Chapter 9
Probiotics

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9.1 Introduction

The human gastrointestinal tract (GIT) is a complex ecosystem and its bacteria inhabitants can achieve very high densities. A delicate balance exists between human intestinal microflora and its host. Upset in this community structure may lead toward the symptoms of acute gastroenteritis, inflammatory bowel disease and colon cancer. It is therefore important to sustain gut microflora in an optimal manner (Gibson and Fuller 2000; Vaughan et al. 2005).

In recent years, the advances in understanding the relationship between human gut microbiota and health have resulted in the development of the concept of probiotics. Probiotics are defined as "live microorganisms, as they are consumed in adequate numbers confer a health benefit on the host" (FAO/WHO 2001). Bacterial strains selected as probiotics are predominantly from the genera *Bifidobacterium* and *Lactobacillus* (Saarela et al. 2000), which form a part of normal human intestinal ecosystem (Backhead et al. 2005) and play a pivotal role in maintenance of healthy human gut (Gomes and Malcata 1999).

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9.2 Morphology and Physiology of Probiotic Bacteria

Bifidobacteria are gram-positive, strictly anaerobic, non-motile, non-spore-forming pleomorphic rods with a particular cell morphology ranging from regular rods to various branched and club-shaped forms (Leahy et al. 2005). Morphologic heterogeneity of cells from developing populations of bifidobacteria correlates with ultrastructure peculiarities (Fig. 9.1).

Active proliferating cells in exponential phase are characterised by formation of intracytoplasmatic membrane complex represented by lamellar, myelinoform, vesicular structures. Nucleoid is localised as the central polybranched or disperse osmophobic zone. Nucleoid distribution is determined by morphogenesis processes—exobudding, branching or multiseptation. Electronograms reveal multiple polyphosphate and polysaccharide inclusions. Ageing of bifidobacterial populations is accompanied with ultrastructural changes: cell wall hypersynthesis, reorganisation and increased size of intracytoplasmatic membrane complex, altered morphology and compactness of nuclei and formation and dissimilation of inclusions (Novik et al. 1994). Populations of *Bifidobacterium*, growing on liquid and agar media, are represented by highly ordered mycelial structures. Their topography depends on mutual arrangement of polymorphic cells and the way of their daughter cells' separation after division. Evidence obtained by scanning electron microscopy (SEM) of total preparations and by transmission electron microscopy (TEM) of ultrathin sections correlate well. The data showed the existence of morphologically varied intercellular contacts that ensure the stability of such microbial consortia during adaptation to ambient conditions (Fig. 9.2).

Intercellular contacts with the aid of different extracellular structures—microfibrillae, knob-like juts, cell wall evaginations and capsule form stuff (glycocalyx)—are the result of genetically determined self-regulating development of microbial populations as multicellular systems (Novik and Vysotskii 1995). Figure 9.3 shows a scheme of morphological transformations of bifidobacterial cells in the developmental cycle of populations. Multiplication occurs via reversion of transitory rod-shaped and coccoid forms into repeatedly budding and dichotomously branching multiseptate filaments, which, under certain conditions, fragment with the formation of differentiated reproductive forms (Novik 1998).

Bifidobacteria are generally described as strictly anaerobic, although some strains can tolerate oxygen. The sensitivity to oxygen, however, can differ between species and between different strains within a species (de Vries and Stouthamer 1969; Talwalkar et al. 2001). Most human isolates of bifidobacteria grow at an optimum temperature of 36–38 °C, whereas animal strains appear to have slightly higher optimum growth temperature of 41–43 °C. The notable exceptions are *Bifidobacterium thermacidophilum* which exhibits a maximal growth temperature of 49.5 °C and *Bifidobacterium psychraerophilum* which has been shown to grow at temperatures as low as 4 °C (Dong et al. 2000; Leahy et al. 2005; Simpson et al. 2004).

