Chapter 1

Review of Literature
1.1 Mammalian gut microbiota and probiotics

INTRODUCTION

The majority of epithelial surfaces of mammalian body, such as the skin and mucosa, are colonized by a vast number of microorganisms called normal microflora or the microbiota (Tlaskalova-Hogenova et al., 2011). The microbiota primarily comprises bacteria; however, viruses, fungi and protozoans are also present. Human microbiota contains trillions of bacterial cells, 10 times more cells than the number of cells constituting our body. Most of the commensal bacteria are symbiotic; however, under specific conditions, such as immunodeficiency, commensal bacteria could cause pathology. These bacteria are present at anatomical locations that provide suitable conditions for their growth and proliferation. Skin is predominantly colonized by bacteria in the skin folds. The upper airways, particularly the nasopharynx, and some mucosal surfaces of the genital tract harbor bacteria, although the greatest numbers of bacterial cells are found in the digestive tract. The oral cavity (tongue, teeth and periodontal tissues) also harbors high numbers of bacteria \(10^{12}\). The stomach has only \(10^3\)–\(10^4\) bacteria, the jejunum harbors \(10^5\)–\(10^6\) bacteria and the terminal ileum harbors \(10^8\)–\(10^9\) of bacterial cells. However, the largest number of bacterial cells is found in the large intestine (\(10^{11}\) per gram of intestinal content). **Figure 1.1** shows the spatial distribution of different bacterial communities in the human GIT. A considerable portion, about 70%, of this microbial cosmos inside our body is composed of bacteria that cannot be cultivated by current microbiological methods.

Our microbiota represents a complex ecosystem with enormous microbial diversity (Eckburg et al., 2005). It is important to note that the number of genes of human colonic microbiota exceeds the number of genes present in the human genome by 150 times (Qin et al., 2010). There are more than 50 bacterial phyla on Earth, but human gut-associated microbiota is dominated by four main phyla: Firmicutes, Bacteroidetes, Actinobacteria and Proteobacteria (Eckburg et al., 2005). Fundamental comparative studies of human fecal microbiota have revealed the astonishing fact that each individual has a unique microbiota fingerprint (i.e., there are considerable
differences between the compositions of the microbiota within individuals). It is known that the main bacterial populations comprising our gut microbiota stabilize during the first years of life. During this time, the microbiota develops and subsequently remains stable throughout our life in terms of the major bacterial populations, even after antibiotic treatments (Dore et al., 2010). Microbial diversity and functional genomics in human genome has been well reviewed by Morgan et al. (2013). Current techniques followed in Metagenomic profiling and emerging methods have also been discussed well.

Figure 1.1 Distribution of bacterial communities in different parts of human gastrointestinal tract with varying pH.

SIGNIFICANCE AND FUNCTIONS OF GUT MICROBIAL COMMUNITY

Microbiome produces an enormous amount and diversity of molecules and metabolites, which interact with the host; however, the role of the majority of these remains to be elucidated. The existence of enormous population of bacteria in the large intestine and their fundamental functions in nutrition and metabolism (fermentation of nondegradable oligosaccharides, metabolism of xenobiotics and activation or destruction of mutagenic metabolites) makes the colonic microbiota a large fermentative organ of the body (Martin et al., 2009). The different microbial communities (Bacteria, viruses and eukaryotes) interact with themselves and host, and alter the outcome of disease (Clemente et al., 2012) (Figure 1.2). Kamada et al. (2012) summarizes complex host microbiome interaction during homeostasis and pathogenic conditions. The commensal microbiota has coevolved with host and is
essential for several host physiological processes such as enhancement of intestinal barrier function, development of immune system and acquisition of nutrients. Varying pH and physiological role of gut, varies the presence of dominant bacterial groups along the intestine. The major dominant groups of commensal bacteria and their major role is represented in Figure 1.3.

Figure 1.2 Development of the intestinal microbiota (Clemente et al., 2012). The gastrointestinal tract of the fetus is sterile until birth. Depending on delivery mode, the initial communities tend toward a skin-like (caesarean section) or a vaginal-like (vaginal delivery) configuration. During the first weeks of life, there is a reduced activity of TLRs, potentially allowing the necessary formation of a stable bacterial community in the gut. As the infant grows, and with the introduction of solid foods, the microbiota diversity increases, and the community converges toward an adult-like state. At the same time, the immune system “learns” to differentiate between commensal and pathogenic bacteria. By adulthood, a relatively stable community composition (but varying between different individuals) is achieved, dominated mostly by Bacteroidetes and Firmicutes. Different diseases are characterized by significant changes in the microbiota and associated changes in the production of cytokines.
Figure 1.3 Localization of dominant bacterial groups in the intestine (Kamada et al., 2012). The small intestine is rich in nutrients used by both the host and the microbe for growth. Proteobacteria spp. (mainly Enterobacteria), Lactobacillales and Erysipelotrichales (especially Turicibacter spp.) are dominant group of microbes present in the small intestine. In contrast, the large intestine is poor in such nutrients and therefore has fewer of these bacteria, whereas Bacteroidetes and Clostridia, which can use host-indigestible fibers as energy sources, are in abundance.

Metagenomic approaches demonstrate that that the main functions of the small intestine microbiota differs from the function of the colonic microbiota. The microbiota present in the small intestine is enriched in pathways and functions related to carbohydrate uptake and metabolism (Kleerebezem, 2010). The small intestine contains the majority of immune cells in the body and is substantially involved in proper functioning immune system; the small intestinal microbiota thus plays a very important role in development and maintenance of mucosal and systemic homeostasis. Dietary interventions and the administration of probiotics are the effective means of changing the composition of the relatively simple microbial community present in the small intestine and thereby significantly affect this community’s metabolic and immunomodulatory functions. Along with molecular biological analysis of microbiota, functional studies concentrating on intensive analysis of the effects of the microbiota on the host are also proceeding in parallel.
Gnotobiological methods on experimental animals (rats and mice) are an essential methodological tool in the study of the significance of the microbiota and the consequences of their colonization in the mammalian gut.

**Commensal microbes as intestinal immune modulators**

Commensal microbes in the mammalian intestine are one of the most impressive examples of mutualism in nature, where both microbes and animal host depends on each other for their optimal survival (Ivanov and Honda, 2012). These commensals are involved in development and maintenance of healthy host immune system. Exploiting the property of these commensals to interact the host immune system, is considered as one of the important therapeutic strategy against several immune related pathological conditions such as Inflammatory Bowel Disease (IBD), celiac disease, metabolic syndrome, diabetes, and microbial infections. In addition to collective effect of gut microbial community on host immune system, effect of individual commensals have also been deeply investigated (Table 1.1 and Figure 1.4).

**Table 1.1** Nonpathogenic bacterial members of the intestinal microbiota with immune effects (Ivanov and Honda, 2012)

<table>
<thead>
<tr>
<th>Concept</th>
<th>Examplesa</th>
<th>Association with Host</th>
<th>Immune Effects</th>
<th>Mechanismsa</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Probiotics</strong></td>
<td>● Confer health benefit to the host when administered in adequate amount ● Not necessarily part of the “normal microbiota” ● May affect beneficial microbiota (indirect effects)</td>
<td>Bifidobacterium spp; Lactobacillus spp</td>
<td>Transient</td>
<td>Innocuous, immunostimulatory</td>
</tr>
<tr>
<td><strong>Autobionts</strong></td>
<td>● Direct influence on host immune cell homeostasis or function ● Part of the “normal microbiota”</td>
<td>Bacteroides fragilis, Clostridia XIVa and XIV, SFB, Faecalibacterium prausnitzii</td>
<td>Permanent, host dependent, symbiotic</td>
<td>Immunomodulatory</td>
</tr>
<tr>
<td><strong>Pathobionts</strong></td>
<td>● Do not cause disease in the presence of normal microbiota in healthy host ● Cause disease when microbiota or host immunity is perturbed</td>
<td>Helicobacter hepaticus, Clostridium difficile, Prevotella spp., Klebsiella spp., Biophila wadsworthiae</td>
<td>Permanent, parasitic/infectious</td>
<td>Innocuous, detrimental</td>
</tr>
</tbody>
</table>

(7) speculative mechanisms. 

*Examples and mechanistic studies have been performed in mice (with the exception of F. prausnitzii and C. difficile). See text for details and references.*
Mutualistic commensal microbiome possessing immunomodulatory effect sustains healthy immune homeostasis (Ivanov and Honda, 2012). Autobionts are permanent members of the normal commensal microbiota. They control immune homeostasis in the lamina propria by, for example, inducing different subsets of effector T cells (control of T cell homeostasis). The relative proportions of Th17 and Treg cells depend on the relative presence of different autobionts, e.g., in different individuals, different intestinal locations, or at different stages in ontogeny. These mutualistic interactions sustain the healthy steady state. Loss of autobionts and general dysbiosis perturbs also the immune balance of the host. Dysbiosis may occur in multiple ways. Invasive intestinal pathogens cause transient infections but may lead to long-term perturbations of the microbiota due to strong inflammatory responses against the pathogen. Antibiotic (Abx) treatments or inflammation caused by physical damage to the mucosa may also lead to dysbiosis and the outgrowth of pathobionts, which are permanent members of the microbiota but do not cause disease in the presence of autobionts. Host genetic factors may also initiate or perpetuate dysbiosis. Dysbiosis leads to loss of the immunomodulatory effects of autobionts and results in a perturbed immune balance, which under appropriate conditions may manifest itself in disease. The disease state augments dysbiosis in a vicious circle. Autobionts and conventional probiotics are both microorganisms with beneficial effects. Probiotics have transient effects and can boost host immunity. Autobionts are part of the normal microbiota and have developed evolutionary adaptations to colonize the host, regulate host immunity, and establish a healthy immune state. Autobionts can therefore reverse dysbiosis as well as immune homeostasis.

**Figure 1.4** Mutualistic commensal microbiome possessing immunomodulatory effect sustains healthy immune homeostasis (Ivanov and Honda, 2012). Autobionts are permanent members of the normal commensal microbiota. They control immune homeostasis in the lamina propria by, for example, inducing different subsets of effector T cells (control of T cell homeostasis). The relative proportions of Th17 and Treg cells depend on the relative presence of different autobionts, e.g., in different individuals, different intestinal locations, or at different stages in ontogeny. These mutualistic interactions sustain the healthy steady state. Loss of autobionts and general dysbiosis perturbs also the immune balance of the host. Dysbiosis may occur in multiple ways. Invasive intestinal pathogens cause transient infections but may lead to long-term perturbations of the microbiota due to strong inflammatory responses against the pathogen. Antibiotic (Abx) treatments or inflammation caused by physical damage to the mucosa may also lead to dysbiosis and the outgrowth of pathobionts, which are permanent members of the microbiota but do not cause disease in the presence of autobionts. Host genetic factors may also initiate or perpetuate dysbiosis. Dysbiosis leads to loss of the immunomodulatory effects of autobionts and results in a perturbed immune balance, which under appropriate conditions may manifest itself in disease. The disease state augments dysbiosis in a vicious circle. Autobionts and conventional probiotics are both microorganisms with beneficial effects. Probiotics have transient effects and can boost host immunity. Autobionts are part of the normal microbiota and have developed evolutionary adaptations to colonize the host, regulate host immunity, and establish a healthy immune state. Autobionts can therefore reverse dysbiosis as well as immune homeostasis.
Mucosal barrier function: Role of commensal gut microbiota

Intestinal epithelial surface are composed of single layer cells and have evolved protective mechanisms to restrict bacterial invasion. Both, intestinal mucosa and skin are the barriers between the host and the external environment (Tlaskalova-Hogenova et al., 2011). At this barrier, organism encounters many antigenic, mitogenic and toxic stimuli present in food, normal microbiota and air. Most of the ‘exogenous’ pathogenic infections enter their host by the mucosal route. The internal environment of the organism is protected by mucosa which comprises of intense and effective innate and adaptive immune system. Almost 80 % of the immunologically active cells of the body belong to Mucosa Associated Lymphoid Tissue (MALT). Majority of these cells are present in the gastrointestinal tract, where antigens derived from food or microbiome is at highest abundance. The barrier function of intestinal mucosa is ensured by complex mechanisms acting on several levels. Microbiota itself is an important integral part of that system. At its optimal composition, microbiota prevents attachment, multiplication and invasion of pathogenic bacteria to the mucosal surface. Thus, the intestinal microbiota plays a vital role in pathogen resistance both by direct interaction with pathogens and by interacting with host immune system (Tlaskalova-Hogenova et al., 2004; Turner 2009). Mucins (highly glycosylated macromolecules) secreted by specialized epithelial cells (goblet cells) form the first barrier between the gut contents and epithelial cells, protecting them from direct contact with commensal bacteria and their components (Linden et al., 2008). Change in amount and/or composition in mucus has been shown to elicit immune response. The epithelial layer of the gut mucosa is reinforced by junctions (tight junctions, adherens junctions and desmosomes) in the paracellular spaces between epithelial cells forming an interconnected network. Tight junctions have been shown to act as a dynamic and strictly regulated port of entry that open and close in response to various signals such as cytokines and bacterial components, originating in the lumen, lamina propria and epithelium.

In general, innate immune mechanisms are affected mainly by phagocytic cells such as macrophages, neutrophils and dendritic cells. These
cells can produce cytokines essential for inflammatory reactions and factors critical for the subsequent initiation of adaptive immunity. These cells initiate innate immune responses to microbes via the sensors called pattern recognition receptors (Medzhitov and Janeway 2000). These sensing structures, the Toll-like receptors (TLRs), C-type lectin receptors, RIG-I-like receptors and nucleotide-binding domain (NODs) and leucine-rich repeat containing proteins, sense pathogen motifs and transmit activation signals to their target cells.

