CHAPTER – II
REVIEW OF LITERATURE

Status of Probiotics:

*Eschericia coli* Nissle 1917 played a major role in probiotic field after *Lactobacillus*, *E.coli* Nissle 1917 isolated in 1917 at the time of second world war based on its potential to protect from presumably infectious gastroenteritis, intestinal disorders, urinary tract diseases initial therapeutic success was noted and then it is used to chronic inflammatory conditions (Schultz, 2008; Krishnamoorthy *et al*., 2012). Isolated two lactic acid bacteria from 20 fermented samples of plant fermented beverages (PFB) were analyzed for probiotic properties. Acid tolerant (pH 3), thirty six *Lactobacillus* sp. and thirty *Streptococcus* sp. showed good resistance (2%) in bile salt even after exposure for 48 h. The organisms showed high specific growth rate and inhibitory action against potent food borne pathogenic bacteria. According to Resta-lenert *et al.* (2003) probiotic *Streptococcus thermophilus* and *Lactobacillus acidophilus* can prevent invasion of entero invasive *E.coli* enhance intestinal epithelial barrier function by amplifying phosphorylation of occluding and ZO-1(zonula ocludens-1) in vitro. *E.coli*Nissle has evolved into one of the best characterized probiotics, and its therapeutic efficiency and safety have convincing been proven (Kruis *et al*., 2004; Westendorf *et al*., 2005; Henker *et al*., 2007). The work on these lines is promising, even though their mechanism of action is still under investigation. A potential mechanism by which probiotics may exhibit their beneficial activities is modulation of epithelial barrier function (Dotan *et al*., 2005).

Probiotics and Gut Microflora:

Gut microflora controls several aspects of bodily function including certain type of cancer (khan *et al*., 2012). Normally large number of *Lactobacilli* is observed in the intestine, but they rapidly decline after infancy (Balamurugan *et al*., 2008). Majority of the organisms are strictly anaerobic. They comprise the main part of the human normal gut microflora and appear in the stool a few days after birth and subsequently raise the number (Matto *et al*., 2004). Living microorganisms that enter the gastrointestinal tract (GIT) in an active state and exert a positive influence on the host tissues are called probiotics (Bohm *et al*., 2006). The potential therapeutic role of probiotics in the prevention or treatment of GIT
diseases is earmarked (Mach, 2006). At present *E.coli* Nissle is contained in a probiotic drug called Mutaflor. In recent years, there has been considerable progress in understanding the mechanisms of probiotic action and in the future this should help to select suitable bacterial strains which could beneficially affect mucosal barrier function, immune responses, and suppression of inflammation (Meijerink *et al.*, 2013). The knowledge of the effects of simultaneous administration of drugs and probiotics on drug pharmacokinetics is still very limited. The complexity of mechanisms by which the fate of orally administered drugs could be affected by probiotics is recently reported (Stojancevic *et al.*, 2013). Role of both human and commensal microbiota components in drug efficacy and toxicity was recently documented and pointed out (Haise *et al.*, 2013). Some scientists say that great quote “Life without gut bacteria would be extremely unpleasant, if not possible” (Gibson *et al.*, 1999).

The study showed that the application of proteomic tools provided an overview of the proteins present in *E.coli* Nissle under *Cocos nucifera* sap and wine stress conditions. It confirmed that proteins belonging to the vascular system are involved in various biological functions like stress and defense reactions, redox reactions, signal and the transport of the substances and sugar metabolism.

When the cell is under stress arising from oxidation, heat, infection, toxic contamination or any other stressful condition, proteins may unfold and expose residues in their structure that under normal conditions are hidden and shielded from chemical reactions. As a consequence of stress, these residues can easily interact and form aggregates which may harm or even kill the cell (Krebs *et al.*, 2003). Under such conditions all cells produce stress proteins to protect the cell from damage.