Bifidobacteria are acid-tolerant microbes and their optimum pH for growth is between 6.5 and 7.0. Strains of *Bifidobacterium animalis* ssp. *animalis* and

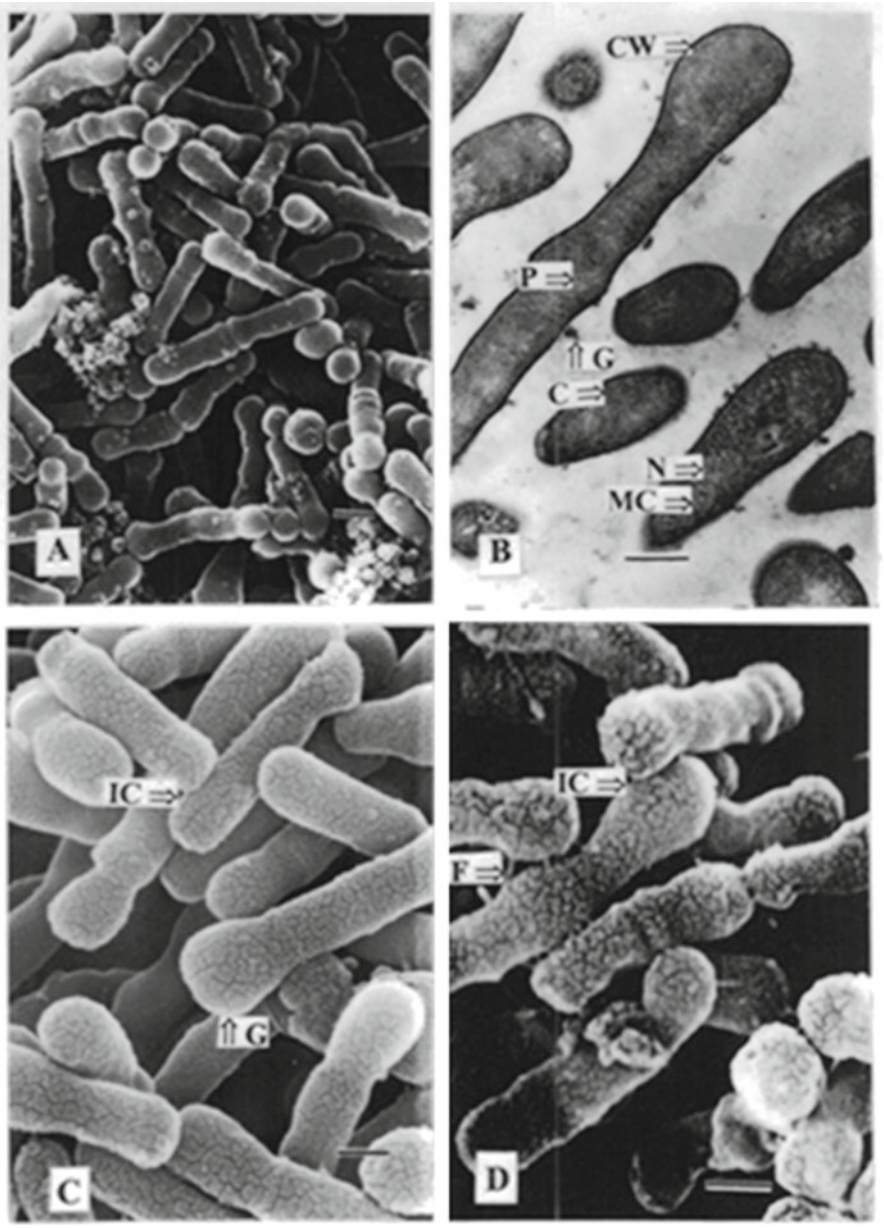

Fig. 9.1 Morphological analysis of bifidobacterial cells by means of electron microscopy; (**a**) rod-shaped forms, having wide ends; (**b**) fine structure of cells; (**c**, **d**) visualisation of intercellular contacts in some cells. *CW* cell wall, *C* cytoplasm, *MC* membrane complex, *N* nucleoid, *P* polyphosphate granules, *IC* intercellular contacts, *F* microfibrillae, *G* capsule (Photo by Galina Novik)

