FERMENTATION ASSISTED FUNCTIONAL FOODS.

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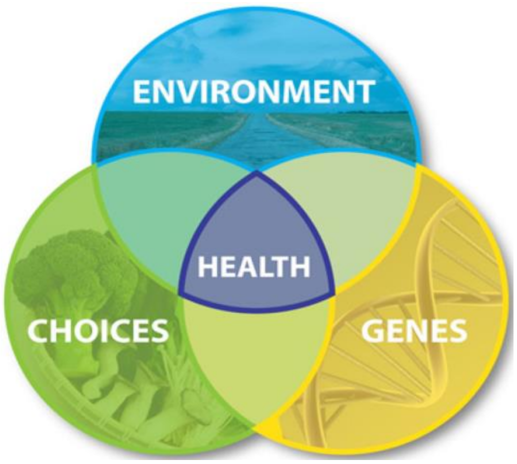
Introduction

Food is the utmost basic need of life that meets the nutritional requirement of an individual. Fats, carbohydrates, proteins ,vitamins and minerals are the nutrients needed for growth and maintenance, whereas dietary fiber, phytochemicals, antioxidants, probiotics, prebiotics, etc.are considered as non-nutrient factors that boost human health by positively influencing the physiology of host and global epigenetic imprints . Dietary intake of selected categories of foods called as nutraceuticals or foodiceuticals or functional foods or medifoods regulate the disease controlling mechanisms either as prophylactics or therapeutics. Nutraceuticals or functional foods or foodiceuticals or medifoods are the food categories that may act as prophylactics or therapeutics and whose dietary inclusion may regulate the disease controlling mechanisms .

Food particularly the traditional can optimize human health and environmental sustainability if provided in right quantity and quality. It is a great challenge to provide healthy diets using indigenous food processing techniques. Since time immemorial, fermented foods have been traditionally valued and are usually categorized as “functional foods” owing to their health benefits. Functional foods are reported to support basic nutrition, affirming good health and longevity with natural ingredients as influential drivers . Fermentation brings about desirable changes in food constituents by enhancing the palatability, organoleptic properties and bioavailability of nutrients and altering the functional properties of food and also facilitating the viability of beneficial probiotic bacteria. Fermented foods comprising live microorganisms is now considered as a paramount dietary strategy to improve human health. Bacteria, yeast and fungi generally facilitate food fermentations. A considerable confusion exists on which fermented foods actually includes live microorganisms and also the understanding about impact of these microbes on the gut microbiome. Introducing the potentially beneficial microbes to the gastrointestinal tract via intake of microbe-containing foods seem to be an easiest and suitable option.

Most microbes related to fermented foods may survive digestion, reach the gastrointestinal tract intact, and eventually provide health benefits . The vast benefits of such fermented foods has paved way in the development of synbiotic foods comprising of prebiotics and probiotic bacteria . Further novel functional foods with positive health effects are in huge demand among health conscious public and in the population influenced by changing eating patterns induced by urbanization .



Diet , Gut Microbiome and Microbiota

Sedentary lifestyle or various morbidities can impair the composition of human gut microbiota(HGM) , eventually contributing to metabolic disorders ,gut diseases and neurological diseases. A diversified healthy diet containing limited amounts of refined grains and highly processed foods and added sugars should augment health and provide ideal nutrient intake. Although global consumption of fruits, vegetables, nuts and legumes has increased with a decreased consumption of red meat and sugar among health conscious consumers, processed foods still continue to be a part of diet. Dietary modification can prevent, treat, or ease some of the obesity, celiac disease or gastrointestinal(GI) disorders associated symptoms and improve general health.

Microbiome implies to the sum total genome of the microorganisms in a specific ecology, whereas microbiota is the community of microorganisms .A microbiome originating from a normal healthy gut is complex and consists a highly diverse collection of microorganisms (approximately 100 trillion)consisting of bacteria, viruses, fungi, parasites, viruses, protozoa, bacteriophages , archaea and other tiny organisms . The “human microbiome project” has reported the number of other bacterial cells in the human body to be about 10 times the number of human cells , with more than 1000 different metabolically active bacteria (*Bacteroides* and *Firmicutes* being the main dominant flora) .This flora ably works with the host immune system , resisting stress associated changes and thus strongly impacting control of energy metabolism, metabolic functions , immune system , nutrition and intestinal functions of the host . Additionally, microbial residual exopolysaccharides , building blocks of microbes , proteins, or metabolites, may directly or indirectly interact with human physiology along with the human gut microbiome, thus influencing human health. There is an ever-growing research with commercial concern in various aspects of prebiotic and probiotic functional foods to counteract "dysbiosis" by replacing harmful microflora with beneficial microorganisms .

Various conditions like obesity, autoimmune diseases, and infections may cause predominance of harmful bacteria. Probiotics with its direct interaction with immune cells may maintain the immune balance in the GI tract, thus can be regarded as potential adjuvant therapy . There has been recent advancements in significant treatment of neurodegenerative illnesses owing to microbiome gut–brain axis . Quick gut lumen communication with brain, post meals and the significance of microbiota in regulating homeostasis and suitable signaling with the discovery of "neuropod cells" has been supported by research. An encouraging association has been reported in mothers consuming fermented foods and sleep duration in year-old infants , attributed to the changes in the gut microflora of mothers. The conversion of dietary flavonoids is determined by gut microbiota and its interaction with intestinal microbiota may not only regulate the HGM composition and functional enhancement of probiotics but it significantly increases the bioavailability and bioactivity of flavonoids thus regulating the intestinal diseases.

Primary and secondary metabolites which are considered as bioactive compounds are produced during the fermentation process, which include antibiotics, antimicrobial peptides, carbon dioxide, alcohol, vitamins, folates, and organic acids like short chain fatty acids (SCFAs) (butyrate, acetate, and propionate). Bioactive molecules with its therapeutic potential, are reported to be advantageous to the gut, they improve the intestinal barrier, inhibit development and the production of pathogens,toxic elements and help prevent metabolic disorders. Dairy and non-dairy fermented foods could serve as carriers of probiotics, prebiotics, and/or bioactive compounds (phenolic compounds, antioxidants, bioactive peptides, etc.) and significantly impact the gut microbiota . Biotics that include probiotics, prebiotics, postbiotics, synbiotics, paraprobiotics, oncobiotics, pharmabiotics and psychobiotics are emerging in recent years and have gained much attention .

<u>Food Fermentation</u>: *<u>Benefits, changes brought about in food and types of food fermentation</u>*

Food fermentation is an ancient practice known to human kind around the globe. “Fermentation” is derived from the Latin word “fermentum” and when applied to foods, it has a much wider meaning as against its biochemical definition as “an ATP-generating process in which organic compounds act as both donors and acceptors of electrons”. Foods made by preferred microbial growth along with enzymatic alterations of food components are addressed as fermented foods and beverages. An estimated of over 5,000 types of fermented foods and beverages are manufactured and consumed globally from varied raw materials (substrates) such as cereals, roots and tubers, vegetables meat, dairy products, fish, fruits and legumes. Fermentation is much used in the making of probiotics, prebiotics and synbiotics with high microbial viability and functionality.

