Chapter 1

Review of Literature

1 Introduction

1.1 Sugar consumption global picture

Recently United Nation declared greater worldwide health burden of chronic non-communicable diseases such as heart disease, cancer and diabetes than infectious diseases, contributing to 35 million deaths annually **(Lustig et al., 2012)**. This is not limited only to developed world, countries adopting western diet- one dominated by low-cost, highly processed food — has witnessed increased prevalence of obesity and related diseases. Obese people are 30% more than who are undernourished. Many people think that root cause of these diseases is obesity which is not true because 20% of obese individuals have metabolism within normal range. Conversely, up to 40% of normal-weight people develop metabolic syndrome. This suggests that obesity is not the cause; rather it is marker for metabolic syndrome.

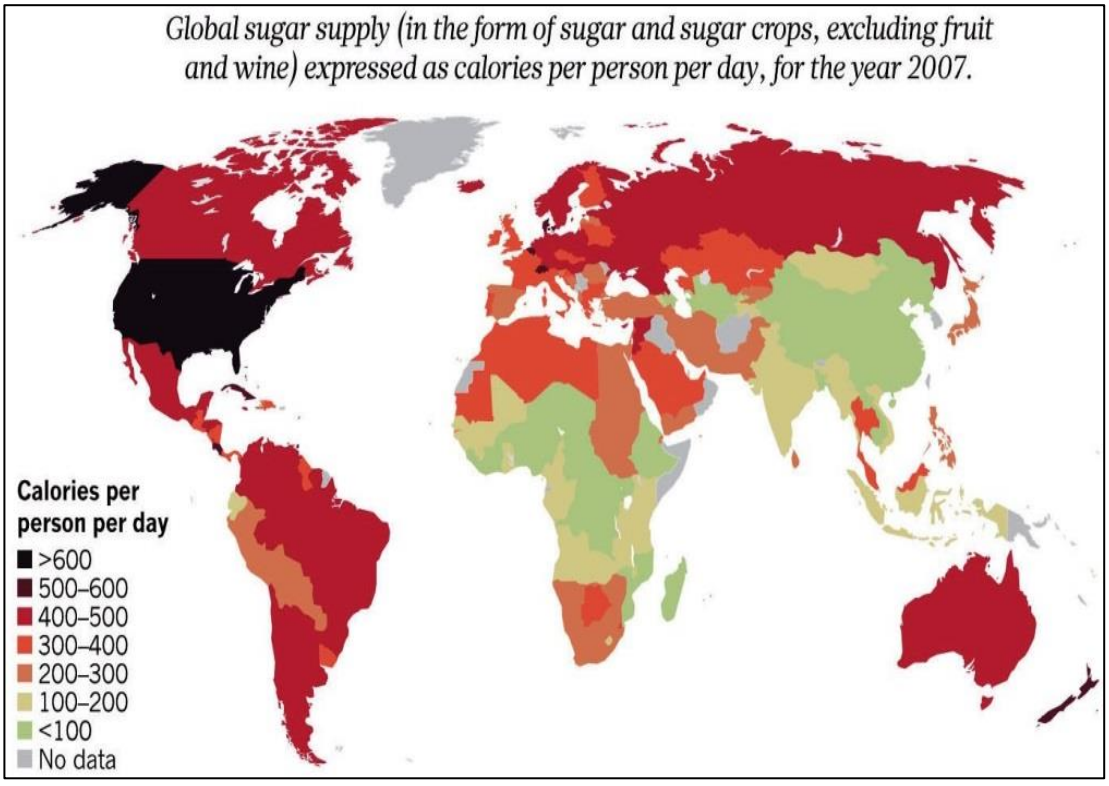

Fig. 1.1 Global sugar consumption (Lustig et al., 2012).

Over the past 5 decades, sugar consumption has tripled worldwide. There is fierce controversy over the pervasive use of High-Fructose Corn Syrup (HFCS) in the United States. HFCS is manufactured from corn syrup, processed to yield a roughly equal mixture of glucose and fructose. Therefore, developed countries eschew HFCS,

relying on sucrose as naturally occurring sugar which also consists of equal parts glucose and fructose. Global sugar supply expressed as calories per person per day across the globe is shown in **Fig. 1.1**. Sugar consumption in India and disease association Per capita consumption of sugar in India during last five decades has been increased by more than ~ 3 folds **Fig. 1.2**. Excessive consumption of sucrose or fructose is associated with metabolic syndrome like symptoms including; Hypertension (Uric acid), Myocardial infraction (dyslipidaemia, insulin resistance), Dyslipidemia (de-novo lipogenesis), Pancreatitis (Hypertriglyceridaemia), Obesity (Insulin resistance), Malnutrition (Obesity), Hepatic dysfunction (non-alcoholic steatohepatitis) and Habituation. There are accumulating evidences, both in animal models and in humans which support the fact that hyperenergetic high sucrose diet produce adverse metabolic effect. These metabolic effects appear to be more enhanced in overweight and insulin resistance patients.

1.2 Sugars metabolism

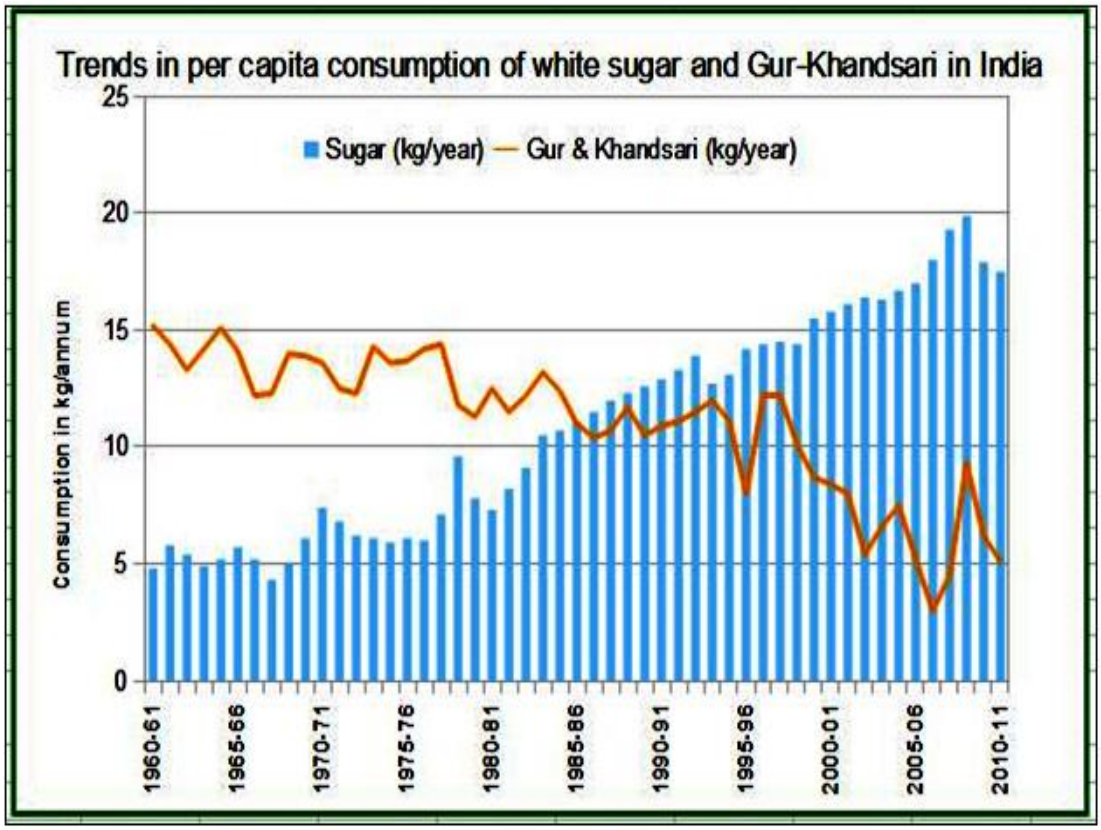

Fig. 1.2 Trends in per capita consumption of white sugar and Gur-Khandsari in India.

Dietary sugar encompasses several carbohydrates i.e. starch, sucrose, fructose and high-fructose corn syrup, each of which is composed of glucose with or without fructose **(Lyssiotis et al., 2013)**. From an energetic standpoint caloric values of glucose and fructose are same. However, these carbohydrates are treated by the human body quite differently. In general, glucose utilization is directly by muscle and brain tissues.

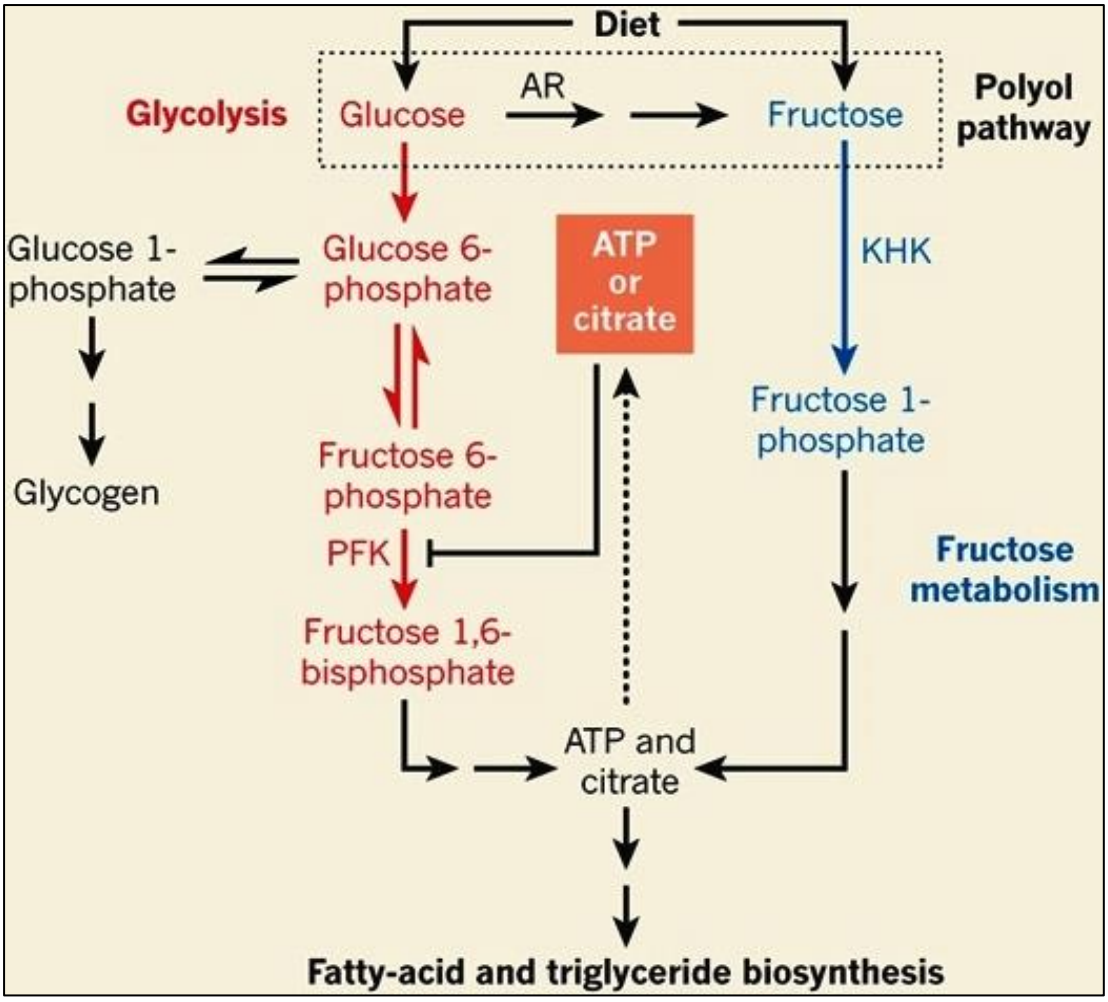

Fig. 1.3 Glucose and fructose are dietary sugars (Lyssiotis et al., 2013).

Excess glucose is stored as energy reserves in the liver but can also be metabolized by the polyol-biochemical pathway to form fructose Fig. 1.3. By contrast, fructose metabolism takes place exclusively in liver. In liver, fructokinase-a liver-specific fructose-metabolizing enzyme, traps fructose in liver cells as fructose 1-phosphate. Fructose-1-phosphate unlike that of fructose-6-phosphate can bypass a major regulatory step in glycolysis that generates fructose 1, 6-bisphosphate through

the action of phosphofructokinase-energy-sensitive enzyme. Fructose is a hexose sugar having chemical formula identical to glucose **(Tappy et al., 2010)**. Presence of keto group in position 2 of its carbon chain distinguishes it from glucose having aldehyde group at position 1glucose carbon chain.

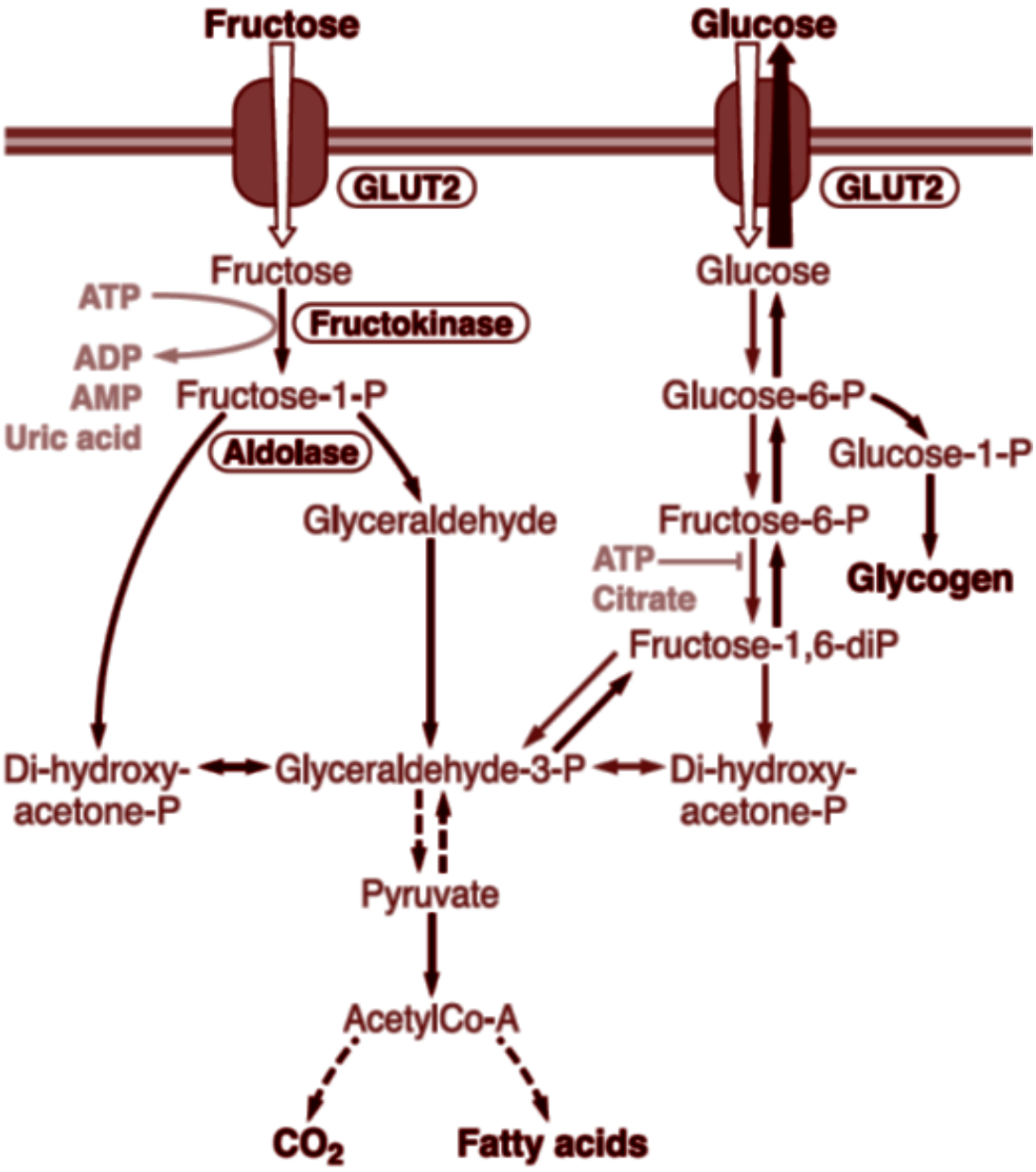

Fig. 1.4 Hepatic metabolism of Glucose and Fructose (Tappy et al., 2010).

Fructose ingested in from of pure fructose or of HFCS or from digestion of sucrose by disaccharidases present at brush border membrane, is transported into the enterocytes via GLUT5 receptor specific for fructose **(Fig. 1.4)**. This transport does not require ATP and it is also independent of sodium absorption. In contrast, transport

of glucose via enterocytes is ATP and sodium absorption dependent. Once transported in enterocytes, 12% of absorbed fructose in converted in lactate and released into the portal circulation as evident from study on miniature swine. However, in case of glucose only 2% of ingested glucose is converted to lactate. In addition to lactate formation by fructose, it can also result in small rise in intestinal glucose production suggesting presence of glucose-6-phosphatases activity in rodent and human intestine.

After absorption of fructose from enterocytes it is efficiently extracted by the liver through the glucose transporter GLUT2. Majority of ingested fructose is rapidly metabolized into fructose-1-phosphate under the action of the enzyme fructokinase, which is highly specific for fructose. Deficiency of fructokinase is attributed to a rare, benign condition called hereditary fructosuria. Fructose-1-P is further metabolized into triose-P through the action of aldolase B and subsequent enzymatic reaction leads to formation of Acetyl Co-A and fatty acid.

The difference in hepatic metabolism of fructose to that of glucose is responsible for fructose being considered as bad sugar. These differences are glucose metabolism via glycolytic pathway is under the control of hexokinase IV, or glucokinase enzyme characterized by a high Km for glucose. Therefore phosphorylation of glucose varies with the changes in portal glucose concentration. In addition, phosphofructokinase activity-enzyme responsible for conversion of Glucose-6-P to fructose-6-P, is inhibited by ATP and citrate allowing regulation of the reaction according to the energy status of the cell. Moreover, conversion of glucose to pyruvate is also regulated by insulin levels. In contrast, fructose metabolism is insulin independent and is also a rapid process due to low Km of fructokinase. Absence of ATP and citrate feedback inhibition results in a transient depletion of free phosphate and a decrease in ATP in liver cells in response to fructose.

1.3 Metabolic effects of high intake of sugars

1.3.1 Dyslipidemia

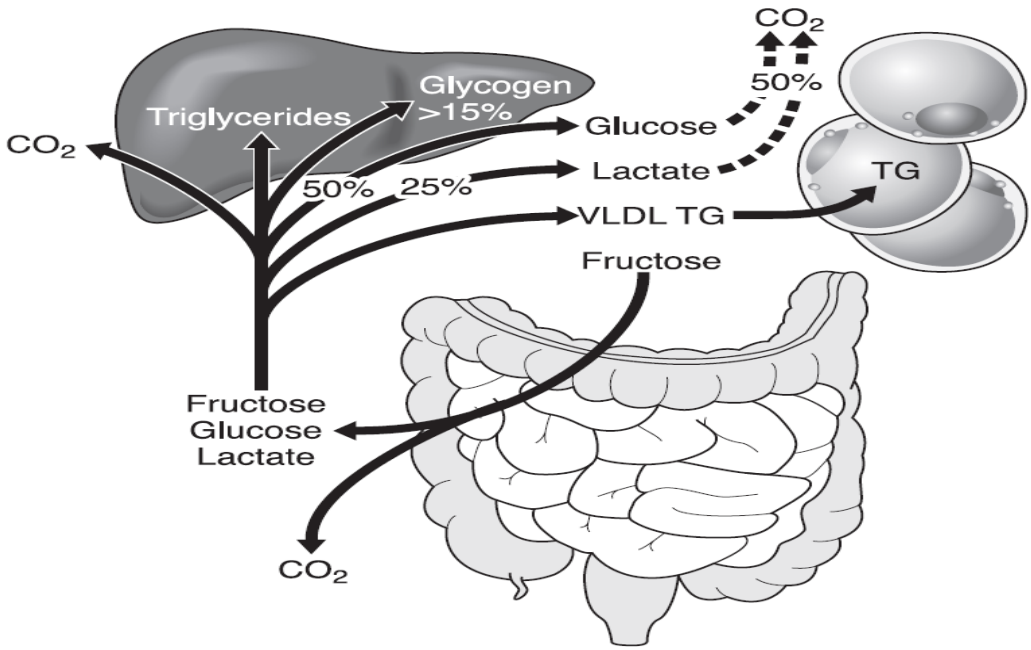

Fig. 1.5 Mechanism of fructose induced dyslipidemia (Tappy et al., 2010).