**Metabolic functions of the gut microbiota**

Gut microbiota metabolizes wide range of luminal compounds and produce metabolites. These metabolites play a major role in the detoxification of ingested toxic compounds, but in some cases these metabolites can be harmful for the host (Figure 1.5; Backhed et al., 2012). Thus composition of healthy vs. pathogenic bacteria determines production of beneficial or harmful secondary metabolites in the gut lumen.

**Figure 1.5** Metabolic conversion of luminal substance by gut microbiota (Backhed et al., 2012).
Gut microbiota derive their energy by fermentation of carbohydrates including large and small complex polysaccharide such as oligosaccharides, unabsorbed alcohols and sugars (Tremaroli and Backhead, 2012). Apart from carbohydrates they can also digest proteins such as glycoproteins. These gut microbiota produces Short chain fatty acids (SCFAs) as end products of complex carbohydrate and ammonia, amines and phenolic compounds as end product of protein digestion. Additionally, gut microflora facilitates the metabolism of bile acids and choline.

**Fermentation of polysaccharides.** Bacteroides thetaiotaomicron and B. ovatus harbour more than double amount of genes than the human genome which codes for glycosidase and lyase (Xu et al., 2007; Martens et al., 2011). These enzymes act on all types of plant polysaccharides and host glycan such as mucus associated glycoproteins.

**Bile acid metabolism.** Primary bile acids, including cholic acid and chenodeoxycholic acid, are synthesized in liver from cholesterol (Tremaroli and Backhead, 2012). Bile acid is important factor for digestion of fat constituent of the food and absorption of fat soluble vitamins from intestine. Primary bile acid conjugated with glycine in the mice and with taurine in the humans, reaches the distal part of ileum and is then transported back to the liver. Some amount of bile acid which escapes from intestinal uptake is taken up by gut microbiota. Further they convert primary bile acid into secondary bile acid, hence; germ-free rodents have more bile acid and a less diverse profile than their conventionally raised counterparts. These secondary bile acids also serve as signalling molecules and bind to cellular receptors such as Farnesoid X-receptor (FXR), which is involved in the regulation of bile acid synthesis and Transmembrane G protein-coupled receptor (TGR5). These receptors are involved in the regulation of glucose metabolism in the mice. FXR impairs whereas TGR5 stimulates glucose homeostasis. In contrast to FXR (receptor for primary bile acids), TGR5 binds secondary to bile acids such as deoxycholic acid (Derived from cholic acid) and lithocholic acid (formed from chenodeoxycholic acid). TGR5 signalling releases GLP-1 from enteroendocrine-L cells, which further results in improvement of liver and pancreatic function and promotes glucose tolerance in obese mice (Thomas...
et al., 2009). The bile acids in blood circulation activate FXR and TGR5 signalling in peripheral organs and affects overall metabolism of the host. In addition, activation of TGR5 signalling in adipose and muscle tissue increases energy expenditure and thus protects from diet-induced obesity (Watanabe et al., 2006). Hence, microbiota regulates lipid and glucose metabolism by regulating levels of bile acid through FXR and TGR5 signalling. Bile acid receptors have been demonstrated as target for drug development against Non-alcoholic steatohepatitis (NASH) (Figure 1.6; Schapp et al., 2014).

**Choline metabolism.** Choline is an important constituent of cell membrane and widely present in the food such as red meats and eggs. It can also be synthesized by humans. Choline plays a major role in lipid metabolism and very-low-density lipoprotein (VLDL) synthesis in liver (Vance et al., 2008). Diet lacking in choline changes the gut microbial community and causes liver steatosis in mice and the humans. Microbial and host enzymatic activities together involved in the conversion of choline into toxic methylamines. Trimethylamine (TMA) that is synthesized by gut microbiota may get converted into the trimethylamine-N-oxide (TMAO) in the liver (Dumas et al., 2006). Decreased bio-availability of choline due to these conversions triggers non-alcoholic fatty liver disease (NAFLD) in mice. Thus, shift in gut microbial composition and its effect on choline metabolism may have an important influence in modulating NAFLD as well as glucose homeostasis.
Probiotics

Probiotics are a very important group of microorganisms and have been studied from a very long time. The probiotics literally mean “for life” and have been defined in several ways. The definition that tries to give a proper meaning to the probiotics is “live organisms which when administered in adequate amounts confer a health benefit upon the host” (Reid et al., 2003).

Significance of probiotics

The gastrointestinal microbiota presents a significant barrier that must be overcome for a pathogen to initiate an infection (Tlaskalová-Hogenová et al., 2011). The concept of preventing or ameliorating intestinal infections through dietary interventions, which manipulates commensal bacteria in the gut, or as a means of introducing colonizing probiotic strains, has received great attention in recent years. Such strategies possibly decrease antibiotic use and associated problems of antimicrobial resistance. Further, an increase in stress due to modern day living makes a major demand on the host immune system which disturbs the homeostasis of the GIT. Consumption of pharmaceutical compounds, particularly antibiotics which kill bacteria, can alter the gut microflora in such a way that numbers of beneficial microorganisms decrease and of pathogenic increases which leads to disease (Parracho and Gibson, 2007). Therefore, it is of considerable benefit to the host to maintain homeostasis in the gut through increasing the levels of beneficial bacteria.

Probiotic therapy is a disease-prevention strategy used in humans and domesticated animals. This also enhances the growth rate of livestock and poultry. This method ensures the establishment of ‘good’ bacteria in the GIT which can prevent the establishment of bacterial pathogens. One of the most important attributes of a ‘good’ probiotic strain is its ability to produce antimicrobial compounds.
Clinical significance of probiotics

Probiotics are now clinically proven to have a number of health benefits including usefulness in irritable bowel syndrome (Reid et al., 2003; Saggioro, 2004; Fan et al, 2006). And, have been known to prevent or ameliorate pathological conditions such as allergies, skin health, dental health, blood pressure impairments, immune functions, liver functions and pain relief. Moreover, they have been used in management of vaginal infections, as anti-inflammatory agents, in supporting cardiovascular health and wellness. Probiotics has also been shown to reduce stress in the Gut and improve antioxidant status (Konturek et al., 2009; 2011).

**Figure 1.7** A schematic representation of major functions of probiotics in humans.

Mechanisms of Probiotic function

Probiotics stimulate the immune system in such a way that the host can respond to potential pathogens rapidly (Edwards and Parrett, 2002). They release antimicrobial substances like bacteriocins, which inhibit the colonization of pathogens (Jack et al., 1995). Increasing the numbers of
friendly bacteria by way of a probiotic may thereby decrease the nutrient availability for other bacterial populations, especially pathogenic ones (Fooks and Gibson, 2002). Probiotic bacteria compete with pathogens for attachment sites to the intestinal brush border epithelium and prevent their entry into the cells (Bernet et al., 1994; Guarner and Malagelada, 2003). Probiotic bacteria interacts with intestinal epithelium and maintains its integrity through direct interaction or secretory substances (Ukena et al., 2007; Khailova et al., 2009; Gareau et al., 2010).

**Microorganisms used as probiotics**

Firmicutes and Bacteroidetes are the two phyla representing major proportion of gut flora and several species have been demonstrated as probiotics. The most common probiotics belong to heterologous groups of *Lactobacillus*, *Enterococci* and *Bifidobacteria* (Ouwehand et al., 2002). In particular, Lactobacilli are most common used probiotics, this might have historical reasons, since Metchnikoff first proposed that Lactobacillus present in yogurt has many health benefits. Also, fermented milk products are the most common means of probiotic administration. However, other microorganisms including yeasts have been demonstrated as potent probiotics (Table 1.2).
Table 1.2 Commonly used probiotic strains and their beneficial characteristics (Ouwehand et al., 2002).

<table>
<thead>
<tr>
<th>Genus</th>
<th>Species</th>
<th>Example strain</th>
<th>Health benefits</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lactobacillus</td>
<td><em>acidophilus</em></td>
<td>La5</td>
<td>Reduced antibiotic associated diarrhoea</td>
</tr>
<tr>
<td></td>
<td><em>casei</em></td>
<td>Shirota</td>
<td>Shortening of Rotavirus diarrhoea, Reduced recurrence of superficial bladder cancer, Immune modulation</td>
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<td></td>
<td><em>fermentum</em></td>
<td>KLD</td>
<td>Improved oral vaccination</td>
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<td></td>
<td><em>johnsonii</em></td>
<td>La1</td>
<td>Reduced colonization by Helicobacter pylori</td>
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<tr>
<td></td>
<td><em>Paracasei</em></td>
<td>F19</td>
<td>Relief of irritable bowel syndrome (IBS), Reduction of LDL cholesterol</td>
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<td></td>
<td><em>plantarum</em></td>
<td>299v</td>
<td></td>
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<tr>
<td></td>
<td><em>reuteri</em></td>
<td>SD2112</td>
<td>Shortening of Rotavirus diarrhea</td>
</tr>
<tr>
<td></td>
<td><em>rhamnosus</em></td>
<td>GG</td>
<td>Shortening of Rotavirus diarrhoea, Immune modulation, Relief of IBD, Treatment and prevention of allergy</td>
</tr>
<tr>
<td></td>
<td><em>salivarius</em></td>
<td>UCC118</td>
<td>Reduced symptom of IBD,</td>
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<tr>
<td>Bifidobacterium</td>
<td><em>breve</em></td>
<td>--</td>
<td>Reduced symptom of IBD</td>
</tr>
<tr>
<td></td>
<td><em>longum</em></td>
<td>BB536</td>
<td>Treatment of allergy, Shortening of rotavirus diarrhoea, Reduced incidence of travellers diarrhoea, Improved vaccination.</td>
</tr>
<tr>
<td></td>
<td><em>lactis</em></td>
<td>Bb12</td>
<td></td>
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<tr>
<td>Propionibacterium</td>
<td><em>freudenreichii</em></td>
<td>JS</td>
<td>Fewer relapse of IBD.</td>
</tr>
<tr>
<td>Bacillus</td>
<td><em>subtilis</em></td>
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<td></td>
<td><em>cereus</em></td>
<td>Toyoi</td>
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<tr>
<td>Enterococcus</td>
<td><em>faecium</em></td>
<td>SF68</td>
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<tr>
<td><strong>Saccharomyces cerevisiae</strong></td>
<td><strong>boulardii</strong></td>
<td><strong>Escherichia coli</strong></td>
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<td>Reduced symptoms of IBD, Maintenance of intestinal tight junctions, Treatment of diarrhoea, Efficient delivery system for molecules in the gut as well as blood. Successful human clinical trials against IBD and IBS.</td>
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<tr>
<td></td>
<td></td>
<td>Immune modulation and attenuation of murine colitis. Reduced symptoms of IBS and IBD. Successful human clinical trials against IBS.</td>
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<td></td>
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<td>Reduced cytokine markers of inflammation in rodents, Antibacterial activity, and efficient secretory machinery exploited to deliver specific molecules in the gut.</td>
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</table>

*(Fitzpatrick et al., 2008)*

*(Kumar et al., 2009; 2013; 2014)*
Escherichia coli as probiotics

There are a very few number of gram-negative probiotics. However, recently less fastidious microorganisms have gained importance. *E. coli* is a Gram-negative, non-acid-fast, non-sporing rods or bacilli, 0.3-1.0µm in diameter and 1.0-6.0µm in length. This species is a natural inhabitant of the mammalian gut and generally in numbers around $10^8$ to $10^9$ per gm. of large intestinal contents (Bettelheim and Thompson, 1987). In humans, babies were found to have *E. coli* in their feces within the first few days of life, before they left the maternity ward (Nowrouzian et al., 2003).

*E. coli* M-17 strain isolated long back in Russia is one of the examples of probiotics used in treatment of human diseases (Fitzpatrick et al., 2008). It has been shown to modulate host immune system in order to prevent inflammatory symptoms in Irritable Bowel Syndrome (IBS) and Inflammatory Bowel Disease (IBD). *E. coli* CFR-16 was isolated by Kumar et al. (2009) from rat fecal matter. It has been shown to possess antibacterial activity, pH tolerance and reduces inflammatory cytokines in the serum when fed to animals. It has efficient mechanisms to secrete high amount of heterologous proteins (Kumar et al., 2013). *E. coli* CFR-16 expressing vgb and pqq gene cluster has been found to be effective in CCl$_4$ and 1,2-dimethylhydrazine induced toxicity in rodents respectively (Kumar et al., 2014; Pandey et al., 2014).

*E. coli* Nissle 1917 was isolated from the fecal sample of a soldier in a camp. The peculiar thing that was observed was that when other soldiers were suffering from diarrhoea or bowel disorders, he was the only one who was not. Hence, from then on *E. coli* 1917 was considered as a probiotic.

Major features of *E. coli* Nissle 1917

- Six different iron-uptake systems for energy generation through ATP (Grozdanov et al., 2004; Groβe et al., 2006; Valdebenito et al., 2006)
- Antagonism toward other members of the intestinal microbiota through colicin production (Patzer et al., 2003; Westendorf et al., 2005).
• Reduced secretion of pro-inflammatory cytokines and up-regulation of regulatory cytokines via TLR (Sturm et al., 2005; Grabig et al., 2006; Helwig et al., 2006).

• Restoration of a damaged epithelial barrier function through up-regulation of tight junction protein including ZO-1, ZO-2 and human β-defensin ( Schroeder et al., 2006; Zyrek et al., 2007).

• Lack of virulence factors (E.g. enterotoxins, haemolysins, cytotoxins, invasins, pathogen-specific fimbriae), with the presence of fitness factors (E.g. microcins, iron uptake systems, typical adhesins) which enable the micro-organism to survive and colonise in the intestine ( Grozdznov et al., 2004).

• *E. coli* strain Nissle 1917 is well used probiotic strain in treatment of diarrhoea, inflammatory bowel disease, constipation and ulcerative colitis ( Krammer et al., 2006; Henker et al., 2007).

• *E. coli* strain Nissle 1917 could be easily genetically modified to incorporate additional features.