<u>Benefits of food fermentation</u>:

Fermented foods and ingredients have appealed interest as they can facilitate HGM and stimulate human well-being. Science and technology face a major challenge in the creation, development and improvement of foods with additional health properties, the so-called functional foods, to meet consumer’s expectations. Meeting the expectations of consumers to make available functional foods with added benefits, is a big challenge to food science and technology. Food fermentation considered as an ancient biotechnology, is considered as a good method for enhancing nutritional and sensory properties. Consumption of fermented foods is promoted in Indian food pyramid affirming their significance in actual human nutrition framework.

Fermentation helps break down large organic molecules via the action of microorganisms into simpler ones and provide a solid guarantee to improve quality, nutritional value, organoleptic properties of many perishable foods and extend shelf life owing to the production of secondary metabolites with high biological value. Fermentation provide an assured guarantee to improve quality, nutritional and biological value and organoleptic properties of perishable foods with the breakdown of large organic molecules into simpler ones via the microbial action. The characteristic taste, flavor, color, consistency, appearance, texture, improved vitamins, essential amino acids, proteins, reduced anti-nutrients, extended shelf life and functional properties and health benefits due to the effects of microbial assimilation, metabolites production and enzymatic activities. Fermented foods can be considered as a dietary source of live organisms.

Bacteria (e.g. lactic acid bacteria (LAB) such as *Lactobacillus, Streptococcus, Enterococcus, Lactococcus* and *Bifidobacterium*), molds (e.g. *Aspergillus oryzae, Aspergillus sojae, Penicillium roqueforti* and *Penicillium chrysogenum*), and yeasts (e.g. *Saccharomyces cerevisiae, Andida krusei* and *Candida humilis*) are used in the production of fermented foods and beverages. Fermentation is brought about with mono or mixed cultures such as bacteria, fungi and yeasts, which act parallel or sequentially altering dominant microbiota. For instance, sugars and starches convert to alcohol by yeast enzymes, while proteins get converted to peptides/amino acids. The microbial or enzymatic actions tend to ferment food and causing desirable biochemical changes that significantly modifies the food. For eg;degradation of complex phytochemical compounds to smaller ones, release of more bioactive polyphenols prove advantageous for the microbiome , reduce inflammatory reactions and cytokine production. The plausible mechanisms for additional benefits of fermentation include reduction of free radicals or its neutralized effects and subsequent reduction in oxidative stress, controlling antioxidant enzyme activity and increase in activity of immune system. Reduced occurrence of asthma and atopic dermatitis, improved fasting blood glucose and other metabolic syndrome symptoms were observed in overweight and obese Korean adults consuming kimchi and other fermented vegetables. Similarly, intake of phytoestrogens and bioactive peptides rich fermented soybean foods showed reduced risks of Type 2 diabetes, hypertension with improved serum triglyceride levels in Japanese adults. Strong evidence in improvement of lactose tolerance with yogurt, owing to microbial release of β-galactosidase has been documented. Beyond the potential of fermentation, the type of raw material or substrate (cereal, pseudocereals, and legumes) used is seen of key importance for the optimal delivery of bioactive compounds for human nutrition. The nature of raw material or substrate (cereal, pseudocereals, and legumes) used in fermentation is of significant importance in the ideal delivery of bioactive compounds for human nutrition.

Changes on fermentation in food: *microbiological activities, biochemical modifications and health impact*

Fermentation is a natural way of improving nutritional quality and organoleptic properties of food. Making informed food choices and the acceptance of novel foods is influenced by sensory properties of food, thus the prospects of fermentation in creating edible and pleasant flavors and textures will be of great relevance in changing eating habits. Changes happening during the fermentation that includes chemical transformation of primary compounds to new secondary metabolites may be categorized into five stages (Figure 1). First stage results into transformation of sugars to end products like simpler acids, carbon dioxide and alcohols that further enhances the nutrient bioavailability . Second stage, leads to hydrolysis of polymers with enzymatic synthesis of bioactive compounds like antioxidants. In third stage, there is enhanced bioavailability of phytochemicals and minerals and higher concentration of vitamins and amino acids. Stage 4 results in degradation of the toxic and anti-nutritional factors . Finally, the promoters get synthesized for improving absorption and uptake of compounds.

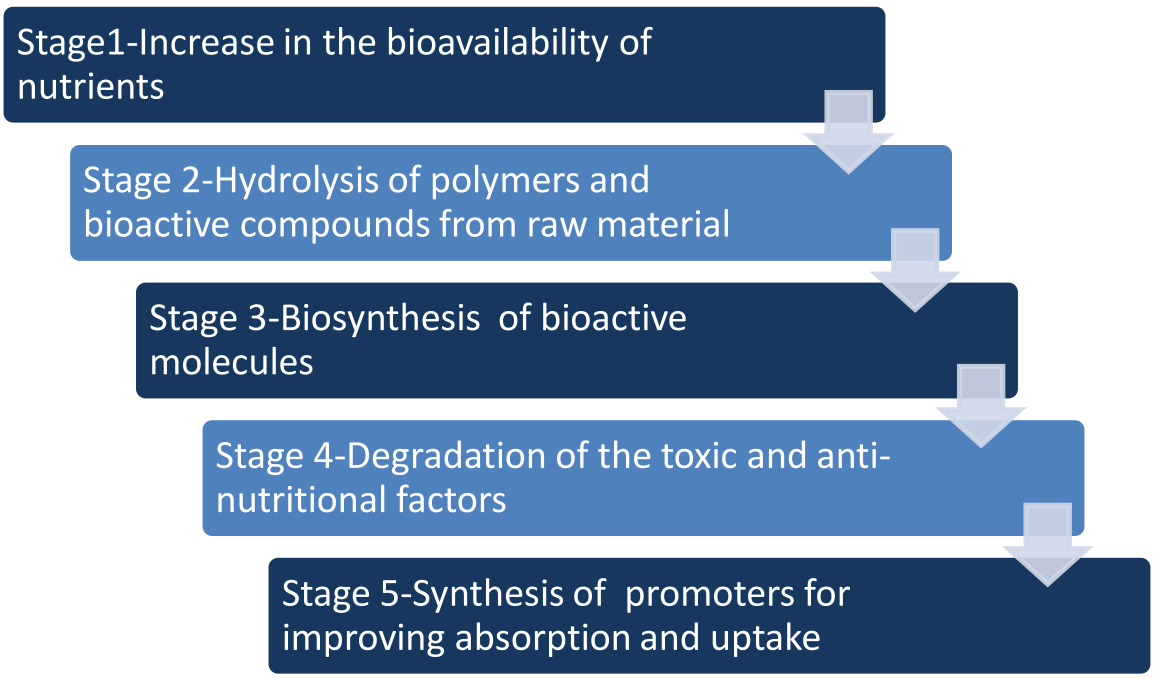


Fig.1. Five stages indicating changes during food fermentation.