After ingestion of fructose, metabolic fate of fructose is depicted in **Fig. 1.5.** High-fructose diet feeding for more than 1 week increases triglycerides in healthy volunteers and in patients with insulin resistance or type-2 diabetes **(Macdonald et al., 1986)**. Moreover, some studies also reported increase in total cholesterol levels. Evidence supports the fact, plasma triglyceride kinetics in rats fed high-sucrose, glucose or fructose diets showed increases triglyceride production and decreases triglyceride clearance in sucrose and fructose fed rats compared with glucose fed rats **(Kazumi et al., 1986)**. Fructose is highly lipogenic as it provides large amounts of hepatic triose-phosphate as precursors for fatty acid synthesis. Hepatic de novo synthesis is stimulated after acute fructose ingestion with increased synthesis of both the glycerol- and the fatty-acyl parts of VLDL-triglycerides **(Chong et al., 2007; Parks et al., 2008)**. Increase in the expression of key lipogenic enzymes and transcription factor SREBP is mediated by peroxisome proliferator-activated receptor co-activator 1 α (PCG-1α) in the liver. These effects were independent of changes in insulin levels **(Nagai et al., 2002)**. Expression of hepatic transcription factor

Carbohydrate-Responsive Element Binding Protein (ChREBP) is also induced by fructose. ChREBP up-regulates the expression of fatty acid synthase and acetyl-CoA carboxylase **(Denechaud et al., 2008)**.

1.3.2 Ectopic lipid deposition

Fructose may also modulate ectopic lipid deposition-deposition of triglyceride in the cytoplasm of non-adipose cells, such as hepatocytes, muscle fibers, or endocrine cells which is closely linked to tissue-specific insulin resistance **(Unger et al., 2003)**. In animal models, intrahepatic fat deposition has shown marked increase after 1 week of high sucrose administration **(Pagliassotti et al., 2008)**. This effect of fructose is attributed to enhanced intrahepatic synthesis of triose-phosphate precursors promoting de novo lipogenesis and an increased lipogenic gene expression. Deposition of intrahepatic fat after fructose administration requires PGC-1α, which act as co-activator of SREBP-1c **(Fig. 1.6)**. Interestingly, fat deposition and insulin resistance in response to high-fructose diet can be prevented by inhibiting PGC-1ß **(Nagai et al., 2002)**.

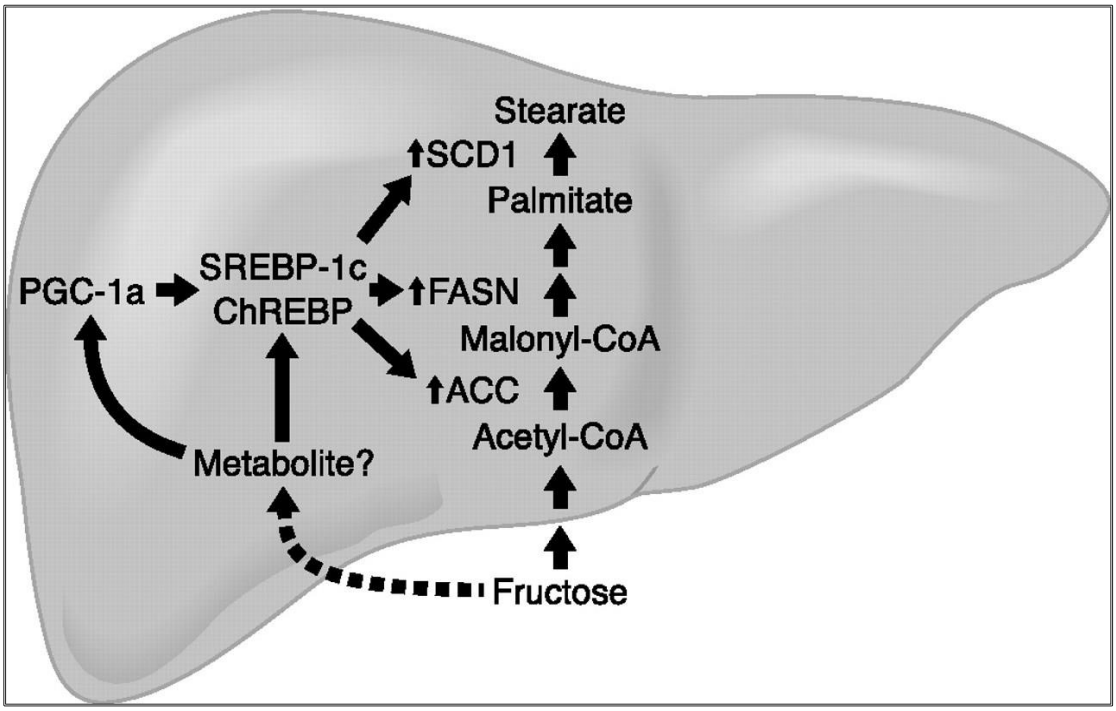

Fig. 1.6 Mechanisms for fructose-induced de novo lipogenesis: fructose acutely and chronically increases intrahepatic de novo lipogenesis (Tappy et al., 2010).

In rodent model of early stage sucrose overfeeding, significant alterations in hepatic metabolism and in hepatic insulin sensitivity, with relatively little alterations of glucose homeostasis and no significant alterations of extra-hepatic insulin sensitivity was observed. However, sustained high-sucrose diet over weeks results in accumulation of intramyocellular lipids and muscle insulin resistance **(Pagliassotti et al., 2008)**.

1.3.3 Impaired insulin resistance and glucose homeostasis

Sir Philip Randle work in the 1960s established relationship between disturbed lipid metabolism and insulin resistance **(Wilkin et al., 2004)**. During those days increased non-esterified fatty acids (NEFA) concentration was recognized as prime factor in lipid-induced insulin resistance. In recent times, role of both high NEFA and high plasma triglyceride concentrations are manifest to cause insulin resistance **(Shulman et al., 2000)**. Human studies on insulin-resistant subjects with higher ectopic fat deposition have shown increased lipid derived metabolites; diacylglycerol, fatty acyl CoA, and ceramides **(Liu et al., 2006)**. Intracellular accumulation of these metabolites may abolish insulin signaling by higher serine/threonine phosphorylation of insulin receptor substrate-1 (IRS-1). Development of insulin resistance and disturbed glucose metabolism is undoubtedly associated in high fructose or sucrose fed animal models **(Shulman et al., 2000)**.

1.3.4 Uric Acid metabolism

Rapid phosphorylation to fructose-1-phosphate in liver drastically stimulates ATP hydrolysis, with a subsequent increase in AMP levels attributing to uric acid synthesis **(Reiser et al., 1985)**. Plasma uric acid levels are repeatedly appears to increase after high dietary fructose intake. Moreover, consumption of fructose has been associated with occurrence of diseases related to uric acid metabolism i.e. gout and kidney stones **(Choi et al., 2007; Taylor et al., 2008)**. According to recent hypothesis link between fructose intake, hyperuricemia, and insulin resistance was proposed. Utilization of glucose by key metabolic pathways is induced by insulin not only in insulin sensitive cells albeit it also increases blood flow and nutritive circulation to the major insulin-sensitive tissue, skeletal muscle. This physiological

effect of insulin is attributed to activation of endothelial enzyme nitric oxide synthase (eNOS) **(Steinberg et al., 1994)**. Ability of insulin to induce muscle vasodilation is impaired in obese subjects who were predicted to have altered glucose homeostasis through "prereceptor" insulin resistance **(Steinberg et al., 1996)**. Since uric acid is potent inhibitor of eNOS, it was proposed that fructose-induced insulin resistance may involve inhibition of vascular effects induced by insulin. This hypothesis was supported by study in which rats fed with high-fructose diet developed both hyperuricemia and insulin resistance simultaneously. These symptoms can be prevented by using uricosuric agent **(Taylor et al., 2008)**. Interestingly, new putative fructose transporter SCL2A9 (GLUT9) bears relationship with uricemia. These transporters may possibly modulate renal uric acid excretion as they are expressed in renal tubules. In addition, increased fractional excretion of uric acid has been shown to be linked with polymorphisms **(Steinberg et al., 1994)**.

1.3.5 High blood pressure

Development of hypertension has been reported in rats fed with high fructose/ sucrose diet **(Hwang et al., 1987)**. This effect can be explained on the basis of couple of mechanisms. As mentioned above fructose feeding is associated with insulin resistance and hyperinsulinemia which is linked to high blood pressure. Hyperinsulinemia triggered increased sympathetic nervous system activity has been invoked as possible mechanism. In addition, it can also increase blood pressure by increasing kidney sodium reabsorption **(Rocchini et al., 1989)**. High fructose intake results in intracellular accumulation of glyceraldehyde and dihydroxyacetone phosphate **(Fig. 1.7).** These products can be metabolized into methylglyoxal, a highly reactive ketoaldehyde **(Vasdev et al., 2004)**. Under low level of fructose metabolism, the methylglyoxal so formed is not accumulated in the liver **(Fig. 7)**. It gets converted to D-lactate by glutathione-dependent glyoxalase system **(Zaidi et al., 1989; Murphy et al., 1990)**. However, this process gets disturbed when the fructose amount is high, leading to excess of methylglyoxal accumulation **(Phillips et al., 1993)**. These pyruvaldehydes have the property to react with sufhydryl groups of proteins i.e. with the sulfhydryl groups of L-type calcium channels **(Zaida et al., 1989; Murphy et al., 1990)**. These calcium channels are responsible for the excitation-contraction of skeletal muscles and cardiac muscles. Disruptions of these L-type calcium channels

raise cytosolic calcium ion concentration, increase peripheral vascular resistance and hypertension **(Vasdev et al., 1996)**. Therefore, fructose fed Sprague-Dawley rats have been demonstrated to show elevated levels of methylglyoxal followed by the occurrence of hypertension **(Hwang et al., 1987; Wang et al., 2007)**. Another cause of hypertension and elevated blood pressure could be hyperuricemia **(Heinig et al., 2006; Hikita et al., 2007)**. In rats, hyperuricemia triggers hypertension and impairs nitric oxide synthesis by decreasing endothelium-dependent relaxation **(Mazzali et al., 2002)**. This can further lead to complications in renal functioning by decreasing Glomerular Filtration Rate (GFR) due to diminished blood flow **(Sanchez-Lozada et al., 2007)**.

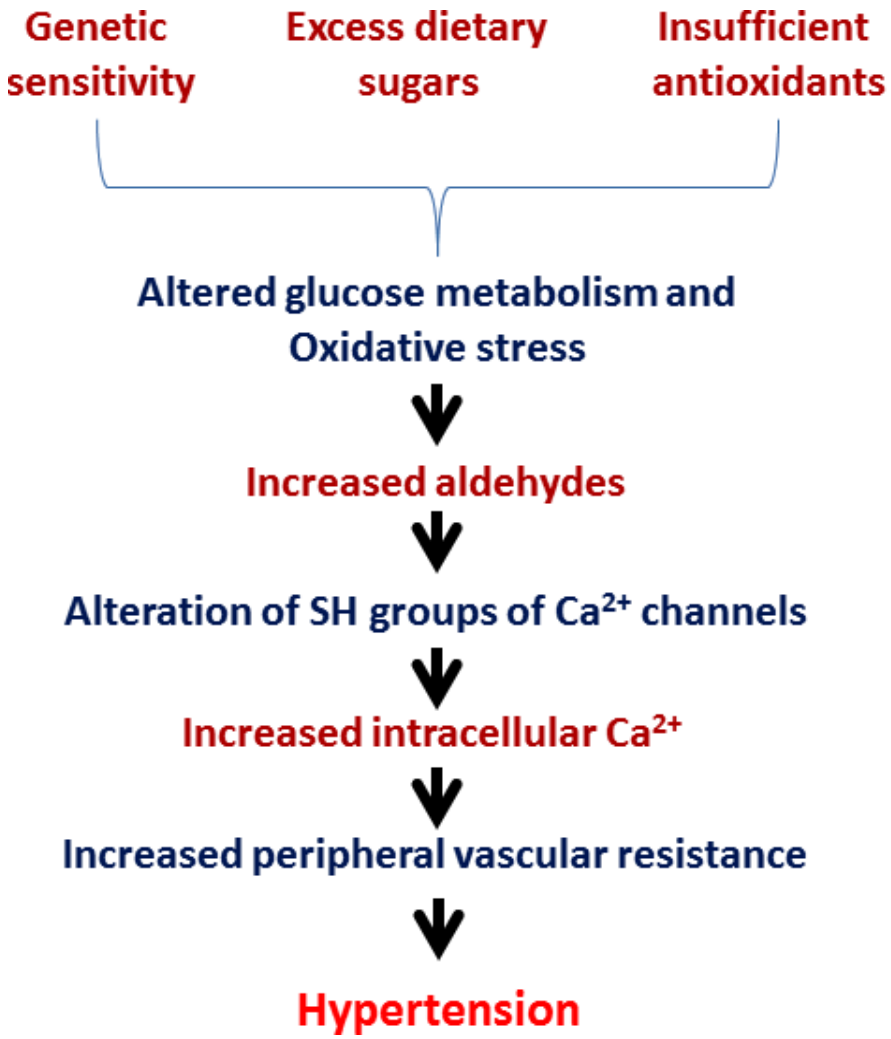

Fig. 1.7 Effect of genetic sensitivity, sugar and insufficient antioxidants on hypertension (Vasdev et al., 1996).

1.3.6 Sucrose forming advanced glycation products (AGEs)

AGEs are produced when reducing sugars react non-enzymatically with proteins, lipids and nucleic acids forming Schiff bases and Amadori products **(Bucala et al., 1992; John et al., 1993; Goldin et al., 2006)**. This reaction is also termed as Maillard's reaction. The most vulnerable proteins to such kind of conversions are collagen, myelin, elastin, plasminogen activator and fibrinogen **(Boel et al., 1995; Vlassara et al., 1996; Mikulikova et al., 2007)**.

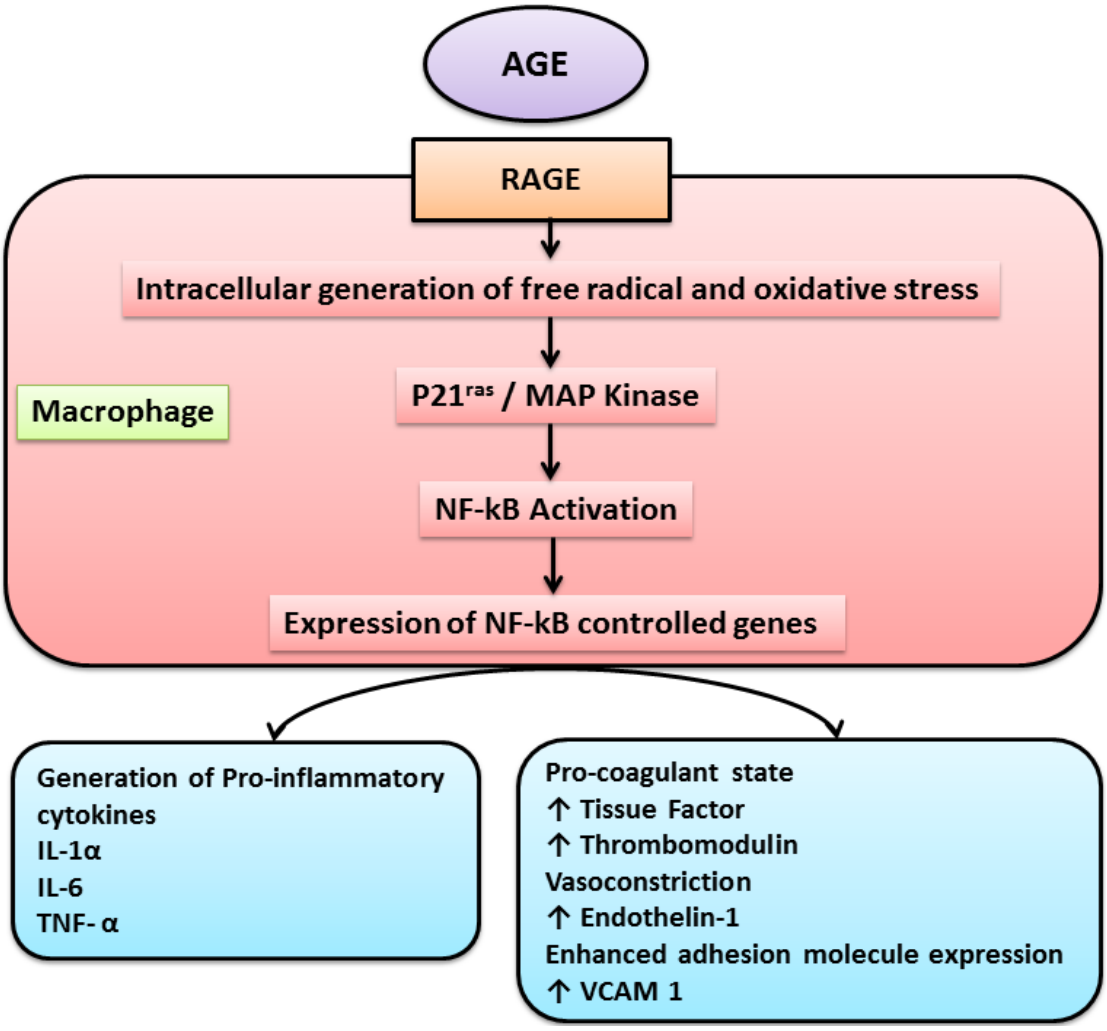

Fig. 1.8 Activation of Signalling Cascade in Response to AGEs in smooth muscle (Anderson et al., 1999).

Such reaction decrease solubility of these proteins and is attributed to development of atherosclerosis and influence diabetes related vascular disease. Activation of signalling cascade in response to AGE is represented in **Fig. 1.8**.

Accumulation of excessive glycation products leads to formation of vascular lesions and reduce vascular permeability **(Anderson et al., 1999)**. Their interaction with specific receptors, RAGE (Receptor for AGE) mediates inflammatory pathways and is correlated with coronary artery disease **(Nakamura et al., 2007)**. Additionally, these glycation products are shown to accumulate in macrophages, fatty streaks, lesions and in smooth muscle cells.

In vivo and in vitro studies implicate that, AGE products affect signaling pathways, activate transcription factors and affect gene expression **(Singh et al., 2001)**. Fructose moiety of sucrose can indirectly generate AGE products through its metabolic intermediate, methylglyoxal, which is associated with increased plasma triglycerides and insulin resistance **(Jia et al., 2007)**. Previous studies indicate; AGE products are also associated with nitric oxide depletion, lipid peroxidation, and generation of oxidative stress. Glucose has the slowest glycation rate in contrast fructose can produce 10 times more such products than glucose **(Ardestani et al., 2007)**. A common AGE product, Pentocidine, is found in severe cases of diabetes mellitus and it is also demonstrated to be induced by fructose **(Goldin et al., 2006)**. Apart from these observations, higher concentrations of AGE products can cause nerve damage as evident from its association with Alzhiemer's disease **(Loske et al., 1998)**. Glycation of amyloid-ß-protein precursor of senile plaque is seen in such conditions.