E. coli Nissle use and safety

Probiotics can be used in food, dietary/nutritional supplements, drugs and medical foods. Each product has country specific legal requirements for allowed claims of efficacy, target populations, safety and risk/benefits assessments. E. coli Nissle 1917 is one of the best and extensive studied probiotic (Schultz, 2008). EcN isolated in 1917 based on its potential to protect from presumably infectious gastroenteritis. Initial therapeutic use was focused in management of gastrointestinal infectious disorder and infections affecting urinary tract; later on focus shifted to chronic inflammatory conditions. It has unique combination of fitness and survival factor to support intestinal survival. Lack of virulence and probiotic properties make this microorganism safe and effective candidate in treatment of chronic inflammatory bowel disease. Metaflor is being used extensively in Ulcerative colitis, chronic constipation, Crohn’s disease, Pouchitis, Irritable bowel syndrome, Antibiotic-associated/ pseudo-membranous and prophylaxis against colonization of pathogens and enhanced immunity of new born infants.

EcN is best investigated therapeutically used E. coli strain in the world. Its genome is fully sequenced and stocked in German collection of microorganism and cell cultures (DSM 6601). It has history of close to 100 years of safe, uninterrupted medical use. It has no antibiotic resistance gene and possesses genetic stability (No endotoxin production, no cytokine production, no hemolysin production, no pathogenic adhesion factors, no invasivity, no immunotoxicity, no serum resistance, no uropathogenecity and no toxicity in germ-free and conventional keeping of animals).

Genetic modification of probiotics

Probiotic research has now moved to the next level. Incorporating desired characteristics in the desired probiotics using genetic engineering tools has raised the opportunities to treat certain diseases or disorders where conventional native probiotics are ineffective. Table 2 summarizes major modifications of probiotics and clinical outcomes.

Chapter 1
<table>
<thead>
<tr>
<th>Sr. No.</th>
<th>Strain name</th>
<th>Modification</th>
<th>Disease condition</th>
<th>Functional outcome</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td><em>E. coli</em> Nissle 1917</td>
<td>Expressing human EGF-LARD3</td>
<td>Disruption of mucosal barrier in response to necrotizing and/or ulcerogenic agents like aspirin, bile acids and alcohol.</td>
<td>Wound healing capacity of the recombinant probiotic was established and its efficacy was observed in vitro.</td>
<td>Choi et al., 2012</td>
</tr>
<tr>
<td>2</td>
<td><em>E. coli</em> Nissle 1917</td>
<td>Expressing CAI-1</td>
<td>CAI-1, cholera autoinducer 1, is a quorum sensing molecule which determines the cell density of <em>Vibrio cholerae</em> in the intestine. At low cell density, the virus is supposed to express both cholera toxin and a critical colonization factor.</td>
<td>Significant reduction of intestinal colonization by the pathogen and reduced amount of cholera toxin bound in the epithelium</td>
<td>Duan et al., 2010</td>
</tr>
<tr>
<td>3</td>
<td><em>Lactobacillus jensenii</em></td>
<td>Expressing cyanovirin-N (CV-N)</td>
<td>HIV-1 infection</td>
<td>Significant protection from repeated HIV-1 infection was observed</td>
<td>Lagenaur et al., 2011</td>
</tr>
<tr>
<td>4</td>
<td><em>E. coli</em> CWG308:lgtA-lgtB-lgtE</td>
<td>Expressing mimic of lacto-N-neotetrose (LNT)</td>
<td>Enterotoxigenic <em>E. coli</em> disease (traveler's diarrhea) associated heat labile exotoxin LT.</td>
<td>Co-administration neutralized &gt;93.8% LT activity and protected rabbits from LT induced fluid secretion. Also, protected mouse from hemorrhagic enteritis</td>
<td>Paton et al., 2006</td>
</tr>
<tr>
<td>5</td>
<td><em>E. coli</em> CWG308:lgtE-cstII-cgtA-cgtB</td>
<td>GM1 receptor-mimic probiotic</td>
<td>Cholera induced by <em>V. cholerae</em> resulting in massive diarrhea and electrolyte imbalance.</td>
<td>Oral administration post infection exhibited 99% efficacy in protecting the infant mice against cholera</td>
<td>Paton et al., 2006</td>
</tr>
<tr>
<td>6</td>
<td><em>E. coli</em> CWG308: lgtD</td>
<td>expressing mimic of globotetrose</td>
<td>Diarrhea and fluid accumulation in tissues of stomach and large bowel.</td>
<td>98.4% capacity to neutralize Stx26 (shiga toxin) crude extrates</td>
<td>Paton et al., 2006</td>
</tr>
<tr>
<td>7</td>
<td><em>E. coli</em> CWG308:lgtC-lgtE</td>
<td>Expressing mimic of galactosyltransferase</td>
<td>Mild non-bloody diarrhea to hemorrhagic colitis accompanied by vomiting and nausea.</td>
<td>Oral administration showed 100% effectiveness in the mice models against STEC infection</td>
<td>Paton et al., 2006</td>
</tr>
<tr>
<td>No.</td>
<td>Organism</td>
<td>Modification/Expression</td>
<td>Disease/Effect</td>
<td>Related Studies</td>
<td></td>
</tr>
<tr>
<td>-----</td>
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<td>--------------------------------------------------------------</td>
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<td></td>
</tr>
<tr>
<td>8</td>
<td>Lactococcus lactis</td>
<td>Genetically modified to express mature human IL-10</td>
<td>Chronic intestinal colitis</td>
<td>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., 2006; 2012.</td>
<td></td>
</tr>
<tr>
<td>9</td>
<td>Lactococcus lactis</td>
<td>Genetically modified to express human Trefoil-factor 1 (hTFF1)</td>
<td>Epithelial damage cause by chemotherapy or radiation in cancer patients.</td>
<td>Formulated as mouthwash, hTFF1 efficiently reduced the severity and course of radiation induced oral mucositis in hamster models Caluwaerts et al., 2010</td>
<td></td>
</tr>
<tr>
<td>10</td>
<td>Lactobacillus casei</td>
<td>Genetically modified to express interleukin-10 (IL-10)</td>
<td>Chronic intestinal colitis and related inflammation.</td>
<td>Combined with 5-aminosalicylic acid (5-ASA), 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 Qiu et al., 2013</td>
<td></td>
</tr>
<tr>
<td>11</td>
<td>Lactococcus lactis</td>
<td>Genetically modified to secrete murine IL-10</td>
<td>Food induced systemic anaphylaxis</td>
<td>Diminished anaphylaxis and inhibited antigen specific IgE and IgG1 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</td>
<td></td>
</tr>
<tr>
<td>12</td>
<td>Lactobacillus gasseri</td>
<td>Genetically modified to express SOD</td>
<td>Intestinal colitis</td>
<td>Significantly attenuated inflammation and infiltration of neutrophils and macrophages in IL-10 deficient mice. Carroll et al., 2007</td>
<td></td>
</tr>
<tr>
<td>13</td>
<td>Lactococcus lactis</td>
<td>Genetically modified to secrete anti-mouse TNF-α nanobody (single domain antibody fragment)</td>
<td>Chronic colitis</td>
<td>Reduced intestinal inflammation. Improved colitis in IL-10 -/- mouse model. Could lead to effective and safer management of IBD in humans Rottiers et al., 2013</td>
<td></td>
</tr>
</tbody>
</table>
Chapter 1

1.2 Reactive oxygen species and oxidative stress

Life on earth started in anaerobic environment and progressed towards aerobic. The complexities of organisms increased and they started utilizing oxygen to fulfill their energy needs (Lane, 2002; 2005). With the emergence of oxidative phosphorylation came the negative consequence of oxidative damage. Free radicals, such as Reactive Oxygen Species (ROS), generated as a result of normal metabolism causes oxidation of biomolecules such as proteins and nucleic acids present within the cell. These damages ultimately results in either compromised cellular function or total loss of function (cellular death). To cope up with the harsh situation, evolution of antioxidant molecules became imperative to the existence and homeostasis. Oxygen utilizing organisms evolved with antioxidants either produced by them or scavenged from the environment in the form of food or nutrient.

Antioxidant defense can be divided in to 5 categories:

1) Avoidance (sheltering)
2) Antioxidant enzymes (prevention)
3) Free radical scavenger (containment)
4) Repair mechanisms (Fist aid)
5) Stress response (entrenchment).

Some organisms, particularly those that evade oxygenic environment rely on only couple of these mechanisms. Higher eukaryotic organisms such as mammals (especially humans) have high capacity metabolic processes running within their body. Free radicals generated from oxidative phosphorylation as well as metabolism of dietary products are challenge. Consequently, complex antioxidant defense system comprising of enzymatic (antioxidant enzymes) and non-enzymatic antioxidants (self-generated and dietary) were evolved to effectively control the deleterious effects of ROS.

ROS are a class of free radicals which are produced within a cell that is metabolically functional (Bolisetty et al., 2013). Metabolic activities occurring within the cell are a good source of ROS. Mitochondria, peroxisomes, chloroplasts, phagosome, lysosomes etc. are the main centers of ROS
production within a cell. Molecular oxygen, is a biradical in its ground state, has two unpaired electrons in its outermost shell (hence, it is called the triplet state). These two unpaired electrons have the same spin, hence, oxygen can react with only one electron at a time, so it is relatively a slow reacting molecule. On the other hand, when one of the unpaired electrons is excited then the spin changes for one of them and its reactivity increases. The product is called singlet oxygen and it is known to be a powerful pro-oxidant as the two electrons with the opposite spins have the ability to react with other bonds especially double bonds. Other ROS are superoxide anion, hydrogen peroxide, hydroxyl radical etc. Longevity of the ROS varies from microseconds to seconds to minutes. Chemical generation of free radicals take place through a variety of reactions like Fenton reaction, Haber Weiss reaction which involves Fe$^{+2}$ which is an extremely reactive oxidant. Now, there are separate pathways for ROS production within biological systems, like ageing, reperfusion injury, increased partial pressure of oxygen, chemicals or drugs, radiation or even normal metabolism (Murphy, 2009). The following diagrams represent the basic pathways and reactions for ROS formation.

**Figure 1.9** summarizes different pathways of ROS formation, the lipid peroxidation process and the role of glutathione (GSH) and other antioxidants (Vitamin E, Vitamin C, lipoic acid) in the management of oxidative stress (equations are not balanced) (Valko et al., 2007).
Figure 1.9 Pathways of ROS formation (Valko et al., 2007) **Reaction 1:** The superoxide anion radical is formed by the process of reduction of molecular oxygen mediated by NAD(P)H oxidases and xanthine oxidase or non-enzymatically by redox-reactive compounds such as the semi-ubiquinone compound of the mitochondrial electron transport chain. **Reaction 2:** Superoxide radical is dismutated by the superoxide dismutase (SOD) to hydrogen peroxidase. **Reaction 3:** Hydrogen peroxide is most efficiently scavenged by the enzyme glutathione peroxidase (GPx) which requires GSH as the electron donor. **Reaction 4:** The oxidised glutathione (GSSG) is reduced back to GSH by the enzyme glutathione reductase (G-red) which uses NADPH as the electron donor. **Reaction 5:** Some transition metals (e.g. Fe2+, Cu+ and others) can breakdown hydrogen peroxide to the reactive hydroxyl radical (Fenton reaction). **Reaction 6:** The hydroxyl radical can abstract an electron from
polyunsaturated fatty acid (LH) to give rise to a carbon-centered lipid radical (L•). **Reaction 7:** The lipid radical (L•) can further interact with molecular oxygen to give a lipid peroxy radical (LOO•). If the resulting lipid peroxy radical LOO• is not reduced by antioxidants, the lipid peroxidation process occurs (reactions 18–23 and 15–17). **Reaction 8:** The lipid peroxy radical (LOO•) is reduced within the membrane by the reduced form of Vitamin E (T-OH) resulting in the formation of a lipid hydroperoxide and a radical of Vitamin E (T-O•). **Reaction 9:** The regeneration of Vitamin E by Vitamin C: the Vitamin E radical (T-O•) is reduced back to Vitamin E (T-OH) by ascorbic acid (the physiological form of ascorbate is ascorbate monoanion, AscH−) leaving behind the ascorbyl radical (Asc•−). **Reaction 10:** The regeneration of Vitamin E by GSH: the oxidised Vitamin E radical (T-O•) is reduced by GSH. **Reaction 11:** The oxidised glutathione (GSSG) and the ascorbyl radical (Asc•−) are reduced back to GSH and ascorbate monoanion, AscH−, respectively, by the dihydrolipoic acid (DHLA) which is itself converted to lipoic acid (ALA). **Reaction 12:** The regeneration of DHLA from ALA using NADPH. **Reaction 13:** Lipid hydroperoxides are reduced to alcohols and dioxygen by GPx using GSH as the electron donor. Lipid peroxidation process: **Reaction 14:** Lipid hydroperoxides can react fast with Fe2+ to form lipid alkoxyl radicals (LO•), or much slower with Fe3+ to form lipid peroxy radicals (LOO•). **Reaction 15:** Lipid alkoxyl radical (LO•) derived for example from arachidonic acid undergoes cyclisation reaction to form a six-membered ring hydroperoxide. **Reaction 16:** Six-membered ring hydroperoxide undergoes further reactions (involving scission) to from 4-hydroxy-nonenal. Reaction 17: 4-hydroxynonenal is rendered into an innocuous glutathyl adduct (GST, glutathione S-transferase). **Reaction 18:** A peroxy radical located in the internal position of the fatty acid can react by cyclisation to produce a cyclic peroxide adjacent to a carbon-centred radical. **Reaction 19:** This radical can then either be reduced to form a hydroperoxide (reaction not shown) or it can undergo a second cyclisation to form a bicyclic peroxide which after coupling to dioxygen and reduction yields a molecule structurally analogous to the endoperoxide. **Reaction 20:** Formed compound is an intermediate product for the production of malondialdehyde. Reactions 21, 22, 23: Malondialdehyde can react with DNA bases Cytosine, Adenine, and Guanine to form adducts M1C, M1A and M1G, respectively.

