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Review Paper

MICROBES AS BIOTHERAPEUTICS

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ABSTRACT

Probiotics are live microorganisms which when administered in adequate quantities render health benefits to humans and animals. A bacteria would be considered as probiotic if it possess several characteristics as generally regarded as safe (GRAS) proposed by USFDA Regulations. Similar terms like Prebiotics and Synbiotics are often used in association with probiotics but they are often different. Several species of bacteria like lactobacillus, bifidobacterium, streptococcus, enterococcus etc are widely used as probiotics for human and animal applications. Each Probiotic strain is unique in its origin and mechanism of action, hence only a few probiotics which has good market potential are manufactured commercially. Depending upon the strain selected for manufacturing, a unique media, a separate process for fermentation and downstream would be applied to ensure the cell viability, stability and potency for a longer duration. Microorganisms should confirm to the guidelines laid down by the Food and Agricultural Organization (FAO) or US Food and Drugs Administration (USFDA) to be conferred as Probiotics. However products manufactured in specific country should confer to the guidelines of food and drugs administration of their respective country. Recently (2011), Department of Biotechnology (DBT)) and Indian Council of Medical Research (ICMR), Government of India had laid down certain guidelines for evaluation of probiotics in food. However, no stringent regulatory guidelines are available for animal applications. There is an ever increasing demand in the consumption of Probiotics due to increasing awareness of their beneficial effects. One of the key requirements for regulators is the labelling of the product in a legible and truthful manner and this labelling applies to viable cells as well as their functional mechanism. There are instances of mislabelling the strain due to the recent changes in their nomenclature and classification and the appropriate way of representing the presence of Probiotics in their product

would be to include the genus, species and strain designation for each product and the presence of viable cells in terms of CFU/ml (colony forming units) or MSG (million spores per gram) and the expiry date. Apart from this they could also provide additional information through a separate sheet or brochure along with the product. Maintenance of cell viability and shelf life is very critical and it depends on several factors like method of manufacturing and storage etc. Another way to convince the regulators and research bodies is by advertising the appropriate methods of assaying the viable cell count and certificate of conformation by an independent third party validation.

Number of Tables: 2 **Number of Figures: 1** **Number of References: 50**

INTRODUCTION

Probiotics are live microorganisms which when administered in adequate quantities render health benefits to humans and animals (Gregor, 2006). Microbes are used in various processes of fermentation for preparation of foods and alcohol since several years. Almost a century ago, Eli Metchnikoff proposed a revolutionary idea of consuming microorganisms for promoting health (Francesca *et al.,* 2010). It was Vergin who first introduced the term Probiotic while comparing the detrimental effects of antibiotics and other antimicrobial substances on the gut microbial population (Jankovic *et al*, 2010). However the term "Probiotic" was first coined by Lily and Stillwell in 1965 in reference to substances produced by protozoans (Indu *et al.,* 2001; katerina *et al., 2012*). Dead bacteria or products derived from bacteria or end products of bacterial growth may also impart certain health benefits, but these organisms are not considered as probiotics because they are not alive when administered (Wojciech *et al.,* 2010). Similar terms like Prebiotics, Probiotics and Synbiotics are often used in association with probiotics but they are often different and could be defined as described below in table no.1 (Harish *et al.,* 2006).

Table 1

Probiotics	Live microorganisms which are used as food ingredients to render health benefits
Prebiotics	An indigestible food ingredients which render beneficial effects to the host by stimulating the growth or activity of host bacteria to improve the health benefits
Synbiotics	A mixture of probiotics and prebiotics which beneficially effects the host by improving the survival & implantation of live microbial dietary supplements in the Gastro Intestinal Tract (GIT)

A bacteria to be considered as Probiotics should possess characteristics as generally regarded as safe (GRAS) proposed for Probiotics by USFDA regulations (Allgeyer *et al.,* 2010).

