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Safety Aspects of Fermented and Probiotic Foods

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Abstract

Fermentation is an age old technique that has been progressing continuously for better shelf life, safety, digestibility and for better nutritional value of fermented milk products. Lactic acid bacteria are most important in onset of fermentation which directing health benefits conferred by them. These health benefits are strain specific as genetic, biochemical and physiological differences among the strains of the same species. Safety evaluation and assessment of local and undefined cultures are utmost important otherwise, it may promotes public allegations succeeding into confidence loss in functional foods. Conventional techniques of toxicology and safety evaluation are not sufficient since a probiotic is meant to survive and grow in human colon in order to benefit humans. Hence, there is a need for *in vivo* assessment in support of *in vitro* confirmation for an adequate safety of currently undefined cultures before their use in fermented milk products preparations.

Keywords: Probiotic, *in vitro* methods, safety assessment, health benefits, evaluation of LAB

Fermented foods have very long history and are part of our diet since antiquity. They are produced by lactic acid bacteria through fermentation of lactose. Fermentation as a technique has been in practice as a means of improving the shelf life, safety, digestibility and nutritional value since ages (Guarner *et al*., 2008). Many fermented products with different name but similar in content can be found worldwide (Yerlikaya, 2014). Fermentation became popular with the dawn of civilization because it not only preserved food but also gave tastes, forms and awesome sensory sensations to foods. Nearly every civilization has developed fermented milk products of some type. The terms *dahi*, butter milk, yogurt, leben and acidophilus milk are highly accepted by the people around the world. Tough many products are region specific; their popularity did not hide from anyone.

India has rich knowledge of fermented foods prepared from milk, cereals, pulses, vegetables, fruits, fish *etc*. Fermented milks like dahi, butter milk (chhash), *lassi* are popular all over the country (Prajapati and Nair, 2008). The fermented cereal legume based products like *dhokla, khaman* are very popular in western parts and *idli*, *dosa* are popular in southern parts. The eastern part of the India enjoys the tradition of sweetened *dahi* and fermented rice based food and beverages. In the Northern part of the India *Nan, Bhatura, Kulcha, Jelebee etc*., are very common (Prajapati, 2003). Globally several ranges of non dairy probiotic products have been developed and existed in market from past few decades. These include fruits and vegetable, juices, non dairy beverages, cereal based products, chocolate based products, meat and many more (Farnworth *et al*., 2007; Gupta and Sharma, 2016).

As a process, fermentation consists of the transformation of simple raw materials into a range of value added products by the action microbes and their activities on various substrates. This means that knowledge of microorganisms is essential to understand the process of fermentation. Subsequently, food fermentation processes underwent through a continuous improvement and microbial cultures particularly lactic acid bacteria (LAB) became essential component of food production. These cultures are characterized taxonomically, physiologically, biochemically and genetically. Most of the LAB come under the category of GRAS as they are historically associated with foods and have been found to be safe. However, when strains are isolated and are promoted as probiotics, their safety needs to be established by *in vitro* as well as *in vivo* tests.

Fermentation may be the most simple and economical way of improving nutritional value, sensory properties and functional qualities of food. Lactic acid fermentation in case of cereals has been used as a strategy to decrease the anti nutritional content, such as phytates and tannins, and for improving the bioavailability of micronutrients (Hotz and Gibson, 2007). Many bacteria associated with fermented foods produce antimicrobial bioactive molecules, such as hydrogen peroxide, organic acids and bacteriocins that make them effective bio preservatives. Similarly they enhance functional properties of food and increase bioavailability of nutrients (Toma and Pokrotnieks, 2006; Mokoena *et al*., 2015).

Probiotics and health benefits

Probiotics are live microorganisms that when administered in adequate amounts are intended to confer health benefit to the host (FAO, 2001). The use of probiotics in food is directed by the health benefits conferred by them. These health benefits are strain specific as genetic, biochemical and physiological differences among the strains of the same species (Schmid *et al*., 2006; Senders, 2007). Some of the ways by which probiotics impact on the host are as follows (Aguirre and Collins, 1993).