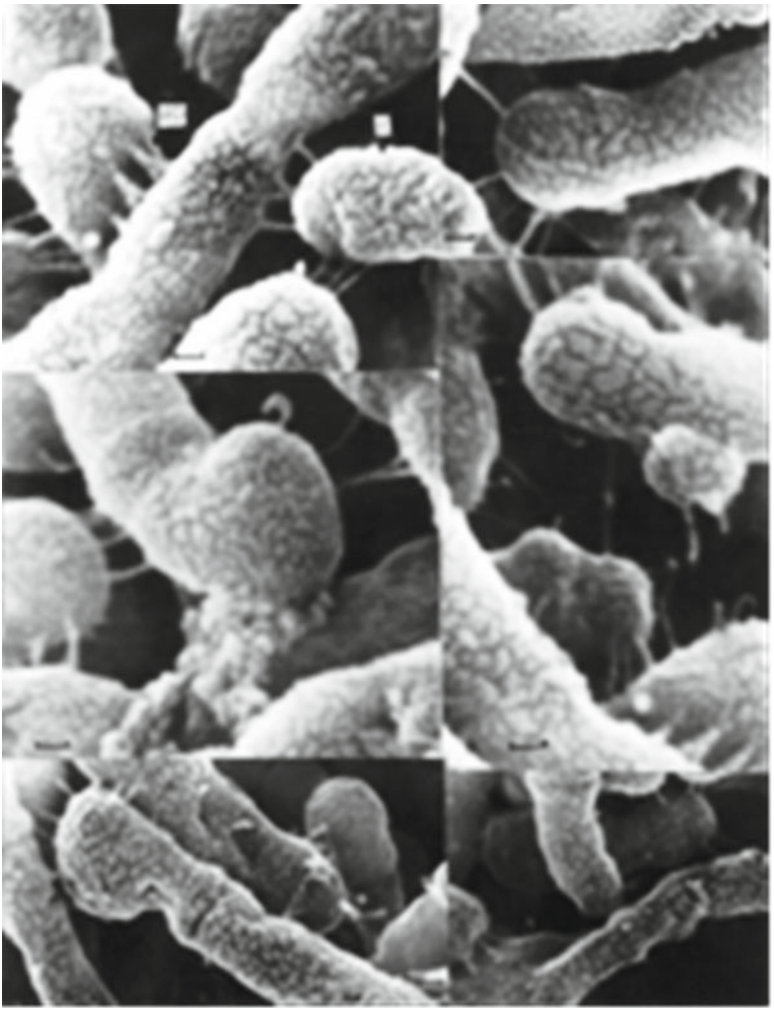

Fig. 9.2 Morphology of bifidobacterial cells by means of electron microscopy with visualisation of intercellular links in some cells (Photo by Galina Novik)

B. animalis ssp. *lactis* can survive exposure to pH 3.5, whereas most of *Bifidobacterium* strains do not survive at pH 8.5 (Leahy et al. 2005).

Bifidobacteria are strictly fermentative bacteria, in which hexose metabolism occurs through a unique fructose-phosphate pathway also called "bifidus shunt" (Biavati and Mattarelli 2006; Leahy et al. 2005). They ferment glucose to lactic acid and acetic acid in molar ratio 2:3 without carbon dioxide. Variations in growth conditions, such as quality and quantity of carbon source, may result in the production of varying amounts of fermentation products. Bifidobacteria possess an array of enzymes that allow them to utilise a great variety of monosaccharides, disaccharides