Normally a Probiotic strain should possess the following characteristics: Should have a demonstrable beneficial effect on the host Should be non-pathogenic; Should be non-toxic; Shouldn't have any adverse effects on humans and animals; Should be able to survive the gastro intestinal tract environment; Should retain the stability during the intended shelf life of the product; Should contain an adequate number of viable cells to confer the health benefit; Should be compatible with product storage containers to retain the stability (Indu *et al.,* 2001; Harish *et al.,* 2006)

List of few important species of microbes used as Probiotics

1. **Genus :** Lactobacillus
   Species: *acidophilus, brevis, delbrueckii, fermentum, gasseri, johnsonii, paracasei, planatarum, reuteri, rhamnosus, salivarius*
2. **Genus:** Bifidobacterium
   Species: *adolescentis, animalis, bifidium, breve, infantis, longum*
3. **Genus:** Streptococcus
   Species: *thermophilus, salivarius*
4. **Genus:** Saccharomyces
   Species: *cerevisiae, boulardii*
5. **Genus:** Bacillus
   Species: *coagulans, clausii*
6. **Genus:** Escherichia
   Species: *coli*
7. **Genus:** Enterococcus
   Species: *faecium*

(Koji *et al.,* 2005; Julien *et al.,* 2010)

MANUFACTURING OF PROBIOTICS

Each Probiotic strain is unique in its origin and mechanism of action. Not all probiotics are manufactured commercially. The strains which are used for commercial exploitation should be ensured by the manufacturer that they are qualified under the Generally Regarded as Safe (GRAS) category (Julien *et al.,* 2010). Usually all ingredients used in production should be certified by respective food and safety assessment authorities of respective countries (Gregor *et al.,* 2006). It would be a good idea for the manufacturers to conduct few tests to assess the safety and efficacy of the strains they are producing .Traditional manufacturing of Probiotics starts initially with isolation of the desired probiotic strain in its pure form. Efforts should be taken to isolate effective and efficient probiotic strain (Jon *et al.,* 2003). There are several literatures citing various methods and sources (gut, intestine, faeces etc) for isolation of probiotic strain in pure form.

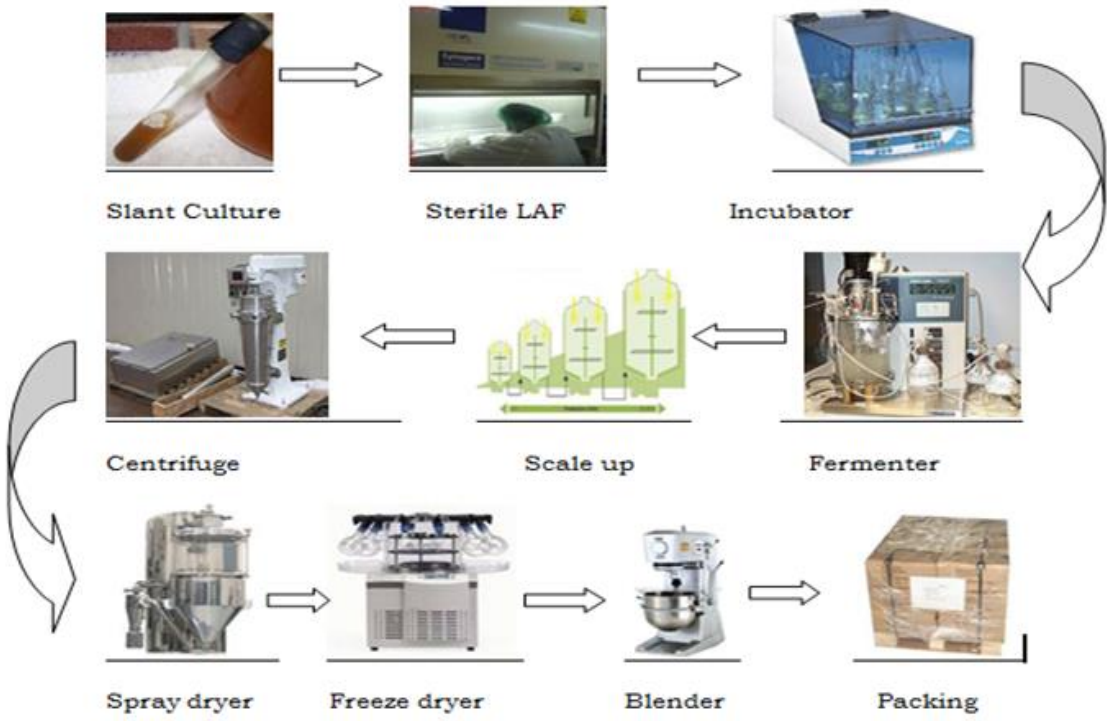
Isolated strain is then assayed and pure colony of the strain is preserved mostly in slants or in petri plates for regular usage and regular revival of the strain is necessary to keep the cell viability at its peak. Later a synthetic medium is designed specifically to meet the requirements of each strain. Inoculum preparation is a very crucial step in any manufacturing process so care should

