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# Probiotics

دراسات عليا / ماجستير/ فرع الأحياء المجهرية
الفصل الدراسي الثاني

مدرس المادة:
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# Contents

# Lec.1: Introduction into probiotics

- The term '-biotics' (derived from the Greek word biōtikós, meaning 'pertaining to life') refers to nutritional strategies that can be utilized to direct the gut microbiota towards a more favorable state for host health.
- The family of "biotics" include; probiotics, prebiotics, synbiotics and postbiotics.
- Probiotics are living microorganisms that confer health benefits to the host when administered in adequate amounts.
- Probiotic as a term is derived from the Latin and Greek languages (Latin *pro* meaning "for" and Greek *bios* meaning "life").
- In 1965 the word probiotic introduced for the first time into scientific literature by Lilly and Stillwell, but Parker in 1974 is considered the first one used this term as we know it today.
- Interest in the role of probiotics for human health began as early as 1908 when the Ukrainian professor of biology, the father of probiotics, Elie Metchnikoff (1845-1916) associated the intake of fermented milk with prolonged life. However, the relationship between intestinal microbiota and good health and nutrition has only recently been investigated.
- Normal microbiota denotes the population of microorganisms that inhabit the skin and mucous membranes of healthy normal persons. The genomes of the microbiota are collectively defined as the microbiome.

**How do microbial populations within each of us vary across a lifetime?**

- The amniotic fluid that surrounds the growing fetus contains some microbial species. On the list are Firmicutes, particularly lactic acid bacteria.

- On the first and second days after birth, coliforms, enterococci, clostridia and lactobacilli have been shown to be present in infants' feces.
- Within three to four days, bifidobacteria begins colonization and becomes predominant around the fifth day. Simultaneously, coliform counts decrease. So, microbes that aid in milk digestion, such as Bifidobacterium and some lactic acid bacteria, dominate the microbiota during the first few months after birth.
- Once a solid diet is introduced, Bifidobacterium is replaced with Bacteroides and Firmicutes, as they are required to help with the breakdown of more complex carbohydrates and the production of vitamins.
- By the time children reach the age of 3 years, their microbiota is fully established and thought to remain relatively stable for life.
- Microbiota in the GIT can also change because of the food and health conditions of an individual. For example, use of antibiotics can damage the equilibrium of intestinal microbiota, reducing counts of bifidobacteria and lactobacilli and increasing clostridia. The ensuing imbalance can cause diarrhea in elderly and immunocompromised people.

➢ **What can we do to help improve the balance of intestinal microbiota?**

## Selection of probiotic

In 2002, FAO/ WHO published the "Guidelines for Evaluation of Probiotics in Food," which established safety and effectiveness standards for probiotics. In this guideline are suggested several criteria for the selection of probiotics (fig.1-1). So, the microorganisms that used as probiotics must be have the following properties:

### General properties:

- Able to survive in the intestinal tract under gastric conditions by exhibiting acid and bile tolerance as well as pancreatic digestion.
- Able to adhere to intestinal epithelial surfaces, proliferate, and colonize the gut.

- Have antimicrobial activity.
- Some authors have suggested that probiotic bacteria should be of "human origin".
- Safety; nonpathogenic, nontoxic, and generally recognized as safe (GRAS).
- Antibiotics resistant.

## Technological properties:

Remain viable and stable after culture, during use and storage before consumption;

- Before probiotic strains can be delivered to consumers, they must first be able to be manufactured under industrial conditions.
- They must then survive and retain their functionality during storage as frozen or freeze-dried cultures, as well as in the food products into which they are finally formulated.
- Moreover, they must be able to be incorporated into foods without producing off-flavors or textures.
- The preparation should remain viable for large-scale production

## Physiological (Functional) properties:

- Give direct and indirect beneficial effects after consumption (stimulate immune system, decrease cholesterol levels…etc.).

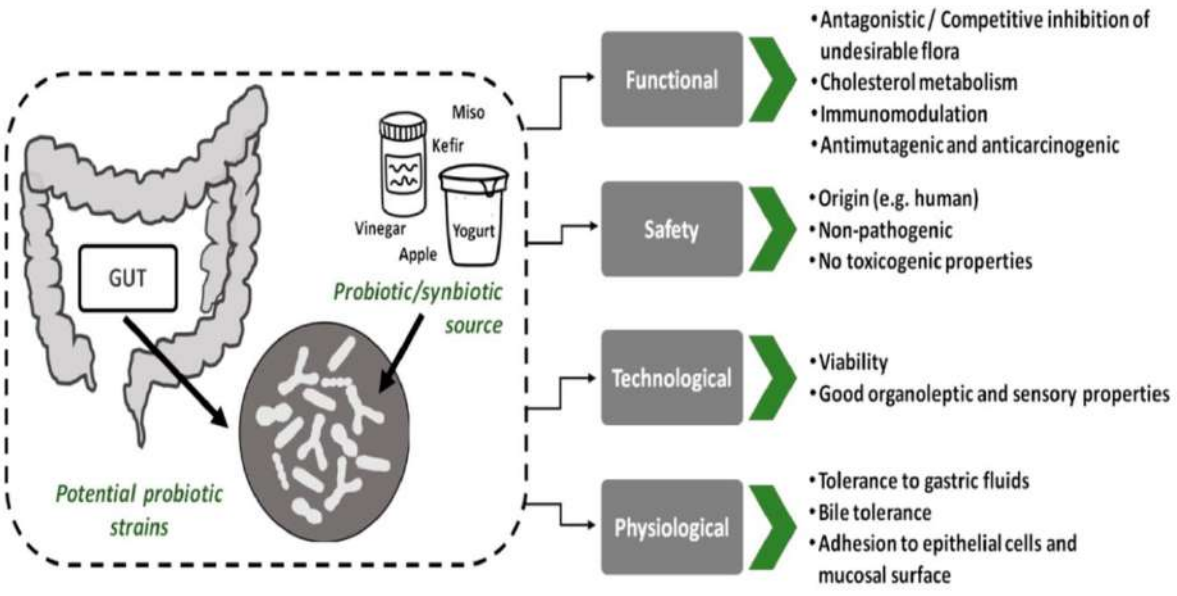



**Figure 1-1: Theoretical basis for selection of probiotic MO**

## Types of probiotics

The microorganisms that used as probiotics are strains of different bacterial species and nonpathogenic yeast Saccharomyces (table1-1). Strains of *Bifidobacterium*, *Enterococcus, Lactobacillus, Saccharomyces boulardii*, and *Escherichia coli* Nissle 1917 are the most widely used probiotic bacteria. However, other strains such as *Lactococcus, Leuconostoc, Pediococcus,* and *Streptococcus* are also used as probiotics. The most studied probiotics are; *Lactobacillus rhamnosus*, *Bifidobacterium lactis*, and *Streptococcus thermophilus*. Many probiotic supplements combine different species together in the same supplement. These are known as broad-spectrum probiotics, or multi-probiotics.

**Table 1-1: The microorganisms that used as probiotics**

| | | |
|---|---|---|
| Gram positive bacteria | Lactic acid bacteria (LAB) | *Lactobacillus*; including: *L. acidophilus*, *L.rhamnosus*, *L. plantarum*, *L. johnsonii*, *L. crispatus*, *L. paracasei*, *L. casei*, *L. gasseri*, *L. fermentum*, *L. salivarius*,*L. delbrueckii*, *L. helveticus*, *L. gallinarum*, *L. mylovarus*, *L. reuteri* , *L. brevis*, *L. bulgaricus*, *L. cellobiosus, L. crispatus*, *L. curvatus*, *L.lactis*, *L. sporogenes* and *L. sakei* |
| | | *Lactococcus lactis* |
| | | *Streptococcus*; including: *S.thermophiles*, *S. salivarius subsp.thermophiles*, and *S. diaacetylactis* |
| | | *Enterococcus* including: *E. faecium and E. durans* |
| | | *Pediococcus pentosaceus* |
| | | *Leuconostoc cremoris* |
| | *Bifidobacterium* including: *B. infantis*, *B. adolescentis*, *B. animalis* subsp *animalis*, *B. animalis* subsp *lactis*, *B. longum*, *B. breve* , *B. thermophilum* and *B.bifidum* | |
| | *Bacillus* including: *B. subtilis*, *B. coagulans*, *B. licheniformis* , *B.clausii* and *B. cereus* | |
| | *Propionibacterium freudenreichii subsp.shermanii* | |
| Gram negative bacteria | *Escherichia coli* Nissle 1917 (EcN) | |
| Nonpathogenic yeast | *Saccharomyces* including: *S. cerevisiae, S. bayanus* ,*S. florentinus* and *S. boulardii* | |

# Lec.2: Bacterial probiotics

## Lactic acid bacteria (the Lactics)

LAB are highly diverse group of commensal and environmental microbes historically defined by the ability to ferment hexose sugars to lactate.
They are:

- Gram-positive bacteria united by a constellation of morphological, metabolic and physiological characteristics.
- Non-sporing, non-motile and do not reduce nitrite
- Carbohydrate-fermenting lactic acid producers
- Acid tolerant of non-aerobic habitat
- Catalase negative
- Generally associated with habitats rich in nutrients, such as various food products (milk, meat, vegetables), but some are also members of the normal microbiota of the mouth, intestine and vagina of mammals.

Dietary LAB are GRAS owing to their long history or association with humans and food and are therefore often selected as candidate probiotics. The fraction of LAB in the GIT is low (< 1% in most individuals). The highest fractions of LAB are found in the oral and vaginal cavities.

## Classification at genus level

The classification of LAB into different genera (table 2-1) is largely based on the criteria used by Orla-Jensen (1919);

1. Cellular Morphology, rods (Lactobacillus and Carnobacterium) and cocci (all other genera)
2. Mode of glucose fermentation:
   - **Homofermentative**, which convert glucose almost quantitatively to lactic acid.
   - **Heterofermentative**, which ferment glucose to lactic acid, ethanol/acetic acid, and $CO_2$.
3. Growth at different temperatures

4. Configuration of the lactic acid produced
5. Ability to grow at high salt concentrations
6. Acid or alkaline tolerance

| Table 2-1: Genera of lactic acid bacteria (LAB) | |
|---|---|
| 1 | ***Lactobacillus*** |
| 2 | *Enterococcus* |
| 3 | *Carnobacterium* |
| 4 | *Lactococcus* |
| 5 | *Streptococcus* |
| 6 | *Leuconostoc* |
| 7 | *Oenococcus* |
| 8 | *Pediococcus* |
| 9 | *Tetragenococcus* |
| 10 | *Vagococcus* |
| 11 | *Weissella* |
| 12 | *Alloiococcus* |
| 13 | *Globicatella* |
| 14 | *Aerococcus* |
| 15 | *Dolosigranulum* |

## Current Taxonomic Position of LAB

They belong to the:

Phylum: Firmicutes
Class: Bacilli
Order: Lactobacillales
Families: Aerococcaceae, Carnobacteriacea, Enterococcaceae, Lactobacillaceae, Leuconostocaceae, and Streptococcaceae.

## *Lactobacillus*

- The genus Lactobacillus is by far the largest of the genera included in LAB encompass **261 species** .It is very heterogeneous group with unstable taxonomay, encompassing species with a large variety of phenotypic, biochemical, and physiological properties. The genus *Lactobacillus* belongs to the family Lactobacillaceae.

- The typical species composition of the intestinal *Lactobacillus* population varies among subjects and geographical regions; examples of typical species include *L. rhamnosus, L. acidophilus, L. plantarum, L. paracasei,* and *L. reuteri*.
- *Lactobacillus* was isolated for the first time by Döderlein in 1892 from the acid vaginal secretion of pregnant woman, this was termed Döderlein's bacillus, but it is now accepted as a strain of *Lactobacillus acidophilus*.
- *Lactobacillus* is low GC Gram – positive rods, usually occurring singly or in short or long chains, anaerobic, microaerophilic or facultatively aerobic.
- The temperature range is (2 – 53)°C with optimum generally (30 – 40)°C.
- *Lactobacillus* grows best in slightly acidic media with an initial pH of (4.5 – 6.4) but the optimal pH is usually between 5.5 and 6.2. The aciduric properties of *Lactobacillus* species are one of their most characteristic features.

## Sources of *Lactobacillus*

*Lactobacillus* species are widely distributed in different ecosystems and are commonly found in silages, raw milk, dairy products, fermented milk, meat and meat products, pickled vegetables, sourdoughs, fermented tea leaves, beverages, sewage, on plants, and also in the genital and intestinal tracts of man and animals.

## Probiotic lactobacilli

Probiotics are used as:

1. Dietary supplements; commonly administered as capsules or sachets,
2. Probiotic foods; may be fermented foods such as yogurts, but they are also used in nonfermented foods and beverages such as probiotic ice cream, probiotic snacks, and probiotic juices.

Clinically documented probiotic *Lactobacillus* strains include, among others, *L. acidophilus* NCFM, *L. acidophilus* La-5, *L. casei* Shirota, *L. casei* DN-114 001, *L. rhamnosus* GG, *L. rhamnosus* HN001, *L. rhamnosus* GR-1, *L.plantarum* 299v, and *L. reuteri* ATCC 55730. Two strains, *L. rhamnosus* GG and *L. plantarum* WCFS1 have been studied in great detail, and mutants of these strains have greatly aided in our understanding of the interaction with the host.

In addition to human probiotics, lactobacilli are also used as probiotic ingredients in feed for farm animals and companion animals such as pigs, poultry, and cattle.

### *Lactobacillus rhamnosus* GG

- *L. rhamnosus* is commonly found in a variety of ecological habitats, including dairy products, the oral cavity, the intestinal tract, and the vagina.
- *L. rhamnosus* strain GG (LGG), ATCC 53103 was originally isolated in 1985 from fecal samples of a healthy human adult by Sherwood Gorbach and Barry Goldin; the GG is derived from their names. It is one of the most widely studied probiotic strains and its effects on human health have been examined in numerous clinical trials, such as the diarrhea, atopic eczema, and respiratory infections.
- LGG is a Gram-positive rod (fig. 2-1) with a distinct buttery odor when cultured and its morphology is that of a creamy white colony. On Gram stain, it forms a palisading structure unlike other lactobacilli, which helps in identifying it.
- LGG produces a bacteriocin, with antimicrobial activity against anaerobic bacteria, such as *Clostridium, Bacteroides*, and *Bifidobacterium*, as well as *Escherichia coli*, *Pseudomonas, Staphylococcus, Streptococcus*, and *Salmonella*. It has been shown elsewhere that LGG is able to greatly inhibit the growth of Salmonella typhimurium specifically.
- Not all of the various secreted antimicrobials made by LGG have been definitively identified, though lactic acid is a major antimicrobial

substance made by all lactobacilli. Lactic acid may facilitate the activity of other compounds with antimicrobial action.

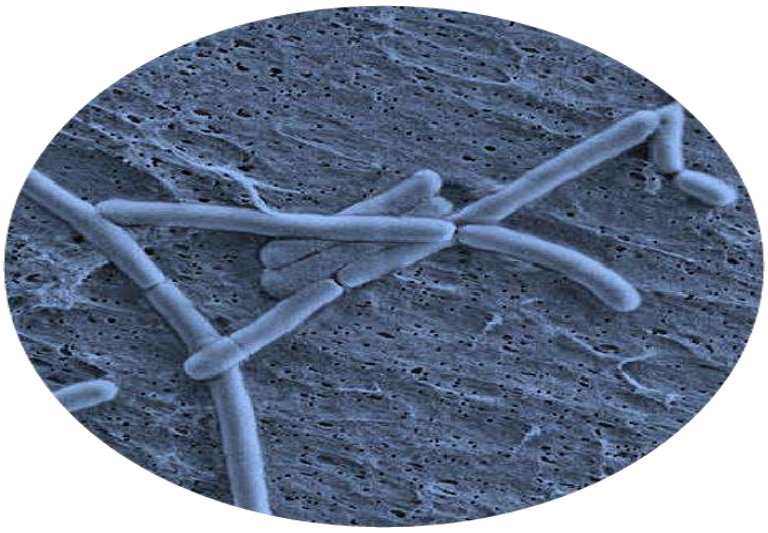


**Figure 2-1: *Lactobacillus rhamnosus* GG**

### *Lactobacillus plantarum*

- *L. plantarum* (fig. 2-2) is a versatile and flexible species of LAB that is found in a wide range of different ecological niches including vegetable, meat and dairy substrates and the GIT of humans and metazoans.
- At the genetic level, *L. plantarum* has a relatively large genome compared to other *Lactobacillus* spp. It has a 3.3 Mb circular chromosome consisting of 3052 protein-encoding genes. The genome diversity of *L. plantarum* is high and explains its flexibility and versatility, which allow this species to succeed in diverse niches and applications.
- It is frequently isolated from the human intestinal lumen and noted for being a species that is capable of surviving the low pH of the stomach and duodenum, adept at resisting the effect of bile acids in the small intestine, and transiently occupying the GIT by binding to the intestinal and colonic mucosa.
- Enteral ingestion of *L. plantarum* decreases bacterial groups with gas-producing ability, such as *Veillonella* spp. and *Clostridia* spp.

- The most important *L. plantarum* strains as probiotic are; *L. plantarum* 299v, *L. plantarum* CECT 7315, *L. plantarum* CECT 7316 and *L. plantarum* 423.
- In summary, *L. plantarum* has been widely used as a single probiotic, as in the case of *L. plantarum* 299v, or in symbiotic formulations.
- The positive influence of *L. plantarum* in health and disease extends across diverse physiological processes, including IBS, cardiovascular disease, pancreatic, respiratory tract infections, modulation of immunity in the GIT, as well as metabolic and dermatological influences.

➢ ***L. plantarum* is a very attractive probiotic candidate for clinical use?**

Because of the capacity of *L. plantarum* to efficiently survive transit through the stomach and duodenum.