Probiotics may stimulate immunity, regulate immune signaling pathways, and produce anti-pathogenic factors. Probiotics may produce secreted factors that stimulate or suppress cytokines and cell-mediated immunity. These factors may also interfere with key immune signaling pathways such as the NF-κB and MAP kinase cascades. Probiotics may produce that factors inhibit pathogens and other commensal bacteria, effectively enabling these microbes to compete effectively for nutrients in complex communities. Microbes that produce antipathogenic factors may be regulated by master regulatory genes in particular
classes of bacteria. Probiotics mainly present in gastrointestinal tract, plays an important role as an interface between the host and the environment. It is colonized by about 10 trillion microbes of many different species (O’Hara et al., 2006). Intestinal epithelial cells have the capacity to distinguish pathogenic from non-pathogenic bacteria on the basis of their invasiveness and the presence of flagella, although the exact mechanisms that allow them to do this have not been elucidated fully (Borchers et al., 2009). The importance of the intestinal microflora composition in physiological process in the GIT is becoming more evident and has led to new possibilities for prevention and therapy of diseases (Dominguez-Bello et al., 2008; Sekirov et al., 2010; Kau et al., 2011). There is a growing interest in probiotics as a safe way of changing the intestinal bacterial flora. It is possible to increase the proportion of Lactic acid bacteria (LAB) and Bacillus sp. In the gastrointestinal microflora by consumption of probiotics or by oral administration of specific non-digestible substrates, such as oligofructose, termed as prebiotics (Parracho et al., 2007).

**E. coli Nissle 1917- A model organism:**

*E.coli* is the most commonly used bacteria *E.coli*Nissle 1917 is one of the oldest, most well-characterised probiotic agents and has shown promising results in treatment of various intestinal diseases (Sonnenborn et al., 2009). At the time of World War II it is isolated from feces. Completed genomic studies on *E.coli*Nissle 1917 including sequencing, DNA-DNA hybridisation, t-RNA screening (Grozdanov et al., 2003) and even low-coverage genomic shotgun sequencing (Sun et al., 2005). Until now, however, the whole genome sequence has been inaccessible. Serotyping of *E. coli*Nissle 1917 has identified the presence of a K5 antigen, which is known to be composed of N-acetyl heparosan (a precursor to the anticoagulant pharmaceutical heparin), a group 2 capsular polysaccharide (CPS) consisting of a repeating [\(\beta 4\)] -D-glucuronic acid (GlcA) (1 → 4) N-acetyl-D-glucosamine (GlcNAc) disaccharide unit. Under certain growth conditions, *E. coli*Nissle 1917 produces significantly more CPS than *E. coli* K5, making the organism attractive as a production strain for bioengineered heparin.
**Important aspects when select as a probiotic strain:**

The significance of human origin has been debated recently, but currently successful strains are indicated to be of human origin. It can also argue that a probiotic strain can function better in a similar environment like human gut to where it was originally isolated from; safety aspects include the following specifications.

1. Strains for human use are preferable of human origin.
2. They are isolated from healthy human GI Tract
3. They have a history of association with diseases such as infective endocarditis or GI disorders
4. They have history of being non-pathogenic.
5. They do not deconjugate bile salts
6. They do not carry transmissible antibiotic resistance genes.

The *E. coli* is a common bacterium present in our gut, it is a gram-negative rod shaped bacteria, about 1.1–1.5 μm x 2.0 – 6.0 μm in size. It grows under aerobic and anaerobic conditions (facultative anaerobic), because it possesses two different redox systems (menaquinone and ubiquinone) which enable it to derive energy from catabolic metabolism under both aerobic and anaerobic conditions. Under optimal growing conditions, the rate of cell division of the *E. coli* bacteria is very fast, the number of bacterial cells can double every 20 minutes. However, the circumstances that are ideal for this population dynamics are not achieved in the bacteria are normal environment. Midtvedt, in 1998 reported that doubling of cells in the caecum of the rat after about 100 minutes, while in the human gut it may take 30 hours. Various strains of *E. coli* have been classified serologically on the basis of their surface antigens O, K and H. O antigens represent the heat-stable constituents of the lipopolysaccharide complex (LPS) of the outer cell membrane, K antigens represent polysaccharides of the capsule and H antigens represent whip or flagellar antigens.

*E.coli* M-17 is a novel probiotic drug with beneficial effects on the GI tract. EC-M17 is believed to be a direct descendant of the M17 strain first identified by the Russian bacteriologist L. G. Peretz in 1933 (Fitzpatrick *et al*., 2008). This strain used extensively in humans as a therapy for GI diseases such as colitis, inflammatory bowel disease and
infections. Anti-colitis action of EC-M17 is mediated by modulation of immune processes attributed to an inhibitory effect on NF-kB signaling.

**Cocoti sap and wine:**

Isolation of microorganisms from palm wine *Saccharomyces cerevisiae* dominated in yeast, *Lactobacillus plantarum, Leuconostoc mesenteroides* were the dominated organisms. Acetic acid bacteria were isolated after third day when levels of alcohol had become substantial. The pH, lactic and acetic acid concentrations during the tapping were among 3.5 -4.0 %, 0.1-0.3% and 0.2-0.4% respectively, while the alcohol contents of samples collected within the day were between 3.24% to 4.75% and palm wine held for 24 h, over 7.0% and in palm oil wine alcohol content is 1.4% and 2.82%.