1.3.7 The two hit theory

Added-sugar diets and beverages are associated with hepatic steatosis independent of the degree of obesity **(Assy et al., 2008; Abid et al., 2009)**. Presence of lipid droplets within hepatocytes along with inefficient clearance, results in chronic liver disease **(Fig. 1.9) (Lim et al., 2010)**. This condition is known as Non-Alcoholic Fatty Liver Disorder (NAFLD). It arises mainly from the intake of carbohydrate rich food and exhibit damages similar to that obtained from alcohol intake. In past three decades prevalence of NAFLD along with Type-2 Diabetes, obesity and other metabolic syndromes **(Roberts et al., 2007)**. NAFLD mainly involves two distinct interrelated 'Hits' on the liver **(Lim et al., 2010)**.

The first hit comprise of hepatic lipid accumulation and hepatic steatosis is accompanied by reduced rate of hepatic lipid clearance by fatty acid catabolism or lipoprotein export (VLDL), in comparison to the rate of de novo lipogenesis and lipid influx **(Koteish et al., 2001; Bradbury et al., 2004)**. Free fatty acids from adipocytes, that circulate the liver, also contribute to the hepatic lipid pool **(Anstee et al., 2006; Roden et al., 2006)**. Extreme cases of dyslipidemia are linked to Cardiovascular Disorder (CVD) **(Stanhope et al., 2009)**.

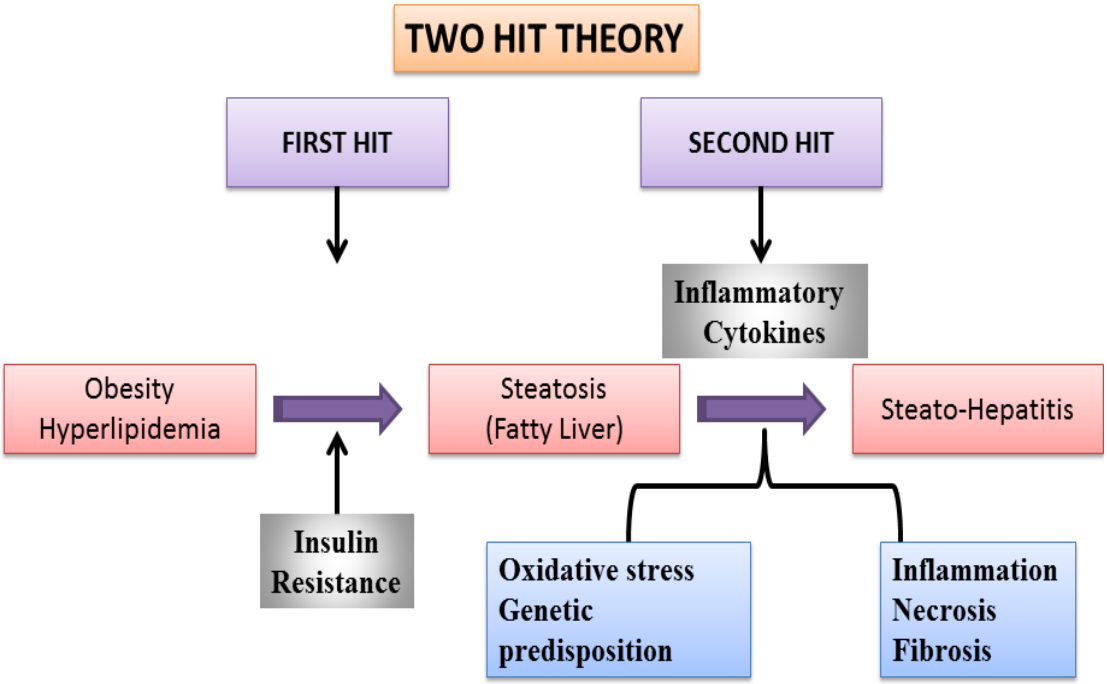

Fig. 1.9 Two Hit Theory (Lim et al., 2010).

The second hit is inflammation, evidently as a consequence of oxidative stress, lipid peroxidation and cytokine activity **(Lim et al., 2010)**. Excessive inflammation may further lead to lobular ballooning degeneration and perisinusoidal fibrosis followed by cell death **(Syn et al., 2009)**. Moreover, inflammation also increases free fatty acid influx by TNF mediated upregulation of hepatic fatty acid translocase **(Shoelson et al., 2003)** attributed to triggering steatosis **(Memon et al., 1998; Ravikumar et al., 2005)**.NAFLD patients are diagonosed with dyslipidemia, hypertension and hepatic steatosis **(Deivanayagam et al., 2008; Fabbrini et al., 2009)**. The VLDL (Very Low Density Lipoprotein) release is Microsomal Triglyceride Transfer Protein (MTTP) dependent and via ApoB100. In rodents, long

term exposure to dietary fructose inhibit PPAR-α (Peroxisomal Proliferation-Activated Receptor-α) **(Taghibiglou et al., 2002; Jurgens et al., 2005; Roglans et al., 2007)**. PPAR-α is required to stimulate MTTP expression **(Ameen et al., 2005)**. Hence, in absence of PPAR-α, VLDL release from hepatocytes is diminished. In hamster models, elevated VLDL concentration was observed after administration of high sucrose diet **(Taghibiglou et al., 2002)**.

Animal experimentations on rats showed increased de novo lipogenesis, attributed to a decreased pyruvate dehydrogenase kinase (PDK) activity and an increased pyruvate dehydrogenase (PDH) activity **(Park et al., 1992)**. In addition to this, fructose has been proposed to increase citrate availability for lipogenesis by activating Acetyl CoA Carboxylase-1 (ACC1). ACC1 gives rise to malonyl-CoA that inhibits Carnitine Palmitoyl Transferase-1 (CPT1) and thereby reducing ß-oxidation **(Cave et al., 2007)**. Furthermore, laboratory studies on humans supported the two hit hypothesis, wherein a marked increase in serum triglyceride level was observed after fructose consumption **(Teff et al., 2004; Chong et al., 2007)**. Moreover, adipocytokines and intestinal endotoxins also increase intrahepatic lipid levels and bring rise to hepatic dysfunction **(Lim et al., 2010).**

1.3.8 Fructose and gut microbe

Fructose consumption in excess has been proposed to alter gut microbial population, affecting phylogenetic diversity as well as an increased intestinal permeability **(Payne et al., 2012; Thuy et al., 2008)**. The increased intestinal permeability is attributed to the presence of fructokinases in intestinal epithelia. A very minor percentage of fructose gets metabolized in the intestinal epithelial, resulting in depleted intracellular phosphates **(Diggle et al., 2009; Lim et al., 2010)**. This condition often affects intracellular protein stability and synthesis. As a result, generation of local inflammation and increased intestinal permeability is observed in various lab models **(Bergheim et al., 2008)**. Non-human primates displayed a transient increase in endotoxemia and microbial translocation, in correlation with altered gut wall permeability **(Kavanagh et al., 2013)**. Decreased expressions of claudin-4, occludin and ZO-1 have is associated with chronic fructose ingestion and studied in vitro **(Richard et al., 2013)**. Furthermore, studies state that fructose has the

ability to perturb the regulation of host energy balance associated with gut and adipocytes **(Payne et al., 2012)**.

1.3.9 Postnatal and prenatal fetal health consequences

Maternal food intake during crucial developmental stages of pregnancy exhibits distinct metabolic effects on the offspring **(Fig. 1. 10) (Alzamendi et al., 2010)**. Intake of sucrose rich diets before and during pregnancy has shown a possible onset of obesity in the offspring **(Goran et al., 2013)**. The obesogenic effect is postulated to be the direct or indirect actions of developing hypothalamus, adipose tissues and disrupting neuroendocrine signaling between adipose and hypothalamus **(Elmquist et al., 2005; Goran et al., 2013)**. Prenatal and postnatal health for children aged 13 months to 9 years, is considered to be the most sensitive states towards nutritional perturbations **(Niinikoski et al., 2012)**. Moreover, studies on primates indicated that maternal nutrition during these developmental stages in gestation tends to develop metabolic malfunctioning **(McCardy et al., 2009)**. Based on these facts it becomes evident that there exists a positive correlation between poor maternal diet and development of a similar food preference in offspring **(Brion et al., 2010; Ong et al., 2011)**.

Sucrose fed pregnant rats produced over-weight offspring, which were affected with a reduced hypothalamic sensitivity towards leptin and increased retroperitoneal adipose tissue **(Jen et al., 1991; Alzamendi et al., 2010)**. On exposure to maternal added-sugar diet in the course of pregnancy, lactation and post-weaning period, young ones displayed an elevated level of hepatic triglyceride and hepatic oxidative stress **(Bayol et al., 2010)**. Besides these effects they also exhibit 2 fold increase in fasting serum insulin level, in comparison to glucose administered mothers **(Rawana et al., 1993)**. Additionally, the liking for sweetened foods can develop before weaning suggesting sucrose can affect the offspring through breast milk **(Bayol et al., 2008)**.

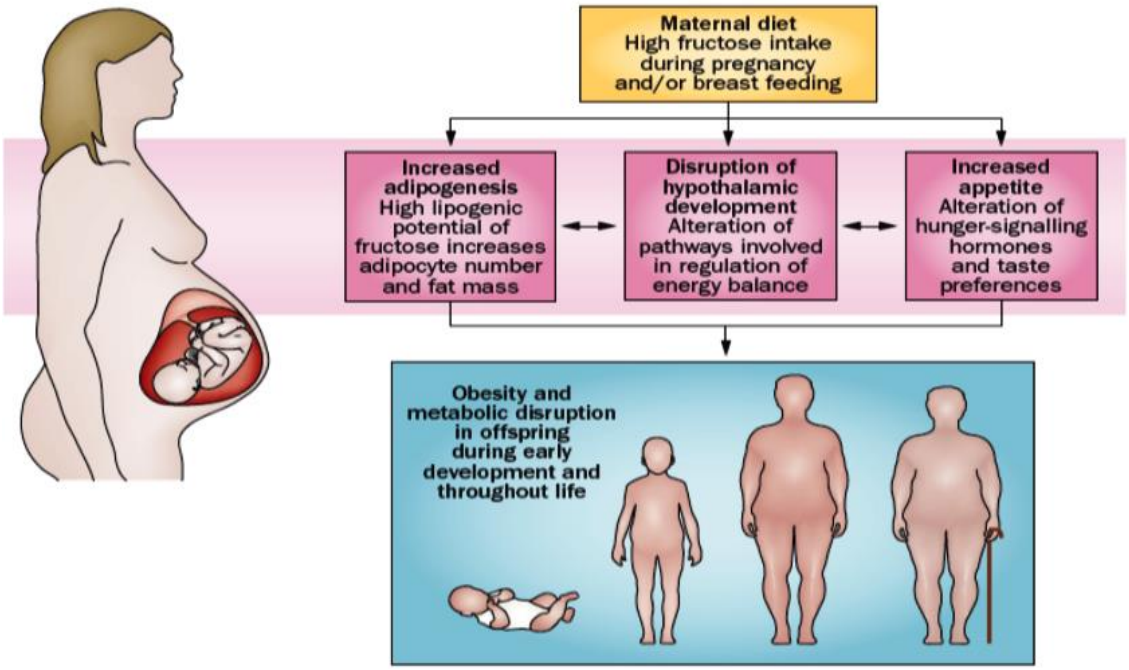

Fig. 1.10 Link between obesity and fructose exposure during critical development period (Goran et al., 2013).

Some reports based on in vitro studies predicted, fructose can play an important role in implantation since fructose activates Target of Rapamycin (mTOR) in addition to trophectoderm cell proliferation **(Kim et al., 2012)**. Whether placental transfer of fructose plays a role in the metabolic malfunctioning in offspring or not, is not known yet. However, some placental transfer studies suggests an increased concentration of fructose in fetus, compared to that present in maternal circulation **(Hagerman et al., 1952; Holmberg et al., 1956; Goran et al., 2013)**. Moreover, GLUT-9- transporter for both glucose and fructose, has shown to be expressed more in placenta of mothers suffering from hyperglycemic condition. However, this particular observation was contradicted by the fact that, GLUT-5 fructose transporter is hardly expressed in fetal intestine despite of maternal fructose intake **(Burant et al., 1994; Douard et al., 2008)**. These findings indicate that, fructose metabolism in infants is normally very low to almost negligible at prenatal state **(Goran et al., 2013)**.

Furthermore, the effect of sucrose was observed to be sex specific. In rat maternal fructose intake resulted in reduction in female fetus weight and not that of males, leaving aside birth weight **(Vickers et al., 2011; Gray et al., 2013)**. Currently,

the obesogenic nature of added-sugars is the rising topic of interest and requires further strong experimental evidences.

1.3.10 Sucrose and cognitive impairment

Sucrose induced metabolic syndrome in long term may be attributed to memory impairment **(Darling et al., 2013)**. High sucrose diet has been shown to reduce expression of synaptic plasticity associated proteins such as, synaptophysin and synapsin I **(Agarwal et al., 2012)**. Alternatively, these changes are observed more in people suffering from obesity. Insulin signaling impairment in obese individuals has been associated with altered electrophysiological property of neurons, reduced synaptic input density and afflicted hypothalamic neurons controlling energy balance **(Horvath et al., 2010)**. The role of insulin in memory loss was further confirmed in rat models receiving sucrose rich diet **(Kanoski et al., 2011)**. Acute administration of rats with exogenous insulin enhanced their memory and a similar outcome was obtained with Alzheimer's models **(Craft et al., 1999)**. However, human studies are yet to be carried out and a very less evidences are currently available to support this finding **(Darling et al., 2013).**

In obese individuals, impaired cognition is one of the observed issues **(Kloiber et al., 2007; Farr et al., 2008)**. This has been further studied on rodent models, where high sucrose consumption led to impaired hippocampus-dependent learning and memory **(Ross et al., 2009; Kanoski et al., 2010)**. High sucrose consumption also exhibited an association between weight gain, visceral fat deposition and deficit in long term spatial memory **(Jurdak et al., 2008)**. Moreover, it reduced the level of Brain-Derived Neuro-Trophic Factors (BDNF) and dendritic spine density **(Stranahan et al., 2008)**. Apart from these, fructose and sucrose is known to increase plasma uric acid level **(Nakagawa et al., 2006)**. This is further associated with ROS generation and decreased nitric oxide synthesis. Since nitric oxide acts as a signaling molecule and is also synthesized in brain using neuronal NOS, effect of fructose involves vascular dementia and memory impairment **(Matsumoto et al., 2006; Nakagawa et al., 2006)**.

Moreover, fructose has been reported to induce nocturnal hypertension and sympathetic nervous system changes **(Farah et al., 2006)**. Disruption of neuronal

plasma membrane is another major effect of high sucrose diet **(Agrawal et al., 2012)**. Excess of sucrose can induce structural changes in the Nuclear Tractus Solitarius (NTS) of rats within 30 days and reduce sensitivity of baroreceptor reflex **(Ai et al., 2010)**.

1.3.11 Appetite signals

Hypothalamus comprises of a set of neurons involved in the expression of endo-cannabinoid receptor-1 (CB1), which is associated with the rewarding aspect of feeding **(Arnone et al., 1997)**. Fructose and sucrose have been reported to up-regulate CB1 mRNA in rat hypothalamus and decrease peptide YY (PYY) levels in blood **(Lindqvist et al., 2008)**. Studies in rat models have demonstrated, consumption of sucrose in drinking water for 2 weeks decreased blood level of PYY **(Batterham et al., 2002).** PYY is released from lower intestine and hypothesized to suppress feeding **(Lundberg et al., 1982)**. Therefore, decreased level of PYY and increased CB1 indicates sucrose induced hyperphagia **(Lindqvist et al., 2008)**. Considering these reports, it can be concluded that sucrose is capable of modulating normal satiety signal and hence, can induce signals to eat independent of the body's energy need **(Pelchat et al., 2002; Erlanson et al., 2005)**.

Furthermore, appetite is regulated by peripheral and central signals, including ghrelin from the stomach and leptin from the white adipose tissue **(Zhang et al., 1994; Date et al., 2000; Domonville et al., 2001)**. Rat models fed on sucrose rich diets demonstrated a reduced fasting serum ghrelin levels and increased fasting serum leptin levels **(Lindqvist et al., 2005)**. Leptin failed to induce an anorexic response in rats fed with high-carbohydrate diet **(Shapiro et al., 2008)**. Moreover, the fructose and sucrose does not elicit insulin surge, unlike glucose since pancreas lacks fructose transporters. In addition to this, it neither triggers the release of gastric inhibitors that stimulates insulin release **(Elliot et al., 2002)**. Sucrose can infuse into nucleus accumbens (NA) and reduce dopamine receptors and μ-opioid receptors and so it prevents clearance of dopamine from NA **(Spangler et al., 2004)**. NA and ventral tegmental area (VTA) constitutes the "reward" centre for food. Leptin and insulin receptors are present in VTA neurons and modulate feeding stimuli **(Figlewicz et al., 2003)**. This indicates that any deficit in VTA-NA activity, affect the reward center for food **(Shalev et al., 2001)**. Such a condition is most prevalent in subjects suffering

from obesity **(Haschimi et al., 2000)**. Mostly, development of hepatic and muscle insulin resistance in obese patients is proposed to alter the VTA-NA dopamine neurotransmission. In nut shell consuming sucrose rich diet and beverages is attributed to disturbance of metabolism in our system. Overview of the various metabolic disorders that arise from fructose and on long-run can result in syndromes like cardiovascular disease and diabetes are represented in **Fig. 1.11**.

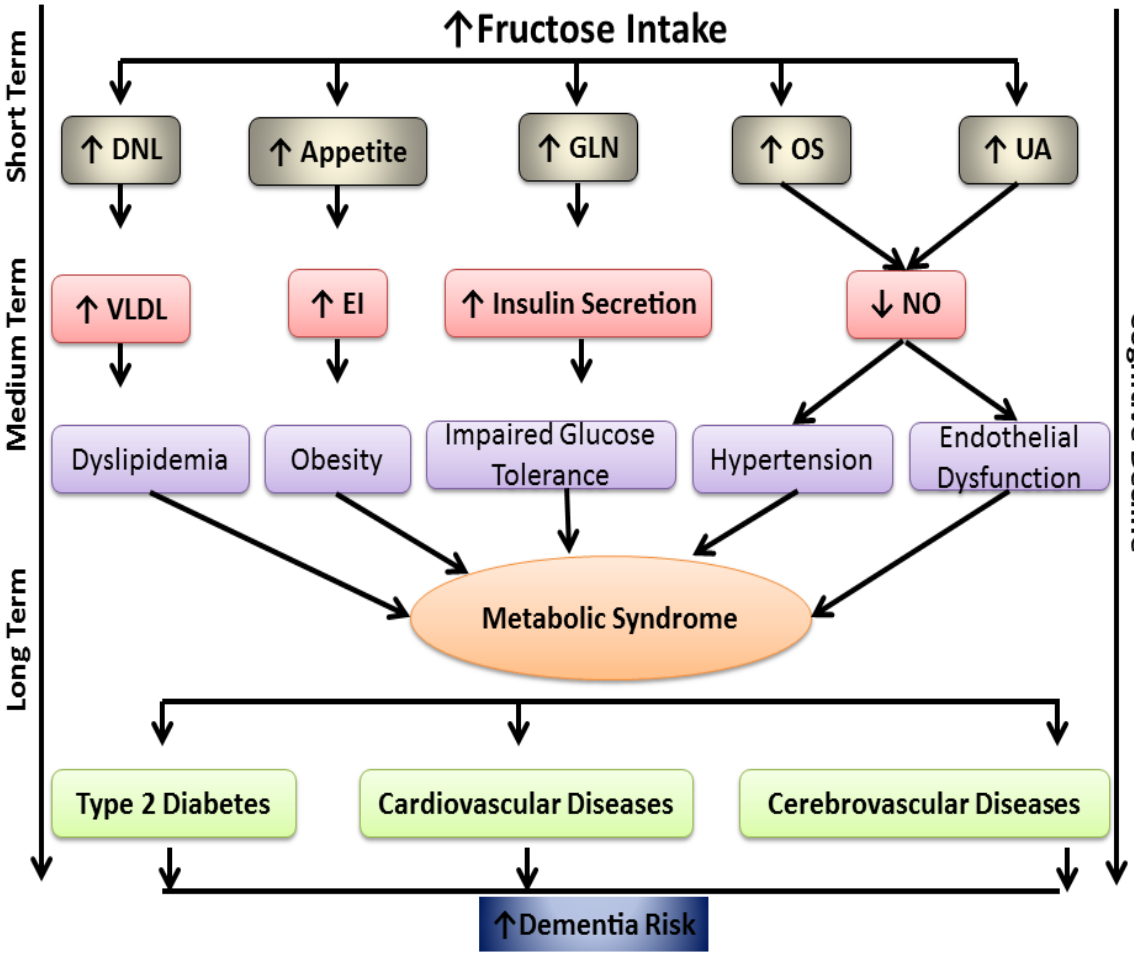

Fig. 1.11 Overall sucrose effect on appetite signals .

