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Probiotics

Biology, Genetics and Health Aspects

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Editor

Probiotics

Biology, Genetics and Health Aspects

Springer

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Preface

Probiotics have a long history of safe use and have been well-documented for their health benefits on hosts. This volume is a collection of various topics on probiotics, ranging from the microbiological aspects of both prokaryotic and eukaryotic probiotic microorganisms to genetic modifications, maintenance, health benefits and claims, detection, genetic modifications, safety and market trends.

The chapter by Daniel O. Otieno discusses prokaryotic probiotics and their classification based on morphology, ability to form spores, method of energy production, nutritional requirements and reaction to the Gram stain. Prokaryotic probiotics are mainly from the genera of *Lactobacillus* and *Bifidobacterium*. *Lactobacillus* has 106 validly described species, out of which 56 species have probiotic potential. On the other hand, *Bifidobacterium* currently has 30 species validly described, with eight having probiotic capabilities. A close study of these microorganisms revealed that probiotic microorganisms are likely to be Gram positive, mostly rod shaped but with fewer spherically shaped ones, nonspore forming and nonflagellated bacteria.

The chapter by Sukanta K. Nayak covers the microbiological aspects of eukaryotic probiotics. Several eukaryotic microorganisms have been identified with probiotic properties and have been consumed as single cell protein and/or as components of food starters for human and animal consumption. A limited number of these eukaryotic probiotics originate from fungi/moulds/yeasts. Among the eukaryotic probiotics, yeasts especially *Saccharomyces* species are dominant and routinely used in a broad range of hosts. This chapter deals with the occurrence, distribution, taxonomic characterization and detail modes of action of eukaryotic probiotics with special reference to yeasts in human and other animals.

The chapter by Alexander G. Haslberger et al. provides an overview of probiotic strain characterization, gut metagenomics and the analytical methods (FISH, PCR, RAPD, DGGE, repPCR, PFGE, RFLP, microarray, high throughput sequencing) required for their study. Molecular microbiological analysis has increased the understanding of the diversity and phylogeny of beneficial strains and their functions. These modern techniques, including genotyping methods, become increasingly

important for species identification and for the differentiation of probiotic strains. The precise classification and identification of probiotic strains give a strong indication of its typical habitat and origin, safety and technical applicability and provides possibilities for monitoring and product quality.

The chapter by Zhibing Zhang and Benjamin D. Huckle discusses the importance and types of protection techniques for probiotic microorganisms, with a particular focus on encapsulation and compression coating. Beneficial effects of probiotic bacteria depend on the viability of cells once delivered to the intestines. Cells tend to lose their viability with time during storage and the passage through acidic gastric fluids. Enteric coating materials are discussed as suitable for compression coating, while also improving cell storage stability and ensuring cell survival during exposure to harsh acidic gastric fluids. Techniques for controlled release of cells to the colon, including the use of hydrophobic disintegrants such as pectin, are also described.

The chapter by Chathuranga T. Bamunuarachchige et al. addresses the issues of genetically engineered probiotic microorganisms. Improved technologies in genomics and proteomics have led to greater understanding on the beneficial characteristics of probiotics that are enhanced via genetic modification (GM). GM probiotics are mainly associated with improved gut survival and persistence, tolerance of packing and storage conditions and as successful delivery vehicles for therapeutics. These are attributed to increased tolerance to osmolarity, bile salt and reduced water activity. With the expression of various molecules such as antigens, enzymes and molecules of immunological importance within probiotic microbes, the use of GM probiotics in the field of therapeutics looks promising. Safety issues of GM probiotics are also discussed.

The chapter by Wai-Yee Fung et al. documents the strong in vivo and in vitro evidences of probiotics on gut health, emphasizing on reestablishing the intestinal ecosystem balance, and alleviating gut and malabsorption disorders such as diarrhea, lactose intolerance and irritable bowel syndrome. Probiotics are also therapeutic against postoperative complications and inflammatory bowel diseases, in addition to exerting antibacterial and anticancer properties in the gut, due to their ability to attenuate the immune system. Mechanisms involved include competitive exclusion of pathogenic bacteria for nutrients and adhesion sites, production of antimicrobial bacteriocins and metabolites, and gut immunomodulation.

The chapter by Narayan C. Mandal and Vivekananda Mandal documents the different strains or species of orally consumed probiotics in conferring various new health benefits beyond gut well-being. New roles include modulation of immunological parameters, allergy and lung emphysema. Probiotics fight invading pathogens by various mechanisms, such as competitive inhibition, production of active principles such as bacteriocins, hydrogen peroxide and organic acids. In addition to inhibiting pathogens, probiotics also contribute to improving the immunological and physiological state of the host by interfering with metabolic processes. As they colonize the vital parts of the human intestines, probiotics are intricately involved with different systems of the human body and alleviate the problems associated with them.

The chapter by Siok-Koon Yeo et al. discusses nondairy new carriers for probiotics. Despite being an ideal substrate for probiotics, the growth of probiotics in dairy products is often inhibited by excessive acidification, antagonistic effect of starter cultures and the presence of oxygen during processing. The drawbacks of milk-based carrier associated with cholesterol contents, and lactose intolerance has prompted the development of alternative carriers for probiotics. Currently, soy-based, cereal-based, fruits, vegetables and meat products are developed as potential probiotic carriers. These nondairy products contain reasonable amounts of carbohydrates, fibers, proteins and vitamins that support the growth of probiotics, and protective components that are able to protect probiotics during gut transit, processing and storage. The challenges of these new carriers are also discussed.

The chapter by Maria G. Cifone et al. documents the clinical and experimental evidences of probiotic benefits at the skin. Scientific and evidence-based reports strengthen the assumption that certain probiotics can contribute to modulate cutaneous microflora, lipid barrier, skin immune system, leading to the preservation of skin homeostasis. In this chapter, recent evidences available from scientific literature as well registered patents have been summarized in relation to actual or potential topical applications of probiotics in the field of dermatology. Altogether the evidences reported in this review afford the possibility of designing new strategies based on a topical approach for the prevention and treatment of cutaneous disorders.

The chapter by Istan Siro addresses the challenges of the various probiotic health claims. Substantiation of claims should be based on scientific evidences, which requires a long and expensive procedure. Different in vitro and in vivo methods are applied for screening and characterizing the putative probiotic strains. Although useful, these assessment tools must be validated by properly designed human clinical studies. Poor prior selection, limited capacity of in vitro tests, and unsuitable animal models often contribute to contradictions between in vitro findings and in vivo feasibility. This chapter reviews the crucial steps of substantiation of health claims associated with probiotics with special emphasis on the related challenges.