be taken to ensure that a pure culture is selected free from contaminants and which is healthy and actively growing (Christine *et al.,* 2010). Media designing for inoculum and production is also very important because in any manufacturing process it is only the project economics which matter. So, efforts should be taken to design a media which is cheap and easily available. Sufficient amount of carbon, nitrogen and hydrogen should be present in the media to ensure that the strain won't starve during fermentation and it is allowed for sufficient growth and then after obtaining the desired amount of growth it is transferred to a pilot scale fermenter which is pre sterilized with media components (Mack *et al.,* 2004). There after depending upon the scale of operation of the plant, sufficient amount of inoculum is generated and transferred to fermenter. Once the culture reaches the production stage care should be taken to ensure for the maintenance of the optimal growth parameters of the strain so as to attain the maximum amount of final product at a faster rate in a quicker time.

Samples are monitored at regular intervals of time so as to make sure that the product is free of contaminants by standard microbial plating techniques. Once the batch reaches the final stage of production it is initially harvested by high speed centrifuges so that product could be easily separated (Gasser *et al.,* 1994). Later it is mixed with liquids and made into slurry and then it is subjected to spray drying (at high temperatures) or freeze drying (at low temperatures) and then due to adverse conditions cells are sporulated into powder form (Koji *et al.,* 2005). Later in blenders specific diluents are added and blended according to the requirement of the customer and packed accordingly (Antonio *et al*., 2010).

Before final packing of the product it is tested in quality control laboratory to ensure the viable cell count and free of contamination (Zihler *et al.,* 2010). Usually viable cell count is measured either in CFU/ml (Colony forming units) or MSG (Million spores per gram) which is later displayed on the label of the packing with date of manufacturing, batch number and expiry date.

Fig 1: *Process Flow Diagram for Manufacturing of Probiotics*



REGULATORY ASPECTS INVOLVED WITH PROBIOTICS

Microorganisms should confirm to the guidelines laid down by the Food and Agricultural Organization (FAO) or US Food and Drugs Administration (USFDA) to be conferred as Probiotics. However products manufactured in specific country should confer to the guidelines of food and drugs administration of their respective country. Even though there is a debate on the term "Probiotic" in USA and worldwide still there is an ever increasing demand in the consumption of these Probiotics due to increasing awareness of their effects. One of the key requirements for regulators is the labelling of the product in a legible and truthful manner and this labelling applies to viable cells as well as their functional mechanism (Mishra *et al.*, 1996). There are instances of mislabelling the strain due to the recent changes in their nomenclature and classification but the important aspect is that the strain should bear an appropriate strain name so that research can be carried out if necessary to investigate appropriate efficiency, efficacy and cell viability of the strain and there are many instances where the manufacturers couldn't keep up their label claims. Maintenance of cell viability and shelf life is very critical and it depends on several factors like method of manufacturing and storage etc. In few instances manufacturers does not claim anything on the label like species and number of viable cells present in their product.

Table 2: *Guidelines for the Evaluation of Probiotics for Food Applications*

I. Genus / Species / Strain Identification	❖ Speciation of the bacteria must be established using phenotypic and genotypic tests Viz: ✓ DNA- DNA Hybridization ✓ DNA Sequences encoding 16S rRNA ✓ Patterns generated from the fermentation of a range of sugars ✓ Final fermentation products obtained from glucose utilization ❖ Combination of genotypic tests with phenotypic tests to be done ❖ Strain typing has to be performed with a reproducible genetic method or using a unique phenotypic trait ✓ Pulsed Field Gel Electrophoresis (PFGE) is Gold Standard ✓ Randomly Amplified Polymorphic DNA (RAPD) ❖ Determination of the presence of extra chromosomal genetic elements, such as plasmids can contribute to strain typing
	All strains to be deposited in Internationally recognized culture collection
III. In Vitro tests to screen potential Probiotics	✓ Resistance to gastric acidity ✓ Bile acid resistance ✓ Adherence to mucus and / or human epithelial cells and cell lines ✓ Antimicrobial activity against potentially pathogenic bacteria ✓ Ability to reduce pathogen adhesion to surfaces ✓ Bile salt hydrolase activity ✓ Resistance to spermicides (applicable to probiotics for vaginal use)
IV. Safety consideration requirements for proof that a Probiotic strain is safe and without contamination in its delivery form	❖ In recognition for assuring safety, even among a group of bacteria that is GRAS, the Probiotic strains be characterized with the following tests: ✓ Determination of antibiotic resistance patterns ✓ Assessment of certain metabolic activities (D-Lactate Production, Bile salt conjugation) ✓ Assessment of side-effects during human studies ✓ Epidemiological surveillance of adverse incidents in consumers ✓ If the strain belongs to a species that is a known mammalian toxin, then it must be tested for toxin production ✓ If the strain belongs to a species that is known for haemolytic potential , then it must be tested for haemolytic activity ❖ Assessment of lack of infectivity by a Probiotic strain in immuno compromised animals would add a measure of confidence in the safety of Probiotics
V. In Vivo studies using	❖ The principal outcome of efficacy studies on Probiotics should prove benefits in human trails, such as statistically and biologically significant improvement in condition, symptoms, signs, well-being or quality of life