Source: R. B. Cuvas-Limon *et al*, (2021).

Changes on fermentation in food that occur are as follows:

1. Food containing high fiber , toxins, anti-nutritional factors(ANFs) such as phytic , oxalic acids, tannins, raffinose, alkaloids, lectins, pyrimidine glycosides (e.g. vicine and convicine), protease inhibitors and trypsin inhibitors ; that chelate metal ions , decrease

digestibility and reduce the bioavailability of nutrients, can be changed into palatable and nutritionally improved foods via fermentation.

2. Substantial enhancement in protein digestibility can be brought about by fermentation.
3. Reduction in cyanide levels in the roots and leaves of the staple cassava plant to acceptable levels and the removal of flatulence factors in legumes on its fermentation has been reported.
4. Fermentation may result in production of biotransforming phytochemicals from dietary polyphenols with a potential to trigger favorable changes to stimulate microbial development .
5. The Short chain fatty acids (SCFAs) produced during fermentation, especially butyrate, are responsible to maintain integrity of the intestinal mucosa, decreasing the risk of developing colon cancer by reducing cell multiplication and encouraging cell differentiation. It is also related with maturation of the enteric neurons. SCFAs have also been identified as important immune system modulators.
6. *De novo* synthesis of delicious flavors and textures can be obtained by microbial fermentation eg: diverse textures of cheese, the flavor of chocolate, soy sauce, wine, beverages, condiments, etc. Fermentation leads to the synthesis and buildup of volatile and non-volatile aroma or aroma-active compounds including those associated with bitter, umami, sweet, sour and salty tastes. The attributing factors for changes in flavor may be due to the production of alcohol, aldehydes, amines, esters, fatty acids (especially those volatile species), ketones, lactones, terpenes, organic acids, phenols, thiophene, diacetyl , sulfur compounds, pyrazines and other nitrogen-containing compounds .
7. Fermentation conditions along with microbial activities may reduce the pathogenic cell count of organisms, either through changes in pH, by nutrient competition, or by the production of preservatives with anti-microbial or anti-fungal activity in fermented foods.
8. Fermentation increases the extractability of bioactive compounds (Table 1) from various raw materials and functional biomolecules which are part of the LAB/yeast metabolism.
9. Fermented milk and yogurt consist of higher in vitro antioxidant properties, owing to release of biopeptides that follow the proteolysis of milk proteins, particularly α-casein, α-lactalbumin, and β-lactoglobulin.

10. Microbial fermentation has also enabled synthesis of GABA (g- Aminobutyric acid) ,a chief inhibitory neurotransmitter of the central nervous system with a role to play in induction of antihypertension, prevention of diabetes, diuresis and tranquilizer effects .
11. Fermented foods could be a food-based alternative for delivering folic acid to at-risk populations in dealing with global deficiency problem.
12. Fermentation enables the reduction of the energy needed for cooking
13. Fermented foods are often safer to consume than their raw counterparts
14. The extended shelf life of most fermented foods leads to the reduction of food loss thereby improving the efficiency and resource use of food production.

Table 1. Functional properties related to bioactive compounds in fermented foods.

Functional Attributes	**Bioactive compounds**	**Microbes**	**Fermented foods synthesized in**	**Health aspects**
Antimicrobial Properties	Antimicrobial compounds Bacteriocin Pediocin Nisin Z	LAB *Lactococcus lactis* BH5, Leuconostoc *citreum* GJ7, *Pediococcus pentosaceus.* *Lactococcus lactis.* *Weisella cibaria.*	Vegetables Milk Kimchi Dahi (India) Fermented cabbage product	GI tract protection , Anticancer effects Antiviral effects
Antioxidant Properties	Antioxidants compounds, Peptides, Enzymes.	*Bacillus, Lactobacillus, Bifidobacterium*	Asian fermented soybean foods Soybean food of India and Nepal	Modulate circulatory oxidative stress Protect cells against carcinogen-induced damage Antioxidant Anti-inflammatory Anticancer
Immunomodulatory Properties	Bioactive peptides, Amino acids.	Proteolytic microorganisms (*Bacillus*), LAB.	Fermented beverages	Maintain the energy balance Contribute to antioxidant effects

				,Anti-inflammatory effects
Inhibitory properties	Bioactive peptides, Amino acids.	Microbial enzyme (ACE)	Kefir Koumiss Yogurt	Minimize the risk of developing chronic or degenerative diseases.
Enhancement of metabolic functions	Lipases, Proteases, Esterases, Amylases b-galactosidase, Lactase	LAB, *Bifidobacterium.*	Fermented beverages	Help to improve the metabolism ,Improve nutrient absorption ,Mitigate lactose intolerance
Production of SCFAs	Acetate, Butyrate, Propionate.	LAB	Fermented beverages	Provides 60–70% of the energy needed by the colon, muscle and brain cells. Therapeutic effects: Anti-inflammatory bowel ,Anti-colon cancer ,Help in prevention of heart diseases ,managing obesity and diabetes.
Production of vitamins	Vitamin B12, Vitamin K, Vitamin B6, Cobalamin, Folate, Thiamin.	*Bifidobacterium, Lactobacillus.*	Fermented beverages	Help to synthesize DNA. Combat cardiovascular disease

Source: R. B. Cuvas-Limon *et al*, (2021).

<u>Types of food fermentation:</u>

Fermented foods are normally referred to as those processed by uncontrolled natural fermentation or with controlled starter cultures. It results in enzymatic changes of primary and secondary components of foods with cost-efficient bioprocess. Food fermentation is impacted by many factors, such as substrate composition, the fermenting microorganisms, food treatment and duration of fermentation whilst processing. Depending on the type of the matrices (substrate) exposed to fermentation, there exists nine main categories of fermented foods: (a) fermented cereals; (b) fermented vegetables and bamboo shoots; (c) fermented legumes; (d) fermented roots/tubers; (e) fermented milk products; (f) fermented and preserved meat products; (g) fermented, dried and smoked fish products; (h) miscellaneous fermented products; and (i) alcoholic beverages. To initiate any microbial metabolism, the substrate used must have a rich chemical composition, including high concentration of mono and di-saccharides and the minerals with no inhibitors like toxins that may inhibit the microbial growth. Environmental conditions also impact the fermentation process.

