CHAPTER 1

Review of Literature

1 Introduction

1.1 Probiotics

The term probiotic is derived from the Greek and literally translates as *for-life*. The probiotic was conceptualized at the end of 19th century by Elie Mechnikoff at the Pasture Institute, Paris. Metchnikoff is regarded as the grand father of modern probiotics. The scientific rationale for the health benefit of lactic acid bacteria were provided in his book. "The Prolongation of Life" published in 1907. He suggested, "The dependence of the intestinal microbes on the food makes it possible to adopt measures to modify the microbiota in our bodies and to replace the harmful microbes by useful and beneficial microbes". Contemporary of Metchnikoff, Henry Tissier observed that children with diarrhoea had in their stools a low number of bacteria characterized by atypical, Y shaped morphology. On the contrary, these "bifid" bacteria were abundant in healthy children. He suggested that these bacteria could be administered to patients with diarrhoea to help restore a healthy gut flora. In 1965, term 'probiotics' was first used by Lilly and Stillwell in a different context to represent the 'Substances secreted by one organism which stimulate the growth of another'. Nine years later, Parker described probiotics as "organisms and substances that contribute to intestinal microbial balance" (Anukam and Reid, 2007). Fifteen years later, Fuller proposed that probiotics were 'Live microbial supplements which beneficially affects the host animal by improving its microbial balance. In 2002, FAO/WHO defined Probiotics as 'live micro-organisms which when administered in adequate numbers confer a health benefit on the host'.

1.2 Current global and national status of Probiotics

The most common form for probiotics are dairy products mainly fortified foods. However, tablets, capsules, powders and sachets containing the bacteria in lyophilized form are available worldwide (**Table 1.1**) (Sander, 2005; Sekhon *et al.*, 2010; Bhadoria *et al.*, 2011). Worldwide market of probiotics is growing fast, however, in India; the market has just started to conceive with leading companies like Yakult, Amul, Nestle, and Mother dairy making the first step (Sekhon *et al.*, 2010; Bhadoria *et al.*, 2011; Raja *et al.*,

2011). In India, these companies have come up with their probiotic products, which are very popular. Some of the products are mentioned (**Table 1.2**).

Table 1.1: Probiotic products marketed worldwide with targeted health benefits (Sanders, 2007; Sekhon *et al.*, 2010)

Strain	Brand Name	Producer	Proven effect in humans
Bifidobacterium animalis DN 173 010	Activia	Danone	Stabilises intestinal passage
Bifidobacterium breve Yakult	Bifiene	Yakult	
Bifidobacterium infantis 35624	Align	Procter & Gamble	Irritable Bowel Syndrome (IBS)
Bifidobacterium lactis HN019 (DR10)	Howaru™ Bifido	Danisco	Immune stimulation
E. coli Nissle 1917	Mutaflor	Ardeypharm	Immune stimulation, IBS, Ulcerative colitis.
Lactobacillus casei F19	Cultura	Arla Foods	Improves digestive health, immune stimulation, reduces antibiotic-associated diarrhoea, induces satiety, metabolizes body fat, reduces weight gain
Lactobacillus casei Shirota	Yakult	Yakult	Immune stimulation
Lactobacillus plantarum 299V	GoodBelly ProViva	NextFoods Probi	IBS, used post-operative
Lactobacillus rhamnosus ATCC 53013 (discovered by Gorbach & Goldin (=LGG))	Vifit and others	Valio	Immune stimulation, alleviates atopic eczema, prevents diarrhoea in children and many other types of diarrhoea
Lactobacillus rhamnosus LB21	Verum	Norrmejerier	Immune stimulation, improves digestive health, reduces antibiotic-associated diarrhoea

Veillonella parvula, Streptococcus and *Lactobacillus*	VSL#3	(VSL Pharmaceutical Inc., Fort Lauderdale, FL)	Effective for the management of remission of pouchitis and colitis

Table 1.2: Probiotic products marketed in India (Sekhon *et al.*, 2010; Bhadoria *et al.*, 2011; Raja *et al.*, 2011)

Sl. No	Probiotic Products	Company
1	Probiotic drugs	Heritage Foods (India) Ltd
2	'b-activ' Probiotic curd	Mother dairy
3	'Nesvista' Probiotic Yougurt	Nestle
4	Probiotic ice creams, 'Amul Prolife' 'Prolite' and 'Amul Sugarfree'	Amul (Brand of Gujarat cooperative milk marketing federation Ltd)
5	Yakult Probiotic curd with *L. casei* strain *Shirota*	Yakult Danone India (YDL) Private Ltd
6	Probiotic drugs	Ranbaxy (Binifit)
7	Probiotic drugs	Dr. Reddy's Laborotries
8	Probiotic drugs	Zydus Cadila
9	Probiotic drugs	UniChem
10	Probiotic drugs	JB Chem
11	Probiotic drugs	GalaxoSmithKline
12	Fructo-Oligo Saccharides, Probiotic drugs	Genmark Alkem Labs

Major factors driving the growth of the global probiotics market are increasing levels of health-consciousness, and the availability of probiotics in the form of dietary supplements. Global industry analysts reported that in 2008, the global probiotics market (including both foodstuffs and supplements) was worth over US$15.7bn, or over 18% of the global functional foods (Starling, 2010). Since 2003, the global probiotics market has more than doubled in value terms, and is currently rising by almost 15% per annum and expecting to reach approximately worth of US$30 bn by 2015.

1.3 Characteristics of Probiotics

Probiotic organisms require certain characteristics to enable them to exert maximum therapeutic effects. These qualities are outlined in **Table 1.3**.

