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Regulatory Framework for the Evaluation of Probiotics: Present Scenario and Future Recommendations

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ABSTRACT

Probiotics "the healthy bugs" are increasingly used as pharmaceuticals now- a- days rather than as a component of our daily diet. This trend calls for proper regulations for them similar to drugs. This review focuses on the need for harmonized evaluator guidelines for probiotic usage across the globe. The paper discusses existing regulations on probiotics and recommendations for future use under the Guidance Document. Though probiotics offer many benefits yet they are contraindicated in certain groups of the population. The safety aspect of probiotics is also taken into consideration. This Guidance Document defines a set of parameters deemed essential for a product to be called as 'Probiotic'. The parameters include identity test, pathogenicity test, viability check, probiotic screening, safety considerations, etc.

INTRODUCTION

With arising trend in probiotic market value, the chances of the proliferation of misbranded, counterfeit products, and products with inferior quality increases manifold. The global probiotics market had increased by the US $ 31.1billion in 2015. Europe and Asia account for nearly 42 and 30% of the total revenues respectively as per the report published by Markets and Markets during 2009-2014.[1] With an increase in awareness for good health, there's a surge in the use of probiotics. Probiotics can be easily obtained as over-the-counter products. This makes the safety, purity, potency, stability, and efficacy assessment a mandate requirement.[2] There is a strong need for submission of evaluatory data to the regulatory authority (FDA) for INDA, NDA to get approval for manufacturing marketing and sale of the product. Further, evaluatory tests are essential for scientific validation of health claims. The present review aims to generate and recommend criteria and methodology for evaluation of probiotics including preclinical and clinical data for documentation of safety and efficacy via different *in-vivo* and *in-vitro* studies. It also identifies and outlines the minimum requirements needed for the declaration of probiotic status.

SCOPE

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This guidance document is prepared in context to all age groups including adults, geriatric, and pediatric population. This document addresses the quality of probiotics in foods and pharmaceuticals. It involves many *in-vitro* and *in-vivo* tests for safety and efficacy assessment, in animal and human models. This document also provides a systematic approach to deal with the concept of quality presumption of safety along with the inventory of microbes with a documented safe history of use.

Basic probiotic evaluation starts with the evaluation of probiotic culture media followed by microbial identification, pathogenicity test for safety considerations, and *in-vitro* and *in-vivo* tests for safety and efficacy.

PRESENT SCENARIO

To date, there are no harmonized regulatory guidelines for probiotics across the globe. They are regulated under different categories in different countries like Natural Health Products in Canada, Dietary Supplements in the U.S.A, FOSHU in Japan, and SFDA in China. In India, probiotics are regulated as functional foods as they are mainly used as a food component.

Probiotics are gaining acceptance as pharmaceuticals. They are being used in the treatment of numerous clinical disorders. They are largely replacing antibiotics from the market shelves due to their negligible side effects accompanied with low cost. Hence, it is the need of the hour to adopt harmonized regulations on probiotics' identification, safety, evaluation, GMP, approval process; health claims and labeling etc.

Presently, the internationally accepted guidelines containing all the parameters necessary for the evaluation of probiotic formulations are provided by FAO/WHO. Indian organizations such as ICMR, ILSI, and NDRI also provide the guidelines for the same. Many loopholes in these existing guidelines led to generate a revised document containing all the necessary parameters for their evaluation.

PRODUCTION MEDIA

Probiotic microbes need to be grown on an appropriate culture media before conduction any specific tests. The following points must adhere while selecting the culture medium:

- The culture medium meant for probiotic growth should be devoid of toxic or allergic potential, especially in human subjects.
- The use of animal-derived products should be discouraged.
- The materials should comply with current policy on Transmissible spongiform encephalopathy (TSE).[3-8] A TSE risk assessment must be included for the materials of the culture medium as per the revised WHO guidelines on TSE about biological and pharmaceutical products.[3]

IDENTITY TEST

The probiotic microbe in the master record as well as in working seed lots whether an NME (New Microbial Entity) or a microbe with a long history of safe use should be identified using microbiological techniques. Identification testing can be carried out by using a polyphasic approach which may include phenotypic tests in combination with the molecular biology-based genotypic techniques (e.g. PCR test, RAPD, AFLP, 16SrRNA sequencing, etc.) for identification of the specific probiotic strain as given in Table 1. Relevant information to ensure genetic consistency in production, from master seed through working seed and to final product is also provided by the said techniques.