Food fermentations are classified either based on added or naturally present starter cultures as **controlled** or **uncontrolled** (Natural/ spontaneous) fermentation respectively and based on the product produced. Based on the dominant metabolic process, three fermentation processes include: acidic, alkaline, and alcoholic fermentation, each contributing in a unique way. Acid fermented products or alcoholic beverages are widely documented for its nutritional and health benefits as compared to alkaline fermented foods (AFFs), thus the focus here will be on AFFs. Active research in the area of "**alkaline food fermentation**" began in the late 1980s, whereas lactic acid fermentation had benefited from detailed scientific research dated as far back as in 1950.

1. **Acidic fermentation**: is chiefly carried out by lactic acid bacteria(LAB), acetic acid bacteria (AAB) and yeast with lactic acid as main metabolite in addition to different types of organic acids, thus called as LAB fermentation. Curd/ Yogurt and sourdoughs are produced by lactic acid and yeast fermentation respectively. AAB (mainly *Acetobacter*) oxidize carbohydrates, alcohols and sugar alcohols (polyhydric alcohols or polyols) to their corresponding acetic acid (vinegar) as the primary product, organic acids, aldehydes, or ketones in oxidative fermentation.
2. **Alkaline fermentation** is carried out by different species of *Bacillus* and fungi and often occurs during seed fermentation. Eg. AFFs such as dawadawa, ugba, bikalga, kinema, natto, iru ogiri and thua-nao.
3. **Alcoholic fermentation**, with AAB and yeasts (predominant organisms), results in the primary production of ethanol and CO_2.Eg. alcoholic beverages such as beer, wine, cider.

Alkaline-Fermented Foods (AFFs): ***Nutritional significance of AFFs***

Alkaline-fermented foods made from various raw ingredients, are a lesser known food product in developing and a few developed countries. In alkaline-fermentation the pH of the substrate (usually protein-rich foods) increases to alkaline values (pH 8–10). This fermentation is either due to alkali-treatment during production or due to protein degradation/hydrolysis (proteolysis) to peptides, amino acids (essential) and further degradation of amino acids to ammonia by dominant *Bacillus spp*. The increase in pH contributes to a strong ammoniacal smell which can be restricted by limiting the growth and metabolism of the bacteria without inhibiting the action of flavor generating proteolytic enzymes. Holding the fermented beans at appropriately low temperature during maturation can also enable this restriction and is also possible by adding glycerol as humectant to partially fermented beans or by limiting oxygen supply. The most important metabolic activity considered during alkaline fermentation is proteolysis.

Traditionally, AFFs of Africa and Asia are usually processed by spontaneous fermentation (depends on autochthonous or resident microorganism present in the raw substrate and/or surrounding environment) but can also be developed by controlled fermentation . Predominant microorganisms in AFFs include *Bacillus subtilis, Bacillus subtilis var. natto, Bacillus cereus, Bacillus megaterium, , Bacillus thuringiensis, Bacillus endophyticus, Bacillus licheniformis, Bacillus borstelensis, Bacillus pumilus, Bacillus coagulans, Bacillus circulans, Bacillus amyloliquefaciens, Bacillus firmus, , Bacillus megaterium, Paenibacillus polymyxa, Lysinibacillus sphaericus,* and *Lysinibacillus fusiformis*, but other secondary microorganisms such as *LAB, staphylococci,* and *micrococci* are also involved. *Bacillus spp.* are used as probiotic in humans.

AFFs as functional foods: *Nutritional significance and health benefits of AFFs*

Plant-based substrates are usually used with soybean being the most common in AFFs. Important features of alkaline-fermentations are changes in the texture and color of the products on softening. Softening of plant based foods leading to the desired texture may be attributed to pectic and proteolytic enzymes that readily hydrolyze the pectin and protein. Non-enzymic browning reactions involving the amino groups of amino acids and the non-reducing sugar constituents and microbial activity may contribute to the color development. Many alkaline fermentations are characterized by the widespread production of sticky whitish mucilaginous polymers assumed to be polysaccharides. AFFs can immensely achieve dietary sufficiency in several traditional communities where they are produced and consumed. Two main features of alkaline fermentation process include;

1. **Hydrolysis of macronutrient molecules** i.e. of complex carbohydrates like oligosaccharides, proteins and fats by the enzymes produced from predominant bacteria, thereby augmenting nutrient bioavailability and digestibility as compared to unfermented substrate. For example, fermentation with B. *subtilis* can effectively hydrolyze soybean proteins and polysaccharides into low-molecular-weight, water-soluble products requiring minimal further degradation by gastrointestinal enzymes. Increased levels of free amino acids with increased concentration of cysteine, methionine, leucine, isoleucine, tyrosine, phenylalaline and even lysine (limiting amino acids in plant foods), non-protein and soluble nitrogen content during alkaline fermentation is reported.

Most plant based sources used for AFFs are rich in carbohydrates, large proportion being non-digestible oligosaccharides such as galactamannan, stachyose, raffinose, verbascose, sucrose and arabinogalactan. They act as functional prebiotics and enable the action of beneficial microbes. Some of these non-digestible carbohydrates cause abdominal distension and flatulence in humans. Fermenting microorganisms hydrolyze starch and non-digestible carbohydrates into readily digestible sugars (melibiose, fructose, galactose) and additionally positively influencing the product texture by softening the tissue. *Bacillus spp.* produce enzymes like amylase, galactanase, galactosidase, glucosidase, and fructofuranosidase, that degrade carbohydrates during alkaline fermentation.

Alkaline fermentation also results in the change of crude fat content and fatty acid profiles attributed to the lipolytic activity of dominant microbial species involved in the alkaline fermentations. Most studies have reported increase in free fatty acid levels with very few reporting contradicting results. The composition of the free fatty acids like saturated fatty acids, polyunsaturated fatty acids and monounsaturated fatty acids varies. The presence of palmitic, stearic, arachidic, behenic, lignoceric, linolenic, palmitoleic, myristic acids and gadoleic acids and high concentrations of linoleic and oleic acids have been reported in AFFs. The presence of essential linoleic and oleic acids in human nutrition can be converted into polyunsaturated fatty acids and enhance nutritive value of the products.

2. **Enzymatic degradation:** This results in substantial reduction of naturally occurring toxic components, allergens and ANFs in the raw food substrate, thus converting otherwise inedible, difficult to digest or toxic raw material into palatable food products. During fermentation, protease and

amylase enzymes act upon protein and insoluble sugar, and therefore improve the nutritional value of fermented products.