Table 1.3: Desirable properties of probiotics (Ouwehand *et al.*, 2002)

Property	Benefit
Resistance to pancreatic enzymes, acid and bile	Survival of passage through the intestinal tract
Adhesion to the intestinal mucosa	Immune modulation Pathogen exclusion Enhanced healing of damaged mucosa Prolonged transient colonization (?)
Human origin	Species specific interactions with the host
Documented health effects	Proposed health effects are 'true'
Safe	No health risk to consumer
Good technological properties	Strain stability Production at large scale Oxygen tolerance

1.4 Groups of Probiotics

The major groups of probiotics are *Lactobacilli*, *Bifidobacteria* and minor group is represented by *Saccharomyces, Streptomyces, E. coli*, although it is important to remember that there are many other types of bacteria that are also classified as probiotics. Each group of bacteria has different species and each species has different strains.

1.4.1 Lactic acid bacteria (LAB)

Lactic acid bacteria are characterized as Gram-positive, aerobic to facultative anaerobic, asporogenous rods and cocci which are oxidase, catalase, and benzidine negative, lack cytochromes, do not reduce nitrates to nitrite, gelatinase negative, and are unable to utilize lactate. They are used in the manufacture of dairy products such as acidophilus milk, yogurt, buttermilk, and cheeses. They are sub-divided into four genera *Streptococcus*, *Leuconostoc*, *Pediococcus* and *Lactobacillus*. Beneficial effects of lactic acid bacteria include alleviation of lactose intolerance (Batista *et al.*, 2008; Granato *et al.*, 2010; Pathan *et al.*, 2011), prevention and treatment of diarrhea (Guarino *et al.*, 2009; Pathan *et al.*, 2011), maintenance of normal intestinal flora (Khurana *et al.*, 2007), antagonism against pathogens (Zoumpopoulou *et al.*, 2008), stimulation of the immune system (Dı´az-Ropero *et al.*, 2006), anticarcinogenic activity (Commane *et al.*, 2005), and reduction of serum cholesterol levels (Nguyen *et al.*, 2007; Pan *et al.*, 2010; Parnami *et al.*, 2011).

1.4.2 Bifidobacteria

Bifidobacteria are characterised as gram-positive, obligate anaerobes, non spore-forming, nonmotile, distinct Y-shaped sometimes in the form of club-shaped. They optimally grow in anaerobic conditions while some species can only tolerate O_2 only in the presence of CO_2. The optimum growth temperatures are 37-41°C; minimum growth temperatures 25-28°C and a maximum at 43-45°C. Optimum pH for initial growth is 6.5-7.0 with no growth at pH 4.5-5.0 or 8.0-8.5. Genus *Bifidobacteria* contains approximately 30 species (Leke *et al.*, 2007). A number of health benefits have been claimed for *Bifidobacterium* spp.. and therefore inclusion of these organisms in the diet is considered to be important in maintaining good health. *Bifidobacteriun* spp.. has anticarcinogenic properties, a specific probiotic effect, which are of three types: (1) elimination of procarcinogens; (2) modulation of procarcinogenic enzymes; and (3) tumour suppression (Wollowski *et al.*, 2001; Shah, 2007). Furthermore, consumption of these organisms is an ideal method to re-establish the balance in the intestinal flora after antibiotic treatment (Quigley *et al.*, 2006). Many health promoting affects have been attributed to certain

Bifidobacterium spp.. These include stimulation of the immune system, alleviation of lactose intolerance and prevention of gastrointestinal (GI) disorders (Shah, 2007).

1.4.3 Others

1.4.3.1 *Saccharomyces boulardii*

Saccharomyces boulardii is a probiotic, non-colonizing yeast species closely related to Brewer's yeast but not related to *Candida*. It aggressively displaces problematic yeast species in the GI tract and has been used to prevent acute diarrhea (Billoo *et al.*, 2006; Czerucka *et al.*, 2007). Oral consumption of *S. boulardii* results in its establishment in GI tract and secretes lactic acid and some B vitamins. It is eliminated after supplementation is discontinued. During use, friendly probiotic bacteria are allowed to colonize in the GI tract, supporting micro-ecology. *S. boulardii* increases the levels of secretory IgA and is effective against specific unfriendly microbes (Czerucka *et al.*, 2007).