1.3.12 Mineral metabolism

Intestinal absorption and bioavailability of minerals can be modulated by fructose, as it can form complexes with metal ions **(O'Dell et al., 1993)**. Studies have demonstrated that absorption of copper is decreased in high fructose and sucrose fad rats in comparison to starch fed rats **(Johnson et al., 1986)**. There was specific concern that fructose consumption can impact calcium balance and bone negatively

(Tsanzi et al., 2008). Overall metabolic effect of Hyperglycemic diet is represented in **Fig. 1.12.**

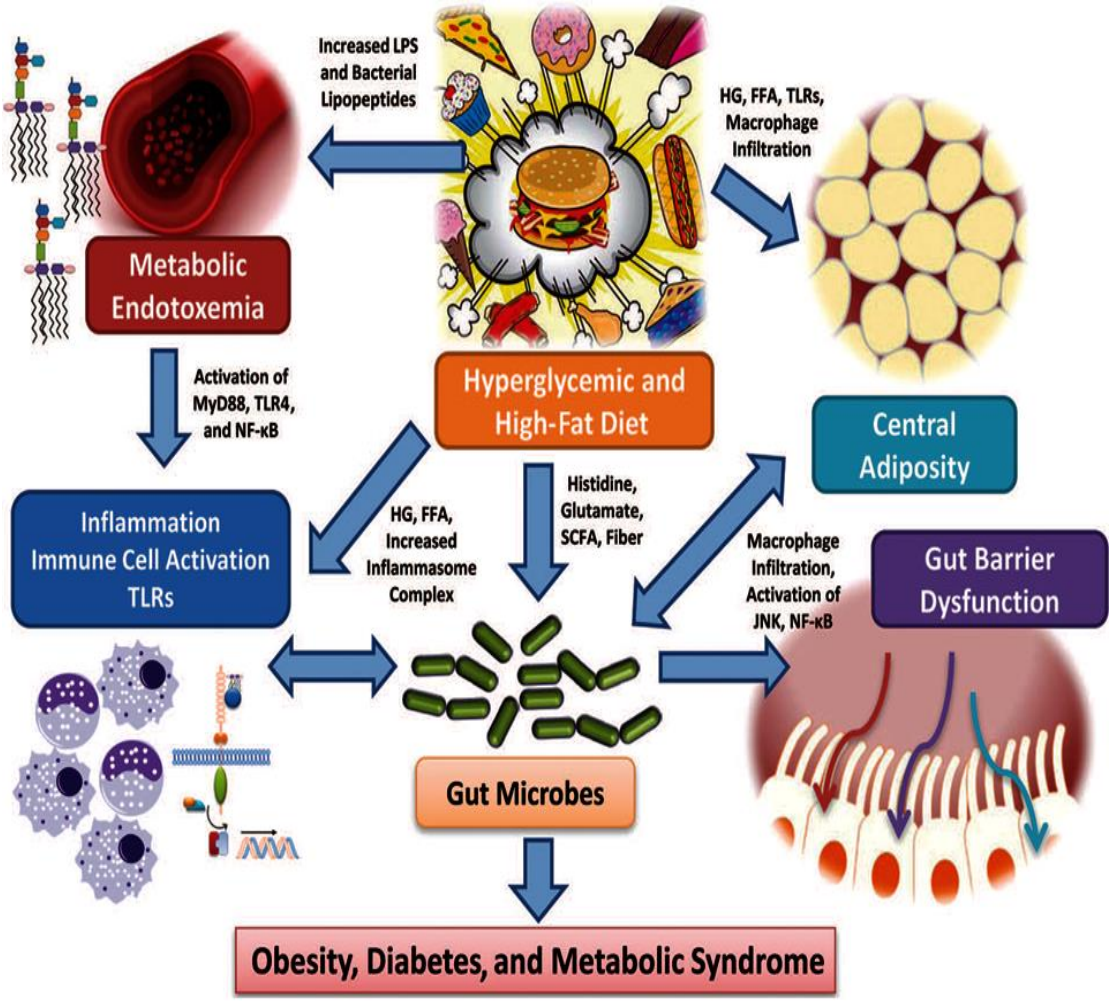

Fig. 1.12 Overall metabolic effect of hyperglycemic diet (Devaraj et al., 2013).

1.4 Strategies employed for management of sugar induced metabolic syndrome

Among the earliest pharmacological agents used for weight loss, centrally acting sympathomimetics, such as the amphetamine derivatives desoxyephedrine, phentermine and diethylpropion are most common **(Colman et al., 2005; Wilding et al., 2007)**. These drugs were very popular in 1960s but increased cardiovascular risk associated with them has led down there use by 1970s. Serotonin (5-HT)-releasing agents fenfluramine and dexfenfluramine were next series of drugs used during 1980s. Later in 1990s use of combined therapies gained significant consideration. **Table. 1.1** summarizes current status all the anti-obese drugs. There is evidence which suggests that Captopril and Allopurinol are able to retards fructose-induced metabolic syndrome.

Name or code	Company	Type of agent or combination	Current status
Monotherapies			
Lorcaserin (ADP359)	Arena Pharma	5-HT_{2C} receptor agonist	FDA approved 2012, following re-file
ATHX-105	Athersys	5-HT_{2C} receptor agonist	Phase II
BVT.74316	Biovitrum	5-HT_6 receptor antagonist	Phase I
PRX-07034	EPIX Pharma	5-HT_6 receptor antagonist	Phase I
S-2367	Shinogi	Neuropeptide Y5 receptor antagonist	Phase II; abandoned 2011
TM30339	7TM	Neuropeptide Y4 agonist	Phase I
Cetilistat	Alizyme/Takada	Lipase inhibitor	Phase III; abandoned?
Amylin analogue	Amylin	Amylinomimetic	Phase I
KRP-204	Kyorin	Selective β3-adrenoceptor agonist	Phase II
Remogoflozin etabonate (GSK 189075)	GlaxoSmithKline	Sodium glucose transporter-2 (SGLT-2) antagonist	Phase I; abandoned 2010
TKS 1225	Thiakis	Oxyntomodulin analogue	Phase I; sold to Wyeth 2008*
SLx-4090	Surface Logix	Mitochondrial transfer protein inhibitor	Phase II; abandoned 2010
Polytherapies			
Tesofensine	NeuroSearch	5-HT/DA/NA reuptake blocker	Phase III
Dov 21947	Dov Pharmaceuticals	5-HT/DA/NA reuptake blocker	Phase II
Obinepitide	7TM	Neuropeptide Y2 + Y4 receptor agonist	Phase II
Contrave	Orexigen	Bupropion + naltrexone	Declined FDA 2011; cardiovascular concerns; company re-file probable
Empatic	Orexigen	Bupropion + zonisamide	Phase II
Qnexa	Vivus	Phentermine + topiramate	FDA approved 2012, following re-file
Pramlintide/metreleptin	Amylin	Amylinomimetic/leptin	Phase II; programme terminated 2011; antibody generation

Table 1.1 Drugs previously used as therapeutic for Obesity (Rodgers et al., 2012). DA: Dopamine; NA: Noradrenaline; 5-HT: 5-hydroxytryptamine.

1.5 Human Gut microbiota

Human gastrointestinal tract harbors various microbial ecosystems along its length in several rich oases **(Flint et al., 2012)**. These microbes are outcome of lengthy and complex coevolution with the mammalian host. The primary challenge for the host must be to defend against microorganisms in the gut as they are constant threat of infection. Conversely, nutrients supplied from resident microbiota, and the development of the gut and of the immune system is attuned to the presence of a complex microbiota and is beneficial for mammals.

The gut micro-environment varies markedly in terms of physiology, digesta flow rates, substrate availability, host secretions, pH and oxygen tension. Therefore, these microbes should be viewed as semidiscrete communities. In comparison to large bowel, micro-environment of small bowel is more challenging with small transit time (3-5hr) and high bile concentration **(Booijink et al., 2010; Zoetendal et al., 2012).**

1.6 Diversity, distribution and functionality of gut microflora

Intestinal microbial diversity up till recently culture based method was adopted which led to successful isolation, culture and characterization of more than 400 bacterial species **(Rajilić-Stojanović et al. 2007)**. Since large fraction of the intestinal microbiota remains uncultivated, culture-based methods have proven to be inadequate in determining the true microbial diversity **(Gerritsen et al., 2011)**. Therefore, recent strategies to determine intestinal microbiota diversity has focused on culture independent approaches by targeting highly conserved 16S ribosomal RNA (rRNA) gene sequences of bacterial and archaeal microorganisms. These techniques include quantitative polymerase chain reaction (qPCR), temperature or denaturing gradient gel electrophoresis (TGGE or DGGE), terminal-restriction fragment length polymorphism (T-RFLP) and fluorescent in situ hybridisation (FISH). In addition, most recent development in high-throughput technologies, such as next generation sequencing and phylogenetic micro-arrays has allowed in-depth analysis of the complete phylogenetic diversity of the intestinal microbiota **(Zoetendal et al. 2008; Van den Bogert et al. 2011)**.

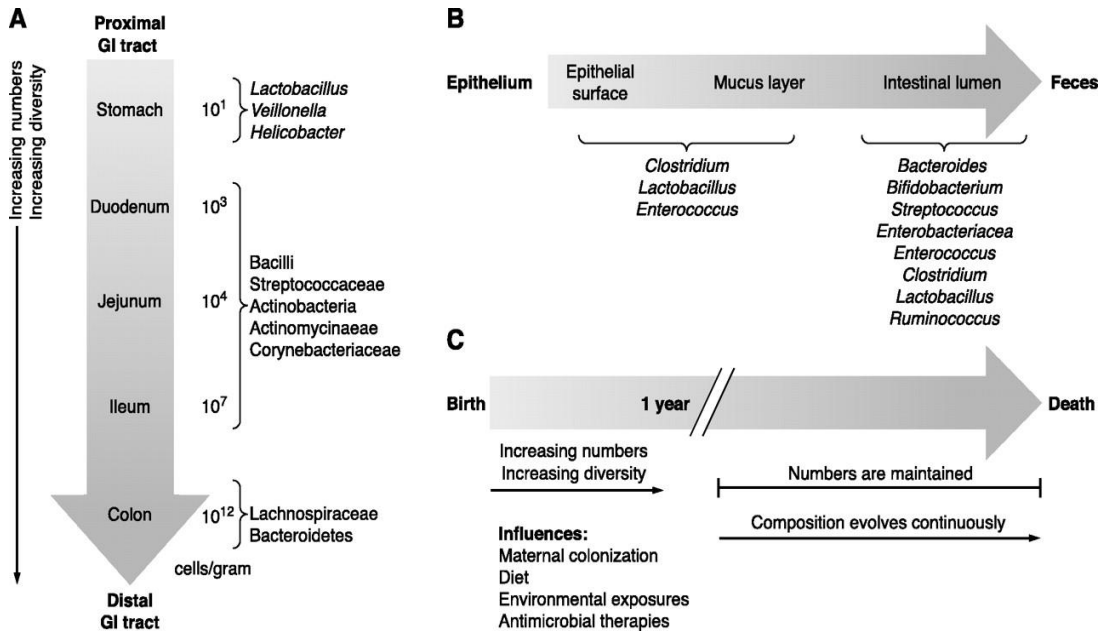

Fig. 1.13 Diversity and distribution of microflora throughout GI tract (Sekirov et al., 2010).

Most of microbial cells in human GI tract are bacteria of which majority of them belong to two phyla, the *Bacteroidetes* and the *Firmicutes* as demonstrated by culture dependent and independent studies **(Fig. 1.13) (Mariat et al. 2009)**. Among *Bacteroidetes, Bacteroides* and *Prevotella* are well studied. In contrast, *Firmicutes* is largest bacterial phylum encompassing more than 200 genera. *Firmicutes* detected in the GI tract belongs to two main groups, the *Clostridium coccoides* and the *Clostridium leptum* (Collins et al. 1994; Mariat et al. 2009). These groups contain members of the genera *Clostridium*, *Eubacterium* and *Ruminococcus*. Apart from the *Bacteroidetes* and *Firmicutes* phyla, members of other phyla such as *Proteobacteria, Actinobacteria, Fusobacteria, Spirochaetes, Verrucomicrobia* and *Lentisphaerae*, have been detected **(Rajilić-Stojanović et al. 2007; Zoetendal et al. 2008)**.

GI tract ecosystem also harbors archaeal domain including methanogens, *Methanobrevibacter smithii* and *Methanosphaera stadtmanae* being by far the most dominant archaeal groups **(Gill et al. 2006; Mihajlovski et al. 2008)**. Moreover, eukaryotic microorganism can also be members of the intestinal microbiota including fungi belonging to the two fungal phyla Ascomycota (which includes the genera

Candida and *Saccharomyces*) and *Basidiomycota* **(Ott et al. 2008; Scanlan et al., 2008)**.

Intestinal microbiota and host physiology are intimately connected as evident from the fact that each distinct anatomical region along the GI tract is characterized by its own physicochemical conditions. Composition of intestinal microbiota is influenced by physicochemical conditions including intestinal motility, pH, redox potential, nutrient supplies, host secretions (e.g. hydrochloric acid, digestive enzymes, bile and mucus), and the presence of an intact ileocaecal valve **(Booijink et al. 2007)**. Different region of the GI tract harbors many distinct niches along the GI tract, each containing a different microbial ecosystem. The microbial density increases along the GI tract ranging from 10^1–10^4 microbial cells in the stomach and duodenum, 10^4–10^8 cells in the jejunum and ileum, to 10^{10}–10^{12} cells in the colon and faeces per gram intestinal content **(Dethlefsen et al. 2006; Booijink et al. 2007)**.

In recent time collective genome of the human intestinal microbiota was found be ~150 times (3.3 million genes) more genes than the human genome **(Qin et al. 2010)**. In addition to our own genome presence of this wide range of genes reflects profound influence of these microorganisms on humans. The extent to which the intestinal microbiota involved in expanding the metabolic, nutritional, physiological and immunological functions of host is largely unknown. However, metagenomic studies has demonstrated that almost 40% of the microbial genes present in each human individual were shared with at least half of the human individuals in the studied cohort **(Qin et al. 2010)**. First step in assessing the functional capacity of intestinal microbiota is Function-driven metagenomics. Metagenomics involves comparing the assembled sequences to reference databases, such as the COG (clusters of orthologous groups) and KEGG (Kyoto encyclopedia of genes and genomes) databases. In addition, this approach can assign function to predicted gene product and can contribute to gene discovery **(Cowan et al. 2005; Tasse et al. 2010)**. Pathways that are highly represented in human microbiome include metabolism of energy, amino acids, nucleotides, carbohydrates, cofactors and vitamins, terpenoids and polyketides, and the biosynthesis of secondary metabolites. These pathways influence the host in addition to providing the microbes to generate energy, to grow and proliferate.

1.7 Probiotics

The term probiotics and its use for beneficial health effect in humans started a century ago by Russian Nobel laureate, Elie Metchnikoff who postulated that Lactic acid bacteria (LAB) can increase longevity **(WGO report 2008)**. According to him intestinal toxicity is attributed to ageing and it can be suppressed by modifying gut flora and replacing harmful species like Clostridium which are known to produce toxic products i.e. phenols, indoles, and ammonia by beneficial microbes. He used "Bulgarian bacillus" to prepare diet with fermented milk. In 1917 Russian Nobel laureate, Elie Metchnikoff who postulated that Lactic acid bacteria (LAB) can increase longevity. According to him intestinal toxicity is attributed to ageing and it can be suppressed by modifying gut flora and replacing harmful species like Clostridium which are known to produce toxic products i.e. phenols, indoles, and ammonia by beneficial microbes. He used "Bulgarian bacillus" to prepare diet with fermented milk. In 1917 another organism possessing ability to have significant health benefit *E. coli* Nissle 1917, was isolated by Sir Alfred Nissle during First World War from soldier who did not developed enterocolitis during a severe outbreak of Shigellosis. Yet another organism, *Bifidobacterium* having probiotic potential was isolated by Henry Tissier from breast fed infant and he named it *Bacillus bifidus* communis. He claimed that this organism was capable of displacing proteolytic bacteria that cause diarrhea. The term probiotic was first coined by Lilly and Stillwell in 1965 which unlike that of antibiotic are known to stimulate the growth of other organisms. Proposed health benefits of probiotics are listed in **Table 1.2.** Among probiotic commercially available in the market *Lactobacillus* and *Bifidobacterium* species are predominant and are listed in **Table 1.3.**

Health benefits	Proposed mechanisms	References
Immune modulation	Influence secondary bile salt concentration and deactivate carcinogens; Prevent antigens from translocating in blood stream	Sanders and Veld, 1999
	Strengthen specific and non-specific defense against infection and tumors by increasing cytokine release and activating macrophages and natural killer cells	Barbe et al., 2006; Wei et al., 2008
	Alleviate food allergy symptoms in infants	Kalliomaki et al., 2001
	Enhance secretion of IgA production	Qamar *et al*., 2001; Parkes *et al*., 2009
	Suppress allergen induced inflammatory response	Feleszko et al., 2007
	Adjuvant effect in antigen-specific immune response	Brudnak, 2002
Metabolic effects	Cell wall component act as inhibitors of angiotensin converting enzymes	Hata et al., 1996; Nakamura et al., 1996
	Inhibit urease producing gut flora	Lunia et al., 2013
	Lower serum cholesterol levels	Taylor and Williams, 1998
	Assimilation of cholesterol within bacterial cell	Tahri et al., 1996; Lin and Chen, 2000
	Improve lactose tolerance	Garvie et al., 1984; Mcdonough et al., 1987
	Antioxidant property	Ljungh et al., 2006
	Lower levels of toxigenic/mutagenic reactions in gut	Pool-zobel et al., 1996; Mcintosh et al., 1999
Colonization resistance	Supply short chain fatty acids and vitamins to the colonic epithelium	Sandus et al., 2013
	Suppress exogenous pathogens and antibiotic associated diarrhea	Floch et al., 2011; Guandalini et al., 2011
	Alter toxin binding sites	Paton et al., 2006
	Upregulate intestinal mucin production and interfere with pathogen attachment to intestinal epithelial cells	Tao *et al*., 2006; yan *et al*., 2007
	Influence gut flora populations	Martin et al., 2008
	Alter conditions to be less favourable to overgrowth flora activities	Quigley and Quera, 2006
	Control of irritable bowel syndrome	Kruis *et al*., 1997
	Production of inhibitors of *H. pylori*	Ryan *et al*., 2009

Table 1.2 Proposed health benefit of Probiotics.