The chapter by Fumiaki Abe documents the safety aspects of probiotics, rising from issues including bacterial translocation causing sepsis and horizontal transfer of acquired antibiotic resistance gene. To resolve these concerns, manufacturers have to demonstrate safety of probiotics on a strain by strain basis because not all probiotics are the same. Also, probiotics harboring acquired antibiotic resistance genes should not be used to avoid the possibility of gene transfer. A high hygienic standard to prevent contamination by pathogenic bacteria or allergen during the production of probiotics is another requirement to assure the safety of probiotics. Safety regulation of various countries including the United States, Canada, Australia, New Zealand and Japan are also highlighted.

The chapter by Carlos R. Soccol provides an insight on the current probiotic market trends and future directions. Current trends in the consumption of probiotics are associated with increased levels of health-consciousness, and the availability of probiotics in the form of dietary supplements. Several companies have profited by marketing these products in different forms, with different purposes, and with

recommendation for all ages. Important aspects in maintaining the viability and bioactivity of probiotic strains during processing and storage are also discussed in this chapter. The probiotic consumption by infants and the elderly has been supported by scientific evidences and represents a new niche market.

Penang, Malaysia Min-Tze Liong

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Biology of Prokaryotic Probiotics

Daniel Obed Otieno

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Abstract Bacteria and archaea are two distinct phyla of the prokaryotic kingdom containing many different species of microorganisms. Prokaryotic probiotics are single-celled nonnucleated organisms which when consumed live in adequate numbers confer a health benefit to the host. They can be classified based on

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morphology, ability to form spores, method of energy production, nutritional requirements, and reaction to the Gram stain. Currently, there are no known probiotic archaea but they have an important potential in the synthesis of prebiotics and other bioproducts due to their unique characteristics. There are however, probiotic bacteria mainly coming from the genera of *Lactobacillus* and bifidobacteria. *Lactobacillus* has 106 validly described species, out of which 56 species have probiotic potential. On the other hand, Bifidobacteria currently has 30 species validly described, with 8 having probiotic capabilities. A close study of these microorganisms reveal that probiotic bacteria are likely to be Gram positive, mostly rod shaped but with fewer spherically shaped ones, nonspore forming and non-flagellated bacteria.

1 Introduction

Prokaryotes are a group of microorganisms that do not possess a cell nucleus or any other membrane-bound organelles. An *organelle* refers to structures within the cell membrane that perform functions analogous to what an organ does in the body. Previously, prokaryotes were thought not to have organelles; however, some examples have recently been identified (Kerfeld et al. 2005) that possess these structures. They are not as structurally complex and for a long time were thought not to have any internal structures enclosed by lipid membranes. They were also previously thought to have little internal organization. Some prokaryotes have been found to have microcompartments such as carboxysomes, which are the subcellular compartments measuring 100–200 nm in diameter and are enclosed by a shell of proteins (Kerfeld et al. 2005). In addition, there have been descriptions of membrane-bound magnetosomes in bacteria (Komeili et al. 2006; Scheffel et al. 2006) as well as the nucleus-like structures of the *Planctomycetes* that are surrounded by lipid membranes (Fuerst 2005). These studies have revealed that a typical prokaryotic cell have important major organelles and cellular structures including nucleoid, ribosome, vesicles, rough endoplasmic reticulum, golgi apparatus, cytoskeleton, smooth endoplasmic reticulum, mitochondria, vacuole, cytosol, lysosome, and centriole. These structures determine the cell's structure, function, growth, origin, survival, and distribution as well as the taxonomic classification of the microorganism. Thus, despite their apparent simplicity compared to eukaryotes, they pretty much can exist in any given optimal environment. Table 1 presents a summary of the most important organelles in prokaryotic bacteria, their corresponding functions, structure component, and the microorganisms they are found in.

There remains two major domains of prokaryotic microorganisms, namely, the bacteria and the archea. This chapter will focus on prokaryotic probiotic microorganisms, which admittedly, only form a very small fraction of the entire population of prokaryotic microorganisms. In spite of this reality, the relatively few prokaryotes, mostly from bacteria and a few archaea are very important in their probiotic functions. Although archaea have an important niche of

Table 1 A summary of prokaryotic cell organelles, components, functions, structure, and the organisms they are found in

Prokaryotic organelles and cell components			
Organelle/ macromolecule	Main function	Structure	Organisms
Carboxysome	Carbon fixation	Protein-shell compartment	Some bacteria
Chlorosome	Photosynthesis	Light harvesting complex	Green sulfur bacteria
Flagellum	Movement in external medium	Protein filament	Some prokaryotes and eukaryotes
Magnetosome	Magnetic orientation	Inorganic crystal, lipid membrane	Magnetotactic bacteria
Nucleoid	DNA maintenance, transcription to RNA	DNA–protein	Prokaryotes
Plasmid	DNA exchange	Circular DNA	Some bacteria
Ribosome	Translation of RNA into proteins	RNA–protein	Eukaryotes, prokaryotes
Thylakoid	Photosynthesis	Photosystem proteins and pigments	Mostly cyanobacteria

applications, most of the well-known probiotics are of the bacterial domain. The highlights in this chapter will be on probiotic prokaryotic bacteria. According to the currently adopted definition by FAO/WHO, probiotic bacteria are: "Live microorganisms which when administered in adequate amounts confer a health benefit on the host" (FAO/WHO, 2001). Lactic acid bacteria (LAB) and bifidobacteria are the most common types of microbes used as probiotics, but certain yeasts and bacilli may also be helpful. Probiotics are commonly consumed as part of fermented foods with specially added active live cultures, such as in yogurt, soy yogurt, or as dietary supplements. Archaea are increasingly finding important application in bioproducts, pharmaceutical, food, and biofuel industry.

2 The Phylogenic Tree of Prokaryotic Probiotic Microorganisms

2.1 Bacteria

The bacteria are a large group of single-celled, prokaryote organisms that are microscopic, typically measuring a few micrometers (10^{-6}), hence referred to as microorganisms. They do not contain a nucleus and rarely harbor membrane-bound organelles. Although the term *bacteria* traditionally included all prokaryotes, the scientific classification changed after the discovery in the 1990s that prokaryotes consist of two very different groups of organisms that evolved independently from

an ancient common ancestor, namely, Bacteria and Archaea (Woese et al. 1990). Therefore both bacteria and archaea are part of the domain considered prokaryotic. The classification of bacteria is generally based on five main criteria, namely, morphology, ability to form spores, method of energy production, nutritional requirements, and reaction to the Gram stain. These aspects will be discussed in detail in the various subsections of this chapter.