Table No. 1: Identification procedure for commonly used probiotics from genus to strain level along with reported primers used in molecular techniques

Bacterium genus/ species	**Morphological identification (Step-1)**	**Phenotypic and biochemical tests (Step-2)**	**Genotypic identification (Step 3)**		**Ref.**
			Primers used (target site) with sequence (5' – 3')	**The technique for species to species /strain level identification**	
Lactobacillus	*Media (Rogosa SL agar), acidic pH, Gram +ve, nonspore forming rods (ranging from coccobacilli to long slender bacilli)*	*Catalase, indole test –ve confirms genus Lactobacilli. Lactic acid production along with small amounts of succinic and formic acid.*	CACCGCTACACATGG AG (16S 683–667)	PCR	9
			HDA1-GC (CGCCCGGGGCGCGCC CCGGGCGGGGCGGGG GCACGGGGGGACTCC TACGGGAGGCAGCAG T-3′) and HDA2 (5′-GTATTACCGCGGCTGC TGGCAC-3′)	16SrDNA gene sequencing of V2 and V3 regions + DGGE	
			AGCAGTAGGGAATCT TCCA (16S 362–380)	FISH	10
			ATTY*CACCsGCTACA CATG (16S 705–688)		11
Lactobacillus acidophilus	Rod-shaped occurs in small chains and is usually 0.5 to 0.8 micrometers across by 2 to 9 *mm* in length.	*Ferments D-glucose, D-fructose, D-mannose, saccharose, cellobiose and esculin. Confidence interval 71.1%.*	GAATCTGTTGGTTCAG CTCGC (16S 86-66)	DBH	12
			AGCTGAACCAACAGA TTCAC (16S 70-89)	PCR + DGGE	10 13
L. fermentum	Short, single, and paired square bacilli in MRS Broth colonies are smooth and convex.	Gas production from glucose ferments D-raffinose, saccharose, melibiose, Lactose, maltose D-fructose, D-glucose, galactose, ribose.	GTTGTTCGCATGAACA ACGCTTAA (16S 160–183)	PCR ribotyping	14 17

Bacterium genus/ species	Morphological identification (Step-1)	Phenotypic and biochemical tests (Step-2)	Genotypic identification (Step 3)		Ref.
			Primers used (target site) with sequence (5' – 3')	A technique for species to species /strain level identification	
L. fermentum	Short, single, and paired square bacilli in MRS Broth colonies are smooth and convex.	Gas production from glucose ferments D-raffinose, saccharose, melibiose, Lactose, maltose D-fructose, D-glucose, galactose, and ribose.	GTTGTTCGCATGAAC AACGCTTAA (16S 160–183)	PCR ribotyping	14
			GCCGCCTAAGGTGGG ACAGAT CTGATCGTAGATCAG TCAAG (16S–23S IS)	PCR DGGE	14 17
L. salivarius	Gram +ve, non spore forming	Ferments D-raffinose, lactose, there lose, saccharose, melibiose, maltose, N-acetyl glucosamine, sorbitol, mannitol, inositol, rhamnose, D-mannose, D-fructose, D-glucose, galactose.	ATTCACTCGTAAGAA GT (16S 95–111)	PCR ribotyping	14 13
L. brevis	Rod-shaped, smooth surface with thick cell wall with differentiated cytoplasmic Organization.	Gas production from glucose, Ferments 5-keto-gluconate, D-xylose, D-ribose, D-arabinose, D-glucose.	TGTTGAAATCAGTGC AAG (16S 107–90)	Dopamine β-hydroxylase DBH	15 13
L. paracasei	Rods, 0.8-1.0 µm, single or chains, small round creamy-yellow	Ferments D-Tagatose, β gentiobiose, melizitose, threalose, saccharose, lactose, maltose,	CACCGAGATTCAACA TGG (16S 67–84)	PCR RAPD	16
			CCGAGATTCAACATG G (16S 88–103)	PCR	17
			GCGATGCGAATTTCTT TTTC(16S–23S IS)	PCR	18 20
			GGCCAGCTATGTATT		