In fermented food products the claimed benefits of probiotics are primarily focused on intestinal health mainly dietary management of patients with an ileoanal pouch, infectious diarrhea, enhance gastrointestinal tolerance to antibiotic therapy, the

Table 1: Metabolites of lactic acid bacteria which may be inhibitory to other pathogenic and food spoilage organisms

Product	Main target organisms
Organic acids	
Lactic acid	Putrefactive and gram negative bacteria, some fungi
Acetic acid	Putrefactive bacteria, clostridia, some yeasts and some fungi
Hydrogen peroxide	Pathogens and spoilage organisms, especially in protein rich foods
Enzymes	
Lactoperoxidase system with hydrogen peroxide	Pathogens and spoilage bacteria (milk and dairy products)
Lysozyme (by recombinant DNA)	Undesired gram positive bacteria
Low molecular weight metabolites	
Reuterin	Wide spectrum of bacteria, yeasts, and molds
Diacetyl	Gram negative bacteria
Fatty acids	Different bacteria
Bacteriocins	
Nisin	Some LAB and gram positive bacteria, notably endospore formers
Other	Gram positive bacteria, inhibitory spectrum according to producer strain and bacteriocin type

(Breidt and Fleming, 1997)

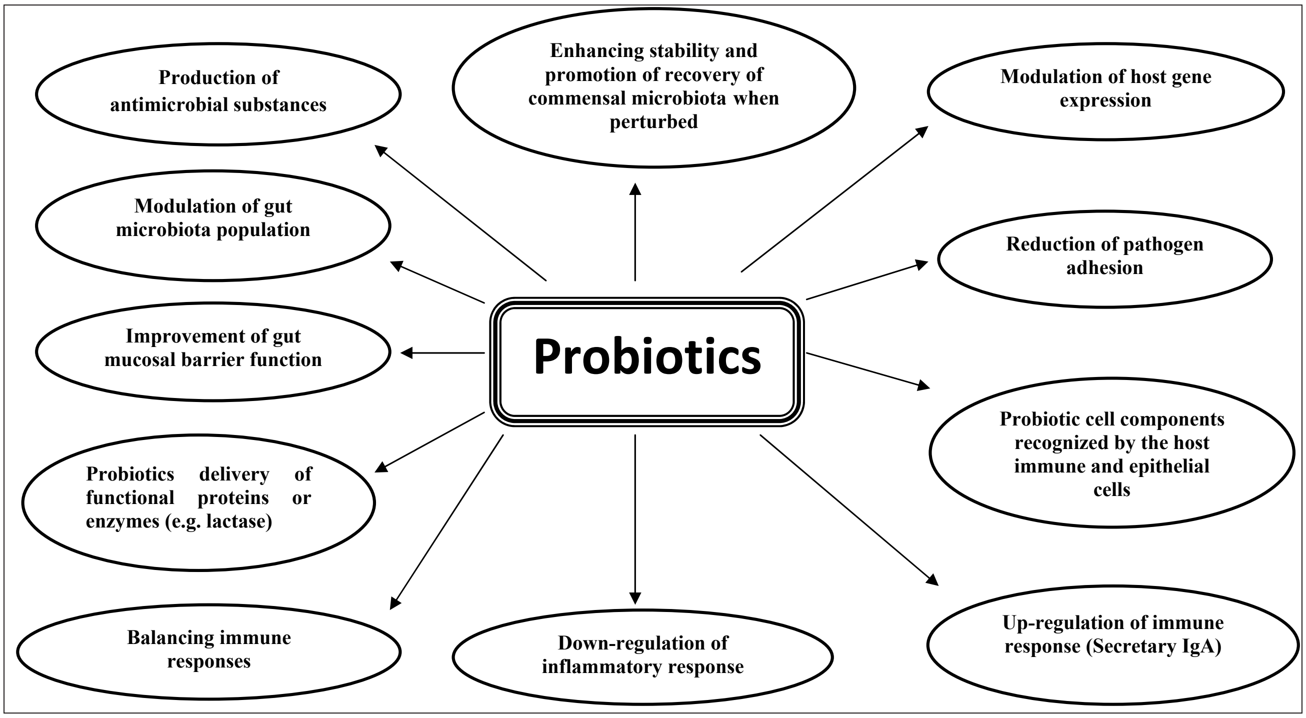

Fig. 1: Some of the ways by which probiotics impact on the host

lactose intolerance, helps in the control of irritable bowel syndrome and inflammatory bowel diseases, suppression of endogenous/exogenous pathogens by normalization of the intestinal microbial composition, alleviation of food allergy symptoms in infants by immunomodulation (Mokoena *et al*., 2015) and reducing risk factors for colon cancer by metabolic effects (Saarela, *et al*., 2002a; Fitton and Thomas, 2009), have use as drug delivery vehicles, novel applications in mental and emotional well being of humans and promoting overall health because of their ability to occupy pathogens adhesion sites (Kore *et al*., 2012; Mokoena *et al*., 2015). Some specific strains of *L. acidophilus* have hypocholesterolemic effects (Buck and Gilliland, 1994) as cholesterol can precipitate with free bile salts especially in an acidic environment (Klaver and Meer, 1993; Saarela, *et al*., 2002a).