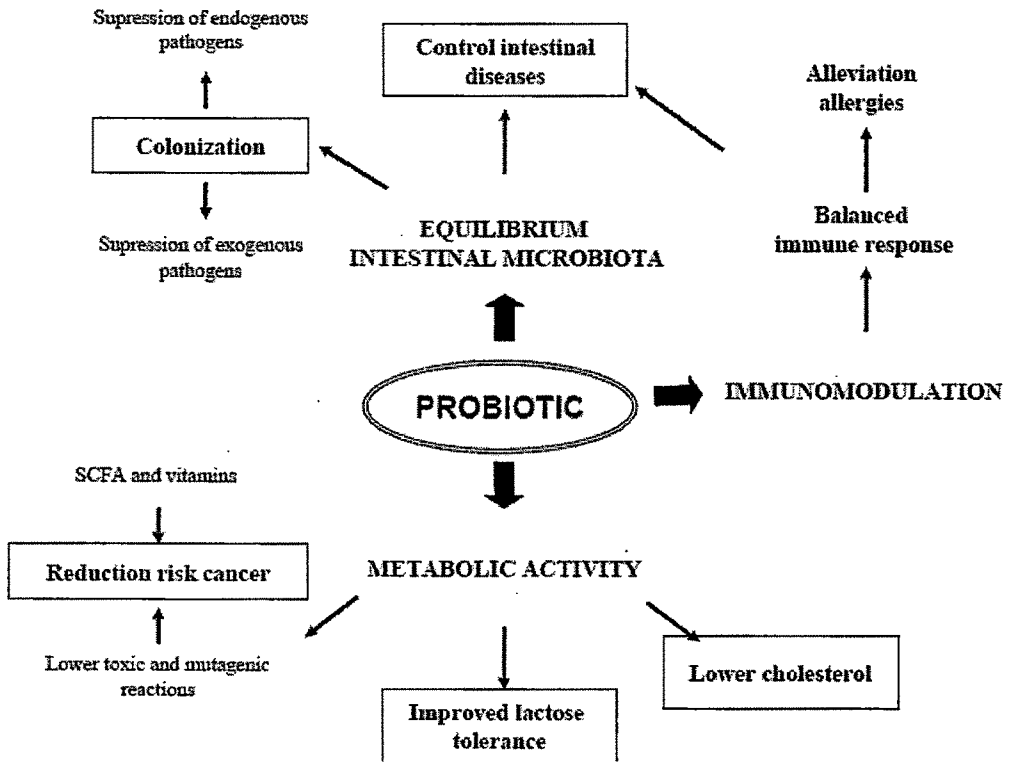
1.4.3.2 *Streptococcus gordonii*

Streptococcus gordonii belong to the viridian group of *Strepotococci*, nonpathogenic and commensal, which are integral members of the human oral flora. These organisms colonize tooth surfaces by creating biofilms in the human mouth, also known as dental plaque. *S. gordonii* plays an integral role in initiating colonization by creating surfaces for other colonizers to adhere to. The efficiency of *S. gordonii* in colonizing the oral cavity creates great potential for the stimulation of the mucosal immune system (Magliani *et al.*, 1997). *S. gordonii* is a good candidate for a "live bacterial mucosal vaccine vector". *S. gordonii* can also be easily controlled genetically to produce a "fusion construct" that will enhance its stability. Another advantage is that the activation can be attributed to multiple immunostimulatory components present within *S. gordonii* bacterial cells thereby expressing a number of viral and bacterial antigens.

1.5 Mechanism of action of Probiotics

The mechanism by which Probiotics exert its effects are schematically represented in **Fig 1.1**: (i) The indigenous GI tract microflora stimulate the immune system of the host to respond more rapidly against potential pathogens (Collado *et al.*, 2009); (ii) Probiotics can inhibit the growth of their competitors by producing antimicrobial substances called bacteriocins (Collado *et al.*, 2009); (iii) Compete for adhesion to the intestinal brush border epithelium surface. Adherent non-pathogenic bacteria (probiotics) can prevent attachment and subsequent entry of pathogenic enteroinvasive bacteria into the epithelial cells (Guarner and Malagelada, 2003) and (iv) Bacteria compete for nutrient availability in ecological niches (Fooks and Gibson, 2002). Increasing the numbers of friendly bacteria by way of a probiotic may thereby decrease the substrate availability for other bacterial populations, especially pathogenic ones.

Fig. 1.1: Mechanism by which probiotics safeguard our health (Collado *et al.*, 2009)



1. 6 Application of Probiotic

Clinical studies of Probiotic proved their potential application in GI disorders, including diarrhea, IBS, urogenital infection, *Helicobacter pylori* infection, stimulate the immune system, reduction in serum cholesterol and lactose intolerance **Table 1.4.**

Table 1.4: Selection of probiotic strains fulfiling the FAO/WHO Guidelines (Sanders *et al.*, 2005; Carmen *et al.*, 2010)

Strains	An examples of Clinical evidence showing Probiotic effects
Lactobacillus casei Shirota (Yakult, Japan)	Improvement in treatment of constipation
L. casei DN114 001 (Danone, France)	Reduced duration of winter infections in elderly subject
Lactobacillus rhamnosus GG (Valio, Finland)	Various benefits including improved treatment of diarrhoea and management of atopy
L. rhamnosus HN001 (Danisco, Denmark)	Enhanced immunity in the elderly, as measured by in vitro phagocytic capacity of peripheral blood polymorphonuclear leukocytes and tumoricidal activity of natural killer cells, following 3 weeks intake of probiotic
FL. rhamnosus 19070-2 and *Lactobacillus reuteri* DSM 12246 (Chr. Hansen, Denmark)	Reduction in acute diarrhoea in children following twice daily treatment
L. rhamnosus GR-1 and *L. reuteri* RC-14 (Chr. Hansen, Denmark)	Reduction in, and better treatment of, urogenital infections in women taking oral lactobacilli daily for 2 months
L. salivarius spp. *salivarius* CECT5713	Recovery of inflamed tissue in TNBS model of rat colitis, increase in TNF-α and iNOS (inducible NO synthase) expression
L. reuteri ATCC 55730 (BioGaia, Sweden)	For treatment of diarrhoea and to produce CD4-positive T- lymphocytes in the ileal epithelium

