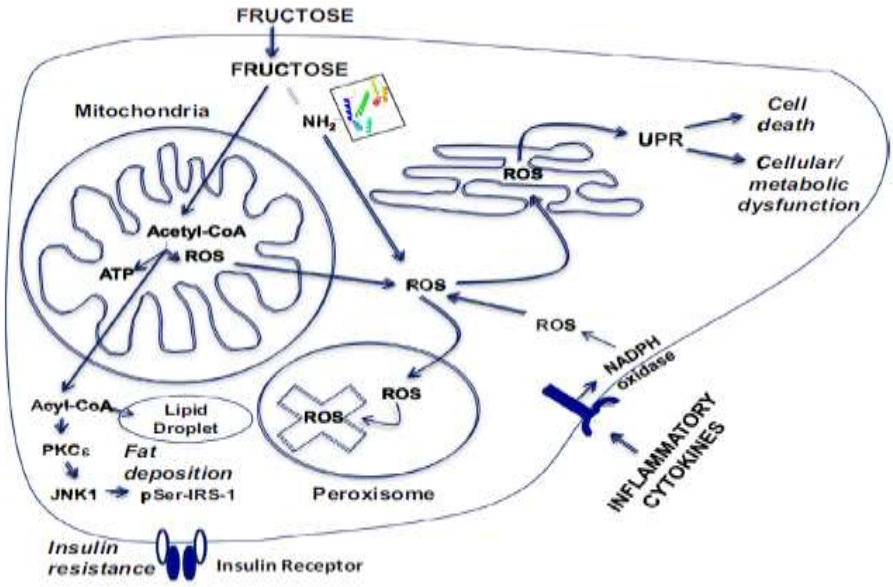

Figure 1.7: Hepatic fructose metabolism and generation of oxidative stress (Lustig *et al*., 2016)

1.3.5 Non-alcoholic Fatty Liver Disease

Lipogenesis stimulated by overconsumption of fructose leads to increased hepatic load. This excessive accumulation of fat in hepatocytes (>5%) is called NAFLD. It is one of the clinical characteristics associated with metabolic syndrome which occurs even in the absence of significant alcohol consumption in almost 11% of adults and adolescents (Younossi *et al.*, 2011; Welsh *et al*., 2013). These ultimately cause cardiovascular diseases. But overconsumption of substrates is not the only reason of lipogenesis (Moore *et al.*, 2014). Altered transcriptional regulation of lipogenic enzyme expressions including sterol regulatory element-binding protein 1(SREBP-1c) and carbohydrate responsive element-binding protein (ChREBP) also are responsible for lipogenesis (Fig.1.8). In addition, fructose metabolism produces uric acid which may trigger DNL and lipid disposition in hepatocytes by further activating fructokinase gene expression and causing mitochondrial stress. Fructose may promote a net intrahepatic fat accumulation by two ways; directly through DNL and indirectly through DNL induced inhibition of fatty acid oxidation (Cox *et al.*, 2012). This outcome may also depend on the ratio of saturated to unsaturated fatty acids (SFA:UFA) in blood lipids, affected by dietary fat as well as by the relative activities of the enzymes involved in synthesis, elongation, and desaturation of newly generated fatty acids (Miyazaki *et al.*, 2007; Siddiqui *et al.*, 2015). Fructose consumption was associated with NAFLD in general and NASH in particular in case controlled studies (Ouyang *et al., 2*008; Thuy *et al., 2*008). Recent studies show consumed carbohydrates to be responsible for hepatic DNL, directly contributing to NAFLD than dietary fat intake (Basaranoglu *et al., 2*015**).**

Patients with NAFLD have an altered intestinal microbiota as compared with healthy lean or obese individuals (Schnable and David, *2*014). Tight junction protein occludin is also lower in duodenal biopsy specimens (Miele *et al*., 2009). In patients with NAFLD, lactulose/mannitol ratio, an indicator of intestinal permeability, is found to be significantly increased, and it also tightly linked with the severity of the disease (Giorgio *et al., 2*014). Fructose is absorbed in the small intestine primarily through glucose transporter family and malabsorbed fructose would pass on to the

colon and interacts with bacteria (Vos, 2014). Fructose has been demonstrated to increase intestinal translocation of bacterial endotoxin and subsequent activation of Kupffer cells through Toll-like receptor-dependent mechanisms, leading to hepatic steatosis. Administration of antibiotics decreased fructose-induced hepatic steatosis by improving endotoxin levels. Alteration in gut microbiome is at least partly, responsible for metabolic disorders such as obesity, inflammatory bowel disease and colon cancer (Feldstein *et al., 2*003).

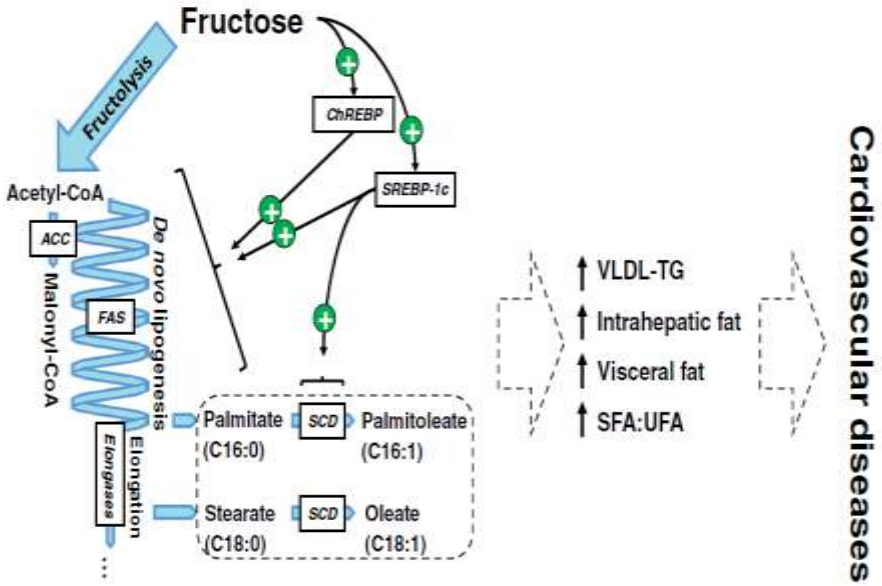

Figure 1.8: Role of fructose derived de-novo lipogenesis on cardiovascular health (Rosset *et al.*, 2016)

1.4 Metabolic Syndrome and Mercury

The main risk factors of metabolic syndrome development were mainly sedentary lifestyle, genetic predisposition and excessive caloric consumption (Zhang *et al*., 2009). However, research has also indicated a significant role of environmental factors in the development of this syndrome (Lind *et al*., 2013). Studies have shown a significant interrelation between heavy metal exposure and metabolic syndrome development (Moon, 2014). A strong relationship between metabolic syndrome and oxidative stress has been demonstrated (Furukawa *et al*., 2004; Roberts and Sindhu, 2009; Youn *et al*., 2014). Commercial HFCS blamed for metabolic syndrome development has been made using “mercury-grade” caustic soda, contaminated with

0.2 to 0.3 parts per million (ppm) of mercury, and perhaps as much as 1 ppm, in some cases (Hansen *et al.*,1996; Dufault *et al., 2*009). HFCS also contains citric acid used as a preservative derived from mercury cell chlor-alkali plants. Due to its Physico-chemical properties, mercury is an inducer of oxidative stress in biological systems.

1.4.1 Mercury and oxidative stress

Mercury cause oxidative stress through mitochondrial damage, lipid peroxidation, production of ROS and accumulation of neurotoxic molecules (Patrick, 2002) (Fig 1.9). The primary intracellular antioxidant, reduced glutathione (GSH) was shown to be depleted in Hg toxicity thereby suggesting that oxidative stress might be involved in Hg-induced toxicity (Quig, 1998). Persons being occupationally exposed to mercury are prone to oxidative stress development identified by an increase in 8-hydroxy-20-deoxyguanosine (8-OH-dG), a marker of oxidative DNA damage and a decrease in antioxidant levels in serum (Chen *et al*., 2005). Intraperitoneal injection of 1 mg/kg methylmercury resulted in increased lipid peroxidation (LPO) in various brain regions (Zahir *et al.,* 2006). Subcutaneous injection of mercury resulted in a dose-dependent amplification of LPO in rat organs and tissues (Yonaha *et al.*, 1983; Lin *et al.,* 1996; Huang *et al.,* 1996). Studies involving intragastric administration of mercury compounds showed similar pattern with the above mentioned studies (Mahboob *et al*., 2001). In Wistar rats, pre oral administration of mercuric chloride also resulted in an increase in blood plasma TBARS content (Hijova *et al*., 2005).

Glutathione is one of the main antioxidants being impaired by mercury exposure because of its high affinity to thiol groups. Studies using cell cultures have indicated that mercury treatment results in a significant decrease in GSH levels (Lee *et al*. 2001; James *et al*. 2005; Chang *et al., 2*008). Likewise, in rats administered with prolonged pre oral methylmercury resulted in increased activity of the rate-limiting enzyme in glutathione synthesis, c-glutamyl cysteine synthetase along with elevated glutathione level in kidney cortex(Woods *et al.,* 1995). Precisely, methylmercury has been demonstrated to decrease GPx activity in rodent liver (Hirota *et al*., 1980; Farina *et al*., 2004), brain (Franco *et al*., 2009), and cell cultures (Farina *et al*., 2004; Franco

et al., 2009). Mercuric chloride also reduced GPx activity in different organs of laboratory rodents (Wada *et al*., 1976; Black *et al*., 1979).

Mercury intoxication leads to inactivation of the SOD, that catalyses the dismutation of superoxide leading to generation of hydrogen peroxide (McCord *et al*., 1969; Benov *et al*., 1990). Additionally in mercury-treated mice, a decrease in cytosolic along with mitochondrial Cu, Zn-SOD levels has been detected (Garcı´a-Sevillano *et al*., 2014). Studies including human subjects have confirmed the influence of mercury on SOD activity. Specifically, individuals exposed to mercury for a period of 7–32 months were characterized by a significant decrease in erythrocyte SOD activity as compared to the control subjects which has also been proved by later studies (Zabin´ski *et al*., 2000; Abdel-Hamid *et al*., 2001). Mercury-treatment in animals showed a decrease in erythrocyte catalase activity (Barcelos *et al*., 2011). Additionally, women living in contaminated Amazon areas had decreased catalase activity in comparison to the respective control group (Pinheiro *et al*., 2008). Conversely, studies have shown a positive association between mercury levels and catalase activity in organisms (Queiroz *et al*., 1998; Perrin-Nadif *et al*., 1996).