2.2 *The Archaea*

The archaea are also a group of single-celled microorganisms and forms an important component of prokaryotic microorganisms. A single individual or species from this domain is called an *archaeon* (sometimes spelled "archeon"). Like bacteria they also have no cell nucleus or any other membrane-bound organelles within their cells. In the past they were regarded as an unusual group of bacteria and named archaebacteria; however, after studies of the sequencing of their ribosomal RNA, it became obvious that they bore no close relationship to the bacteria (http://users.rcn.com 2010). Because of their independent evolutionary history (Zuckerkandl and Pauling 1965) and differences in their biochemistry from other forms of life, they are now classified phylogenetically as a separate domain in the three-domain system consisting of Archaea, Bacteria, and Eukarya as shown in Fig. 1. The archaea are divided into four recognized phyla, but it is well established that many more phyla may exist. Of these groups, the Crenarchaeota and the Euryarchaeota are most intensively studied, and to date, more than 250 species have been discovered. Classification of archaea has only been made possible through analysis of their nucleic acids in samples from the environment. Based

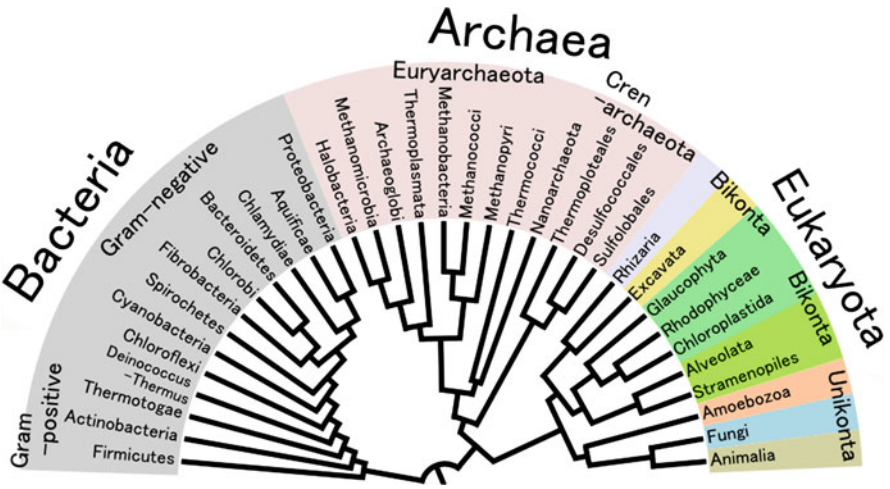

Fig. 1 Phylogenetic tree showing the diversity of prokaryotes, compared to eukaryotes (Woese 1998)

on these studies, more evidence of their differences from bacteria was established (Pace 2006).

Archaea and bacteria are visually similar in size and shape, although a few archaea have very unusual shapes, such as the flat and square-shaped cells such as those of *Haloquadra walsbyi*. Despite this similarity to bacteria, archaea possess genes and several metabolic pathways that are more closely related to those of eukaryotes such as the enzymes involved in transcription and translation, as well as reliance on ether lipids in their cell membranes. The archaea exploit a much greater variety of sources of energy for metabolic processes than bacteria and eukaryotes; ranging from familiar organic compounds such as sugars, to inorganic ones including ammonia, metal ions, sun light, and even hydrogen gas as nutrients. There are also species of archaea that fix carbon into the soil. Their mode of reproduction is asexual and divides by binary fission, fragmentation, or budding. In contrast to some bacteria, they do not form spores.

Initially, archaea were regarded as extremophiles that lived in harsh environments, such as hot springs, salt lakes, and other extremes of temperature, pH, and radiation, but they have since been found in a broad spectra of habitats, including soils, oceans, and marshlands. Archaea are particularly numerous in the oceans, and the archaea in plankton may be one of the most abundant groups of organisms on the planet. Archaea are mostly recognized for their major role in both the carbon cycle and nitrogen cycle. There are currently no known examples of archaeal pathogens or parasites, but they are often mutualists or commensals. The only known archaea with probiotic potential are from the methanogens, which inhabit the gut of humans and ruminants, where their vast numbers aid digestion. Figure 2 shows branches of archaea highlighting those increasingly useful in the biotech industry such as methanogens and hyperthermophiles. If functions by intestinal microflora such as maturation of the immune system, protection against cell injury, and regulation of energy balance (O'Hara and Shanahan 2006) are considered probiotic, then the complex ecosystem which also includes archaea as constituent of the 400–1,000 gastrointestinal species from at least nine different phyla (Rajilic-Stojanovic et al. 2007) is probiotic. Most of these species especially of archaea are still uncultured and our knowledge of the microbial diversity still remains incomplete. In humans, the archaeal methanogens have been found in the anaerobic population of the oral cavity, the vagina, and the large intestine (Chaban et al. 2006). Gut methanogenic archaea that have been well determined are two species belonging to Methanobacteriales which are *Methanobrevibacter smithii* and *Methanosphaera stadtmanae* (Fricke et al. 2006). Methanogens are also useful in biotechnological processes such as in industrial catalyzation of thermochemical process such as the application of archaeal β-galactosidases in the synthesis of prebiotics such as galactooligosaccharides (GOS) from lactose at elevated temperatures. They are also useful in biotechnological processes such as biogas production and sewage treatment. Due to superior stable properties of the glycosidases from thermophilic methanogens, they have become an attractive interest for transgalactosyl reactions for GOS synthesis at high temperatures (Otieno 2010). Examples of archaea currently being used in this respect include

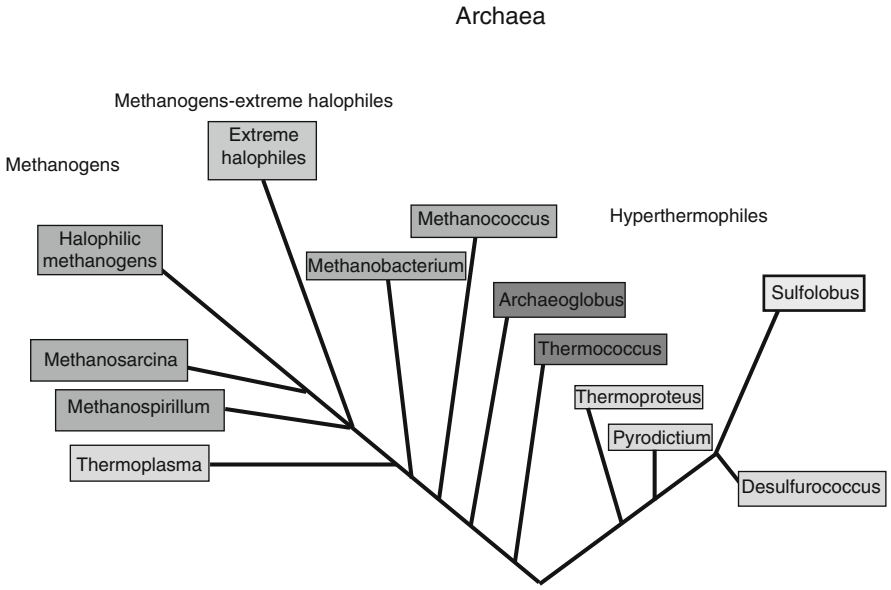

Fig. 2 Branches of archaea showing methanogens and hyperthermophiles useful in biotechnology (Baross and Holden 1996)

Thermus sp. Z-1 (Akiyama et al. 2001), *Pyrococcus furiosus* (Petzelbauer et al. 2000; Bruins et al. 2003), and *Sulfolobus solfataricus* (Reuter et al. 1999; Hansson et al. 2001) in search of GOS production at high temperatures. The rationale of using thermostable enzymes is that transgalactosyl reactions proceed more efficiently at high substrate concentrations due to the improved solubility of lactose at higher temperatures. As a result of their ability to exist and adapt in different extreme environments, they can also be grouped in various classes such as thermophiles (high temperatures), hyperthermophiles (very high temperatures such as 121°C), psychrophiles (in cold conditions and may grow best at 4°C), halophiles (in very saline environments), acidophiles (at low pH, i.e., as low as pH 1 and die at pH 7), alkaliphiles (at a high pH).