Lactobacillus plantarum 299V (Probi, Sweden)	Decreased incidence of infections in liver transplant patients
Lactobacillus acidophilus La5 (Chr. Hansen, Denmark)	Suppression of *Helicobacter pylori* with use of La5 and B. lactis BB12 yogurt given twice daily after a meal for 6 wks
L. acidophilus L1 (Campina Melkunie, Holland)	Fermented milk containing this organism was found to lower serum cholesterol – this would translate to 6–10% reduction in risk for coronary heart disease
Lactobacillus johnsonii La1 (Nestle, Switzerland)	A moderate but significant difference in *H. pylori* colonization was detected in children receiving live La1
Lactobacillus paracasei LP-33 (Uni-President Enterprise Corp., Tainan, Taiwan)	Effectively and safely improved the quality of life of patients with allergic rhinitis
Lactobacillus brevis CD2 (VSL Pharmaceuticals, Inc., Fort Lauderdale, FL).	Decreases *Helicobacter pylori* colonization, thus reducing polyamine biosynthesis
Lactobacillus gasseri OLL 2716 (Meiji Milk Products, Tokyo, Japan).	Yogurt containing this organism suppressed *H. pylori* and reduced gastric mucosal inflammation
L. fermentum, L.reuteri	Improvement of histology in a TNBS model of rat colitis, decreased levels of TNF-α and i-NOS expression
VSL#3 (VSL Pharmaceuticals, Inc., Fort Lauderdale, FL)	Effective for the management of remission of pouchitis and colitis
Saccharomyces cerevisiae boulardii lyo (Biocodex, France)	250 mg treatment for 5 days reduced the duration of acute diarrhoea and the duration of hospital

Bifobacterium animalis/lactis BB12 (Chr. Hansen, Denmark/Nestle, Switzerland)	Various effects including prevention and treatment of diarrhoea
B. animalis/lactis DN-173 010 (Danone, France)	Two to three servings per day helps with regularity
Bifidobacterium longum BL1 (Morinaga, Japan)	3 x 100 mL per day low-fat drinking yogurt prepared with the two starter cultures plus *B. longum* BL1 resulted in some evidence of lower serum cholesterol
Bifidobacterium lactis HN01	The ex vivo phagocytic capacity of mononuclear and polymorphonuclear phagocytes and the tumoricidal activity of natural killer cells were elevated
Bifobacterium infantis 35624 (Ardeypharm, Germany)	Taken in a malted milk drink for 8 weeks, shown to relieve abdominal pain/discomfort,bloating/distention, and bowel movement.
BIFICO (3 bifidobacteria species)	Prevention of flare-ups of chronic ulcerative colitis, inactivation of NF-κB, decreased expressions of TNF-α and IL-1β and elevated expression of IL-10
L. rhamnosus GG, L. rhamnosus Lc705, P.freudenreichii spp.. *shermanii JS and B. breveBb99*	Alleviating irritable bowel syndrome symptoms
E. coli Nissle 1917	use in treatment of colitis

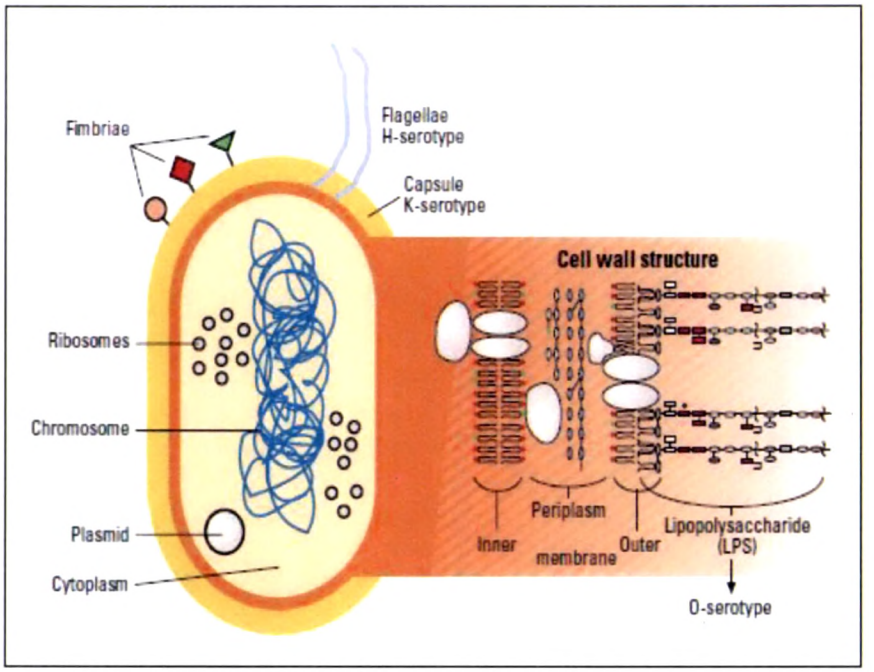
1.7 *Escherichia coli*

Theodor Escherich delivered a lecture to the Society of Morphology and Physiology in Munich bearing the title "The intestinal bacteria of the neonate and infant"

in 1885. He reported on his research results meconium of the neonate is sterile, but is colonised by microorganisms in the first few hours of life. He noticed mainly two types of bacteria in the infant faeces, which he described as *"obligate species of the faeces of milk-fed infants"*. One of these was found particularly frequently in the lower sections of the gut – as he described them as typical "colonic bacteria" and gave them the species name *"Bacterium coli commune"*, the common colonic bacterium later rename as *Escherichia coli* in 1919. On the basis of his findings, Escherich described the colonisation of the upper sections of the intestine as monotonous and sparse, and stated that the rapid increase in bacteria begins at the ileocaecal valve. He postulated that bacterial multiplication was not dependent on the supply of food and that the bacteria would meet their nutritional requirements from the metabolisation of intestinal secretions.

The *E. coli* bacterium is a gram-negative rod of about 1.1–1.5 μm x 2.0 – 6.0 μm in size. It grows under aerobic and anaerobic conditions (facultatively 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's 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 (**Fig. 1.2**).

Fig. 1.2: Diagramatic longitudinal cross-section and cell wall structure of a gram-negative *E. coli* serotype O6:K5:H1 (Schulze *et al.*, 2006).



According to Srivastava *et al.*, (2009) around 50,000- 100,000 different serotypes may occur in nature arising from the combination of different antigen structures. To date 173 surfaces (O), 103 capsular (K) and 56 flagellar (H) antigens are known in *E. coli*. In addition, there are also more than 100 adhesin variants cause further differences in serological behaviour and exhibit differences in receptor recognition. However, the number of pathogenic serotypes is limited.