Sr. No	Probiotic strain	Products containing them	Comments	References
1	*Escherichia coli* Nissle *1917*	Mutaflor : Gastro-resistant gelatin capsules	Available for patients suffering from Ulcerative colitis and other inflammatory bowel syndromes in Canada and USA. Fewer relapse in Crohn's disease patients in comparison to control. Helpful in reducing symptoms in diverticulitis. Significant improve in stool frequency.	Schütz 1989; Bruckschen *et al.* 1994; Fric *et al.* 2003; Kruis *et al.* 2004
2	*Lactobacillus rhamnosus* GG	Dielac Pedia (Infant formula); Nutramigen 2 Lipil with Enflora LGG® (Infant formula for allergic babies); Nichmen (probiotic capsules); Hydra Choice (ORS + LGG powder); Bifiform sachets (Powder with vitamin); Lacto GG (hard gelatin capsules)	Effective for travellers' diarrhea. Not recommended for pregnant women. Allergic side-effects.	Kalliomaki *et al.*, 2001; Tao *et al.*, 2006; Yan et al., 2007

3	*Saccharomyces boulardii*	Florastor capsule	Yeast allergy can prevent ingestion of this probiotic.	Klein *et al*., 1993;
4	*Lactobacillus casei*	Yakult cultured milk		Shirota 1930
5	*Lactobacillus gasseri*	Naturally found in breast milk and Puba or carima, staple food of Brazil. In market available as DR Caps™ gastroresistant capsules.	Use of *Lactobacillus gasseri* and other probiotics may trigger such side effects as gas and bloating. In addition, there's some concern that taking probiotics in combination with immunosuppressive drugs may be extremely harmful. A pregnant woman needs doctor's prescription before use.	Itoh *et al*., 2011; Kang *et al*., 2013
6	*L acidophilus, B. bifidum*	Active Balance High Potency Probiotic (Active Balance) - capsules	Boosts up immune system and enhances weight loss.	Active Balance Probiotics

7	*Lactobacillus acidophilus* NAS, *Bifidobacterium bifidum* Malyoth, and *Lactobacillus bulgaricus* LB-51.	Healthy Trinity (Natren): three-in-one probiotic capsules	No GMOs and artificial colour used. No adverse side-effects observed since past 30 years.	Natren
8	*L. acidophilus, B. longum, B. bifidum, B. lactis*	Probioplus DDS (UAS Laboratories): Vegetarian capsules	Reduces chronic acid reflux and could reduce occasional gas and stomach cramps	UAS Labs
9	*L. salivarius, L. rhamnosus, B. bifidum, B. longum, B. infantis, L. acidophilus, B. lactis*	Super 10 Probiotic Complex (GNC): Tablets		GNC Probiotics
10	*Saccharomyces boulardi, S. thermophilus, L. fermentum, L. acidophilus, L. brevis, L bulgaricus, L casei, L helveticus, L paracasei, L pantarum, L rhamnosus, L salivarius, L lactis, Bacillus coagulans, B bifidum, B breve, B infantis, B lactis, B longum, Pediococcus acidilacti*	Nexabiotic (Bioprosper labs): enteric coated capsules	Provides relief from irritable bowel syndrome. Improves immune system within an year of regular dosage. Improves conditions of chronic diarrhoea within a week.	Bioprosper Labs

Table 1.3 Commercially available probiotic in market.

1.8 *Escherichia coli* as a probiotic

There are very few probiotic *E. coli* available in the market which are M-17, H22 and Nissle 1917 strains. *E. coli* M-17 was first identified by the Russian bacteriologist L. G. Peretz in 1933 **(Fitzpatrick et al., 2008)**. It has been used extensively to treat GI diseases such as colitis, inflammatory bowel disease and infections in humans. Protective effect of EC-M17 is believed to be via modulation of immune processes by inhibitory effect on NF-kB signaling. *E. coli* H22 is known to inhibit at least seven genera of pathogenic or potentially pathogenic strains of family *Enterobacteriaceae* (*Enterobacter*, *Escherichia, Klebsiella, Morganella, Salmonella*, *Shigella* and *Yersinia*) in both in vitro and in vivo conditions. In-vitro inhibition of these strains is believed to be mediated by production of microcin C7 **(Smajs et al., 2008)**, colicins E1 and Ib, aerobin and an unidentified phage **(Cursino et al., 2006)**. However, in-vivo mechanism needs to be investigated. Simultaneous administration of *E. coli* H22 and enteric pathogen *Shigella flexneri* to germ-free mice leads to inhibition of the pathogen attributing to its microcin production. *E. coli* strain H22 has been beneficial for both livestock and humans.

1.9 *Escherichia coli* Nissle 1917 (EcN)

EcN is a gram negative bacterium of the *Enterobacteriaceae* family. It is among one of the very few gram negative probiotics available in the market. EcN belongs to common O6 serogroup (serotype O6:K5:H1) **(Wolf et al., 1997)** which includes both non-pathogenic commensals as well as pathogenic variants (mainly diarrhoeagenic and uropathogenic) **(Blum et al., 1995)**. This particular *E. coli* strain appeared to provide protection against the intestinal disorder. Since then beneficial health benefit of EcN has been reported in various gastrointestinal disorders, ranging from childhood diarrhoea to Inflammatory Bowel Diseases (IBD) by many independent studies. In recent time basic science research on EcN has revealed few characteristics which enable it to survive in hostile environment of gastrointestinal tract. In addition, studies have also demonstrated underlining mechanisms responsible for beneficial effects in clinical trials.

S. No.	Effects
1.	Six different iron-uptake systems for energy generation through ATP
2.	Lack of virulence factors
3.	Antagonism towards other members of the intestinal microbiotia through microcin production
4.	Restoration of a damaged epithelial barrier function by induction of expression of human β-defensin 2
5.	Protection against *Salmonella, Candida albicans, Yersinia enterocolitica, Shigella flexneri, Listeria monocytogenes* and pneumophila, adherent-invasive *E. coli*
6.	Reduced secretion of pro-inflammatory cytokines and upregulation of regulatory cytokines via TLR
7.	Detection of viable EcN in feces after oral administration
8.	Limited amelioration of experimental acute DSS-induced colitis but significant improvement of chronic colitis

Table 1.4 Probiotic effect, mechanisms and fitness factors of EcN (Schultz et al., 2011).

EcN unlike to that of pathogenic strains lack prominent virulence factors i.e. *E. coli* α-haemolysin, P-fimbrial adhesins **(Grozdanov et al., 2002; Blum-Oehler et al., 2003; Grozdanov et al., 2004; Nagy et al., 2005)**. Moreover, they also exhibits specific fitness factors which promotes there survival in competitive environment of intestine. These fitness factors are summarized in **Table 1.4.**

1.10 Probiotics in remission of diseases

Over 100 years, the use of probiotics has become more effective to treat a vast range of diseases, ranging from metabolic syndromes to infectious enteric diseases **(Ahmed *et al.*, 2003)**. Majority of the natural probiotics available are dominated by the genus of *Lactobacillus* and *Bifidobacterium*. Although not all bacteria present in fermented foods of yogurts, kefir resides human gut, but some species of these lactic acid bacteria (LAB) have been found to associate with human gut, nevertheless the strains

might vary. Phylogenetic analysis has shown 18 species of *Lactobacillus* and 11 species of *Bifidobacterium* could be utilized as probiotics for humans **(Klaenhammer *et al.*, 1999).** In 2003, **Reid** and his colleagues supported the fact that human gut microbiota exhibits a positive correlation with the host metabolic activities. Additionally, *Lactobacillus* sp. were studied for their influential effect on the host biochemical environment. **Martin *et al*. (2008)** demonstrated the efficacy of *Lactobacillus rhamnosus* and *Lactobacillus paracasei* in improving metabolic health of germ-free mice. These strains exhibit direct and beneficial effect as a probiotic by altering the composition of gut microbiota as well on the host metabolic process and suggest future therapies oriented to an individual's specific metabolic make-up. Probiotic strain *Bifidobacterium animalis* subspecies *lactis* 420 has been shown to be effective in ameliorating the condition of metabolic syndrome mainly by reducing tissue inflammation and preventing endotoxemia **(Mallapa *et al.*, 2012).** Furthermore, **Kadooka** and co-scientists **(2010**) conducted a double blinded, randomized placebo-controlled clinical trial on 87 obese patients showing high body mass index. *Lactobacillus gasseri* SBT2055 strain could significantly reduce abdominal adiposity. List of probiotics used to treat obesity is given in **Table 1.5**.

Enteric infections such as antibiotic associated diarrhea (AAD) and *Clostridium defficle* associated diarrhea (CDAD) along with Inflammatory Bowel Disease affecting a large percentage of adult and infant populations have been demonstrated to show remission by using probiotic strains of *E. coli, Lactobacillus* and *Bifidobacterium spp.* **(McFarland *et al.*, 2006)**. Suppressing colonization of pathogens, maintaining carbohydrate fermentations and stimulating immune factors are the key mechanisms conferred by these probiotics. *S. boulardii* and *L. rhamnosus GG* are most effective strains against infectious diseases. Furthermore, *Escherichia coli* Nissle 1917 have demonstrated excellent management in the cases of Ulcerative colitis and Crohn's disease **(Kruis *et al.*, 1997).** This strain is well-characterized as the only probiotic to maintain remission of Inflammatory Bowel Disease.

In addition to infections and metabolic diseases, now-a-days upcoming therapeutics focus on remission of allergies and maintaining brain physiology. List of natural and modified probiotics used in infection, allergy and inflammation model are

listed in **Table 1.6**. In this context, *L. rhamnosus* (JB-1) has been demonstrated to reduce anxiety like behaviors in mice models by influencing GABAergic receptors **(Bravo *et al.*, 2011)**. **Sian Lewis** in 2011 supported these findings of probiotics by stating the fact that bacterial strains have the ability to influence brain physiology and these effects are at part mediated by the vagus nerves.

Despite of these success stories, the reliability and compatibility of these probiotic strains still remain a conundrum for the researchers. **Parkes and colleagues (2009)** could not successfully reestablish the therapeutic potentials of *S. boulardii* and *L. rhamnosus* in the cases of *Clostridium difficile*–Associated Disease (CDAD). These findings raise risk concern associated with these strains and therefore it affects the possibility of these probiotic strains being recommended by physicians. Furthermore, despite of the fact that the US Food and Drug administration classifies the probiotic strains as generally recognized as safe (GRAS) a handful of cases strictly put these bacterial species for further examination and extensive trials **(Teitelbaum *et al.*, 2002)**. As an illustration, probiotics *Lactobacillus gasseri* has been associated with the development of gas and bloating in patients. Therefore, this strain has been strictly restricted from being administered along with immunosuppressive drugs **(Kang *et al.*, 2013)**. Furthermore, *L. gasseri* and *L. rhamnosus* GG are strictly not recommended to pregnant women.

The genetically engineered probiotics act as delivery models for effector molecules at physiological sites that are otherwise difficult to reach **(Paton *et al.*, 2012)**. IL-10 treatment to reduce inflammation in Crohn' diseased patients was found to be more effective when delivered through engineered *Lactococcus lactis* rather than systemic administration. *L. lactis* expressing human Trefoil Factor 1 (hTFF1) significantly reduced the severity and course of radiation induced oral mucositis in hamster models **(Caluwaerts *et al.*, 2010)**. Besides probiotics expressing immunomodulators, *Lactobacillus jensenii* has been manipulated to express cyanovirin-N, a naturally occurring cyanobacterial protein possessing anti-viral activity against HIV **(Lagenaur *et al.*, 2011)**. The effect of this strain was demonstrated in *Rhesus macaques* as well as in humans. Vaginal secretions did not induce antibody production against the molecule and the patients were protected from vaginal infections with simian HIV. These modified

probiotics could help abate the viral infections and subside the use of antibiotics **(Paton et al, 2012).** The development of receptor mimic probiotics involve modification of lipopolysaccharide (LPS) or oligosaccharide receptors to mimic the host receptors blocking toxin or adhesions involved in enteric infections **(Paton *et al*., 2005).** Modification of E. coli has been demonstrated to combat the effect of STEC (Shiga toxigenic *E. coli*), a food-borne infection which can prove to be fatal if remained substantially untreated. A nonpathogenic *E. coli* strain having truncated lipopolysaccharide (LPS) is manipulated to express two *Neisseria* galactotransferase genes and this receptor mimic could significantly neutralize Shiga toxins with extraordinary avidity **(Paton et al., 2010)**.

Cholera is another life-threatening enteric infection occurring globally caused by the ingestion of infected food and water with *Vibrio cholera*. **Focareta *et al*. (2006)** demonstrated that by neutralizing cholera toxin (Ctx) recovery in infants could be enhanced. Receptor mimics of nonpathogenic *E. coli* strains for Ctx has been developed and termed as GM_1 probiotics. *Bifidobacterium infantis* and *Escherichia coli* Nissle 1917 have proven substantial recovery from inflammatory bowel disorders. Our laboratory results suggests that modified probiotic *E. coli* Nissle 1917 can be used as continuous and sustained release delivery system for pyrroloquinoline quinine (PQQ) in 1,2-dimethy hydrazine (DMH) induced oxidative stress and ethanol induced toxicity in rats **(Pandey et al., 2014; Singh et al., 2014)**. Moreover, gluconic acid production by PQQ-dependent glucose dehydrogenase (GDH) by recombinant *E. coli* Nissle 1917 secreting PQQ acts as synbiotic. Gluconic acid acts as prebiotic molecule which is fermented by colonic microflora to release short chain fatty acid (SCFA) as end product **(Biagi et al., 2006).** These SCFA have been demonstrated to decrease blood glucose, serum insulin levels and serum triglyceride levels, characteristics of metabolic syndrome **(Pereira et al., 2002).**

1.11 Possible advantage of target based probiotic development

Implementation of probiotics in the field of therapies is sustaining in the market since decades. Against the various drug based treatments available in the market, probiotics proves to be significantly safe and efficient towards the most commonly

prevailing clinical outcomes **(Reid *et al.*, 2003)**. Enteric infections, chronic inflammation and viral cancers, are some of the major infectious disease states attacking the world population. However, besides the innovation of science and treatment strategies, the pathogenic micro-organisms exhibit a trend of developing resistance to the conventional antibiotics. Moreover, a limited range of anti-viral agents are halting the development of ways to control these infectious diseases **(Paton *et al.*, 2012)**. Conventional probiotic approach mainly utilizes the wild type strains of the microorganisms typically observed to be identical or related to species/strains persisting in the gastro-intestinal tract. These so- called natural probiotics exploits their inherent properties in obstructing the pathogenic invasion. However, they often carry antibiotic resistance gene, which pose a threat for their colonization as well as an increased risk of horizontal transmission of the resistance genes into pathogenic strains **(Pinyon *et al.*, 2004)**. Moreover, natural probiotics often become disease specific and might not be effective in combating critical infection states. Increased knowledge about molecular mechanisms of the pathogenesis and the response conferred by our body towards these diseases, have enabled intelligent designing of novel therapeutic ways. Introducing recombinant DNA technology in addition to the massive advances in genomics announces new era therapeutic use of the microorganisms through target based manipulations of bacterial traits **(Paton *et al.*, 2012)**. These genetically modified probiotics could be targeted to remote sites in human body for treating diverse health affecting conditions such as infectious diseases, cancers, metabolic disorders and chronic inflammations.

Recombinant probiotic approach provides both biosynthesis machinery and an assembly display platform. It is safe and more efficacious. It is supported by low- cost batch preparation, increased shelf life and stability, ease of administration, low delivery cost and facilitated technology transfer **(Medina *et al.*, 2001)**. One of the important features of these genetically modified probiotics is their mode of administration. These anti-infectives could be orally administered to patients. Specific receptor structures recognizing key toxins and adhesions could block the adherence capacity of pathogens and also neutralizes the toxins **(Paton *et al.*, 2006)**. The receptor-mimic probiotics development was aided by the fact that bacteria produce a diverse range of sugars, oligo- and polysaccharides, providing a vast repertoire of genes that could be exploited. At the

same time, the natural strains such as *N. gonorrhoea*, *N. meningitis* and *C. jejuni*, whose LPS sequences has been used to construct the receptor-mimics in *E. coli CWG308,* could also be administered as probiotics. However these genes are subjected to random switch on and off in the wild type strains **(Yang *et al.*, 1996; Linton *et al.*, 2000; Gilbert *et al.*, 2002)**. A major advantage of these receptor-mimic probiotics is that it does not apply significant selective pressure for evolution of resistance by the targeted pathogen. These genetically modified strains would not interfere with the capacity of the pathogen to survive and reproduce in the environment. Meanwhile, if at all spontaneous mutation in the toxin or receptors is structured in the pathogen, it will simply prevent binding to receptor mimics and logically with their natural targets, too **(Paton *et al.*, 2012)**. Finally, this would just comprise the capacity of the pathogen to cause disease. Apart from anti-infectives, genetically modified probiotics are also implemented to ameliorate various metabolic disorders. Target based probiotics expressing molecules that can modulate our metabolism can help in managing certain ill-health cases such as obesity, hypertension and cardio-vascular diseases. In this case, the natural probiotics also exhibits significantly well amelioration capacity **(Pereira *et al.*, 2002; Ma *et al.*, 2008; Pandey *et al.*, 2014)**. Development of target based probiotics for metabolic disorders still requires better manifestation. A similar strategy is also applied for modulating immune response of the body against infective agents, allergens. These probiotcs provide an *in situ* delivery of immunomodulatory agents at sites of inflammation that are otherwise difficult to reach **(Paton *et al.*, 2012)**.

Despite of the efficacy provided by the recombinant probiotics, regulatory issues concerning with their release into the environment needs to be pondered upon. Most of these genetically engineered strains face a lot of market resistance when it comes to their commercialization. One of the major concerns regarding the genetically modified organisms is the risk of losing control over these probiotic strains. Introducing foreign genetic material into non-pathogenic commensals or environmental microorganisms might increase their virulence capacity. The recombinant probiotics needs to be refined to improve their stability. Plasmid based expression confer a greater risk of gene loss via horizontal gene transfer phenomenon **(Renault *et al.*, 2002)**. Moreover, antibiotic resistance is not suitable for clinical use. Hence, using chromosomally integrated genes

would prove to be a better approach. Finally, recombinant bacteria mimicking host cell-surface epitopes can increase the risk of developing autoimmune diseases. This is further supported by the fact that normal micro-flora do express wide range of host receptor mimics, however maintains a low concentration and is well managed by our immune system **(Moran *et al*., 1996; Karlsson *et al*., 1998)**. But, ongoing exposure to these self-antigens is believed to trigger autoimmune responses. Introduction of nutrient-dependent auxotrophs as delivery vectors or programmed elimination of genetically engineered microbes when they are no longer required, are some of the strategies that can help before releasing the recombinant probiotics in the environment **(Paton *et al*., 2012)**.

Sr.NO.	Probiotic strains	Experiment models	Obesity concerned effect	Reference
1	*Lactobacillus paracasei*	Germ free *Fiaf* -/- mice demonstrating total body weight gain as that observed in conventional Fiaf (angiopoietin like protein 4) suppressed mice	Significant reduction in total body fat and increased circulating levels of Fiaf (Fasting induced adipose factor)	Backhed *et al*., 2004; Aronsson *et al.*, 2010
2	*Bifidobacteria* L66-5	Sprague-Dawley rats fed on high fat diet	Lower serum and liver triglyceride, total cholesterol and body weight gain, and also alleviated liver lipid accumulation	Yin *et al.*, 2010
3	*Bifidobacteria longum*	Rat and human models of obesity	Hypocholesteremia effect	Xiao *et al*., 2003
4	*Lactobacillus acidophilu*s ATCC 43121	Rat and human models of obesity	Hypocholesteremia effect and reduced body weight	Park *et al.*, 2007
5	*Lactobacillus gasseri*	Rat and human models of obesity	Hypocholesteremia and reduced body weight	Usman *et al.*, 2000
6	VSL# 3 (combination of four *Lactobacilli and three Bifidobacteria* spp. and *S. thermophilis*)	Rat models fed on high fat diet	Improving high fat diet induced hepatic steatosis by lowering liver inflammatory signalling, increasing expression of PPAR-alpha and increasing hepatic NK T cell numbers	Ma *et al.*, 2008; Esposito *et al*., 2009
7	*Lactobacillus fermentum* KCb5	*in vitro* experiment using batch fermentation of cholesterol	Particularly effective in removing the cholesterol from batch fermentation	Pereira *et al*., 2002

8	*Lactobacillus paracasei* NFBC338 : *pai*	BALB/c mice orally administered with recombinant probiotic expressing *Propionibacterim acnes* isomeras (PAI)	t10, c12 CLA (conjugated linoleic acid) content in the adipose tissue was elevated by 4-fold. Modulation of the fat composition of the host was observed.	Rosberg-Cody *et al.*, 2011
9	*Escherichia coli* Nissle 1917*::vgb-gfp* secreting PQQ and inulosucrase enzyme	Charles Foster rats fed on 20% sucrose rich diet	Significant decrease in serum and live triglyceride and glucose levels, and significant amelioration of oxidative stress as well as lowering of body fat content	Pandey *et al.*, 2014
10	*Escherichia coli* Nissle 1917*::vgb-gfp* secreting PQQ	Charles Foster rats fed on 20% sucrose rich diet	Significant decrease in serum and live triglyceride and glucose levels, and significant amelioration of oxidative stress as well as lowering of body fat content	Pandey et al., 2014; Singh *et al*., 2014
11	*Lactobacillus rhamnosus* PL60	Male C57BL/6J mice, Diet induced obesity	Production of trans-10, cis-12-conjugated linoleic acid	Lee *et al*., 2006
12	*Lactobacillus gasseri* BNR17	Rats, Diet induced obesity	Prevented increase in body weight and adipose tissue.	Kang *et al*., 2010
13	*Lactobacillus gasseri* SBT2055	Human subjects (n=87) with high body mass index, Multicenter, double-blind, randomized, placebo controlled intervention trial	Reduced the abdominal adiposity	Kadooka *et al*., 2010

Table 1.5 List of natural probiotics used for management of obesity.

