CHAPTER 2

REVIEW OF LITERATURE

2.1. HISTORY OF PROBIOTICS:

Elie Metchnikoff (a Russian scientist) postulated that the health benefiting bacteria which can afford a long life as well as antagonistic effect is offered by a lactic acid bacteria (LAB) group. It was his suggestion that this group of bacteria can replace "intestinal autointoxication" by accumulation of good bacteria which improves the gut health of the host. The non-disease forming strains of *E. coli* which was isolated from the fecal matter of a soldier by a German professor Alfred Nissle during World War I and he confirmed that this *E. coli* strain Nissle 1917 is one of probiotic bacteria but do not belong to the LAB group. The other example of LAB includes Bifidobacterium which was discovered by Henry Tissier from an infant that consumes breast milk, and is recognized as *Bacillus bifidus communis*. He also claimed that ingestion of these probiotic by the diseased host can replace the pathogenic bacteria and will show improvement to its health.

The term probiotic was taken from Greek word 'pro' and 'biotos' that actually means 'for life'. Lilly and Stillwell (1965) first narrate this as a substance or the living organism itself secreted by any of the microorganism that promotes their growth. Fuller used the term probiotic as a living food supplement derived from bacteria that can improves its intestinal microbial balance [Fuller, 2012]. Scientists also evaluated the probiotics as a living microorganism which can contribute a health upliftment when administrated in requisite proportions [Hill *et al.* 2014].

Table Definitions used by the international scientific associations for probiotics and prebiotics	
Probiotics	Live microorganisms that confer a health benefit on the host when administered in adequate amounts
Prebiotic	Selectively fermented ingredients that result in specific changes in the composition and/or activity of the gastrointestinal microbiota, thus conferring benefit(s) upon host health
Synbiotics	Products that contain both probiotics and prebiotics

2.2. PROBIOTICS:

Several different types of product which is formulated as drugs or other dietary supplements that are originated from live microbes i.e. probiotics. Several different micro-organisms are considered as a potent probiotic based on their biological and clinical aspects. The majorities of these organisms is of human origin and belong to the LAB group. The LAB predominantly includes *Lactobacillus* and also *Bifidobacterium* but from varied species and strains which altogether is considered as potential probiotic [Kleerebezem and Vaughan, 2009]. Moreover, the other species of bacteria which is included into this LAB group are *Streptococcus*, *Propionibacterium*, *Enterococcus* and *Bacillus*. Whereas, bacteria not included in the LAB groups but are potentially a probiotic are *Escherichia coli* Nissle 1917 and *Saccharomyces* [Gareau *et al.* 2010].

LAB has the ability to colonize the gut of humans. It belongs to anaerobic bacteria group, which is generally Gram positive in nature and non- spore former. These are the bacteria that are found in a different plant source, gastrointestinal tract of mammals and other animals too, surface soil and water, some fermented foods etc. [Vrese and Schrezenmeir, 2008]. The LAB group is the combination of different bacteria such as, *Lactobacillus, Enterococcus, Lactococcus, Streptococcus, Leuconostoc, Pediococcus, Macrobacterium, Bifidobacterium,* and

Propionibacterium [Holzapfel *et al.* 2002]. This group of bacteria has gained high attention in the field of pharmaceutical, fermented food and many other eras [Harzallah and Belhadj, 2013]. They are also used in the food industry to increase the texture, aroma and acidic property of the product [Leroy and Vuyst, 2004]. The two important Lactic Acid Bacteria which restricts the proliferation of the pathogenic and spoilage bacteria are *Lactococcus lactis* and *Streptococcus thermophilus* which exerts and preserve nutritive value of the food [Klaenhammer *et al.* 2002].