Escherich was convinced that *E. coli* is a "harmless commensal" (Tenaillon *et al.*, 2010). He was right because most of the approximately 50,000 serotypes mentioned must be counted among the commensal gut organisms. However, Escherich reported *"on cystitis in children provoked by E. coli.* He hypothesised that the intestinal bacteria could be considered as a source of urinary tract infections (bladder and kidney infections).

Pathogenic *E. coli* variants are characterised by the presence of various virulence factors, such as various toxins, particular fimbrial adhesins, invasins or secretion systems. *E. coli* pathogenic in the intestine are divided at present, on the basis of their virulence factors discovered in recent years, into 6 classes, which cause diarrhoeal illnesses with different clinical manifestations (Kaper *et al.*, 2004). Pathogenic *E. coli* present in the small intestine are divided into six classes' based on their virulence factors, which cause diarrhoeal illnesses.

1.7.1 Intra- and extra-intestinal pathogenic *E. coli* variants (Kaper *et al.*, 2004).

Enterotoxic *E. coli* (ETEC):

These *E. coli* cause diarrhoea in infants and travelers (travelers' diarrhoea) in underdeveloped countries or regions of poor sanitation. ETEC infections manifest themselves as minor discomfort to a severe cholera-like syndrome. Enterotoxins produced by ETEC include the LT (heat-labile) toxin and/or the ST (heat-stable) toxin which cause damage to enterocytes.

Enteraggregative *E. coli* (EAggEC):

These *E. coli* cause persistent watery diarrhoea in young children. EAggEC adhere to the intestinal mucosa and cause non-bloody diarrhoea without invading or causing inflammation. The organism produces a toxin, which resembles the heat stable toxin of ETEC.

Enteroinvasive *E. coli* (EIEC):

Closely resemble *Shigella* in their pathogenic mechanisms and the kind of clinical illness they produce. They penetrate and multiply within epithelial cells of the colon causing widespread cell destruction. The clinical syndrome is identical to *Shigella* dysentery and includes dysentery-like diarrhoea with fever. Like *Shigella*, EIEC are invasive organisms, but they do not produce LT or ST toxin and, unlike *Shigella*, they do not produce the shiga toxin.

Enteropathogenic *E. coli* (EPEC):

Induce watery diarrhoea similar to ETEC, but they do not possess the same colonization factors and do not produce ST or LT toxins. They make use of specific

fimbrial adhesins called bundle-forming pili (*bfp*) to dock onto the intestinal mucosa and inject signal proteins into the epithelial cell that leads to destruction of the cell.

Enteroheamorrhagic *E. coli* (EHEC):

These are the causative agent of bloody diarrhoea. They enter the mucosal cell and release toxins similar to the shiga toxin and enterohemolysin, leading to bloody lesions in the intestine.

Diffusely Adherent *E. coli* (DAEC):

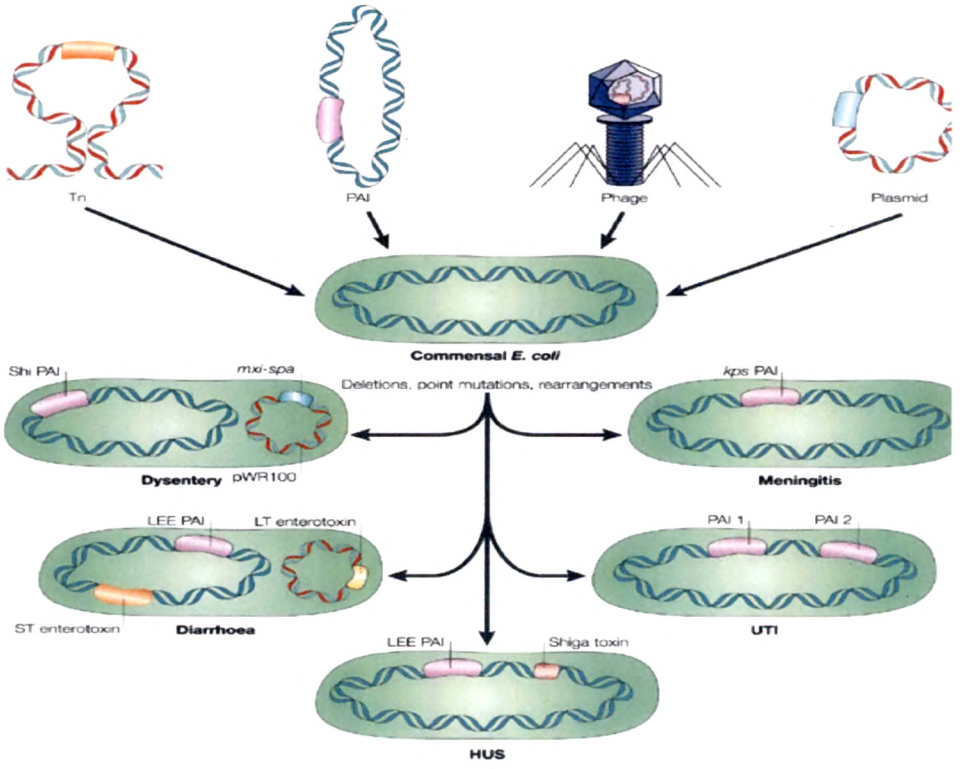
E. coli (DAEC) strains are a major cause of urinary tract infections worldwide, but its role as a causative agent of diarrhoea is controversial.