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	colonies	cellobiose, salicin, N-acetyl glucosamine sorbitol, mannitol, glycerol, ribose, D-fructose, D-glucose.	CACTGA(23S)	PCR	
L. paracasei	Rods, 0.8-1.0 µm, single or chains, small round creamy-yellow colonies	Ferments D-Tagatose, β gentiobiose, melizitose, threalose, saccharose, lactose, maltose, cellobiose, salicin, N-acetyl glucosamine, sorbitol, mannitol, glycerol, ribose, D-fructose, D-mannose, D-glucose.	CACCGAGATTCAACA TGG (16S 67–84)	PCR RAPD	18 20
			CCGAGATTCAACATG G (16S 88–103)	PCR	
			GCGATGCGAATTTCTT TTTC(16S–23S IS)	PCR	
			GGCCAGCTATGTATT CACTGA(23S)	PCR	
			Primers used (target site) with sequence (5' – 3')	**A technique for species to species/strain level identification**	
L. plantarum	Small, 0.5 – 1.2 x 1.0 – 10 µm, elongated rod-like bacilli assembled in pairs or chains of variable length; colony: punctiform, convex	Lactose and free amino acids not fermented produces hydrogen peroxide.	ATCATGAT TTACATTT GAGTG (16S 96–117)	PCR	211 4
			TTACCTAA CGGTAAAT GCGA (16S–23S)	16S rDNA sequencing	10
			CCTGAACT GAGAGAAT TTGAIS ATTCATAG TCTAGTTG GAGGT (23S)	PCR assays using group- and species-specific primers derived from the 16S-23S rRNA intergenic spacer region and its flanking 23S rRNA	19
Lactococcus lactis	Staining with 2% (w/v) uranyl acetate on freshly prepared carbon	Ferments fructose, galactose, glucosamine, glucose, lactose, maltose, mannitol,	8F (5′-AGAGTTTG ATCMTGGC TCAG-3′; positions 8 to 27) and 520R (5′-	Sequencing of 16S rRNA gene	22 23

	films.	mannose, ribose, sucrose and trehalose acidification ability (Strain level discrimination). proteolytic activity (o-PA{o-phthaldialdehyde method}): peptidase activity. antimicrobial activity. methanithiol formation. Fermented *L. lactis,* produces lactic acid.	ACCGCGGCTGCTGGC-3′; positions 531 to 517) for amplification of the V1 to V3 variable regions of the 16S rRNA gene		
Bacillus cereus	Gram +ve, rod-shaped, motile, spore-forming, anaerobic growth, cells were grown on glucose agar contain intracellular globules, unstainable by fuchsin.	Citrate utilization +ve; gelatin and casein hydrolysis +ve; Voges-proskauer +ve; reduces nitrate; ferments glucose, maltose, threalose, glycerol, sucrose, lactose; hemolytic activity on blood agar using *Bacillus cereus;* enterotoxin reversed passive latex agglutination using toxin detection kit (BCET-RPLA).	GGTATGCGACAGAGCTTA GGTATGCGACAGAGCTTC GGTATGCGACAGAGCTTG and GGTATGCGACAGAGCTTT S-D-BACT-1494-a-s-20-F L-D-Bact-0035-a-A-*15R*	PCR AFLP Species-level identification by amplifying the 16S-23SrDNA intergenic spacer region.	24 18
Bacterium genus/ specie	**Morphological identification**	**Phenotypic and biochemical tests**	**Genotypic identification (Step 3)**		**Ref.**
			Primers used (target site) with	**The technique for species to species/strain level identification**	