Scientific community have been showing interest in potential areas including some allergic diseases, initiation of colon cancer (Fernandes and Shahani, 1990; Kampman *et al*., 1994), dental caries (Bonifait *et al*., 2009), respiratory infections (Douglas and Sanders, 2008; Schmid *et al*., 2006;) candidal vaginal infections (Hilton *et al*., 1992); prevention of stomach ulcers caused by *H. pylori* (Brassart *et al*., 1995) and use in stimulating brain function (Rajan, 2015).

LAB as a probiotics

The role of LAB in improving the shelf life and nutritional quality of fermented foods and beverages, in conferring therapeutic and nutritional benefits have been well established now. LAB comprises a significant component of the human gut flora and has several beneficial roles in the gastrointestinal tract. Thus, a better understanding of the intestinal microbial populations will contribute to the development of new strategies for the prevention and/or treatment of several diseases.

Fermented foods are the main vehicle of administration of probiotic organisms and, among them; dairy products are by far the most important vehicles for the delivery of these LAB. These products containing living microorganisms have been traditionally used to restore gut health. However, there is an increasing consumer demand for nondairy based probiotic food and are being incorporated into drinks or marketed as dietary supplements in the form of tablets and freeze dried preparations (Wedajo, 2015).

Safety concerns of LAB for use in fermentation

It is clear that the right selection and application of a probiotic strain in food materials exhibits fundamental impacts on qualitative aspects of final products, namely safety (related to strain/s used), health benefits (conferred by probiotic for any specific function) and sensory attributes (Mortazavian *et al*., 2012). The incorporation of inaccurately identified probiotic bacteria in functional food products may promotes public allegations which undermine the efficiency of probiotics and consumers confidence loss in functional foods (Huys *et al*., 2006). The use of suitable tools in proper strain selection, clearing legal requirement and in developing any new product is essential. Meanwhile tracking probiotics during food production as well as during their intestinal transit are recommended for effective evaluation (Lee and Salminen, 2009).

Probiotic strains such as *Lactobacillus spp*., *Bifidobacterium spp*., *Streptococcus spp*. and other lactic acid bacteria have GRAS status and additionally their use have long history of safe use in fermented product manufacturing. Lactobacilli and Bifidobacteria have been rarely associated with human clinical infections which are likely to be a result of opportunistic infections especially in immuno compromised individuals (ICMR-DBT, 2011). Rare cases of local or systemic infections including septicemia and endocarditis, dental infections (Saarela *et al*., 2002) due to lactobacilli, bifidobacteria or other LAB have been reported. Most *Lactobacillus* strains isolated from clinical cases belong to the species *L. rhamnosus, L. casei* or *paracasei* and *L. plantarum* (Saxelin *et al*., 1996; Marteau, 2001). However, some reports of clinical pathological conditions such as bacteraemia and endocarditis due to LAB associated fermented products consumption have been in testimony (Lara-Villoslada *et al*., 2007). These reports have raised concerns about the safety and use of probiotic bacteria in fermented foods consumption. Safety evaluation of probiotic products is a difficult, but a very important task. For the products involving ingestion of live microbes zero risk can be applied.

Safety of probiotics their use has been judged by selective screening and by various methods (Donohue and Salminen, 1996; Donohue *et al*., 1998; Heller, 2000). Straub *et al*. (1995) suggested biomarkers for screening for potential virulence factors (Franz *et al*., 1999) and for enzyme activities involved in the formation of putatively genotoxic metabolites, including β-glucuronidase, nitroreductase, and azoreductase (Heller, 2000). Some *In vitro* mucin degradation like model which detect any damage or disturbance of the mucin layer is considered to compromise the host's mucosal defense function (Ouwehand *et al*., 2002; Edelman *et al*., 2003). Another risk is antibiotic resistance, which may rise with the possibility of exchange of antibiotic resistance markers between pathogens and food microorganisms (Teuber *et al*., 1999; Schmid *et al*., 2006). Safety assessment is much essential for newly identified cultures before recommending its use in food production (SKLM, 2010).