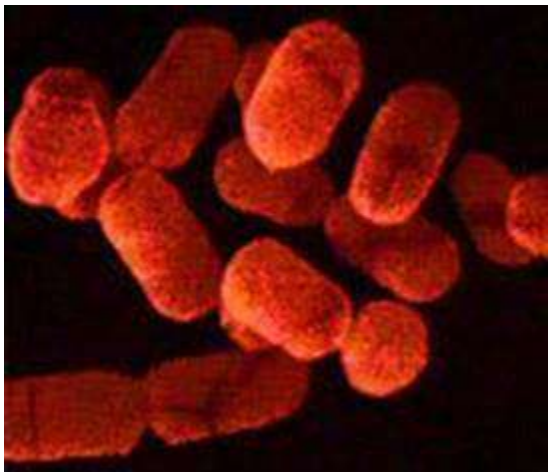


**Figure 2-2: *Lactobacillus plantarum***

## *Streptococcus thermophilus*

- Some researchers have turned to the development of replacement therapy strategies using relatively harmless indigenous streptococci as oral and nasopharyngeal probiotics, since (it is reasoned) these should have greater colonization potential than lactobacilli and bifidobacteria for these target tissues.
- Streptococcal probiotics are generally considered most likely to confer health benefits to sites other than the intestines, since their

numbers within the adult human gut microbiota, either in health or in disease, are relatively small.

- *S. thermophiles* (fig.2-3), however, is a nominated component of some "intestinal" probiotic mixes, and since large numbers of its cells are often consumed in yogurts, this species could potentially contribute to systemic or local immune stimulation in the intestines as well as conferring health benefits for subjects either exhibiting lactose intolerance (due to its high content of beta-galactosidase) or having inflammatory bowel disease (part of the VSL-3 probiotic mix).
- While *Streptococcus thermophilus* is likely to be one of the leading bacteria consumed by humans, it is less often utilized as a single strain treatment with regards to probiotics.
- In probiotic use, *S. thermophilus* is typically found in multicombinational species/strain formulations and has often been included in studies.
- Given its excellent record of safety and similarity to less desirable streptococcal bacteria, it is perhaps an organism that has been underutilized in the area of probiotics.
- Including safety, it has a number of attributes that make it a useful probiotic for humans. *S. thermophilus* has the distinction of being one of the most consumed organisms in fermented food and probiotics, with it being present in the millions of tons of yogurt and cheese produced each year. Given that yogurt containing a live culture typically has $1 \times 10^8$ bacteria present; it is likely that it contains *S. thermophilus.*
- *S*trains of *S. thermophilus* are traditionally paired with *Lactobacillus delbrueckii* and *Lactobacillus helveticus,* or *L. delbrueckii* spp. *bulgaricus*. During fermentation by *S. thermophiles* formate is produced as a by-product, which synergistically enhances the growth of lactobacilli. In these dairy products, *S. thermophilus* rapidly produces acid and subsequently a coagulum of proteins by utilizing the abundant lactose for metabolism with its highly beta-galactase production. This is a benefit for people with low levels of intestinal lactase who are lactose intolerance.

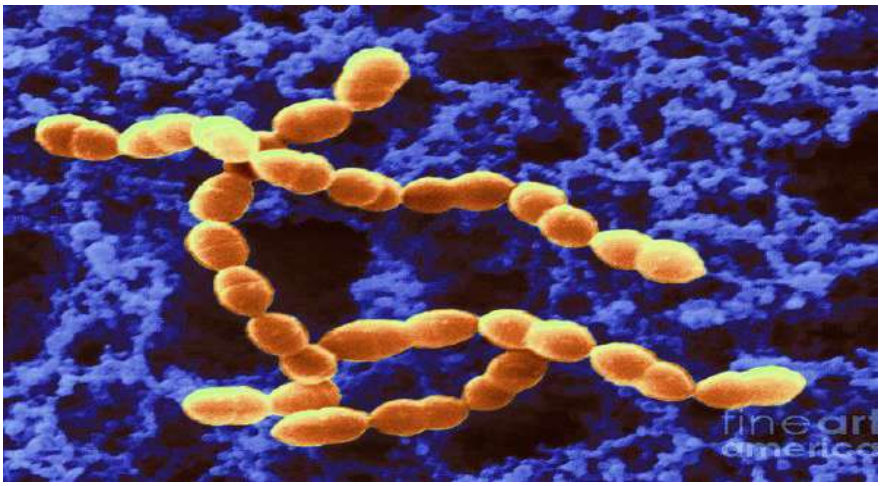


**Figure 2-3: *Streptococcus thermophilus***

- Patients with chronic *Clostridium difficile* infections are key targets for *S. thermophilus* probiotic treatment as their last resort is bowel removal. It has been shown in mice that even simple lactic acid production by *S. thermophilus* alters *C. difficile* infection and *in vitro* toxin production.

## *Bacillus*

- *Bacillus* species are ubiquitous in nature but found in higher concentrations in soil, water, and food products that have a plant origin.
- Strains of *Bacillus* are very good potential candidates to be used as probiotics. Metabolically, *Bacillus* species are very active and previous research has identified a number of useful enzymes and numerous antibiotics they produce. In addition to these secreted products, *Bacillus* remains stable in probiotic products much longer than conventional probiotics due to their ability to form endospores. Most survive the rigors of food processing, including those designed to deplete microorganisms such as pasteurization.
- They have a history of use in fermented foods (like Natto and Kinema) largely in Africa and Asia, but are becoming more prominent in global probiotics relatively recently. While there are pathogenic species of *Bacillus*, including *B. cereus* and *B. anthracis*, the more

benign members have a good record of safety and their appearance in randomized controlled studies in humans is increasing.

- The longer survival of *Bacillus* strains is quite different to other bacterial types that are used as probiotics (lactobacilli, streptococci, and bifidobacteria). These organisms, when in their vegetative states, have short lives. Most are only able to survive for a few weeks when refrigerated in dairy products. Many probiotic supplements; however, rely on lyophilisation to extend the lives of these cultures. But as a comparison, the freeze-dried products produced under optimal manufacturing and storage conditions can survive in very dry products for up to several years. Due to the requirement of these to remain dry and be protected from even atmospheric moisture, and locked in protective packaging, limited the use of these probiotics to a limited number of products. Endospores of *Bacillus* can easily attain this viability in nonideal conditions and therefore show great promise for probiotic application in a much wider array of applications.
- The main species of *Bacillus* used as probiotics include *B.subtilis* (fig.2-4)*, B. coagulans, B. clausii*, *B. pumilus*, *B. licheniformis,* and *B. cereus*, the latter has been associated with foodborne outbreaks of disease due to its potential toxin production.
- The awareness of *Bacillus*-containing probiotics has risen dramatically, as has their use and scientific credibility. More recent studies have added further insight on the composition of *Bacillus* species in the human intestinal tract belonged to *B. clausii, B. fordii,B. licheniformis, B. pumilus, B. simplex, B. sonorensis, B.thermoamylovorans,*

➢ **Can an endospore former be metabolically active and be an effective probiotic?**

It is possible that nongerminated spores still provide an immunologic benefit to the host but it is likely that the spore must germinate and grow within the gut to be fully active.

*Bacillus* species have been widely used in different countries with various regulatory approvals. *B. coagulans* was added by the European Food Safety Authority (EFSA) to their Qualified Presumption of Safety (QPS) list while the *B. coagulans* BC30 strain has undertaken the generally recognized as safe (GRAS) affirmed status. Given its prior use in foods and dietary supplements, it is becoming an acceptably safe probiotic for adults and even children, even though its efficacy is not always established. However, the evidence for *Bacillus* as a probiotic is not yet as comprehensive as for the typically used bacterial species.

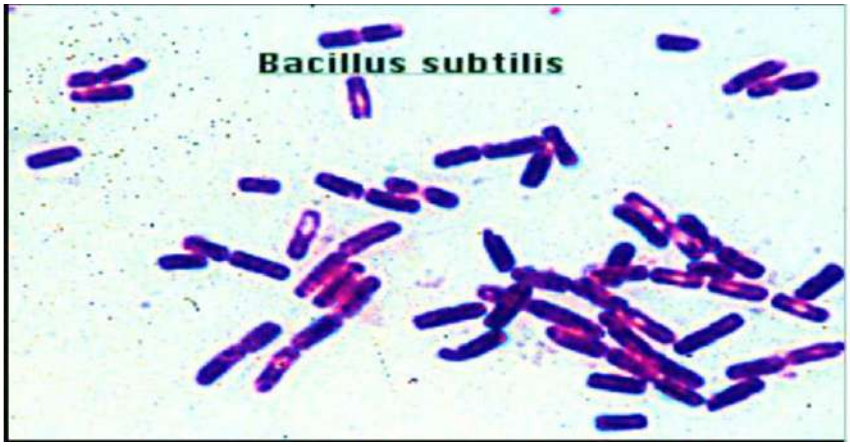



**Figure 2-4: *Bacillus subtilis***

## *Escherichia coli* Nissle 1917 (EcN)

- *E. coli* strain Nissle 1917 (fig.2-5) is probably one of the most exciting probiotic strains, both from a historical as well as a scientific and clinical point of view.
- *E. coli* strain Nissle 1917 (O6:K5:H1) is a typical example of a nonpathogenic, commensal fecal *E. coli* isolate.
- It is certainly one of the most extensively studied probiotic bacteria worldwide.
- EcN is the active component of Mutaflor (Ardeypharm GmbH, Herdecke, Germany), which since 1917 has been used to successfully treat a variety of gastrointestinal disorders.
- It is named after Professor Alfred Nissle (1874–1965), a German physician and hygienist who observed that various *E. coli* strains differed in their ability to inhibit the growth of typhus pathogens when

cultured together in a Petri dish. He isolated an antagonistically very strong *E. coli* strain from the feces of a soldier who, on the battlefields of south-eastern Europe during World War I, was not affected by the then-rampant *Shigella*-induced diarrhea. In early studies at the Institute for Hygiene of the University of Freiburg, Germany, he successfully treated patients suffering from diarrhea with this *E. coli* strain. Based on this success, he began filling gelatine capsules with the bacteria, grown on agar plates, and in 1917 applied for a patent for *Mutaflor*. Since then, Mutaflor, unchanged in its composition and containing $2.5–25\times10^9$ CFU of lyophilized viable *E. coli* Nissle strain 1917 bacteria, has been manufactured and marketed as a treatment for a number of gastrointestinal disorders.

- Despite decades of research however, the fundamental mechanisms by which EcN exerts its probiotic benefits are still incompletely understood.
- While EcN is by far the most studied probiotic *E. coli* strain, the probiotic effects of several other *E. coli* strains have also been reported like *E. coli* M-17 and *E. coli* H22.

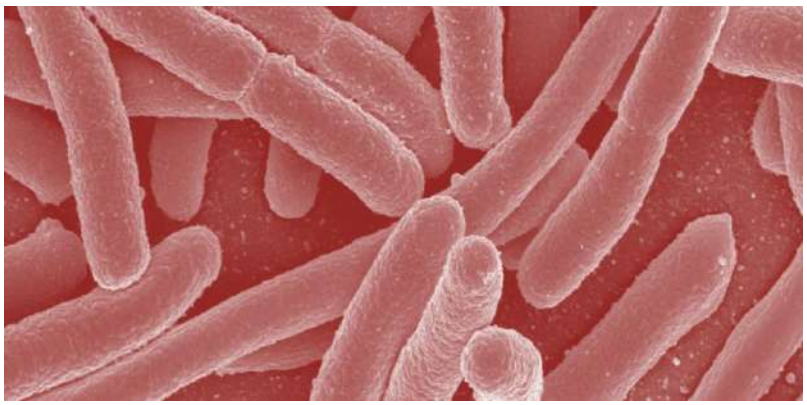


**Figure 2-5: *Escherichia coli* Nissle 1917 (EcN)**

# Lec.3: Bacterial probiotics; *Bifidobacterium*

## History

The bifidobacteria were for the first time recognized and isolated by Henry Tissier in 1899 (*Bacillus bifidus*). His observations on the microflora in the feces of breast fed infants led to the identification of certain Y-shaped irregular bacteria (bifids) which we now know as the bifidobacteria.

## Classification

It is included in the phylum Actinobacteria, class Actinobacteria, subclass Actinobacteridae, order Bifidobacteriales, and family Bifidobacteriaceae.
Currently, this genus contains more than 50 species, isolated majorly from the GI tracts possessing the physiology and morphology that characterizes them, including several subspecies; this number rises every year.
There are seven phylogenetic groups within the genus:

1. *Bifidobacterium pullorum* group (birds)
2. *Bifidobacterium asteroides* group (insects)
3. *Bifidobacterium boum* group
4. *Bifidobacterium longum* group
5. *Bifidobacterium bifidum* group
6. *Bifidobacterium adolescentis* group
7. *Bifidobacterium pseudolongum* group (human)

## Description

Taxonomy microorganisms of the genus *Bifidobacterium* are:

- High G+C Gram positive,
- Obligately anaerobic bacteria,
- Not classified with LAB, but which occupy similar habitats and produce lactic acid as a sole end-product,
- Optimum temperature for growth is 37–41°C,
- Non-spore forming, nonmotile, non-gas-producing,

- Nonfilamentous rods, which can display various shapes (fig.3-1), with slight bends or with a large variety of branching, from which the most typical ones are slightly bifurcated club-shaped or spatulated extremities. They can be found singularly, in chains, in aggregates, in "V," or palisade arrangements when grown under laboratory conditions, and under stressful conditions their cell morphology becomes pleomorphic.

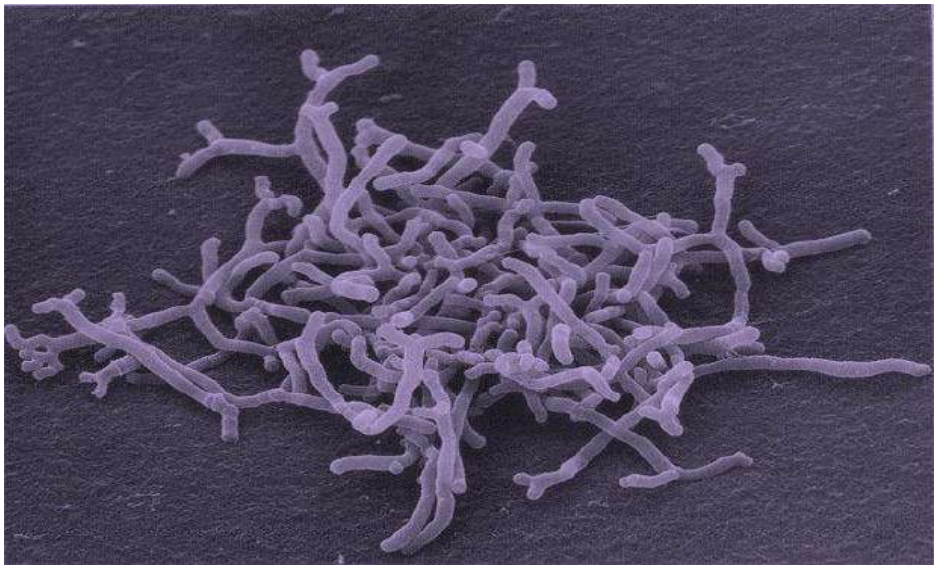


**Figure 3-1: Bifidobacterium**

From a metabolic point of view, the more typical trait of this genus is the catabolism of monosaccharides. Bifidobacteria use a particular route for monosaccharide degradation, the so-called **fructose 6-phosphate pathway, or bifid shunt**. The fructose 6-phosphate phosphoketolase (Xfp) is the main enzyme of this path. Xfp possesses a dual-substrate specificity on fructose 6-phosphate or xylulose 5-phosphate. The end metabolites of the pathway are acetate, lactate, and ethanol. Xfp activity on fructose 6-phosphate is the most common phenotypic test for bifidobacteria, and for many years it has been the main taxonomic test to identify this genus, since this activity is present in members of the family Bifidobacteriaceae, but it is not present in other Gram-positive intestinal bacteria. However, currently, DNA-sequencing-based analyses are the standard techniques for identification and typing of bifidobacteria.

## *Bifidobacterium* habitats

The recognized habitats of bifidobacteria include the human intestine, the human oral cavity, food, the animal (nonhuman) GIT, the insect intestine, and sewage.

- Bifidobacteria are among the dominant bacterial populations in the gastrointestinal tract (GIT) of humans.
- Among the bifidobacterial species described so far, *B. catenulatum, B. pseudocatenulatum, B. adolescentis, B. longum, B. breve, B. bifidum, B. animalis*, and *Bifidobacterium dentium* are commonly detected in feces of healthy subjects.
- *B. bifidum*, *B. longum* subsp. *infantis* and *B. breve* are the most abundant species in breast-fed neonates. Human colostrum and milk contain high concentrations of human milk oligosaccharides (HMOs) that enriches for the growth of the bacteria. With increasing age the relative abundance of the bifidobacteria decreases which might explain some of the age related disorders in the elderly population and needs to be replaced with proper probiotic supplements containing the right combination and dosage of the bacterial strains.
- The typical species isolated from functional foods is *B. animalis* subsp. *lactis.*

Until now there have been no clear relationships between type of diet (Western, Asian and Mediterranean) and the enrichment in the gut of particular *Bifidobacterium* species, but differences have been reported between different human groups and countries.

## Probiotic bifidobacteria

There have been numerous reports of the health benefits provided by the *Bifidobacterium* strains. Their role have been found to be important to the health of infants, adolescents, youths and aged. There is also no such distinction in the efficiency of their beneficial effects according to the sex of the individuals.

However, the probiotic efficacy of bifidobacteria showing positive effects on gastrointestinal functions after human intervention trials has only been studied for a few strains, normally supported by multinational food

companies, most of them belonging to the species *B. animalis* subsp. *lactis*, *B. breve, B. longum* and *B. bifidum.*

The low oxygen tolerance of bifidobacteria hampers their use as probiotics because stabilization of *Bifidobacterium* products to meet requirements for shelf-life is difficult compared with, for example, lactobacilli.

Beneficial effects that have been demonstrated for *Bifidobacteria*, in general, include:

- Protection against pathogens,
- Enhancement of the gut barrier,
- Synthesis of water-soluble vitamins,
- Digestion of plant oligo- and polysaccharides,
- Suppressing the production of potentially toxic and carcinogenic metabolites,
- Promoting an antiinflammatory environment.

## *Bifidobacterium animalis* subsp. *lactis*

- *B. animalis* is a normal inhabitant of the mammalian colon and has been isolated from dairy cultures.
- *B. animalis* contains two subspecies: *animalis* and *lactis. B. animalis* subsp. *lactis* is very resistant to acidity and oxidative stress, adheres to intestinal mucin, and grows in milk-based media; important characteristics given its frequent formulation as a yogurt and its need to survive transit through the gut.