Limited consumption of Date sap was found to improve the treatment of haemoglobin deficient anaemic patients and to supplement vitamin-B 12 levels in the Vitamin deficient patients (Debmalya et al., 2008). The organisms *Saccharomyces cerevisiae, Debaryomyces hansenii, geotrichum lactis* and *Zygosaccharomyces rouxiiare* isolated from freshly tapped palm wine (Boboye et al., 2008). Kadere et al., (2008) has isolated *Acetobacter* and *Gluconobacter* in coconut toddy. Palm sap is a rich medium capable of supporting the growth of various types of micro-organisms as high number of aerobic mesophiles, lactic acid bacteria, yeasts and acetic acid bacteria were found in palm wine (Amoa-Awua et al., 2007). It has been reported that fermented palm wine exposure could cause prenatal osteo-inhibitory effects on bones (Eluwa et al., 2010).

Palm wine caused changes in body weight of rats after treatment for 30 days this suggests that palm wine was not toxic as well as non-androgenic in nature, since androgens are known to possess anabolic activities. Several reports suggests that palm wine shows negative effect on rat reproductive system (Verma et al., 2002; Gonzales et al., 2006; Das et al., 2009). The reason could be due to increased hydrostatic pressure, reduced oncotic pressure, lymphatic obstruction and sodium retention (Kumar et al., 1999). In rats Palm wine (10 mL kg-1 bw) caused significant decrease in testosterone level (0.12±0.02 ng mL-1; relative to control 1.38±0.52 ng mL-1). Palm wine (10 mL kg-1 bw) caused significant
decrease in sperm counts, motility and viability but no significant change in morphology (Oyedeji et al., 2012).

Palm wine causes hypoglycemia, was seen in treated rats when compare to ethanol palm wine causes more effect on gestation. According to Lal et al. (1997) key enzymes of citric acid cycle and gluconeogenesis were inhibited on administration of both alcohol and toddy. It shows effect on carbohydrate metabolism. Palm wine increased activity of glycolytic enzymes.

In previous studies, composition of sugars analysis of fresh cocoti sap contains sucrose was the major sugar component in sugars content varied from 9.40g/100ml to 12.24g/100ml. It contains 0.36- 1.5% of proteins and minerals when compared to the date palm wine revealed that sugars are the major components (92-95% dry matter basis) with the dominance of sucrose. It contains also 2.7-5% of proteins and 2.3-2.6% of minerals (Ben Thabet et al., 2009). The nutrients – rich in coconut sap comes right out of the tree naturally abundant in 17 Amino acids, broad- spectrum B- Vitamins, Vitamin C as well as FOS. The protein quantification assays revealed that the proteins content was about 0.2g/ml which was comparable with that of the coconut palm exudates 0.1g/ml but was lower than the proteins concentrations of cucumber (60g/l) and of pumpkin (35g/l) phloem sap ( Walz et al., 2002; Nakamura et al., 2004).

Physico - chemical stress on *E.coli*: 

*E.coli* in response to physical (heat) and chemical (benzyl alcohol) stress elucidate the common and differing elements of the stress response originating in cellular membranes caused by external stress signals of a different nature (high temperature and membrane fluidizing agent), by observing overlapping changes at the membrane level. It is expected that signals generated within the membranes might cause HSR and acquisition of cellular thermotolerance in a similar manner independently from the nature of the membrane perturber. The present study addressed the validity of the membrane sensor hypothesis in *E.coli*, which was chosen as our model organism due to its different cellular simplicity and because it is biochemically and genetically well characterised. A reporter system was also developed to study the transcription of heat shock genes, including heterologous promoter
sequences of cyanobacterial heat shock genes recognised in an *E.coli* host (Georgopoulos *et al*., 1993). In proteome analysis total 93 proteins are identified that are phosphorylated in E.coli upon heat shock. These are include chaperones, signaling molecules, ion-channels, proteins involved in transcription and translation process, in amino acid biosynthesis, oxi-do-reduction, energy metabolism, cell motility and cell membrane structure. Changes in stress signaling pathways are achieved mostly through the activation of protein-tyrosine kinases (Kim *et al*., 2002).