Certification for safety assessment of LAB

Many probiotic strains in use for several decades have been validated for their safety and efficacy and are therefore, safe to use. Even though any new strain if used as a probiotic, it should be evaluated for safety and efficacy. Internationally, the LAB for use in foods is regulated in different ways by different regulatory body of different countries. To provide international consensus on methodology to assess efficiency and safety of probiotics, the FAO and WHO undertook work to compile and evaluate the scientific evidence on functional and safety aspects of probiotics and have provided these as a guidelines (FAO/WHO, 2002).

In the US, a manufacturer can apply for Generally Recognized as Safe (GRAS) status from the Food and Drug Administration. In addition, the producer voluntarily can submit a file to the FDA and if the FDA does not object, it is assumed that organism is safe and can be marketed. In European Union, European Food Safety Agency (EFSA) has developed a QPS (Qualified Presumption of Safety) (EFSA, 2005a) system for certification of specific microorganisms or groups of microorganism. The QPS approach allow the establishment undertook specific safety assessment steps that should be fulfilled for each taxonomic unit (genus, species or strains depending on the microorganisms) which must be suitable for QPS status granted (Gueimonde *et al*., 2006; Ashraf and Shah, 2011). QPS is based on the four pillars of pathogenicity, taxonomy, familiarity and use with basic knowledge of the organism. The confirmation of a QPS status is a case by case decision made on the basis of a decision tree (EFSA, 2005b).

The Indian Council of Medical Research (ICMR), along with the Department of Biotechnology Govt of India have proposed 'Guidelines for Evaluation of Probiotics in Food in India', which articulates the base for the law to govern the use of probiotics in various applications (ICMR-DBT, 2011).

Safety evaluation of a novel probiotic strain

Safety evaluation of novel probiotic strain needs a multidisciplinary approach necessary to assess the toxicological, immunological, gastroenterological, pathological, infectivity, the intrinsic properties of the microbes, virulence factors comprising metabolic activity, and microbiological effects of probiotic strains (Holzapfel *et al*., 1998; FAO/WHO, 2002). Conventional toxicology and safety evaluation is not sufficient, since a probiotic is meant to survive and/or grow in human colon in order to benefit humans. It needs to consider more aspects which can provide absolute protection against the use of virulence strains. Several methods have been developed for evaluation of the safety of LAB through *in vitro* and *in vivo* studies (Aguirre and Collins, 1993). Evaluation of strain on intrinsic properties and interactions between the host and probiotic bacteria can be used as means to assess probiotic safety (Holzapfel *et al*., 1998). Evaluation of the acute, sub acute and chronic toxicity of ingestion of extremely large quantities of probiotic bacteria should be carried out for potential strains. Such assessment may not be necessary for strains with established documented use or safe history of long use (Marteau, 2001; Bourdichon *et al*., 2012).

Safety of probiotic organism can be ascertained by following four major approaches:

(i) ***Characterization*** of the genus, species and strain and its origin that will provide an initial indication of the presumed safety in relation to known probiotic and lactic starter strains (Salminen *et al*., 2000)

(ii) ***Intrinsic characteristics of strains*** *viz*. enzymatic properties, deconjugation of bile salts, degradation of mucus, growth profile during processing, survival and viability during transport and storage, general physiological aspects like resistance against environmental stress and to the antimicrobial factors prevailing similar conditions as in the upper GIT as encountered by probiotic food during the stomach duodenum passage.

(iii) ***Pharmacokinetics of probiotics*** can be measured *in vivo* using faecal collection, intestinal intubation techniques or behaviour of specific strain on mucosal biopsies. Similarly several *in vitro* models can help to predict the fate of ingested strains. Functional and beneficial features can be ascertain by recording adhesion to cell line, colonization potential of the mucosa, competitiveness, specific antimicrobial antagonism against pathogens, stimulation of immune response, selective stimulation of beneficial autochthonous bacteria, restoration of the normal gut biota and for safety aspects point of view can confirm by non invasive potential, no transferable resistance against therapeutic antibiotics and non transmittable virulence factors (Holzapfel, 2006; SKLM, 2010)

(iv) ***Adverse interactions*:** It can be ascertained by conducting animal studies, clinical trials on healthy volunteers and by conducting an epidemiological surveillance study (Marteau, 2001; Saarela *et al*., 2002).