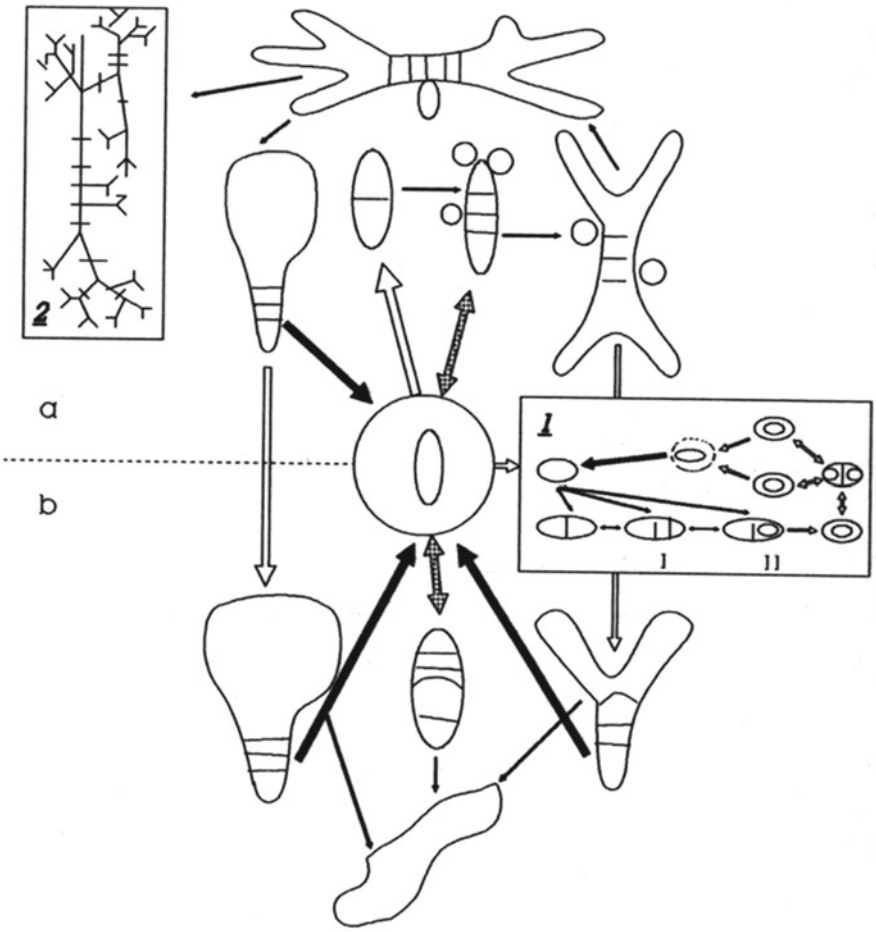

Fig. 9.3 Morphological and structural differentiation of cells in a cycle of development of bifidobacteria populations: (**a**) reproductive forms; (**b**) ageing of bifidobacterial cells; *1*—stage of transitory rod-shaped and coccoid forms; *2*—stage of branching filaments (Scheme by Galina Novik)

and complex carbohydrates as carbon sources. This feature should give bifidobacteria an ecological advantage to colonise the intestinal environment where complex carbohydrates, such as mucin, are present in large quantities either because of production by the host epithelium or introduction through the diet. The selective stimulation of the growth of bifidobacteria by simple or complex carbohydrates is the basis of prebiotic concept (Biavati and Mattarelli 2006).

Lactobacilli are a broad, morphologically defined group of gram-positive, catalase-negative, non-spore-forming bacteria. They usually occur as rods that may differ in length between the various species. Some species grow as coccobacilli or appear curved or coryneform. Some heterofermentative lactobacilli may appear

coccoid and can be confused with leuconostocs. Some homofermentative anaerobic lactobacilli from intestinal sources may resemble morphologically certain bifidobacteria. Morphological variations may occur within some *Lactobacillus* species depending on growth conditions (Hammes and Hertel 2003).

Lactobacilli are strictly fermentative, aerotolerant or anaerobic, aciduric or acidophilic bacteria (Kandler and Weiss 1986). Based on the type of fermentation, *Lactobacillus* species are divided into three groups: homofermentative, facultatively heterofermentative and obligately heterofermentative. Homofermentative lactobacilli are able to ferment glucose almost exclusively to lactic acid via the Embden-Meyerhof-Parnas (EMP) pathway, while pentoses and gluconate are not fermented as they lack phosphoketolase. Facultatively heterofermentative lactobacilli degrade hexose to lactic acid by the EMP pathway and are also able to degrade pentoses and often gluconate as they possess both aldolase and phosphoketolase. Obligately heterofermentative lactobacilli degrade hexoses by the phosphogluconate pathway producing lactate, ethanol, acetic acid and carbon dioxide; moreover, pentoses are also fermented through this pathway (Hammes and Vogel 1995; Pot et al. 1994).

Generally, lactobacilli grow well at temperatures above 20 °C and below 42 °C, though some strains of these microorganisms can grow at up to 44 °C and down to 15 °C (Hammes and Hertel 2003; Savoie et al. 2007). Lactobacilli are mostly microaerophilic, but many strains of these microorganisms grow better either anaerobically or in the presence of increased CO_2 tension, particularly on first isolation (Hammes and Hertel 2003).

Lactobacilli have complex nutrient requirements—they grow in the presence of carbohydrates, amino acids, peptides, nucleic acid derivatives and vitamins. Lactobacilli grow in a variety of habitats, wherever high levels of soluble carbohydrate, protein breakdown products, vitamins and a low oxygen tension occur. Large amounts of lactic acid and small amounts of other compounds are the products of their carbohydrate metabolism, which lowers the pH of the substrate and suppresses the growth of many other bacteria (Kandler and Weiss 1986).

9.3 Taxonomy of Probiotic Bacteria

According to current taxonomy, bifidobacteria belong to the phylum *Actinobacteria*, class *Actinobacteria*, order *Bifidobacteriales*, family *Bifidobacteriaceae*, genus *Bifidobacterium* and are represented by over 32 species (Biavati and Mattarelli 2006; Holzapfel et al. 2001; Leahy et al. 2005) with type species *Bifidobacterium bifidum*. In the phylogenetic tree of bacteria, *Bifidobacterium* cluster is in the subdivision of high G+C gram-positive bacteria together with other genera such as *Propionibacterium*, *Actinomyces* and *Streptomyces*, so they form a part of the so-called *Actinomycetes* branch (Leahy et al. 2005). The species belonging to the genus *Bifidobacterium* form a coherent phylogenetic unit and show generally over 93 % similarity of 16S rRNA sequences with other members of the genus. A number of phylogenetic studies carried out during the past decade, mainly based on

sequencing of 16S rRNA gene and housekeeping genes, have grouped the bifidobacterial species in six groups, namely, *B. boum* group, *B. asteroids* group, *B. adolescentis* group, *B. pullorum* group, *B. longum* group and *B. pseudolongum* group (Matsuki et al. 2003; Sakata et al. 2006; Ventura et al. 2004; 2006).