**ROS induced oxidative damage**

**Damage to DNA**

ROS is known to cause DNA damage to a great extent. At high concentrations, the purine and pyrimidine bases get damaged and along with that ROS can also inflict damage upon deoxyribose backbone of DNA. The highly reactive radical, hydroxyl radical reacts with the double bonds of DNA bases via addition reactions and via abstraction of a hydrogen atom from the
methyl group of thymine along with each of the C-H bonds of 2-deoxyribose (Valko et al., 2006). The addition reaction occurs at a rate that is near to the diffusion controlled rates that include rates of 3-10x 10⁹ M⁻¹s⁻¹, while the rates for the reactions involving hydrogen abstraction is around 2x10⁹ M⁻¹s⁻¹. The DNA oxidation products include 8-hydroxy guanine, thymidine glycol, 5-hydroxymethyl uracil etc. while the oxidation of deoxyribose results in the production of apurinic or apyrimidinic sites or aldehyde products or even breakage of strands. The addition reactions involving C5-C6 double bonds of pyrimidine results in the formation of C5-OH and C6-OH adduct radicals while the hydrogen abstraction reaction that involves thymine shall result in the formation of allyl radicals. The resulting radicals that are formed during the course of the reactions involving ROS, are different in terms of their redox potentials, hence, some are oxidizing (C5-OH) while some are reducing (C6-OH) (Evans et al., 2004). All these processes result in mutagenesis, carcinogenesis and even ageing. The DNA adducts, strand breakage, apurinic and apyrimidinic site creation results in the depletion of the energy reserves (like PARP), imbalanced induction of DNA repair enzymes and also induction of error prone polymerases (Cooke et al., 2003). The 8-OH-dG formed in situ can cause a transition of G→T, while 8-OH-dGTP may get incorporated rather misincorporated opposite a dA which results in an A→C conversion (Graziewicz et al., 2002). Increased damage in cells has been implicated to increased ROS levels and lower antioxidant levels in tumors. Multiple studies established a direct correlation between increased ROS damage and cancer. Some tumour cell lines have the ability to create high levels of H₂O₂, without any external stimulation. This, perhaps, accounts for the high amount of DNA damage. But, there have been increasing evidences which suggest that elevated levels of ROS production and consequently high amount of ROS damage are not pathologically related to carcinogenesis. There are a number of pathological conditions where ROS damage and carcinogenesis are not happening simultaneously (Evans et al., 2004). Inflammatory diseases are caused due to ROS damage but do not induce carcinoma. This supported by the fact that many inflammatory diseases like arthritis, systemic lupus erythematosus, vasculitis or Behcet’s Disease have
elevated levels of 8-OH-dG. Also, the lymphocytes that have been isolated from the patients suffering from these diseases were found to be extra sensitive to the cytotoxic levels of hydrogen peroxide (Bashir et al., 1993). **Figure 1.10** represents the different methods by which ROS inflict damage upon DNA.

**Damage to proteins**

Damaged proteins are ubiquitinated and eliminated by degradative pathways involving 26S proteasomes. The ROS produced within a cell oxidation of amino acids (**Table 1.4**). Protein carbonylation is promoted by ROS (Suzuki et al., 2010). It is a process which forms reactive ketones and aldehydes which can be reacted to 2,4-dinitrophenylhydrazine (DNPH) to form hydrazones. Direct oxidation of side chains of amino acids like lysine, arginine, proline and threonine in “primary protein carbonylation” reaction produces products which are detectable with DNPH. Chain reaction initiated by oxidative decomposition of PUFA forms a variety of carbonyl compounds (3 to 9 carbon in length). The most reactive and cytotoxic of them are α, β unsaturated aldehydes (4-hydroxy-trans-2-nonenal and acrolein), di-aldehydes (malondialdehyde and glyoxal), and keto-aldehydes (4-oxo-trans-2-nonenal).

A basic characteristic of cytosolic and the secretory proteins is that the former has to maintain its cysteines in the reduced form while the latter with disulfide linkages (Aslund et al., 1999). Hence, unwanted generation of disulfide linkages in the cytosolic environment due to the creation of oxidative stress may lead to problems pertaining to protein folding. This has been referred to as the “disulfide stress” and such a situation has proven to be deleterious for the cellular survival. Many proteins have been modified by both enzymatic and non-enzymatic metal catalyzed oxidation (MCO) in vivo and in vitro (Cabisco et al., 2010). The protein modifications due to oxidative stress lead to modification in amino acid side chains which leads to thermal instability, loss of catalytic activity, generation of protein-protein cross links,
protein fragmentation, formation of protein adducts, increased susceptibility towards proteolysis etc.

Table 1.4 ROS mediated production of Oxidized products of Amino acids (Suzuki et al., 2010).

<table>
<thead>
<tr>
<th>Amino Acids</th>
<th>Oxidized Products</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cysteine</td>
<td>Disulfides, Cysteic Acid</td>
</tr>
<tr>
<td>Arginine</td>
<td>Glutamic semialdehyde</td>
</tr>
<tr>
<td>Tyrosine</td>
<td>3,4-Dihydroxyphenylalanine, Tyr-Tyr cross-linkages</td>
</tr>
<tr>
<td>Tryptophan</td>
<td>Kynurenine, Nitrotryptophan</td>
</tr>
<tr>
<td>Histidine</td>
<td>2-oxohistidine, Asparagine</td>
</tr>
</tbody>
</table>

**Damage to lipids**

Lipids constitute an integral part of the biological world. They are in the biological membranes; they are one of the most potent sources of energy. The double bonds of polyunsaturated fatty acids (PUFA) are highly susceptible for damage mediated by ROS. Lipid peroxidation has increased drastically with the increase in oxidative stress (Halliwell et al., 1993). PUFA undergoes lipid peroxidation and generates highly reactive radical species called lipoperoxyl radical (LOO.). This lipoperoxyl radical in turn reacts with lipids and results in the formation of lipid radical and lipid hydroperoxide (LOOH). This lipid hydroperoxide is unstable and forms new peroxyl and alkoxy radicals, thereby generating secondary products which induce more lipid peroxidation (Spiteller et al., 2001).

The free radicals generated during the process of lipid peroxidation, tend to have local effects, mainly due to their short half-life and extremely reactive nature. But, the formation of lipid hydroperoxides tends to act as “Secondary Messengers” of lipid peroxidation, as they can diffuse away from
their site of generation, accompanied by the fact that they have along half-life, 
they can induce a lot more damage than their free radical counterparts 
(Barrerra et al., 2008). The products that are generally formed after the 
breakdown of these lipid hydroperoxides are aldehydes - malondialdehyde, 4-
hydroxynonenal (HNE), hexanal, acrolein, etc. Lipid peroxidation alters 
membrane fluidity and permeability leading to the loss of cellular integrity. 
HNE is a highly electrophilic compound, and it can easily react with 
glutathione, proteins, and at very high concentrations, even with DNA 
(Esterbauer et al., 1993). The aldehyde group, the C=C double bond and the 
hydroxyl group are the primary reacting participants. They can induce 
modifications of the macromolecules and thereby assert alteration of the 
biological functions. HNE has the ability to form protein adducts through 
Schiff's base formation or even Michael adduct formation. This conjugation 
with proteins occurs via lysyl, cysteinyl or even histidyl residues; glutathione 
also becomes the victim by forming conjugates with HNE, which are known as 
GS-HNE and their formation is characterized by the enzyme glutathione-S-
transferase (Forman et al., 2008). Lipid peroxidation products, such as 
malondialdehyde and HNE, are harmful for the cell which is known to induce 
cancer (Zanetti et al., 2003).

Figure 1.10 Targets of free radicals (Carocho et al., 2013)
Antioxidants

Exposure to ROS has led the organisms to develop antioxidant defense systems. Enzymatic antioxidants includes superoxide dismutase (SOD), catalase (CAT) and Glutathione peroxidase (GPx), whereas glutathione (GSH), ascorbic acid (vitamin C), α tocopherol (vitamin E), flavonoids, carotenoids and other antioxidant molecules represents non-enzymatic antioxidant group (Carocho et al., 2013). The balance between intracellular levels and activities of these antioxidants is very crucial for cell functioning.

Glutathione is the major soluble antioxidant molecule of the cell. It is highly abundant in cytosol (1-11 mM) and mitochondria (5-15 mM) (Masella et al., 2009). It is synthesized in cytosol and transported to mitochondria by carrier proteins. GSH is taken up in mitochondria from the cytosol against its concentration gradient. GSH in the nucleus maintains redox states of several proteins involved in DNA repair and gene expression. Oxidized glutathione (GSSG) accumulates in the cell and GSH/GSSG ratio reflects oxidative stress (Sen and Packer, 2000). Too high concentration of GSSG damages many proteins by oxidizing them.

Major protective roles of glutathione are enlisted below.

- Cofactor for several ROS detoxifying enzymes (GPx, glutathione transferase etc.).
- Participates in amino acid transport through plasma membrane
- Scavenges hydroxyl radical and singlet oxygen directly.
- Detoxify hydrogen peroxides and lipid peroxides using catalytic activity of glutathione peroxidase.
- Regenerate other antioxidants such vitamin C and vitamin E.

Variety of enzymatic antioxidants such as SOD, CAT etc. reduces oxidative stress majorly by enzymatic conversion of free radicals in to other. Redox state is generally reflected by paired species of GSH (oxidized and reduced) and TRX (oxidized and reduced) (Kohen and Abraham, 2002). However, glutathione (GSH/GSSG couple) represents major cellular buffer. Under normal conditions, the redox state of the cell is kept within a narrow
range just like pH. Redox state change of 30 mV represents 10 fold changes in the ratio of oxidants and reductants. Glutathione reductase and TRX reductase maintains high ratios of reduced to oxidized GSH and TRX in the cell, respectively. Apart from antioxidant functioning, GSH and TRX are also involved in signal transduction process. Figure 1.11 summarizes all major natural antioxidants and their classification.

Figure 1.11 Natural antioxidants and their classification (modified from Carocho et al., 2013). Blue words represent exogenous antioxidants, while blood red ones represent endogenous antioxidants.

Various low molecular weight molecules also act as antioxidants such as free amino acids, peptides, proteins and polyphenols. Several synthetic food antioxidants have also been used as described by Carocho et al., 2013 (Table 1.5).
Table 1.5: Chemical structure and application of common synthetic antioxidants

<table>
<thead>
<tr>
<th>Compound name</th>
<th>Structure</th>
<th>Application</th>
</tr>
</thead>
<tbody>
<tr>
<td>BHA (butylated hydroxyanisole)</td>
<td><img src="image" alt="Structure" /></td>
<td>Food antioxidant</td>
</tr>
<tr>
<td>BHT (butylated hydroxytoluene)</td>
<td><img src="image" alt="Structure" /></td>
<td>Food antioxidants</td>
</tr>
<tr>
<td>TBHQ (tert-butylhydroquinone)</td>
<td><img src="image" alt="Structure" /></td>
<td>Animal processed food antioxidant</td>
</tr>
<tr>
<td>PG (propyl gallate)</td>
<td><img src="image" alt="Structure" /></td>
<td>Food antioxidant</td>
</tr>
<tr>
<td>OG (octyl gallate)</td>
<td><img src="image" alt="Structure" /></td>
<td>Food and cosmetic antioxidant. Antifungal property</td>
</tr>
<tr>
<td>2,4,5-Trihydroxy butyrophenone</td>
<td><img src="image" alt="Structure" /></td>
<td>Food antioxidant</td>
</tr>
<tr>
<td>NDGA (nordihydroguaiaretic acid)</td>
<td><img src="image" alt="Structure" /></td>
<td>Food antioxidant</td>
</tr>
<tr>
<td>4-Hexylresorcinol</td>
<td><img src="image" alt="Structure" /></td>
<td>Prevention of food browning</td>
</tr>
</tbody>
</table>
Physiological functions of ROS in mammals

Free radicals play important physiological functions like control of erythropoietin production, control of ventilation and other hypoxia inducible functions, signal transduction from various receptors and even enhancement of immunological functions (Droge, 2002). The general response of a cell in stress is to leave cellular division and enter G0 phase. Continuous exposure of ROS may trigger apoptosis. In response to oxidative stress or pro-oxidants, p21 gets activated and that results in blocking of cell cycle progression. Similar to p21, p27 also can arrest cells in G1 phase (Gartel and Radhakrishnan, 2005). The cells may also get arrested in the S-phase due to the dephosphorylation of retinoblastoma (RB) in response to long term exposure to oxidative stress or as a result of activation of p53 and p21 induced by oxidative stress (Burhans et al., 2009). Foxo transcription factors are also responsible for the expression of p27, thereby playing a role in apoptosis as well as cell cycle. Cells that are not involved in cell division, like neurons, have mechanisms to cope up with the oxidative stress, which involve Foxo, as it induces the transcription of manganese, based SOD. Figure 1.12 summarizes major roles of ROS in mammals.

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**Figure 1.12** Sources and cellular response of ROS (Finkel and Holbrook, 2000)
1.3 Ethanol and associated metabolic disorders in humans

Frequent associations of human chronic alcoholism and diverse hepatic lesions, grouped under Alcoholic liver disease (ALD) have been known since 19th century (Koch et al., 2004). Malnutrition and hepatotoxic effect of ethanol are the main contributors of liver disease in alcoholics (Lieber, 2004). Ethanol's hepatotoxicity is linked to its metabolism by two different pathways namely, alcohol dehydrogenase (ADH) and Cytochrome P450 2E1 (CYP2E1) which results in production of toxic acetaldehyde. Acetaldehyde is the major culprit of ethanol associated toxicity while ethanol by itself is toxic at very high concentrations. After intake, ethanol is completely absorbed by GI tract (20% by stomach and 80% by small intestine) (Figure 1.13). Ethanol, being highly soluble in water, gets mixed with the aqueous phase in the entire body. The percentage ethanol in the body depends upon the water content of the fluid.