S.NO.	Strain name	Experimental models	Disease condition	Functional out come	Reference
1	*Lactococcus casei*	Cultured human intestinal epithelial cells infected with *Shigella flexneri*	*S. flexneri* induced inflammation	Attenuation of pro-inflammatory signalling by inhibiting NF-kB signalling pathway	Tien *et al.*, 2006
2	*Saccharomyces boulardii*	Mice models demonstrating *Clostridium difficile*-associated diarrhea	Cytotoxicity induced by toxin A	Up-regulation of antitoxin A secretory IgA	Qamar *et al*., 2001; Parkes *et al*., 2009
3	*Lactococcus salivaris*	Cultured human gastric epithelial cells infected with *Helicobacter pyroli*	Inflammation and peptic ulceration	Down regulation of virulence factors associated with *H. pyroli*	Ryan *et al.*, 2009
4	*Bacillus infantis*	PBMCs (peripheral blood mononuclear cells) derived from patients suffering from inflammatory bowel disease	Chronic inflammatory bowel disease is associated with abnormal immune responses to the enteric microbial environment	Patients exhibited lower symptom scores on treatment with the probiotic strain. Also, the ratio of IL-10/IL-12 in PBMCs was brought to normal ratio.	Spiller *et al*., 2005
5	*Escherichia coli* Nissle 1917	Caco-2 intestinal epithelial cells	Inflammatory bowel disease associated with intestinal epithelial and mucosa immune cell dysfunction	Induce expression of anti-microbial human beta-defensin 2 contributing to the increased mucosal barrier to luminal bacteria	Wehkamp *et al*., 2004
6	*Escherichia coli* Nissle 1917	Human models suffering from Ulcerative colitis	Ulcers and inflammation in the large bowel	Significantly reduced symptoms and maintained remission of the disease	Kruis *et al*., 1997

		double-blinded, randomized and placebo-controlled	accompanied with increase invasion of pathogenic bacterium		
7	*Escherichia coli* Nissle 1917	TLR-2 knockout mice models	Inflammatory bowel disease associated with enhanced inflammation and Tcell activation	Inhibited T cell proliferation and induced apoptosis of peripheral blood T cells. Regulates decreased expression of TNF-alpha, IL-2 and interferon gamma. Induced increased IL-10 in peripheral blood T cells.	Sturm *et al*., 2005
8	*Lactobacillus reuteri, lactobacillus casei, lactobacillus rhamnosus* GG	Rotavirus infected children	Acute diarrhea	Significant reduction in the diseased condition. Reduced duration of diarrhea.	Shornikova *et al.*, 1997
9	*Lactobacillus salivarius* UCC118	Mice models infected with *Listeria monocytogenes* demonstrating listeriosis	In pregnant women, listeriosis results in miscarriage, premature delivery, stillbirth or severe infection in the newborn	Inability to protect mice against listeriosis when administered with bacteriocin negative *L. salivarius* wild type strain. Indicated the antimicrobial activity confered by *L. salivarius* UCC118	CorrSC *et al*., 2007
10	*Lactobacillus rhamnosus* GG	*in vitro* experiment on intestinal epithelial cell culture	Pathogenesis or diseased condition results in epithelial barrier disfunction	Modulation of epithelial barrier function through prevention of cytokine induced apoptosis in the epithelial cell. Activity induced by two proteins expressed by *L. rhamnosus*, p75 and p40	Tao *et al*., 2006; Yan *et al*., 2007
11	*Lactobacillus rhamnosus* JB-1	Healthy mice	Depression and anxiety	Reduced levels of anxiety and depression like behaviour. Also, induced changes in the	Bravo *et al*., 2011;

				GABAergic system in regions of brain involved in these behavioural control	Lewis *et al*., 2011
12	*Bacteroides fragilis*	Pregnant mice injected with viral like particles to induce autism like symptoms in offsprings	Autism associated with enhanced anxiety and shift od gut barrier functions.	Elevated serum level of 4-ethylphenyl sulfate resulting a subset of the behavioural abnormalities so observed in the autistic offsprings	Hsiao *et al*., 2013
13	*Esherichia coli* Nissle 1917 expressing human EGF-LARD3 (Recombinant probiotic)	HCT-8 intestinal epithelial cells and nontransformed intestinal epithelial cells (IEC-18)	Disruption of mucosal barrier in response to necrotizing and/or ulcerogenic agents like aspirin, bile acids and alcohol	Wound healing capacity of the recombinant probiotic was established and its efficacy was observed in vitro.	Choi *et al.*, 2011
14	*Escherishia coli* Nissle 1917 expressing CAI-1 (Recombinant probiotic)	Infant mice models prior to *V. cholerae* infection	CAI-1, cholera autoinducer 1, is a quorum sensing molecule which determines the cell density of *Vibrio cholerae* in the intestine. At low cell density, the virus is supposed to express both cholera toxin and a critical colonization factor.	Significant reduction of intestinal colonization by the pathogen and reduced amount of cholera toxin bound in the epithelium	Duan *et al*., 2010
15	*Lactobacillus*	*in vitro* human vaginal	HIV -1 infection	Significant protection from repeated HIV-1	Lagenaur *et*

	jensenii expressing cyanovirin-N (CV-N) (Recombinant probiotic)	tissue HIV-1 infection model and *in vivo* demonstration in Rehsus macaques		infection was observed	*al*., 2011
16	*Escherichia coli* CWG308:*lgtA-lgtB-lgtE* expressing mimic of lacto-N-neotetrose (LNT) (Recombinant receptor-mimic probiotic)	Rabbit Ligated Ileal Loop Model; Mouse models demonstrating traveller's diarrhea	Enterotoxigenic *E. coli* disease (traveller's diarrhea) associated heat labile exotoxin LT	Co-administration neutralized >93.8% LT activity and protected rabbits from LT induced fluid secretion. Also, protected mouse from hemorrhagic enteritis	Paton et al., 2001; Paton et al., 2005; Paton *et al*., 2006
17	*Escherichia coli CWG308:lgtE-cstII-cgtA-cgtB (* GM1 mimic) (Recombinant receptor-mimic probiotic)	Infant mice models infected with *Vibrio cholerae*	Cholera induced by *V. cholerae* resulting in massive diarrhea and electrolyte imbalance	Oral administration post infection exhibited 99% efficacy in protecting the infant mice against cholera	Focarota *et al.*, in Press; Paton *et al.*, 2006
18	*Escherichia coli CWG308: lgtD* expressing mimic of globotetrose (Recombinant	*in vitro* experiments on crude extracts of STEC (Shiga toxigenic *E. coli*) toxin Stx2e	Diarrhea and fluid accumulation in tissues of stomach and large bowel	98.4% capacity to neutralize Stx26 (shiga toxin) crude extrates	Paton *et al*., 2001; Paton et al., 2006

	receptor-mimic probiotic)				
19	*Escherichia coli CWG308:lgtC-lgtE* expressing mimic of galactosyltransferase (Recombinant receptor-mimic probiotic)	Mice models challenged with fatal dose of STEC	Mild non-bloody diarrhea to hemorrhagic colitis accompanied by vomiting and nausea	Oral administration showed 100% effectiveness in the mice models against STEC infection	Paton et al., 2000; Paton et al., 2006
20	*Lactococcus lactis* (Engineered to express mature human IL-10)	Human models suffering from Crohn's disease, under placebo-uncontrolled trials	Chronic intestinal colitis	Patients experienced reduced disease outcome by the anti-inflammatory activity of Il-10 mediated by dendritic cells and suppression of Th cells.	Braat *et al.*, 2006; Huibregtse *et al*., 2012
21	*Lactococcus lactis*(Engineered to secrete murine TNF-neutralizing antibody)	Mice models demonstrating dextran-sulfate induce chronic colitis	Chronic intestinal colitis	Oral administration reduced inflammation in the mice	Vandenbroucke *et al*., 2010
22	*Lactococcus lactis* (Engineered to express human Trefoil-factor 1 (hTFF1))	Hamster models demonstrating radiation induced oral mucositis	Epithelial damage cause by chemotherapy or radiation in cancer patients	Formulated as mouthwash, hTFF1 efficiently reduced the severity and course of radiation induced oral mucositis in hamster models	Caluwaerts *et al*., 2010
23	*Lactobacillus casei*	Female BALB/c mice	Chronic intestinal colitis	Combined with 5-aminosalicylic acid (5-ASA),	Qiu et al.,

	(Engineered to express interleukin-10 (IL-10))	treated with dextran sodium sulfate (DSS) to induce colitis	and related inflammation	genetically modified *L. casei* was more effective than native probiotic. Effective against DSS treatment possibly by blocking NF-kB pathway and thus suppressing release of inflammatory factors	2013
24	*Lactococcus lactis* (Engineered to secrete murine IL-10)	Mouse model of food allergen	Food induced systemic anaphylaxis	Diminished anaphylaxis and inhibited antigen specific IgE and IgG_1 production very efficiently. Induced IL-10 secretion by Peyer patches cells with elevated IL-10 levels in plasma. Provide and option to prevent IgE-type sensitization to common food allergens	Frossard et al., 2007
25	*Lactobacillus gasseri* (Engineered to express SOD)	IL-10 deficient mouse model	Intestinal colitis	Significantly attenuated inflammation and infiltration of neutrophils and macrophages in IL-10 deficient mice.	Carroll et al., 2007
26	*Lactococcus lactis* (Engineered to secrete anti-mouse TNF-α nanobody (single domain antibody fragment))	DSS induced colitis mouse model. And, IL-10 $^{-/-}$ mouse model of enterocolitis	Chronic colitis	Reduced intestinal inflammation. Improved colitis in IL-10 $^{-/-}$ mouse model. Could lead to effective and safer management of IBD in humans	Vandenbrouce et al., 2010
27	*Escherichia coli* CFR 16 (Transfected with *pqq* gene cluster of *Pseudomonas*	DMH induced colon cancer rat model	Intestinal colitis	Prevented intestinal oxidative stress and colonic damage. Elevated colonic antioxidant enzyme activities and reduced lipid peroxidation	Pandey et al., 2014

	fluorescens to secrete PQQ)				
28	*Escherichia coli* Nissle 1917 (Transfected with pqqABCDE gene cluster of *G. oxydans* to secrete PQQ)	Rat model of chronic alcoholism	Chronic ethanol induced oxidative stress and hyperlipidemia (Alcoholic liver disease)	Ameliorated acute as well as chronic ethanol induced oxidative stress. Elevated hepatic enzymatic and non-enzymatic antioxidants and prevented tissue damage caused by chronic ethanol ingestion. Lowered hepatic and blood lipid levels through modulating hepatic lipid metabolizing genes	Singh et al., 2014

Table 1.6 List of natural and recombinant probiotic strains used in infection, allergy and inflammation model.

1.11.1 Gut microbiota maturation and nutrition

The microbial stability is affected by any major change in lifestyle or diet reflecting extremely complex interactions between diet and the gut microbiota in mammals. Rite from first stage of life prime factor behind development of the microbiota colonization pattern is diet. Accumulating evidence reveals the role of microbiota in the obesity development. Therefore, use of probiotics and prebiotics are the proposed for therapeutic for the obesity and metabolic syndrome management **(Fig. 1.14) (Kovatcheva-Datchary et al., 2013).**

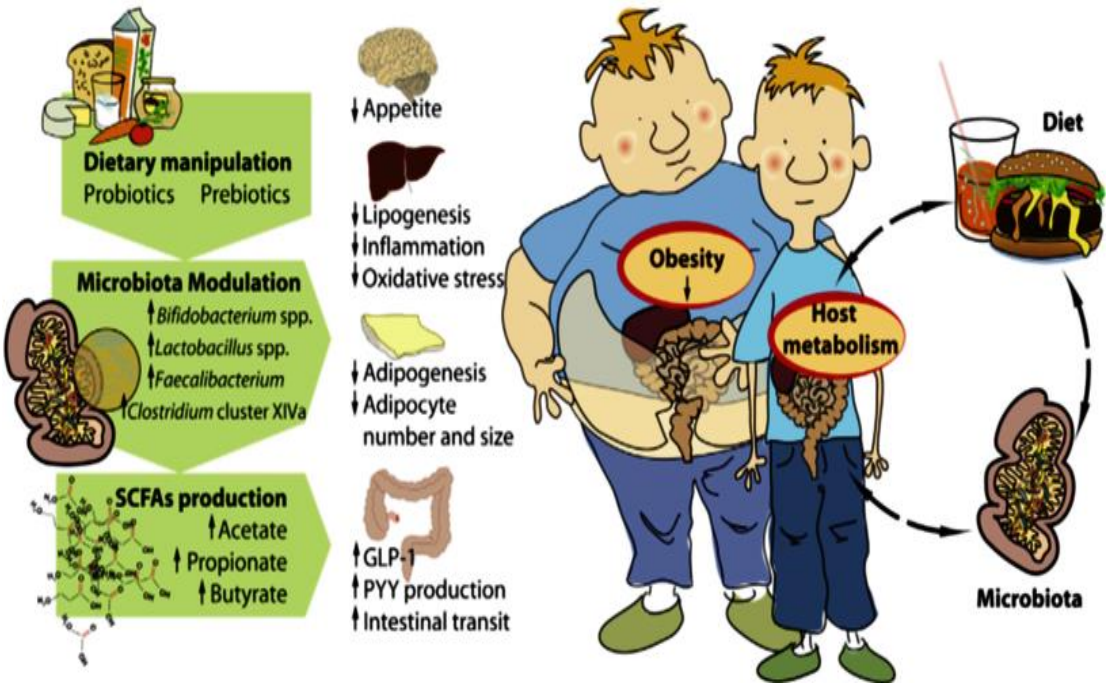

Fig. 1.14 Interaction between diet and gut microbiota affect host metabolism (Kovatcheva-Datchary et al., 2013).

Studies provided evidence that exclusive breastfeeding results in enriched Bifidobacteria and lactic acid bacteria in infant gut. In contrast, formula feeding is characterized by more diverse community dominated by bifidobacteria, *Bacteroides* spp., *Clostridium* spp., and facultative anaerobes **(Palmer et al., 2007; Wall et al., 2009).** This observation is supported by fact which revels, breast milk is rich in oligosaccharides, which serve as prebiotic substrate for fermentation in the distal gut and promote the growth of *Bifidobacteria* **(Wall et al., 2009)**.

1.11.2 EcN in the treatment of Inflammatory Bowel Diseases (IBD)

IBD (Crohn's Disease (CD) and Ulcerative colitis (UC)), is chronic recurrent diseases mainly characterized by inflammation, ulceration and in the case of CD structuring and fistulising of the gastrointestinal tract. IBD has significant impact on life quality as it mainly affects young patients with a 2nd peak later in life **(Wietersheim et al., 2006)**. There are few clinical studies which supports effectiveness of EcN in treatment of IBD **(Table 1.7)**.

Diagnosis	Study design	Number of patient per treatment group
Crohn's Disease		
Active and Inactive Crohn's, Disease Predominantly in the Colon (CDAI >150)	Double-blind, randomized, placebo-controlled	Group 1 (n = 16): Prednisolone + EcN (5 × 1010 viable bacteria) Group 2 (n = 12): Prednisolone + placebo
Ulcerative Colitis		
Inactive Ulcerative Colitis (CAI≤4)	Double-blind, randomized, placebo-controlled	Group 1 (n = 50): EcN (2.5 × 1010 viable bacteria) Group 2 (n = 53): Mesalazine (1.5g/d)
Active and Inactive Ulcerative Colitis	Double-blind, randomized,	All patients received 80 mg gentamycin tds initially for one week and hydrocortisone enemas or oral prednisone 30–60 mg. Group 1 (n = 59): Mesalazine (2.4g/d—reduced to 1.2g/d after 12 weeks) Group 2 (n = 57): EcN (5 × 1010 viable bacteria—reduced to 2.5 × 1010 viable bacteria)
Inactive Ulcerative Colitis (CAI≤ 4)	Double-blind, randomized, placebo-controlled	Group 1 (n = 162): EcN (initially 2.5 × 1010 viable bacteria, from day five 5 × 1010 viable bacteria) Group 2 (n = 165): Mesalazine (1.5g/d)
Inactive Ulcerative Colitis	Open-label	Group 1 (n = 24): EcN (5 × 1010 viable bacteria) (CAI ≤4) Group 2 (n = 10): Mesalazine (1.5g/d)

Table 1. 7 EcN in treatment of IBD (Rembacken et al., 1996; Kruis et al., 1997; Malchow et al., 1997; Kruis et al., 2004; Henker et al., 2008)

Western societies have relatively higher incidence and prevalence of IBD but in recent time it is also increased in low-incidence areas such as southern Europe, Asia, and much of the developing world **(Loftus et al., 2004)**. The exact cause of IBD is still puzzling, but it is generally accepted that genetically susceptible individuals have inappropriate immune response to the normal intestinal bacteria **(Sartor et al., 2008)**.

1.12 Oxidative stress and ROS

ROS encompass a wide range of chemical species including superoxide anions, hydroxyl radicals and hydrogen peroxide **(Finkel et al., 2000)**. Among these species superoxide or hydroxyl radicals are extremely unstable and hydrogen peroxide is freely diffusible and relatively long-lived. These ROS molecules can be generated from several different endogenous and exogenous sources **(Fig. 1.16).** Cytosolic enzyme systems contributing to oxidative stress include a family of NADPH oxidases, a superoxide-generating system. This NADPH oxidase can either be phagocytic NADPH oxidase (expressed by neutrophils) or non-phagocytic NADPH oxidase (e.g. epithelial cells). Depending on the specific NADPH oxidase expressed, can either trigger cellular transformation or replicative senescence **(Suh et al., 1999; Geiszt et al., 2000)**. Widely differing outcome of these NADPH oxidases reinforces the complexity in determining the cellular response to oxidants. Factors contributing towards cellular responses may include the cell type, the absolute level and duration of oxidant production, the species of ROS generated, and the specific intracellular site of ROS production. Like the widely characterized nitric oxide synthase (NOS) family, NADPH oxidase family of enzymes also illustrates the apparent purposeful and deliberate use of oxidant generation in normal cellular signalling and homeostasis. Majority of intracellular ROS production is derived from the mitochondria as evident from most estimates **(Turrens et al., 1997)**. Within the mitochondria electron transport chain, namely complex I (NADH dehydrogenase) and complex III (ubiquinone–cytochrome c reductase) are the site of superoxide radicals production. Complex III is the main site of ROS generation under normal metabolic condition **(Fig. 1.15)**. In in-vitro conditions, evidence suggests that mitochondria convert 1-2% of the oxygen molecules consumed into superoxide anions **(Boveris et al., 1973).** These estimates were made on isolated mitochondria in presence of non-physiological

concentration of oxygen, although in-vivo rate of superoxide production seems to be considerably less.