1.7.2 *E. coli*: Transition between commensalism and pathogenicity

Host and the environment determine the genetic structure of commensal *E. coli* (Tenaillon *et al.*, 2010). After sequencing the complete genome of the *E. coli* K-12 strain MG 1655 in 1997, genomes of non-pathogenic and pathogenic variants of the species were decoded. Analysis of the genome sequence data led to the realisation that bacterial genomes frequently consist of a core genome, but possess DNA sequences that have possibly been integrated into the genome by horizontal gene transfer. Mobile genetic elements are frequently involved in horizontal gene transfer of virulence-associated genes. Acquisition of genetic information as a result of lateral spread of mobile genetic elements contributes to constant and rapid evolution of bacterial pathogens. The integration of foreign DNA by means of mobile genetic elements (e.g.plasmids, bacteriophages, transposons), mutations and intra-genomic relocations could make a significant contribution to the creation of a flexible gene pool (Kaper *et al.*, 2004). When pathogenic variants come into being by horizontal gene transfer what are known as the "pathogenicity islands" (PAIs - large chromosomal DNA regions with DNA sequences which differ greatly in their composition from the core genome) also play a crucial role **Fig 1.3**. Large clusters of virulence PAIs can be found on plasmids or integrated into the chromosome in pathogenic bacteria but they are not found in

commensal bacteria (Hacker and Kaper, 2000). PAIs are usually flanked by mobile genetic elements such as bacteriophages, insertion sequences or transposons and often insert near tRNA genes.

Fig.1.3: *E. coli* virulence factors encoded by mobile genetic elements (Tn): Heat stable enterotoxin (ST) of ETEC), plasmids (for example, heat-labile enterotoxin (LT) of ETEC and invasion factors of EIEC), bacteriophage (for example, Shiga toxin of EHEC) and pathogenicity islands (PAIs) — for example, the locus of enterocyte effacement (LEE) of EPEC/EHEC and PAIs I and II of UPEC. Commensal *E. coli* can also undergo deletions resulting in 'black holes', point mutations or other DNA rearrangements that can contribute to virulence. These additions, deletions and other genetic changes can give rise to pathogenic *E. coli* forms capable of causing diarrhoea (EPEC, EHEC, EAEC, and DAEC), dysentery (EIEC), haemolytic uremic syndrome (EHEC), urinary tract infections (UPEC) and meningitis (MNEC). HUS, haemolytic uremic syndrome; UTI, urinary tract infection (Kaper *et al.*, 2004).



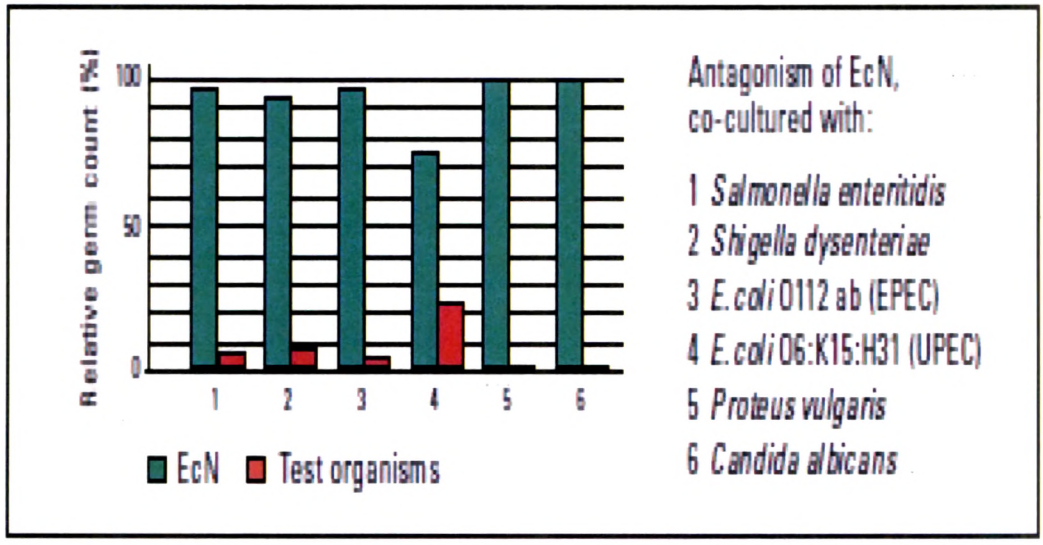
1.8 *Escherichia coli* as probiotic and its clinical significance

1.8.1 *Escherichia. coli* Nissle 1917

During the end of the 19th century Medical Microbiology experienced a tremendous upswing, mainly due to the pioneering discoveries of Pasteur, Koch, Escherich, Ehrlich, Metchnikoff and many others. However the question remained unanswered whether the intestinal flora as a whole or certain intestinal micro-organisms tend to have a positive influence, or more of a negative influence, on the host, and equally unanswered was the question concerning the mechanisms regulating the bacteria that co-exist in the GI tract (Sonnenborn and Schulze, 2009). Alfred Nissle, a bacteriologist, raised further questions on the basis of his studies of intestinal micro-organisms. "Why and how do bacteria mutually influence each other in their growth in a mixed culture such as is present in the intestine?" or "Why do the intestines of just a few people remain healthy in the face of epidemic-like diarrhoeal diseases?" After working experimentally on these questions for several years, Nissle presented his lecture entitled "On the principles of a new causal control of pathological intestinal flora" Nissle had developed an "Antagonism Index" to identify these intestinal micro-organisms and found "more strongly" and "more weakly" antagonistic strains among the *E. coli* strains he had isolated from the intestinal tract. He also used this method to determine the antagonistic activity of various *E. coli* bacteria against each other. During the First World War he found *E. coli* strains with a particularly strong inhibitory action in two patients who had *"never shown any inclination to intestinal diseases and had specifically not contracted any infectious intestinal process when a fairly large proportion of those around them had contracted such infections, and who were exposed to infection to a most substantial degree as a consequence of living in close contact with those already sick"*. Nissle tested these strains, which also retained their antagonistic effectiveness under laboratory conditions, firstly on himself and on healthy individuals in order to establish their safety to humans following oral administration. Subsequently this, he treated patients experimentally, firstly those with infectious diarrhoea, paratyphoid fever B, shigellosis and chronic salmonella carriers, and later patients with post-dysenteric intestinal