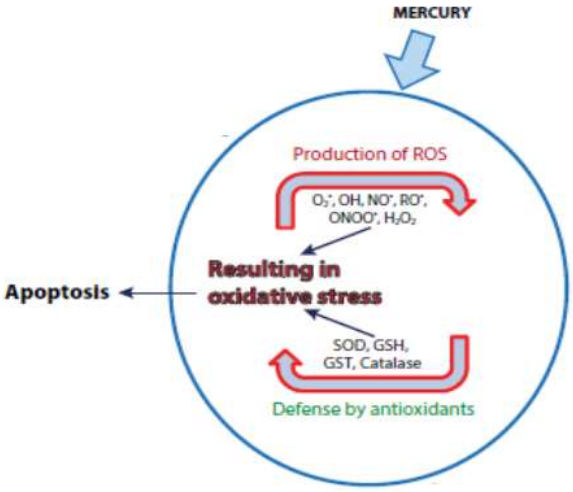

Figure 1.9: Effect of mercury on cell and balance between ROS production and antioxidant defence mechanism (Jaishankar *et al*., 2014).

1.4.2 Mercury and obesity

Blood mercury levels are significantly intertwined with overweight and obesity (Cho *et al*., 2014). Moreover, a study involving 1,853 persons in KNHANES 2010 has indicated an association between blood Hg and BMI and waist circumference values (Kang *et al., 2*013). Mercuric chloride administration in mice was followed by a reduction in adipose tissue content, decreased adipocyte size and leptin secretion coupled with a significant inhibition of both peroxisome proliferator-activated receptor PPARα mRNA expression in adipocytes (Kawakami *et al*., 2012). A significant mercury-induced decrease in adipose tissue content may play an important role in the development of obesity associated-pathology.

1.4.3 Mercury and insulin resistance

Various studies have demonstrated the effect of mercury on development of insulin resistance and type 2 diabetes mellitus. Mercury content of hair was found to be higher in Ontario inhabitants suffering from DM2 in comparison to the control group (Pal *et al*., 2013). Moreover, an elevated level of blood serum mercury concentrations was observed in a cohort simultaneously exposed to dioxins and mercury (Chang *et al*., 2011). Pre oral administration of mercuric chloride and methylmercury resulted in an increase in serum TBARS concentration and decrease in glucose intolerance, serum insulin levels and hyperglycaemia. These observed changes were reversed by N-acetylcysteine administration demonstrating that oxidative stress might play a role in mercury-induced glucose dyshomeostasis (Chen *et al*., 2006a, b, c, and d).

1.4.4 Chelators for metal induced toxicity

The most commonly used therapeutic strategy for heavy metal poisoning is chelation therapy to promote metal excretion. Chelators such as $CaNa_2EDTA$ and meso-2,3-dimercaptosuccinic acid (DMSA) have been demonstrated to be protective against Hg toxicity. Nevertheless, in subjects with previous history of renal toxicity, $CaNa_2EDTA$ can cause kidney damage at the proximal tubule by recurrent high doses treatment above 75 mg/kg (Porru and Alessio, 1996). Other essential metals such as

iron, manganese and zinc are excreted and depleted following $CaNa_2EDTA$ therapy, because of lack of relative specificity, (Aposhian *et al.*, 1995). DMSA also has side effects such as appetite loss, nausea and diarrhea (Liebelt *et al.*, 1994). A study of children being treated with DMSA showed that 12% had mild gastrointestinal symptoms and 5% experienced general malaise (Mann and Travers, 1991).

1.5 Fructose and Iron

Iron deficiency anaemia is responsible for affecting an estimated 2 billion people which corresponds to 24.8% of the population worldwide and in many developing countries it is responsible for lowered productivity, overall death rates, maternal haemorrhage and reduced school performance (Fig.1.10) (WHO, 2008; Kassebaum *et al*., 2014).

Iron plays an important role in many metabolic processes in plants, microbes and animals, including oxygen transport and its storage, electron transfer, substrate oxidation-reduction, hormone synthesis, DNA replication, repair and cell cycle control, nitrogen fixation, and protection from reactive oxygen species , neuron signalling (Soetan *et al.*, 2010; Dlouhy and Outten, 2013; Abbaspour *et al.*, 2014).

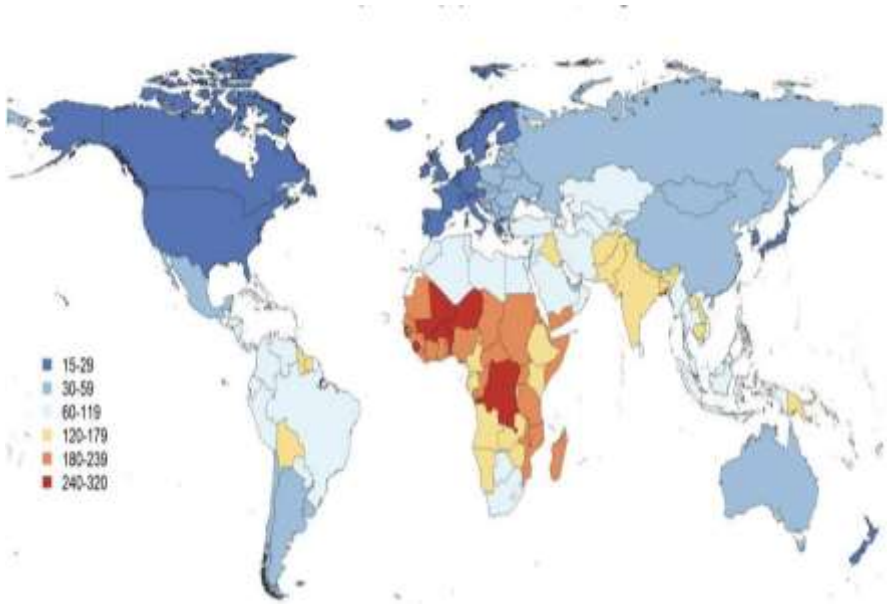

Figure 1.10: Prevalence of iron deficiency worldwide (WHO, 2008)

Consumption of plant-based diets is the cause of low iron bioavailability because of low absorption of non-heme iron compared with heme iron predominantly present in animal tissues (Hurrell and Egli, 2010). High levels of phytic acid present in cereals and legumes, iron-binding phenolic compounds, and calcium, are responsible for inhibition of the absorption of iron in animals because they form non absorbable complexes with the dietary inorganic iron (Kumar *et al*, 2010; Bothwell and MacPhail, 2004; Rossander *et al*., 1992, Ma *et al*., 2010). Since calcium is absorbed by same mechanism as that of iron, high calcium in milk inhibits iron absorption.

Saccharides, ascorbic acid, carotenoids and mineral chelating peptides are found to enhance the bioavailability of iron (Citelli *et al*, 2012; Guo *et al*., 2014). Although excess fructose is blamed for modern health problems, fructose has been proved to enhance dietary non-heme iron absorption, possibly by chelating and/or reducing iron to the ferrous form, increasing ferrozine - chelatable ferrous iron levels (O'Dell, 1993, Christides *et al*., 2013).Additionally, fermented carbohydrates promote the growth of bacteria that produce propionate which improves iron absorption mediated by iron transporters present in the cecum and right colon (Levrat *et al.*, 1991).

1.6 Human Gut Microbiota

Gut bacteria which outnumber somatic and germinal cells has an intense impact on human physiology, immunology, and nutrition (Eckburg *et al.,* 2005). The intestinal microbiota distributed throughout the gut in different numbers due to varying microbial ecosystems primarily belongs to five microbial phyla: *Firmicutes*, *Bacteroidetes*, *Actinobacteria*, *Proteobacteria* and *Fusobacteria* (Rajilic *et al., 2*007). In the adult human, the predominant phylum, Firmicutes is present up to 90 % of the total gut microbiota (Turroni *et al., 2*012).

Host factors such as pH, bile acids, transit time, and mucus, environmental factors such as nutrients and medication and microbial factors such as adhesion capability, bacterial enzymes, metabolic strategies, and bacteriocin production and

bacteriophage influence the diversity of microbial gut communities (Reyes *et al.*, 2010; Prakash *et al.*, 2011; Ventura *et al.*, 2011). Microbial density influences the various functions of small and large intestine (Fig.1.11). Only few intestinal bacterial strains reside in the proximal part of the small intestine where an important metabolic function of uptake of dietary glucose, lipids, and proteins takes place. Majority of intestinal bacterial strains reside in the distal portion in the colon where water is absorbed from feces and SCFAs are produced via fermentation.

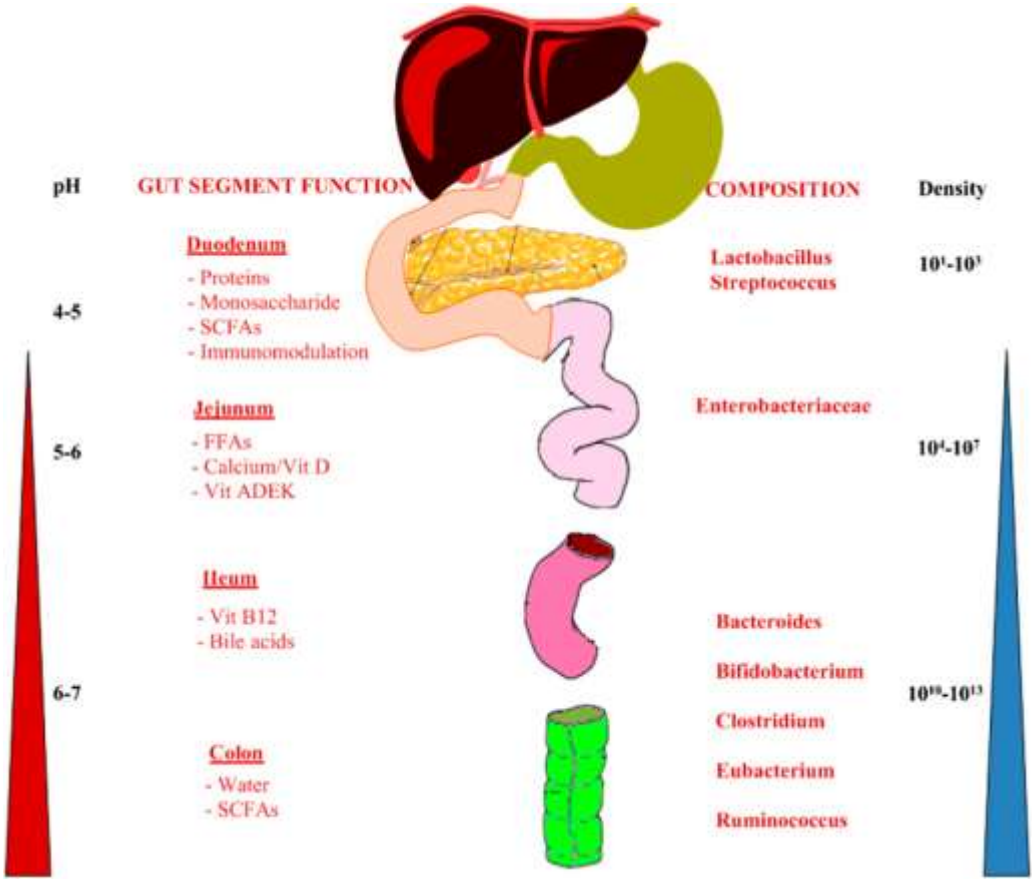

Figure 1.11: Differential functions of small and large intestine in relation to microbial density (Hartstra *et al.*, 2015).