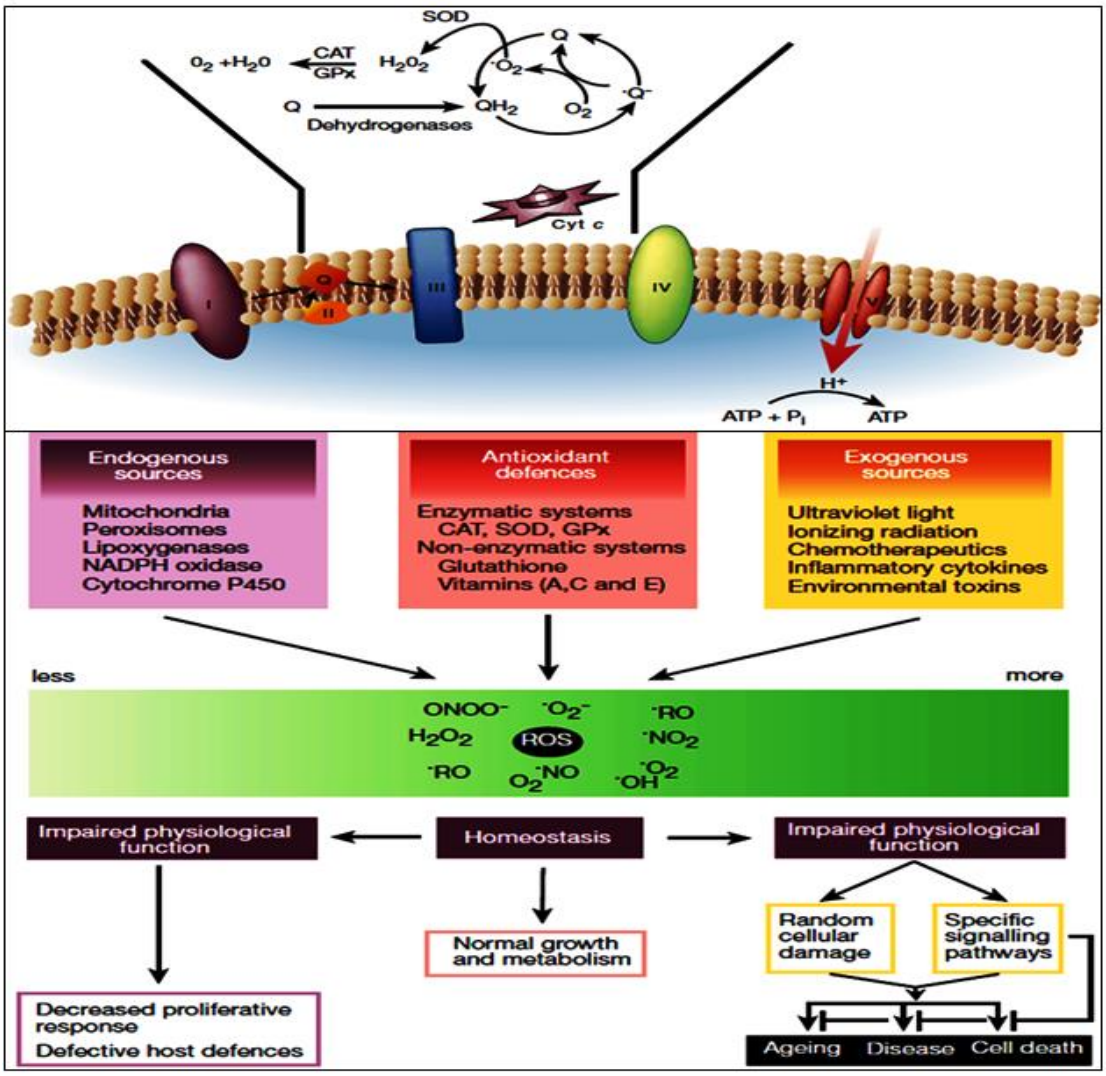

Fig. 1.15 Source of ROS and antioxidant defences (Finkel et al., 2000)

ROS produced from the mitochondria is taken care by the protective mechanisms which limits oxidant production and release. Increasing the rate of metabolic uncoupling is one of the postulated mechanisms to reduce mitochondrial oxidant production **(Skulachev et al., 1997)**. This uncoupling and resultant heat production is mediated by family of uncoupling protein (UCP-1,-2 and -3). Recent studies has made evident, increase in uncoupling reduces mitochondrial ROS release. Conversely, targeted deletion of UCP-3 is attributed to increased levels of mitochondrial oxidants.Intricate antioxidant defence system, including enzymatic scavenger SOD, catalase and glutathione peroxidases counteracts the burden of ROS

(Finkel et al 2000). SOD converts superoxide to hydrogen peroxide, whereas, catalase and glutathione peroxidase convert hydrogen peroxide to water. In addition, five peroxiredoxins-new families of peroxide scavengers have been isolated **(Chae et al., 1999)**. A variety of other non-enzymatic, molecules are also important in scavenging ROS including ascorbate, pyruvate, flavonoids, carotenoids and perhaps most importantly, glutathione, which is present in millimolar concentrations within cells. Degree of oxidative stress is determined by balance between ROS production and antioxidant defences. Elevated level of ROS is attributed to modification of proteins, lipid and DNA **(Stadtman et al., 1992)**. Among these modifications formation of carbonyl derivative is widely studied in oxidative stress. Carbonyl formation can occur through either direct mechanisms including direct oxidation of certain amino-acid side chains and oxidation-induced peptide cleavage. Oxidative stress can potentially modify all organs and all proteins albeit certain tissues and specific protein targets may be especially sensitive **(Yan et al., 1997; Goto et al., 1999).**

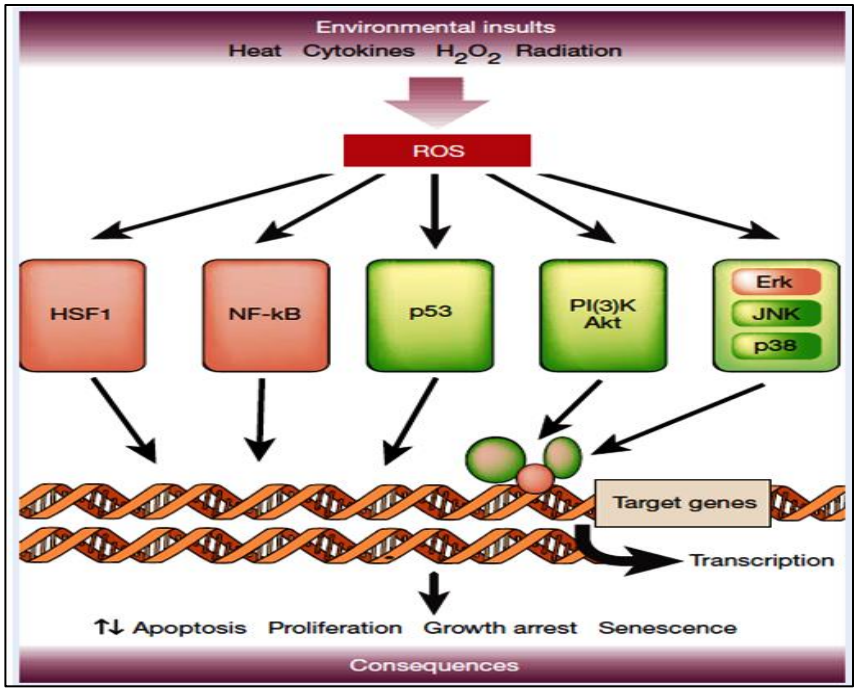

Fig. 1. 16 Major signaling pathways of ROS (Finkel et al., 2000).

Organisms accumulate increased levels of ROS over time causing DNA damage. Limited DNA repair system in mitochondria makes is vulnerable to oxidative damage **(Finkel et al., 2000)**. Two recent studies have shown convincing results,

deletion of Mn-SOD or adenine nuclear transporter results in mitochondrial superoxide scavenging capacity defect and increased levels of mitochondrial DNA rearrangement. In response to ROS, major signaling pathways can also be induced **(Fig. 1.16)**. Among these, Heat-shock transcription factor 1 (HSF1), NF-kB and p53 are themselves transcription factors while the PI(3)K/Akt and MAPK pathways regulate transcription factors through phosphorylation.

1.13 Antioxidants

Antioxidants can be broadly classified into two categories, Natural antioxidants and synthetic antioxidants. **Halliwell** et al. **(2007)** defined antioxidants as ‘‘any substance that, when present at low concentrations compared with that of an oxidizable substrate, significantly delays or inhibits oxidation of that substrate’’. Few years later they defined them as ‘‘any substance that delays, prevents or removes oxidative damage to a target molecule’’. **Khlebnikov** et al. defined antioxidants as ‘‘any substance that directly scavenges ROS or indirectly acts to up-regulate antioxidant defences or inhibit ROS production’’. Another property which compound much possess in order to be defined as antioxidant is the ability after scavenging the radical to form a new radical that is stable through intramolecular hydrogen bonding on further oxidation **(Halliwell et al., 1990)**.

Over time as humans evolved, endogenous defences system also developed gradually to maintain a balance between free radicals and oxidative stress. The antioxidant activity by an molecule or compound can be displayed in various ways including: inhibition of free radical oxidation reactions, by limiting the formation of free lipid radicals, by interfering with the propagation of the autoxidation chain reaction; as a quencher of singlet oxygen; by synergistic effect with other antioxidants; conversion of hydroperoxides into stable compound by acting as reducing agents; by acting as metal chelators that convert metal pro-oxidants (iron and copper derivatives) into stable products; and finally acting as inhibitors of pro-oxidative enzymes (lipooxigenases) **(Darmanyan et al., 1998; Heim et al., 2002; Min et al., 2002; Pokorny, 2007; Kancheva, 2009).**

Antioxidant system of humans can be classified into two main groups, enzymatic antioxidants and non-enzymatic oxidants **(Fig. 1.17) (Carocho et al.,**

2013). Enzymatic antioxidants are further divided into primary and secondary enzymatic defences. Primary defence encompasses three important enzymes preventing the formation or neutralize free radicals: glutathione peroxidase, eliminates peroxides as potential substrate for the Fenton reaction and also donates two electrons to reduce peroxides by forming selenoles; catalase, has one of the biggest turnover rates known to man (one molecule of catalase to convert 6 billion molecules of hydrogen peroxide) and converts hydrogen peroxide into water and molecular oxygen; and finally, superoxide dismutase from hydrogen peroxide as a substrate for catalase from superoxide anions **(Rahman, 2007)**. Secondary enzymatic defense consists of glutathione reductase (reduces glutathione from its oxidized to its reduced form, thus recycling it to continue neutralizing more free radicals) and glucose-6-phosphate dehydrogenase (regenerates NADPH creating a reducing environment) **(Gamble et al., 1984; Ratnam et al., 2006)**. The secondary enzymatic defence does not neutralize free radicals directly, albeit they support the other endogenous antioxidants.

Among non-enzymatic endogenous antioxidants, there are number of them including vitamins (A), enzyme cofactors (Q10), nitrogen compounds (uric acid), and peptides (glutathione). Vitamin A or retinol is produced in the liver from the breakdown of β-carotene and it has beneficial impact on the skin, eyes and internal organs. Coenzyme Q10 play very important role in all respiratory chain and other cellular metabolism. It is present in all the cells and prevents the formation of lipid peroxyl radicals. In addition, it has ability to regenerate vitamin E. However, some groups describe this process to be more likely than regeneration of vitamin E through ascorbate (vitamin C) **(Turunen et al., 2004)**. Purine nucleotide metabolism end product in humans, uric acid is known to prevent the overproduction of oxo-heme oxidants that result from the reaction of hemoglobin with peroxides. Additionally, it also prevents lysis of erythrocytes by peroxidation and is a potent scavenger of singlet oxygen and hydroxyl radicals **(Kand'ár et al., 2006)**. Glutathione, an endogenous tripeptide protects the cells against free radicals by donating a hydrogen atom or an electron. Moreover, it is crucial for the regeneration of other antioxidants like ascorbate **(Steenvoorden et al., 1997)**.Despite of remarkable efficiency of the endogenous antioxidant system, humans depend on various types of antioxidants present in the diet to maintain free radical concentrations at low levels **(Pietta et al.,**

2000). Vitamin C and E are absorbed through the gastrointestinal tract and are effective in scavenging the superoxide radical anion, hydrogen peroxide, hydroxyl radical, singlet oxygen and reactive nitrogen oxide **(Barros et al., 2011)**. Vitamin K belongs to the group of fat-soluble compounds and is important for posttranslational conversion of protein-bound glutamates into carboxy-glutamates in various target proteins **(Carocho et al., 2013)**. Flavonoids are group of compounds composed of flavonols, flavanols, anthocyanins, isoflavonoids, flavanones and flavones possessing antioxidant activity. Phenolic acids are ubiquitous to plant material and sometimes present as ester and glycosides. These are composed of hydroxycinnamic and hydroxybenzoic acids and have antioxidant activity as chelators and free radical scavengers with special impact over hydroxyl and peroxyl radicals, superoxide anions and peroxynitrites.

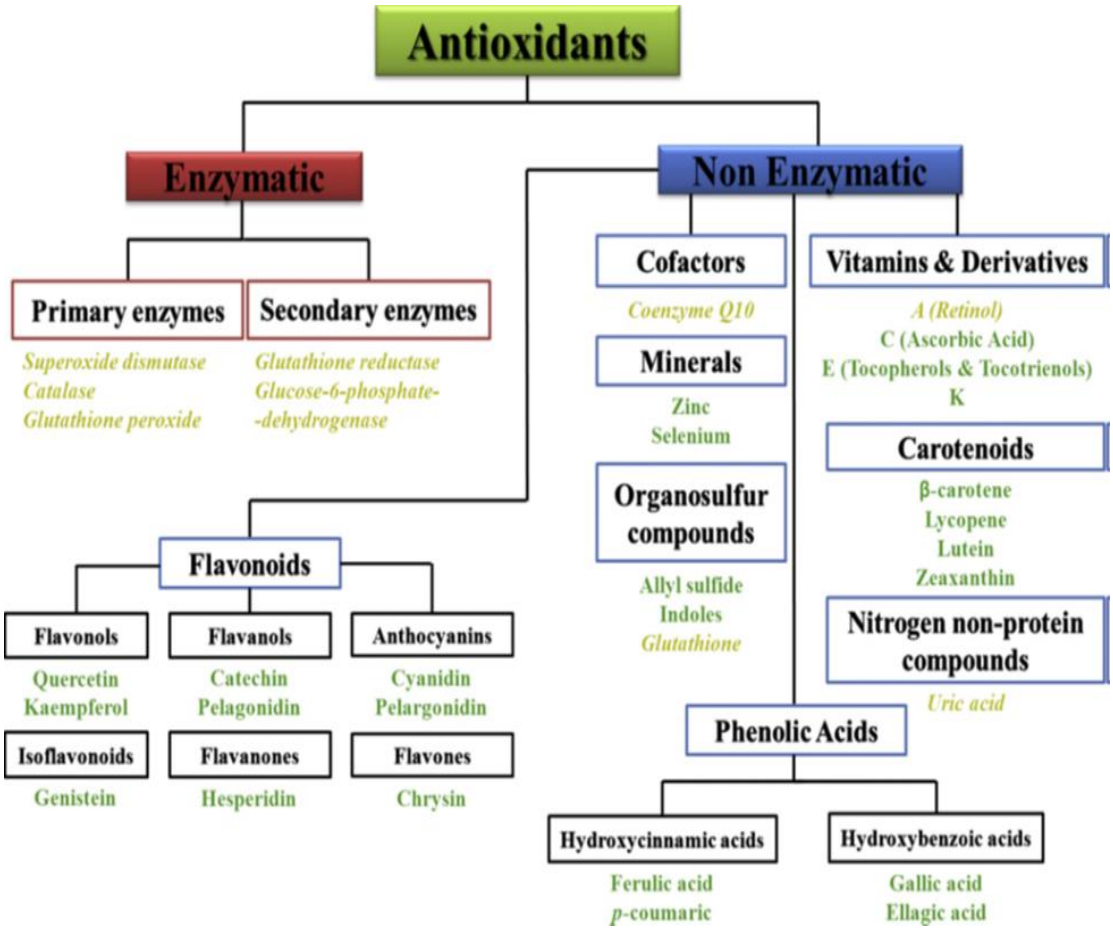

Fig. 1.17 Enzymatic and non-enzymatic antioxidants (Carocho et al., 2013).

Carotenoids are a group of natural pigments synthesized by plants and microorganisms. Antioxidant property of carotenoids is based on quenching of singlet

oxygen which results in excited carotenoids that dissipate the newly acquired energy through a series of rotational and vibrational interactions with the solvent, thus returning to the unexcited state and allowing them to quench more radical species. Some of the minerals found in the human system have antioxidant activity i.e. selenium and zinc. These minerals do not directly act on free radicals but is an indispensable part of most antioxidant enzymes (metalloenzymes, glutathione peroxidase, thioredoxin reductase) that would have no effect without it.

Synthetic antioxidants have been developed to measure the antioxidant activity of natural antioxidants and to incorporate them into food **(Carocho et al., 2013)**. Almost all the processed food contains synthetic antioxidants, which are reported to be safe, although some studies contradict this statement. BHT (butylated hydroxytoluene) and BHA (butylated hydroxyanisole) are the most widely used chemical antioxidants and European food safety authority (EFSA) have revised acceptable daily intakes (ADIs) of 0.25 mg/kg bw/day for BHT and 1.0 mg/kg bw/day for BHA after looking at the contradictory results (EFSA, 2011; EFSA, 2012). Apart from this TBHQ (tert-Butylhydroquinone), known to stabilize and preserves the freshness of animal food products and NDGA (Nordihydroguaiaretic acid) are also used as synthetic antioxidants in the food products.

1.14 Pyrroloquinoline quinone (PQQ)

Pyrroloquinoline-quinone (PQQ) was first identified as a coenzyme for methanol dehydrogenase in methylotrophic bacteria **(Rucker et al., 2009)**. It can covalently interact with different proteins. Proteins interacting with PQQ are termed as quinoproteins. Majority of these proteins are bacterial dehydrogenases. In addition, amine oxidase/dehydrogenase, Ser/Thr protein kinases from *Escherichia coli*, and *Deinococcus radiodurans*, 2-aminoadipic 6-semialdehyde dehydrogenase from mammalian system and a signalling protein having AMP binding and phosphopantetheine-binding domains and six PQQ binding motifs also use PQQ as co-factor **(Rajpurohit et al., 2010)**. PQQ is heat stable, water soluble and it can undergo 20, 000 oxidation reduction cycles (Rucker R et al., 2009). Wide variety of foods has PQQ ranging from 0.19-7.02 ng per g fresh weight or per ml of liquid food **(Noji et al., 2007)**. Although, role of PQQ in growth and development is evident in all

organism studied so far, its biosynthesis gene is absent in higher organisms. Higher organisms are dependent on microbial population as source of PQQ.

Different microorganisms can synthesize varying amount of PQQ depending on media composition. PQQ biosynthesis gene clusters have been identified and studied in many bacteria including *Acinetobacter calcoaceticus*, *Enterobacter intermedium* 60-2G, *Gluconobacter oxydans*, *Klebsiella pneumoniae*, *Methylobacterium extorquens* AM1 and *Pseudomonas fluorescens* CHA0 **(Misra et al., 2012)**. *Escherichia coli* are deficient in PQQ biosynthesis gene cluster and therefore it is used as host for the expression of *pqq* gene cluster from different organisms. Naturally occurring quinones have antioxidant property and they are used as drug to protect cells from oxidative stress in-vivo. These quinoes produce oxidative products which results in depletion of free glutathione upon reacting with reactive oxygen species (ROS). As all antioxidant molecules can be pro-oxidant, cytotoxicity mechanism underlining PQQ have been extensively studied. Upto 10 μM PQQ behave as antioxidant molecule and above 50 μM it can be pro-oxidant **(He et al., 2003)**. Antioxidant property of PQQ in response to oxidative stress has been extensively studied in bacteria and in mammalian cells. Previous studies has supported the fact that antioxidant property of PQQ is implicated in various beneficial effects; protection of neurological cells by suppressing peroxynitrile formation, blocks SIN-1-evoked ATP depletion and nitration of bovine serum albumin by scavenging superoxide radical, prevents the neurotoxin 6-hydroxydopamine (6-OHDA)-induced cell death and DNA fragmentation in SH-SY5Y cells **(Misra et al., 2012)**. In addition, *E. coli* cells producing PQQ could tolerate the mixed ROS several fold higher than the control. Moreover, sciatic-nerve-deficit model created in rats showed PQQ can induce nerve cells regeneration of peripheral nerves, enhance mitochondrial respiratory ratios in ischemic and nonischaemic myocardium which are clinically relevant. Differential phosphorylation of signaling protein in NIH3T3 mouse fibroblast can be induced by PQQ via activation of Ras signaling pathways (Kumazawa et al., 2007). Additionally, it can increase the phosphorylation of Rb and c-Jun by quick activation of ERK and PKC epsilon. Conversely, presence of PQQ can abolish the expression of growth inhibitory molecules like IκB and p27. Moreover, the effect of soluble NSF attachment proteins (SNAP) and effect of growth inhibitors, and activates Ras pathway kinases, which lead to a dynamic shift in G0/G1 population

to S and G2/M population can be counteracted.by PQQ. This observation make it evident, PQQ mediates cell proliferation through Ras mediated signaling pathways.