The most popular alkaline-fermented soybean products is Natto that has distinct odor, flavor and stringy material (a polypeptide of glutamic acid and fructan) . Natto contains nattokinase, a polypeptide composed of a total of 27 amino acid residues. During natto production, protein, lipids and minerals such as iron, zinc and calcium tend to increase with decreased lipid content. Another remarkable compositional change in natto is the significant increase in vitamin K content that has been attributed to vitamin K2 (menaquinone-7 or MK-7) synthesis by *B. subtilis natto* . A positive correlation has been reported on increased consumption of Japanese natto with bone density in Japanese females with increase in serum vitamin K and ϒ-carboxylated osteocalcin, both being bone mineralization facilitators. Fermentation also revealed 3-fold increase in thiamine and riboflavin content and a 5-fold increase in vitamin B12 content compared to the raw substrate. Fermented soybeans are rich in isoflavone genestein and gamma-polyglutamic acid (PGA), which is an amino acid polymer responsible for characteristic sticky texture to the product, acts as dietary fiber to reduce serum cholesterol level and increases calcium absorption. Isoflavone genestein acts as a chemo preventive agent against cancer.

Formation of aroma compounds in AFFs:

Flavor of traditional AFFs has been mainly attributed to various volatile compounds viz: Pyrazines (acetoin,2,5–dimethylpyrazine, tetramethylpyrazine, 3-methylbutanal, 2-decanone, 3,5-dimethylphenylmethanol, ethyl linoleate , chlorobenzene), aldehydes, ketones, esters, alcohols, acids, alkanes, alkenes, benzenes, phenols, sulphurs, amines , pyridines and furans groups produced through the metabolic activities of *Bacillus* spp. during fermentation . The amino acid, particularly glutamate as well as peptides may lead to flavor enhancement. Free fatty acids are also known to positively influence the production of characteristic flavors in food but their high levels may easily cause rancidity. The heat treatment during the production contributes to the flavor by formation of pyrazines originating from reactions between sugars and amino acids. However, sun-drying of AFF products may cause loss of some volatile aroma compounds. The profiling of volatile compounds produced during controlled microbial alkaline fermentation of legumes may help in providing information for the future choice of starter cultures.

Food safety of alkaline fermented products

Alkaline-fermentation are generally known to produce safe products. For most AFFs, raw materials take a long cooking time (up to 40 h) prior to fermentation, thus contributing in elimination of non-spore–forming pathogenic bacteria. In addition, the AFFs are stable and well-preserved due to the antimicrobial effects of the dominant microorganisms towards harmful bacteria and molds. *B. subtilis* and *B. pumilus* isolated from AFF was able to inhibit and inactivate both Gram-positive and Gram-negative bacteria including *Micrococcus luteus, Staphylococcus aureus, Bacillus cereus, Enterococus facium, Listeria monocytogenes, Escherichia coli, Salmonella typhimurium, Shigella dysenteriae, Yersinia enterocolitica*, as well as *Aspergillus* mold. *Bacillus* strains isolated from some AFF is found to produce antifungal substances (iturin A, surfactin). Addition the high pH and free ammonia of AFFs makes it difficult for spoilage microorganisms to grow .

Although AFFs can be generally considered safe as already mentioned, they can at times involve a risk of food borne disease, a problem common to all types of traditional fermented foods. Further with household production using basic equipment under unhygienic conditions and hardly any training on Good Manufacturing Practices (GMP), can contribute to unsafe AFFs . This allows a persistent recontamination of the fermented seeds. Thus positive actions like adequate GMP guideline based training on hygienic conditions and hazard analysis critical control point (HACCP), better constructed equipment like improved fermentors easy to maintain, sanitation and cost effective operating cost and production of desirable starter cultures for controlled fermentation can improve the safety of traditional AFFs .

Spontaneous Versus Controlled Fermentation:

The fermentation process can occur spontaneously with uninoculated cultures (**Natural /Uncontrolled fermentation**) or can be triggered by adding starter cultures (**Controlled fermentation**).

Spontaneous Fermentation

Fermentation can occur as a natural (spontaneous phenomenon) as long as there is a substrate available, either animal or vegetable, and a microorganism consortium naturally present in the substrates to begin fermentation activity. For so-called natural or spontaneous fermented foods, the microorganisms are autochthonous and are naturally present in the raw material, equipment or manufacturing environment.

The microorganisms may participate in parallel or act in a sequential manner with a changing dominant flora during the course of the fermentation. The common fermenting bacteria are species of *Leuconostoc, Lactobacillus, Streptococcus, Pediococcus, Micrococcus,* and *Bacillus*.The fungi genera *Aspergillus, Paecilomyces,Cladosporium*, *Fusarium,Penicillium,* and *Trichothecium* are most frequently found in certain products. Lactic acid bacteria (LAB), yeasts, and/or fungi ferment various cereals and legumes based substrates to produce variety of food by natural fermentation .They classically result in products with comparable microorganisms (even the same species), regardless of provenance. For example, cabbage and other green leafy vegetable fermentations are all started by *Leuconostoc mesenteroides* followed by *Lactiplantibacillus* species and *Levilactobacillus brevis,* irrespective of whether the product is known sauerkraut (Europe and North America), kimchi (Korea), suan-cai (China) or sinki (Nepal). Such a fermentation microbiota which is highly reproducible in spontaneous plant based fermentations reveals a steady association between organisms with raw materials. With such consistency in the raw materials and environmental conditions with classic practices and factors such as salt concentrations, pH, atmosphere or other expected control measures used for making that food are in place, any erratic events likely to cause fermentation failure, are relatively rare . Spontaneous fermentations can also create a potential to isolate strains having industrially relevant attributes, including unique flavor reduction. Integrating these strains in mixed fermentations can improve the flavour of products, in addition to achieve more control over the process. As the attractiveness of spontaneous fermentations rises, it becomes important to characterize and understand the dynamics of fermentation to gain control over the process for achieving improved quality attributes.

Spontaneous fermentations involve a complex consortium of microorganisms, thus the phenotypic and genotypic nature of an individual microorganism is very much dependent on the community assembly, strongly influenced by biotic and abiotic factors that govern the fermentation ecosystem .Despite the great utility of spontaneous fermentation in safeguarding plant based foods, the chance of fermentation failure, in terms of inappropriate inhibition of spoilage or pathogen microbes, and nutrients composition, unwanted sensory traits and the risks of the implantation/domination of microbial strains dangerous for the human health, are significant. In the framework of heterogeneous microbiota of spontaneous fermentation, it is possible to find microbial pathogens and/or strains liable to synthetize toxic by-products such as mycotoxins, ethyl carbamate and biogenic amines. These microbial contaminants can reduce the safety of the corresponding fermented product. Thus relying on spontaneous uncontrolled fermentations poses serious challenges for the safety and the quality of fermented foods.

Controlled fermentation

Safety of fermented food associated with microbes in spontaneous fermentation can be conciliated with tailored starter cultures for specific productions in such a way to mimic protechnological microbial diversity associated to spontaneous fermentation .Selected starter cultures are used to bring about fermentation, the use of autochthonous cultures isolated from fermented products which are also regarded as probiotics, may speed up the fermentation process, guarantee prolonged shelf life and improved functional and organoleptic properties of the fermented foods, provide uniform quality products and render health benefits .