**Cellular cross-protection by stress:**

Cellular cross-protection occurs when the stress response induced by one specific type of stress, gives cells increased resistance to other types of stress (Mary, 2003; Vattanaviboon, 2003). An example of this kind of protection is demonstrated with stress-induced thermo-tolerance, where *Escherichia coli* cells given a non-lethal heatshock (42 °C) down-regulated normal protein production and begin production of HSPs, and so are later able to survive what would otherwise be a lethal heat shock (46 °C). This is due to the up-regulation of stress proteins at many levels (e.g. mRNA synthesis and stability, translational efficiency) that can protect cells from other stress. Cross-protection is not universal, and it can also occur in specific ways. For example, heat shock may protect against hydrogen peroxide.

Booth (2002) proposed that “Stress is any change in the genome, proteome or environment that imposes either reduced growth or survival potential”. The cellular response depends on the severity of the stress. Under slight stress, growth continues at the same rate and cells fully adapt to the new conditions. Under severe stress the growth rate is reduced but cells adapt and tolerate the conditions while under extreme stress, growth cease and cells switch to a survival mode (Storz *et al*., 2000). The key aspects to surviving environmental stress are the cell’s ability to maintain the integrity of the cell membrane, the integrity of DNA and the ability to properly fold proteins (Booth, 2002). An understanding of the physiological, biochemical and molecular mechanisms involved in response of *E.coli* to environmental stresses is essential for assessing, predicting, and minimizing the health risks and can offer insight into designing effective methods to control their growth.
When the bacteria expose to high temperature into less time period it produce heat shock response proteins these proteins are unfolded and damaged proteins, such as exposure to harmful chemicals (antibiotics, solvents) or overproduction of endogenous and recombinant proteins. In E.coli, heat shock response consists of the induction of more than 20 different heat shock proteins (HSPs), the majority of proteases that degrade misfolded and abnormal proteins. Bacterial cells exposed to one type of stress it can also condition them against other, seemingly unrelated, stresses, when bacteria are challenged with high osmolality (Fletcher et al., 2001).

**Proteomics of stress responses of potentially probiotic bacteria:**

Proteomics is an excellent approach for studying changes in bacterial metabolism and, e.g., stress responses during the progression of growth. The proteome of the potential probiotic L. plantarum WCFS1 was mapped at mid- and late-exponential and early- and late-stationary phases, and growth phase-dependent differences were detected in the abundances of 154 protein spots (Cohen et al., 2006). In a study of L. plantarum REB1, isolated from fermented feed, and the potential probiotic L. plantarum MLBPL1, isolated from white cabbage, both the growth phase (lag, early exponential, late exponential, and early stationary phases) dependent and strain-dependent differences in the proteomes were compared (Koistinen et al., 2007). Proteome maps of L. casei Zhang cells grown until the exponential and stationary phases were also compared. Forty-seven protein spots showed growth phase-dependent production, and the major up-regulated proteins in the stationary phase were stress proteins and proteins involved in carbohydrate and energy metabolism, and they were suggested to be involved in the stress response mechanisms of L. casei (Wu et al., 2010).

**Current Status of the E.coli Proteome:**

*E.coli* proteome study can be classified into two main types i.e., Proteomics for biology and Proteomics for biotechnology. An enormous number of *E.coli* proteome studies have focused on improving our biological knowledge proteins and findings members of regulations and stimulations under particular conditions (VanBogelen, 2003) and these studies are referred to as proteomics for biology. Other groups have studied the *E.coli* proteome under various genetic and environmental perturbations in an effort to develop
strategies for improving cellular properties and enhancing the production of bio-products based on comparative proteome profiles and these studies are referred to as proteomics for biotechnology.

Proteomic studies of gut microflora explain the molecular mechanisms, expression patterns of proteins and enzymes in response to dietary components and therapy provide a rationale for the development of new active ingredients. For instance, a nano-high-performance liquid chromatography/mass spectrometry (nano-HPLC/MS) system was established to separate proteins of *E. coli* in a two-dimensional manner by combining strong cation exchange (SCX) and reverse phase (RP) chromatography. Peptides were eluted online to an ion trap MS instrument and further analyzed by tandem MS fragmentation for identification using the Swissprot database. Differentially expressed proteins on glucose and lactose were identified. Similarly, lactic acid bacteria that are widely used in the agro-food industry have been characterized by proteomic techniques as reviewed in Champomier-Verges et al., (2002). More recently, the proteome of *Bifidobacterium* has received considerable attention. Adaptation to Low-pH tolerance response and tolerance of bile stress are among the main limiting factors to ensure survival of *Bifidobacterium* in the intestinal environment of humans. Comparing protein patterns of strains grown with or without bile showed 34 different proteins whose expression was regulated (Sanchez et al., 2005; Sanchez et al., 2007).