Commensals present in the human gut produce molecules that mediate healthy immune response conferring protection from inflammatory disease (Fig.1.12) (Mazmanian *et al.*, 2008). Mammals depend on the metabolic products of gut microbiota for immunological development. Apart from this, metabolic functions of the gut microbiota include bile acid biotransformation and synthesis of vitamins (Yatsunenko *et al.*, 2012). The major short-chain fatty acids (SCFA) produced by the

gut microbiota such as acetate, butyrate, and propionate have trophic effect on the intestinal epithelium (Bergman *et al.*, 1990; Wong *et al.*, 2006; Claesson *et al.*, 2012) Butyrate is used as the preferred energy source for epithelial cells. Additionally, they have been reported to affect brain located beyond their site of production (Bravo *et al.*, 2011; Cryan and Dinan, 2012). Changes in the gut microbiota are closely linked with metabolic disorders such as obesity and type 2 diabetes (Greiner *et al.*, 2011; Kau *et al.*, 2011; Khan *et al.*, 2014).

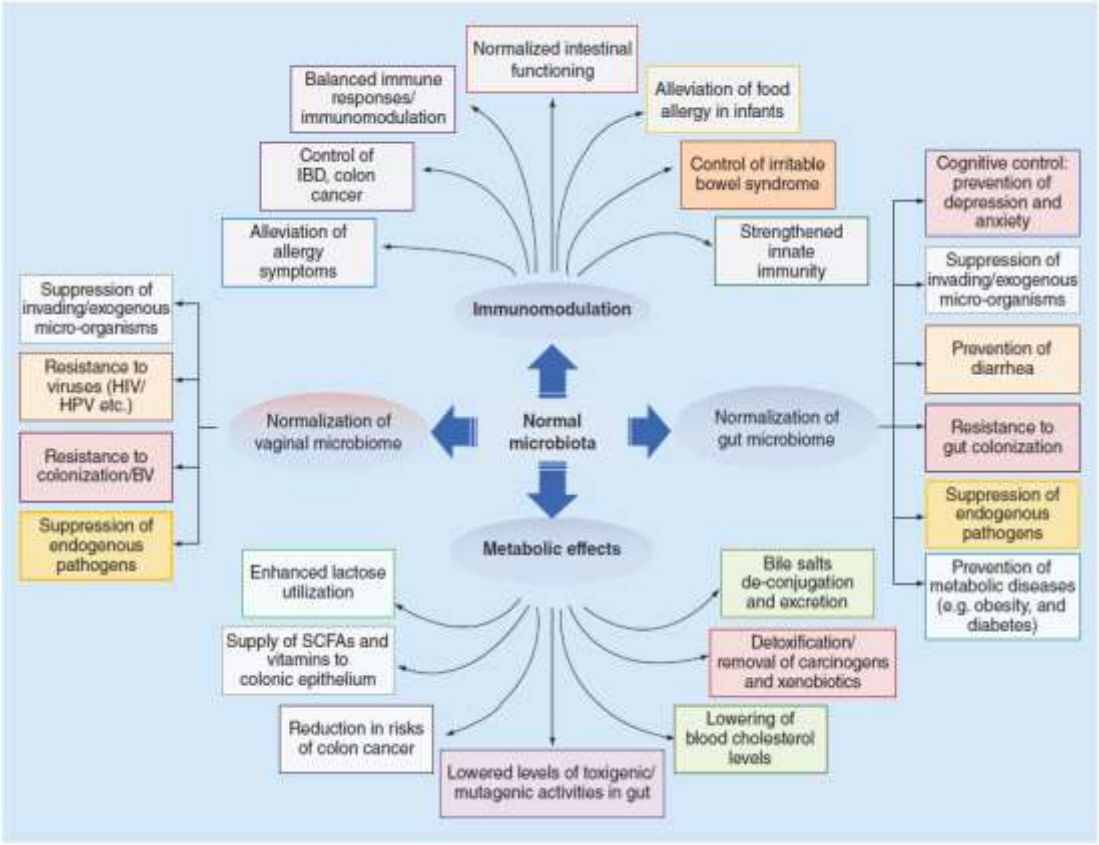

Figure 1.12: Functions of normal microbiota (Kumar *et al*., 2016).

Functional foods comprising of probiotics (microorganisms), prebiotics (compounds as fibers) and antioxidants have become increasingly popular in functional food markets (Saad *et al.*, 2013). The health-beneficial properties, physiological or metabolic benefits like boosting the immune system, counteracting diseases and degenerative disorders are provided by ingredients that are naturally present in or added to food or feed (Delgado *et al*., 2010; Watzl *et al*., 2005; Grajek *et al*., 2005).

1.7 Probiotics

Probiotic bacteria confer one or more health benefits to the host when consumed as live dietary supplements (FAO/WHO, 2006). There are minimum scientific criteria set for use of probiotics for human beings (Fig.1.13) (Sanders, 2003). The initial screening and selection of probiotics include testing of the phenotype genotype and plasmid stability; protein and carbohydrate utilization patterns; intestinal epithelial adhesion properties; ability to inhibit known pathogens; production of antimicrobial substances; antibiotic resistance patterns (Harzallah and Belhadj, 2013). Probiotic bacteria beneficially affect human health through different mechanisms involving modulation of the immune response, antagonism of pathogens by strengthening of the intestinal barrier or either by the production of antimicrobial compounds or through competition for mucosal binding sites (Daliri *et al.*, 2015). Different species of *Lactobacillus* or *Bifidobacterium*, the yeast *Saccharomyces cerevisiae* (*boulardii*) are commonly used in probiotic products. Some other bacteria, including strains of *Escherichia*, *Bacillus* and *Enterococcus* are also used but only as supplements (Douglas and Sanders, 2008). Currently used probiotics belong to bifidobacteria, lactic acid bacteria, dairy propionibacteria, yeasts (*Saccharomyces boulardii*), *Bacillus*, and the gram-negative *Escherichia coli* strain Nissle 1917 (Gareau *et al*., 2010).

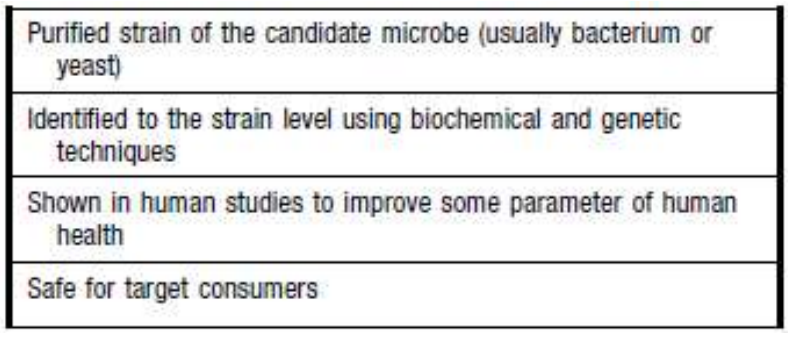

Figure 1.13 : Minimum criteria to be considered for use by human beings (Douglas *et al*., 2008)

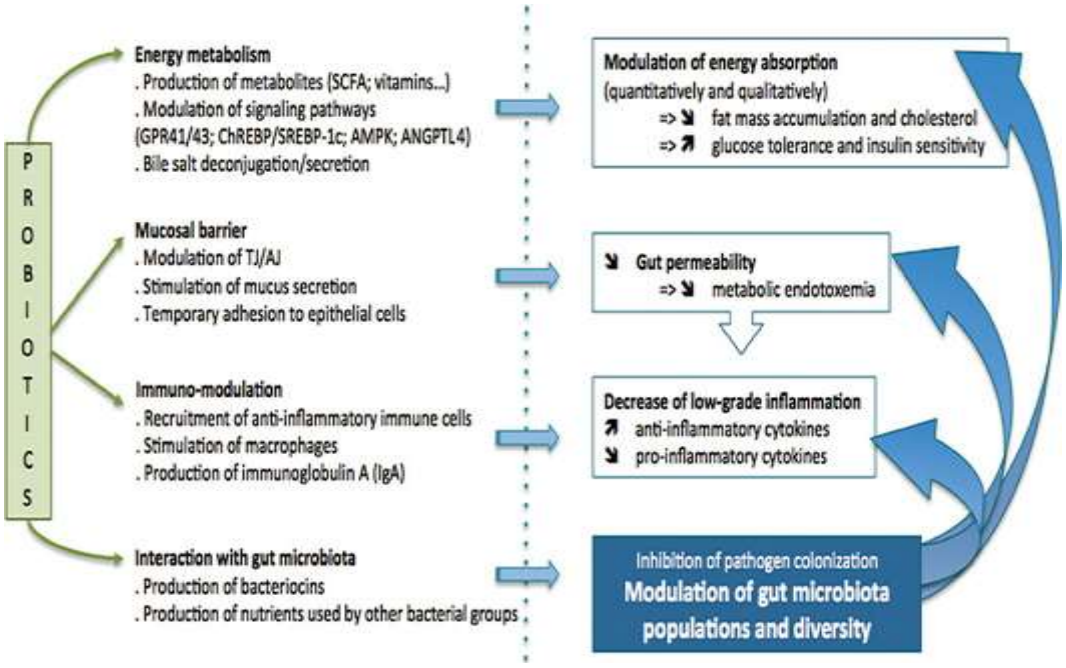

Figure 1.14: Potential beneficial effects of probiotic supplementation against metabolic disorders (Barz *et al.*, 2015).GPR, G protein-coupled receptor; SCFA, short-chain fatty acid; ChREBP, carbohydrate-responsive element-binding protein.