3 Morphology of Prokaryotic Probiotic Bacteria

Prokaryotic bacteria have various shapes and morphological features that are important in their classification and identification. The four basic shapes are cocci (spherical), bacilli (rod shaped), spirocheate (spiral shaped), and vibrio (comma shaped). Probiotic prokaryotes well known in the genera such as *Lactobacillus* and bifidobacteria are mostly rod shaped with fewer strains having spherical ones.

3.1 Coccus

The coccus (plural cocci) can be used to describe any bacterium that has a circular shape. They can be solitary in nature, but also group together to form different patterns in clusters. *Diplococci* are two-celled pairs of bacteria, while *Streptococci* are those arranged in a chain formation. The *Sarcina* are those having a cuboidal cell arrangement, while the tetrad ones are arranged in a square formation. The *Staphylococcus* genus usually contains cells arranged in large clusters of bacterial cells. Figure 3 shows the arrangements of Cocci into different patterns from which they derive their names. Whereas the arrangements of the bacterial cells take different forms, the bacterial cells themselves remain circular. Most of the bacteria having spherical shapes are known to be disease causing or pathogenic in nature, e.g., *Staphylococcus aureus*. However, there are a few well-known prokaryotic bacteria that have probiotic function such as *Streptococcus thermophilus* and *Enterococcus faecium*. *S. thermophilus* is a probiotic well known for its use in yogurt production. It uses its endogenous lactase to break down lactose into glucose and galactose. This enables a wider consumption of dairy products which also includes consumers who are lactose intolerant. Other Streptococcus strains which are probiotic include *Streptococcus cremoris* and *Streptococcus infantis*. *E. faecium* has shown in studies to be helpful for diarrhea through shortening the duration of disease symptoms. It also inhibits pathogenic microbes, such as rotavirus and lower LDL or bad cholesterol. Although *E. faecium* is considered a transient guest, i.e., does not occur naturally in the GIT, it is a welcome natural resident in the human body.

3.2 Bacilli

These are bacteria that are rod shaped and the singular form is referred to as *bacillus*. They are found in many different taxonomic groups of bacteria; however,

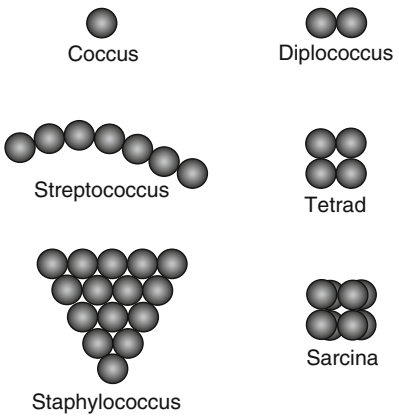

Fig. 3 Different patterns of spherically shaped prokaryotic bacteria. (Kaiser 1999) http://student.ccbcmd.edu/~gkaiser/goshp.html

the name *Bacillus*, capitalized and italicized, refers to a specific genus of bacteria. The name Bacilli, capitalized but not italicized, can also refer to a more specific taxonomic class of bacteria that includes two orders, one of which contains the genus *Bacillus*. They are also usually solitary, but can combine in clusters to form diplobacilli, streptobacilli, and palisades (http://www2.nemcc.edu). The most studied and well-known probiotic prokaryotic bacteria are from the genera of lactobacilli and bifidobacteria. A majority of the currently used probiotic prokaryotes are the Gram-positive rod-shaped bacteria.

3.3 Spirochaete

They are also spelled spirochetes, and belong to a phylum of Gram-negative bacteria, which have long, helically coiled (spiral-shaped) cells known as the flagella which is sometimes referred to as axial filament (Ryan and Ray 2004). They are not known to be probiotic, hence will not be discussed into greater detail in this chapter.

3.4 Vibrio

Vibrio is also a genus of Gram-negative bacteria that distinctly possess a curved rod shape, (Faruque and Nair 2008). They too are not known to be probiotic.

4 Reaction of Prokaryotic Bacteria to the Gram Stain

Gram staining is important in the identification of bacteria and is named after the nineteenth-century Danish bacteriologist named Hans Christian Gram who developed it. In the Gram staining process as shown in Fig. 4, the bacterial cells are first stained with a purple dye known as crystal violet. The cells are then treated with alcohol or acetone, followed by counterstaining with a dye of a different color (such as safarin or fuchsine) which is red or pink in color. Gram-positive bacteria are those that are stained dark blue or violet by Gram staining. This is in contrast to Gram-negative bacteria, which cannot retain the crystal violet stain, instead taking up the counterstain (safarin or fuchsine) and appearing red or pink. Gram-positive organisms are able to retain the crystal violet stain because of the high amount of peptidoglycan in the cell wall. Gram-positive cell walls typically lack the outer membrane found in Gram-negative bacteria. Figure 5 shows the marked difference in the wall structure of Gram-positive and Gram-negative bacteria, specifically indicating a thicker peptidoglycan layer among the Gram-positive bacteria, which influences their ability to retain or lose the crystal violet stain.

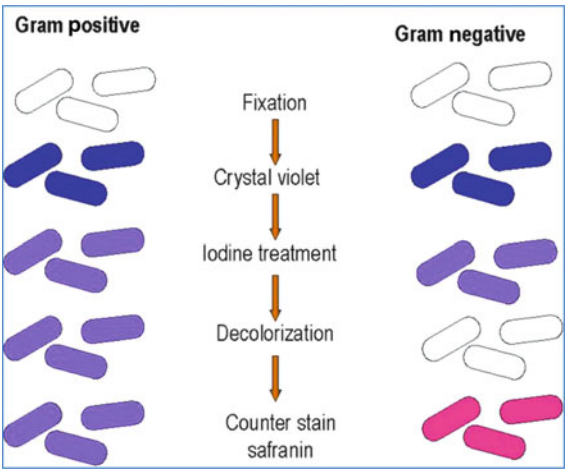

Fig. 4 Gram staining procedure in the identification of bacteria (Fox 2010). Generated by Alvin Fox, USC School of Medicine http://pathmicro.med.sc.edu/fox/culture