s	(Step-1)	(Step-2)	sequence (5' – 3')		
Bifidobacterium longum	Gram +ve, strictly anaerobic, non-motile, non spore forming, pleomorphic rods ranging from regular rods to various branched and club-shaped forms.	Ferments melizitose, hexose; F6PPK key enzyme of hexose fermentation; β-D-xylosidase absent.	TTCCAGTT GATCGCAT GGTC (16S 182–201)	PCR Sequence analysis of conserved genes other than 16SrDNA such as recA (enzyme involved in recombination) and ldh coding for lactate *dehydrogenase.*	25
			GGGAAGCC GTATCTCT ACGA (16S 1028–1008)	DNA- DNA Hybridization or DNA-DNA reassociation studies.	
			GGCCGCAA GATTCCTC (16S 103-120)	PCR targeting of transaldolase gene and subsequent separation of amplicons by DGGE (Species level discrimination)	26
			TAGCCTCG GCGGTCTC CCGTGA (16S 308-326)	RAPD and PFGE for strain level discrimination	
B. animalis	Isolated using TPY agar modified by the addition of mupirocin other medias include Wilkins-chalgren, RCM, Rogosa, or MRS.	Do not ferment melizitose; β-D-xylosidase present; grows only at pH 6.5-7, no growth below pH 4.5 and above 8.5, genus-level discrimination by detection of fructose-6-phosphate phosphoketolase activity.	For tuf gene : Forward primer GTGTCGAG CGCGGCA reverse primer ACTCGCAC TC ATC CAT CTG CTT BGB probe ATCAACAC GAACGTCG AGA	Species level discrimination by fluorescence *in-situ* hybridization and PCR amplification with genus and species specific primers. Identification of a highly conserved single-copy *tuf* gene encoding the elongation factor Tu involved in bacterial protein biosynthesis, used as a marker for *Bifidobacterium animalis* subsp. *lactis* (strain Bb12) and *Bifidobacterium animalis* subsp. *animalis* differentiation	27
Bacterium	**Morphological**	**Phenotypic and**	**Genotypic identification (Step 3)** Primers used	**Technique for**	**Ref.**

genus/ species	identification (Step-1)	biochemical tests (Step-2)	(target site) with sequence (5' – 3')	species to specie/strain level identification	
Saccharomyces cerevisae	Cylindrical, dimensions 2-3µm x 5-8µm Produce no filaments in slide cultures on potato glucose medium.	Assimilate D-glucose, glycerol, D-saccharose, and D-raffinose. API-ZYM tests reveal lipolitic and proteolytic activity of yeast; All strains exhibit alkaline phosphatise, acid phosphatise, and napthol-AS-BI phosphohydrolase activity; All strains hydrolyse 2-napthyl butyrate and 2-napthyl caprylate indicating esterase and esterase lipase activity; Strong leucine arylamide activity.	OPA-07 (GAAACGGGTG)	Clearcut discrimination between *S. cerivisae* and *S, boulardii* was achieved by DNA-DNA reassociation studies. PCR with species specific primers. Real time PCR	28
Saccharomyces boulardii	Ascospores stained with Kinyoun stain and ascospore stain, ascospores Gram-ve and vegetative cells Gram +ve on YPD media, cylindrical, dimension 2-3 µm × 5-8 µm	Unable to adhere humen intestinal cells *in vitro*, enhanced ability for pseudohyphal switching in response to nitrogen limitation Assimilate glucose, maltose, raffinose does not use galactose as a carbon source.	For 5.8S rRNA gene amplification ITS1 (5′-TCCGTAGGTGAACCTGCGG-3′) and ITS4 (5′-TCCTCCGCTTATTGATAT GC-3′)	PCR (ITS-PCR) ribotyping (Species level identification) RFLP using enzymes *Mae*I, *Hae*III, *Cfo*I, *Dde*I, *Bgl*II, *Bam*HI, *Hind*III, *Eco*RI, *Sma*I, or *Pst*I	30 29