Probiotics are effective in metabolic disorders by improving various parameters such as plasma and liver cholesterol, free-fatty acids, hepatotoxicity markers including alanine and aspartate transaminases, gene and protein expressions of inflammatory and metabolic pathways (Table 1.1).Probiotic supplementation may help to reduce hyperphagia (Yadav *et al.*, 2013), improve control of weight gain, fat mass loss and glucose tolerance in case of obesity and metabolic disorders without modulation of caloric intake (Yoo *et al.*, 2013; Kang *et al.*, 2013; Everard *et al.*, 2014; Stenman *et al.*, 2014; Everard *et al.*, 2014;Wang *et al.*, 2015; Wu *et al.*, 2015).

Probiotic strains	Dose	Host organism	Diet	Treatment	Principal findings	Refer ence
Lactobacillus acidophilus La5; *Bifidobacterium lactis* Bb12	300 g of low fat (2.5% fat) yogurt/day corresponding to more than 5.10^8 cfu/dose of each strain	NAFLD patients	Own regular lifestyles (without other yogurt)	8 wk	↓Body weight and BMI; ↓serum ALT and AST, total chol and LDL-C	Nabavi *et al.*, (2014)
Lactobacillus rhamnosus CGMCC1.37 24+ prebiotics	1, 6.10^8 cfu/capsule with oligofruct-	Healthy overweight men and women	Energy restriction	Phase 1: 12 wk of dietary restriction	Prebiotics improve probiotic	Sanche z *et al.*, (2014)

	ose and inulin (2 capsules/day)			+/– probiotic Phase 2: 12 wk of weight maintenance +/probiotic	survival; ↓of body weight gain and body fat mass in women; ↓Lachnospiraceae family in women but not in men; ↓leptin concentration in plasma	
Lactobacillus curvatus HY7601; *L. plantarum* KY1032	0, 5.10^{10} cfu/day of each strain in 2 g of powder	Non-diabetic and hyper-triglyceridemic subjects	Own regular lifestyles	12 wk	↓Serum TG; ↑plasma apolipoprotein A–V and LDL particule size	Ahn *et al*., (2015)
Lactobacillus casei Shirota	65 mL of Yakult Light twice each day	Healthy human subjects	HFD (only 7 day)	4 wk (normal diet during 3 wk followed by a high-fat high-energy diet during 7 day)	Trend to reduce body weight gain; prevention of ↓insulin sensitivity induced by HFD; preservation of glycaemia and insulin action	Hulston *et al*., (2015)
L. plantarum A7	200 mL soy milk/day	Type 2 diabetes patients	Own regular lifestyles (without consumption of other dairy products)	8 wk	↓Systolic and diastolic blood pressure	Hariri *et al*., (2015)
Bifidobacterium breve B-3	5.10^{10} cfu/day	Overweight human subjects	Own regular lifestyles	12 wk	↓Fat mass accumulation; ↓plasma HbA1c; ↑γ-GTP; ↓hCRP levels	Minami *et al*., (2015)
cfu, colony forming unit; NAFLD, non-alcoholic fatty liver disease; BMI, body mass index; ALT, alanine transaminase; AST, aspartate transaminase; chol, cholesterol; LDL-C, low density lipoprotein cholesterol; *L. plantarum*, *Lactobacillus plantarum*; TG, triglyceride; HFD, high fat diet; HbA1c, glycosylated hemoglobin; γ-GTP, γ-glutamin transpeptidase; hCRP, human C-reactive protein.						

Table 1.1: Recent studies of probiotics effects on metabolic disorders in humans (Barz *et al*., 2015).

1.7.1 Colonisation and survival of probiotics

The daily dose of 10^{10} viable ingested bacterial cells is recommended to impart beneficial effect on humans and induce an intense population shift that temporarily overcrowds resident communities proved to likely impact the host's immune and

neuroendocrine functions (Aidy *et al*., 2015). Fig.1.15 depicts the distribution of microflora along the gastrointestinal tract, their abundance and relative abundance of ingested microflora compared to resident flora. Major changes occur mostly in the stomach and small intestine thereby influencing the metabolic capacities, microbe–host crosstalk, and host physiology.

	Taxonomy (phylum level)	Resident bacteria (number/ml or g)	Transit time[a]	Relative abundance of ingested bacteria compared to resident bacteria[b]
Stomach[c]		10^2–10^4	15 min–3 h	100 to 10 000-fold
Small intestine (ileum)[d]		10^6–10^8	2–5 h	0.01 to 1-fold
Colon (feces)[e]		10^{10}–10^{11}	12–24 h	0.0001 to 0.00001-fold

Table 1.2: Bacteria community and ingested bacteria mediated microbiome alteration along the GI tract (Kumar *et al*., 2016).

Ingested bacteria can influence resident communities through trophic interactions, a direct alteration in fitness or an indirect alteration in fitness through altered production of host-derived molecules (Figure 1.16). Many ingested bacteria have the ability to rapidly metabolize simple carbohydrates to lactic, acetic, or propionic acids thereby influencing and integrating pathways of dietary carbohydrate degradation altering metabolic outputs (McLaughlin *et al*., 2015; Derrien *et al*., 2015). Additionally, ingested bacteria can directly impact the abundance of pathogens through a decrease in pH resulting from the production of SCFA, niche competition, or through EPS and bacteriocins (Walker *et al*., 2005).Some ingested bacteria degrade mucin that liberate sugars, amino acids, sialic acids, and sulfate that serve as substrates for the resident commensals (Caballero *et al*., 2007). Finally, some strains

may impact the resident bacteria indirectly by interaction with host epithelium and the epithelial immune system (Reid *et al*., 2011; Bron *et al*., 2012; Lebeer *et al*., 2010). Taken together, human gut microbiome is altered with a transient community depending on recent diet and environmental exposure (Derrien *et al*., 2015).

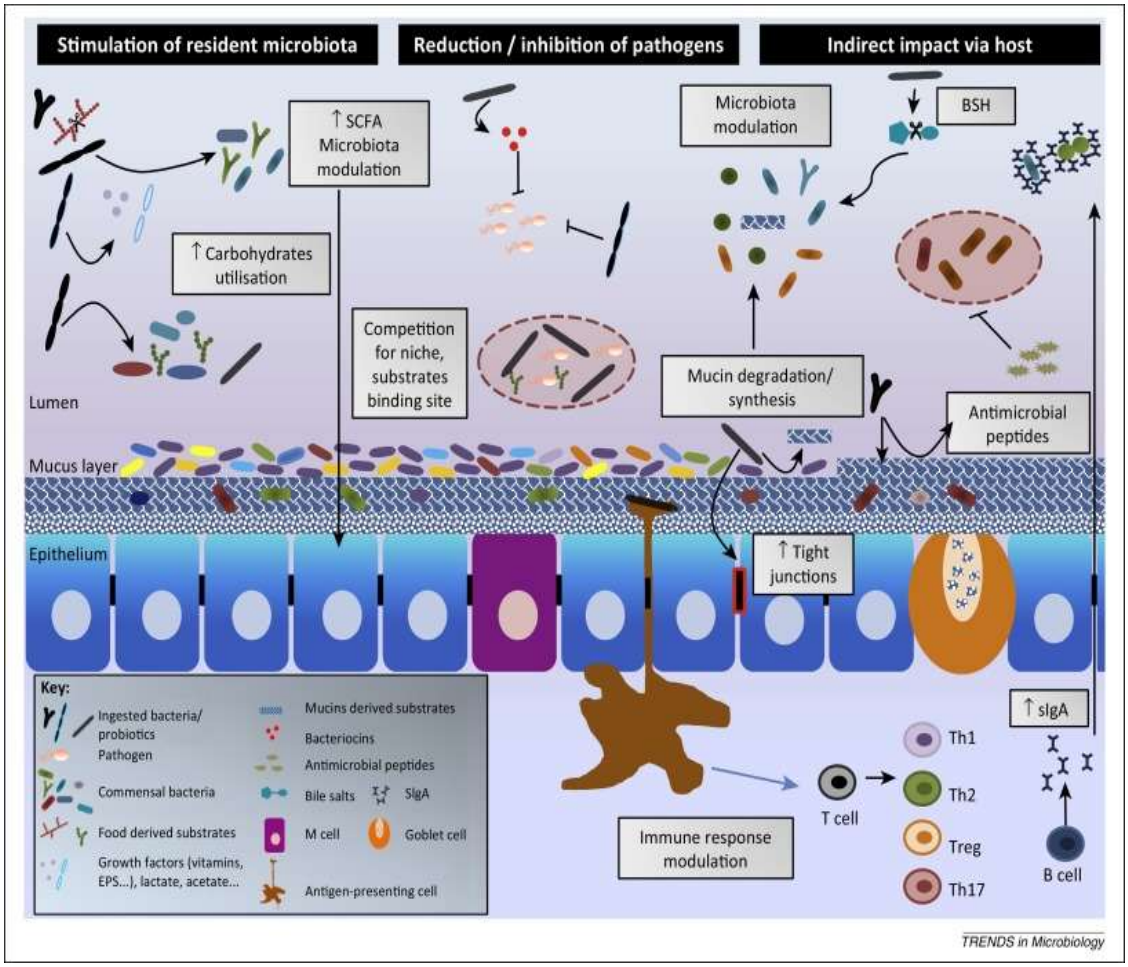

Figure 1.15: Different mechanisms by which ingested bacteria can impact the resident microbiota (Derrien *et al*., 2015).

1.7.2 *Vitreoscilla* hemoglobin

Vitreoscilla hemoglobin (Vhb) was the first bacterial hemoglobin discovered (Wakabayashi *et al*., 1986) and since its discovery it has been a widely used to enhance production of a variety of bioproducts, increase growth and survival of engineered organisms and stimulate bioremediation (Stark *et al*., 2015) (Fig 1.17).

Organisms	Applications
Crop plants (e.g., rice)	Improved growth (e.g., improved submergence tolerance for rice)
Animals used as food	Improved growth of food fish
Recombinant bacteria	Improved production of various biomolecules (enzymes, polymers, pharmaceuticals, insecticides)
Recombinant microbes (bacteria, yeast)	Improved production of biofuels
Aerobic wastewater bacteria	Efficient wastewater treatment at low aeration

Table 1.3: Applications of VHb technology (Stark *et al*., 2015).