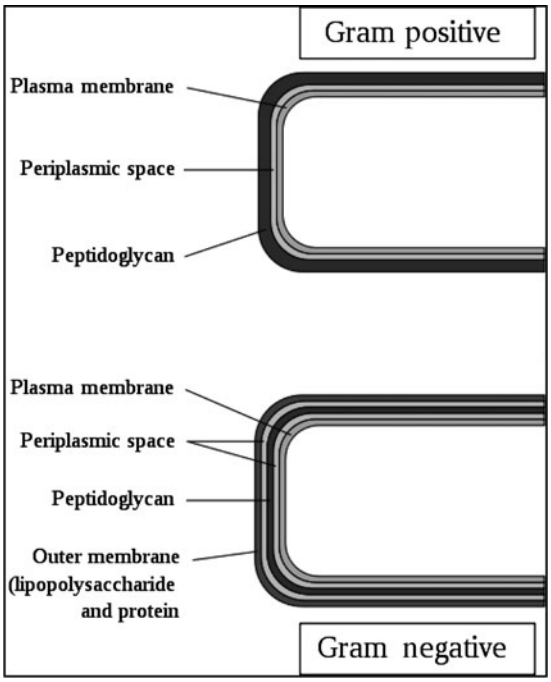

Fig. 5 Gram-positive and Gram-negative cell wall structure (Madigan and Martinko 2005)

5 The Cellular Structures of Prokaryotic Bacteria

The cellular structures are important for specific biological functions and are divided into two main classes, namely, (a) the structures external to the cell wall and (b) the structures internal to the cell wall.

5.1 The Structures External to the Cell Wall

The capsule is a very large structure of Gram-negative prokaryotic cells which lies outside the cell wall of the bacteria. It is basically composed of polysaccharides but may also include polypeptides among certain bacterial strains. Probiotic prokaryotes do not possess a bacterial capsule.

A flagellum is a tail-like projection that protrudes from the cell body of Gram-negative prokaryotic cells and helps in locomotion. Probiotic prokaryotic bacteria do not have a flagellum.

Fimbriae and pili are structures made of the protein pilin. The fimbriae are shorter and thinner than flagella and are used for attachment and not movement. They are found on some Gram-negative pathogenic bacteria and they enable the bacteria to attach to surfaces, such as mucous membranes. The pili on the other hand are comparatively longer than the fimbriae and much fewer in number, i.e., only one or two per cell. Some are used to join bacterial cells in preparation for the process of conjugation (transfer of DNA), hence sometimes called conjugation pili. There are no known probiotic prokaryotic bacteria possessing fimbriae and pili.

The cell wall is also located outside the cell membrane and it is a tough, usually flexible but sometimes fairly rigid layer that surrounds some types of cells. Its main functions are to provide the cells with structural support and protection, while also acting as a filtering mechanism. Perhaps its most important function is to act as a pressure vessel, preventing overexpansion when water enters the cell. Prokaryotic probiotic bacteria are known to have cell walls. There are known strains within species of probiotic bacteria having cell walls are *Bifidobacterium longum, Bifidobacterium animalis, Lactococcus lactis*, *Lactobacillus johnsonii, Lactobacillus planetarium, Lactobacillus acidophilus,* and *Lactobacillus casei* amongst others.

Prokaryotic cell walls are mainly made of the peptidoglycan material. However, the archaea do not contain peptidoglycan, instead, their cell wall contains pseudomurein. Peptidoglycan consists of disaccharide units connected by polypeptides to form a lattice. The many layers of peptidoglycan form a thick rigid structure that provides shape and protection. There are also teichoic acids in the cell walls, which consist of an alcohol and phosphate and the exact structure of the acids varies among species of bacteria. This is the part that often acts as an antigen and is used for identification. Some cell walls contain large amounts of mycolic acid thus making the bacteria to be regarded as acid-fast bacteria. The mycolic acid is in a layer outside the peptidoglycan.

As shown in Fig. 6, the peptidoglycan layer in the bacterial cell wall is a crystal lattice structure formed from linear chains of two alternating amino sugars, namely, *N*-acetylglucosamine (GlcNAc or NAG) and *N*-acetylmuramic acid (MurNAc or NAM). The alternating sugars are connected by a β-(1, 4)-glycosidic bond. Attached to the *N*-acetylmuramic acid is a peptide chain of three to five amino acids. Peptidoglycan layer is important in serving as a structural role in the bacterial

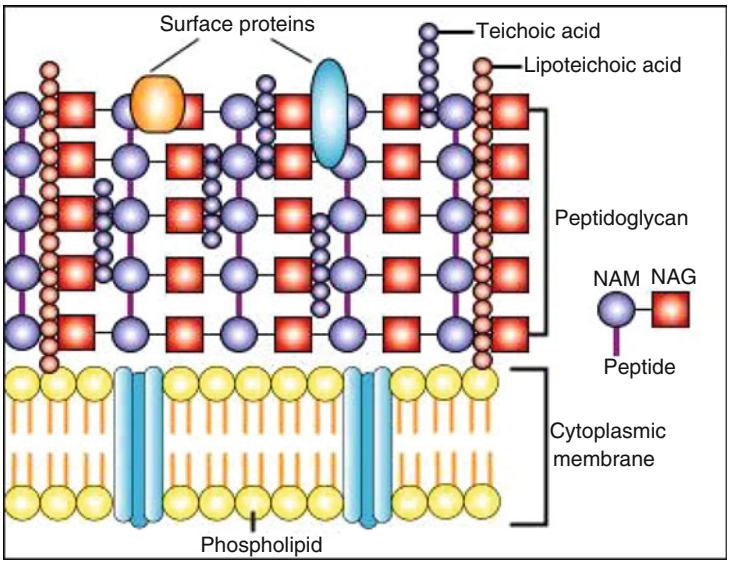

Fig. 6 The structure of Gram-positive prokaryotic cell wall (Kaiser 1999) http://student.ccbcmd.edu/~gkaiser/goshp.html

cell wall, giving structural strength, as well as counteracting the osmotic pressure of the cytoplasm. The peptidoglycan layer is substantially thicker in Gram-positive bacteria (20–80 nm) than in Gram-negative bacteria (7–8 nm), with the attachment of the S-layer. Peptidoglycan forms around 90% of the dry weight of Gram-positive bacteria but only 10% of Gram-negative strains. In Gram-positive strains, it is important in attachment roles and stereotyping purposes (Salton and Kim 1996). For both Gram-positive and Gram-negative bacteria, particles of approximately 2 nm can pass through the peptidoglycan (Demchick and Koch 1996). The functions of peptidoglycan (glycocalyx) include protecting the probiotic bacteria from phagocytosis, making them protected from digestive enzymes along the gastrointestinal track, allowing the bacterial cell to attach to a surface, for example, the intestinal linings, formation of a biofilm which protects the bacterial cells, often making body defenses, antiseptics, and antimicrobials less effective and may serve as a source of nutrients and protect against cell dehydration. The surface proteins on the peptidoglycan have different roles which include acting as enzymes for metabolic processes within the cell, adhesions, to enable the bacterium to attach intimately to the host and other surfaces in order to colonize and resist flushing, and for resisting phagocytic destruction.