B. bifidum	Rod or club shaped, 0.5-1.3 µm×1.5-8 µm V shaped colonies	Ferments maltose, mannose, fructose and tagatose; no gas production.	CCACATGATCGC ATGTGATTG (16S 184–204) CCGAAGGCTTGC TCCCAAA (16S 475–442)	PCR G-C content 55-67%	31
			GCTTGTTGGTGA GGTAACGGCT (16S 245–266)		32
Bacterium genus/ species	**Morphological identification (Step-1)**	**Phenotypic and biochemical tests (Step-2)**	**Genotypic identification (Step 3)**		**Ref.**
			Primers used (target site) with sequence (5' – 3')	**Technique for species to specie/strain level identification**	
Bacillus coagulans	Gram-positive rod (0.9 µm by 3.0 µm to 5.0µm in size); catalase positive, spore-forming, motile peritrichous flagella, spores are ellipsoidal or sometimes spherical, subterminal or terminal occasionally paracentral colonies are 1-3mm in diameter, white to cream convex with entire margins and	Positive results for catalase, starch hydrolysis, acid production from glucose, glycerol, starch, N-acetyl-D-glucosamine, D-mannose, fructose, galactose, and melibiose; -ve results for indole, urease, H_2S production and lysine decarboxylase, do not grow in 7% NaCl.	16S1 (5'-GAG TTT GAT CCT GGC TCA-3') and 16S2 (5'-ACG GCT ACC TTG TTA CGA CTT-3')	PCR amplification	33

	smooth surfaces				
Streptococcus thermophilus	Isometric, hexagonal and assumptively icosahedral capsids, 47-74 nm in diameter and non-contractile tails 182-290 nm long and 7-14 nm wide. None of the phag-es displayed collar, tail plaque, or fiber structures.	Ferments lactose, glucose, galactose but not mannose.	5XD9 (5′GAAGTCGTCC) 5’-	RAPD PCR	34
			TGGGCAGAAACT CAAGA-3’ 5’- AACACCACCACC GATAAC-3’	PCR amplification	35
L. delbrueckiis. Sp. bulgaricus	Optimal media: Milieu Proche du Lait (MPL), Specific stain: Acridine orange. The capsids were 47 - 73 nm in dm and isometric hexagonally shaped.	Metabolize lactose and case amino acids to D-lactate and free amino acids.	Primers LB1 (5′-AAAAATGAAGTT GTTTAAAGTAGG TA-3′) and LLB1 (5′-AAGTCTGTCCTC TGGCTGG-3′),	PCR	36

PATHOGENICITY TEST

Pathogenicity testing involves the testing of any kind of pathogen. This can be tested via:

1. Initial microscopic examination, visual examination of culture media followed by genotypic techniques for pathogen detection.

2. Pyrogen testing in suitable animal models.

3. Determination of hemolytic potential

VIABILITY CHECK

Viability check is a key factor to check the number of culturable organisms in a starter culture[37]The probiotic product should contain the expected viable probiotic count till the mentioned date i.e. "Best before" date/Expiry date. An appropriate method approved by the NRA should be used to determine the number of culturable particles on a solid medium of each final bulk. Viability assessment can be tested by opting any of the following methods:

- Plate counting;[38,39]

- Reverse transcriptase-polymerase chain reaction (RT-PCR) and nucleic acid sequence-based amplification (NASBA);[40]

- Real time PCR;[41]

- Flowcytometry;[42]; Fluorescence *in situ* Hybridization (FISH) and fluorescence techniques that use two fluorochromes with different emission wavelength to discriminate between intact and viable cells, injured or damaged or dead cells;[43]

- Viability test kits such as LIVE/DEAD® Baclight™ are commercially available which contains two nucleic acid stains: the green fluorochrome SYTO 9 (small molecule that can penetrate all membranes) and fluorochrome propidium iodide (large molecule penetrates only compromised membranes). Cells render to be green when they are viable and red when they are dead;[43,44]

Assessment of intracellular esterase activity and maintenance of intracellular pH, membrane integrity are some of the other methods to check cell viability.