Important criteria for selection of starter culture(s) to be used in the manufacture of functional foods include probiotics properties, antimicrobial properties, antioxidant , peptide production , fibrinolytic activity , poly-glutamic acid , degradation of antinutritive compounds, etc. The use of appropriate starter cultures would improve product quality through rapid accelerated metabolic activities, improved and more predictable fermentation processes, desirable organoleptic characteristics as well as improved safety and reduced hygienic risks. Starter culture technology represents a cornerstone in the assurance of quality and the safety of fermentation. Generally, the importance of the adoption of a starter culture regimen is to minimize the risk of food-borne diseases , to pursue personalized nutrition, to reach new health targets, to sustainably increase shelf life, particularly of artisanal, traditional, typical, organic and biodynamic productions, yield increase, and food security .

Functional foods :Prebiotics , probiotics , synbiotics and postbiotics in food fermentation

The interest in functional foods continues to increase rapidly around the world and synbiotic foods play important role in nutrition due to their health benefits. The demand for the food formulations containing nutrients such as dietary fibers, fatty acids, proteins, vitamins, minerals and flavonoids that are naturally present in foods or added later is increasing.

<u>Definitions :</u>

Prebiotics are defined by the International Scientific Association for Probiotics and Prebiotics (ISAPP),2010 as "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" . The definition of prebiotic was quite recently revised as "*a substrate that is selectively utilized by host microorganisms conferring a health benefit*"

Probiotics can be defined as "*live microorganisms that, when administered in adequate amounts, confer a health benefit on the host.*"

Synbiotics are a combination of prebiotics and probiotics , having a synergistic effect by inhibiting the growth of pathogenic bacteria and enhancing the growth of beneficial organisms

Post-biotics are the bioactive soluble by-products released following either through the metabolic processes of probiotics or through lysis of probiotics . A technical definition of post-biotics, as proposed by The International Scientific Association of Probiotics and Pre-biotics (ISAPP) is "*preparation of inanimate microorganisms and/or their components that confers a health benefit on the host*"

<u>Prebiotics</u>

Prebiotics are categorically functional foods defined as the non-digestible food ingredients. Their consumption as dietary ingredients, may positively affect the human gut microbiota (HGM) composition and metabolic functions at the level of small intestine and colon . Different prebiotics are responsible for modification of different bacteria at strain or species level.

Prebiotics, reach large intestine in an intact form as they cannot be broken down by digestive enzymes in the gut thus serving as a substrate (as a carbon or energy source) for the growth of gut probiotic bacteria, resulting in an increase in the probiotic count. Prebiotics act synergistically with probiotics with symbiotic action resulting in optimal repopulation of the flora and suppressing the pathogens, which consequently enhance the gastrointestinal functions and immune system. Prebiotics maintain the optimal pH in the intestine which is essential for the existence of the probiotics, stimulate the peristalsis and reduce the formation of gases . They increase the absorption of calcium and magnesium, influence blood glucose levels and improve plasma lipids .Diets with prebiotics, besides increasing the HGM diversity, influence both microbial metabolic activities and the formation of determined fermentative end products like SCFA's, branched-chain fatty acids, organic acids, peptides, ammonia, amines, phenolic compounds and gases . Prebiotics are also extensively studied as protectants for encapsulation of probiotic strains to enhance the probiotic viability

Plant based prebiotics

- Inulin
- Fructooligosaccharides (FOS)
- Galactooligosaccharides (GOS)
- Trans-galactooligosaccharides (TOS)
- Mannan- oligosaccharides (MOS)
- Xylo-oligosaccharides (XOS)
- Lactulose
- Dietary fiber(soluble and insoluble)
- Gums
- Lafinose

Dietary Sources

- Garlic, onions, guar gum ,leeks, shallots, asparagus, spinach, Jerusalem artichokes, jicama, chicory root , agave, legumes peas, beans, lentils, raw oats, soyabeans, unrefined wheat, unrefined barley , yacon and bananas

Figure 2 Sources of prebiotics

Most of the prebiotics are plant products, well recognized being carbohydrates (examples given in fig.) while some peptides, proteins and certain lipids are potential prebiotics . The length of the chemical chain; the short chain, the middle chain or the long-chain determines where in the GI tract the prebiotic has an effect and how the host can feel the benefits. Breast milk oligosaccharides are reported as original prebiotic. however, other dietary sources include Plant cell wall polysaccharides and gentio-oligosaccharides have also gained attention as novel prebiotics. Marine poly-/oligo-saccharides, sugar alcohols(erythritol, xylitol, sorbitol, mannitol,lactitol, isomalt, maltitol , polyglycitols) .Algae and microorganisms are extensively studied for their prebiotic potential ,the most well known algal polysaccharides being alginates, laminarins and fucoidans (from brown algae), carrageenans and agar (from red algae) and ulvans (from green algae) . Food-grade bacteria are also a potential source of non-digestible carbohydrates and potentially bifidogenic prebiotics, hetero or homo-polysaccharides, as well as oligosaccharides such as dextran, mucan, and levan are produced by several species of *Lactobacillus, Streptococcus,* and *Leuconostoc* .

A **dietary substance** to meet the definition of prebiotics must fulfill specific criterias by *in vitro* and *in vivo* proven tests of non-digestibility (resistance to low pH gastric acid, enzymatic digestion, and intestinal absorption); fermentation by the intestinal microbiota; and selective stimulation of growth and activity of intestinal bacteria and should be unaffected by the food processing conditions and remain unchanged, non-degraded, or chemically unaltered and be available for bacterial metabolism in the intestine .*Currently,* ***fructooligosaccharides, galactooligosaccharides, lactulose,*** *and* ***non-digestible*** *carbohydrates are the prebiotics that fulfill these criterias* .

<u>Probiotics :</u>

The science of probiotics covers aspects from the field of microbiology to food processing. Probiotics are microorganisms providing health benefits to the host when consumed in sufficient amounts. They reduce gastrointestinal infections, improve lactose metabolism, regulate the functions of intestinal epithelial cells and have antimutagenic and anti-carcinogenic properties. Probiotic supplementation has a potential to reduce the intensity or shorten the duration of the infections with stimulating effects on the immune system. Right type and levels of exogenous dietary supplementation of probiotics of a species/strain can temporarily colonize the intestinal tract and stabilize the native microfloral composition, thereby restoring vital physiological function of a commensal flora . The multitude of health benefits of probiotics, are through their biological mechanisms in the body.