List of microorganisms studied as probiotics

***Bifidobacterium* sp.**	***Lactobacillus* sp.**	***Bacillus* sp.**	**other LAB**	**other microorganisms**
B. animalis, *B. animalis* subsp. *lactis*, *B. adolescentis*, *B. bifidum*, *B. longum* subsp. *infantis*, *B. longum* subsp. *longum*, *B. breve*	*L. acidophilu*, *L. delbrueckii*, *L. casei*, *L. fermentum*, *L. helveticus*, *L. paracasei*, *L. johnsonii*, *L. plantarum*, *L. sakei*, *L. gasseri*, *L. salivarius*, *L. reuteri*, *L. rhamnosus*	*Bac. coagulans*, *Bac. subtilis*, *Bac. cereus*, *Bac. clausii*, *Bac. licheniformis*, *Bac. mesentericus*	*Enterococcus faecalis*, *E. faecium*, *Lactococcus lactis*, *Leuconostoc mesenteroides*, *Pediococcus acidilactici*, *P. pentosaceus*, *Streptococcus salivarius*, *Str. thermophilus*	*Escherichia coli*, *Saccharomyces cerevisiae* subsp. *cerevisiae*, *Saccharomyces cerevisiae* subsp. *boulardii*

Adapted from Foligne et al. (2013)

2.3. SOURCES OF PROBIOTICS:

The sources of probiotic bacteria are widely distributed in different niches. Milk along with milk products are key sources of probiotics. The potential sources of probiotic also include traditional fermented food products that mostly include *Lactobacillus* genera. Human breast milk is now a primary source of LAB which has been studied extensively. A group of bacteria viz. Lactobacilli, Bifidobacteria, Micrococci, Streptococci, Lactococci, Enterococci and Staphylococci are generally present in human milk [Martin *et al.* 2003]. Other than the human

milk, the other primary source of probiotic micro-organism includes gastrointestinal tract, fecal matter of the mammals and many others [Butel *et al.* 2014].

2.4. SELECTION CRITERIA FOR PROBIOTIC:

The selective qualities of the microbes to be considered as a probiotics are tolerance to adverse condition of the gastrointestinal conditions, adherence property to the intestinal mucosa, antagonism against pathogens etc. Thus, based on all the following qualities microbes are considered as a probiotic [Fontana *et al.* 2013];

- Must have the potential to resist the adverse condtions of the gastrointestinal tract.
- Must not have any pathogenicity as well as toxicity to the host cells.
- Must have a defined beneficial role on the intestinal ecosystem.
- Must have longer viability in all the products if commercialized.
- Must be safe to the human host if administered externally.
- Must attain the ability to be preserved for maintaining the quality of the products containing probiotic.

Thus it can summarize as the fact that selection criteria of a bacteria to be a potent probiotic must possess human trials along with *in vitro* & *in vivo* tests before formulating it as an alternative therapeutics as per the guidelines of ICMR and DBT [Ganguly et al. 2011].

The guidelines suggested that the following criteria must be attained by the microbes that are putative probiotics and are considered as so:

(a) Resistivity to gastric juice.

(b) Resistivity to bile.

(c) Resistivity/antagonistic activity against the potential pathogenic bacteria.

(d) Competitive adherence ability with the pathogens.

(e) Resistivity towards low pH.

(f) Capability to adhere to the intestinal wall.

All these parameters are stipulated on the basis of the harsh condition of the gut environment that is investigated in *in vitro* conditions and also in *in vivo* trials in different animal models (mammals) prior to apply to humans.

To consider a particular strain safe for consumption it must go through different toxicological tests. The experiments which are indispensible for the mentioning a strain as a probiotic are;

- The effect of the bacteria on animal models as well as cell lines.
- Investigation of short term or long term side effects on application of the probiotics.
- The determination of the antibiotic susceptibility.
- Identification of toxin production or hemolytic activity.
- Evaluation of the strains on humans to determine its safety.

2.5. IDENTIFICATION CRITERIA OF PROBIOTIC:

For selecting a bacterium as a potent probiotic the most important criteria of the identification is its taxonomic classification. The taxonomic classification is based on phenotypic and genotypic classification [Vandamme *et al.* 1996]. Some traditional phenotypic characteristics include sugar fermentation profiling, different enzyme assay, optimum condition determination and many other biochemical characteristics [Fontana *et al.*2013]. However, the most widely used phenomenon is 16S rDNA sequencing and construction of the phylogenetic classification of organisms. The sequence further obtained was used for analysis to those available in databases like GenBank, DDBJ, EMBL etc. [Amar *et al.* 2007].