Two commercial preparations contain strains of *B. animalis* subsp. *lactis*:

- *B. animalis* subsp. *lactis* DN-173 010, often referred to a *B. lactis* DN-173 010, is commonly available in a fermented milk product combined with two yogurt starter cultures: *Streptococcus thermophilus* and *Lactobacillus bulgaricus.*
- *B. animalis* subsp. *lactis* BB-12 is available either on its own or in combination with other probiotics or a prebiotic (as a synbiotic) in a variety of preparations (dietary supplements, infant formula, and fermented milk products).

➢ **Why *B. animalis* subsp. *lactis* is the most widely used *Bifidobacterium* species in probiotic products?**

## *Bifidobacterium bifidum*

- *Bifidobacterium bifidum* (GRAS) was first isolated from the infant intestine. *B. bifidum* is an important constituent of the colonic microbime and is especially prevalent in the colon of breast-fed infants which, along with *Bifidobacterium longum* and *Bifidobacterium breve*, constitutes one of the dominant commensal bacterial species.
- The probiotic potential of number of strains have been evaluated in the laboratory (BB-06, mimbb-75, YIT 4007, YIT-10347, G9-1, R0071, NCFB 1454, BbVK3, BGN4, and PRL 2010) and some have been formulated as probiotics and studied in man.
- *B. bifidum* strains have been studied in isolation, in conjunction with another putative probiotic organism, with a prebiotic, or in a probiotic cocktail, such as the formulation that includes *Lactobacillus acidophilus*, *Lactobacillus casei*, and *B. bifidum* or the probiotic Dahi containing *L. acidophilus* LaVK2 and *B. bifidum* BbVK3.

Some clinical effects of *B. bifidum:*

- Reduce the occurrence of upper respiratory infections
- Benefits in eczema and *Clostridium difficile* infection
- The management of radiation-related diarrhea
- Prevention of rotavirus-related diarrhea in children
- Metabolic and neuromodulatory effects in hyperlipidemia
- Affecting both the upper and lower gastrointestinal tract.

## *Bifidobacterium breve*

- It was first isolated from the feces of healthy infants; indeed, *B.breve* is regarded as one of the first colonizers of the infant gut.
- A number of *B. breve* strains have been evaluated in man: BBG-001, BR-03, B632, M-16V, BB536, CNCM I-4035, C-50, and strain Yakult, either as a single organism, in combination with another probiotic (typically a *Lactobacillus* or a *Streptococcus*) or as a synbiotic in

combination with a prebiotic (such as a galactooligosaccharide). *B. breve* has also been a component of multiorganism probiotics, such as VSL#3 and a commercially available preparation that includes *Lactobacillus rhamnosus* GG, *L. rhamnosus* LC705, *B. breve* Bb99, and *Propionibacterium freudenreichii* subsp. *shermanii* JS.

Various strains and preparations of *B. breve* have been evaluated in clinical studies;

- Positive immunological effects; an enhancement of transforming growth factor (TGF) , increases in secretory IgA and the anti-inflammatory cytokines interleukins (ILs) 10 and 4 , reduction in the proinflammatory cytokine IL-12.
- Benefits have been demonstrated in cow's milk allergy, in preventing allergic disorders in children through its administration to their mothers during pregnancy, in atopic dermatitis, and asthma. However, it is important to note that other studies have shown no benefits in atopic dermatitis.
- A formulation incorporating *B. breve*, *Lactobacillus casei* and the prebiotic galactooligosaccharide was shown to reduce the occurrence of infectious complications following liver transplantation.

## *Bifidobacterium longum*

- *Bifidobacterium longum* contains three subspecies: *longum*, *infantis*, and *suis* which. *B. longum* spp. *infantis* and *longum* have been isolated from the infant and adult intestine and several strains from both subspecies have been studied in the laboratory, as well as in clinical trials as probiotics.
- Unlike some pharmacological agents or even other probiotics, this organism was similarly effective among all IBS subjects regardless of subtype (i.e., diarrhea-predominant, constipation-predominant, or mixed) in a dose of $10^8$ CFU/ml but not in a dose of $10^6$. Furthermore, it was well tolerated and not associated with any major adverse events.

# Lec.4: Non- Bacterial probiotics (yeasts)

The yeasts constitute a large and heterogeneous group of eukaryotic microorganisms that are widespread in natural environments, including the GIT of humans, plants, airborne particles, and food products.
The high content of proteins, vitamin B, traces minerals, and various immune-stimulant compounds (proteases, β- glucans, and mannan oligosaccharides) has increased the interest in the use of yeasts as a probiotic. Yeasts also have the advantages of non-susceptibility to antibiotics and good tolerance for industrial processing conditions (i.e., lyophilization and high temperatures).
Fungal probiotics have been shown to improve gut barrier function and decrease the inflammation tone, reducing body weight, fat mass, and hepatic steatosis in obese and type 2 diabetic mice.

## Probiotic yeast selection

The criteria adopted by the most studies for the selection of yeasts are essentially those established for bacteria:

- The ability to survive during the passage through the human GIT
- Tolerance to low pH and bile salts
- Epithelial adhesion capacity
- Control of the growth of pathogenic microorganisms

Based on these criteria, a significant number of potential probiotic yeasts, have been isolated from different sources , including:

- *Saccharomyces*
- *Rhodotorula*
- *Pichia*
- *Candida*
- *Meyerozyma*
- *Yarrowia*
- *Torulaspora*
- *Debaryomyces*
- *Kluyveromyces*

## *Saccharomyces*

It is a genus of fungus that encompasses numerous species and strains used for a wide diversity of purposes. *Saccharomyces cerevisiae* strains have a long history of use in baking and brewing preparations, but have only infrequently been investigated for probiotic properties.

### *Saccharomyces boulardii*

*S. boulardii* (fig.4-1) is not normally found in humans, was discovered by a French microbiologist, Henri Boulard in 1920 when he was in IndoChina searching for new strains of yeast that could be used in fermenting processes. He was visiting during a cholera outbreak and noticed that some people who did not develop cholera were drinking a special tea. This tea was made by taking the outer skin from a tropical fruit (lychee and mangosteens) and cooking them down to make tea. He succeeded in isolating the agent responsible. It was a special strain of yeast he named "*S. boulardii.*" The patent for this yeast was bought by Laboratories Biocodex in 1947, which began research and manufacturing protocols.

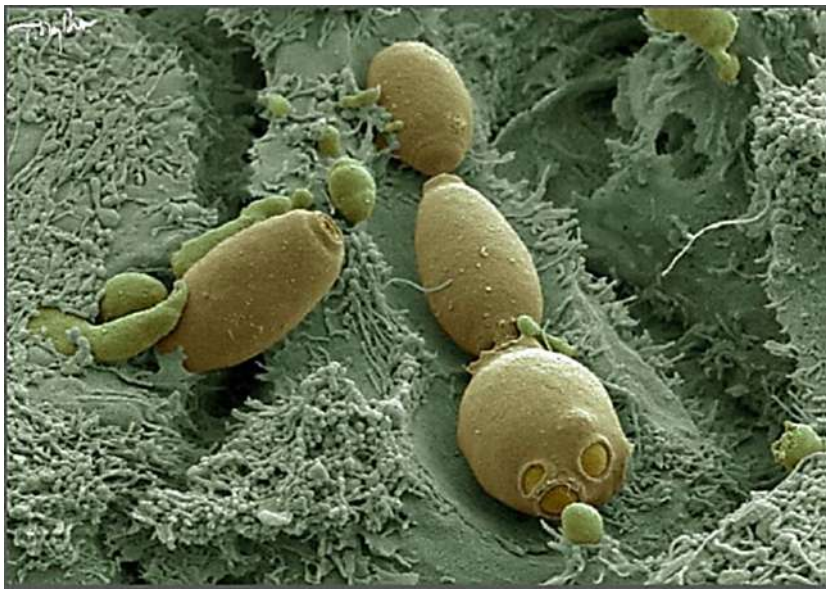


**Figure 4-1: Photograph of *Saccharomyces boulardii***

Advances in typing methods opened a debate as to whether this strain should be reclassified as a variant of *S. cerevisiae* or remain as a separate species. *S. boulardii* and *S. cerevisiae* strains were found to be similar, but

*S. boulardii* has different behavior and growth profiles. *S. boulardii* is different physiologically and metabolically in that its optimum growth temperature is 37°C, and it is resistant to low pH and is tolerant of bile acids; whereas other strains of *S. cerevisiae* prefer cooler temperatures (30–33°C) and do not survive well in acid pH ranges. Microsatellite polymorphism analysis and retrotransposon hybridization analyses showed that *S. boulardii* has a unique and specific microsatellite allele that differs from *S. cerevisiae* isolates.

## *Saccharomyces boulardii* as probiotic

- *S. boulardii* is the most studied yeast probiotic with 359 published studies (from January 1975 to October 2015) on a wide variety of topics (fig.4-2). Its properties of growing at 37°C and being intrinsically antibiotic-resistant have led to its use as a probiotic in humans.
- Other strains of *Saccharomyces* may have probiotic properties, but clinical efficacy evidence for these other strains is currently lacking. So, it is the only yeast preparation formally recognized and commercialized as probiotic for humans.

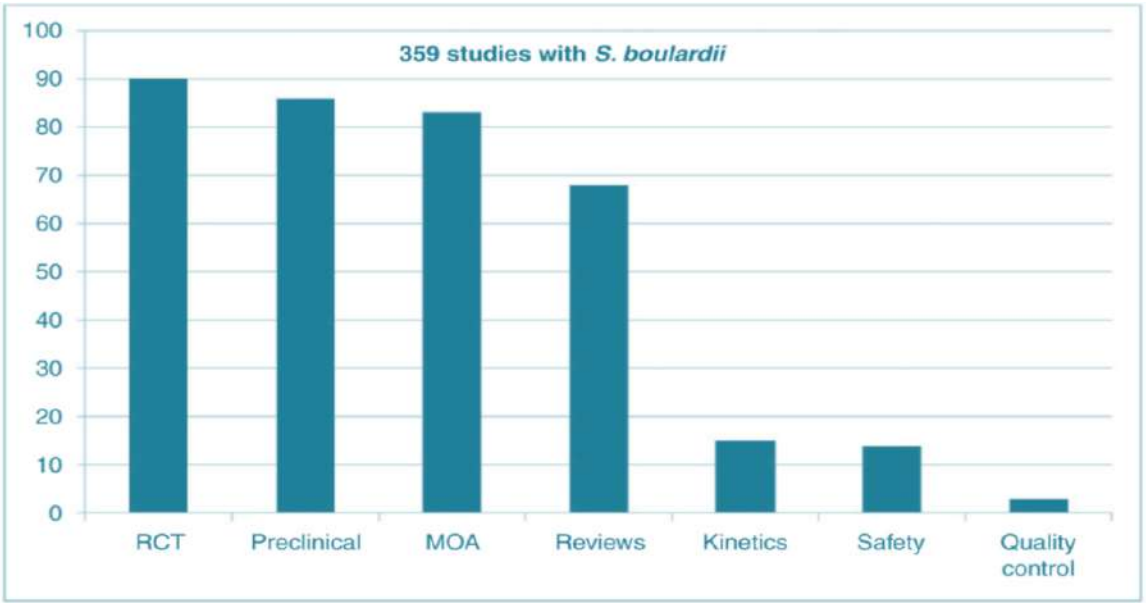



**Figure 4-2: The types of published studies (1975–2015) for the *S. boulardii* CNCM I-745 strain. *MOA*, mechanism of action; *RCT*, randomized controlled trials**

The use of *S*. *boulardii* as a therapeutic probiotic is supported by its mechanisms of action, pharmacokinetics, and efficacy from animal models, and clinical trials. The overall safety profile for *S*. *boulardii* is beneficial. *S*. *boulardii* can be recommended for several diseases. *S. boulardii* is effective for the:

- Treatment of acute pediatric diarrhea
- Prevention of antibiotic-associated diarrhea (AAD)
- Reduction of side effects of *H. pylori* treatment

Although less well studied, *S. boulardii* shows promise for the treatment of IBD and for the prevention of enteral nutrition related diarrhea. More clinical trials are encouraged for the treatment of chronic diseases (HIV related diarrhea and IBS) and the prevention of *C. difficile* disease recurrences and other diseases.

Guidelines for the most effective use of *S. boulardii* is generally, giving a daily dose of $>10^9$ per day. The duration of treatment can vary from 7 days to 6 months and it may be given alone or as an adjunctive treatment, depending upon the disease indication.

Currently, this yeast probiotic is used in over 80 countries spanning Europe, North and South America, the Middle East and Asia.

# Lec.5: Genetically modified probiotics (GMPs)

While the search continues for nonpathogenic organisms with therapeutic potential, genetic engineering of an organism to produce an identified desirable bioactive molecule may represent a technically more efficient and attractive approach.

Administration of therapies by the systemic route is recognized to cause unwanted side effects at sites other than those of interest. The concept of localizing delivery to a region of the intestinal mucosa where synthesis of a bioactive molecule in situ may bring about the desired effect without the disadvantages of systemic side effects has appeal.

### Genetic manipulation offers the potential to:

- Enhance the existing probiotic properties of an organism
- Imbue an organism with probiotic properties

Consumer resistance to genetically modified organisms (GMO) is such that GMO probiotic foods are unlikely in the near future, but clinical applications to ameliorate or prevent chronic intractable diseases may be more readily accepted.

### The organism will need to be "biologically contained" to prevent:

- Its undesirable release and accumulation into the environment
- Transmission of the genetic modification to other bacteria

## Safety Considerations

A major challenge for using engineered probiotics in humans is the fact they are GMOs and in many cases will be expressing proteins derived from humans. Therefore, the utmost care must be taken to ensure that these organisms are not released into the environment, where they could be adsorbed by another person or possibly transfer the therapeutic cassette to another bacterium. Another concern is the deleterious effect that genome manipulation as well as population numbers of a nontraditional microorganism might have on microbiome functioning and

overall human health. Proper and rigorous characterization of the safety of these GMOs should help to prevent the occurrence of this type of issue.

## Genetically manipulate LAB

The development of strategies to genetically manipulate LAB dates back to the 1970s when the first studies of *Lactococcus lactis* plasmids began, which was facilitated by the development of recombinant DNA technology in the1980s. *L. lactis* was the genetic workhorse of the LAB field, with a wide variety of gene knockout/ editing technologies and regulated expression systems being developed over the past 30 years.

## Engineered probiotics for the heterologous expression of therapeutic proteins

In addition to being used as traditional probiotics, lactobacilli also have been investigated as delivery systems for therapeutic proteins. While many lactobacilli and *L. lactis* have been engineered to secrete human proteins, relatively few have been tested in humans.

Synthetic biology, next generation sequencing, and improved genome-editing technology have made it possible to explore novel ways of engineering microbes to produce therapeutic proteins and compounds to improve human health.

## Conceptual Challenges

One of the biggest hurdles scientists face in developing therapeutic delivery systems is engineering organisms that have evolved to live in the intestinal environment rather than lab-adapted strains that are conditioned to grow on laboratory media. Lactobacilli offer an attractive avenue for such platforms, because many strains isolated from humans have long been used in probiotic preparations, and the necessary tools are being developed.

Many Lactobacillus species have also been vetted for their survivability in animal and human intestinal tracts, indicating that several strains have the ability to traverse the complex environments of the intestine and remain viable to be able to deliver proteins to sites of pathology.

- ***L. lactis*, which despite a plethora of strong genetic tools for protein expression and secretion lacks the ability to survive in the human GIT?**

  Because *L. lactis* is involved in milk fermentation and has an optimum growth temperature of 30°C, an environment that is far from the harsh environment of the intestinal tract, and this has limited the utility of *L. lactis* in humans.

Now that genetic tools for human-associated lactobacilli are becoming more readily available, combined with emerging concepts from synthetic and systems biology, the time is right for engineering precision therapeutic delivery vehicles for humans.

**Criteria that consider key to generating an ideal delivery vehicle were proposed. The organism must:**

1. Survive in the intestinal tract
2. Have a remarkable safety profile
3. Deliver the therapeutic payload only when in the presence of pathology
4. Turn off the therapeutic payload when pathology is resolved
5. Self-destruct upon or before exiting the body
6. Have stable integration into the chromosome to prevent horizontal gene transfer
7. Be unable to stably colonize the intestine
8. Have a stable system that will deliver a reliable output
9. Remain viable and functional when grown in an industrial format for dissemination to patients

These criteria will be especially important for the delivery of proteins that may induce deleterious effects when released for prolonged times, such as is the case of several therapeutic targets including cytokines and hormones that stimulate cell growth and could potentially promote cancer when administered constantly.

- **The scenarios in which the application of these guidelines will be redefine depending on the therapeutic goal. How?**

  For instance, when the delivery of proteins for long periods is needed (such as for treatment of chronic gastrointestinal diseases) the use of microorganisms that colonize the GIT might be more beneficial than organisms that transiently inhabit the intestinal tract. In these special cases, it will be necessary to have strategies to promote biocontainment as well as to control the colonization of the GMO to reinforce the safety of the system.

# Lec.6: Isolation and identification of probiotics

Several genera of bacteria (and yeast) have been proposed as probiotic cultures, the most commonly used are Lactobacillus and Bifidobacterium species. However, the selection of a strain to be used as an effective probiotic is a complex process (fig.6-1). The work begins with the source of screening of strains, the most suitable approach being the natural intestinal environment.

According to FAO/WHO guidelines it is necessary to identify the microorganism to species/strain level given that the evidence suggests that the probiotic effects are strain specific. It is recommended to employ a combination of phenotypic and genetic techniques to accomplish the identification, classification, and typing.

## Phenotypic techniques

They are including:

- Morphological examinations (macroscopic and microscopic)
- Physiological and biochemical tests

In general, although phenotypic tests provide some evidence of metabolic capabilities, there are some problems such as a lack of reproducibility and a lack of discriminatory power.