dysfunction ("irritable colon") or chronic habitual constipation. Nissle obtained another *E. coli* isolate with a particularly strong "antagonistic potency" from the faeces. He thereby created the basis for a medicine containing live *E. coli* bacteria as its active principle. Nissle registered the name "MUTAFLOR" for this medicine, and it acquired protection as a trademark on 1st March 1917. Later the strain of *E. coli* contained in Mutaflor® was given the strain designation "Nissle 1917", abbreviated to "EcN", acted *in vitro* against various pathogenic *E. coli* strains (**Fig 1.4**).

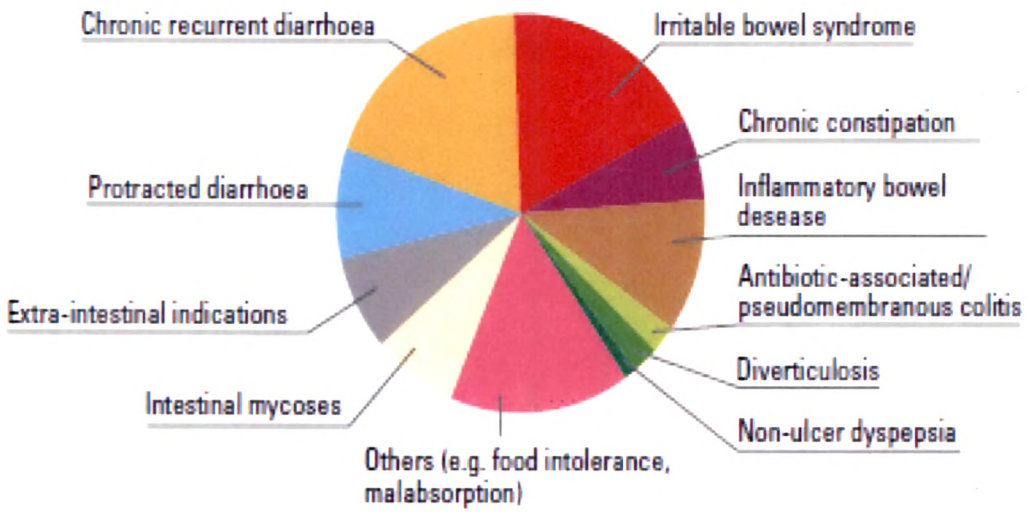
Fig.1.4: Antagonism of *E. coli* Nissle 1917 (EcN) against various micro-organisms under *in vitro* condition (Schulze *et al.*, 2006)



Determination of non-pathogenicity or non-virulence is of particular importance in therapeutic use of *E. coli* as probiotic. Advances in molecular genetics differentiation and identification methods enabled to distinguish pathogenic *E. coli* variants unequivocally from non-pathogenic strains. In addition, non-pathogenic strains such as *E. coli* Nissle 1917 exhibit no harmful effects in toxicological studies of both conventionally kept and germ-free animals (Schulze *et al.*, 2006). *E. coli* Nissle 1917 (EcN), fulfils all characteristics of probiotic mentioned in **Table 1.3**. Genome sequence revealed the

absence of pathogenicity factors (e.g. of enterotoxins, haemolysins, cytotoxins, invasins, pathogen-specific fimbriae), combined with the presence of "fitness factors" (e.g. microcins, iron uptake systems, typical adhesins) which enable the micro-organism to survive in and colonise the intestine (Grozdznov *et al.*, 2004). *E. coli* strain Nissle 1917 has been used widely in human as a therapy for dirrahoea, inflammatory bowel disease, constipation and ulcerative colitis (**Fig 1.5**).

Fig 1.5: Therapeutic potential of Mutaflor® (Schulze *et al.*, 2006)



1.8.2 *Escherichia coli* strain M-17 (EC-M17) 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 signalling.

1.8.3 *Escherichia coli* H22 inhibits pathogenic or potentially pathogenic strains of at least seven genera of the family Enterobacteriaceae (*Enterobacter*, *Escherichia*, *Klebsiella*, *Morganella*, *Salmonella*, *Shigella*, and *Yersinia*) in both *in vitro* and *in vivo*

conditions. Although the nature of *in vivo* inhibition remains unclear, the *in vitro* inhibition of these strains has been shown to be mediated by production of microcin C7 (Smajs *et al.*, 2008), colicins E1 and Ib, aerobin and an unidentified phage (Cursino *et al.*, 2006). Simultaneous administration of the probiotic and the enteric pathogen *Shigella flexneri* to germ-free mice resulted in a strong inhibition of the pathogen, which was attributed to its microcin production. Thus *E. coli* strain H22 acts as one of the most potent probiotic strain for livestock and humans.

1.9 Significance of *E. coli* in Genetic engineering

The importance of *E. coli* to international scientific advance is also underlined by the fact that 14 Nobel prizes have been awarded in the last 50 years for work on and with *E. coli*. Human insulin could be manufactured with many improvisations in the genetic modifications of *E. coli* (Miralles *et al.*, 2009). Genetic engineering and biotechnology has now succeeded in manufacturing recombinant medicines (Garcia *et al.*, 2009). Genetic engineering tools are well standardized for *E. coli* making it a preferred organism for metabolic engineering and developing computational programs or algorithms for the metabolic and regulatory networks (Baumler *et al.*, 2011).