animals and humans	❖ Standard methods for clinical evaluation are comprised of the following ✓ Phase I (Safety) ✓ Phase II (Efficacy) ✓ Phase III (Effectiveness) ✓ Phase IV (Surveillance)
VI. Health Claims and Labelling	❖ Specific health claims should be permitted on the label and promotional material, where sufficient scientific data is available ✓ Genus, species and strain designation. Strain designation should not mislead consumers about the functionality of the strain ✓ Minimum viable numbers of each Probiotic strain and end of shelf life ✓ Serving size must deliver the effective dose of Probiotics ✓ Health claim (s) ✓ Proper storage conditions ✓ Corporate contact details for consumer information

Joint FAO/WHO Working Group Report on Drafting Guidelines for Evaluation of Probiotics in London, Ontario,Canada, 2002

Appropriate way of representing the presence of Probiotics in their product would be to include the genus, species and strain designation for each product and the presence of viable cells in terms of CFU/ml (colony forming units) or MSG (million spores per gram) and the expiry date. Apart from this they could also provide additional information through a separate sheet or brochure along with the product .Another way to convince the regulators and research bodies is by advertising the appropriate methods of assaying the viable cell count and certificate of conformation by an independent third party validation.

Since the field of Probiotics is witnessing rapid growth due to the developments in molecular biology which have aided in better understanding of the genome sequences of the strain. There is a difference in regulatory guidelines and procedures of Probiotics in comparison to pharmaceutical drugs which are highly regulated with proof of efficiency and safety apart from mandatory analysis of every batch with purity conformation to the regulatory guidelines. In order to address these issues FDA refuses the usage of unsafe ingredients in their formulations, implementation of good manufacturing practices and to display the labelling of the product ingredients with expiry date. However as per the literature published in Council for Agricultural Science and Technology (CAST), 2007 the following considerations could be made before launching new Probiotic product into the market.

To adopt a standard to identity for usage of the term Probiotic through well documented efficacy of the strain.;To conform to the regulatory guidelines laid down by the concerned departments;To carry out a third party validation of the product to ensure the efficacy of the label claims with respect to cell viability and efficacy. ;The usage of Probiotics for clinical applications should be performed after consultation and approval by the ethics approval committee of the concerned nations.

The evaluation of the Probiotic strain should be carried out effectively through publication in journals and papers before launch of the product into the market.;To perform effective assessment of the benefits and risks of genetically engineered Probiotics for their safety and environmental exposure risks.;To provide complete and sufficient information to consumers about their cell viability, safety and shelf life.

APPLICATIONS OF PROBIOTICS IN HEALTH

Probiotics have several applications in rendering health benefits to the humans. Some of the key health benefits bestowed by Probiotics are listed below.

A. Infantile Diarrhoea

Several clinical studies have proved the efficiency of Probiotics in treating various adverse effects related to gastro intestinal tract. One of the widely used applications of Probiotics in health is to treat diarrhoea especially in infants aged between 6 months to 2 years (Gregor *et al.*, 2001; Maria 2012). This is mainly caused by a Rotavirus which is evidenced by continuos vomiting and rapid watery diarrhoea (Harish *et al.*, 2006). Various researchers have carried out several experiments and suggested a simple remedy of fluid replacement in the body to compensate the fluid loss during dehydration apart from intake of high nutritional diet (Zvi *et al.*, 2010). Literatures suggest that different strains like *Lactobacillus rhamnosis, Bifidobacterium, Streptococcus thermophilus* could be used for the treatment of this diarrhoea. A dose of up to 10 billion colony forming units could be given to infants to treat this diarrhoea.