Generally, for identification 16S rDNA was extracted and amplified to obtain a result with the technology used are polyacrylamide gel electrophoresis (PAGE), DGGE (Denaturing Gradient Gel Electrophoresis), RFLP (Restriction Fragment Length Polymorphism) and FISH (Fluorescence *in situ* Hybridization) [Mohania *et al.* 2008].

Thus, these are the most useful and popular tools used to identify and characterize the potent strains. The genotypic characterization is a technique to identify the strains of the culture at the molecular level.

2.6. PHENOTYPIC CHARACTERIZATION OF PROBIOTIC:

Probiotics are classified based on some pre-determined phenotypic properties which include morphological arrangement, glucose or other carbohydrate utilization, determination of optimum temperatures, pH and salt concentrations [Mohania *et al.* 2008]. There are numerous limitations of genomic identification which leads to molecular categorization as the confirmatory test.

2.7. MOLECULAR CHARACTERIZATION OF PROBIOTIC:

In recent days, the advanced technologies used for the identification of the bacteria in strain level are important and significant. Phenotypic characterization mat show a variety of flaws, thus molecular methods is an alternative tool of it. Some of the primers specific identification genes are 16S rDNA and 23S ribosomal RNA have been used for identification. The techniques used for such identifications are RAPD, DGGE, RFLP, PFGE, restriction enzyme analysis and AFLP [Mohania *et al.* 2008].

Among this all techniques mentioned earlier, 16S rRNA is the most familiar gene that is generally aimed for identification due to its highly conserved sequences which became the universal marker for determining the phylogenetic diversity [Kimura *et al.* 2014]. However, due to the presence of the conserved sequence of the genus available the target sequence were matched and evaluated. 16S rDNA is an authentic tool commonly used for the probiotic identification [Naser *et al.* 2007].

2.8. SURVIVALITY OF THE PROBIOTICS IN GASTROINTESTINAL TRACT CONDITIONS:

The screening method that is widely used for the screening of probiotic bacteria are basically *in vitro* screening that allows the catagoeoging of a large number of isolates [Papadimitriou *et al.*, 2015]. Based on the definition the entire probiotic organism must contain the tolerance to bile and gastric acid [Salminen *et al.* 1999]. A group of studies reportedly claimed that these probiotic organisms are able to tolerate the simulated gastric fluid conditions. It was observed that after ingestion the probiotic must tolerate the acidic environment. The stipulated pH of the stomach varies from 1-3 whereas, after consumption of the food it varies from 4-8

[Papadimitriou *et al.* 2015]. Different popular microbiological techniques are used to identify the potent putative probiotic by assaying optimum parameters of it. Other than simulated gastric fluid tolerance identification, the probiotic are also assayed by its survival ability on pancreatic juices (containing pepsin and pancreatin) [Fernandez *et al.* 2003].

Bile present in the small intestine initiates disruption of the cell membrane which induces protein folding and denaturation of the DNA [Papadimitriou *et al.* 2015]. The tolerance of bile upto 0.5% has been widely accepted for the identification of the probiotic. [Gorbach and Goldin, 1992] The probiotic bacteria use the ability to reduce the toxicity of bile by producing bile salt hydrolase enzyme [Begley *et al.* 2005].

2.9. ADHESION:

One of the primary properties of the probiotic is its adhesion to the epithelial cells which reflect its ability to colonize and impact its health improvement on the host. The competitive ability of the probiotic bacteria with the pathogenic organisms on the basis of their adhesion to the target sites, such as, epithelial cells is it main criteria. The adhesion property is categorized as a beneficial characteristic but some conflict to this suggestion remained which says that this is a potential risk for immune suppressed individuals [Sanders *et al.* 2010]. There are several *in vitro* assays for determining the adhesive nature of a culture being assessed for probiotic which include hydrophobicity on the cell surface, auto-aggregation property, adhesion to cell lines, adhesion to intestinal fragments, adhesion to mucus etc. One of the most conventional methods is that the cell surface hydrophobicity is used to determine the hydrophobic nature of the bacteria [Kaushik *et al*. 2009].