Vhb serves as one protein with many different functions including electron transfer ability (Dikshit *et al*., 1992), antioxidant (Geckil *et al*., 2003; Wang *et al*., 2009), stimulate nitric oxide dioxygenase (NOD), oxygenase activities (Fish *et al*., 2000; Kaur *et al*., 2002; Demirtas *et al*., 2004) (Fig 1.18). VHb expression has significant effects on host gene expression and thus metabolism (Roos *et al*., 2004; Ayudhya *et al*., 2008) such as shifting metabolism to an energetically more efficient aerobic state (Ramachandran *et al*., 2012). Apart from this it has been used in the transformation of inorganics such as ammonia nitrogen, sequestering of metals, and solubilization of phosphate by increased production of organic acids (Arnaldos *et al*., 2013, 2014; Liao *et al*., 2014; Yadav *et al*., 2014; Kumar *et al*., 2014; Kahraman *et al*., 2014). Therefore in the present thesis work we have used *Ec*N containing the *vgb* for enhanced production of biomolecules and efficient survival in the intestine.

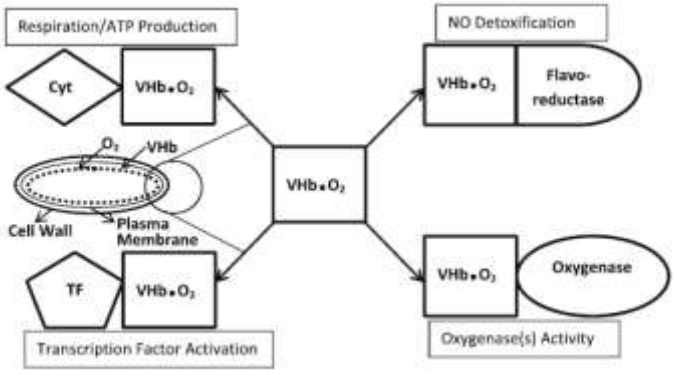

Figure 1.16: Probable roles of VHb in enhancing performance of heterologous bacterial hosts (Stark *et al*., 2015).

1.7.3 Bioengineering of probiotic bacteria

Probiotics can be effective in the preventing and treating of many diseases, but probiotic action is non-specific and non-discriminatory in certain hosts because of broad mode of action and strain variability (Bomba *et al., 2*002). The beneficial attributes of one strain or a cocktail of strains may not be reproducible as they differ from one another (Karimi *et al., 2*008). Additionally, the efficacy of a probiotic also depends on the probiotic strain, the formulation of probiotic preparation dose and route of administration (Morrow *et al., 2*008). Production of exopolysaccharide by the probiotics has been shown to be modified by the manufacturing process and probiotic delivery system thereby modifying their efficacy (Salazar *et al., 2*008; Grzeskowiak *et al., 2*011). Probiotic efficacy is also influenced by variability in the indigenous flora among different populations (Barzegari *et al., 2*012). These limitations develops need to genetically modify probiotic strains solely for drug delivery or target a specific pathogen or toxin to be used either as a vaccine (Culligan *et al., 2*009; Bhunia *et al.,* 2012). Oral recombinant probiotics offer several advantages such as increased shelf-life and stability, low delivery costs, direct *in vivo* delivery of active molecule to the mucosal surface without the need for bio-separation of the active molecules. This use of oral administration of live recombinant microorganisms for prevention and treatment of various diseases has resulted into development of "Biodrug" concept (D'Silva, *2*011).

The use of recombinant designer probiotics for mucosal delivery of therapeutic and prophylactic molecules including DNA, peptides, single-chain variable fragments, cytokines, enzymes, and allergens could be a safer and novel strategy against chronic intestinal diseases (Fig.1.19) (Wells, *2*011; Sleator *et al.,*2008). Apart from being invaluable sources of gene delivery vectors, delivering tumor-specific anticancer genes, toxins, polysaccharides for synthesis of nanodrugs, bacteria have emerged as important candidates for preventing cancer both in tumor diagnosis and therapy due to the ease of genetic manipulation and ability of certain bacteria to colonize solid tumor (Panteli *et al*., 2015; Van Dessel *et al*., 2015).

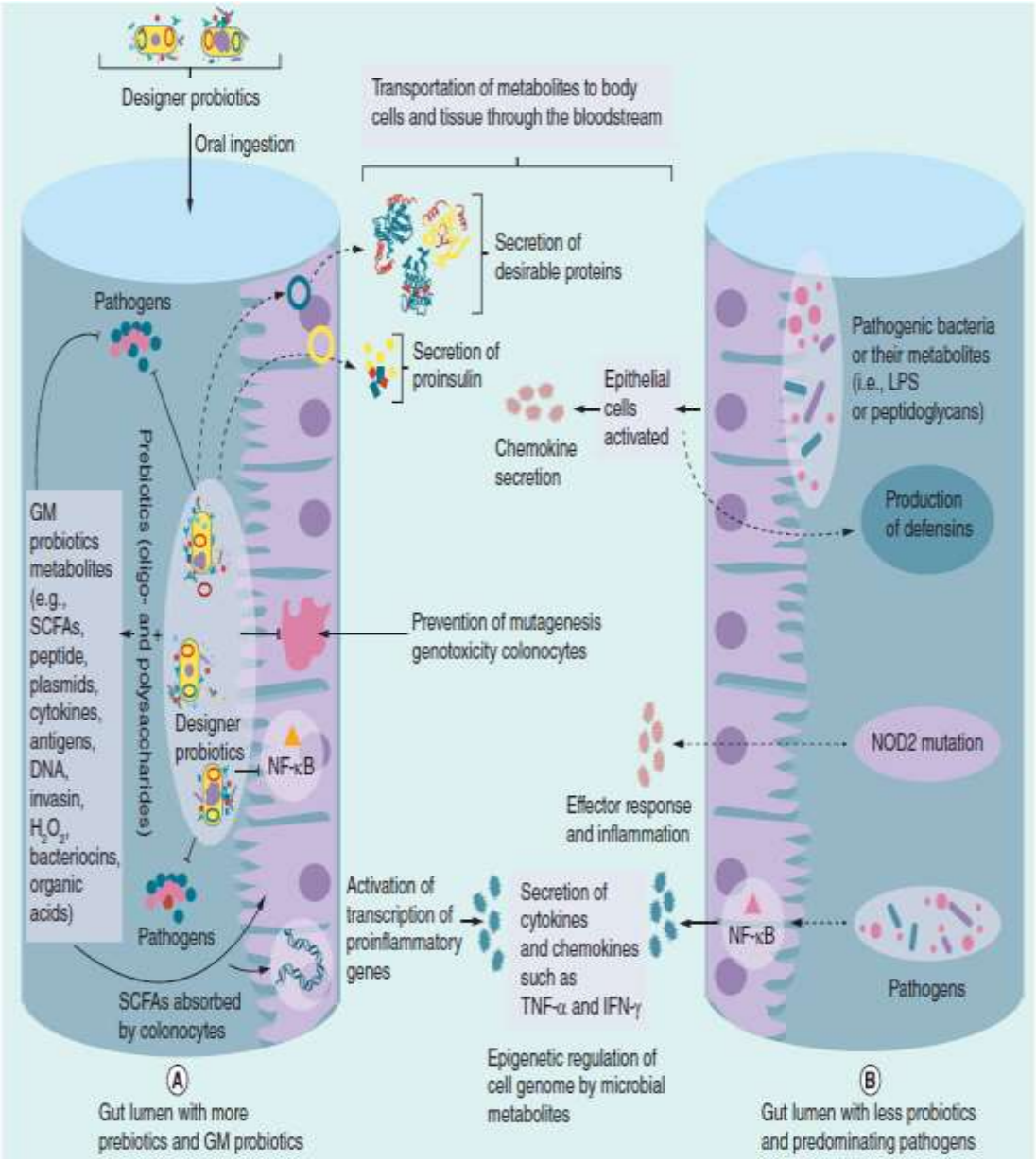

Figure 1.17: Effects of bioengineered probiotics (Kumar *et al*., 2016).

Orally administered engineered acylphosphatidyl ethanolamines (NAPE)-expressing *E. coli* Nissle 1917 in drinking water for 8 weeks had reduced the levels of obesity in mice fed a high-fat diet (Chen *et al*., 2014). Table 1.2 shows further examples of bioengineered probiotics used as designer probiotics.

Microbial species(origin)	Modification induced	Model	Inferences/remarks	Ref
Bacillus subtilis	*B. subtilis* expressing human IL-1 receptor antagonist (IL-1RA)	Rat and rabbit	Expression of intact and active IL-10 IL-1RA protein mucosal administration of recombinant *B. subtilis* released cytoplasmic recombinant protein with biological activity *in vivo* that prevented endotoxininduced shock and death.	Porzio *et al*., 2004
Bacillus subtilis subtilis	Expression of *Helicobacter pylori* urease B protein on *B. subtilis* spore coat protein CotC as fusion reporter	Mice	Prolonged colonizatic of recombinant *subtilis* in GI tract mice, significant (84% reduction in *H. pylo* load in the stomac indicating that oral administered urease I producing spores beir immunogenic couldprovide protectic against *H. pylo* infection	Zhou *et al*., 2015
Escherichia coli Nissle 1917	Expression of CAI-1 in *E. coli* Nissle 1917, termed as (Nissle-cqsA)	Infant mice	Pretreatment for 8 h with Nissle-cqsA increased survival of mice against *Vibrio cholerae*. The strategy was suggested to be an inexpensive approach to use bioengineered commensal bacteria to prevent humans from invading bacterial pathogens	Duan *et al*., 2010
Lactobacillus jejuni 1153 (Human vaginal strain)	Surface-anchored two domain CD4 (2D, CD4) linked to a peptidoglycan in the cell wall of *L. jejuni* 1153	*In vitro* modelling	Uniform expression of recombinant protein on *Lactobacillus* cell surface. The recombinant protein adopted a native functional conformation	Liu *et al*., 2008