Safety assessment

In vitro assessment of risk factors

The best choice to assess the safety of a novel or existing strain *in vitro*, is to search for the presence properties that are known to be virulence or risk factors associated. This may be affirmed by some tests like platelet aggregation test, haemolysis, resistance to complement mediated killing, adhesion to extra cellular matrix proteins, antibiotic resistance, resistance to gastric acidity, bile acid resistance, adhesion to mucus and or human epithelial cells and cell lines, antimicrobial activity against potentially pathogenic bacteria and ability to reduce pathogen adhesion to surfaces (FAO/WHO, 2002).

Metabolic end products

In addition to intrinsic properties of microbes, the metabolic activity is also important in screening of LAB.

Production of D Lactic acid

The risk for D lactic acidosis appears to be mainly limited to children with short bowel syndrome (SBS). Human tissue contains the enzyme D-2-hydroxy acid dehydrogenase that also converts D-lactate to pyruvate and reduces the risk for acidosis. However, if absorption of D-lactate exceeds metabolism, *e.g.* during over growth of lactobacilli in SBS patients, acidosis may occur. On the other hand, D-lactic acidosis can be treated by using an L-lactate producing probiotic ex. *Lactobacillus* GG (Gueimonde *et al*., 2006).

Production of biogenic amines

Biogenic amines may get produced in fermented dairy products due to ripening for longer periods of time. This is of minor concern for probiotics as this is happen in some ripened type of cheeses (Gueimonde *et al*., 2006). However, conversion of intestinal proteins and their digested products into ammonia, idol, phenols and biogenic amines by some gut bacteria may happen (histamine, tramline, putrescence, *etc*.) (Drasar and Hill, 1974). Secondary bile acids produced by intestinal bacterial actions are harmful and may exhibit carcinogenicity by acting on the mucous secreting cells and promoting their proliferation (Cheah *et al*., 1990).

Biogenic amines such as histamine and tyramine are of concern as they may get produced in high amounts by microorganisms through the activity of amino acid decarboxylases. Intake of high amounts of biogenic amines can be lead for allergic reactions with occurrence of the signs and symptoms of facial flushing, sweating, rash, burning taste in the mouth, diarrhea and cramps with severe reactions including respiratory distress, swelling of the tongue and throat and blurred vision (Sanders *et al*., 2010).

Antimicrobial resistance

Transferable antibiotic resistance/plasmids mediated gene transfer

Colonic bacteria normally residing in colon act as reservoirs for resistance genes that can be acquired from ingested bacteria. Commensal bacteria in the gut including an opportunistic and those that are truly non-pathogenic, exchange genetic material with one another (Salyers *et al*., 2008).

Lactic starter cultures used in food products could also be a source for spread of antibiotic resistance. Hence, a strain under screening should be systematically monitored for resistance (Ammor *et al*., 2007).

Evaluation of side effects in human studies

Probiotics may theoretically be responsible for four types of side effects *i.e.* systemic infections; deleterious metabolic activities; excessive immune stimulation in susceptible individuals and by gene transfer. Recorded reports which could be used for co-relating between systemic infections and probiotic consumption are few and all occurred in patients with underlying medical conditions. These side effects terminated into bacteremia, septicemia and cholangitis in all patients which were undergoing treatments (FAO/WHO, 2002).

Pathogenicity/toxicogenicity

Lactobacillus or Bifidobacterium species used as probiotics have been identified for no genes associated with pathogenicity. It is difficult to assess what might exist. Some researchers have suggested that resistance to host innate defense mechanisms should be considered in the safety assessment of Lactobacillus strains, but research in this area still needs further explorations (Sanders *et al*., 2010). On the contrary, numerous virulence factors in enterococci have been reported, including hemolysin, gelatinase or DNAse activities, or the presence of structural genes *cylL, ace, asal* and *esp* (Eaton and Gasson, 2001).

In vivo assessment of risk factors

Animal model studies

Safety assessments of probiotics have been done using several animal models. One of the important risk factor is translocation and it can be studied in animals.