2.10. ANTI-MICROBIAL ACTIVITY:

The disturbances in intestinal microbiota may cause the gastrointestinal diseases. Probiotics have been suggested by many researchers to elevate different conditions to maintain the microbial balance of the intestine [Hickson, 2011]. The probiotic bacteria are useful in acting against infectious diarrhea [Vrese and Marteau, 2007], respiratory tract infections [Kechaou *et al.* 2013], infection of *H. pylori* [Chenoll *et al.* 2011] and urogenital dysfunction [Strus *et al.* 2012]. The anti-microbial property of the probiotics include synthesis of different organic acids as well as antimicrobial compounds called bacteriocins which is active against all group of bacteria [Devi and Halami, 2013]. Different test were considered for assayinf the anti-microbial activity, they are, paper disk diffusion and agar cup well diffusion assay are most extensively used for determining antimicrobial activity [Balouiri *et al.* 2016]. However, the antimicrobial spectrum activity against pathogens and normal microflora needs to be evaluated and already reported in many studies.

2.11. ANTIOXIDANT ACTIVITY:

The free radicals that were generated during passage of nutrients through the gastrointestinal tract are highly toxic for the host. These free radicals cause cirrhosis, atherosclerosis, cancer and other chronic diseases [Ljungh and Wadstrom, 2006]. Reportedly, different probiotics were found to prevent linoleic acid peroxidation and thus aid in scavenge free radicals [Lin and Chang, 2000]. Radical scavenging activity of *Lactobacillus* was found to be closely dependent [Wang *et al.* 2009]. It was further claimed by different researchers that some cell surface components may also be responsible for the anti-oxidative nature of the probiotics [Wang et al. 2009].

2.12. HEALTH BENEFITS OF PROBIOTICS:

On the basis of their antimicrobial activity, intestinal microbial modulation, protection of mucosal barrier and immune regulation probiotic can be considered as a potent alternative of therapeutics against different health disorders. The beneficial health effects of probiotics are broadly categorized into three sections, namely maintenance of the gut icrobial ecosystem, metabolic effect on the intestine and immune-modulation [Bermudez-Brito *et al.* 2012]. Besides that, these bacteria also help in maintaining the microbial balance either by destroying the pathogens or by blocking the adhesion sites. Thus in these ways they are capable of acting against human GI disorders. Sometimes, it is observed that probiotics help in the digestion process by secreting short chain fatty acids (SCFAs), vitamins etc. Though in recent times, several beneficial roles of probiotics have been highlighted such as treatment against kidney stones, modulation of the nervous system and psychological effects [Hemarajata and Versalovic, 2013]. Some other prominent health disorders are as follows;

2.12.1. GASTROINTESTINAL DISORDERS:

Probiotics elevates the therapeutic use of the live bacteria against different human pathogens as mentioned. It helps to flow out intestinal malabsorption by secreting several enzymes into the gut lumen and thus aiding in digestion [Parvez *et al.* 2006]. The low pH acts against different other bacteria to grow [Sanders and Klaenhammer, 2001].

2.12.2. LACTOSE INTOLERANCE:

Lactose intolerance or lactose mal-absorption is triggered by the absence of the lactase enzyme which leads to incomplete breakdown of lactose of milk [Lomer *et al.* 2008]. In humans the

same took place for the similar reasons [Marteau *et al.* 2002]. Lactic acid produced by LAB in fermented milks aids in increase of the lactase enzyme activity in the gastrointestinal tract thereby relieving symptoms of lactose intolerance [Swallow, 2003].

2.12.3. BOWEL HEALTH:

Probiotics host's immune system was regulated by producing secretary IgA. Also, it has the ability to compete the attachment on epithelial cells which is also binding receptors of the pathogens [Sullivan *et al.* 1992]. In intestinal mucin the expression of MUC2 and MUC3 showed sharp inhibition towards pathogens [Mack *et al.* 1999]. This enhanced intestinal mucin prevents the attachment of the entero-pathogens. The inhibition of the growth of pathogenic bacteria can also be achieved by producing bacteriocins as well as production of lactic acid resulting in lowering of pH [Lebeer *et al*. 2008].