B. Antibiotic Induced Diarrhoea (AID):

Consumption of antibiotics for short term as well as long term induces antibiotics associated diarrhoea (Hans *et al.*, 2005; Gerald., 2012). Supplementation of antibiotics with Probiotics has shown a decrease in incidence of AID, particularly administration of *S.boulardii* and *Lactobacillus rhamnosis* . Wide consumption of antibiotics like erythromycin, penicillin, vanomycin etc causes an imbalance in the normal intestinal flora and thereby subsequently leads to diarrhoea (Mishra *et al.*, 1996). Investigations have revealed that various toxins exerted by *Clostridium difficile* as a primary cause of this type of diarrhoea. Studies have revealed that effects caused by Clostridium and antibiotics could be successfully treated with the administration of few Probiotics like Saccharomyces boulardii and Lactobacillus species.

C. Traveller's Diarrhoea:

This type of diarrhoea is observed mainly in travellers who travel from one region to another region especially tropical and subtropical (Indu *et al.*, 2002). Several investigations have stated that

administration of *Lactobacillus or S.boulardii* could be an effective remedy for this disease (Arthuro *et al.,* 2010). Much research needs to be done in this sort of diarrhoea due to varied regions and climatic conditions all over the world.

D. Radiation induced Diarrhoea:

Continuous exposure of the body to radiation especially while treating cancer patients causes adverse effects to the patients in the form of radiation diarrhoea. Intake of Probiotics could render health benefits to patients. Much research needs to be done in this area as treatment of side effects of radiation could open new areas for research (Jankovic *et al.,* 2010).

E. Lactose Maldigestion:

Lactose is a disaccharide sugar carbohydrate molecule containing glucose and galactose. An enzyme called Lactase is required to breakdown this lactose molecule in the intestine which is usually not found in infants. When people consume lactose based products, they are prone to develop gastrointestinal abnormalities like pain, flatulence and diarrhoea. A number of studies revealed that consumption of dairy products with live cultures of *Lactobacillus delbrueckii, Streptococcus* etc have demonstrated better lactose digestion as well as decrease in gastrointestinal symptoms (Julio *et al,* 2010).

F. Inflammatory bowels syndrome:

Several chronic conditions of intestine are characterized by persistent inflammation. Evidences have revealed that abnormal activation of the mucosal immune system against the gut micro biota is the crucial factor that leads to abnormal inflammatory disease (Sheil *et al*., 2007). Probiotics like *Lactobacillus* have proven to reduce the effects of this phenomenon (Julio *et al.,* 2010; Eamon *et al., 2012*).

G. Ulcerative Colitis

Probiotics have shown some positive results in the treatment of ulcerative colitis. Strains like *Bifidobacterium, Lactobacillus acidophilus* have shown their effect in prevention of relapse (Tadashi *et al.,* 2010., Yolanda *et al, 2013*).

H. Crohn's Disease

Probiotics have been used for the treatment of adverse effects of Crohn's disease, however well documented evidence and more research have to be done to prove their effects. Strains of *Lactobacillus* are widely used to cure this disease (Koji *et al.,* 2005).

I. Pouchitis

Long term complication of Ileal Pouch Anal Anastomosis (IPAA) is an acute chronic inflammation in Pouchitis. Studies proved that supplementation of Probiotics had some positive effect against Pouchitis (Kim *et al.,* 2003). Still sufficient data needs to be generated to prove the efficacy of the probiotics.

J. Bowel Transit

Regular consumption of Probiotic strains *Bifidobacterium* have revealed the decrease in the amount of time taken for the food to travel from mouth to anus of the people (Sheil *et al*., 2007; Mary, 2012).

K. Irritable Bowel Syndrome (IBS)

Patients with IBS usually suffer with abdominal pain, bloating and flatulence the reasons may be attributed to the gas formation taking place in the intestine due to fermentation. Few of the clinical studies carried out by *Lactobacillus* and *Bifidobacterium* revealed positive results in the treatment of IBS (Gareth *et al.,* 2010; Eamon *et al., 2012*).