## Genotypic techniques

They are powerful even between closely related species and including:

1. Hybridization techniques
2. Polymerase Chain Reaction (PCR)
3. 16S rDNA Sequencing
4. Random Amplified Polymorphic DNA (RAPD)
5. Ribotyping
6. Restriction fragment length polymorphism (RFLP)
7. Amplified Fragment Length Polymorphism PCR (AFLP-PCR)
8. Amplified rDNA (Ribosomal DNA) Restriction Analysis (ARDRA)
9. Rep-PCR

10. Denaturing/Temperature gradient gel electrophoresis (DGGE/TGGE)
11. Pulsed Field Gel Electrophoresis
12. Fluorescence in situ hybridisation (FISH)
13. DNA micro-arrays

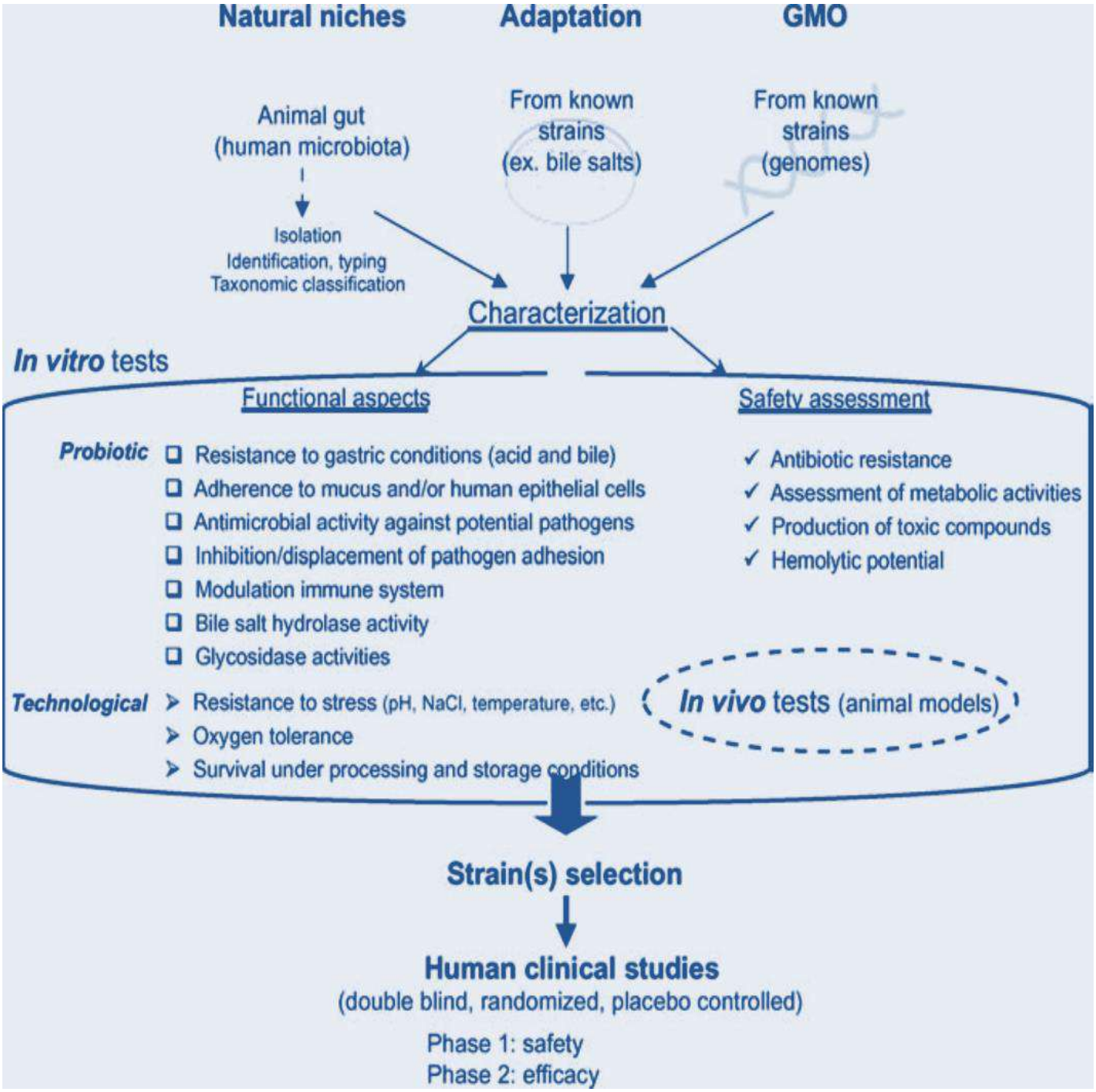



**Figure 6-1: Procedure for the isolation and characterization of novel strains with putative probiotic status**

For the nomenclature of bacteria, scientifically recognized names must be employed and it is recommended to deposit the strains in an internationally recognized culture collection.

Further characterization of strains must be undertaken taking into account the "functional" or probiotic aspects and safety assessment. *In vitro* tests, some of them summarized in figure 1, are useful to gain knowledge of both strains and mechanisms of the probiotic effect. In addition, even if these genera have a long history of safe consumption in traditionally fermented products and several species have been awarded a "General Recognised As Safe" (GRAS) status by the American Food and Drug Association or a qualified presumption of safety (QPS) consideration by the European Food Safety Authority (EFSA), some characteristics must be studied to ensure the safety of the novel lactobacilli and bifidobacteria strains. Several of the *in vitro* tests can be correlated with *in vivo* studies with animal models, but probiotics for human use must be validated with human studies covering both safety (phase 1 trials) and efficacy (phase2 trials) aspects. Phase2 studies should be designed as double-blind, randomized, and placebo-controlled to measure the efficacy of the probiotic strain compared with a placebo and also to determine possible adverse effects.

## Sources for the isolation of probiotic bacteria

### Clinical samples:

- Fecal samples from healthy adult or infant volunteers
- Vaginal samples from healthy women volunteers
- The terminal ileum or colonic mucosa
- The oral cavity
- Human milk

### Non-Clinical samples:

- Food and beverages
- Plants
- Soil

For the isolation of novel strains, classical cultivation techniques must be employed. Enrichment, selective media, and specific culture conditions are employed for the isolation of strains from human samples.

➢ **Even though essentially all animals contain strains of both *Lactobacillus* and *Bifidobacterium* genera, it is well accepted that an effective human probiotic should be of human origin. Why?**

Because human intestines are sufficiently different from those of animals, such that the isolates suited to those environments would not necessarily be suited to the human intestine. The human GIT is a very complex ecological niche and its bacteria inhabitants can achieve the highest cell densities recorded for any ecosystem. Nonetheless, diversity at a division level is among the lowest and the lactobacilli and bifidobacteria comprise less than 5% of the total microbiota.

## *Lactobacillus*

The primary methods for identificaton of *Lactobacillus* species and still in use are:

- Morphology
- Gram staining
- Biochemical tests such as;

  - Fermentation of carbohydrates
    Miniaturized biochemical test kits API 50 CH (bioMerieux, France) were used to study the carbohydrate fermentation profiles of probiotic lactobacilli. The API 50 CH carbohydrate kit comprising 49 different carbohydrate tests is used routinely in biotyping, taxonomy and identification related research studies. It is found to be one of the rapid versatile techniques with ease of operation and interpretation of results.
  - Growth at different temperatures
  - Salt concentration

Phenotypic analyses are time consuming and require technical skill and standardized assays and reading conditions, in order to avoid subjective results.

*Lactobacillus* species and strains display an inherent high level of phenotypic variability. Thus, phenotypic heterogeneity makes classical

microbiological methods ambiguous and unreliable. In fact, many studies emphasize that the phenotypic classification of lactobacilli is unsatisfactory.

At present, a majority of the molecular identification methods of *Lactobacillus* strains rely on the analysis of rRNA genes, mostly after their partial or complete amplification by the PCR technique (Table 1), such as ribotyping, RAPD, PFGE, TAP-PCR, AFLP, REP elements PRC amplification (REP-PCR), ERIC-PCR , etc.
Comparison of the rRNA gene sequences (mainly 16S rRNA) allows a precise identification and, at the same time, tracking of the evolutionary relationships among the distinct species. At present, specific primers are available for targeting most *Lactobacillus* species.

Polyphasic approach which integrates phenotypic, genotypic, and phylogenetic information is a new tool for the description of species and for the revision of the present nomenclature of some bacterial groups.

## *Bifidobacterium*

Traditionally, bifidobacteria have been identified on the basis of phenotype investigations. The host from which the bifidobacteria was isolated (e.g. animalis, adolescentis, pullorum, dentium, etc.) often represented the first identification criteria for many of these bacteria.

**The most commonly analyzed phenotypic characteristics for this genus;**

- Cell morphology
  Their distinct cell morphology can be helpful in differentiating bacteria belonging to this genus. For example, *B. bifidum* appear as flask-shaped cells, while *B. asteroides* are star-shaped.
- Colonies formed on agar plates are convex, creamy or white, glossy, smooth, neat-edged, sticky, and soft.
- Determination of metabolites
- Enzyme activities
- The ability to utilize sugars

- The presence of fructose-6-phosphate phosphoketolase (F6PPK or Xfp) activity

Until the 1960s these criteria were the only identification criteria used. However, several problems become apparent when the identification is carried out at species level, and the classical phenotyping, such as sugar fermentation profiles, transaldolase serotyping, cell-wall composition, and the study of the F6PPK isoforms, is clearly not discriminative enough to reach species, subspecies, and biotype level identification with confidence.

Molecular tools have been developed for identifying probiotics (Table 1). The study of ribosomal rRNA genes (rDNA) is the most common methodology for bifidobacteria identification up to date.

➢ **The phenotypic methods suffer from a certain lack of reproducibility, why?**

Due to the culture conditions, metabolic status of the cells, and sometimes the lack of stability of the genetic determinants responsible for such phenotypes. As a matter of fact, most cases of probiotic misidentifications stem from the use of inappropriate phenotypic methods.

## Characterization of Probiotic Properties in Bifidobacterium and Lactobacillus Strains

Several criteria have been used for the selection of probiotic strains (Figure 1), the most commonly employed being the:

- Survival of the stressful GIT conditions (low pH and high bile salts concentrations)
- Ability to transitory colonize the GIT, which is related with the adhesion to mucus and/or intestinal epithelium
- Antimicrobial activity through the production of antimicrobial molecules or the ability to inhibit/displace the adhesion of pathogens

# Lec.7: Manufacture of probiotics

It is not uncommon that academic researchers isolate a promising strain that in the end appears to be difficult to grow on a large scale.

A probiotic strain manufacturing process can vary from supplier to supplier, and a simplified version (post strain selection) of the process is provided in fig.7-1.

Because probiotic strains can vary widely in their tolerance to processing conditions, a consideration of the inherent probiotic traits along with an understanding of finished product matrix formulation can be critical in developing a suitable process.

One of the critical factors to consider is whether activity of the probiotic is expected in the finished product. For example, in most dietary supplement or powder applications, a long shelf-life, tolerance to desiccation, and elevated storage temperatures and cell dormancy are required. In a different product example, like a dairy matrix, cell activity and resistance to low pH and oxygen are required.

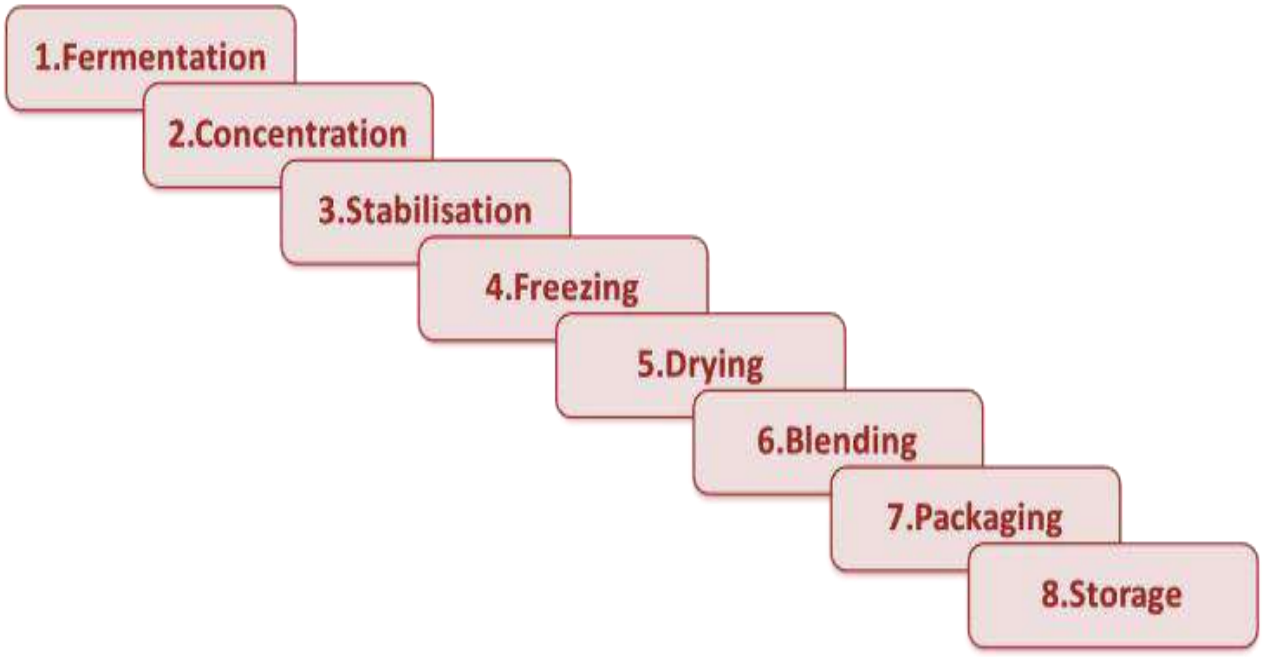



**Figure 7-1: Schematic representation of the manufacture of probiotics**

## 1. Fermentation

When selecting fermentation ingredients, an understanding of the allergen status requirements of the final product is important to properly select for alternative ingredients. Additional parameters, such as the pH set-point the organism is grown at, the temperature of growth, the oxygen tension, agitation speed in the fermenter and even light, all need to be tailored to the particular organism.

## 2. Concentration

The traditional methods of concentration would involve centrifugation and/or membrane filtration. The concentration step should be done under cold conditions in order to better preserve the physiological state of the cells.

## 3. Stabilization and Freezing

Freezing probiotic cells damages cell membranes and is detrimental to survival. Protectants are usually added to cultures to be frozen or dried in order to prevent, or at least mitigate, cell injury. The most common protectants used at industrial scale are lactose or sucrose, monosodium glutamate, milk powders, and ascorbate. Once frozen, probiotics can survive well over long shelf lives in products such as frozen yogurts and ice-cream. Use of slow cooling rates, or conditioning cells with prefreezing stress, can significantly improve cell survival. Repeated freeze–thawing cycles are highly detrimental to cell survival and should be avoided.

## 4. Drying

Optimization of the freeze-drying step is a critical process involving careful consideration of ramp-up temperature, chamber vacuum, finishing temperature, and desired residual moisture.

## 5. Blending

A variety of commercial blending options are available for use in designing a mixing process that maximizes homogeneity and minimizes mixing time. Careful blending tests help determine the minimum time needed to ensure adequate product homogeneity.

## 6. Packaging

Selection of the final packaging material should be made with the following considerations in mind:

- To minimize oxygen and moisture transfer into the product, the use of material with low moisture vapour transmission rate is preferred.
- Inclusion of a desiccant can be beneficial, especially for packaging with tablets and veggie-caps.
- Process in a cool room with humidity control (<40% is a must, <35% preferred).
- Avoid prolonged exposure to the packaging operation.
- Avoid creating a powder aerosol.
- Be aware of manufacturing activities in the area with regard to scheduling, e.g. avoid proximity to wet cleaning operations.

## 7. Storage

Regarding probiotic stability (the ability to survive under given circumstances) in the dry state, it is important to emphasise the two fundamental factors that impact probiotic viability during probiotic powder manufacturing and storage; temperature and water activity (Aw).

## Probiotics supply

Probiotics organisms are usually supplied by manufacturers of these ingredients as either:

- As dry powders (freeze-dried or spray-dried) at $10^{10}$–$10^{12}$ CFU /g.
- As frozen "direct vat set" concentrates at $10^{9}$–$10^{10}$ CFU /g.

When received as ingredients, it is important that the probiotic be correctly stored as per the manufacturer's instructions in order to avoid rapid losses in probiotic viable counts. For dried powders, this means storing the probiotics cold and avoiding moisture or humidity, while for frozen cultures is it important to maintain constant temperatures and to avoid repeated freeze–thawing.

## Producing products containing probiotic

### ➢ Food as vehicles of probiotics (Probiotic Foods)

Product taste, composition, and marketing determine the success of the product in the marketplace. The majority of foods containing probiotic bacteria are dairy foods, fermented milks, yogurts, soft and semi hard cheeses, ice cream, frozen dairy desserts, quark cheese, and cottage cheese as well as fermented cereals, infant formulas, beverages, salami, sausages, and bread. Over 70 products all over the world including sour cream, buttermilk, yogurt, powdered milk, and frozen desserts contain bifidobacteria and lactobacilli.

Probiotics can be incorporated into foods and beverages in a variety of ways.

- Dry blended into foods and powders such as infant formulas.
- Dispersed into liquid or semiliquid products such as juice or ice-cream.
- Inoculated into fermented products such as yogurts and fermented milks.

In the first two cases, the probiotics do not multiply in the product and are generally added at doses in the order of $10^7$–$10^8$ CFU /g. For a standard probiotic freeze-dried powder at $10^{11}$ CFU /g, this represents addition of the probiotic at 0.01–0.1% (w/w) of the final product.
In fermented products there may be some growth and increase in probiotic numbers during fermentation, allowing a lower number of organisms to be initially added (for example $10^6$ CFU /g). The number of viable probiotic organisms then usually declines during product storage, with the rate of decline dependent on a range of factors. Ensuring losses in probiotic viability are minimized is the one of the main goals for food technologists developing foods containing probiotics (fig.7-2).

## Factors affecting the viability of probiotics in foods

A number of intrinsic and extrinsic factors influence the survival of probiotics in foods. It is important to consider these factors at all stages between addition of the probiotic to the food and delivery of the probiotic to

the gut of the consumer. These include manufacturing processes, food formulations and matrices, packaging materials, and environmental conditions in the supply chain and during self-storage.