- **Bio-luminescence;**

A rapid test for viability; Bio-luminescence is a biochemical method that can be used as an alternate to colony counting method for viability test; provided that the method is properly validated against the culturable particles. If such tests are properly validated, they may be considered by the NRA to replace the other methods used for culturable particle test. The bioluminescence reaction takes place due to the presence of adenosine triphosphate (ATP), luciferin luciferase, oxygen, and magnesium ions. This reaction can be reproduced by mixing

these components *in vitro*. If all components except ATP are present in excess, the amount of light emitted is proportional to the amount of ATP coming from the probiotic microbial preparation. ATP proves to be a reliable marker for living cells since ATP is present in all living cells and is immediately destroyed when the cell dies.[45]

Viability of probiotics is affected by a large number of variables during various steps as given in Figure 1:

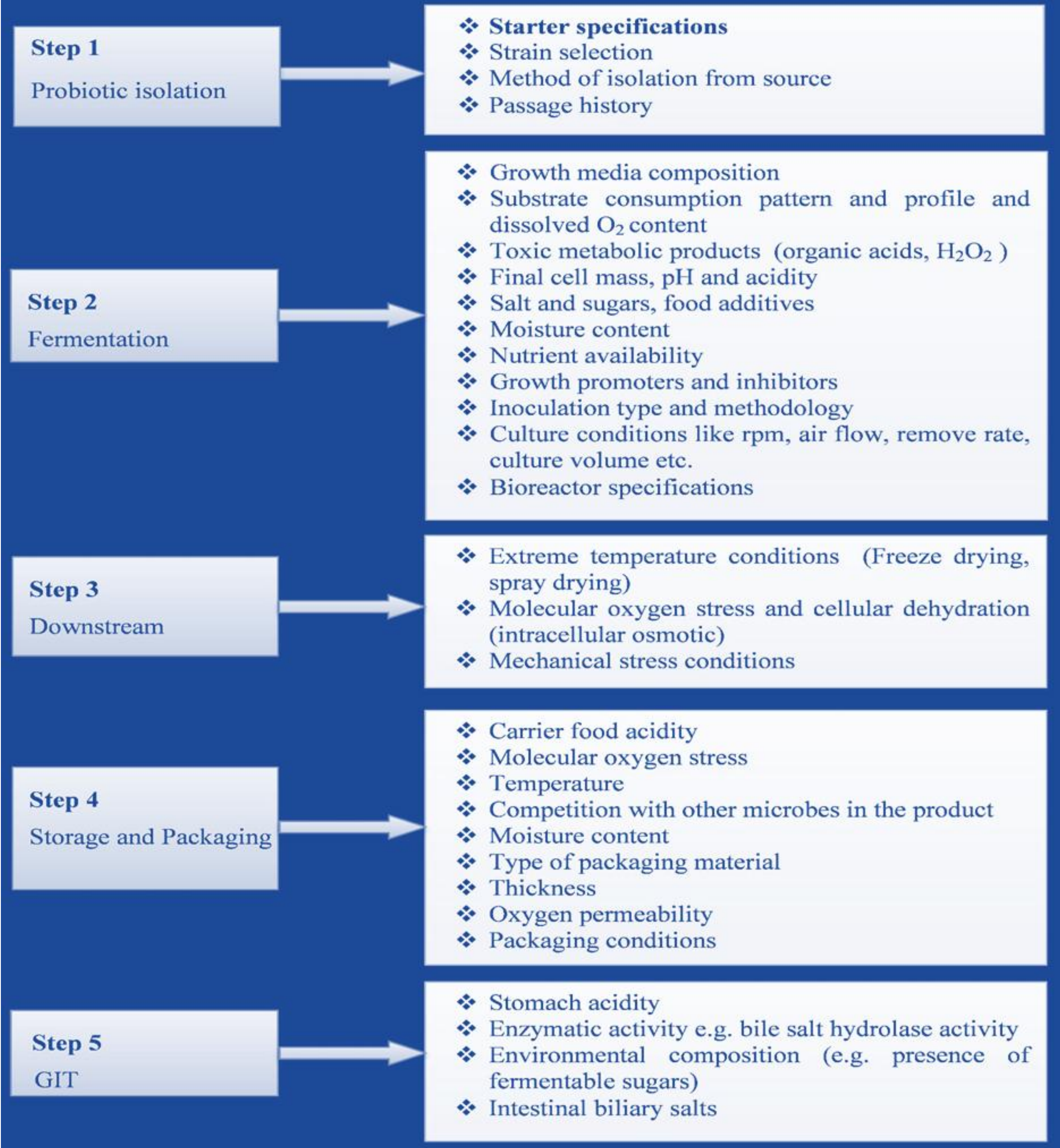

Figure No. 1: Factors affecting viability at different stages related to probiotics[46]