Lactobacillus jensenii (Human vaginal strain)	Secretion of 2D, CD4 proteins, anti-CD4 recognizing on formation dependent antibody, and bound HIV-1 gp120	HeLa cells	Inhibition of HIV-1 entry into target cells in a dosedependent manner.The study represents an important step toward development of engineered commensal bacteria within vaginal microbiota to inhibit heterosexual transmission of HIV	Chang *et al*., 2003
Lactobacillus jensenii (Human vaginal strain)	Expression of anti-HIV chemokine RANTES and C1C5 RANTES	CD(+) T cells and macrophages	Inhibition of HIV in CD4+ cells and macrophages by both the variants	Dey *et al*., 2013
Lactobacillus jensenii 1153	Expression of potent HIV-inhibitor cyanivirin-N (CV-N), inhibition of CCR5-HIV (BaL), infectivity *in vitro* with 50% inhibitory concentration of 0.3 nM	Mice	Successful colonization of vaginal epithelium by the engineered strains administered to mice in estrus phase. The study was reported to be an expensive and durable approach to prevent HIV infection in women	Liu *et al*., 2006
Lactobacillus jensenii (Human vaginal strain)	Expression of HIV1-entry inhibitor, modified cyanovirin-N (mCV-N) in *L. jensenii* (LB-mCV-N)	Rhesus macaque model SHIVSF162P3	Detection of higher IL-1RA, lower load of Simian HIV, indicating the potential of engineered LB-mCV-N as a safer microbiocide	Brichacek *et al*., 2013
Lactobacillus jensenii 1153 (Human vaginal strain)	Expression of HIV-entry inhibitor modified cyanovirin N (mCV-N)	Human cervical	Expression of mCV-N with anti-HIV activity conserved in epithelial cell lines, expression of higher immunomodulatory potential by recombinant *L. jensenii* activity compared with control strains of *L. jensenii* 1153. Recombinant *L. jensii* 1153 were	Yamamoto *et al*., 2013

			recommended for clinical trials in humans	
Lactococcus lactis	Expression of Der p2 in *L. lactis* in different cell components (extracellular, intracellular and cell wall)	Mouse model	Oral pretreatment of mice with live recombinant *L. lactis* prevented the development of allergen-induced airway inflammation by induction of specific mucosal immune tolerance	Ai *et al*.,2014
Salmonella enteritica sv *typhimurium*	Eukaryotic expression of plasmids encoding Cu-Zn SOD and MCP-1 to intestinal cells	Male Balb/c mice	Bactofection-mediated improved total antioxidant capacity, reduced histological colitis score compared with untreated controls	Palffy *et al*., 2011
Streptococcus gordonii (oral origin)	Recombinant *S. gordonii human* IL-10 composed of amino acid residues RVFP of transporter at N-terminus	Mice model	Display of full biological activity by RFVP/IL-RA *in vitro*, recombinant strain was proposed to be useful as delivery system for selective targeting of mucosal surface	Ricci *et al*., 2003

Table 1.4: Bioengineered probiotics for use as designer probiotics in humans (Kumar *et al*., 2016).

1.7.4 *E. coli* as probiotic

Most probiotic bacteria are Gram-positive strains mostly lactic acid bacteria (LAB), such as *Lactobacilli, Lactococci, Bifidobacteria* and *Streptococci* because of their ability to persist within the gut ecosystem and produce organic acids such as lactate and acetate (Gill *et al., 2*008). However, stability within the product is the major problem with most probiotics. Bifidobacteria are strictly anaerobic and that leads to processing difficulties. To overcome this problem, less fastidious micro-organisms have come into picture and reports have cited the use of *E. coli* as a probiotic. Most of the studies on probiotic *E. coli* centres around one particular strain, known as Nissle 1917 (McFarland *et al.*, 1993; Fric *et al.*, 2002; Schulze *et al.*, 2008).

E. coli strain Nissle 1917 (EcN) is the active component of the pharmaceutical preparation Mutaflor. This drug has been traditionally used since 1917 (Nissle *et al.,*1918; 1925) to treat various diseases and dysfunctions of the intestinal tract (Hamilton *et al.,* 2001; Fric *et al., 2*002; Kruis *et al., 2*004; Krammer *et al.,* 2006; Schultz *et al.,* 2008).

The *E. coli* strain Nissle 1917 has been thoroughly analysed by means of microbiological, biochemical, and molecular genetic methods (Table.1.3). The strain does not possess any virulence factors but has gene clusters located on genomic islands (GEIs), its chromosome responsible for the synthesis of several so-called 'fitness factors', which contribute to the strain's probiotic nature. Serologically, EcN belongs to the *E. coli* O6 group and is of serotype O6:K5:H1 (Blum *et al.,* 1995; Grozdanov *et al., 2*002). *Ec*N is a typical Gram-negative enterobacterium containing lipopolysaccharide (LPS) as a structural component of its outer cell membrane. The O6 surface antigen represents the outer part of the strain's LPS and exhibits some peculiar features like the O6 polysaccharide side-chain is very short, consisting of only one single 'repeating unit' of the oligosaccharide building block typical of the O6 antigen, giving the strain a so-called 'semi-rough' phenotypic appearance when grown on solid nutrient medium (Rietschel *et al.,* 1996). The specific features of the LPS of *Ec*N are likely to explain the phenomenon whereby the strain exhibits immunomodulating properties without showing immunotoxic effects (Grozdanov *et al., 2*002). Despite its capability to form a capsule, in the classic serum resistance test (Hughes *et al.,* 1982) the *Ec*N strain is nevertheless serum-sensitive and is rapidly killed in the presence of human serum or sera of other mammalian species. *Ec*N possesses flagella of serotype H1 and is thus quite mobile. The possession of flagella enables the microbe to actively move within the viscous intestinal mucus layer, e.g. in the direction of the intestinal mucosa, which serves as an oxygen source, useful for aerobic catabolism of substrates by *E. coli*. Besides their function as driving apparatus, the flagella are important in bacterial crosstalk with the epithelium and have also been described as bacterial sensors for humidity (Cario *et al., 2*005; Wang *et al., 2*005).

	***Ec*N**	**Ref.**	**Symbio-flor 2**	**Ref.**	**Colinf-ant newbo-rn**	**Ref.**
Information about the product						
Descript-ion	Single *E. coli* strain		Six *E.coli* genotypes		Single *E.coli* strain	
Information about the *E. coli* present in the product						
Isolation date	"1917"	Jacobi *et al*., 2011	1954	Zschüttig *et al*., 2012	Data not availa ble	
Serotype	06:K5:H1	Reister *et al*., 2014	Variable including 035,129, 0:169, rough, all H–	Wassenaar *et al*., 2015	083:K 24:H3 1	Mandal *et al*., 2008
Plasmid content	2 Cryptic plasmids	Reister *et al*., 2014	12 Plasmids	Zschüttig *et al*., 2012	No plasm-ids	Lodinová-Zádníková *et al*., 1998
Microcin product-ion	Microcin M, H47	Patzer *et al*., 2003	Microcin S	Zschüttig *et al*., 2012	Data not availa-ble	
Motility	Motile (flagella present)	Jacobi *et al*., 2011	Nonmotil e (flagella absent)	Wassenaar *et al*., 2015	Data not availa-ble	
No. of genes	5324 Genes	Reister *et al*., 2014	28,180 Genes belonging to 6486 gene families	Wassenaar *et al*., 2015	Data not availa-ble	
Closest relatives	CFT073, ABU8397 2 (UPEC)	Vejborg *et al*., 2010	K12, ATCC873 9 (commens -als)	Wassenaar *et al*., 2015	CFT07 3, 536 (UPE C)	Hejnova *et al*., 2006

Table 1.5: Details of *E. coli* strains present in market (Wassenaar *et al*., 2016).

*Ec*N possesses three different types of fimbriae which mediate adhesion to intestinal epithelial cells facilitate colonization of the gut. Fitness factor enable them

to compete with other strains in the ecological system of the gut and to effectively communicate with the host organism have been detected in the *Ec*N strain (Hacker *et al., 2*001; *2*003). *Ec*N produces an unexpectedly wide array of siderophores which are iron-chelating substances needed for bacterial iron uptake. *Ec*N also possesses an elemental ferrous iron uptake system (EfeU) (Große *et al., 2*006).

1.7.5 Probiotics for sugar mediated disorders

Obesity and Type 2 diabetes are associated with a dysbiosis, altered bacterial genes and respective metabolic pathways (Tilg and Moschen, 2014). Butyrate-producing intestinal bacteria (e.g. *Roseburia intestinalis* and *Faecalibacterium prausnitzii*) are reduced in Type 2 diabetes patients along with alteration in *Akkermansia muciniphilia* lining the intestinal mucus by potent antidiabetic drugs, like metformin, develop a need for developing novel microbial therapeutic principles. Probiotics can counteract the negative effect of obesogenic diet by interaction with commensal bacteria and altering expressions of microbial enzymes, especially those involved in carbohydrate metabolism or butyrate synthesis pathways (McNulty *et al., 2*011; Veiga *et al.,* 2014). The microbiome in obese individuals is thought to display an increased capacity to harvest energy from the diet along with a decreased ability to stimulate the production of gut factors that inhibit fat deposition (Turnbaugh *et al., 2*006). *Lactobacillus rhamnosus* GG protects against NAFLD through specifically reducing liver fat mass in association with modulation of the carbohydrate-responsive element-binding protein pathway (Ritze *et al*., 2014). The VSL#3 probiotic promotes the release of the hormone glucagon-like protein-1, resulting in reduced food intake and improved glucose tolerance, which was correlated with SCFA production leading to L-cell stimulation (Yadav *et al*., 2013). Probiotic treatment significantly improved insulin resistance, glucose intolerance, oxidative stress, fatty liver, reduced lipogenesis and increased β-oxidation in rats fed with high fructose diet (Park *et al*., 2013; Hsieh *et al., 2*013).

1.8 Prebiotics

Dietary prebiotics are a selectively fermented ingredient consequently resulting in specific changes in the composition and/or activity of the gut microbiome, thereby conferring benefits upon host health not only in the colon, but also in the urogenital tract, the oral cavity, and on the skin thus conferring a beneficial physiological effect on the host (Gibson *et al.,* 2010) (Fig. 1.20). The effects linked with optimized colonic function and metabolism are an increase in the faecal weight, expression of the binding proteins associated with mineral absorption, decrease in nitrogenous end products and reductive enzymes, a mild reduction in luminal colon pH, and immune system modulation (Douglas *et al.,* 2008). Additionally, prebiotic exert health benefits by the change in the colonic microbiota, their products, and their effects on host biochemistry and histology. Colonocytes derive most of the energy by the localized fermentation of this prebiotics (Priebe *et al.,* 2002). Prebiotics prevent obesity and characteristics of T2D (Cani *et al*., 2005; 2006; 2009; Delme´e *et al*., 2006; Dewulf *et al*., 2011), epithelial barrier dysfunction (Johnson-Henry *et al*., 2014) and modulate hepatic lipogenesis (Beylot, 2005) (Fig. 1.16).