A few prokaryocytes such as members of the genus *Mycoplasma* do not have a cell wall. As a result, they are also extremely tiny and their plasma membranes need extra strength which is provided through incorporation of sterols. Bacteria known to have atypical cell walls are pathogenic or potentially pathogenic.

5.2 *The Structures Internal to the Cell Wall*

Various internal structures to the cell wall exist for cellular physiological functions and maintenance. These include the cytoplasmic membrane, cytoplasm, nucleiod, ribosomes, plasmids, and endospores.

5.2.1 Cytoplasmic Membrane (Cell Membrane)

It refers to selectively permeable membrane that encloses the cytoplasm of a cell. It is composed of a bilayer of phospholipids embedded with proteins which determines what enters and leaves the cell. It is also known as a plasma membrane or cell membrane.

As shown in Fig. 7, the prokaryotic cell membrane separates the interior of prokaryotic cells from the outside environment. The cell membrane is selectively permeable to ions and organic molecules such as sugars and lipids, thus facilitating the transport of materials needed for survival. The movement of compounds i.e nutrients across the membrane can be passive, that is, occurring without the input of cellular energy, or active, requiring the cell to expend energy in moving it. The cell membrane also helps the cell to maintain the cell potential. It consists of the phospholipid bilayer with embedded proteins, which are involved in a variety of cellular processes such as cell adhesion, ion conductivity, and cell signaling. The plasma membrane also serves as the attachment surface for the extracellular

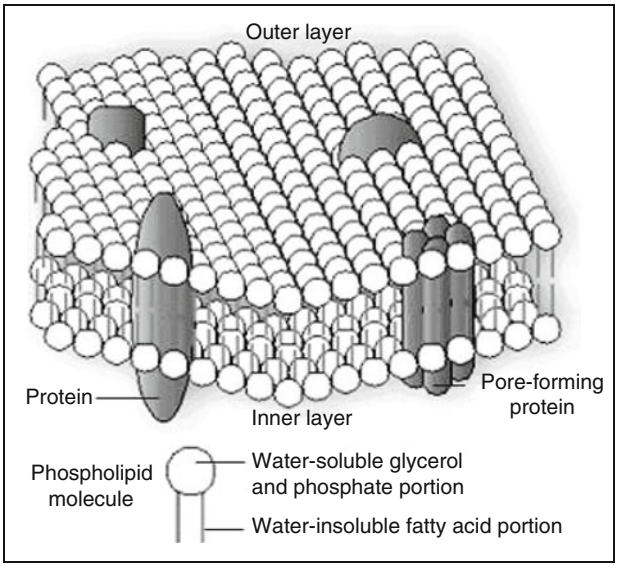

Fig. 7 Details of the phospholipid bilayer and phospholipid in a prokaryotic cell membrane (Kaiser 1999) http://student.ccbcmd.edu/~gkaiser/goshp.html

glycocalyx and cell wall and intracellular cytoskeleton. Cell signaling is part of a complex system of communication that determines basic cellular activities and coordinates cell actions. The ability of bacterial cells to perceive and correctly respond to their microenvironment is the basis of development, tissue repair, and immunity as well as normal tissue homeostasis. Communication also occurs between the cells. In the human gastrointestinal tract, bacteria exchange signals with each other and also with human epithelial and immune system cells. Many cell signals are carried by molecules that are released by one cell and move to make contact with another cell. In bacteria, the plasma membrane is also associated with the breakdown of nutrients and the synthesis of ATP.

5.2.2 Cytoplasm

The cytoplasm is a thick, semitransparent and gel-like liquid residing inside the plasma membrane and holds membrane organelles. It contains dissolved and suspended materials such as proteins (mostly enzymes), carbohydrates, lipids, and organic ions. It is the site of most cellular activities such as metabolic pathways including glycolysis, and processes such as cell division. The inner, granular mass is called the endoplasm and the outer, clear and glassy layer is called the cell cortex or the ectoplasm. Species of probiotic prokaryotes like any other cell do posses a cytoplasm and include some of the best known strains used such as *B. animalis* ssp. *lactis* BB12 and *L. acidophilus* NCFM.

Constituents of cell cytoplasm – The cytoplasm has three major elements: the cytosol, organelles, and inclusions. The cytosol is the portion within membrane-bound organelles, making up about 70% of the cell volume and is composed of water, salts, and organic molecules. The cytosol is a translucent fluid in which the plasmic elements are suspended. It also contains the protein filaments that make up the cytoskeleton, as well as soluble proteins and small structures such as ribosomes, proteasomes, and the mysterious vault complexes (van Zon et al. 2003). The inner, granular and more fluid portion of the cytoplasm is referred to as endoplasm. Some major organelles that are suspended in the cytosol are the mitochondria, the endoplasmic reticulum, the Golgi apparatus, vacuoles, and lysosomes. The cytoplasmic inclusions are small particles of insoluble substances suspended in the cytosol. A huge range of inclusions exist in different cell types but the most prominent in prokaryotic cells are the lipid droplets, also known as adipocytes, where lipids (fatty acids), proteins, and sterols are stored (Murphy 2001). The lipid droplets make up much of the volume of adipocytes, which are the specialized lipid-storage cells. All prokaryotic probiotic bacteria do possess cell cytoplasm from which all metabolic processes take place.

Cytoplasmic inclusions – These are reserve deposits of chemicals within the cytoplasm that tend to accumulate when nutrients are plentiful. They include:

(a) Metachromatic granules (volutin granules) – they are phosphate reserves that can be used in synthesizing ATP for metabolic processes within the cell.

(b) Polysaccharide granules – they are glycogen granules and starch granules.
(c) Lipid inclusions – the most common is poly-β-hydroxybutyric acid (PHB) which is found in *Bifidobacterium, Bacillus,* and others.
(d) Sulfur granules – these are found in bacteria whose metabolism involves oxidation of sulfur thus depositing these as energy reserve. Some *Bacilli* are an example of sulfur-containing granules.
(e) Carboxysomes – are found in bacteria that use carbon dioxide as their only carbon source from these granules, requiring an enzyme to perform this process.
(f) Gas vacuoles – these contain gases within the bacterial cell. These may aid floatation.
(g) Magnetosomes – inclusions of iron oxide that act like magnets. Function uncertain, but it is thought they can decompose hydrogen peroxide.

5.2.3 Nucleiod

Prokaryotic cells do not have a nucleus. Instead, they have a *nucleus-like* structure in the irregularly shaped region of the cytoplasm known as nucleoid. It is therefore the nuclear body of a prokaryotic (bacterial) cell, which is usually composed of a single molecule of circular, chromosomal DNA. As shown in Fig. 8, the nucleoid has a nuclear material without a nuclear membrane and it is where the genetic material is localized (Thanbichler et al. 2005).