All the currently known *Bifidobacterium* species were isolated from limited number of habitats, including human and animal gut, insect intestine, food, sewage and breast milk (Felis and Dellaglio 2007; Ventura et al. 2004). Strains which are the most typical for human GIT belong to species *B. catenulatum*, *B. pseudocatenulatum*, *B. adolescentis*, *B. longum*, *B. breve*, *B. angulatum* and *B. bifidum*, and the most frequent species isolated from dairy products is *B. animalis* ssp. *lactis* (Masco et al. 2005). Therefore these species are the most widely used probiotics (Biavati et al. 2001).

The genus *Lactobacillus* belongs to the phylum *Firmicutes* (gram-positive bacteria with low G+C content), class *Bacilli*, order *Lactobacillales*, family *Lactobacillaceae*, and its closest relatives are the genera *Paralactobacillus* and *Pediococcus*, being grouped within the same family (Felis and Dellaglio 2007). Lactobacilli form the largest group among the lactic acid bacteria (LAB), containing at present more than 120 species; the type species is *Lactobacillus delbrueckii* (Felis and Dellaglio 2007; Vaughan et al. 2005).

Phylogenetic structure of the genus *Lactobacillus* is quite complicated. According to the results of the first phylogenetic analysis of lactobacilli, they were divided into three groups, *L. delbrueckii* group, *L. casei–Pediococcus* group and *Leuconostoc* group, which also contained some lactobacilli (Felis and Dellaglio 2007). In 1995 *L. delbrueckii* group was given the name of *L. acidophilus* group and the *L. casei–Pediococcus* group was split into further four subclusters (Schleifer and Ludwig 1995). Recently, due to the description of a large number of species and the following re-examination of the genus, this strategy of grouping was updated. Nowadays, genus *Lactobacillus* includes *L. delbrueckii* group, *L. salivarius* group, *L. reuteri* group, *L. buchneri* group, *L. alimentarius–L. farciminis* group, *L. casei* group, *L. sakei* group, *L. fructivorans* group, *L. coryniformis* group, *L. plantarum* group, *L. perolens* group, *L. brevis* group, *Pediococcus dextrinicus* group, *Pediococcus* group, couples (e.g. *L. rossiae–L. siliginis*) and single species (*L. kunkeei*, *L. malefermentans*, *L. pantheris*, etc.) (Felis and Dellaglio 2007; Hammes and Hertel 2003).

The dominant species isolated from human gut are those belonging to the *L. casei* group (*L. casei, L. paracasei, L. rhamnosus, L. zeae*); obligatory homofermentative species *L. gasseri, L. crispatus and L. johnsonii*; and heterofermentative species *L. reuteri* (Dunne et al. 1999; Morelli et al. 1998; Song et al. 2000; Tannock et al. 2000).

9.4 Identification of Probiotic Bacteria

The correct identification of probiotic bacteria is the first prerequisite of their microbiological safety. Thus, the use of adequate tools to provide proper strain identification is strictly necessary (FAO/WHO 2002; Saarela et al. 2000).

Traditionally, bifidobacteria have been identified on the basis of phenotype characteristics. Cell morphology, determination of metabolites, enzyme activities and ability to ferment sugars are used for routine bifidobacteria identification. Genus *Bifidobacterium* can be distinguished from other bacterial groups such as lactobacilli, actinomycetes and anaerobic corynebacteria by the peculiar metabolic pathway of glucose fermentation, the bifidus shunt, the key enzyme of which is fructose-6-phosphate phosphoketolase (F6PPK). This enzyme was considered a taxonomic marker for identification on the genus level (Tannock 1999; Vlkova et al. 2002), but, due to the reclassification of *Bifidobacterium* species into new genera, it can be used as taxonomic character of the family *Bifidobacteriacea* (Felis and Dellaglio 2007). Since fermentation of glucose by the bifidus pathway produces acetic and lactic acids in a theoretical ratio of 3:2, gas liquid chromatography of fermentation products provides another means of differentiating bifidobacteria from other bacterial types (Tannock 1999).