![Figure 1.13 Absorption and distribution of ethanol in humans.](image-url)

Approximately 20% of ingested ethanol is absorbed by stomach, rest by small intestine. Ethanol present in blood after absorption is distributed to all major organs including Liver, large intestine (esp. colon), Kidney, brain etc. Colonic microbiota metabolizes ethanol it in to acetaldehyde and further
acetate. Ethanol along with acetaldehyde causes gut barrier disruption and as a result luminal antigens, endotoxins are diffused into the blood and causes inflammatory response and ROS in major organs such as blood and liver. Moreover, liver being major metabolizing organ of ethanol, produces massive amount of acetaldehyde resulting in further oxidative damage of macromolecules.

**Metabolism of ethanol in mammals**

Ethanol is majorly metabolized in liver and the metabolic product acetaldehyde is converted into acetate which serves as energy source. However, ethanol metabolism is also associated with enormous amount of ROS production and consequently oxidative damage to macromolecules. In humans and as well as other mammals such as rodents, ethanol is metabolized by 2 major systems (Lieber, 1999) namely Alcohol dehydrogenase (ADH) and Microsomal Ethanol Oxidizing System (MEOS). ADH is present in cytosolic compartment of the cells. In humans ADH 1B catalyzes the formation of acetaldehyde using NAD⁺ as cofactor. But adhB enzyme alone does not explain well the phenomenon of ethanol tolerance and various associated physiology developed in alcoholics. These were then explained with emergence of knowledge that MEOS plays important role in ethanol metabolism. Microsomal system in general comprises gluconeogenesis using ketones, fatty acid metabolism and detoxification of several Xenobiotics such as ethanol (**Figure 1.14 and Figure 1.15**).

![Figure 1.14](image_url) **Figure 1.14** Metabolism of ethanol in mammals. Ethanol is metabolizes in to acetate in two step reaction as depicted above. The acetate then enters in to central metabolic pathway for generation of energy and lipids.
MEOS is a general term collectively used for those enzymes which catalyzes oxidation of ethanol on Smooth Endoplasmic Reticulum (SER). This includes:

1. Ethanol specific cytochrome P450 called CYP2E1.
2. Catalase present in SER coupled with Xanthine oxidase and NADPH oxidase.

Various isozymes of CYP2E1 have been characterized in humans as well as rodents. They differ in induction level and activity, which is responsible for differential ethanol tolerance (Kunitoh et al., 1993). In addition to CYP2E1, ethanol can also be oxidized by liver microsomes through hydroxyl radicals (OH\(^\circ\)), including those originating from iron catalyzed H\(_2\)O\(_2\) degradation (Rashbastep et al., 1993).

**Figure 1.15** Reactions involved in ethanol oxidation (Lieber, 1999). (1) alcohol dehydrogenase (ADH) and nicotinamide adenine dinucleotide (NAD); (2) the hepatic microsomal ethanol oxidizing system [(MEOS), which involves cytochrome P450 2E1 and the reduced form of nicotinamide adenine dinucleotide phosphate (NADPH)]; (3) a combination of NADPH oxidase and catalase; and (4) xanthine oxidase and catalase.
Ethanol, a naturally occurring carbon source, is considered to provide health benefits if consumed in small doses (Lusting, 2010). But it is largely recognized as neurotoxin in acute large quantity and hepatotoxic in chronically in large quantities. Although, few studies have associated moderate ethanol consumption with increased insulin sensitivity and reduced cardiovascular risk, other cross-sectional and prospective studies link dose dependent chronic consumption of large quantities of ethanol with insulin resistance and metabolic syndrome.

Metabolism of ethanol majorly occurs in liver, but other peripheral organs such as kidney, muscle and GIT also metabolizes to certain extent (Lusting, 2010). On oral consumption of 120 Kcal of ethanol, 10% is metabolized by stomach and intestine during first pass and another 10% by kidney and muscle. Thus, 96 Kcal reaches liver, which is 4 times more than glucose. Ethanol does not stimulate insulin secretion and enters in hepatocytes through osmosis. In the liver cells, it is metabolized by alcohol dehydrogenase (ADH) in to acetaldehyde, which is very strong pro-oxidant. Acetaldehyde causes intensive cellular damage if not quenched by antioxidants (Vitamin C, GSH etc). Acetaldehyde is then metabolized to acetate by acetaldehyde dehydrogenase 2. Acetate thus formed in then converted to acetyl-CoA by acyl-CoA synthetase. And, acetyl-CoA enters in to mitochondrial TCA cycle, or in presence of other energy sources it is most likely diverted to fatty acid synthesis through De Novo lipogenesis (DNL). Furthermore, acetaldehyde also stimulates SREBP-1c, which is responsible for activating enzymes for DNL. The absolute rate of DNL (metabolized to VLDL) of ethanol relatively small, however, it increases from 1% baseline to 31% after an ethanol bolus. Thus, liver is primed to convert ethanol to lipids. Malonyl-CoA, formed in excess as intermediate in the process of DNL, is a steric inhibitor of the mitochondrial enzyme carnitine palmitoyl transferase-1 (CPT-1) which regulates mitochondrial β-oxidation. It is regenerated for trans-esterification and transport of fatty acids in to the mitochondrial matrix to generate 2 carbon products for ketone formation. Furthermore, ethanol inhibits peroxisome proliferator activated receptor-α (PPAR-α) and adenosine monophosphate activated protein kinase (AMP activated protein kinase), and
thus blocks fatty acid β-oxidation. This inhibition leads to decreased phosphorylation and resulting increased activity of acetyl-CoA carboxylase, increased levels of Malonyl-CoA and decreased activity of CPT-1. Thus, increase in DNL as a consequence of ethanol consumption, blocks intrahepatic β-oxidation leading to further lipid buildup. The synthesized lipids in the liver take their route to the blood in the form of VLDL. Hepatic VLDL synthesis depends upon microsomal triglyceride transfer protein (MTP) for correct apoB100 protein folding before exporting. Reduction in PPAR-α by ethanol down regulates MTP activity and thus reduced rate of clearance of VLDL in the plasma. This suppression of VLDL production and dysfunctioning lipid export machinery contributes to hypertriglyceridemia. The accumulation of hepatic diacylglycerol (DAG) and triglycerides (TG), and resultant activation of c-jun N-terminal Kinase 1 (JNK-1) results in hepatic insulin resistance (Figure 1.16).
Chapter 1

Figure 1.16: Hepatic ethanol metabolism (Lustig, 2010). Of an ingested load, 80% reaches the liver. Ethanol induces: de novo lipogenesis and dyslipidemia; c-jun N-terminal kinase (JNK-1) activation, which serine phosphorylates hepatic insulin receptor substrate-1 (IRS-1), rendering it inactive, and contributing to hepatic insulin resistance, which promotes hyperinsulinemia and influences substrate deposition into fat; hepatic lipid droplet formation, leading to steatosis; and stimulation of the reward pathway, promoting continuous consumption. ACC acetyl CoA carboxylase. ACL adenosine triphosphate citrate lyase. CPT-1 carnitine palmitoyl transferase-1. FAS fatty acid synthase. FFA free fatty acids. IR insulin resistance. IRS-1 insulin receptor substrate-1. JNK-1 c-jun N-terminal kinase 1. LPL lipoprotein lipase. MTP microsomal transfer protein. PKCε protein kinase C-ε. SREBP-1c sterol regulatory element binding protein-1c. TCA tricarboxylic acid. TG triglyceride.

Effect of ethanol and acetaldehyde on human physiology

Pathogenesis of alcoholic liver disease

Ethanol and malnutrition: Ethanol, unlike other drugs, has enormous amount of energy. It gives 7.2 Kcal (29.7KJ) of energy per gram, which is higher than other carbohydrates and proteins (Lieber, 2004). Ethanol displaces normal nutrients in alcoholics and accounts for on an average half of the calorie intake and thus causing malnutrition including folate deficiency, thiamine deficiency, and deficiency of other vitamins (Figure 1.17).

Interaction of ethanol with malnutrition. Malnutrition may be primary or secondary. Secondary malnutrition may be caused by either (1) maldigestion or malabsorption or (2) impaired utilization due to decreased activation or increased degradation of nutrients. Both direct toxicity of ethanol and malnutrition (primary and secondary) may affect function and structure of liver and GI tract (Lieber 2004).

Hepatotoxic effect of ethanol metabolism: Oxidation of ethanol through ADH pathway producing acetaldehyde utilizes NAD$^+$ and results in formation of excess NADH in due course of time. Excess NADH accumulation
causes several metabolic disorders including inhibition of Kreb’s cycle and fatty acid oxidation (Dicker and Cederbaum, 1992; Leiber, 1994). Acetaldehyde produced from ethanol is very toxic and has numerous deleterious effects on macromolecules of cells (Figure 1.18).

Figure 1.18 Schematic representation of various deleterious effects of ethanol and acetaldehyde.

1.4 Ageing and age associated deleterious changes

Ageing is an important phenomenon which has gained popularity in the biological world. Ageing is deteriorative changes with time during post maturational life that underlie an increasing vulnerability to challenges, thereby decreasing the ability of the organism to survive (Masoro, 2006). It is a degrading mechanism that is accompanied by the functional decline of various cellular processes. Thought details of degradation have not been clearly elucidated; the general view of ageing is a process involving free radical generation which accompanies oxidative metabolism. In recent years, many studies have strengthened the free radical theory of ageing.

Harmann (1992), another pioneer in the field of gerontology, had described ageing as the progressive “accumulation of deleterious changes in
the cells and tissues with the advancement of age, which increases the risk of disease and death.” Thus, ageing has been implicated to be accompanied by progressive decline in resistance to diseases and diminished efficiency of biological functions with time. But, even with well-developed scientific machinery and knowledge, ageing is one of the poorest understood mechanisms in the biological world. The interactively complex nature of ageing makes it really complicated to elucidate the inherent processes that underlie in the mechanisms of ageing. Also, it is difficult to differentiate between the consequences of normal ageing and the age related diseases. Hence, a variety of theories have been proposed by different scientists that defines ageing.

Theories of aging

As already mentioned, number theories have tried to answer the question- “What is ageing?” A few of such theories have been described in the subsequent paragraphs.

Free Radical Theory of Ageing (FRTA)

Harmann (1992) had proposed that oxidative stress was the one of the most potential candidates responsible for ageing. ROS generation, their functions and the oxidative stress that has been induced by ROS have already been mentioned in the section 2. He had postulated that the reactions pertaining to the generation of free radicals could be the source of the increasing macromolecular disorganization that underlies the process of ageing. Many researchers observed the free radical basis for ageing in terms of elevated levels of oxidative damage. For example, 2-fold increase in \( \text{H}_2\text{O}_2 \) levels were seen in ageing house fly (Sohal et al., 2002); 23% rise in \( \text{H}_2\text{O}_2 \) production was seen in rats as they grew old (Hagen et al., 1997; Sastre et al., 1996). Overexpression of mice catalase activity showed that the mutants showed 17-21% increase in lifespan with respect to wild type mice (Schriner et al., 2005). However, dietary restriction with a potent antioxidant failed to increase lifespan (Howes et al., 2006). Overexpression of SOD1, SOD2 and catalase have been found to increase lifespan in *Drosophila* but failed to
replicate the same results in mice (Huang et al., 2000; Sun et al., 2002; Orr et al., 2003).

**Vicious Cycle Theory of Ageing**

This theory came in to the limelight just after the FRTA and was in total accordance with the data that were being published at that time (Hiona and Leeuwenburgh, 2008). The fact that the production of the superoxide rises with age, and induces oxidative stress that is responsible for damage of macromolecules belonging to cells and cellular organelles alike (Pinz et al., 1995). The ROS induced mutation of mitochondrial DNA along with inappropriate replication of the same which further results in the generation of more and more ROS that results in a vicious cycle which finally leads to the generation of more and more ROS and these fresh ROS causes more oxidative damage. Many studies have demonstrated that increase in mitochondrial ROS leads to increased oxidative stress in age animals (Sanz et al., 2006; Hiona and Leeuwenburgh, 2008). However, some of the studies do not fit with vicious cycle theory in which H$_2$O$_2$ generation leads to more and more ROS with age.

**Mitochondrial Mutational Theory**

The vicious cycle theory paved the way for Miquel and his co-workers to promulgate the Mitochondrial Mutational theory. As described above, the mitochondrial DNA is highly susceptible to ROS induced oxidative damage which is attributed to lack of excision and recombination repair mechanisms for mitochondrial DNA (Yakes et al., 1997; Miquel, 1998). The new aspect of this theory invokes linking of senescence to the mutations in the mitochondrial DNA (mt DNA) resulting in the accumulation of less functional respiratory proteins which in turn leads to the generation of free radicals and completing the cycle by causing more mitochondrial mutations. This means that loss of physiological performance may also lead to the pathogenesis of many age-related diseases. Recently, Kowlad (2014) using mathematical modeling stated that mutated mitochondria (having shorter DNA than wild type) may have replicative advantage over wild type and in due course of time replaces all the healthy mitochondria of the cell. And thus leads to accumulation in
mitochondrial DNA. However, the time frame for the accumulation of the mutant mt-DNAs is only compatible to aging processes in long lived species such as humans. This theory could not rationally explain aging in short lived animals such as rats and mice. Mitochondrial deletions have been found to be present in senescent liver and brain cells (Gadaletta et al., 1992). Role of mt-DNA mutations in mammalian disease and aging has been well reviewed by Lagouge and Larsson (2013).

**Inflammatory theory of Ageing**

The age related oxidative stress causes the activation of various redox-sensitive transcriptional factors that cause up-regulation of genes involved in pro-inflammatory responses (Chung et al., 2006; Miquel, 2009). The generation of various pro-inflammatory molecules leads to inflammation in various tissues and organs. This inflammatory cascade gets exaggerated during ageing and this has been linked to various age related diseases like cancer, neurodegenerative diseases (Parkinson’s, Alzheimer’s) and even cardiovascular diseases and arthritis (El Assar et al., 2013).