The nucleoid is largely composed of about 60% DNA while the rest are proteins and a small amount of RNA. The latter two constituents are likely to be mainly messenger RNA and the transcription factor proteins found regulating the bacterial genome. Proteins helping to maintain the super-coiled structure of the nucleic acid are known as nucleoid proteins or nucleoid-associated proteins. The chromosome's DNA twists into a tight helix and is connected to the plasma membrane. Proteins of the plasma membrane are believed to be responsible for the replication of DNA. The DNA-binding proteins often use other mechanisms to promote compaction which can lead to the occurrence of DNA bending.

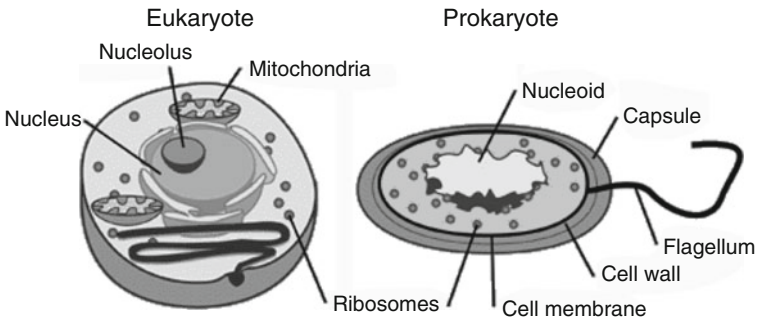

Fig. 8 Prokaryotic cell showing the nucleoid (Browning et al. 2010)

5.2.4 Ribosomes

Ribosomes are the components of cells where the protein synthesis takes place. In prokaryotic cells, the ribosomes are mostly scattered in the cytoplasm. The process involves utilization of DNA to make RNA, and subsequently used to make protein. The DNA sequence in genes is copied into a messenger RNA (mRNA). The information in the mRNA is then read and used to create proteins through a process known as translation in which the mRNA is used as a template for the correct sequence of amino acids in a particular protein. The amino acids are attached to transfer RNA (tRNA) molecules, which enter one part of the ribosome and bind to the messenger RNA sequence. The attached amino acids are then joined together by another part of the ribosome, which then moves along the mRNA, "reading" its sequence and producing a chain of amino acids. Ribosomes are divided into two subunits, one larger than the other and each subunit consist of a protein and a ribosomal RNA. The smaller subunit binds to the mRNA, while the larger subunit binds to the tRNA and the amino acids. When a ribosome finishes reading an mRNA, these two subunits split apart.

5.2.5 Plasmids

These are gene carrying, circular DNA molecules separate from the chromosome which are not involved in reproduction. They are found in most bacteria including those considered probiotic. They usually contain 5–100 genes, which code for proteins that may give the cell resistance to certain antibiotics, to toxic metals, ability to produce certain toxins, or to synthesize enzymes not included in the chromosomal genes. Proteins coded on plasmid DNA are usually not required for cells under ideal conditions, but they can give the cell advantages for survival in a given environment, e.g., in the gut or in the intestinal environment. Copies of plasmids can be transferred from one cell to another and once a cell has a plasmid, it will replicate the plasmid and pass a copy on to each daughter cell during cell division.

5.2.6 Endospores

These are dormant cells, formed when certain genera (mainly *Bacillus* which include species and strains considered probiotic) find themselves facing adverse conditions such as lack of nutrients, drying, presence of toxic materials, or any unfavorable change in the environment. Endospores once formed can withstand extreme conditions and survive for apparently unlimited periods of time. They can perform normal cell functions such as taking in nutrients, carrying out metabolic activities, and reproduction. They too are affected by pH variations, heat, drying, lack of nutrients, and most of them are relatively easy to kill. The process of formation of endospores is referred to as sporulation or sporogenesis.

6 Reproduction of Prokaryotic Probiotic Bacteria

Bacteria and archaea as prokaryotes reproduce through asexual reproduction, usually by binary fission or budding. The genetic exchange and recombination still occur, but through a form of horizontal gene transfer. This is different from the replicative process in which the DNA material is transferred between two cells through bacterial conjugation.

Asexual reproduction is therefore the reproduction which does not involve meiosis, ploidy reduction, or fertilization. Only one parent is involved in asexual reproduction. A more stringent definition is agamogenesis which refers to reproduction without the fusion of gametes. Asexual reproduction is the primary form of reproduction for single-celled organisms such as the archaea, bacteria, and protists. While all prokaryotes reproduce asexually (without the formation and fusion of gametes), mechanisms for lateral gene transfer such as conjugation, transformation, and transduction are sometimes likened to sexual reproduction (Narra and Ochman 2006).

Binary fission, also referred to as prokaryotic fission, is the form of asexual reproduction and cell division used by all prokaryotes. This process results in the reproduction of a living prokaryotic cell by division into two parts both of which have the potential to grow to the size of the original cell as shown in Fig. 9.

Binary fission begins with DNA replication which opens up into a replication bubble (note: prokaryotic DNA replication usually has only one origin of replication, whereas eukaryotes have multiple origins of replication). The replication bubble separates the DNA double strand, each strand acting as template for synthesis of a daughter strand by semiconservative replication, until the entire prokaryotic DNA is duplicated. After this process, cell growth occurs where each circular DNA strand attaches to the cell membrane. The cell then elongates, causing

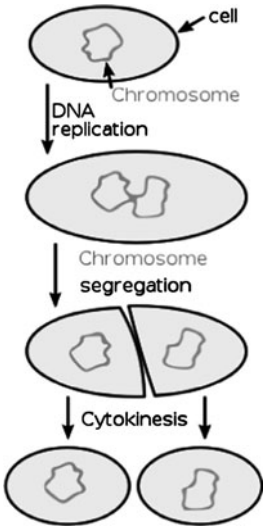

Fig. 9 Process of prokaryotic binary fission (Harry et al. 2006)

the two chromosomes to separate. Cell division in bacteria is controlled by dozen proteins that collect around the site of division known as the FtsZ. There, these proteins direct assembly of the division septum. The cell wall and plasma membrane starts growing transversely from near the middle of the dividing cell and the parent cell separates into two nearly equal daughter cells, each having a nuclear body (Weiss 2004). The cell membrane then invaginates (grows inwards) and splits the cell into two daughter cells, separated by a newly grown cell plate. There are some other forms of asexual reproduction such as budding, vegetative reproduction, sporogenesis, fragmentation, parthenogenesis, agamogenesis, apomixes, and nucellar embryony which does not apply to prokaryotes.

7 The Taxonomy of Prokaryotic Probiotic Bacteria

Taxonomy may be defined as the process of cataloguing biodiversity, as it is the scientific study of the diversity of organisms with the ultimate goal of characterizing and arranging them in an orderly manner (Schleifer and Ludwig 1994). Classification, identification, and nomenclature are the three separate but related subdisciplines of taxonomy. *Classification* is the process of clustering organisms into taxonomic groups (*taxa*) on the basis of similarities or relationships. *Nomenclature* is the assignment of names to the taxonomic groups according to international rules. Finally, *identification* is the process of determining the belonging of a new isolate to one of the established and named taxa (Staley and Krieg 1989). Bacterial taxonomy is driven by the innate human desire to recognize and understand the world around them and this requires a logical ordering of items (Rosselló-Mora 2005).