Currently, biochemical tests for the identification of members of the genus *Bifidobacterium* are largely superseded by the use of the genus-specific PCR primers, which amplify 523 bp or 1.35 kbp regions of the 16S rRNA gene. Genus-specific probes have proved useful in the detection and identification of bifidobacteria in faecal and food samples (Kaufmann et al. 1997; Kok et al. 1996).

With regard to *Bifidobacterium* species identification, they can sometimes be differentiated using the results of fermentation tests together with the electrophoretic mobilities of enzymes such as transaldolase (14 types) or 6-phosphogluconate dehydrogenase (19 types) (Tannock 1999). The most important species may be distinguished to some degree by the fermentation of L-arabinose, D-xylose, D-mannose, salicin, D-mannitol, D-sorbitol and D-mellesitose (Holzapfel et al. 2001). In many cases, phenotypic characterisation is not enough to identify *Bifidobacterium* strains at the species level. So, genotypic approaches hold the most promise for the rapid and accurate identification of bifidobacteria (Gomes and Malcata 1999; O'Sullivan 2000; Satokari et al. 2003).

DNA–DNA reassociation studies have been widely used in the taxonomy of bifidobacteria and currently it is the most reliable method for *Bifidobacterium* species identification (O'Sullivan 2000; Satokari et al. 2003). Species identification by 16S rRNA gene sequence analysis is hampered by the high level of sequence relatedness between closely related bifidobacterial species. Comparison of 16S rRNA gene sequences from 18 species of *Bifidobacterium* showed that they ranged in similarity from 92 to 99 %. This high level of relatedness makes it impossible to differentiate between some species on the basis of 16S rDNA sequence analysis (Leblond-Bourget et al. 1996; McCartney et al. 1996). However, in many cases subtle differences in the 16S rRNA gene sequences have been successfully utilised to design species-specific probes or PCR primers that can be applied in species identification (Langendijk et al. 1995; Matsuki et al. 1999; 2003; Welling et al. 1997; Yamamoto et al. 1992).

The sequence analysis of conserved genes other than 16S rRNA such as *recA*, major enzyme involved in recombination, and *ldh*, coding for L-lactate dehydrogenase, has been proposed as a method for identification of closely related bifidobacteria

(Kullen et al. 1997; Roy and Sirois 2000). Currently, the gene sequence of *hsp60*, heat-shock protein of 60 kDa, is preferentially used to distinguish between different species of *Bifidobacterium* (Jian et al. 1991). Method based on PCR targeting the transaldolase gene and subsequent separation of the amplicons by denaturing gradient gel electrophoresis (DGGE) was developed for the identification of *Bifidobacterium* species (Requena et al. 2002). Multilocus sequencing—sequencing of 16S rRNA and housekeeping genes, such as *tuf, recA, xfp, atpD, groEL, groES, dnaK, hsp60, dnaB* and *dnaJ*—is the highly discriminatory method for bifidobacteria identification, providing unambiguous results (Ventura et al. 2006).

Methods based on the PCR are widely used to differentiate species and even strains of bifidobacteria. Randomly amplified polymorphic DNA (RAPD) profiling is successfully applied to distinguish between strains of *Bifidobacterium* (Määttö et al. 2004; Vincent et al. 1998). Different modifications of repetitive extragenic palindromic PCR (REP-PCR), such as enterobacterial repetitive intergenic consensus sequence PCR (ERIC-PCR), BOX-PCR $(GTG)_5$-PCR, can be considered as promising methods for the identification of bifidobacteria at species, subspecies and even strain level (Krizova et al. 2008; Masco et al. 2003, 2004; Šrůtkova et al. 2011; Ventura et al. 2003). Pulsed field gel electrophoresis (PFGE) protocols have been established for bifidobacteria and the techniques have shown superior discriminatory power in comparison to other typing methods in species and strain differentiation (O'Riordan and Fitzgerald 1997; Roy et al. 1996). A complete survey of methods for bifidobacteria identification has been compiled in reviews (Sidarenka et al. 2008; Ward and Roy 2005).

Members of the genus *Lactobacillus* for a long time were identified on the base of their phenotypic features, including cell morphology, fermentation of carbohydrates, growth at different temperatures and salt concentrations. However, it has been widely recognised that *Lactobacillus* species and strains display a high level of phenotypic variability, making classical microbiological methods of identification unreliable (Hammes and Hertel 2003). Recently, it was demonstrated that the API 40 identification system failed to identify 7 reference strains and 86 freshly isolated *Lactobacillus* strains (Boyd et al. 2005).