Thus, all theories of ageing directly or indirectly involve the concept of ROS induced oxidative stress which increases with age.

**Metabolism in ageing**

Changes in energy metabolism occur in during natural aging, which contributes common phenomenon called senescence. Nicotinamide adenine dinucleotide group of coenzymes plays very crucial role in regulating cellular metabolism and energy balance. NAD+/NADH ratio is very important component of redox state of cell, which reflects metabolic activity and health of the cell (Ying et al., 2006). Numerous evidences have demonstrated that NAD+ and NADH are mediators of several biological processes such as aging. NAD+ and NADH exerts their biological effects by regulating several NAD+/NADH dependent enzymes, including dehydrogenases (e.g. Glyceraldehyde 3 phosphate dehydrogenase and pyruvate dehydrogenase, etc.), poly (ADP-ribose) polymerases, sirtuins, mono (ADP-ribosyl) transferases, ADP-ribosyl cyclases. In healthy mammalian tissue, free NAD+/NADH ratio is around 700. However, ratio of total NAD+/NADH is much lower ranging from 0.05 to 4. In
contrast, NADP+/NADPH ratio is normally 0.005. Thus, NADPH is the dominant form of this coenzyme. These different ratios are the key to different roles of NADH and NADPH (Figure 18 and 19).

**Figure 1.9.** Diagrammatic representation of the metabolism and biological activities of NAD+ (modified from Ying et al., 2006). NAD+ can be generated from the salvage pathway or the de novo pathway, which are mediated by nicotinamide mononucleotide adenylyltransferases (NMNATs). NAD+ produces a number of biological effects through multiple NAD+-dependent enzymes, including NAD+/NADH-dependent dehydrogenases, poly(ADP-ribose) pyimerases (PARPs), sirtuins, mono(ADP-ribose)yltransferases (ARTs), ADP-ribosyl cyclases, and NAD+ kinase. The biological activities in open rectangles are the major NAD+-mediated activities. Abbreviations used: Kyn: Kynurenine; Qa: Quinolinic acid; Nam: Nicotinamide; NA: Nicotinic acid; PARG: Poly(ADP-ribose) glycohydrolase; cADPR: Cyclic ADP-ribose; RyR: Ryanodine receptors.
Figure 1.20. Diagrammatic representation of the metabolism and biological activities of NADH (modified from Ying et al., 2006). NADH is converted from NAD+ via the dehydrogenase-mediated reactions, or generated through a de novo pathway via the action of nicotinamide mononucleotide adenyl transferases (NMNATs). The biological activities in open rectangles are the major NADH-mediated activities. Abbreviations used: NMNH: Reduced form of nicotinamide mononucleotide; RyR: Ryanodine receptors; VDAC: Voltage dependent anion channels; ROS: Reactive oxygen species; XO: Xanthine oxidase; XD: Xanthine dehydrogenase.

Cumulative evidences suggest that NAD+ and NADH are important factors in aging process by regulating PARP-1, sirtuins, tankyrases and oxidative stress (Ying et al., 2005; 2006; 2008). Sir-2 is the key enzyme maintaining life span in invertebrates (Yeast and C. elegans). Decrease or increase in Sir-2 copy results in reduced and extended life span respectively. Calorie restriction modulates Sir-2 levels and increase life span in yeast by decreasing NADH levels. SIRT-6, a human homolog of Sir-2, deficiency leads to aging like symptoms and genetically instability in rodents. NAD+ dependent tankyrases are mediators of telomerase activity and hence NAD+ could also affect aging process through tankyrases activity. There is strong correlation between PARP-1 activity and longevity as a resultant of greater PARP-1 mediated DNA repair capacity (Figure 1.21).
Significant decline in NAD\(^+\)/NADH ratios and intracellular NAD\(^+\) was found in ageing cells (Braidy et al., 2011). Increased activation of PARP-1 in ageing cells causes depletion of NAD\(^+\) levels. Reduced levels of NAD\(^+\) negatively affects the activity of sirtuins (Sirt-1) which can deacetylate tumor suppressor protein p53 (Smith, 2002). Thus, oxidative stress increases in vertebrates with age due to lowered cellular NAD\(^+\) / NADH ratio and NAD\(^+\) levels, which is in consistence with free radical theory of aging. It is also speculated that therapies aimed to maintain high NAD\(^+\) levels may alleviate age associated disorders (Braidy et al., 2011).

**Figure 1.21** Schematic diagram showing association between oxidative stress, PARP mediated and decline in NAD\(^+\)/NADH ratio and NAD\(^+\) dependent functions with aging. (Braidy et al., 2011). Oxidative stress to DNA activates PARP leading to poly(ADP-ribosylation) of proteins in a reaction which consumes NAD+. Depletion of cellular NAD+ stores attenuates the activity of Sirt1 deacetylase leading to hyperacetylation of p53, and consequently tilting the balance to cell death via an apoptotic mechanism.
Pathological implications of ageing

Ageing is a dynamic phenomenon that has intrigued researchers all around the globe. But the complexities of ageing are such that complete understanding of the process has remained elusive to the finest minds of the world.

Cellular senescence or cell death gets intensified with increase in ageing. The oxidative stress that is generated due to ageing, acts as a major factor for causing cellular senescence. Various observations by many researchers all over the world, has demonstrated that ROS acts at multiple steps of cellular senescence. ROS released from various sources acts either upstream or downstream of p53 activation pathway, thereby promoting more and more cellular senescence or cell death (Liu et al., 2008; Vurusaner et al., 2012). ROS induces DNA damage and telomere shortening which increases exponentially with progressive ageing (Herbig et al., 2006). Presence of senescent cells in mitotic tissues of aged animals have been found which are considered as one of the important factors contributing ageing and age related pathologies (Jeyapalan et al., 2007). The development of ataxia telangiectasia syndrome shows premature ageing, elevated levels of ROS, premature cellular senescence and neurodegenerative diseases, thereby promoting the idea that progressive ageing can induce all these kinds of pathological disorders through some way or the other (Valls et al., 2005).

Recently, researchers are trying to find out whether ROS mediated signaling cascade is responsible for the progressive ageing and age related pathologies.

Mitochondrial ROS is the new advancement in field of ageing related studies. Leaky release of ROS from the mitochondrial ETC, is a major cause of mitochondrial ROS generation and damage of mitochondrial macromolecules. It has been postulated that around 2% of the electron flux through ETC results in the partial reduction of molecular oxygen to produce superoxide (O$_2^-$) (Sohal et al., 2002). But, this theory of a “chemo-electric short circuit” revealed only a partial picture of mitochondrial ROS generation and its relation with ageing. The concept of “proton slip” due to the presence
of uncouplers gained a lot of impetus in defining the ageing procedure. There are several “hotspots” in the ETC which can directly reduce the molecular oxygen to produce superoxide. Complex I (NADH dehydrogenase and Fe-S clusters) and complex III (ubiquinol-cytochrome b Qo site) are the major generators ROS (Lambert and Brand, 2009).

The progressive damage caused due to ROS increases with ageing, due to the increase in cellular senescence and reduction in the antioxidant defense mechanism. The manifestations of damage are seen clinically in various kinds of diseases like cardiovascular diseases (atheroscheloris, myocaridal ischemia etc), neurodegenerative disorders (Parkinson’s disease and Alziemer’s disease) and cancer (Andersen , 2004; Halliwell, 2005; Ziech et al., 2011; Selvaraju et al., 2012).

**Ageing and Neurodegenerative Disorders**

Brain utilizes about 25 % of oxygen in the entire body. It is also one of the most metabolically active organs, hence, it is very much susceptible to the damage induced by free radicals (Popa-Wagner et al., 2013). Metabolism of excitatory amino acids and neurotransmitters generates ROS, and the constant use of oxygen by neural mitochondria results in high superoxide levels. Moreover, the antioxidant defense mechanisms in the neurons are inefficient. SOD is found in neurons while GSH is localized in the astrocytes and very less catalase activity in the neurons. The cerebrospinal fluid, globus pallidus, substantia nigra have high concentrations of free unbound Fe$^{3+}$ which is required for the binding of neurotransmitters to receptors. High levels of superoxide generated by these locations reduces Fe$^{3+}$ to Fe$^{2+}$ (Haber-Weiss reaction) which in turn reacts with H$_2$O$_2$ to form highly reactive hydroxyl radical (Sen et al., 2000; Zecca et al., 2004; Moos and Morgan, 2004).

Presence of high concentrations of free unbound iron has been found in Alzheimer’s disease (AD) (Everett et al., 2014). In Parkinson’s disease (PD), high concentrations of iron accumulate in substantia nigra (Mounsey et
High concentration of iron induces oxidative damage thereby causing damage to the macromolecules. AD is caused due to the formation of amyloid plaques, which are formed as a result of misfolding of amyloid β precursor protein (AβPP), generate free radicals and peptide free radicals as well. All these free radicals cause more damage to neurons (Tamagno et al., 2012).

PD is another age-related disease in which substantia nigra neurons undergo damage due to damaged mitochondria or iron mediated free radical damage. The increased release of H$_2$O$_2$ during dopamine metabolism accompanied by excessive iron concentration causes increase in free radical generation which results in the damage of neurons. Reduced GSH levels in the mitochondria of the dopaminergic neurons and excessive concentrations of malondialdehyde are considered as potent markers of PD (Baillet et al., 2010). Thus, age induced oxidative stress has a major role to play in the etiopathology of these neurodegenerative disorders (Jomova et al., 2010; Maes et al., 2011; Taylor et al., 2013; Santos et al., 2013; Kosenko et al., 2014).

Mitochondria targeting antioxidant MitoQ have been shown to improve healthspan of C. elegans Alzheimer’s model (Fang et al., 2014).

**Ageing and Cardiovascular Disorders**

Atherosclerosis is a disease of the arteries, which involves thickening of innermost parts of the vessels, the intima (Harmann et al., 1981). One of the primary types of thickening consists of fatty, slightly raised, narrow, yellow streaks. These streaks are rich in foam cells, which are distorted cells with high lipid concentration that comes from endogenous smooth muscle cells and macrophages. These fatty streaks are likely precursors of fibrous plaques which have the effect of obstructing the arterial lumen. When an arterial lumen gets totally occluded by these fatty streaks, they can cause myocardial infarction, cerebral ischemia etc. The vascular endothelium undergoes damage due to chemical and mechanical reactions and this results in increased permeability, increased serum LDL concentrations and increased
entry of monocytes (Heinecke, 1997; Mudau et al., 2012). The monocytes get activated to form macrophages leading to increased damage by ROS (Stocker et al., 2004). LDL receptors are present on the macrophages get peroxidized and are taken up by the macrophages via acetyl-LDL receptor or scavenger receptors. Intracellular cholesterol levels increase due to increased uptake of LDL which ultimately converts a macrophage into a foam cell.

Increase in age is independently associated with development of atherosclerosis (Wang et al., 2012). Atherosclerotic plaques show evidence of cellular senescence with reduction in cell proliferation, growth arrest, apoptosis, elevated DNA damage and telomere shortening. There is growing evidence that cellular senescence is not only associated with atherosclerosis but it also promotes atherosclerosis.

**Ageing and gastrointestinal tract**

Ageing causes physiological changes in the intestine affecting the absorption of water and electrolytes (Tran et al., 2013; Ren et al., 2014; Saffrey et al., 2014). Aging is also associated with distortion of intestinal epithelial barrier and changes in integrity of tight junctions. The total number of colonic myenteric neurons decrease with age leading to increase in ganglionic cavities or other abnormalities (Hanani et al., 2004; 2012). The colonic regeneration time in humans is around 72 hours (Sipos et al., 2011). Balance of epithelial proliferation and apoptosis are important for normal epithelial regeneration. Alterations from this regulated epithelial cell kinetics may result in a loss of not only structural but also functional integrity of colon. The imbalance of colonic epithelial renewal may lead to either ulcer or carcinoma development of the colonic mucosa. Aging is found to be associated with altered migration and function of regenerative intestinal stem cell and methylation of genes associated with mucosal healing along with alteration in growth factors (Sipos et al., 2011). As a result delayed mucosal regeneration is observed in aged intestine (Figure 1.22).

Telomeres protect the ends of the chromosomes from end to end fusions, degradation and recombination. Telomere length decreases with age in most human tissues, including colon (Takubo et al., 2010; Sputova et al.,
Ulcerative colitis (UC) and colorectal cancer progression is associated with shorter colonocyte telomeres, chromosomal instability and anaphase bridges. Age-related telomere shortening is also accelerated in UC.

Ageing is associated with a progressive alteration of innate and adaptive immune responses (Rodier et al., 2009; Shaw et al., 2010; 2011; 2013). Adaptive immunity significantly declines via a phenomenon called immunosenescence, whereas innate immunity gets activated, which induces a characteristic pro-inflammatory profile and is called inflamm-ageing (Shaw et al., 2011; Cannizzo et al., 2011). An increased release of IL-17 results in the induction of auto-immune diseases and inflammatory diseases like UC or IBD (inflammatory bowel disease) (Fujino et al., 2003). Trefoil factor-3 (TFF-3) is an important colon protecting factor which gets negatively regulated by altered TLR-2 activity which is an outcome of ageing (Fukata et al., 2009; Sipos et al., 2011). Also, the secretion of inflammatory cytokines such as TNF and IL-1β negatively regulates TFF-3 activity which implicates chronic intestinal inflammation and mucosal injury in the elderly (Cario, 2010).