1.8.1 Short Chain Fatty Acid

Dietary fibers that are not completely hydrolyzed by the host enzymes during digestion are fermented by intestinal bacteria leading to production of SCFAs; acetate, propionate and butyrate (Flint *et al., 2*008). These are used for *de novo* synthesis of lipids and glucose, which are the main energy sources for the host (Wolever *et al.,* 1989). SCFAs exert their beneficial effects on metabolic functions such as glucose homeostasis, insulin sensitivity, body weight and food intake, reducing risk of obesity, diabetes, cardiovascular disease, inflammatory bowel disease and colon cancer (Fig.1.21) (Delzenne *et al., 2*005; 2011; Galisteo *et al., 2*008; Besten *et al., 2*013, Harig *et al.,* 1989; Donohoe *et al.,* 2011; Fukuda *et al., 2*011).

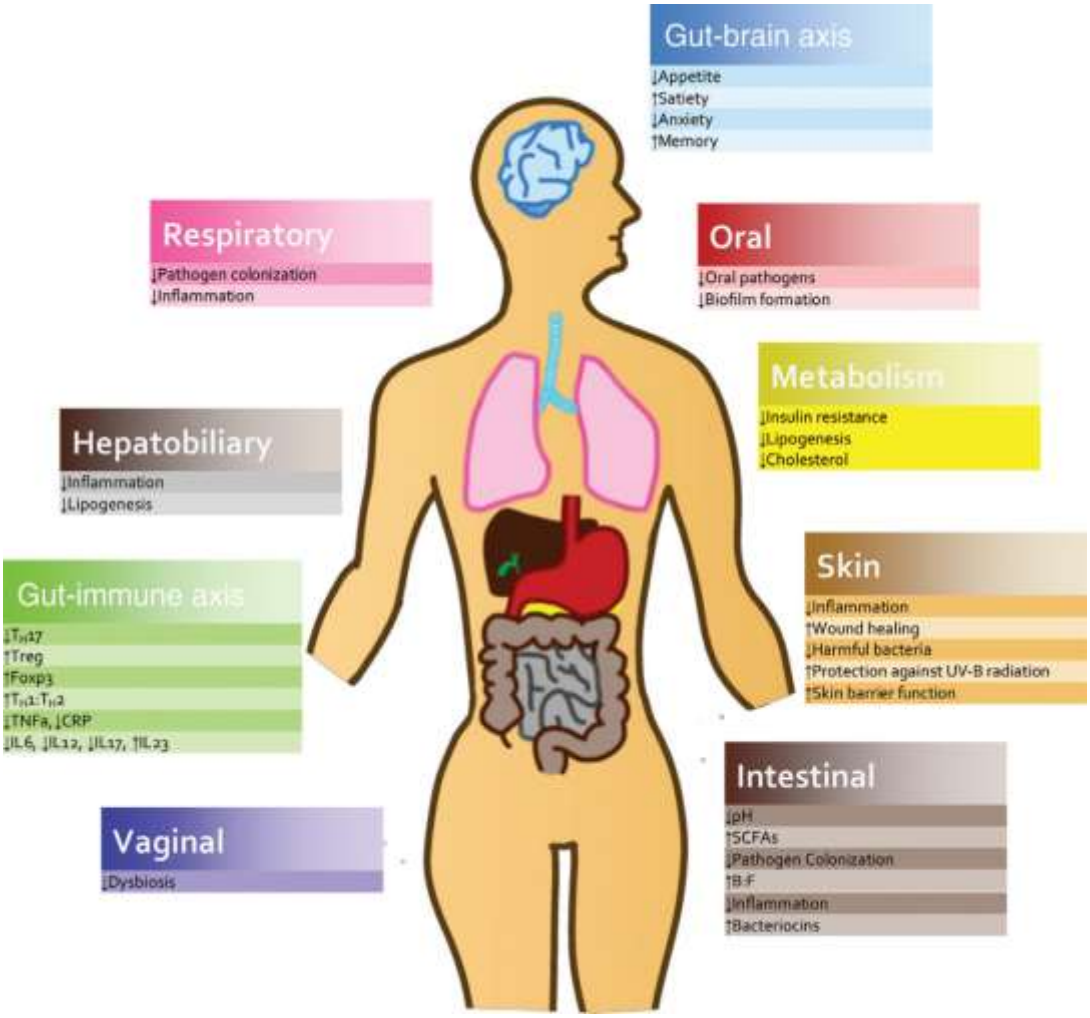

Figure 1.18: Possible affects due to pro-, pre- or synbiotic supplementation (Woloszynek *et al.*, 2016)

Butyrate improved insulin sensitivity and increased the energy expenditure in dietary-obese mice (Gao *et al.*, 2009). Butyrate and propionate were shown to protect against diet-induced obesity and regulated the gut hormones (Lin *et al.*, 2012). The oral administration of acetate improved glucose tolerance and suppresses obesity (Yamashita *et al.*, 2007).High amounts of SCFAs decrease the intestinal pH thereby preventing the growth of pathogenic bacteria and modulating *Bacteroidetes* spp. and *Bifidobacterium* spp as these taxa are not tolerant to low pH (Fig.1.21) (Ghosh *et al.*, 2011; Zimmer *et al.*, 2012; Duncan *et al.*, 2009). SCFAs also help in the absorption of minerals, like calcium, by increasing the expression of calcium binding protein and their solubility (Scholz-Ahrens *et al.*, 2007).

SCFA	Physiological effect
Acetate CH_3-COO^-	Reaches the portal vein and is metabolized in various tissues **Intestinal effects** Is a minor energy source for the colon epithelial cells Decreases the pH of the colon (which decreases bile salt solubility, increases mineral absorption, decreases ammonia absorption, and inhibits growth of pathogens) Has anti-inflammatory effects Increases colonic blood flow and oxygen uptake Is used by cross-feeding species as a co-substrate to produce butyrate **Other effects** Is a substrate for cholesterol and fatty acid biosynthesis in the liver Is an energy source for muscle and brain tissue
Propionate CH_3-CH_2-COO^-	Reaches the portal vein and is subsequently taken up by the liver **Intestinal effects** Is a minor energy source for the colon epithelial cells Decreases the pH of the colon (which decreases bile salt solubility, increases mineral absorption, decreases ammonia absorption, and inhibits growth of pathogens) Prevents proliferation and induces apoptosis of colorectal cancer cells Interacts with the immune system Has anti-inflammatory effects **Other effects** Promotes satiety Lowers blood cholesterol levels Decreases liver lipogenesis Improves insulin sensitivity
Butyrate CH_3-CH_2-CH_2-COO^-	Is mainly taken up by the colon epithelial cells, only small amounts reach the portal vein and the systemic circulation **Intestinal effects** Is the preferred energy source for the colon epithelial cells Decreases the pH of the colon (which decreases bile salt solubility, increases mineral absorption, decreases ammonia absorption, and inhibits growth of pathogens) Stimulates proliferation of normal colon epithelial cells Prevents proliferation and induces apoptosis of colorectal cancer cells Affects gene expression of colon epithelial cells Plays a protective role against colon cancer and colitis Improves the gut barrier function by stimulation of the formation of mucin, antimicrobial peptides, and tight-junction proteins Interacts with the immune system Has anti-inflammatory effects Stimulates the absorption of water and sodium Reduces oxidative stress in the colon **Other effects** Promotes satiety

Table 1.6:Overview of the physiological effects of the short-chain fatty acids (SCFAs) acetate, propionate, and butyrate produced by human colon (Hamer *et al.*, 2008; Al-Lahham *et al.*, 2010; Havenaar, 2011; Macfarlane and Macfarlane, 2012; Chang *et al.*, Louis *et al.*, 2014; Tralongo *et al.*, 2014).

1.8.2 Mannitol

In the human gut, mannitol acts as a prebiotic leading to the formation of SCFAs which have been proved to confer protection against colon cancer and in the prevention and treatment of the metabolic syndrome (Shen *et al.*, 1997; Yadav *et al.*, 2006). These SCFA in the portal vein, can activate the AMPK which phosphorylates and inactivates liver ACC1, ACC2 and HMG-CoA leading to the stimulation of fatty acid oxidation, inhibition of lipogenesis, cholesterol synthesis and glucose production

mainly through the inhibition of gluconeogenic gene expression. (Hardie *et al.*, 2002; Zhang *et al., 2*009).

1.8.3 Medical Uses of Prebiotics

Effective prebiotics are widely available and frequently used in nutrition products whereby they provide short-chain fatty acids to colonocytes via fermentation, maintain colon integrity, normalize and maintain bowel function, and build colonization resistance in a hospital setting (Whelan *et al.*, 2005). These features make prebiotics suitable for use in patients with antibiotic-associated diarrhoea, irritable bowel conditions and for general bowel maintenance (Seidner *et al., 2*005). Prebiotics are also beneficial for renal patients where they lead to an alteration in nitrogen excretion (Younes *et al.,* 1995; *2*001). The compatibility and tolerance of these formulations containing prebiotic ingredient used in a range of enteral products has been established, both empirically and experimentally (Cockram *et al.,* 1998). Prebiotics are used for adult and pediatric patients with a wide range of medical conditions, including cancer, diabetes, renal failure, metabolic stress, pressure ulcers, and immunosuppression (Ross Products Division, 2005).

1.9 Synbiotics

Synbiotics provide the specific substrate to the probiotic organism for its fermentation thereby boosting their growth (Farnworth, 2001). An ideal synergistic synbiotic supplement contains an appropriate single or multi strain probiotic and a mixture of prebiotics. This not only promotes the autochthonous-specific strain of intestinal tract but also stimulates and amplifies the survival of desired probiotics (Fotiadis *et al., 2*008). Synbiotics improve the colonisation and survival of live microbial dietary supplements in the gastrointestinal tract by activating the metabolism of one or a limited number of health-promoting bacteria, selectively stimulating the growth and thus helping in prevention of various diseases like cancer and atopic dermatitis (Gibson and Roberfroid, 1995) (Fig.1.22).