L. Prevention of Colon Cancer

Several experimental data have revealed that the consumption of Probiotics such as *Lactobacillus and Bifidobacterium* and prebiotics such as oligofructose have demonstrated a positive effect on colon cancer patients (Tadashi *et al.,* 2010). Several mechanisms of action are suggested to prove the effects of probiotics by way of alteration of physiochemical conditions of the colon.

M. Allergy

Research investigations have revealed the role of probiotics in atopic disease treatment which is caused by exaggerated or imbalanced immune response to environmental and allergens (Jon *et al.,* 2003; Wojciech *et al.,* 2010). This kind of allergy disease is mostly observed in western societies and is growing at an alarming rate.

N. Hepatic Encephalopathy:

Investigations on the alterations of gut flora have been associated with improvement in hepatic encephalopathy (Mirjam *et al.,* 2010). Studies have revealed that supplementation of probiotics would lower ammonia concentrations specifically by colonization with acid resistant, non urease producing bacteria (Sandhya *et al.,* 2010).

O. Pancreatitis:

Probiotics have potential applications in the treatment of pancreatic necrosis and associated pancreatic infection. Infections to pancreas caused by gram negative organisms could be treated with the regular usage of Probiotics (Jankovic *et al.,* 2010).

P. Sepsis in surgical and critically ill patients:

Infections caused by several microorganisms such as E.coli and other sepsis rely mainly on the translocation of bacterial phenomenon and become a potential cause of sepsis in operated patients (Jose *et al.,* 2010., Seale et al., 2013). Intake of Probiotics like *Lactobacillus* has proven to reduce sepsis in surgical and critically ill patients.

Q. Recombinant Probiotic – Subalin:

A unique and novel category of recombinant Probiotic is made to render health benefits. A plasmid encoding the synthesis of human interferon alpha-2 of *Bacillus subtilis* 2335 is transformed and it is ensured to preserve the antagonistic property of the strain biosporin. This technology is paving newer avenues for research in this sector (Indu *et al.,* 2002).

DISCUSSION:

Probiotics are considered to be “GRAS” (Generally Regarded as Safe”) as they are not expected to cause any adverse effects when administered orally or through other

routes for improving human / animal health. This classification is usually given to products that are composed of ingredients that are natural or have been safely used for years or decades. A study reveals that less than 1% of population administered with Probiotics notice some side effects. Of those that report any side effect, such is usually limited to gastro intestinal related issues, more particularly due to excess gas formation in the stomach. The main reason for such condition is improper and excess intake of Probiotics than prescribed and at times, it's the manufacturer who mislabels the dosage.

Depending on the intended use of a Probiotics either as Drug or Dietary Supplement, regulatory requirements vary greatly. According to the definition of FDA, a drug is an article intended for use in the diagnosis, cure, mitigation, treatment of prevention of disease. If a Probiotic is intended for use as a drug, then it should undergo all the regulatory process of a drug, which is similar to any new therapeutic agent. In contrast to drugs, dietary supplements do not need FDA approval before being marketed but manufacturers needs to notify FDA before marketing a product. According to DSHEA, the manufacturer is responsible for determining that the dietary supplements that it manufactures or distributes are safe and that any representations or claims made about them are substantiated by adequate evidence to show that they are not false or misleading. However, the manufacturer need not provide FDA with evidence that substantiates the safety benefits of their products, either before or after marketing.

Usage of Probiotics as drugs is gaining attention due to wide range of varied positive health attributes offered by these strains. However, long term effects of Probiotic interventions are largely unknown and measures should be taken to conduct studies which would describe how and what are the adverse events were that should be monitored to allow a clear understanding of the presence or absence of adverse effects during intervention studies. To ensure the identity, purity, quality, strength and composition of dietary supplements those who manufacture, package or hold dietary supplements must follow the regulations prescribed by FDA on June 2007 for establishing Current Good Manufacturing Practice Requirements for dietary supplements. Accordingly, Probiotics manufactured under strict GMP conditions and which follow strict safety evaluation and nutritional properties of Probiotics as per FAO/WHO guidelines are absolutely safe to be used as dietary supplements.

It is clearly evident that Probiotics have enormous applications in health sector and could be potentially used for the treatment of various diseases in future. However, the good manufacturing practises needs to be followed strictly as per the guidelines and in conformity to the International and local statutory bodies. As of now in India there are certain guidelines for Probiotics that can be used in food applications, which was jointly drafted by Indian Council of Medical Research (ICMR) and Department of Biotechnology, Government of India which can act as a reference document for manufacturing of Probiotics in India.

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