Food based probiotic functional foods can be divided into dairy products (e.g., cheeses, yogurts, ice cream, milk, acidified milks , creams) and non-dairy products(e.g., meats and meat products, cereal grain based bread or other fiber snacks, chocolates, fruit juices , other fruit preparations). Preserving the efficacy of probiotic bacteria and enhancing its viability during the development of functional food products is vital. International standards require that probiotic functional products claiming health benefits should contain a minimum of 10^6 -10^7 CFU/g viable probiotic bacteria when sold. To protect probiotic survival and viability and help deliver bioactive ingredients both to the food matrix itself and to the GI tract several approaches have been undertaken like immobilization technologies , composite carrier matrix systems, synbiotic development, addition of osmo, thermo, or cryo-protectants to the drying or growth media, lyophilization , microencapsulation(ME) through food matrix modifications and exploitation of the adaptive mechanisms of the living cells for survival under stressful conditions .

Lactobacillus, Lactococcus, Bacillus, Streptococcus, Bifidobacterium, Pediococcus, and *Propionibacterium* are well-known probiotic species. Yeast probiotics include *Saccharomyces cerevisiae*, *S. carisbergensis,* and *S. boulardii* and fungi such as *Aspergillus niger* and *A. oryzae* are also regarded as probiotics. However, the most common probiotics are *Lactobacillus* and *Bifidobacterium* . Probiotics are usually orally administered and are commercially available in the form of functional foods, dietary supplements and medicinal probiotics. Probiotics have also been associated with nutrients to compete with pathogens, inhibit/block pathogenic bacterial adhesion in the colonic lumen, and improve mucus production, which in turn enhances the intestinal epithelial barrier for stimulation of the immune system. Probiotic supplementation has proven useful as a prophylactic regimen in cases of antibiotic-associated diarrhea (AAD) by enabling replenishment of the gut microflora , probiotics has been found to reduce allergies in children , decrease *Helicobacter pylori* colonization of the stomach , manage relapse of some inflammatory bowel conditions , reduce the risk of certain cancers by secreting specific anticarcinogenic and antioxidant metabolites , proven useful in dealing with periodontitis and oral *Candida* infections. Probiotics are reported to reduce toxins through bile salt hydrolase and increase the bioavailability of nutrients in the body through other enzymatic activities .

Specific mechanisms involved in probiotics-linked signaling of nerve functions in the central nervous system can promote potential therapeutic actions on neuropsychiatric disorders and stress-related diseases. Probiotics also relate to brain functioning , help to treat irritable bowel syndrome , and lower serum low-density lipoprotein levels , prevent vaginal and urinary tract infections (yeast/ bacterial), prevent pancreatitis and improve pancreatic health , contribute to respiratory tract health , inhibit tumorigenesis and contribute to metabolic homeostasis to aid in the treatment of metabolic disorders such as diabetes, non-alcoholic fatty liver disease and cardiovascular diseases .Apart from its physiologic effects , probiotics can enhance the bioavailability of micronutrients, can help in the reduction of ferric to ferrous ions thus increasing duodenal absorption of iron as well as calcium uptake and absorption. Probiotics are also involved in vitamin D synthesis and absorption.

Probiotic through their metabolic activity produce soluble factors, called **postbiotics** such as bioactive metabolites through non-specific mechanism such as SCFA's, vitamins (Vit-B_2 and Vit-K_2) , enzymes, lactic acid, bacteriocins , ketones, methyl acetates, carbon disulfides and also secrete antimicrobial peptides that kill pathogenic bacterial strains . Postbiotics exerts a beneficial effect on the host, directly or indirectly and the risks associated with their intake are minimized as postbiotics do not contain live microorganisms.

Table 2 . Probiotic products available commercially

Brand	Description	Producer
Actimel	Probiotic drinking yogurt with L. casei Imunitass cultures	Danone, France
Activia	Creamy yogurt containing BifidusActiRegularis	Danone, France
Gefilus	A wide range of LGG products	Valio, Finland
Hellus	Dairy products containing L. fermentum ME-3	Tallinna Piimatoööstuse AS, Estonia
Jovita Probiotisch	Blend of cereals, fruit, and probiotic yogurt	H&J Bruggen, Germany
Pohadka	PohadkaYogurt milk with probiotic cultures	Valašské Mezirˇícˇí Dairy, CzechRepublic
ProViva	Refreshing natural fruit drink and yogurt inmany different flavors containing L. plantarum	Skåne mejerier, Sweden
Rela	Yogurts, cultured milks and juices with L. reuteri	Ingman Foods, Finland
Revital Active	Yogurt and drink yogurt with probiotics	Olma, Czech Republic

Snack Fibra	Snacks and bars with natural fibers and extraminerals and vitamins	Celigüeta, Spain
SOYosa	Range of products based on soy and oats andincludes a refreshing drink and a probioticyogurt-like soy–oat product	Bioferme, Finland
Soytreat	Kefir-type product with six probiotics	Lifeway, USA
Yakult	Milk drink containing	Yakult, Japan
Yosa	Yogurt-like oat product flavored with natural fruits and berries containing probiotic bacteria (L. acidophilus, B. lactis)	Bioferme, Finland
Vitality	Yogurt with pre- and probiotics and omega-3	Müller, Germany
Vifit	Vifit Drink yogurts with LGG, vitamins, and minerals	Campina, the Netherlands

Source: Adapted from Siró, I. 2008. Appetite, 51, 456–467.

Table 3. Differences between prebiotics and probiotic

Categories	**Prebiotics**	**Probiotic**
Content	Indigestible but selectively fermentable ingredients	Live microorganisms
Functions	Provide food for probiotics; increase number and improve activity of probiotics	Enhance the health and well-being of their host organisms' digestive trac
Health Benefits	Provide supportive function to probiotics	Reduce the number of pathogenic bacteria in the GIT and improve its function; improve immune system function; prevention of cellular damage from oxidative stress
Sources	Asparagus, Jerusalem artichokes, Bananas, Oatmeal, Onion, Garlic , Inulin, Guar gum , Legumes, etc.	Isolated microbial cultures,Yogurt, sauerkraut, Yakult, miso soup, fermented breakfast cereal and snack bars, soft cheeses, kombucha, kimchi, and sourdough
Side Effects	Increase in fermentation, leading to increased gas production, bloating or bowel movement	Possibility of sepsis when given to immune compromised patients

Synbiotic approach towards functional foods:

Synbiotics are a combination of prebiotics and probiotics, displaying a synergistic effect by inhibiting the growth of pathogenic bacteria and augmenting the growth of beneficial organisms. A good synbiotic diet can strongly influence the gut microbiome. A well designed synbiotic formula should have a prebiotic with an enhancing role on the probiotic microorganisms; and the prebiotic should particularly facilitate the growth of probiotics. Synbiotics are formulated to beneficially affect the host by improving survival and colonic implantation of probiotics, selectively stimulating the growth in colon or activating the metabolism of health-promoting beneficial probiotics in addition to maintenance of the intestinal biostructure. Synbiotics should improve the gut microbial composition with production of metabolites and bioactive compounds with the concomitant inhibition of potential pathogens present in the GI tract. Synbiotics are reported to decrease the number of infections in postoperative patients, improve the outcome of irritable bowel disease(IBD), and present anticancer effects . Patients affected by small intestinal bacterial overgrowth (SIBO) treated with antibiotics and synbiotic supplementation (containing *Bacillus coagulans* and prebiotics) obtained better responses than antibiotics alone. Synbiotic are also reported to lower the amount of undesirable metabolites, as well as the inactivation of nitrosamines and carcinogenic substances. Determining the best probiotic and prebiotic combination warrants for a structured approach in each disease setting and in each individual .