1.15 Factors influencing the colonization and survival of probiotics

Colonization and survival of probiotic in gastrointestinal tract is very important factor influencing the efficacy of probiotic **(Drasar et al 1969)**. Following oral administration, probiotics must survive the challenges of transit through the gastrointestinal tract, low pH environment of the stomach (which can be as low as pH 1.5 when fasting), bile stress and elevated osmolarity in the intestine. After passing through all these barriers it must colonize and proliferate to exert beneficial effect on the host. Therefore, potential probiotic strains need to be physiologically robust. Some of the studies have been performed to investigate the survival of probiotics during transit both in-vivo and in vitro **(Berrada et al 1991)**. In same line, 2 strains of *Bifidobacterium* probiotics were challenged to acidity mimicking stomach for 90 min. This resulted in growth inhibition by 0.5 log units in one strain while other showed 4 log units decline. Moreover, similar differences were also observed in humans after administration of these 2 strains in fermented milk. Similarly, another study also evaluated the viability of unspecified *Bifidobacterial* species in-vitro in variable pH and time of exposure **(Pochart et al., 1992)**. There was no change in viability at a pH of 3 for 180 min, declined slowly at a pH of 2, and was zero after 60 min at a pH of 1. Similar studies have also been performed on different *Lactobacillus* and *Bifidobacteriam* strains.

In the small bowel, the most challenging obstacle in probiotic survival is bile salts . In vitro studies evaluating the resistance of different probiotics to bile salts can be divided into 2 types: survival and growth studies. As previously observed in case of pH, exposure to different bile salt concentration for different time period affected the viability of *Lactobacillus* and *Bifidobacterium* strains **(Kailasapathy et al., 1995)**. Growth experiments performed to check the viability in the presence of bile salts includes another variable, the presence of unconjugated bile acids in the medium. *Lactobacilus* and *Bifidobacterium* species can de-conjugate bile salts with the help of bile salt hydrolases. Unconjugated bile acids are much more efficient in lysing bacterial cell than conjugated bile acids. Few experiments have demonstrated that among lactobacillus species, *L. acidophilus* strains 2405 and 2401 are most resistant

to 0.3% oxgall while in *Bifidobacteriaum*, *B. infantis* 1912 and *Bifidobacterium adolescentis* 1920 were most resistant **(Bezkorovainy et al., 2001).**

Another important characteristic to permanently establish a bacterial strain in the host's intestine is its ability to attach to intestinal mucosal cells. Many pathogens do not have ability to attach to mucosal surface which limits there deleterious effects on the gut. In contrast, probiotics have purported ability to interfere with the adherence of pathogens to intestinal mucosal cells. In vitro experiments have demonstrated probiotic interference in adherence of pathogen such as *Salmonella typhimurium* to Caco-2 cells **(Hudault et al., 1997)**. However, in-vivo such scenario is controversial. In a study carried out on human volunteers, antibiotic-resistant strain of *Biofidobacterium* showed recovery rate 29.7 ± 6.0% of the ingested dose, which is consistent with the percentage survival during probiotic passage through the gastrointestinal tract. However, when dose of *Bifidobacterium* was stopped, it was not recovered from the fecal sample suggesting *Bifidobacterium* did not colonize the gut. Similar results were also observed by Kullen et al who on human volunteers fed with a unique *Bifidobacterium* strain followed by examination of fecal *Bifidobacterial* flora. Till the dose of *Bifidobacterium* was continues it showed increases total excretion but this strain disappeared from the feces after the feeding was discontinued. This leads to the conclusion that, although Bifidobacteria are capable of surviving through the passage of gastrointestinal tract but they limit in their ability to colonize in gastrointestinal tract to significant extent and colonization ability may be unnecessary to achieve positive results in probiotic therapy. Alternatively, Fujiwara et al supported this conclusion by demonstrating, Bifidobacteria produce a 100-kDa protein, which prevents the adhesion of pathogenic *Escherichia coli* to their normal receptors in the intestinal tract **(Fujiwara et al., 1997)**. Therefore direct competition between probiotic and *E. coli* may not be necessary for adhesion.

Another very highly effective probiotic *Lactobacillus* GG is said to colonize the human intestinal tract based on the studies showing the ability of this probiotic to adhere to Caco-2 and other enteric cells in vitro **(Bezkorovainy et al., 2001)**. However, this finding is not true in-vivo. Human volunteers fed with *Lactobacillus* GG fermented milk was present in their feces but disappeared in 67% of the subjects within 7 d after stopping its administration. Similar observation was observed in premature infants fed milk formulas containing L. GG. Thus, despite L. GG being of

human origin, no sufficient evidence indicating that it can permanently establish itself to colonize in the intestines of the general population.

1.16 *Vitreoscilla* Hemoglobin

Distinct niches in the GI tract possess different oxygen condition. In contrast, lower part of GI tract is predicted to be hypoxic as demonstrated from whole animal studies showing oxygen concentrations of 2–7% of air saturation **(He et al., 1999)**. This finding is supported by another study exhibiting the contribution of respiratory pathways to *Escherichia coli* survival in the intestine **(Jones et al., 2007)**. Respiratory flexibility is prime requirement for better colonization as well as competitiveness of the organism. Therefore to adapt to the micro-aerophilic condition of the intestine, it switches on and off its aerobic and anaerobic genes involved in the respiration **(Khosla et al., 1989)**. *E. coli* possesses **A**erobic **R**espiratory **C**ontrol system (ARC) and **F**umarate **N**itrate **R**eductase **S**ystem (FNR) to regulate aerobic and anaerobic control respectively **(Jones et al., 2007)**. It has been hypothesized that the presence of facultative anaerobes in the gut makes the environment more and more anaerobic. This statement is supported further by the fact that *E. coli* does not possess polysaccharide-degrading enzyme, ensuring that the nutrients from the mucus is made available by the anaerobes present in the GIT. Hence, *E. coli* tend to couple the oxidation of low nutrient concentrations to the respiration and increase its yield in the intestine. Based on these reports, it can be concluded that in order to develop stable and well colonizing probiotic, we need to improve oxygen availability. Therefore in the present thesis work we introduced the *vgb* gene coding for *Vitreoscilla* hemoglobin.

Vitreoscilla hemoglobin (VHb) is a homodimeric 15.7 kDa bacterial hemoglobin found in an obligate aerobe, which is present in the cytoplasm and get concentrated near the periphery **(Yang et al., 2007)**. Expression of *vgb* gene increases the effective intracellular oxygen concentration under micro-aerobic conditions, and improves growth of *E. coli* under oxygen-limited conditions. The natural promoter of *vgb* gene is oxygen sensitive promoter with ArcA binding site, which regulates VHb expression positively. This regulation helps *vgb* gene to get expressed in microaerobic condition. Evidences so far state VHb's capacity of scavenging and sensing oxygen,

reactive oxygen species and NO, thereby enhancing the function of cytochromes or serving as an oxidase itself. In order to aid to the screening purpose, green fluorescence marker (GFP) was along with *vgb* gene.

1.17 Prebiotics

Prebiotics are non-digestible food ingredients which are metabolized by lower part of gastrointestinal tract and it beneficially affects the host by selectively stimulating the growth and/or activity of one of a limited number of bacteria in the colon, and thus improves host health' **(Pharmaceutiques et al., 1995)**. List of different probiotics are listed in **Table 1.8**. Prebiotics are considered to be important because of following reasons:

(i) It can alter microbiota composition towards more healthy profile,
(ii) It can be beneficial in prevention of diarrhea and immunomodulation,
(iii) Prebiotics like inulin and its derivatives, and galacto-oligosaccharides (GOS) are relatively cheap, can be extracted from plant sources and in addition, they are also valuable functional ingredients in foods with the potential to give fat-based spreads and dairy products improved organoleptic properties.

Based on the last one decade research work published, defining prebiotics has three important aspects of original definition **(Gibson et al., 2004)**:

(i) Resistance to digestion in upper part of gastrointestinal tract,
(ii) Fermentation by the large intestinal microbiota and
(iii) Selective effect on the microbiota having capability of promoting health effects.

According to the most recent definition of prebiotics, they are defined as 'A prebiotic is a selectively fermented ingredient that allows specific changes, both in the composition and/ or activity in the gastrointestinal microbiota that confers benefits upon host well-being and health'.

Name	Composition	Method of manufacture	DP
Inulin	β(2–1) fructans	Extraction from chicory root	11–65
Fructo-oligosaccharides	β(2–1) fructans	Tranfructosylation from sucrose, or hydrolysis of chicory inulin	2–10 3–5
Galacto-oligosaccharides	Oligo-galactose (85%), with some glucose and lactose	Produced from lactose by β-galactosidase	2–5
Soya-oligosaccharides	Mixture of raffinose (F-Gal-G) and stachyose (F-Gal-Gal-G)	Extracted from soya bean whey	3–4
Xylo-oligosaccharides	β(1–4)-linked xylose	Enzymic hydrolysis of xylan	2–4
Pyrodextrins	Mixture of glucose-containing oligosaccharides	Pyrolysis of potato or maize starch	Various
Isomalto-oligosaccharides	α(1–4) glucose and branched α(1–6) glucose	Transgalactosylation of maltose	2–8

DP, degree of polymerization; F, fructose; Gal, galactose; G, glucose.

Table 1.8 Different prebiotics (Macfarlane et al., 2006).

Consumption of prebiotic like Inulin, fructo-oligosaccharides (FOS), trans-GOSs and lactulose in relatively small amounts (5–20 g/day) have been demonstrated in human to stimulate growth of health-promoting species belonging to the genera *Bifidobacterium* and *Lactobacillus* **(Gibson et al., 2004; Roberfroid et al., 2007)**.

1.18 Inulosucrase (InuJ)

Inulosucrase, along with levansucrase are enzymes that polymerize fructose moiety of sucrose into fructans inulin and levan, respectively **(Roberfroid et al., 2000)**. Fructans or fructo-oligosaccharides (FOS) are the polymers of D-fructose joined by ß (2 - 1) linkages in case of inulin and ß (2 - 6) linkage in case of levan; and terminates with a D-glucose molecule linked to fructose by α (1 - 2) linkage **(McKellar et al., 1989; Sangeetha et al., 2005)**. Levan exhibits lower transglycosylation/hydrolysis ratio than inulosucrase **(van Hijum et al., 2006)**. Evidences support the presence of these transfructosylating enzymes in both plants such as *Helianthus tuberous* **(Edelman et al 1966)** and from *Rhodotorula* spp. **(Hernalsteens et al., 2008)**. *Aspergillus niger, Aspergillus oryzae, Aspergillus japonicas* and *Aureobasidium pulurans* have also been observed with FOS producing potential **(Sangeetha et al., 2005; Dorta et al., 2006; Mussatto et al., 2013)**. Also, amongst bacterial species, these enzymes show high level of amino acid sequence

similarity (> 60 %) but no sequence similarity has been observed between plant and bacterial transfructosylating enzymes **(van Hijum et al., 2006; Korakli et al., 2006).**

Inulosucrases (E C 2.4.1.9) are classified as glycoside hydrolase family GH68 **(van Hijum et al., 2006; Anwar et al., 2008)**. In bacteria, it was first isolated from *Lactobacilli reuteri* 121. Subsequently, *Lactobacillus johnsonii*, a member of acidophilus group of intestinal *Lactobacilli* have been observed to possess an open reading frame AAS08734, encoding inulosucrase enzyme (inuJ). The inuJ isolated from *L. johnsonii* shows catalytic property largely similar to the one isolated from *L. reuteri* 121. InuJ exhibits maximum transglycosylation activity in the broad pH range of 4.5 to 7.0 (65 to71 %). Maximum hydrolytic activity has been observed within the same pH range with a peak at pH 7.0 **(Ozimek et al., 2006)**. The optimum temperature for the enzyme activity has been reported to be 55 ^{o}C with a non-Michaelis Menten type of kinetics for the transferase reaction **(van Hijum et al., 2003; Ozimek et al., 2006)**. A drastic decrease in enzyme activity was observed on further increasing pH and temperature.

In addition to this, role of Ca^{2+} in enzyme activity and stability has been determined in *Lactobacillus reuteri* 121 levansucrase (Lev) and inulosucrase (Inu) enzymes **(Ozimek et al., 2006; Anwar et al., 2008)**. *L. reuteri* inulosucrase has been reported to exhibit a Hill-type of kinetics with negative co-operativity **(van Hijum et al., 2003)**. The average molecular mass of the inulin produced by recombinant inuJ has been determined to be 4 X 107 Da **(Anwar et al., 2008)**. Moreover, microbial inulins have been reported with a degree of polymerization of 20 – 10,000 **(van Hijum et al., 2006)**. 3D structure of the active bacterial H68 inuJ, in its apo form with bound sucrose has been determined **(Pijning et al., 2011).** Both levansucrase and inulosucrase exhibits conserved structural framework involved in the binding and cleavage of the substrate, sucrose, in their active site. These enzymes results in polymerization in which fructose units are linked to a fructan polymer and hydrolysis of sucrose into fructose and sucrose **(van Hijum et al., 2003)**. Mature inulosucrases from lactic acid bacteria generally consist of three domains: an N-terminal variable domain, a catalytic core domain of about 500 residues, and a C-terminal variable region that sometimes contains a cell wall anchoring domain **(van Hijum et al., 2006)**. The 3D structure revealed that inuJ has a five-bladed β-propeller fold, with an active site interacting with the donor substrate sucrose in a fully conserved way. The

complex with the first transfructosylation product 1-kestose (Fru- β(2-1)-Fru-α(2–1)-Glc) shows how an inulin-type FOS binds in the active site **(Pijning et al., 2011)**. Cleavage of the glycosidic bond of the donor substrate sucrose has been proposed to result in the formation of a covalent enzyme–fructosyl intermediate **(Chambert et al., 1976)**. Subsequently, the fructosyl unit can be transferred to acceptor substrates bearing a terminal fructose unit, such as sucrose (resulting in the formation of 1-kestose; a growing fructan chain, or various other oligosaccharides (transfructosylation). Alternatively, the fructosyl unit can be transferred to water (hydrolysis) **(Pijning et al., 2011)**. The above schematic diagram represents the donor (negative) and acceptor (positive) binding sub-sites in the active site of inuJ and the reactions hence occurring **(Ozimek et al., 2006)**. Sucrose binding to subsites −1 and +1, results in cleavage of the glycosidic bond (gray arrow), and forms a covalent enzyme–fructosyl intermediate at sub-site −1 (gray line), with the release of glucose. Subsequently, hydrolysis by water or transglycosylation may occur. Transglycosylation results in the synthesis of FOS **(Ozimek et al., 2006; Pijning et al., 2011)**.

1.19 Short chain fatty acid (SCFA)

Dietary carbohydrates which are non-digestible in upper part of gastrointestinal tract are metabolized in colon via microbial fermentation resulting in SCFA production **(Hijova et al., 2007)**. These SCFA has been demonstrated to be associated with few diseases including irritable bowel syndrome, inflammatory bowel disease, cancer and cardiovascular disease. SCFA are principle anions and are composed of 1-6 carbon atoms. Accumulating evidences suggests, SCFA are produced in order of acetate > Propionate > Butyrate. SCFA are absorbed in the cecum and colon with enhanced sodium absorption and bicarbonate excretion. The major SCFAs (Acetate, propionate and butyrate) are metabolized at 3 sites in body:

- By cells of ceco-colonic epithelium as butyrate is substrate for pathways involved in energy production.
- Butyrate and acetate are taken up by hepatocytes
- Muscle cell use acetate for energy generation

In addition, SCFA can activate GPR43 which can regulate white adipose tissue energy uptake **(Kimura et al., 2013)**. More over studies have demonstrated SCFA role in GPR41 activation by activating sympathetic nervous system at the level of ganglion. These studies suggest that SCFA production by gut microbiota can have important role in recognition of postprandial nutrition excess and energy expenditure through activation of GPR 43 and 41 (**Fig. 18).**

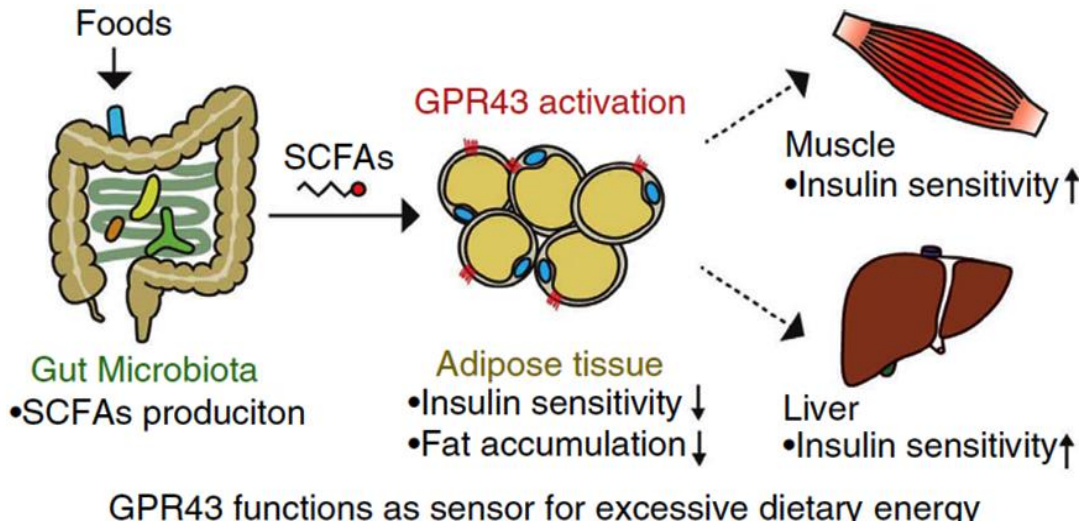

Fig. 1.18 Schematic model for suppression of fat deposition accumulation via GPR43 (Kimura et al., 2013).

In summary, consumption of dietary fructose and sucrose has increased significantly in past 2-3 decades which is attributed to metabolic disorders i.e. hyperlipidemia, insulin resistance, obesity, diabetes and oxidative stress. Numerous strategies including drugs, biologics and herbal therapies have been implicated in the management of metabolic syndrome. An alternative approach to overcome the limitation of these therapies, use of probiotic as natural therapeutic is considered to be safe and effective strategy as reflected by accumulating evidences from animal and human studies. This thesis work is focused on development of probiotic *E. coli* for alleviation of dietary fructose and sucrose induced metabolic syndrome by implementing genetic engineering techniques. For the present work three strategies have been opted: first, improving the colonization ability of probiotic in GI tract; second, incorporation of sucrose metabolizing enzyme (Inulosucrase) in probiotic and improving the antioxidant potential of probiotic by engineering *pqq* gene cluster. Based on the above literature following are the objectives for thesis work:

Objectives

- Evaluating the protective effect of probiotic *E. coli* 16 on 1, 2-di-methyhydrazine (DMH) induced oxidative stress.
- Evaluating the effect of probiotic *E. coli* strains producing pyrroloquinoline quinone (PQQ) on fructose induced metabolic effect and antioxidant status in Charles Foster rats.
- Evaluating the effect of probiotic *E. coli* strains harboring plasmid and genomic integrants encoding pyrroloquinoline quinone *(pqq)* and inulosucrase *(inuJ)* gene cluster on sucrose fed Charles Foster rats.