**Protein identification methods:**

New methods in protein identification have led to the application of mass spectrometry to the identification of proteins by Peptide Mass Fingerprinting (PMF). MS analysis is an effective tool for identification, characterization and quantification of proteins that are integral components of the processes essential for life. Mass spectrometry relies on the formation of gas-phase ions that can be isolated electrically based on their mass-to-charge ratio. Mass spectrometry (MS) has been widely used in forensic science in the identification of compounds, particularly illicit drugs. MS is a technique that allows the detection of compounds by separating ions by their unique mass (mass-to-charge ratios) using a mass spectrometer. The method relies on the fact that every compound has a unique
fragmentation pattern (mass spectrum). The sample is ionized; the sample ions are separated based on their differing masses and relative abundance.

Matrix Assisted Laser Desorption/Ionisation (MALDI) is a soft ionization technique used in spectrometry, allowing to analysis the biomolecules like DNA, protein, peptides. Biomolecules and synthetic polymers have low volatility and are thermally unstable, which has limited the use of MS as a means of characterization. These problems have been minimized through the development of MALDI-TOF MS, which allows for the mass determination of biomolecules by ionization and vaporization without degradation, a Laser beam used to ionize the sample (Wu et al., 1994). Currently high-throughput proteomic tools based on high resolution mass spectrometers and shotgun strategies provide the opportunity to study the physiology of bacterium at high-resolution (Armengaud., 2010; Armengaud, 2013). Recently, efforts have focused on developing new high-throughput techniques for studying microbial cells and complex communities. Among them, proteomics is increasingly being used (Aires et al., 2011). Several studies reported that bile salts can cause stress on probiotic organisms present in gastrointestinal tract, the stress effects are protein misfolding and denaturation, DNA damage, the formation of secondary structure in RNS and intracellular acidification (Begley et al., 2005; Lebeer et al., 2008). The most of the proteomic studies on pH responses have been performed under specific aerobic and/or anaerobic conditions, allowing identification of new classes of acid- and base-dependent regulators and dissection of the relationship between pH and oxygen levels (Wu et al., 2009).

Enterohemorrhagic and enteropathogenic *E. coli* (EHEC and EPEC) strains are human pathogens that are responsible for food-borne epidemics in many countries. Proteomics offers a powerful platform for analyses of the disease process and of bacterium-host interactions at the protein level. Pathogenesis and identifying markers for laboratory diagnoses of these pathogens (Li et al., 2004). Proteomics has been extensively used in understanding and treatment of molecular diseases in humans. The worldwide emergence of antibiotic-resistant bacteria poses a serious threat to human health. For the first time report, potential role of a multi-drug efflux pump system in laboratory *E. coli* strain resistance to piperacillin/tazobactam, and wild type *E. coli* ATCC25922 (Santos et al., 2010). For any
infection, bacteria must first adapt to the host environment and grow. When *E. coli* under hyperosmolarity stress results in rapid loss of water (plasmolysis), loss of turgor, and shrinkage of the cell (Weber *et al.*, 2005). Within the minutes, respiration ceases, both the intracellular ATP concentration and the cytoplasmic pH increases. Many studies reported the cellular membrane of *E. coli* is a vital factor that allows for cells to acclimate to external stresses and is also one of the components highly affected by organic solvents like alcohol (Isaac *et al.*, 2005). Most of the researchers have proposed that the plasma membrane is the most affected target of organic solvents and plays a significant role in adapting to stress, alcohols are sensitive toxins to *E. coli* as tolerances of n-butanol and ethanol are only 0.5-1% and 4-5% respectively.

Proteomics is an excellent approach for studying changes in bacterial metabolism and response. The proteome of the potential probiotic *L. plantarum* WCFS1 was mapped at mid- and late-exponential and early- and late-stationary phases, and growth phase-dependent differences were detected in the abundances of 154 protein spots (Cohen *et al.*, 2006). In a study of *L. plantarum* REB1, isolated from fermented feed, and the potential probiotic *L. plantarum* MLBPL1, isolated from white cabbage, both the growth phases dependent and strain-dependent differences in the proteomes were compared (Koistinen *et al.*, 2007). Proteome maps of *L. casei* Zhang cells grown until the exponential and stationary phases were also compared. 47 protein spots showed growth phase-dependent production and the major up-regulated proteins in the stationary phase were stress proteins and proteins involved in carbohydrate and energy metabolism and they were suggested to be involved in the stress response mechanisms of *L. casei* (Wu *et al.*, 2009).