2.12.4. STIMULATION OF SCFA PRODUCTION:

The dominant role of the probiotics is to normalize the intestinal microbiota that initiates the fermentations by stimulation of the gut fermentation. This stimulation is achieved by the production of SCFAs (short chain fatty acids). Different probiotic have been noticed to increase the indigenous count of LAB in the gut that also produces lactate. This lactate is in turn metabolized to acetate by these probiotic bacteria [Belenguer *et al.* 2006].

2.12.5. INFLAMMATORY BOWEL DISEASE (IBD):

Several studies have shown deletion and alteration in symptoms of IBD with consumption of some specific combinations of the strains of probiotics [Ouwehand *et al.* 2002]. Studies also show that IBD can also be caused by hypersensitivity reaction in the gut which is cell-mediated

[Sartor, 2004]. Both have been reported an elevation in the secretory IgA levels in the gut [Gorbach, 2002]. The improvement of the bowel movement is possibly through a declination of gut pH. The combination therapies of *Lactobacillus*, *Bifidobacterium* and *Streptoccoccus* species relieves the gut from IBD [Snders et al. 2013].

2.12.6. IRRITABLE BOWEL SYNDROME (IBS):

It was observed that around 20% of the world population suffers from IBS [Drossman *et al*. 2002; Guyonnet *et al.* 2007]. The evidence suggests that an imbalance in the gut microbiota can be caused by enteric bacteria that probably can be associated with IBS [Drossman *et al*. 2002]. It reduces abdominal pain, bloating, flatulence, and constipation [MacFarlane and Cummings, 2002].

2.12.7. ANTI-DIABETIC AND ANTI-OBESITY EFFECTS:

It has been already documented that [Ley *et al*. 2005] probiotic has a role in different GI diorders. Intestinal microbiota was found to increase body weight and also show insulin resistance. In addition to it microbiota from obese mice on transmission to germ free mice seemed to transmit the same phenotype [Ley et al. 2006].

2.13. MECHANISM OF ACTION OF PROBIOTICS:

Broadly the mechanism of probiotic action can be broadly classified into three functions namely; enhancement of intestinal barrier function, restriction of pathogenic growth and modulation of the host immunity [Tsai *et al.* 2012]. The digestion of the human body is a composite action occurs by the action of salivary enzymes, action of gastric juices and bile acids followed by processing and absorption in the small and large intestine. Our digestive system itself is not

equipped to breakdown all types of food ingested such as plant material which resists hydrolysis. Thus, the probiotic bacteria in the gut show symbiotic association and a beneficial role too. There are bacteria which produce vitamins in modulating the environment of the gut [Falzon *et al.* 2011].

The 'Barrier effect' referred to the quality of the probiotic to resist the colonization of harmful bacteria may be brought about by competing with binding sites and preventing adhesion of pathogens. *Lactobacillus* strains have gained importance to modulate the metabolic activity of intestinal flora and its composition [Kuisma *et al.* 2003] to compete with the pathogens for binding sites. The probiotics can show potent inhibition against pathogenic adhesion by competitive banning, as shown by different *Lactobacillus*, *Bifidobacterium* and *Propionibacterium* strains in both *in vitro* and *in vivo* models [Collado *et al.* 2006]. The other agents that induce the production of the antibacterial agents such as different organic acids, bacteriocins and hydrogen peroxide that acts against harmful bacteria [Servin, 2004].

Probiotics enhance barrier function by elevating the mucus secretion, enhancing the synthesis of antimicrobial peptides & mucins, preventing apoptosis of epithelial cells and up-regulating cryoprotective heat shock proteins [Johnson-Henry *et al.* 2008]. The alternative expression of tight junction proteins by the strains of *S. thermophilus* and *L. acidophilus* has been showed in *in vitro* as well as *in vivo* studies [Resta-Lenert and Barrett, 2003].

As a summary, the probiotics when ingested have to pass through the adverse conditions of the GI tract. The cells that survive in the gut can transit and adhere to the mucosa of the intestines interacting with the host system.

Cross talk with the gut mucosa by enhancing mucus secretion and promoting the function of tight junction proteins and communicating with the underlying defense system helps in maintaining the barricade for pathogens and also positively influence on the immune response.