1.10 Genetic engineering of probiotics

Genetic engineered probiotic is of dual connotation: the genetic engineering of a previously non-probiotic to acquire probiotic properties and the genetic engineering of a known probiotic to enhance its probiotic properties, both leading to the creation of new, genetically manipulated (GM) probiotics. A number of studies have been reported that utilize a diverse combination of tools to design probiotics. Heterologous protein expression is an obvious approach (Paton *et al.*, 2006). In this, one can make use of expression systems from native cDNA clones as well as of elaborate products of modern DNA technology such as synthetic genes and designed proteins. One can also expand the range of possible active components beyond protein therapeutics by engineering the metabolism of microorganisms through the integration of foreign enzymes. Metabolic engineering allows the use of, for example, modified lipopolysaccharide and anti-

inflammatory via the incorporation of new metabolic pathways (LeBlanc *et al.*, 2010; Paton *et al.*, 2011).

1.10.1 Sequestration of toxins

Shigella dysenteriae causes bacillary dysentery by Stx toxin which is composed of a catalytic A subunit, the actual toxin and a pentameric B subunit that is responsible for binding to its receptor, globotriaosyl ceramide (Gb3, or Galα [1➔ 4]Galβ [1➔ 4]Glc-ceramide, found in all human pathogens) or globotetraosyl ceramide depending on the Stx type. Antibiotic treatment leads to a sudden release of surface-associated Stx from the pathogen, which only aggravates the situation. By introducing the genes *lgt* C and *lgt* E from *Neisseria meningitidis* and *N. gonorrhoeae,* respectively, encoding glycosyl transferases that account for the incorporation of the oligosacharide structure Galα [1➔4] Galβ [1➔4]Glc into *E. coli*, a strain with an altered LPS structure was obtained (Paton *et al.*, 2001). This strain displayed high binding activity for Stx. When administered twice daily to mice infected with STEC, the new strain provided complete protection.

1.10.2 Replacement therapy

Probiotic therapies can be grouped as 'replacement therapies', representing an approach whereby a noxious micro-organism is replaced in its ecological niche by a more potent but harmless competitor. Some of these therapeutic approaches are used in the treatment of various periodontal conditions like gingivitis and oral cavity disorders including tooth decay or periodontal disease (Fernandez *et al.*, 2010). *In-vitro Lactobacillus rhamnosus GG* inhibits colonization by *Streptococcal* cariogenic pathogens, and therefore reduce tooth decay incidence in children (Fernandez *et al.*, 2010). *L. reuteri*, effective in treatment of gingivitis and bacterial plaque deposition in patients with moderate-to-severe gingivitis.

1.10.3 Strategies that involve antibody production

Neutralizing antibodies that are directed towards a pathogen, toxin, cytokine or other agent have proved very valuable and specific tools in medicine (Steidler *et al.*,

2006).With the emergence of single chain (ScFv) antibody technology, it has now become possible to produce neutralizing antibodies from recombinant bacteria. Most of the work in this area relates to the production per se of the antibody for downstream processing and use as a purified protein. Owing to their structure, these peptides suffer from very short half lives *in vivo* hence suitable delivery systems are required to allow their use as therapeutics. A number of applications are now emerging in which the expressor strain itself is used for *in situ* production of the antibody fragment especially to control colonization by pathogens.

Candida albicans is a most frequent causative agent of mucosal inflamation in humans. Infections are seen in the mouth and oesophagus of immune compromised persons such as HIV infected subjects. *C. albicans* also causes acute vaginitis in otherwise healthy women. *S. gordonii* species with good vaginal colonization and heterologous expression potential *in vivo* used for the eradication of *C. albicans* infections (Beninati *et al.*, 2000). Anti-idiotypic ScFv were produced, the surface of which resembled the structure of a wide-spectrum killer toxin of *Pichia anomala.* (Magliani *et al.*, 1997). Two *S. gordonii* strains were constructed: one that expressed the ScFv at its surface and a second that secreted the ScFv. Both surface bound and secreted ScFv showed candidacidal activity over a wide concentration range. Both *S. gordonii* strains successfully colonized the vagina and cleared experimental *C. albicans* infection in rats, this being dependent on the presence of this ScFv.

1.10.4 Immune intervention

Immune intervention strategies are those in which the immune system is actively engaged for the acquisition of health benefits. Genetically modified *Lactococcus lactis* secreting interleukin 10 provides a therapeutic approach for inflammatory bowel disease. IL-10 is a strong anti-inflammatory cytokine that has shown promise in clinical trials for the treatment of IBD (LeBlanc *et al.*, 2010). A probiotic strain of *E. coli*, engineered to secrete HIV-gp41-hemolysin A hybrid peptides, which block HIV fusion and entry into target cells. Administered orally or as a rectal suppository, this "live microbicide" (Lagenaur and Berger, 2005) colonizes the mucosa and secretes the peptide *in situ*,

thereby providing protection at the mucosal barrier, preventing the virus from entering the blood stream and causing a systemic infection. This approach can potentially provide protection in advance of HIV exposure for up to a month. Other anti-HIV probiotics currently in development include a genetically engineered *S. gordonii* which produces cyanovirin-N, a potent HIV-inactivating protein originally isolated from cyanobacterium, and a natural human vaginal isolate of *Lactobacillus jensenii* modified to secrete two-domain CD4 which inhibits HIV entry into target cells (Chang *et al.*, 2003).