PROBIOTIC SCREENING

In-vitro tests to screen potential probiotics

In-vitro tests are needed to assess the safety of probiotic microbes, to gain knowledge of strains, and to understand the mechanism of the probiotic effect. However, the currently available tests are not fully adequate to predict the functionality of probiotic microorganisms in the human body. The data available for particular strains are not sufficient for describing them as probiotics. Probiotics for human use require substantiation of efficacy with human trials by adding appropriate target-specific *in vitro* tests that correlate the performance of these with *in-vivo* results; For example, *in vitro* bile salts resistance was shown to correlate with gastric survival *in-vivo.*[47]

Following is the list of currently used *in vitro* tests for the study of probiotic strains:

- 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).

SAFETY CONSIDERATIONS

Requirements proving a probiotic strain as safe and without contamination in its delivery form

Historically, *Lactobacilli* and *Bifidobacteria* associated with food have been considered to be safe[48] Their occurrence as normal commensals of the mammalian flora and their established safe use in a diversity of foods and supplement products worldwide supports this conclusion. However, probiotics may theoretically be responsible for four types of side-effects.[49]

- Systemic infections
- Deleterious metabolic activities
- Excessive immune stimulation in susceptible individuals
- Gene transfer

The assessment of the safety of probiotics comprises a consideration of a variety of factors as given below:

- ❖ Record of origin, isolation, passage history and taxonomic classification of the candidate probiotic strain;
- ❖ Strict manufacturing controls by strictly following GMP that eliminate contamination (including cross-contamination between batches) of the probiotic with microbes or other substances;
- ❖ Absence of association of the probiotic with infectivity or toxicity such as the absence of hemolytic potential, antibiotic resistance, and delayed hypersensitivity reactions, assessed at the strain level;
- ❖ Absence of transferable antibiotic resistance genes;
- ❖ Absences of allergenic material in the products specially targeted for allergic populations that may stimulate hypersensitivity reactions;
- ❖ Physiological status of the consuming population, Special consideration must be made for use in vulnerable populations, including newborn infants, pregnant or nursing mothers, and the critically ill patients;
- ❖ The dose administered and dosing frequency;
- ❖ Method of administration (oral or otherwise);[50];

However, there are few documented pieces of evidence which indicate adverse effects of probiotics in certain groups of individuals such as immunocompromised patients or patients with the certain diseased condition. Enlisted below are some of the reported cases with the consumption of probiotics.

❖ Two cases of *L. rhamnosus* traced to possible probiotic consumption[51,52]

❖ Thirteen cases of *Saccharomyces* fungemia due to vascular catheter contamination[53]

❖ *Bacillus* infections linked to probiotic consumption include three reports[54,55] detailing seven cases of *B. subtilis* bacteremia, septicemia, and cholangitis, all in patients with underlying disease.

Bifidobacterium is the safest probiotic species used. Enterococcus is increasingly used as a probiotic nowadays but seems to be a major cause of nosocomial infections. It is recognized that some strains of *Enterococcus* display probiotic properties, and may not at the point of inclusion in a product display vancomycin resistance. However, the onus is on the producer to prove that any given probiotic strain is not a significant risk concerning transferable antibiotic resistance or other opportunistic virulence properties. It is recommended that probiotic strains be characterized at a minimum with the following tests even among a group of bacteria that are Generally Recognized as Safe (GRAS) to assure safety:

- Determination of antibiotic resistance patterns;
- Assessment of certain metabolic activities (e.g., D-lactate production, bile salt deconjugation);
- Assessment of side-effects during human studies;
- Epidemiological surveillance of adverse incidents in consumers (post-market);
- If the strain under evaluation belongs to a species that is a known mammalian toxin producer, it must be tested for toxin production. One possible scheme for testing toxin production has been recommended by the EU Scientific Committee on Animal Nutrition (SCAN, 2000);
- If the strain under evaluation belongs to a species with known hemolytic potential, determination of hemolytic activity is required;
- Assessment of lack of infectivity by a probiotic strain in immunocompromised animals would add a measure of confidence in the safety of the probiotic.[56]