Comparative analysis of complete or at least sufficiently informative part (approximately the first 900 bases) of the 16S rRNA gene can be used for the reliable identification of *Lactobacillus* species (Mori et al. 1997; Tannock 1999). It should be noted, however, that in some cases the 16S rRNA gene may be too well conserved to reliably identify closely related species, such as *L. plantarum*, *L. pentosus* and *L. paraplantarum* (99.7–99.9 %); *L. kimchii* and *L. paralimentarius* (99.9 %); and *L. mindensis* and *L. farciminis* (99.9 %) (Fox et al. 1992). Analysis of 16S–23S rRNA spacer region sequences reveals that this region is less conserved compared to 16S rRNA gene and can be considered as powerful tool for genus and species differentiation of lactobacilli (Tannock et al. 1999). Based on the nucleotide sequences of 16S rRNA gene and 16S–23S spacer region, species-specific primers for lactobacilli identification have been derived. Currently, specific primers are available for most *Lactobacillus* species (Berthier and Ehrlich 1998; Kwon et al. 2004; Settanni et al. 2005). Nucleotide differences in 16S rRNA gene can also be

used for the separation by denaturing gradient gel electrophoresis (DGGE) or temporal temperature gradient electrophoresis (TTGE), which are promising tools for the identification of lactobacilli at strain level (Fasoli et al. 2003; Vasquez et al. 2001).

Genes *recA, groES* and *groEL*, coding for highly conserved proteins, are also utilised to identify lactobacilli species (Felis et al. 2001; Torriani et al. 2001; Walker et al. 1999), providing phylogenetic resolution comparable to that of 16S rRNA gene at all taxonomic levels. Comparative analysis of fructose-1,6-biphosphatase (*fbp*) gene has been successfully used for identifying food, newborn and clinical strains of *L. rhamnosus* (Roy and Ward 2004). The powerful multilocus sequencing technique based on the analysis of six genes (*ddl, gyrB, purK1, gdh, mutS, pgm*) has been applied for analysis of *L. plantarum* strains (De las Rivas et al. 2006). Recently, multilocus sequencing variant called multilocus variable-number tandem repeat analysis has been developed for subtyping of *L. casei/L. paracasei* strains (Diancourt et al. 2007).

Many PCR-based typing methods are used for identification of lactobacilli at strain level, including RAPD-PCR (Khaled et al. 1997; Du Plessis and Dicks 1995; Nigatu et al. 2001; Schillinger et al. 2003), REP-PCR (Gevers et al. 2001; Ventura and Zink 2002), PFGE (Tynkkynen et al. 1999; Ventura and Zink 2002; Weiss et al. 2005). Molecular approaches available for *Lactobacillus* identification are described in reviews (Mohania et al. 2008; Singha et al. 2009).

9.5 Criteria for Selection of Probiotic Bacteria

Different in vitro and in vivo approaches have been used to select potentially probiotic strains of bifidobacteria and lactobacilli, as well as to measure their efficacy (Gibson and Fuller 2000). Criteria for the selection of probiotic bacteria have been defined in several reviews (Adams 1999; Bhadoria and Mahapatra 2011; Gibson and Fuller 2000; Saarela et al. 2000; Salminen et al. 1998). They indicate that many aspects, including safety and functional and technological characteristics, have to be taken into consideration in the selection process of probiotic microorganisms.

9.5.1 Safety of Probiotic Bacteria

The safety of probiotic strains is of prime importance. Although vigorous debates continue on what constitutes appropriate safety testing for novel probiotic strains proposed for human use, it generally includes such characteristics as origin, non-pathogenicity and antibiotic-resistance characteristics.

Strains for human use are preferably of human origin, isolated from healthy GIT (Saarela et al. 2000). Probiotic bacteria must be non-pathogenic, with no history of association with diseases such as infective endocarditis or gastrointestinal

disorders. Knowledge on survival of the probiotics within the GIT, their translocation and colonisation properties, is also important for the evaluation of possible positive or negative effect of probiotic consumption (Marteau et al. 1995). From this point of view, lactic acid bacteria and bifidobacteria are widely used in fermented food and dairy products with no case of local or systemic infections occurred, which confirms their GRAS ("generally regarded as safe") status (Sleator 2010). Many findings indicate that the general human population is not at risk from exposure to probiotic bacteria of *Bifidobacterium* and *Lactobacillus* genera. Although the rare cases of infection associated with probiotics have occurred in groups of people whose conditions predispose them to opportunistic infections, in many cases people with serious underlying diseases have benefited from probiotics (Benchimol and Mack 2005; Reid 2006; von Wright 2005).