**The main factors to be considered that may influence the ability of the probiotics to survive in food products include:**

1. The physiological state of the added probiotic; If the food product is dry (e.g., a powdered infant formula) the probiotic will also be dried and in a quiescent state during storage. However, when included in a wet product such as a yogurt, the bacteria will be in a vegetative state and potentially metabolically active.

2. The physical and chemical conditions of food processing;

   - **Temperature:** The optimum temperature for growth of most probiotics is between $37^0$C and $43^0$C. In practical terms, the lower the temperature the more stable probiotic viability in the food product will be. For vegetative probiotic cells in liquid products, refrigerated storage is usually essential. In dried products containing quiescent bacterial cells, acceptable probiotic viability can be maintained in products stored at ambient temperatures for 12 months or more.

   - **pH:** Lactobacilli are able to grow and survive in fermented milks and yogurts with pH values between 3.7 and 4.3. Bifidobacteria tend to be less acid tolerant, with most species surviving poorly in fermented products at pH levels below 4.6.

   - **Water activity:** As moisture levels and water activity are increased the survival of probiotics is substantially decreased. Probiotics can survive well over long shelf lives (12 months or more) at ambient temperatures in dried products as long as the low moisture levels in the products can be maintained (at least below aw 0.2-0.3). In general, the lower the water activity, the better the bacterial survival will be. Maintaining probiotic viability in moderate water activity foods (0.4–0.7) is a major challenge and solutions such as

microencapsulation or incorporation of probiotics into fat phases of products can provide improved survival.

- **Oxygen:** In general, lactobacilli are more tolerant of oxygen than bifidobacteria (do not grow well in the presence of oxygen).

3. The physical conditions of product storage (e.g. temperature);
4. The chemical composition of the product (acidity, nutrients, moisture, oxygen);
5. Interactions with other product components (inhibitory or protective): Interactions between probiotics and other ingredients can be protective, neutral, or detrimental to probiotic stability. Obviously, the inclusion of antimicrobial preservatives can inhibit probiotic survival. Elevated levels of ingredients such as salt, organic acids, and nitrates can inhibit probiotics during storage, while starter cultures can sometimes inhibit the growth of probiotics during fermentation through the production of specific bacteriocins.

**Overall, there are five main points to address when incorporating probiotics into foods:**

1. Select a compatible probiotic strain/food type combination:

   The differences in the technological characteristics of different probiotic species and strains means that care must be taken in selecting the most appropriate strain for a particular food application. Generally, lactobacilli are more robust than bifidobacteria. There is a wider range of probiotic *Lactobacillus* species that are technologically suitable for food applications than bifidobacteria. Common examples include *L. acidophilus, L. johnsonii, L. rhamnosus, L. casei, L. paracasei, L. fermentum, L. reuterii* and *L. plantarum*. Often, the *L. acidophilus* group of organisms, while resistant to low pH, prove less robust than other *Lactobacillus* species in non-traditional probiotic food applications.

   The Bifidobacterium species most commonly used in foods is *B. animalis* subsp. *lactis*. This species is significantly more robust than human intestinal species such as *B. longum* (*infantis*), *B. breve*, and

*B. bifidum*, although certain strains of these species are able to survive well in some foods. *B. adolescentis* is a common species in the intestinal tract of adult humans, but tends to be sensitive to environmental conditions in foods and is rarely used commercially as a probiotic.

2. Use food-processing conditions that are compatible with probiotic survival.
3. If fermentation is required, ensure that the food matrix will support probiotic growth.
4. Select a product matrix, packaging, and environmental conditions to ensure adequate probiotic survival over the product's supply chain and during shelf storage.
5. Ensure that addition of the probiotic does not adversely impact on the taste and texture of the product.

### ➢ Dietary supplements

Some markets prefer supplements over functional foods (such as the USA) or supplements are the most practical form of administration, for example due to lack of cold storage/ transportation or in hospital settings. Probiotic supplements are usually in the form of capsules, but may also be combined with other ingredients (e.g. prebiotics) and packed as sachets.

For stability, it is important that capsules are packed in appropriate bottles, and glass is preferred as it is a better barrier to moisture. Likewise, sachets should preferably be of sealed aluminium-coated foil. In both cases, filling agents should be sufficiently dry. This may be particularly demanding when mixing probiotics with prebiotics, which often have a relatively high Aw (fig.7-2).

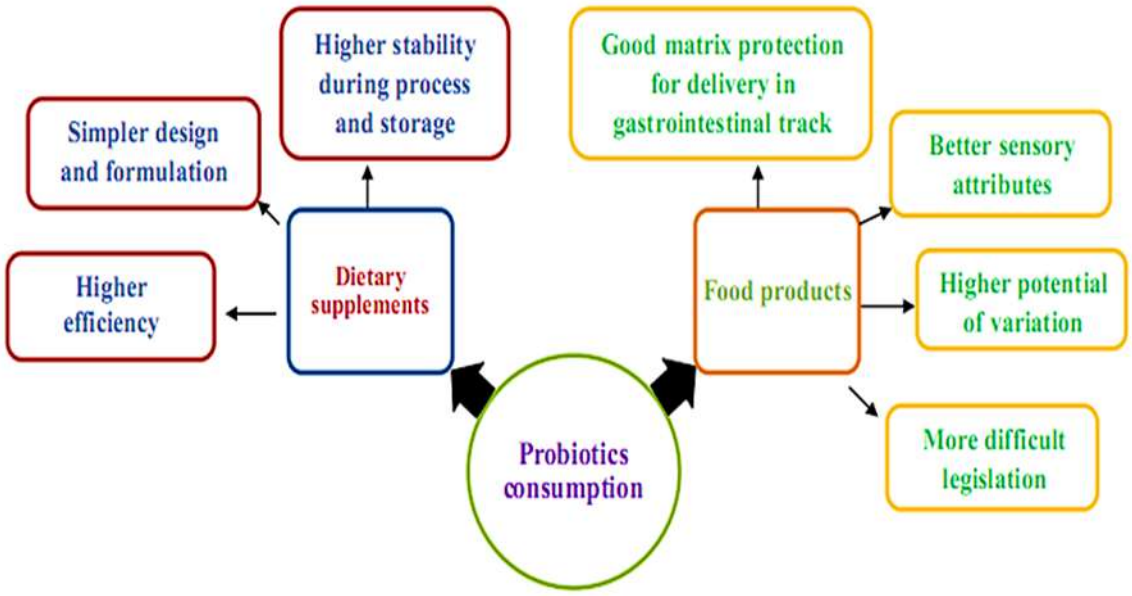



**Figure 7-2: Probiotic consumption as food products and dietary supplements**

**Dosage Form (DF)** is defined as the physical form of a dose of a chemical compound used as a drug or medication intended for administration or consumption.

## Effective dosage for probiotic effects

The probiotics were administered in great variation in accordance to the following:

- Type of probiotics (lactobacilli, bifidobacteria, yeasts, enterococci);
- Daily dose ($10^7$–$10^{10}$ CFU)
- Daily frequency of administration (1–4 times):
  It is not so clear if the frequency of administration has any effect on probiotic efficacy. Microbiologically, $1X10^{10}$ CFU administered four times daily has little different from $4X10^{10}$ CFU administered once a day. Health benefits have usually been attributed in clinical studies to doses of probiotics in excess of $10^8$–$10^9$ viable cells per day. Therefore, food regulatory/advisory bodies generally stipulate that foods containing probiotic organisms need to have $>10^6$–$10^7$ CFU/g at the time of consumption.
- Timing of administration (before, during, and after meal):

There is no information about the best time to administer probiotic preparation. It is logical to assume that probiotics administered orally before meal should have the capability to tolerate the extreme pH condition and digestive enzymes and bile present in the intestinal tract. Probiotics taken together with meal would be diluted by food materials, which could reduce the chances and frequency of physical encounter between the probiotic organisms and the mucosal receptors. Moreover, food matrix may compete with mucosal receptors for probiotic and product binding. Hence it is reasonable to assume that the best period for the administration of probiotics is between meals, and be carried in liquid media.

- Duration of administration (1 day to several months)
- Method of delivery (fermented food, beverage, capsule, tablet, or powder)
- Viability:
  The viable count of probiotic organisms generally declines during product storage (10–100-fold or more). An acceptable viable count can sometimes be achieved by introducing higher numbers of probiotics during manufacture (called overage). The consumption of probiotic organisms at high doses is safe, and so oversupplying consumers does not appear to pose a health risk.

## Delivery Systems

### • Microencapsulation (ME)

Incorporating probiotic bacteria into functional food presents many challenges, particularly with respect to the stability of probiotic bacterial cells during processing and storage and the need to prevent undesirable interactions with the carrier food matrix. Therefore, there is a need to protect the bacterial cells against adverse processing and storage conditions as well as during GI transit. Providing probiotic living cells with a physical barrier against adverse external conditions is an approach currently receiving considerable interest in the food industry. Clearly, obtaining a health benefit requires the viability of the bacterial cells in the

GI tract and their controlled release in the target areas of the GI tract. Microencapsulation (ME) could be useful for this purpose (fig.7-3).

**ME** is an inclusion technique for confining a bioactive substance into a polymeric matrix coated by one or more semipermeable polymers by virtue of which the encapsulated compound becomes more stable and protected than its isolated or free form.

Providing probiotics with a physical barrier to environmental conditions by microencapsulating the bacteria is an approach that has been trialed using a range of materials and techniques. Microencapsulation of probiotics, though, is not a simple undertaking.

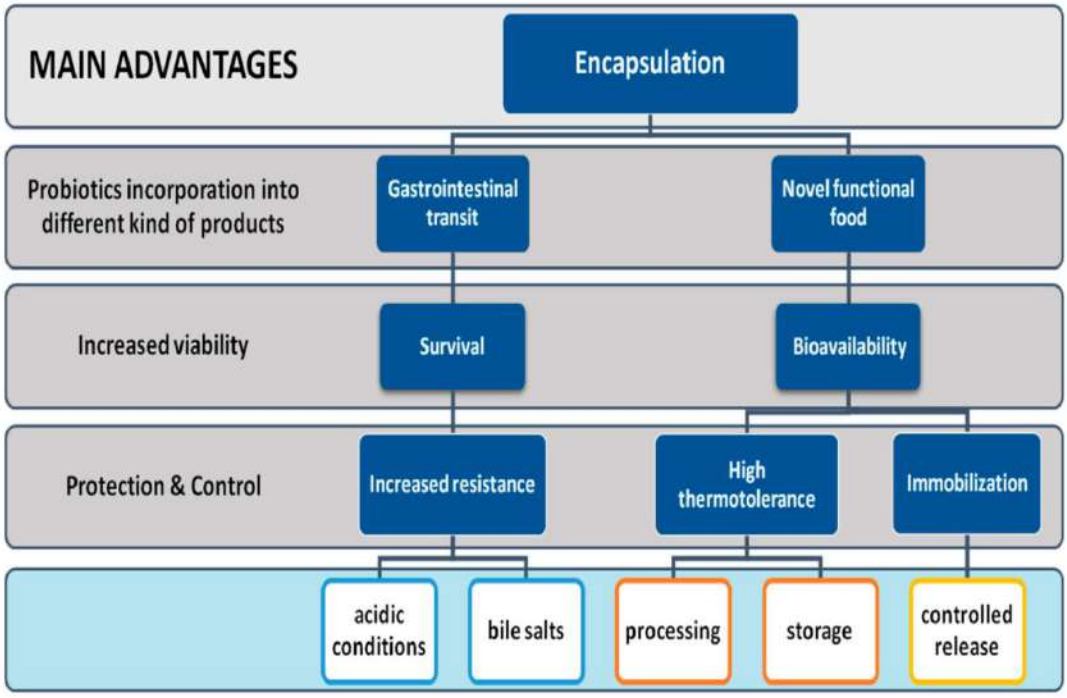



**Figure 7-3: Advantages of encapsulation**

- **Delivery Devices**

Another approach to maintaining the viability of probiotics for long periods at ambient temperatures is to physically separate the probiotics from the food and atmosphere. This can most simply be achieved by keeping dried probiotics in sealed sachets with the food or beverage to be mixed immediately prior to consumption.

# Lec.8: Probiotic mechanisms of action

The benefit effects of probiotics on host may be direct or indirect. The mechanisms of action of probiotics are still a significant question regarding clinical use of them, the broad-based definition of probiotics makes the study of their mode of action difficult, but the scientists during the last decades have studied the mechanisms of action of probiotics and proposed many mechanisms of action for them (fig.8-1);

- Immunologic and non-immunologic mode of action
- Antimicrobial activity, immune modulation, and improvement of mucosal barrier integrity
- Impact of microorganisms or their metabolites on the GIT and microbiota, and interaction with the cells and immune system of the host

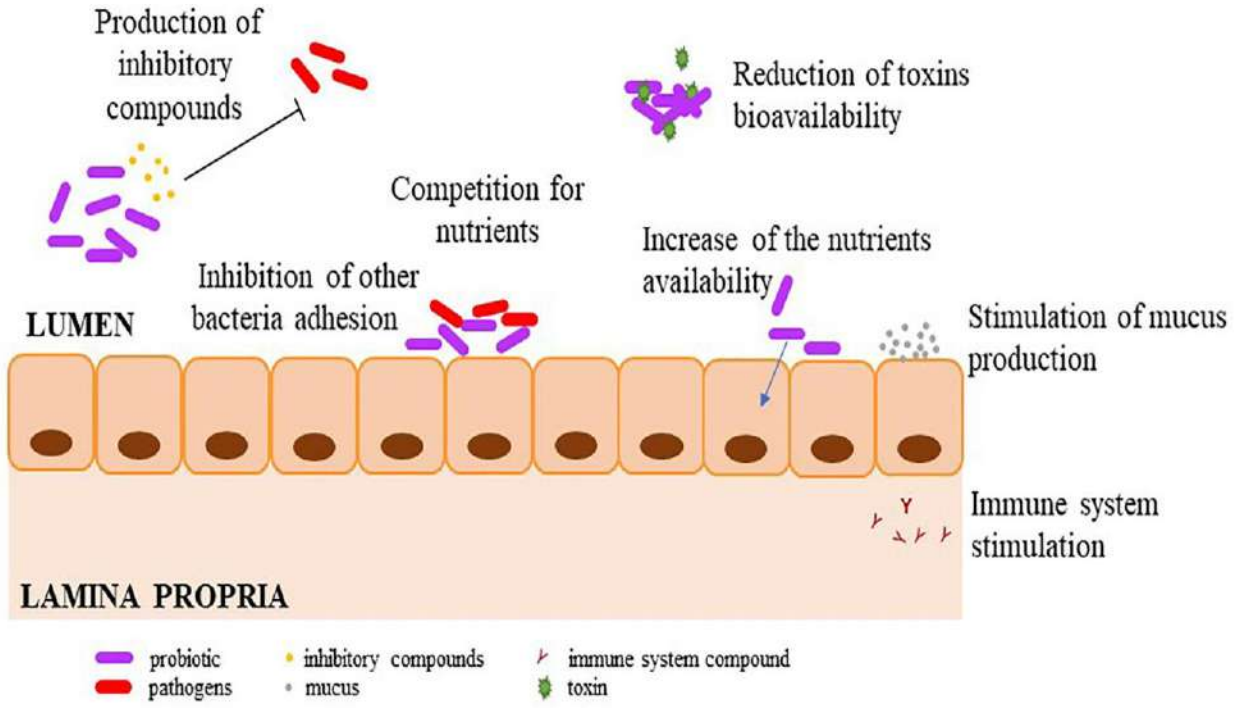



**Figure 8-1: Mechanisms of probiotics action**

However, depending on the function involved, each one of the mechanisms could be further subdivided.
Researchers are working to better understand the specifics of these proposed mechanisms by which probiotics appear to affect the health of humans.

## Enhanced barrier function

The intestinal epithelium is in permanent contact with luminal contents and the variable, dynamic enteric flora. The intestinal barrier is a major defense mechanism used to maintain epithelial integrity and to protect the organism from the environment.

- Mucin is a complex glycoprotein mixture that is the principal component of mucous, thereby preventing the adhesion of pathogenic bacteria.

Defenses of the intestinal barrier consist of the;

1. Mucous layer,
2. Antimicrobial peptides,
3. Secretory IgA,
4. Epithelial junction adhesion complex

- Once this barrier function is disrupted, bacterial and food antigens can reach the submucosa and can induce inflammatory responses, which may result in intestinal disorders, such as inflammatory bowel disease.
- Consumption of non-pathogenic bacteria can contribute to intestinal barrier function, and probiotic bacteria have been extensively studied for their involvement in the maintenance of this barrier.

However, the mechanisms by which probiotics enhance intestinal barrier function are not fully understood;

- Probiotics may promote mucous secretion as one mechanism to improve barrier function and the exclusion of pathogens.
- Probiotics may also enhance the ability of specialized Paneth cells in the small intestine to produce the antibacterial peptides known as defensins.

- Probiotics and prebiotics may affect the barrier function of the epithelium itself by enhancing the resistance of tight junctions, possibly via an effect on tight junction proteins (e.g. occludins and claudins).
- Probiotics help with the proper maintance of the gut barrier function by helping keep intestinal cells healthy.

## Increased adhesion to intestinal mucosa

The effective performance of the probiotic depends on their strong adherence and colonization of the human gut, which in turn improves the host immune system.

Adhesion to intestinal mucosa is regarded as:

- A prerequisite for colonization
- Important for the interaction between probiotic strains and the host
- Important for modulation of the immune system
- Important for antagonism against pathogens

LABs display various surface determinants that are involved in their interaction with intestinal epithelial cells (IECs) and mucus. Additionally, lipids, free proteins, immunoglobulins and salts are present in mucous gel. This specific interaction has indicated a possible association between the surface proteins of probiotic bacteria and the competitive exclusion of pathogens from the mucus.

The most studied example of mucus-targeting bacterial adhesins is mucus-binding protein (MUB) produced by *L. reuteri*. The proteins playing a role in the mucous adhesion phenotype of lactobacilli are mainly secreted and surface-associated proteins, which are either anchored to the membrane through a lipid moiety or embedded in the cell wall.