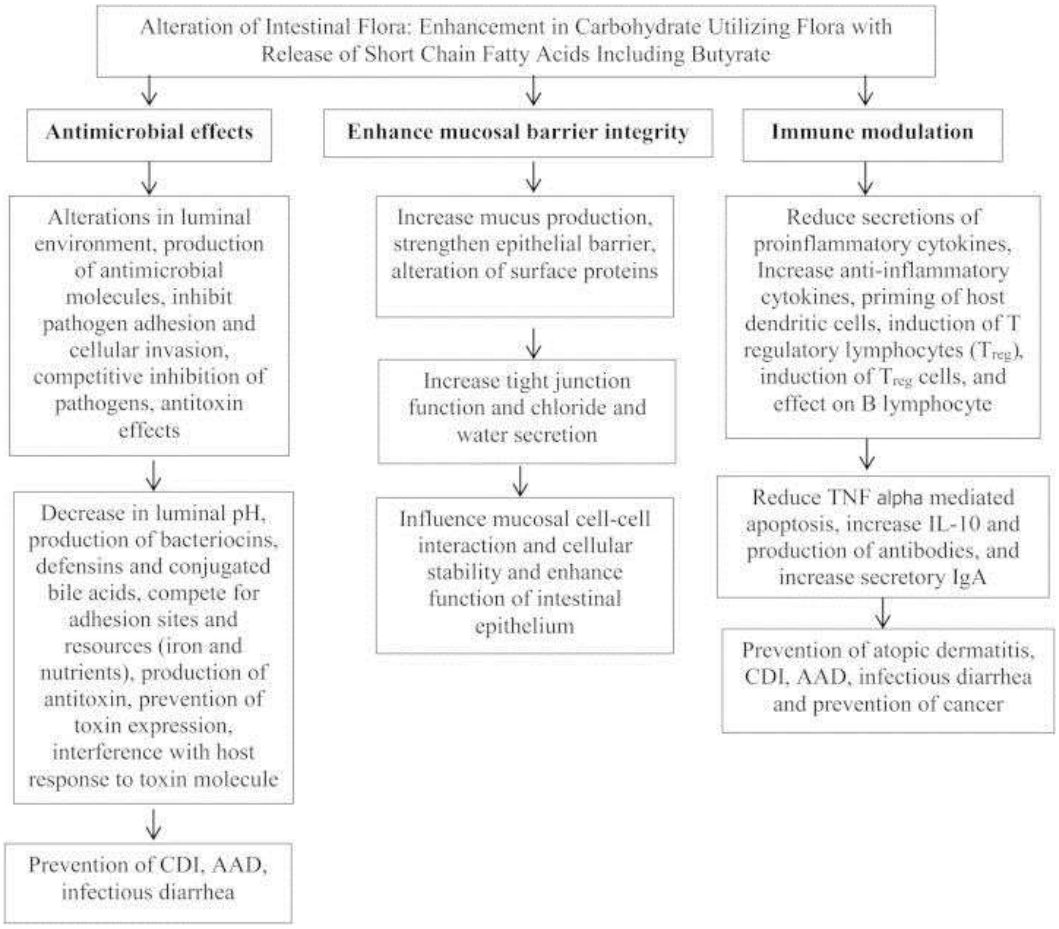

Figure 1.19: Biologic effects and mechanisms of action of prebiotics, probiotics, and synbiotics (Patel *et al.*, 2015). Abbreviations: AAD, antibiotic-associated diarrhea; CDI, Clostridium difficile infection; IgA, immunoglobulin A; IL-10, interleukin 10; TNF, tumor necrosis factor.

1.10 Antioxidants

Reactive oxygen species (ROS) play a key role as secondary messengers in numerous signalling pathways, such as transcriptional regulation, differentiation, proliferation and cellular apoptosis (Bubici *et al.*, 2006). However, free radicals are a major cause of many degenerative diseases, such as atherosclerosis, cancer, cardiovascular diseases, inflammatory bowel diseases, skin aging, old age dementia and arthritis. In the liver, ROS is generated by multiple sources, including the mitochondrial respiratory chain, cytochrome P450 family members, peroxisomes, xanthine oxidase, and NADPH oxidases (Mormone *et al.*, 2011). Persistent production of ROS constitutes a sustained inflammatory response, liver injury and

HSCs activation, at last, resulting in the initiation and progression of fibrosis (Poli *et al.*, 1997; Parola *et al.*, 2001; Sanchez-Valle *et al.*, 2012). Mercury accumulating mainly in the kidneys shows higher binding affinity for endogenous thiol containing molecules such as glutathione and cysteine causing oxidative stress and renal damage by damaging the antioxidant defence system and depleting the thiol reserves (Hultberg *et al.*, 2001; Zalups *et al.*, 2000).Clinical trials have shown that antioxidants play a fundamental role in the prevention of cancer and cardiovascular diseases (Shklar, 1998; Surh, 1999; Kris-Etherton *et al.*, 2002; Ferrari *et al.*, 2003). Antioxidants are thought to play a vital role in neutralizing free radical-induced damage to macromolecules and have been found to repair the free radical mediated cell injury (Arslan *et al.*, 2010).

Due to the potential roles of antioxidants to protect against harmful oxidation, there is a rise in the production and consumption of various antioxidants, and hence, through metabolic engineering, the successful reconstitution of heterologous pathways in reliable bacteria as host is important (Fig.1.23) (Lin *et al.*, 2014).

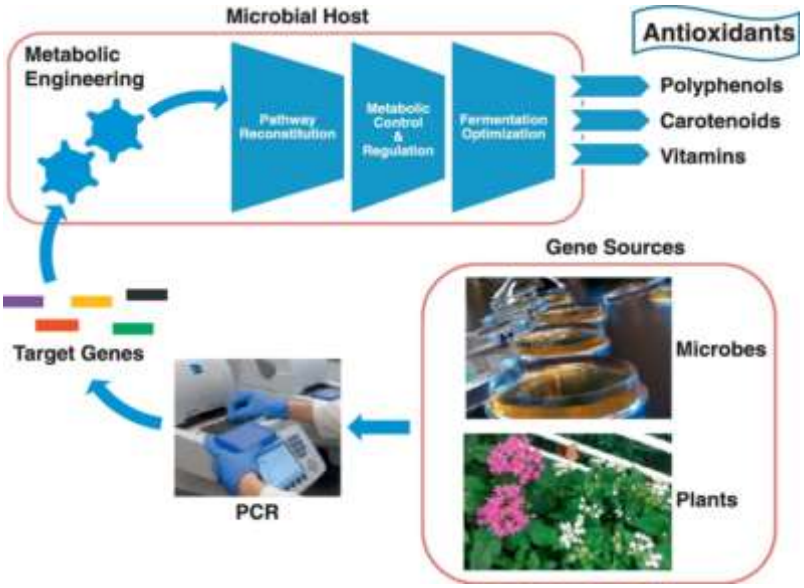

Figure 1.20: Metabolic engineering approach for antioxidant production (Lin *et al.*, 2014).

1.10.1 PQQ

Pyrroloquinoline-quinone (PQQ), was first recognized as a coenzyme for methanol dehydrogenase in methylotrophic bacteria (Salisbury *et al*., 1979; Westerling *et al.,* 1979) and has been detected in a wide variety of foods and other sources (Kumazawa *et al.*, 1995). PQQ is a water-soluble and redox active o-quinone that acts as a redox cofactor of bacterial dehydrogenases, such as alcohol and glucose dehydrogenases (Goodwin and Anthony, 1998; Stites *et al*., 2000). PQQ is synthesized from the two amino acids glutamate and tyrosine encoded in the precursor peptide by a number of microorganisms (Goodwin and Anthony, 1998) and shown to be a potent growth factor in plants, bacteria, and higher organisms (Steinberg *et al.*, 1994). PQQ have been proved to be relatively strong antioxidant by the structural analysis compared to other antioxidants such as indole and pyrrole derivatives as it exhibits comparatively higher reactive electron density (Misra *et al.*, 2004). Though PQQ exhibited little effect on Complex I activity and mitochondrial function (Stites *et al., 2*000), *in vitro* studies revealed that PQQ can scavenge O_2^- and HO efficiently (Urakami *et al.,* 1997). However, the synthesis of PQQ in higher organisms has not been shown, and the major source of PQQ in these organisms is believed to be microbial sources (Misra *et al.,* 2012). Although PQQ is not biosynthesized in mammals, a trace amount of PQQ has been found in human and rat organs or tissues, and an especially large amount occurs in human milk (Stites *et al*., 2000; Mitchel *et al.,* 1999).

PQQ has been receiving much attention owing to its nutritional importance and physiological functions (Rucker *et al.,* 2009). PQQ participates in a range of biological functions, which are related to immune, cognitive, as well as protection from cardiac and neurological ischemic events. PQQ-deficient diets have impaired growth, immunological defects and decreased fertility in mice (Killgore *et al.*, 1989; Steinberg *et al,* 1994; 2003). Accumulating evidence suggest that PQQ plays important roles in regulating cellular signalling and redox balance (Urakami *et al.,* 1997; Felton *et al., 2*005; Rucker *et al., 2*005; Misra *et al.,* 2012).

In summary, fructose consumption and metabolic disorders has increased simultaneously in past few decades. In order to overcome the effects of metabolic syndrome numerous strategies including various drugs and herbal therapy have been implicated. These strategies have their side effects. Therefore, recent research is focussed on use of probiotics in order to overcome these limitations as an alternative natural therapeutic approach considered as a safe and effective approach. Thus, a multidirectional strategy of combining the existing approaches such as antioxidant potential, probiotic, prebiotics and chelation could be effective in preventing metabolic disorders caused by high fructose and iron deficiency. The first part of thesis work deals with the development of probiotic *E. coli* by metabolic engineering approach for alleviation of dietary fructose induced metabolic syndrome and oxidative stress. Contrary to negative effects of fructose, fructose is also known to have property of improving iron deficiency. So, the second part of thesis work deals with the use of metabolically engineered probiotic for countering iron deficiency. Apart from this, fructose which is currently used as HFCS comes with an additional negative effect due to the presence of mercury in it. To deal with this problem, probiotic *E.coli* has been metabolically engineered for alleviation of mercury induced toxicity. Based on the above literature following are the objectives for thesis work:

Objectives:

- Evaluating the effect of genetically engineered *Escherichia coli* Nissle 1917 synbiotics on fructose induced metabolic effect in Charles foster rat model.
- Determining the effect of fructose and prebiotic produced by *Escherichia coli Nissle* 1917 harboring glucose facilitator protein and mannitol dehydrogenase genes on iron absorption.
- Evaluating the efficacy of probiotic *Escherichia coli* Nissle 1917 strain containing citrate synthase and *pqq* operon in amelioration of heavy metal mercury induced metabolic disorders in rat model.
- Evaluating the efficacy of probiotic *Escherichia coli* Nissle 1917 strain containing gluconate dehydrogenase and *pqq* operon in amelioration of heavy metal mercury induced metabolic disorders in rat model.