Bacterial translocation does not occur commonly in healthy specific pathogen free animals but it can be found for a long duration in germ free mice (Ishibashi *et al*., 2001). Tanslocation was observed in sterile born mice; however, lactobacilli did not cause any harm and the organisms cleared in 2 to 3 weeks (Mogensen, 2003). Intestinal microflora of a subject also plays an important role in the prevention of probiotic translocation to internal organs. Animal model could be useful in evaluating the safety of new probiotics in immuno-compromised hosts (Borriello *et al*., 2003). In most of experiments performed in mice, translocation of bacteria is usually observed in immune compromised subjects only but the response may vary with age of the animal. Wagner *et al*. (1997) suggested that the use of probiotic is likely to be safe for immuno-competent and immuno-deficient adults, but they should be tested for safety in immuno-deficient neonates.

Genomic assessment of risk factors

With an increasing number of microbes being sequenced, the available genome can also be used for the detection of potential risk factors (Gueimonde *et al*., 2006). In general, potential probiotic strains should be screened *in vitro* for their interactions with cell lines to investigate possible cytotoxic or cytopathological effects after growth in different media for the presence of known virulence genes (*e.g.* lecithinase activity, toxin genes) and for the presence of mobile genetic elements. After these *in vitro* tests for potentially safe use, *in vivo* toxicity tests and persistence studies would be required.

Functional genomics analyses of these properties will create opportunities to establish direct cause and effect relationships (Reid *et al*., 2003). Functional traits can be targeted for safety assessment by use of specific genomic markers. Feasibility of genomic wide screening approaches was compiled by Prajapati and Senan (2013) are depicted below:

Table 2: Feasibility of genomic wide screening approaches

Genomic markers	Specific function perform in safety assessment
Plasmids	Presence or absence, suggest the acquisition of traits especially antibiotic resistance
Prophages and inedrases	Presence of phage related proteins suggests a history of inactivation or elimination of integrated prophages and development of highly stable genomic integrated systems
Transposases and insertion sequence (IS) elements	These facilitates increased genomic rearrangement, conferring an advantage in variant generations

Clustered regularly interspaced short palindromic repeats (CRISPR)	Represents a family of DNA, repeats shown to provide acquired immunity against genetic elements
Restriction and Modification systems	Natural immune system against phage infections would be crucial in order to devise suitable strategies that avid or limit the negative effects of phage infections and to counteract phage predation upon administration of probiotics
Resistome: antibiotic and heavy metal resistance	Genome is mined for the presence of specific resistance gene. Ex resistance against ampicillin
Acquisition of and tolerance to heavy metals	Co occurrence of genes conferring metal resistance with antibiotic resistance genes which could lead to the selection of antibiotic resistant organism in gut
Adverse metabolic genes	These genes are useful in evaluating the safety and functional characteristics of probiotic strains includes its aminogenic potential and bacterial synthesis of enzyme involved in the formation of putatively genotoxic metabolites including beta-glucosidase, arylsulphatase, beta-glucuronidase, nitroreductase and azoreductase.
Virulence genes	These genes include a putative sortase gene, GroEL, aggregation promoting protein, two copies of fibronectin binding protein, S-layer protein and mucus binding protein
Stress resistance gene	It is an adoptive factor for probiotics to counteract changes in intestinal barrier function, gut motility and ability to cope with digestive stresses. General stress adoptive gene exhibited by the strain includes universal stress protein UspA, chaperonins GroES and GroEL, endopeptidase clpP Clp protease and for some heat shock proteins HtpX, grpE, dnaK, FtsY, DnaJ.

Epilogue

With the increase in demand for safe functional probiotic foods, consumption of new and enriched types of foods has shown growth at higher rates. Fermentation still plays a major role in the establishment and maintenance of food safety. Consumption of fermented foods with the long history of safe use of lactobacilli and bifidobacteria remains the best proof of their safety. Modern days risk assessment of probiotic consumption may be expensive and time demanding but it is essential from legal aspects and for complete assurance to the consumers. When any new starter culture is recommended, it requires relevant information on the efficacy and safety. There is a need for *in vivo* assessment in support of *in vitro* confirmation for an adequate safety of currently undefined cultures. Such assessment can be well-done certified for emerging isolates as well as for already established with development of a framework particularly genome mining for various genes which are credentials for various safety factors of concerns.

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