2.14. IMMUNOMODULATION:

Mammals having a complex barrier of immunity escalate the pathogenic bacteria from the host via different interactions of cytokines and chemokines [Hardy *et al*. 2013]. The interactions between the different microbes and the host are necessary especially in the life's initial stage as they shape the health and host's well-being. Probiotic bacteria are popular in promoting the host's shielding mechanisms. Aditionally, these beneficial bacteria also influence host intestine's defense mechanisms by balancing the normal flora, triggering a antibody-mediated immune response and tutoring the immune response of the host to act against foreign agents, such as; harmful bacteria [Wells, 2011]. The intestinal probiotic bacteria in connection with the lymphoid tissue trigger an immune reaction. The immense changes and influence that probiotic bacteria have on the defense mechanisms has led to the interest of researchers to exploit this trait as future treatment for diseases and ailments. The extreme complexity of the immune system of the host is mediated by humoral as well as cellular immune response against potential infectious agents. The response of the effective beneficial probiotics was extensively studied [McNaught *et al.* 2005] which suggest that all the models including *in vitro* as well as *in vivo* triggers both acquired and innate immunity. The total effect of the host's defense mechanism is mediated via macrophage activation, enhancement of cytokines, activating NK cells and production of antibodies [Ouwehand *et al.* 2002]. Before we venture into the immunomodulatory role of probiotic bacteria, it is important to acquaint with the gut. The selective processing of the luminal contents, the gut is able to differentiate between beneficial entities by mechanism of

tolerance registered by immunity and initiates a protective shielding effect against innate and adaptive immune responses for the presence pathogens. The immune response either towards tolerance or activation is based on the process by which the information of the antigen in contact is transported to the underlying immune cells. The protocol via which the antigens introduced in the host body are responded back is by activating APC cells which includes M cells, epithelial cells, DCs and enterocytes.

2.15. CYTOKINES:

Immune cells which are responsible in deciding the fate of immune response (activation or tolerance) are activated or directed by cell signalling molecules called cytokines. Cytokines can induce pro (IL-1β, TNF-α, IL-6, IL-8, IL-15) and anti (TGF-β, IL-10) inflammatory responses via effector cells such as DCs, granulocytes, macrophages and T & B cells [Hardy *et al.* 2013]. Numerous studies have documented the capability of the probiotics to induce cytokine production through activation or suppression. Modulation of cytokine has a direct impact on the immune function of the host cell and hence use of probiotics as cytokine modulators may be useful in prophylaxis and treatment of immune related conditions. Strains of lactobacilli have shown Th1 induction of cytokines (IFNγ, IL-12) linked to cell mediated response while other strains have shown to elicit Th2 cytokines associated with humoral responses. A number of lactobacilli and bifidobacteria have demonstrated the ability to induce anti-inflammatory cytokines TGFβ and IL-10, thus presenting as anti-inflammatory strains. Probiotics also help in activating the innate immunity against non-specific responses to pathogens and antigens and also exerts a crucial role in regulating chronic inflammation. Species of Lactobacillus and Bifidobacteria have shown to induce the secretion of IL-6, TNF-α and IL-1β in macrophages, DCs, monocytes, PBMCs and epithelial cells [Delcenserie *et al.* 2008]. However, induction of

IL-10 by probiotic bacteria shows varying responses from strain to strain. Species of different bacteria were found to induce different cytokine and chemokine secretion according to their strain specific nature and the kind of cell line used.

2.16. SELECTION OF PROBIOTICS FOR FOOD APPLICATION:

Development of a food product containing first requires the selection of an appropriate strain in adequate amount. Resistance to processing, survival through gastrointestinal tract is some of the criteria used for selecting probiotic cultures [Ventura and Perozzi, 2011]. Survival of cultures during manufacturing conditions is said to be strain specific [Tamime *et al*. 2005]. Lactobacilli tend to survive better than other LAB which showed tolerance to low pH, found naturally in fermented foods and grow better in milk and milk derived substrates [Tripathi and Giri, 2014]. Thus, several lactobacilli have technological properties suitable for food application compared to bifidobacteria.