In the hierarchical way of classification, only two prokaryotic domains are presently recognized, namely, the Archaea and Bacteria. Domains are divided into phyla and the levels below the phylum are classes, orders (or subdivisions, depending on the group), families, genera, and species as shown in Table 1 of chapter "New health potentials of orally-consumed probiotic microorganisms." Different taxonomic levels are characterized by different suffixes in the taxon names. As microorganisms have too simple a structure and too few informative characters compared with higher organisms (e.g., morphology), progress in bacterial taxonomy has always been dependent on advances in technology such as *molecular* data. These data have been made possible with the discovery of DNA as the depositary material of genetic information and the improvement of techniques suitable to deal with the smallest components of cells. However, different methods of analysis have different resolution power, therefore the complete investigation of the identity of a microorganism could be carried out only through the comparison of results of a large number of techniques referred to as the *polyphasic* approach to bacterial systematic. This approach is based on the fact that the more data one has and to compare, the more complete and accurate the identification (Colwell 1970; Vandamme et al. 1996). The definition and naming of

Table 2 An example of hierarchal classification of probiotic bacteria: *Bifidobacterium* and *Lactobacillus*

Taxon	Name	Name
Dormain	Bacteria	Bacteria
Phylum	*Actinobacteria*	*Firmicutes*
Class	*Actinobacteria*	*Bacilli*
Sub-class	*Actinobacteridae*	*Actinobacteridae*
Order	*Bifidobacterales*	*Lactobacillales*
Family	*Bifidobacteriaceae*	*Lactobacillaceae*
Genus	*Bifidobacterium*	*Lactobacillus*/*Paralactobacillus*/*Pediococcus*
Species	*Bifidobacterium animalis*	*Lactobacillus acidophilus*/*casei*

Source: *Taxonomic outline of the prokaryotes* (Garrity et al. 2004)

species is necessary for practical reasons; however, it should be noted that microbiologists work with *strains*, as the strain is the microbial individual. It is possible that properties can be assigned to species when a number of different strains are studied. However, when tests are performed and properties attributed, they are, first of all, characteristics of the strain under study.

Species of the genera *Lactobacillus* and *Bifidobacterium* are some of the most important taxa used in the food industry, feed production, and in human nutrition. They are mostly renowned for the probiotic properties exhibited by some of their strains.

The taxonomic orders for microorganisms are species, genus, family, order, class, phylum, and domain. Except for domain these are rarely used. Table 2 shows the hierarchal taxonomic arrangement and identification of two important and well-studied genera of prokaryotic probiotic, namely, *Bifidobacterium* and *Lactobacillus*. The most significant and well-known prokaryotic probiotic bacteria belong to the two phylums, namely, *Actinobacteria* and *Firmicutes*. Whereas Bifidobacteria, which is the most predominant microorganism in the gastrointestinal tract of humans belong to the phylum *Actinobacteria,* the other known probiotic species such as *L. acidopillus* and *L. casei*, both under the genus *Lactobacillus* belong to the phylum *Firmicutes*. *Acidophilus* is the most well-known probiotic bacteria of which there are many strains that offer health benefits.

7.1 The Genus Lactobacillus

The genus *Lactobacillus* belongs to the LAB, a definition which groups Gram-positive, non-spore-forming, catalase-negative bacterial species able to produce lactic acid as main end-product of the fermentation of carbohydrates. Catalase is a common enzyme found in nearly all living organisms that are exposed to oxygen, where it decomposes hydrogen peroxide to water and oxygen (Chelikani et al. 2004). Catalase has one of the highest turnover numbers of all enzymes, i.e., one molecule of catalase can convert 40 million molecules of hydrogen peroxide to water and oxygen each second (Goodsell 2004).

Morphologically, they present as rod-shaped microorganisms although they can appear circular hence sometimes referred to as coccobacilli. They are fermentative, microaerophylic, and chemo-organotrophic, requiring rich media to grow. Considering DNA base composition of the genome, they usually show a GC content (guanine–cytosine content – which is the percentage of nitrogenous bases on a DNA molecule, from a possibility of four different ones, also including adenine and thymine) of between 32 and 51 mol%. They are almost ubiquitous: they are found in environments where carbohydrates are available, such as food (dairy products, fermented meat, sour doughs, vegetables, fruits, beverages), respiratory, GI and genital tracts of humans and animals, and in sewage and plant material. Based on the *Taxonomic Outline of the Prokaryotes*, given in Table 1 of chapter "New health potentials of orally-consumed probiotic microorganisms," the genus *Lactobacillus* belongs to the phylum *Firmicutes,* class *Bacilli,* order *Lactobacillales*, family *Lactobacillaceae* and its closest relatives, being grouped within the same family, are the genera *Paralactobacillus* and *Pediococcus*. Currently, the genus *Lactobacillus* includes 106 validly described species, making it, the most numerous genus of the order *Lactobacillales*. Moreover, one more species (*Lactobacillus tucceti,* Chenoll et al. 2006) has been described but it has not been validated yet, while few other species have description but are yet to be published including *Lactobacillus composti, Lactobacillus farraginis*, and *Lactobacillus parafarraginis* (Endo and Okada 2007a, b) and *Lactobacillus secaliphilus* (Ehrmann et al. 2007).

Table 3 presents a summary of seven already validated species in the genus *Lactobacillus,* comprising of two subspecies or more. There are other species such as *L. salivarius* which have not been substantially separated in two subspecies, therefore leading to the amendment of the species description (Li et al. 2006).

Table 3 Summary of the genus *Lactobacillus* and its species and subspecies

Genus	Species	Subspecies
Lactobacillus	*Lactobacillus aviarius*	*L. aviaries* subsp. *aviarius*
		L. aviarius subsp. *araffinosus*
	Lactobacillus coryniformis	*L. coryniformis* subsp. *coryniformis*
		L. coryniformis subsp. *torquens*
	Lactobacillus delbrueckii	*L. delbrueckii* subsp. *delbrueckii*
		L. delbrueckii subsp. *bulgaricus*
		L. delbrueckii subsp. *indicus*
		L. delbrueckii subsp. *lactis*
	Lactobacillus kefiranofaciens	*L. kefiranofaciens* subsp. *kefiranofaciens*
		L. kefiranofaciens subsp. *kefirgranum*
	Lactobacillus paracasei	*L. paracasei* subsp. *paracasei*
		L. paracasei subsp. *tolerans*
	Lactobacillus plantarum	*L. plantarum* subsp. *plantarum*
		L. plantarum subsp. *argentoratensis*
	Lactobacillus sakei	*L. sakei* subsp. *sakei*
		L. sakei subsp. *carnosus*