The influx of the neutrophils and macrophages also can generate ROS via respiratory burst of enzymes. Thus, this significant release of ROS mediated damage can cause mucosal erosions, which over the progression of age can cause gastrointestinal diseases like Crohn’s disease and ulcerative colitis (Pravada, 2005). Altered migration and function of regenerative stem cells, the age-related methylation of mucosal healing-associated genes, together with alterations of growth factor signaling with age, may delay mucosal regeneration (Sipos et al., 2011).
Mitochondria and its involvement in aging

Mitochondrial ROS has been known to cause damage to macromolecules of cells and cellular organelles alike. The mitochondrial transition pore gets activated which leads to exit of molecules to cytosol thereby causing cellular damage (Zorov et al., 2006). Although cellular mechanisms control ROS level, the effectiveness of antioxidant systems decline with age results increase mitochondrial damage and leakage of ROS from mitochondria. Increased mitochondrial ROS may lead to chronic inflammation, abnormal homeostatic mechanisms, and excessive apoptosis. Mitochondrial ROS are implicated in neurodegenerative diseases, cardiovascular diseases, cancer and ageing (Kregel et al., 2007; Lesnefsky, 2006).
Throughout the history numerous societies have used calorie restriction (CR) in diet for healthy living (Masoro, 2000; Sinclair, 2005). CR is the widely established way to extend the lifespan in mammals including humans. Early hypotheses claimed that it is a passive response to low diet intake. In addition to passive response, stress induced response also contribute towards increase in life span and is highly conserved in worms, flies, rodents and humans. Harmonis is defined as beneficial action(s) resulting from response to low intensity stress signals in an organism. The theory states that CR is a mild stress that provokes a survival response in the organism boosting resistance to stress and counteracts the causes of aging. The theory unites previously disparate observations about ROS defenses, apoptosis, metabolic changes, stress resistance, and hormonal changes and currently as most accepted explanation for the effects of CR (Figure 1.24).
Pyrroloquinoline Quinone (PQQ)

Pyrroloquinoline Quinone (PQQ) was identified first by Hauge (Rucker et al., 2009). He had identified it as a cofactor in bacteria. PQQ is a heat stable and water soluble compound that is capable of catalyzing oxidative deamination and a number of redox cycles; it is also called methoxatin. These are some of the chemical properties that make it a novel entity in the biological world. PQQ’s stability renders it capable of carrying out thousands of redox cycles with respect to other classes of quinones that tend to self-polymerize (for example Epicatechin) and self-aggregate (for example tannins). Enzymes, that contain PQQ as cofactors, are sometimes designated as “quinoproteins”. The PQQ associated with these enzymes are synthesized in a separate pathway as compared to the pathways producing the eventual target proteins, hence they can be controlled separately as well. PQQ has been quantified in a variety of eatables; Paz and his co-workers had demonstrated that skimmed milk and eggs tend to have a very high concentration of PQQ (of around 574-16500 ng/ml). In fact, Kumazawa et al. (1995) had quantified the amount of free PQQ in human tissues and blood, the value was found to be around 0.8-5.9 ng/gm or ng/ml.
PQQ synthesis

**Pyrroloquinoline Quinone (PQQ)** biosynthesis involves a number of genes that have been characterized in different bacteria (Choi et al., 2008). Some of the bacteria where the genes for PQQ biosynthesis have been characterized include *Acinetobacter calcoaceticus*, *Pseudomonas sp.*, *Klebsiella pneumonia*, *Methylbacterium extroguens*, etc. PQQ is present in human cells and it has been postulated that either the enteric bacteria produce PQQ (which was later falsified) or human diet gives the desired amounts of PQQ. Every possible food starting from fruits, vegetables, skim milk and eggs contained PQQ which suggested that plants might be able to synthesize PQQ but that notion was also proved wrong. So it was finally determined that the symbiotic bacteria that are present along with the plants are able to synthesize PQQ and it is through them that the plants receive PQQ and thus from the plant diet humans are also receiving PQQ (Kumazawa et al., 1995).

The gene cluster involved in the PQQ biosynthesis pathway in *Klebsiella pneumonia* are arranged in an operon system that include the following genes *pqqABCDEF*, while in case of *Pseudomonas aeruginosa*, there are two different operon systems available, where *pqq* ABCDE is separated from *pqqF* operons. Another way in which PQQ gene cluster is arranged in a
bacterium is that of *pqq*ABC/DE operon where *pqq*C and D genes are fused with each other whereas *pqq*FG genes are found in another operon with three different genes. This peculiarity is observed in *Methylbacterium extorquens*, now *Acinetobacter calcoaceticus* possesses another property; it has a *pqq*ABCDEF cluster but this cluster is devoid of any *pqq*F gene (Figure 1.26) (Choi et al., 2008). Though, PQQ biosynthesis pathway has not yet been fully resolved but, structural and functional characterization of some of the proteins have been performed. Table 1.6 illustrates the known functions and some other characteristics of the proteins relevant for the biosynthetic pathway characterization (Puehringer. et al., 2008).

![Figure 1.26 pqq gene clusters in different bacterial species.](image)

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<th>Table 1.6. Representation of different genes involved in PQQ biosynthesis</th>
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B 300 residues 33 kDa  It has a pI of around 5.7. It has similarity with ribonuclease Z. It possesses a putative binding site near Zn atom. Functions as a transporter of PQQ from cytosol to the periplasm.

C 250 residues 29 kDa  It has a theoretical pI of around 6.9. 58kDa homodimer Acts as an acceptor of PQQ from PqqB and catalyzes the formation of PQQ from PqqA polypeptide

D 90 residues 10kDa  pqqD has a theoretical pI of around 5.8. Function not yet Resolved

E 380 residues 43kDa  It belongs to the family of SAM containing enzyme family. Its theoretical pI value is around 5.7 Basically, it catalyzes the linkage of tyrosyl and glutamyl residues in PQQ.

F 760 residues 84kDa  Theoretical pI of around 6.7. 15-20% sequence homology with insulin degrading enzyme. Metalloendopeptidase that functions to process the Tyr and E from pqqA

Functions of PQQ

PQQ has loads of functions which have been demonstrated to a certain extent in vitro as well as in vivo (Rucker et al., 2009; Misra et al., 2012) (Figure 1.27). PQQ, like many other quinones directly react with the ROS and RNS. This results in reduced oxidation of bio-molecules (like proteins) and the manifestation of such modifications on the cellular health. PQQ acts as an antioxidant in a concentration dependent manner. The concentration, in which PQQ is a pro-oxidant, is around 50 μM while the concentration in which it is an antioxidant, is around 10 μM (Rucker et al., 2009). The electrophilic nature of PQQ enables it to react with a host of compounds like – phenylhydrazines, semicarbazides, sulphite, urea, malononitrile. In vitro cell free system suggests that PQQ scavenges singlet oxygen (Mukai et al., 2011), superoxide anions, and hydroxyl radicals (Urakami et al., 1997).

The PQQ has the ability to cause an increment in mitochondrial activity thereby inhibiting the toxins that tend to harm mitochondria. PQQ blocks the formation of peroxynitrite without scavenging it directly as it prevents neurotoxicity by peroxynitrite generator, SIN-1 but not by peroxynitrite itself.
(Zhang et al., 2002). It also confers neuroprotective effects against 6-hydroxydopamine mediated neurotoxicity; also PQQ has been shown to confer NMDA (N-methyl D-aspartate) redox receptor protection by inducing oxidation of the same thereby preventing neurotoxicity (Hirakawa et al., 2009).

PQQ also increases axon density and reduces lesion size. PQQ has the ability to reduce secondary damage by reducing the iNOS expression which comes after a primary damage to the spinal cord (Scanlon et al., 1997). It can also reduce the effective iNOS mRNA concentration, reduce NO induced inhibition, protect NMDA receptors from NO induced damage and promotes cell proliferation (Sanchez et al., 2000).

PQQ activates ERK-MAPK pathway (extracellular signal related protein kinase which is a member of mitogen activated protein kinases), thereby inducing quick phosphorylation of Rb and c-Jun (Tchaparian et al., 2010). On the other hand it causes down regulation of the levels of growth inhibitory molecules like, p27 and IkB, thereby causing the promotion of the cellular proliferation. It gets involved in Ras signaling pathway as well, which subsequently promotes the transition of cell division from G0/G1 stage to S and G2/M phase. PQQ interact with transcriptional pathways mediated by DJ-1, AMPK and CREBP leading to mitochondrial biogenesis. It up regulates transcription of PGC-1α, Tfam, Nrf-1 and Nrf-2 genes (Rucker et al., 2009; Chowanadisai et al., 2010). Apart from inducing mitochondriogenesis, PQQ also enhanced mitochondrial metabolism (Baurley et al., 2011). It has been shown to reduce the myocardial infarct size in rat models of Ischemia and ischemia/reperfusion (Zhu et al., 2004; 2006; Tao et al., 2007).

PQQ has the ability to regulate lipid metabolism and lipid mobilization pathway as well. It influences the m-RNA levels of the genes involved in lipid metabolism which include PPAR-α, carnitine palmitoyl transferase-1 and the mitochondrial complexes I and II. PQQ causes activation of CREB by phosphorylation which causes activation of PPAR-α, and other genes which are associated with lipid metabolism (Baurley et al., 2011). PQQ deprived diet leads to hyperlipidemia and up-regulation of lipid biosynthesis enzymes in mice. When PQQ in the diet is restored, lipid profile and expression levels of genes also get restored to normal levels. Moreover, they showed that
administration of PQQ results in more than 2-fold decrease in triglyceride levels in rat model of type-2 diabetes.

PQQ has been found to be very effective in reducing cardiac ischemia (Zhu et al., 2004; 2006; Tao et al., 2007). It basically reduces depolarization of the mitochondrial membrane potential and oxidative stress in freshly isolated cardiomyocytes (Tao et al., 2007). During cardiac ischemia and reperfusion injury, it causes higher left ventricle pressure and lessens ventricular fibrillation episodes. PQQ has the ability to reduce the myocardial infarct size (a localized area of ischemic necrosis) by reducing the myocardial malondialdehyde, in other words it reduces lipid peroxidation in ischemic heart (Zhu et al., 2004; 2006).

PQQ has been found to influence epithelial cell proliferation by activation of EGFR (Epidermal Growth Factor Receptor) pathway via redox regulation of PTP1B (Protein Tyrosine Phosphatase 1B) by oxidation of the catalytic cysteine of PTP1B thereby producing H$_2$O$_2$ (Kimura et al., 2012). This promotes the downstream pathway where ERK mediated Ras signaling is promoted and it in turn promotes the activation of cellular proliferation via EGFR activation.

As already mentioned that PQQ can inhibit PTP1B activity via redox regulation, hence it has been shown to have an activity in insulin signaling as well. When insulin binds to insulin receptor (IR), there occurs an activation of an intrinsic tyrosine kinase activity becomes activated and that leads to the auto-phosphorylation of several tyrosine residues. The activated IR phosphorylates IR substrate-1 (IRS-1) thereby triggering a signaling transduction by inducing the action of downstream targets such as phosphatidylinositol-3 kinase (PI3K) and Akt. The activation of Akt induces the translocation of glucose transporter 4 (GLUT4) to the plasma membrane from the intracellular compartment vesicle leading to an increased glucose uptake (Witczak et al., 2007). But, PTP1B dephosphorylates IRS-1 and that suppresses insulin signaling (Byon et al., 1998). Hence, the inhibition of PTP1B by PQQ can positively regulate the insulin signal in and therefore the entire human metabolism (Takada et al., 2012). PQQ also influences immune
function accompanied by reduction in the interleukin-2 levels (Steinberg et al., 2003). It can maximize B and T-cell sensitivity towards mitogens as well (Steihberg et al., 1994; Stites et al., 2000).

Thus, PQQ has gained great importance in the world of antioxidants, and is being considered to be a novel antioxidant due to its varied functions. The concise representation of the above functions of PQQ and interaction with signaling pathways has been displayed in Figure 1.27 and Figure 1.28.

Figure 1.27. A representation of summarized functions of PQQ in bacteria, plants and animals (Rucker et al., 2009; Misra et al., 2012).
1.6 Inflammatory Bowel Disease (IBD), ROS and gut microbiota

Innate immune system is the first line of defense against invading pathogens. This is nonspecific response to pathogens and trauma unlike adaptive immune system, it does not confer long-lasting immunity to the host. The magnitude of innate immune response is controlled by activity, orchestrated motility and interactions of various hematopoietic compartments (Wittmann et al., 2012). Inflammation is one of the first response of immune system to infection or injury, which is stimulated primarily by various factors released by injured cells, and hence it serves as physical barrier against the infected or inflamed tissue. This is followed by clearance of pathogen and cell debris and wound healing. All-inclusive, inflammation is considered as critical player in several pathologies such as Inflammatory Bowel Disease (IBD), autoimmune diseases, atherosclerosis, cancer, asthma, thyroiditis, Alzheimer’s and Parkinson’s disease (Coussens and Werb, 2002; Libby, 2002; Van Hove et al., 2008a; 2008b).

Mammalian gastrointestinal tract is highly vulnerable to disease because it is continuously exposed to numerous bacterial species as well as
food-borne environmental toxins. IBD is characterized by acute and chronic inflammation of gastrointestinal tract with multifactorial etiology (Xavier and Podolsky, 2007). It includes ulcerative colitis (UC) and Crohn’s disease (CD). IBD is inflammatory condition which is incurable. Surgery can cure UC if colon is removed and that 2.5 to 3 million people are affected by IBD in Europe with around 4.6 to 5.6 bn Euros/year direct health care cost (Burisch et al., 2012; 2013). IBD was thought to be uncommon for Asian countries particularly India (Ouyang et al., 2006; Bandyopadhyay, 2012; Ng et al, 2013). A growing number of reports suggests that IBD cases are increasing, which may be attributed to increased awareness and improved diagnostics, but may also reflect life style changes.