## Competition with pathogens on adhesion (receptor) sites and nutrients

Probiotics strive to reach the receptor sites along the epithelial cells that line the intestines. Epithelial cells, which line the intestinal tract, mouth and vagina, are host to millions of receptor sites for various microbes. Some probiotics are very effective at attaching to receptor sites, thus preventing bad microbes from finding space. Probiotics literally "crowd out" the bad

microbes. Of note, some probiotics can affect bad microbes' ability to adhere to a receptor;

- Some probiotics can compete with pathogens for receptor sites on epithelial cells or in the mucous layer, thereby preventing pathogens from adhering or translocating.
- In contrast, other probiotics may directly bind to the pathogen, thus reducing its ability to colonize the intestine.

On the other hand, probiotics digest food and compete with pathogens and other microbiota for the nutrients, although there is no evidence that this occurs *in vivo* but probiotics may consume nutrients otherwise utilized by pathogens.

## Probiotics mask receptor sites

When probiotics bind to a receptor site on an epithelial cell, they elicit a number of effects in the cell. One of the proposed effects is that probiotics communicate with the cell, causing the cell and its neighboring cells to alter their lining. When probiotics cause a positive change to the lining, bad microbes are less able to attach.

The cells that line the intestinal tract appear to be able to crosstalk, allowing for a communal exchange. Cells have junctions between them called "tight junctions." Through these junctions scientists think that cells communicate to each other, which enables them to react as a group. When a probiotic tells one cell to change to prevent bad microbes from attaching, the cell can then tell all of its neighbors to do the same. The result is a large protective change that protects the body from infection and creates immunity. When pathogens cannot adhere to the intestinal receptors, they cannot colonize and cause disease. Researchers Lu and Walker have suggested that probiotics strengthen tight junctions between intestinal cells, which ultimately improve immunity.

## Modulation of the mucosal immune system

The most complex proposed mechanisms of action of probiotics are the interaction with GIT immune cells and lymphoid tissue to modulate the immune and inflammatory responses of the host.

Probiotics positively affect the immune system in a number of ways, but we do not yet completely understand how they do what they do. Probiotics affect directly and indirectly ways the function of lymphoids cells, they effect on dendritic cells (DCs), monocytes, macrophages, lymphocytes, T cells, regulatory T (Treg) cells, immunoglobulin A (IgA)–producing B cells, and natural killer (NK)cells. According to the animal studies, probiotics can modulate cytokine profiles, induce hyporesponsiveness to food antigens, and activate local macrophages.

In the intestinal tract, probiotics cause the intestinal lining to be less permeable, which means that harmful microbes and toxins that are not supposed to enter into your bloodstream cannot do so. GIT is lined with its own set of immune cells called the gut-associated lymphoid tissue (GALT) to help prevent these harmful substances from gaining entry to your body.

## Production of antimicrobial agents

Probiotics produce many antimicrobial agents such as, organic acids, hydrogen peroxide and antimicrobial peptides (AMPs) known as bacteriocins. Also, probiotics produce hydrolytic enzymes which contribute increasing amounts of acids and this leads to reduce pH value. The benefits of a lower pH in the colon are that it encourages the multiplication and survival of commensal organisms that prefer acidic conditions and generally inhibits the growth of pathogens.

## Enhancement of digestion and absorption of food, and alteration of the intestinal microbiota

- Probiotics make a variety of enzymes that harmful to bad microbes and offer health benefits to you.
- The enzyme activity of probiotics has been found to help fight infectious disease, lactose intolerance, immune system deficiencies, and urogenital and vaginal diseases.
- Probiotics may also work synergistically to create an environment that promotes probiotic growth, e.g. *L. reuteri* excretes AGGH, a protein that appears to encourage growth of good bacteria in the small intestine.

# Lec.9: Postbiotics, Prebiotics and Synbiotics

- Postbiotics and their synonyms started to appear after 1986.
- In 2014, the International Scientific Association for Probiotics and Prebiotics stated that the development of metabolic by-products, dead microorganisms, or other microbe-based nonviable products has potential; however, these do not fall under the probiotic construct.
- **Postbiotics** are soluble bioactive compounds or components secreted by probiotics, or released after bacterial lysis, that can be used in combination with nutritional components to promote health.
- The term postbiotics can be regarded as an umbrella term for all synonyms and related terms of these microbial fermentation components. Postbiotics comprise metabolites and/or cell-wall components released by probiotics (e.g. organic acids, enzymes, bacteriocins, short-chain fatty acids (SCFAs), microbial cell fractions, vitamins, polysaccharides, cell lysates, peptides, cell surface proteins, teichoic acid, peptidoglycan-derived muropeptides and pili-type structures).

**These postbiotics have drawn attention because of their:**

- Clear chemical structure,
- Safety dose parameters,
- Long shelf life,
- Content of various signaling molecules which may have antimicrobial, anti-inflammatory, immunomodulatory, anti-obesogenic, antihypertensive, hypocholesterolemic, anti-proliferative, and antioxidant activities.

These properties suggest that postbiotics may contribute, to the improvement of host health by improving specific physiological functions, even though the exact mechanisms have not been entirely elucidated.

**Two commonly mentioned types of postbiotics are:**

1. Paraprobiotics or ghost probiotics, non-viable probiotics or inactivated probiotics, are now often defined as 'non-viable or inactivated microbial

cells, which, when administered in sufficient amounts confer benefits to the host'.

Bacterial cell inactivation may be achieved by:

- Physical methods (mechanical disruption, heat treatment, γ-or UV irradiation, high hydrostatic pressure, freeze-drying, sonication)
- Chemical method (acid deactivation).

2. Fermented infant formulas (FIFs) are infant or follow-on formula that have been fermented with lactic acid-producing or other bacteria and in most cases do not contain viable bacteria.

## Bacteriocins

- In 1953; "Bacteriocin" was used by Jacob and his co-workers as a general term for highly specific antibacterial proteins.
- It has been suggested that between 30–99% of the Bacteria make at least one bacteriocin, and the only reason we have not isolated more is that few researchers have looked for them.
- **Bacteriocins** are ribosomally-produced multi-functional substances of a proteinaceous nature, with pronounced antimicrobial activity at certain concentrations.
- Antimicrobial molecules not purified to homogeneity but displaying characteristics similar to bacteriocins, may be indicated as bacteriocin-like inhibitory substances (BLIS).
- In many cases authors have added a terminal "e" to the name of the bacteriocin; e.g., staphylococcine, listeriocine , and corycine (appears most often in European publications).
- Bacteriocin Immunity is a protection system have developed by bacteriocin producers against their own bacteriocin.

### Groups of Bacteriocins

The bacteriocin family includes a diversity of proteins in terms of size, microbial target, mode of action, release, and immunity mechanisms. Bacteriocins can be divided into two main groups:

➢ Bacteriocins produced by Gram-negative bacteria:

- Arise mainly from Enterobacteriaceae.
- They can be divided into 3 groups based on size:

1. Colicins &Colicin-like bacteriocins (CLBs): High Mwt (25- 80) kDa
2. Microcins: Low Mwt (1-10) kDa
3. Phage tail-like bacteriocins (Tailocins): < 1 kDa

➢ Bacteriocins produced by Gram-positive bacteria:

- They are as abundant as and even more diverse than those found in G-ve bacteria and LAB bacteriocins are the best characterized of this group.
- The G+ve bacteriocins resemble many of the AMPs produced by eukaryotes.
- They can be divided into 4 classes:

1. Class I (The lantibiotics or modified bacteriocins)
   - ✓ Small (< 5KDa),Heat-stable, Contain unusual amino acids; as lanthionine and β-methyllanthionine. Nisin is a reference class I bacteriocin.
   - ✓ Have 3 subclasses; (Type A) Linear lantibiotics, (Type B) Globular lantibiotics, and (Type C) Multi-component lantibiotics.

2. Class II (Unmodified bacteriocins)
   - ✓ Large group of bacteriocins
   - ✓ Usually small (< 15KDa),Heat-stable
   - ✓ Pediocin PA-1/AcH produced by *Pediococcus acidilactici*, is a reference class II bacteriocin
   - ✓ Diverse chemical and genetic characteristics.
   - ✓ Divided into 4 subclasses (IIa ,IIb, IIc &IId).

3. Class III (Bacteriolysins)
   - ✓ Large (> 30 KDa),Heat-labile proteins
   - ✓ Hydrolysis of the cell wall
   - ✓ Lesser interest to food scientists, such as acidofilicin A

4. Class IV (Circular bacteriocins)
   - ✓ Circular bacteriocins, peptides covalently linked head to tail.
   - ✓ The most studied circular bacteriocin is enterocin AS-48 from *Enterococcus faecalis*.

Many researchers are now focusing on the bacteriocins of LAB with plenty of applications not only in food industries but also in medical and health applications.

## Bacteriocin Spectrum of Activity

- The antimicrobial spectrum is the set of strains that are sensitive to a given bacteriocin.
- At now bacteriocins are divided into 3 categories:
  1. Narrow inhibitory spectrum, affecting only closely related species.
  2. Wide inhibitory spectrum, affecting G+ve bacteria, such as food-borne pathogens.
  3. Unusual spectrum of activity, affecting G-ve bacteria, viruses and yeasts.

## Bacteriocins mode of action

- Little is known about their mode of action.
- Different mechanisms have been proposed, but the most common one is;

Peptides bond to the plasmatic membrane through electrostatic attractions with phospholipids charged negatively. The monomers of bacteriocin form proteic aggregates that result in the pore formation with the consequent leave of ions, ATP and amino acids (fig.9-1). Therefore, the synthesis of macromolecules is inhibited as well as the production of energy, resulting in cell death.

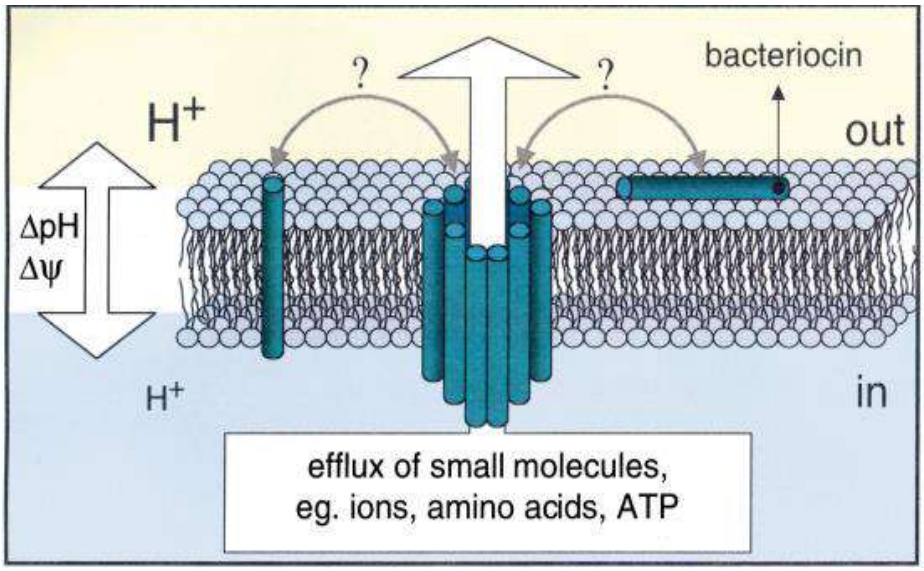



**Figure 9-1: Model for pore formation and mechanism of bacteriocin action**

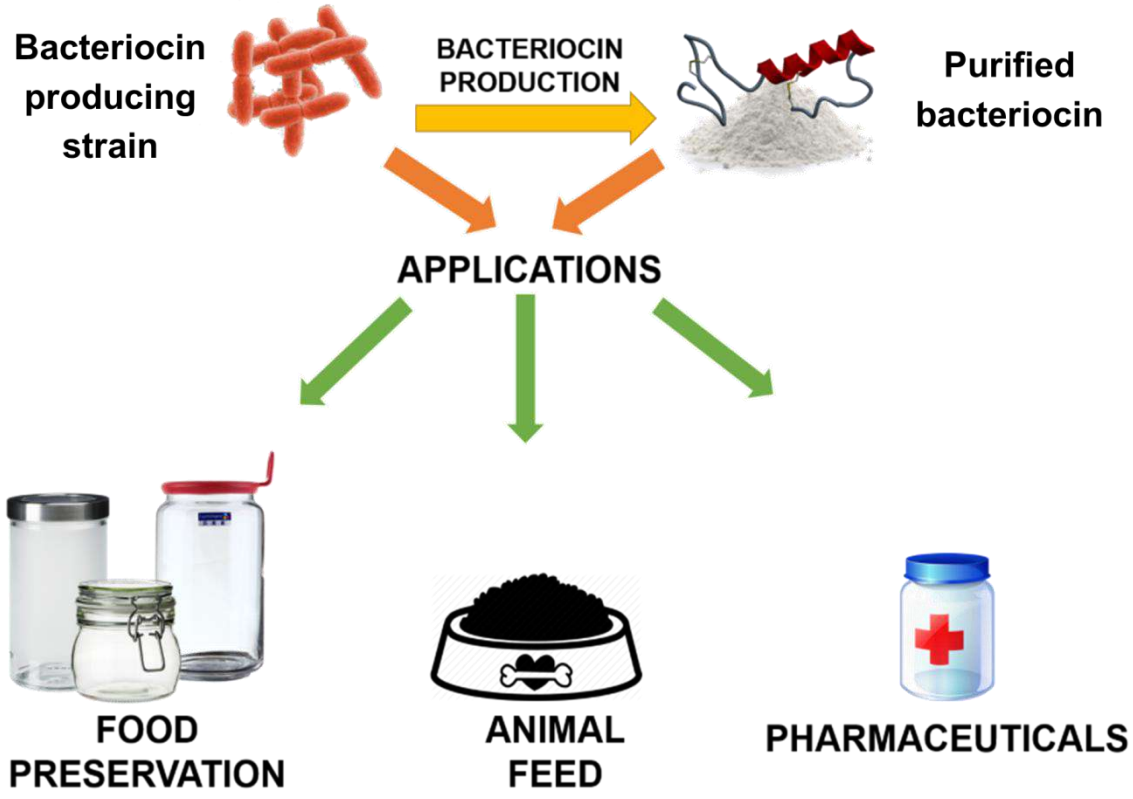
**Advantages of use bacteriocins from LAB in compare with G-ve bacteriocins in food preservation:**

- The usage of bacteriocin producing strains directly in the food matrix as protective cultures.
- The possibility of using LAB as a starter culture in fermented foods.
- No strict purification procedure is required for bacteriocin preparations from LAB unlike G-ve bacteria which may contain LPS and other endotoxins causing deleterious effect, if not removed.

**Three ways in which bacteriocins can be incorporated into a food:**

1. Using a purified /semi-purified bacteriocin preparation as an ingredient in food.
2. Incorporating an ingredient previously fermented with a bacteriocin-producing strain.
3. By using a bacteriocin-producing culture to replace all or part of a starter culture in fermented foods to produce the bacteriocin in situ.

## Applications of bacteriocins

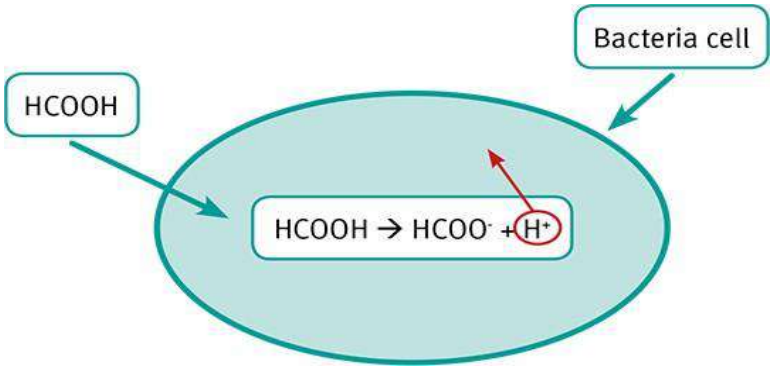


## Organic acids

- An organic acid is a carboxylic acid including fatty acid having the formula RCOOH.
- As a group these compounds primarily include the saturated straight-chain monocarboxylic acids and their respective derivatives (unsaturated, hydroxylic, phenolic, and multicarboxylic versions) and are often generically referred to as fatty acids, volatile fatty acids, or weak or carboxylic acids.
- Most commonly used organic acid are short chain fatty acid e.g. formic acid, propionic acid, butyric acid, acetic acid, citric acid and malic acid which is a dicarboxylic acid.

### Mechanism of action:

- The key basic principle on the mode of action of organic acids on bacteria is that non-dissociated (non-ionized) organic acids can penetrate the bacteria cell wall and disrupt the normal physiology of certain types of bacteria that we call *pH-sensitive.* Among those bacteria are *E. coli*, *Salmonella* spp., *C. perfringens*, *Listeria monocytogenes*, and *Campylobacter* species.
- Upon passive diffusion of organic acids into the bacteria, where the pH is near or above neutrality, the acids will dissociate and lower the bacteria internal pH, leading to situations that will impair or stop the growth of bacteria. On the other hand, the anionic part of the organic acids that cannot escape the bacteria in its dissociated form will accumulate within the bacteria and disrupt many metabolic functions, leading to osmotic pressure increase, incompatible with the survival of the bacteria.



## Applications of organic acids:

- Utilized as food preservatives for preventing food deterioration and extending the shelf life of perishable food ingredients.
- Used as direct additives incorporated into human foods or can accumulate over time as a consequence of the fermentation activity of indigenous or starter cultures added to certain dairy, vegetable, and meat products.
- Lactic acid and its salts sodium lactate and potassium lactate are widely used as antimicrobials in food products, in particular, meat and poultry such as ham and sausages.
- Acid sprays have been incorporated as sanitizers during meat processing.

## Benefits of using organic acid in animal nutrition:

- Killing of microbes especially Gram negative (*Salmonella* and *E. coli*) in drinking water and in the intestine leads to better utilization and absorption of feed.
- Prevent biofilm formation in the drinking systems thus minimize the pathogen load in water.
- Increase the digestibility of nutrients especially proteins by its pronounced pH reducing effect in the stomach which enhance the release of pepsin which is a protein degrading enzyme.
- Increase the pancreatic secretions result in better digestion and metabolism of fat.
- Delay the gastric emptying time of feed result in better nutrient absorption.
- They serve as intermediary substrates (butyric acid and citric acid) in the different cycles of metabolism thus act as a direct energy source for the bird.
- Reduce the incidence of diarrhea and subclinical enteritis.
- Enhance the absorption of minerals (P, Cu, Zn)
- Alternatives to Antibiotic Growth Promoters (AGPs) to reduce medication cost.