Difference Between Fermented Foods and Probiotics:

The practice of traditional food fermentation dates back to human history. The International Scientific Association for Probiotics and Prebiotics (ISAPP) redefined fermented foods and beverages in 2019 as foods made through desired microbial growth and enzymatic conversions of food components.

It is observed that the majority of commercially sold fermented foods sold do not fit in the "probiotic fermented food" category. Fermented foods and beverages are at times labelled as "probiotic foods" or "contain probiotics" by the manufacturers in an attempt to communicate to consumers about the presence of living, health-promoting microorganisms in the product. Term 'probiotic' should only be used when there is established health benefit conferred by characterized live microorganisms and must extend beyond any nutritional benefit of the food matrix. The terms 'fermented food' and 'probiotics' cannot be used interchangeably. In the absence of strain-specific evidence of a health benefit of the live microorganisms in a fermented food, some fermented foods could be aptly labelled as "contains probiotics". Some jurisdictions recognize several common species for which the term 'probiotic' can be used in foods, such as Health Canada recognizes more than 20 species of the *Lactobacillus* genus complex and *Bifidobacterium* provided they are delivered at a minimum of 10^9 CFU per serving . Health claims related to live yoghurt cultures and improved lactose digestion are approved by the European Food and Safety Authority based on the fundamental presence of the lactase enzyme in yoghurt cultures (*Lactobacillus delbrueckii* subsp. *bulgaricus* and *Streptococcus thermophilus*). However, even if the fermented food contains one or more of those species, the label “contains probiotics” should only be used when the strains in the fermented food are defined to the strain level, the genome sequences are known and the strains are present at an appropriate number throughout the shelf-life of product.

It is expected that the majority of fermented foods sold commercially today do not belong in the “probiotic fermented food” category. Instead, fermented foods and beverages often contain undefined microbial consortia, usually at variable levels, and their potential health benefits have generally not been demonstrated, it is suggested that manufacturers should state that their product contains “live and active cultures” provided the food is not processed to remove or kill the fermentation microorganisms and that these microorganisms are present at levels that are expected for foods of that type. For pasteurized fermented foods without live microorganisms in the final product, it is acceptable to label those foods as “foods made by fermentation”. Even when characterized cultures are used to perform fermentations and are understood at the strain level, those microorganisms are mostly selected based on performance characteristics, such as rapid acidification, substrate conversion, and flavour and texture properties, rather than on health-related functions.

Regulatory considerations for fermented foods

Food fermentation guidelines mainly concerned with food safety are covered in International regulations. Whether strains developed by the use of recombinant DNA technology or are genetically modified, their use is regulated and includes criteria for establishing safety. There exists designations like ‘Generally Recognized As Safe’(GRAS) in the USA or the ‘Qualified Presumption of Safety’(QPS) designation assigned by the European Food and Safety Authority to groups of microorganisms. Further identification of vital microbial components in fermented foods has prospects to lead to new food labelling regulations. Regulations ensure that minimum requirements are met relating to the involvement of specific microbial taxa in the fermentation process. Only a few standards exist, mostly for cultured dairy products. Codex Alimentarius states that yoghurt should be made using a combination of *S. thermophilus* and *L. delbrueckii* subsp. *bulgaricus* and that kefir is a fermented milk made using *Lentilactobacillus kefiri* and species of the genera *Leuconostoc*, *Lactococcus* and *Acetobacter*, in addition to lactose-fermenting yeasts (*Kluyveromyces marxianus*) and non-lactose-fermenting yeasts (*Saccharomyces unisporus*, *S. cerevisiae* and *Saccharomyces exiguus*). With identification of newer fermenting microbes with beneficial effects similar standards could emerge like kombucha and water kefir.

Recommendation of fermented foods as part of dietary guidelines

Fermented foods would contribute to meet important macronutrients as well as deliver large numbers of prospective beneficial microbes to the gastrointestinal tract. Recently, the concept of "shared core benefits" was introduced to explain how and why phylogenetically related organisms could deliver similar health benefits. Regular consumption of fermented foods having live microorganisms should be promoted around the globe by health care professionals and should be included in dietary guidelines for all the age groups. The Indian food pyramid clearly promotes the consumption of fermented foods testifying their significance in actual human nutrition. Although by definition ,the microbes in fermented foods cannot be considered probiotic, many of them are largely related to probiotic organisms, often sharing the same molecular mechanisms responsible for health-promoting properties .

Summary:

- The interest in functional fermented foods continues to increase rapidly and probiotic foods are immensely contributing to nutrition due to their health benefits. Fermentation improves quality, nutritional value and organoleptic properties of many perishable foods.
- Fermentation, a biotransformation process involves the added starter culture (in controlled fermentation) or indigenous microorganisms (in natural or uncontrolled fermentation) and the substrate as two prime factors in addition to inherent factors like temperature and the pH conditions. The probiotic properties of many fermented foods are contributed by the presence of starter cultures or indigenous microorganisms
- Fermented food products contribute to a complex rich traditional dietary diversity offering food security and sustainability. Fermentations can be classified as bacterial or fungal according to the

type of microorganisms used. Based on the food used, it is referred to as fermented cereals, vegetables, fruits, legumes, roots/tubers, milk, meat, and fish products.

- Fermentation results in the production of primary and secondary metabolites by microbial action such as antibiotics, antimicrobial peptides (ACE inhibiting peptides}, carbon dioxide, alcohol, vitamins, folates, SCFAs, intracellular and extracellular polysaccharides of microbes, antioxidants compounds, enzymes that degrade complex compounds into simple ones, bacteriocins, and cell wall components, all collectively known as postbiotics and are considered bioactive compounds. These components directly or indirectly can benefit the host.
- Prebiotic components enhance the effects of probiotic cultures when used in the production of probiotic foods. Prebiotics can escalate the nutraceutical properties of foods, facilitate the intestinal growth of beneficial bacteria and play a protective role in the gastrointestinal tract for probiotics. Individual effect of the probiotic microorganisms and prebiotics can be enhanced by using them together as synbiotic.

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