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***In-vivo* studies using animals and humans**

The safety and efficacy data of probiotics is scientifically and statistically proven with benefits in human trials. Probiotics lead to an improvement in condition, symptoms, signs, well-being or quality of life; reduced risk of disease or longer time to next occurrence; or faster recovery from illness. Each should have a proven correlation with the probiotic tested.

Probiotics have been tested for an impact on a variety of clinical conditions. Like drug products, the clinical evaluation parameters are comprised of four different phases i.e. Phase 1, Phase 2, Phase 3 and Phase 4.

A general recommendation for the testing of probiotic foods is that the placebo would be comprised of the food carrier devoid of the tested probiotic. The sample size needs to be calculated for specific endpoints. Statistically, significant differences must apply to biologically relevant outcomes. Probiotics delivered in food generally are not tested in Phase 3 studies, which are concerned with a comparison with standard therapy. The claims on the probiotic products altering a diseased state must be based on scientific substantiation of health claims. In Phase 2 and 3 studies, the value of validated quality of life assessment tools should be validated. It is recommended that human trials be repeated by more than one Center for confirmation of results.

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No adverse effects related to probiotic administration should be experienced when food is considered. Adverse effects related to probiotics should be carefully monitored and incidents reported.

It is also recommended that information accumulated to show that a strain(s) is a probiotic, including clinical trial evidence be published in peer-reviewed scientific or medical journals. Furthermore, the publication of negative results is encouraged as these contribute to the totality of the evidence to support probiotic efficacy.[57]

RECOMMENDATIONS

Regulatory issues

- Recommend a policy for producers/manufacturers to state the scientific basis for their product's strains and clinical effect on all labels.

- Expand dietary regulatory scope for probiotics to include vaginal and skin applications of probiotics.

Clinical issues

- Lobby governments and industry support more studies regarding probiotic safety and efficacy especially related to cardiovascular disease, diabetes, allergy, cancer, and infection.

- Verify mechanisms of action *in-vivo* and prepare an acceptable list of properties needed as minimum requirements for probiotic microbes to confer specific health benefits.

- There is a need for refinement of *in-vitro* and *in-vivo* tests to better predict the ability of probiotic microorganisms to exert definite pharmacological effects in human subjects.

- There is a need for more precise, statistically significant efficacy data in humans.

- Probiotic products shown to confer defined physiological health benefits on the host should be permitted to describe these specific health benefits.

- Further work is needed to address criteria and methodologies for probiotics concerning identification and evaluation.

- Surveillance systems, including trace-back and post-marketing surveillance, should be put in place to record and analyze any risk factors or adverse events associated with probiotics in food, and also such systems could also be used to monitor the long-term health benefits of probiotic strains.

Efforts should be made to make probiotic products more widely available, especially for relief work and populations at high risk of morbidity and mortality i.e. for the geriatric and pediatric population.

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Desirable selection criteria for potential probiotic strains[58]

Selection Criteria for Potential Probiotics			
Functional Criteria	**Technological Criteria**	**Physiological Criteria**	**Safety Criteria**
•Resistance against acid and bile condition •Adhesion to mucosal surface •Clinically validated and documented health effects	•Genetically stable •Good sensory properties •Phase resistance •Large scale production •Desired viability during processing and storage	•Antagonism against enteric pathogens •Lactose intolerance •Cholesterol assimilation •Anticarcinogenic and mutagenic properties •Immunomodulation	•Human origin •Non pathogenic •Resistance to antibiotics

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CONFLICT OF INTEREST

The authors report no conflicts of interest. The authors alone are responsible for the content and writing of this article.

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Abbreviations:

RAPD: Random Amplification of Polymeric DNA

PCR: Polymeric Chair Reaction

FISH: Fluorescent in situ Hybridization

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