### New strategies for organic acids processing include:

- Encapsulation of organic acid; the encapsulation process ensures the targeted release of acid in the small and large intestine where major pathogenic load of microbes are present.
- The use of glyceride of acid; in this process, organic acid is attached (esterified) with glycerol. This combination protects the acid from degradation by the stomach pH. Once these glycerides are reached in the small intestine lipase enzyme breaks the bond and acid is released.
- Use of a combination of the organic acid; the purpose of using combination is to maximize the effects along the tract.
- Use of salts of organic acids e.g. potassium formate is also common; the salt reduces the bitterness and corrosiveness of acid.

## Prebiotics

- The Japanese were the first to recognize the value of fermentable oligosaccharides, initially in feeding piglets and later, during the 1980s, with the identification of human milk oligosaccharides.
- In 1995 the prebiotic concept for modulation of gut microbiota was introduced.
- Although a number of definitions have been proposed, there is as yet no full agreement on a single definition of a prebiotic, the most accepted one is "A dietary prebiotic is a selectively fermented ingredient that results in specific changes, in the composition and/or activity of the gastrointestinal microbiota, thus conferring benefit(s) upon host health." which proposed by International Scientific Association of Probiotics and Prebiotics (ISAPP).

### Criteria for prebiotic selection

- The prebiotic concept is based on the selective stimulation of the host's own beneficial microbiota, the prebiotic being the substrate that is (selectively) fermented, stimulating the growth and activity of the particular micro-organism or group of micro-organisms of interest and thus leading to the desired health effect.

- It is essential to measure the effect of the candidate prebiotic on bacterial growth; *in vitro* tests and human feeding trials are essential in order to demonstrate a health benefit.
- The main site of action for prebiotics is the colon. Thus, a prebiotic should resist the effects of gastric acidity and digestive enzymes in order to reach the colon intact.
- The foremost target genera for prebiotic action are bifidobacteria and lactobacilli.

## Characterization of prebiotic ingredients

- To date only carbohydrate compounds have been studied with regard to prebiotic activity.
- Most research has been carried out on fructans (i.e. the polysaccharide inulin or fructo-oligosaccharides (FOS) derived from various crops or from sucrose) and galacto-oligosaccharides (GOS).
- Candidate or emerging prebiotics require additional evidence in humans before they can be fully established as prebiotic. Such candidate prebiotics include the disaccharide lactulose, further oligosaccharides and resistant dextrins, polysaccharides such as polydextrose, arabinoxylans and resistant starches as well as some polyols such as lactitol and isomalt.
- Some prebiotics occur naturally in foods such as chicory, cereals, agave and milk. However, most foods contain only trace levels.
- Many prebiotics and candidate prebiotics today fall into the nutritional and regulatory definition of dietary fiber and are labeled as nutrients of that category. They share with dietary fiber the properties of resistance to digestion and (for some fibers) fermentability, but established prebiotics are distinguished from dietary fiber by the selectivity of their fermentation. Note that mono- and disaccharides are typically not considered as dietary fiber according to EU and CODEX definitions.

### Commonly known prebiotics are:

- Oligofructose
- Inulin

- Galacto-oligosaccharides
- Lactulose
- Breast milk oligosaccharides

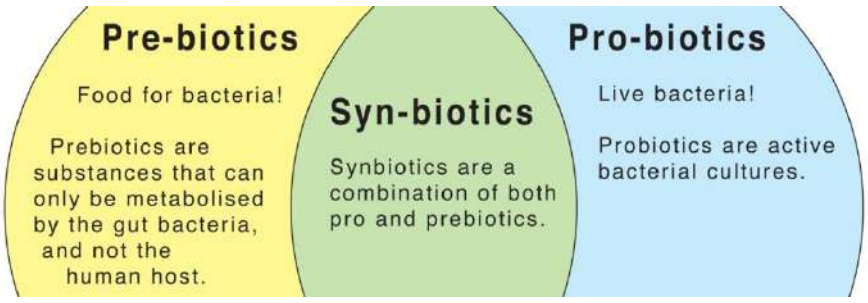
Below is a list of foods that research has confirmed to be prebiotic:

- Bananas
- Onions
- Garlic
- Leeks
- Asparagus
- Whole wheat
- Barely
- Rye

## Application of prebiotics in food

- The commercial prebiotic ingredients GOS and fructans are used in infant foods when their safety and efficacy has been demonstrated; in some countries this may require premarket approval.
- In foods for general consumption, the target level of intake of prebiotic ranges from 2 to 20 g per day, depending on the ingredient and the desired effect. These amounts can be readily incorporated into a variety of foods such as cereals, bread, confectionery, biscuits, yoghurts, table spreads, sauces and drinks.
- Similarly to the case of probiotics, the health benefits of candidate prebiotics need to be demonstrated in clinical trials.

**Synbiotics** are appropriate combinations of prebiotics and probiotics. A synbiotic product exerts both a prebiotic and probiotic effect.




- **How to incorporate and create synbiotic foods?**

# Lec.10: Animal models for probiotics

- Animal models have been used in experimental research to increase human knowledge and contribute to finding solutions to biological and biomedical questions.
- Today, researchers rely on the identification and development of animal models to explore all avenues of medical science to include assessment of pathogenic mechanisms, diagnostic and therapeutic procedures, nutrition and metabolic diseases, and the efficacy of novel drug development.
- Laboratory animal model is "an animal in which normative biology or behavior can be studied, or in which a spontaneous or induced pathological process can be investigated, and in which the phenomenon in one or more respects resembles the same phenomenon in humans or other species of animal."
- Animal experimentation is interventions and manipulations in animals if this is associated with suffering, pain and injury to the animals.

## The 3R's principle

Although animal experimentation cannot be completely avoided in research, there is a general consensus that it must be restricted to the necessary minimum. The 3R's principle, devised by W. Russell and R. Burch in 1959, can be taken as the guideline for animal experiments. The 3R's Principles are now universal, and guide animal research in many countries.

The 3R's stand for;

► Refinement (avoid or minimize animal suffering)
► Reduction (reduced number of animals and experimentation length)
► Replacement (cannot be replaced by *in vitro* alternatives)

The consistent and responsible implementation of the 3R's principle accommodates ethical concerns against the use of animals, and also improves the quality of the test results.

## Types of animal models

Animals used in biological researches are broken down into the following categories:

1. **Exploratory;** Animals are used to gain an understanding of fundamental biological mechanisms, whether normal or abnormal.
2. **Explanatory;** Animals are used to gain an understanding of complex biological problems.
3. **Predictive;** Animals are used to discover and quantify the impact of investigative treatments whether for diseases or chemical toxicities.

## Selecting an animal model

The selection of an animal species is based on the similarities between the animal species and humans in aspects such as:

- Pharmacodynamics (safety pharmacology),
- Pharmacokinetics,
- Physiology and pathophysiology

The choice of animal species also depends on practical considerations, such as species availability and the ease with which they can be used in standardized laboratory environments and procedures.

Animals used in research are mainly small mammals such as mice, rats, guinea pigs and rabbits; fish and birds are used for specific investigations.

## The right model for the right purpose

- Final assessment of probiotic functionality should ideally be performed directly in the target population, the pre-selection of strains need to be made using appropriate *in vivo* models.
- The prototype worm is currently proposed to screen probiotics, or to establish antitumor activity. In a similar way, the fly is useful to explore metabolic, immune and antioxidant effects of the Lactobacillus-host mutualism.
- Quite recently, the zebra fish (*Danio rerio*) has garnered intense interest as a human disease model due to its many advantages as an

experimental vertebrate. It now appears that the zebra fish can be used for high-throughput screening (e.g., of drug libraries) in the discovery process of promising new therapeutics. The latter was successfully developed for probiotics, showing that probiotic administration may enhance the zebra fish welfare by modulating the innate immune response and improving hepatic stress tolerance, involving stress and apoptosis genes, and genes from the innate immune system. Of note, zebra fish can also partly mimic characteristics of IBDs when larvae are subjected to chemicals such as trinitrobenzene sulfonic acid (TNBS) or when encountering unfavorable conditions, including dysbiosis of the intestinal microbiota. Modifications include colitis susceptibility genes like *NOD1* and *NOD2*, enabling the routine evaluation of anti-inflammatory compounds.

## Rodents as the necessary compromise

- The small animal models meet the needs for cost effective and public-acceptable screening but are still far away from an integrated mammalian physiology. Therefore more pertinent experimental models are required for the evaluation of probiotic functionality (fig. 10-1). Accuracy of the results of animal models is not always in perfect accordance with human outcomes. Whether animals can be used to predict human response to drugs, chemicals or foods (including probiotics) is apparently a contentious issue.
- Small animals such as rats and mice will inevitably be continued to be used as models to address numerous research questions related to probiotics, including the evaluation of immune and metabolic responsiveness, regulatory processes or neuro-endocrinological and nutritional aspects, which all play important roles in the complex microbiota–host relationships.
- Clearly, the choice of a model depends on the putative mode of action of the probiotic used (a prophylactic or therapeutic).

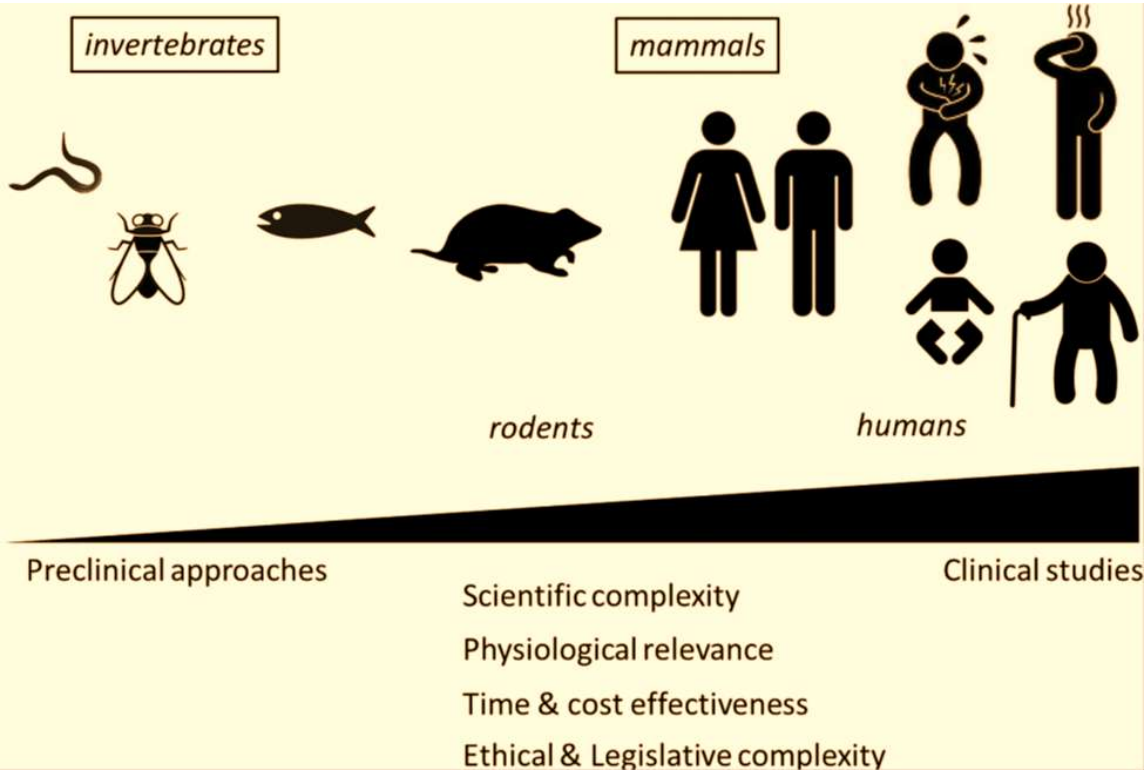



**Figure 10-1: Progressive complexity of *in vivo* models used to support probiotic health effects.**

## Future perspectives for the use of *in vivo* tests in probiotic research

- To increase the accuracy of animal models, multi-humanized mice can be considered, carrying functional human genes, cells, tissues, or organs. These humanized mouse models may assist to model the human immune system in various scenarios of health and disease, and may enable the evaluation of therapeutic candidates in an in vivo setting more close to human physiology.
- While those specific humanized mice are commonly used in biological and medical research for human therapeutics, they do not frequently appear in probiotic research yet.

# Lec.11: Safety of probiotics

Probiotics have been used safely in foods and dairy products for over a hundred years. Most LAB strains used in the food supply are nonpathogenic, nonvirulent, and nontoxigenic microorganisms. Recently, there has been increasing interest in their use to prevent, mitigate or treat specific diseases. To date, the products that contain probiotics seem to be safe for human health.

The absolute essence of probiotics to be considered as safe is the absence of pathogenicity and infectivity. In fact, the safety of probiotics has not been studied scientifically. Because they regulated as dietary supplements rather than as biological products or pharmaceuticals, there is no requirement to demonstrate purity, safety, or potency for probiotics before marketing,

## Probiotics regulation

- In Japan, probiotics are regulated under Food for Specific Health Use (FOSHU), which permits labeling with health claims on food or ingredients that meet scientific evidence required for safety and efficacy.
- In the European Union, probiotics are regulated via the Novel Food Regulation (258/97/EC).
- In the United States, a probiotic is regulated by FDA's Center for Food Safety and Applied Nutrition. It can be classified as an additive, in which case it has to be approved by the Food and Drug Administration (FDA) on the basis of safety and efficacy data, or it can be considered "generally recognized as safe" (GRAS). The GRAS status is given to a probiotic when it has a history of safe use in food dating before 1958 or has been identified as safe by expert judgment under the conditions of intended use.

## Evaluation approaches for safety and efficacy of probiotics

The safety and functional evaluation system of probiotics is not perfect, some clinical trials of probiotics are not designed to adequately address questions about safety.

- The approach proposed by FAO and WHO may be useful in evaluating newly discovered probiotics. According to this scheme (fig.11-1):
  1. Establishment of phenotype and genotype of probiotic strains
  2. Assessment of safety, efficacy and functional characterization of probiotics (*in vitro* assays and animal studies).
  3. Testing of probiotics using standard methods in two clinical evaluations: phase 1 (safety assessment) and phase 2 (efficacy assessment) studies. If these clinical studies confirm efficacy and safety of a probiotic strain, then that strain can be marketed as a probiotic food. When a claim is made that a probiotic can alter a disease state, then a phase 3 study must be performed.

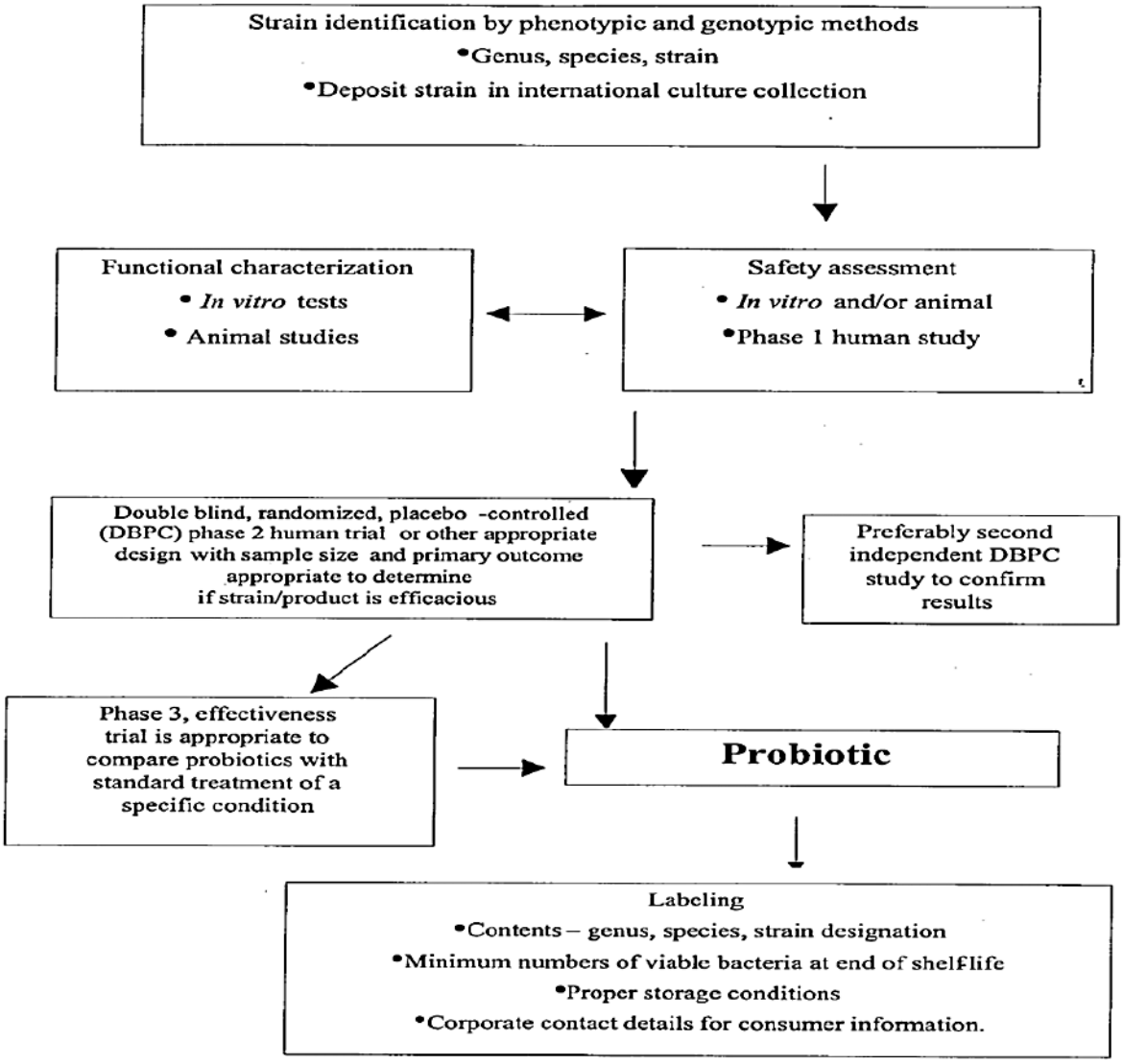



**Figure 11-1: FAO/WHO guidelines for the evaluation of probiotics for food use**

- Salminen and his team recommended that the safety of probiotics should be assessed in three steps (fig. 11-2):
  1. The intrinsic properties of the strain should be studied.
  2. Safety and stability should be evaluated.
  3. Interactions between strain and host should be studied.