CD and UC are the two major forms of IBD. These two also share some common characteristics at clinical and genetic level (Xavier and Podolsky 2007). UC majorly affects mucosal lining of colon and rectum (i.e., lower large intestine). Key feature of UC includes diffused mucosal inflammation extending proximally from rectum to a varying degree. Severe inflammation and subsequent production of inflammatory mediators along with extensive superficial mucosal ulceration are common in UC. Recruitment of enormous amount of neutrophils in lamina propria and crypts forms micro-abscesses. Reduction in goblet cell mucin is often observed in UC patients. On the other hand, CD may affect whole intestinal wall and potentially can extend to any part of gastrointestinal tract. It is characterized by aggregation of macrophages that frequently form non-caseating granulomas. Although any intestinal site could be affected, but commonly terminal ilium is affected by earliest mucosal lesions and often these lesions appear over Peyer’s patches. Unlike UC, CD are often patchy and segmental in appearance and have typical transmural inflammation (Figure 1.29).
Figure 1.29 Histological hallmarks of Crohn’s disease and Ulcerative colitis. **Left panel** (Crohn’s disease—biopsy from a terminal ileum with active disease). This figure shows presence of discrete granuloma composed of compactmacrophages, giant cells and epithelioid cells. Surrounding the nodule thereis marked infiltration of lymphoid cells, plasma cells and other inflammatorycells, but there is no necrosis. **Right panel** (Ulcerative colitis—colonicmucosal biopsy taken from a patient with active disease). The crypt abscess composed of transmigrated neutrophils and the surrounding epithelium exhibiting features of acute mucosal injury.

Genome-wide search for IBD susceptible loci resulted in better understanding of role host genetic factors in IBD pathogenesis. Moreover, enormous evidences supports that dynamic balance between commensal microbiota and host response against them at the host mucosal interface plays a crucial role in initiation and pathogenesis of IBD (Xavier and Podolsky 2007). This can be further supported by several studies which demonstrates the therapeutic benefits of antibiotics and probiotics in, at least, subsets of IBD patients (**Figure 1.30**). Impact of gut microbiota of pathogenesis of IBD has been extensively studied using murine models. Unfortunately, our understanding of mammalian gut microbiota is itself not complete, and thus insights into host-microbe interaction in hampered by both, limited knowledge of gut microbial diversity and complexity, and by limitation of available tools which can delineate the characteristics.
Figure 1.30 Inflammatory Bowel Disease etiopathogenesis patterns (Xavier and Podolsky 2007). Alteration in interaction between intestinal microbiota and host results in inflammation seen in IBD. This is dependent on environmental and/or host factors which vary depending on host genetic makeup and inheritance at several susceptible loci. Host genetic factors majorly affects barrier function and innate and adaptive immunity. New evidences also suggests role of host genetics in shaping gut microbial diversity and hence affecting the nature of their interaction with host.

Mucosal immune responses to gut flora are multifactorial. Innate immune response seems to be essential and pre-requisite for activation of adaptive immunity. However, adaptive immunity is the major factor which drives tissue damage that is manifest in IBD patients (Xavier and Podolsky 2007). Adaptive immune response is mediated by combination of resident and recruited cell populations to the site of inflammation. These cell populations comprises of Mucosal B cells producing secretory immunoglobulin A and immunoglobulin G, complex mixture of T cells dominated by T_H1, T_H2 or T_H17 phenotype, and regulatory T/B cell populations. Microbes trigger T_H1 response and further stimulates production of Interferon-γ and IL-12p40 which signals through STAT1, TBX21 and STAT4. STAT6 is required for enhancement of T_H2 cell differentiation and GATA3 for induction of IL-4, IL-5 and IL-13. In simple terms UC seems to exhibit T_H2-type-like cytokine profile, and CD, a T_H1 like profile (Figure 1.30). Role of autophagy has also been well
illustrated in protecting against pathogens and its importance in IBD like disease.

Figure 1.31 Mucosal immune response in IBD (Modified from Xavier and Podolsky 2007). (A) Mucosal immune response to dietary antigens. DC, dendritic cell; IgA, immunoglobulin A; E, enterocyte; G, goblet cell; L, lymphocyte; IDC, immature dendritic cell; TN, T cell (naive); MLN, mesenteric lymph node; Mw, macrophage; P, Paneth cells; PP, Peyer’s patches. (B) Mucosal immune response initiated by bacterial sensing system further activated adaptive immune responses. Commensal microbes as well as pathogenic bacteria disrupt epithelial barrier function and triggers recruitment and activation of innate immune responses and colitogenic CD4+ T cells in genetically susceptible mice. Involvement of multiple components in controlling mucosal immune responses in inflammation is implied by depiction of multiple cells and cytokines in the figure. NK, natural killer. (C) Differentiation of CD4+ T cells. Intestinal immunity and inflammation is determined by relative balance of effector T cells and regulatory T cells. The differentiation of T_h1, T_h2, T_h17, T_reg present in intestine is determined by cytokines, chemokines, self-ligands and microbial products present locally as well in systemic milieu. nT_reg, natural T_reg; iT_reg, induced T_reg; Tbet, TBX21. (D) Potential roles of autophagy in IBD. Autophagy is essential for cellular homeostasis. It provides all cell types a mechanism of response to minimize the harmful effects of exogenous and endogenous stresses. Schematic flowchart depicts the multiple stages at which autophagy may play a role in intestinal physiology, acute phase of injury and inflammation, and resolution phase of IBD. PRR, pattern recognition receptor; ROS, reactive oxygen species.
Medical treatment of IBD is individualized depending upon type, distribution, genetics and severity of disease. Anti-inflammatory steroids, TNF-α inhibitors, immunosuppressant and antibiotics are generally used for treatment. Fecal bacteriotherapy (FBT) is relatively new treatment option, which has been used to treat IBD in several studies. Probiotics including several strains of *E. coli*, *Lactobacillus* and *Bifidobacteria* have also shown to prevent and improve intestinal inflammation and disease in IBD and related murine models, and currently many of them are under clinical trials. Murine models have been extensively used to demonstrate the physiology of disease and efficacy of various therapies aimed against IBD. These studies indicate that changes in host recognition and response to bacteria as well as alteration of microbiota communities are hallmarks of IBD development.

**Murine models for Inflammatory Bowel Disease**

Several murine models mimicking major pathologies of IBD have been used depending on aim of the experiment. Genetic mutations and knockdown strategies have been employed to understand their role in IBD pathology. **Table 1.7** summarizes key murine models used for colitis in different studies (modified from Xavier and Podolsky 2007).

<table>
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<tr>
<th>Model</th>
<th>Known defects</th>
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<tr>
<td>Multidrug-resistant 1a&lt;sup&gt;-/-&lt;/sup&gt; (also known as ABCB1&lt;sup&gt;-/-&lt;/sup&gt;)</td>
<td>Altered epithelial barrier</td>
</tr>
<tr>
<td>Gα&lt;sub&gt;i2&lt;/sub&gt;&lt;sup&gt;-/-&lt;/sup&gt; (GNAI2&lt;sup&gt;-/-&lt;/sup&gt;)</td>
<td>Defective epithelial barrier; defective regulatory B cells.</td>
</tr>
<tr>
<td>Macrophage-PMN Stat3&lt;sup&gt;-/-&lt;/sup&gt;</td>
<td>Increased response to lipopolysaccharide (LPS); resistance to IL-10 regulation</td>
</tr>
<tr>
<td>Bone marrow Stat3&lt;sup&gt;-/-&lt;/sup&gt;</td>
<td>Increased response to LPS; impairment of innate immune function</td>
</tr>
<tr>
<td>A20&lt;sup&gt;-/-&lt;/sup&gt; (TNFAIP3&lt;sup&gt;-/-&lt;/sup&gt;)</td>
<td>Increased response to LPS</td>
</tr>
<tr>
<td>II10 and II10Rb&lt;sup&gt;-/-&lt;/sup&gt;</td>
<td>Lack of Trl (Tr1; Treg cells) activity; lack of TGF-b signalling</td>
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Clinical appearance of human IBD is heterogeneous which is reflected by increasingly numbers of transgenic or gene targeted murine strains displaying IBD like phenotype (Wirtz et al., 2007). Hence, these models must be chosen appropriately to understand pathophysiological mechanisms pertaining to IBD. Moreover, these models are valuable tools in testing emerging therapeutic strategies in pre-clinical phase. Out of several stated IBD/colitis models, chemically induced mouse colitis models are used. Their action is more generalized and reflects majorly the role of environmental factors in IBD. However, they also reflect major immunological and histopathological symptoms seen in human IBD patients.

Colitis can be induced to susceptible strains of mice and rats by intrarectal instillation of haptenating substances in ethanol, 2,4,6-Trinitrobenzenesulfonic acid (TNBS) and oxazolone (Wirtz et al., 2007).
Ethanol breaks the barrier and TNBS or oxazolone haptenize colonic autologous or microbiota proteins rendering them immunogenic to the host immune system. CD4+ T cells play a major role in TNBS induced colitis, and hence this model is useful to study T helper cell mediated immune response. In mouse, the symptoms are highly dependent on strain and dose of TNBS. The response is mediated by Th1 cells. On the other hand, oxazolone mounts Th2 response which is correlated to symptoms seen in UC patients.

**Dextran Sodium Sulfate (DSS)** is another chemical widely used to induce acute colitis in rodents and rabbits. DSS polymers are toxic by itself affecting epithelial and cryptic cells and hence disrupting integrity of mucosal barrier. As adaptive immune system does not play major role in acute phase, DSS induced colitis in largely used to understand the role of innate immune response and related factors in colitis (Wirtz et al., 2007). DSS induced colitis model has some advantages over TNBS and other chemically induced colitis models. For example, acute, chronic or relapsing model can be easily produced by changing the concentration of DSS to be administered (Perse and Cerar, 2012). It is less stressful and painful for experimental mice, because DSS can be administered in bottle fed along with drinking water. Moreover, dysplasia that is observed in clinical course of human UC, occurs frequently in chronic phase of DSS-induced colitis. DSS-induced models are also valuable in studies involving colitis associated carcinogenesis. Therapeutic strategies and molecules used for human IBD has been validated by several studies on DSS-induced colitis. These studies further validates that DSS-induced colitis can be used as relevant model for translation of mice experimental knowledge to human disease. **Figure 1.32** schematically represents impact of several factors which can affect onset of DSS-induced colitis in mice.

**Key lessons learned from IBD models**

(a) A compromised epithelial layer has been shown to be sufficient in triggering intestinal inflammation.

(b) Inappropriately activated autoreactive effector T-cell population have been implicated in disease outcome.
(c) A variety of other haematopoietic cells have been shown to mediate or regulate intestinal inflammation.

(d) Many studies have elucidated the role of the different cytokines in different models of colitis. Chemoattractant cytokines may have a unique role in IBD pathogenesis. And,

(e) In case of spontaneous colitis, the resident enteric flora appears to initiate colitis.

Figure 1.32 Schematic representation of various factors that can influence the susceptibility, onset, severity, and responsiveness to DSS-induced colitis.

**NADPH oxidases and redox response in gastrointestinal tract**

NADPH oxidases are multimeric protein complexes involved primarily in ROS generation and innate immune response (Babior, 2004). Members of this family, Nox2 and Nox1/Duox2 are expressed in neutrophils/macrophages and the intestinal mucosa, respectively. Clinical observations point to increased oxidative stress in IBD. Nox2 deficiency leads in up to 40% of patients deficient in Nox2 (Chronic granulomatous disease; CGD) to the intestinal disease reminiscent of IBD (Campbell et al., 2014). Nox2 KO mice show exacerbated disease in an IBD model. DUOX is source of non-phagocytic ROS in intestinal epithelial cells and it is believed that DUOX dependent Hydrogen peroxide generation followed by hypothiocyanate
formation in mucosal fluids acts as microbicide and is part of robust antimicrobial defense network in mammalian epithelial cells (Kim et al., 2013). DUOX mediated production of H$_2$O$_2$ has also prevented Helicobacter infection in mice (Grasberger et al., 2013). Additionally, severe host defense defect against pathogens was observed in DUOX deficient Drosophila gut (Ha et al., 2005a; 2005b). H$_2$O$_2$ is non-radical Reactive oxygen species which is produced by family of NADPH oxidases, Xanthine oxidases and 5-lipoxigenases (Demiryurek and Wadsworth, 1999; Pritsos, 2000; Rojkind et al., 2002). DUOX enzymes are directly capable of generating H$_2$O$_2$, whereas NOXes 1-5 generate superoxide which is converted to H$_2$O$_2$ spontaneously or by superoxide dismutase (Rada and Leto, 2008). NADPH oxidase mutant and specific gene knockout mice strains may provide a valuable platform for development of murine IBD, infection and inflammatory models which mimic the disease physiology. Figure 1.32 summarizes redox responses in gastrointestinal tract under normal and disease conditions (Brown et al., 2014). There is balance between free radical production and antioxidant system in healthy intestine under steady state. DCs samples antigens and present them to immune cells which maintain tolerance against commensal microbiota. NOX-1 and DUOX 1-2 present in intestinal epithelial cells (IECs) produces physiological oxidative stress which is neutralized by antioxidants such glutathione. Functional Tight Junction (TJ) proteins maintains protective barrier between commensal microbiota and host.

However, under acute and chronic intestinal disease conditions, there is marked imbalance between ROS production and antioxidant defense system. Pathogenic bacteria cross epithelial barrier, activate immunity and trigger production of NO and RNS in epithelial cells and macrophages by inducible NOS (iNOS). Both acute and chronic gut diseases, allow increased bacterial translocation through leaky gut and promotes inflammation mediated by activated macrophages which secrete free radicals through NADPH oxidase-2 (NOX-2). Macrophages also secrete chemokines such as monocyte chemotactic protein-1 (MCP-1) which signals for more cell infiltration at the site of inflammation. Increased accumulation of macrophages at particular site results in robust oxidative stress response which disrupts the balance
between free radicals and antioxidant system. Although free radicals secreted by IECs and macrophages destroys the pathogens, the continuous secretion of enormous amount of free radicals also damages IECs and Goblet cells which further impairs barrier function and dysregulates TJ proteins leading to more bacterial infiltration and perpetuation of oxidative stress.

Figure 1.33 Redox responses in GIT. (A) Physiological redox response under normal steady state (homeostasis). (B) Redox response during intestinal disease.