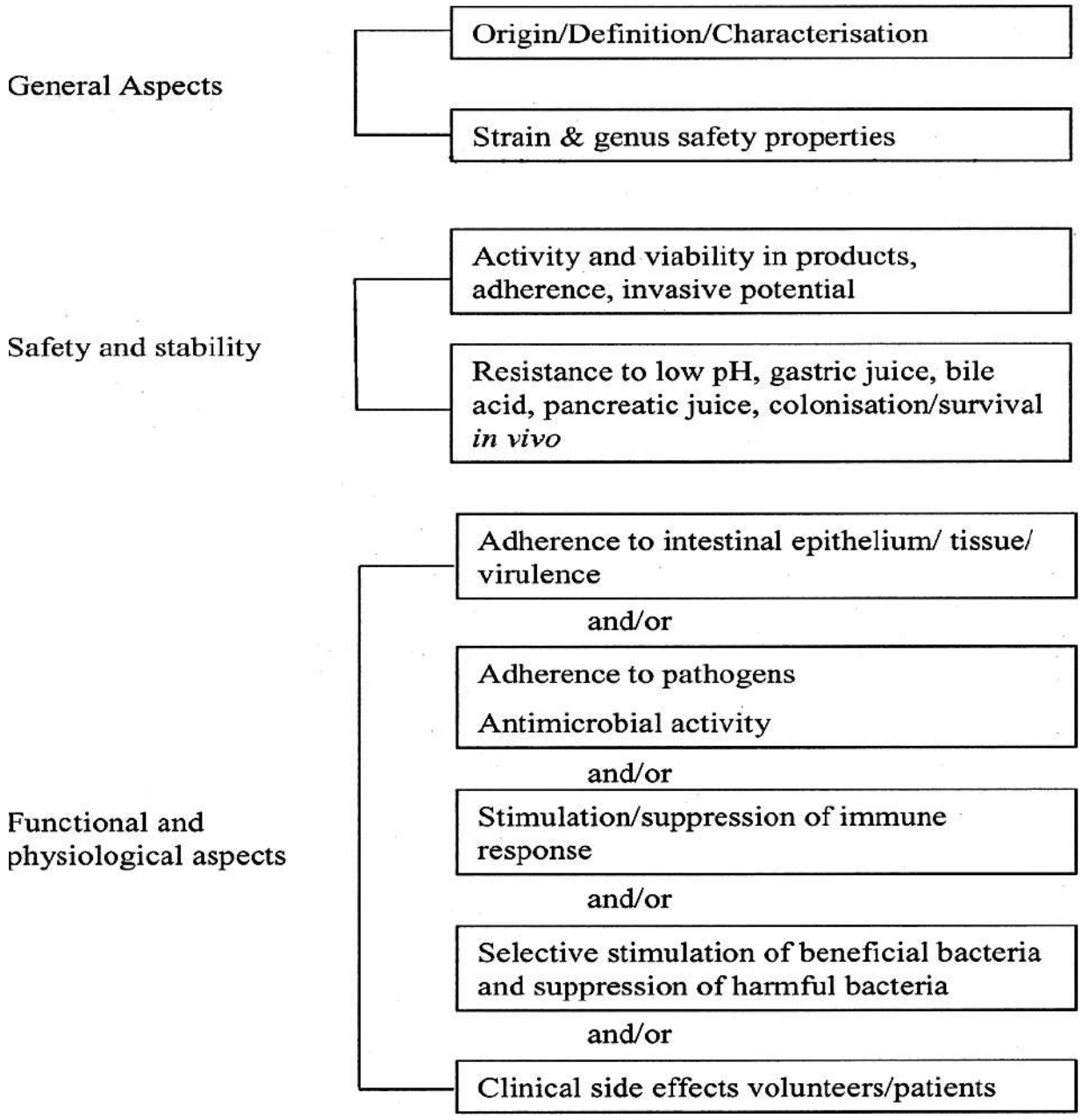



**Figure 11- 2: Decision scheme for safety evaluation of probiotics**

- A system for a pre-market safety assessment was proposed that leads to a 'Qualified Presumption of Safety (QPS)' in the European Community. In summary, a safety assessment of selected groups of micro-organisms from a defined taxonomic group (e.g. genus or group of related species) can be made on the basis of four pillars of information (identity, body of knowledge, possible pathogenicity and end use). If the taxonomic group and characterization to strain level do not raise safety concerns or if any safety concerns can be defined and excluded, the organism may be granted QPS status. Thus, for any strain of micro-organism that can be unequivocally demonstrated to be from a qualified QPS group (such as Lactobacillus or Bifidobacterium), further safety assessment is limited to tests for antibiotic resistance. If a microbe is not covered by QPS, then a comprehensive assessment of safety is likely to be required before it can be used in the food supply.

## Probiotics risk

Probiotics may theoretically be responsible for these types of side effects:

1. Systemic infections
2. Deleterious metabolic activities
3. Excessive immune stimulation in susceptible individuals
4. Gene transfer
5. Minor gastrointestinal symptoms

Probiotics are grouped into two classes based on their risk to health:

1. Risk group 1(No risk) consists of *Lactobecillus* and Bfidobacteria.
2. Risk group 2 (small risk) contains *L. rhamnosus* and *Bfidobacterium dentium*.

# Lec.12: Health effects of probiotics

The potential health benefits of probiotics are the focus of a great deal of scientific research. The beneficial effects of probiotics are strain specific. Numerous probiotics may have similar function with respect to their health effects.

Depending on the results of animal models studies, Probiotics can be used for treatment or prevent a wide range of human diseases and disorders (fig.12-1) such as; types I and II diabetes, respiratory diseases, oral diseases, urinogenital infections in women, *Helicobacter pylori* infections, allergic diseases, hypertension, constipation, mineral metabolism, Lactose intolerance, liver diseases, cancers, diarrhea, obesity, inflammatory bowel disease(IBD) and joint diseases. Also probiotics can be used to promote emotional behavior and may influence underlying brain mechanisms. As well as, probiotics also can be used to remove the microbial toxins from solutions, such as; aflatoxins, ochratoxin A, Shiga toxin, microcystin-LR and cholera toxin.

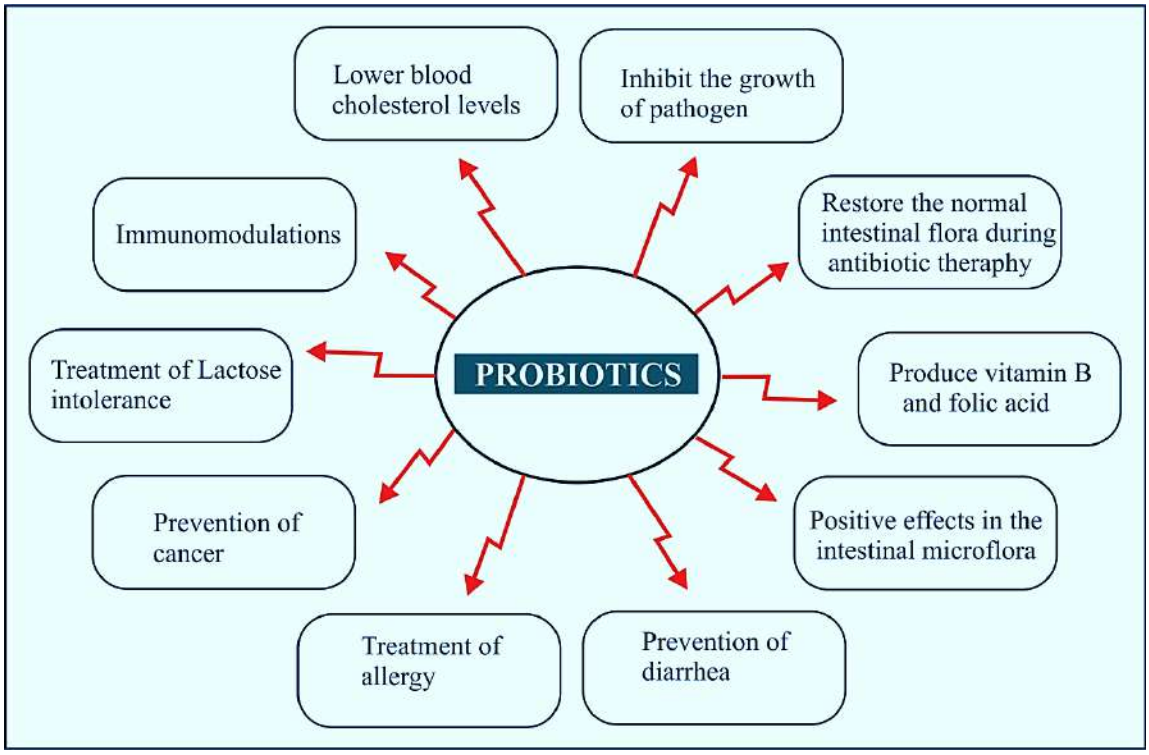



**Figure 12-1: Some health benefits of probiotics**

The health benefits of probiotic depend on many factors, such as:

- Probiotic strain
- Probiotic dose
- Delivery method
- Factors related to the host, such as age, diet and health condition

## Examples on the use of probiotics to prevent or treat some health conditions:

### Atopic dermatitis (AD)

It is the most common form of eczema, is also one of the most common chronic inflammatory skin disorders, affecting approximately 15% to 20% of children and 1% to 3% of adults worldwide.

Numerous probiotic studies have evaluated the effects of various species and strains of bacteria on the prevention of AD, the exposure to probiotics during pregnancy and in early infancy might reduce the risk of developing AD in infants and children. It was suggest that starting probiotic treatment during gestation and continuing through the first 6 months of the infant's life may be of benefit in the prevention of AD.

The effects of probiotic treatment vary depending on probiotic strain. For example, supplementation with either *L. rhamnosus* or *L. paracasei* significantly reduced the incidence of AD, whereas supplementation with *L. reuteri* or *L. acidophilus* did not.

Probiotics containing *Lactobacillus* might reduce AD symptoms in infants and toddlers, but those containing *Bifidobacterium* did not.

Overall, the available evidence suggests that the use of probiotics might reduce the risk of developing atopic dermatitis and lead to significant reductions in atopic dermatitis SCORAD scores, but these products might provide only limited relief from the condition. Furthermore, the effects of probiotics vary by the strain used, the timing of administration, and the patient's age, so it is difficult to make recommendations.

### Pediatric Acute Infectious Diarrhea

Acute diarrhea is usually defined as loose or liquid stools and/or an increase in the frequency of bowel movements (typically at least three in 24

hours). Acute diarrhea can be accompanied by fever or vomiting, and it usually lasts no more than 7 days.

Single- and multi-strain probiotics significantly shortened the duration of acute infectious diarrhea by about 25 hours. These supplements also decreased the risk that the diarrhea would last 4 or more days by 59% and led to approximately one less bowel movement on the second day in patients who received probiotics compared with patients who did not.

*L. rhamnosus* GG is most effective in treating infectious diarrhea at a daily dose of at least $10^{10}$ CFU. Also, patients who aged 1 month to 15 years found that *S. boulardii* (most commonly $10^{9}$ -$10^{10}$ CFU/day for 5–10 days) reduced both duration of diarrhea and stool frequency. In both of these analyses, *L. rhamnosus* GG and *S. boulardii* reduced the duration of acute infectious diarrhea by approximately 1 day.

The European Society for Pediatric Gastroenterology, Hepatology, and Nutrition identified two probiotic supplements, *L. rhamnosus* GG (typically at ≥$10^{10}$ CFU/day for 5–7 days) and *S. boulardii* (typically at 250–750 mg/day [$10^{9}$–$10^{10}$ CFU] for 5–7 days), for which evidence supported use as adjuncts to rehydration for managing acute infectious diarrhea in pediatric patients. However, recent studies suggest that probiotics might not be efficacious in developed country emergency departments because most episodes of acute infectious diarrhea are self-limiting and require no treatment other than rehydration therapy. Therefore, the cost-effectiveness of the use of probiotic supplements to manage acute viral diarrhea lacks consensus.

## Antibiotic-Associated Diarrhea (AAD)

Antibiotics are another common cause of acute-onset diarrhea. Antibiotic treatment often disturbs the intestinal microbiome and, by decreasing microbial diversity, can lead to a loss of microbial metabolism (resulting in osmotic diarrhea due to excessive fluid in the intestine), loss of colonization resistance (resulting in increased numbers of infections by other pathogens), and increased intestinal motility. Up to 30% of patients who use antibiotics experience AAD.

Children younger than 2 years and seniors older than 65 years are at greater risk of developing AAD than other children and adults. Some

antibiotics (e.g., erythromycin and penicillin) are associated with AAD more often than others.

The use of any of a few species and strains (described below) of probiotics might reduce the risk of AAD by 51%. However, the benefits of probiotic use to prevent AAD depend on the type of antibiotic that caused the AAD, the strain(s) of probiotic used, the life stage of the user (child, younger adult, or older adult), and whether the user is receiving inpatient or outpatient care.

Overall, the available evidence suggests that starting probiotic treatment with *L. rhamnosus* GG ($4 \times 10^8$ to $12 \times 10^{10}$ CFU) or *S. boulardii* (various doses) within 2 days of the first antibiotic dose helps reduce the risk of AAD in children and adults aged 18 to 64, but not in elderly adults. There is no evidence to suggest that the benefits are greater when more than one probiotic strain is used.

## Irritable bowel syndrome (IBS)

It is a common functional disorder of the GIT characterized by recurrent abdominal discomfort or pain, bloating, and changes in stool form or frequency. Although the causes of IBS are not completely understood, growing evidence suggests potential roles for intestinal microbiota in its pathophysiology and symptoms; IBS has also been linked to stress. Proinflammatory bacterial species, including *Enterobacteriaceae*, are abundant in patients with IBS, who typically also have a corresponding reduction in amounts of *Lactobacillus* and *Bifidobacterium*. Probiotic products commonly contain *Lactobacillus* and *Bifidobacterium* and, therefore, have the potential to restore some missing microbial functionality and, consequently, help manage IBS symptoms.

Multi-strain probiotic products had beneficial effects on IBS symptoms, some combinations of probiotics were superior to individual strains, but no specific combination was superior to another.

Overall, the available evidence indicates that probiotics might reduce some symptoms of IBS. However additional high-quality clinical trials are needed to confirm the specific strain, dose, and duration of treatment required as well as the type of IBS (such as with predominant diarrhea or constipation) that can be treated effectively with probiotics.

## Hypercholesterolemia

High levels of cholesterol in the blood or cholesterol trapped in arterial walls are a risk factor for cardiovascular disease (CVD).

Researchers have studied the use of probiotics to improve lipid profiles. The mechanisms of their effects on cholesterol concentrations include catabolism of cholesterol by increasing:

- Bile salt hydrolase activity,
- Binding of cholesterol in the small intestine,
- Assimilation and incorporation of cholesterol into bacteria,
- Production by lactobacilli and bifidobacteria of short-chain fatty acids.

Overall, research suggests that the use of multiple probiotic strains in combination as well as of probiotics containing *L. acidophilus*, a mixture of *L. acidophilus* and *B. lactis*, or *L. plantarum* might reduce total and LDL cholesterol levels. However, more research is needed to confirm these findings.

## Obesity

The gut microbiota play an important role in nutrient and energy extraction from food. Research in mice suggests that the gut microbiota affect not only use of energy from the diet, but also energy expenditure and storage within the host. Whether these effects translate to humans is unknown.

Results of clinical trials that assessed the impact of probiotics on obesity-related endpoints have been inconsistent.

- Probiotics promote loss of a mean of 0.54 kg in adults, gain of a mean of 0.20 kg in children, and no significant weight loss or gain in infants.
- Probiotics (mostly *Lactobacillus* administered at various doses for 3 weeks to 6 months) significantly decreased body weight and/or body fat in nine trials, had no effect in three trials, and increased body weight in two trials.
- Supplementation with various doses and strains of probiotics for 3 to 12 weeks resulted in larger reductions in body weight, body mass index, and fat percentage.

Taken together, these results indicate that the effects of probiotics on body weight and obesity might depend on several factors, including the probiotic strain, dose, and duration, as well as certain characteristics of the user, including age, sex, and baseline body weight. Additional research is needed to understand the potential effects of probiotics on body fat, body weight, and obesity in humans.

# Main References


- Andersen,ML and Winter,LMF (2019) Animal models in biological and biomedical research – experimental and ethical concerns . Anais da Academia Brasileira de Ciências, 91(Suppl. 1): e20170238.
- Binns N (2013) Probiotics, Prebiotics and the Gut Microbiota. ILSI Europe Concise Monograph Series.
- Boyle RJ, Robins-Browne RM, Tang MLK (2006) Probiotic use in clinical practice: what are the risks? Am J Clin Nutr. ,83:1256–1264.
- DFG (2016) Animal Experimentation in Research.
- Fijan S (2014) Microorganisms with claimed probiotic properties: An overview of recent literature. Int. J. Environ. Res. Public Health, 11: 4745-4767.
- Floch MH (2014) Recommendations for probiotic use in humans. Pharmaceuticals, 7: 999-1007.
- https://isappscience.org
- Ipek G, Vijay KJ and Mohamed A (2006) Probiotics in Food Safety and Human Health. CRC Press.
- Ng SC, Hart AL, Kamm MA, et al. (2009) Mechanisms of action of probiotics: Recent advances. Inflamm Bowel Dis., 15:300 –310.
- Rijkers GT, de Vos WM, Brummer RJ, et al. (2011) Health benefits and health claims of probiotics: bridging science and marketing. Br J Nutr. , 106: 1291-1296.
- Robert AB and Patrice DC (2018) Bugs as Drugs: Therapeutic Microbes for the Prevention and Treatment of Disease ASM Press.Canada.
- WHO/FAO (2002) Guidelines for the Evaluation of Probiotics in Food.
- Yuan KL and Seppo S (2009) Handbook of Probiotics and Prebiotics, 2nd ed. John Wiley & Sons. Inc., Hoboken, New Jersey.