Another aspect of safety consideration is antibiotic resistance of probiotic bacteria strains. The resistance of bacteria to antibiotics is an increasingly important public health problem worldwide. There is a pressing need to limit the spread of resistance genes, since these could be transferred to opportunistic and pathogenic bacteria (Ammor et al. 2008; Blazquez et al. 2002). Antibiotic resistance could be "intrinsic" and "acquired." Intrinsic resistance is inherent to bacteria species and involves the absence of the target, presence of low-affinity target, low cell permeability or presence of efflux mechanisms. The acquisition of antibiotic resistance occurs through the mutation of pre-existing genes or by horizontal transmission, i.e. acquisition of foreign DNA from other bacteria. Therefore attention is currently being paid to probiotic LAB and bifidobacteria with respect to their potential role in the spread and transmission of antibiotic-resistance determinants (Ammor et al. 2008; Saarela et al. 2000).

Most bifidobacteria are intrinsically resistant to nalidixic acid, neomycin, polymyxin B, kanamycin, gentamicin, streptomycin and metronidazole (Charteris et al. 1998). Their resistance to other antibiotics differs depending on strain and in some cases may be due to the presence of genetic determinants. Indeed, microarray analysis revealed presence of *tet*(*W*) genes in *B. longum* and *B. bifidum* strains, as well as *aph*(*E*) and/or *sat*(*3*) genes in *B. bifidum*, *B. longum*, *B. catenulatum* and *B. pseudocatenulatum* strains (Ammor et al. 2008). Screening of 26 *B. animalis* subsp. *lactis* strains isolated from different sources revealed the presence of *tet*(W) in all isolates. Moreover, in all strains a transposase gene upstream of *tet*(W) gene was detected, which is cotranscribed in tandem. Transposases have been found to be involved in the horizontal gene transfer of genetic elements among bacteria, but to date there is no evidence that *tet*(W) in *B. animalis* subsp. *lactis* is transmissible (Gueimonde et al. 2010). Presence of the resistance determinant *erm*(*X*) was demonstrated in six erythromycin- and clindamycin-resistant *B. thermophilum* strains during investigation of a large collection of bifidobacteria that could be potential probiotics (Mayrhofer et al. 2007). Analysis of additional bifidobacteria revealed that this antibiotic-resistance gene was also present in *B. animalis* subsp. *lactis* strains (Määttö et al. 2007). It was demonstrated that the *erm*(*X*) gene from erythromycin-resistant *Bifidobacterium* strains was part of transposon Tn5432 and was nearly identical to *erm*(*X*) determinants present in several opportunistic pathogenic

corynebacteria and propionibacteria (van Hoek et al. 2008). Although most of the antibiotic-resistance genes were located on bacterial chromosome, studies on the genetics of antibiotic resistance of bifidobacteria are guarantee their safe application.

Lactobacilli display a wide range of antibiotic resistance, and antibiotic susceptibility patterns vary greatly between different species of these microorganisms (Charteris et al. 1998). Thus, *L. delbrueckii* strains as components of yogurt cultures showed intrinsic resistance toward mycostatin, nalidixic acid, neomycin, polymyxin B, trimethoprim, colimycin and sulphonamides. Susceptibility to cloxacillin, dihydrostreptomycin, doxycycline, novobiocin, oleandomycin, oxacillin and streptomycin was prominent while resistance to kanamycin and streptomycin varied. Many lactobacilli carry intrinsic resistance toward vancomycin (Marthur and Singh 2005). In most cases antibiotic resistance of lactobacilli is not of the transmissible type (Saarela et al. 2000), and such strains do not usually form a safety concern. Although plasmid-linked antibiotic resistance is not very common among lactobacilli, they do occur (Rinckel and Savage 1990). R-plasmids encoding tetracycline, erythromycin, chloramphenicol or macrolide–lincomycin–streptomycin resistance have been reported in *L. reuteri*, *L. fermentum*, *L. acidophilus* and *L. plantarum*, isolated from raw meat, silage and faeces. Most of these R-plasmids had a size smaller than 10 kb (Marthur and Singh 2005). The presence of 5.7 kb plasmid carrying *erm* gene conferring high-level erythromycin resistance was demonstrated in *L. fermentum* isolated from pig faeces (Fons et al. 1997). Plasmid-encoding tetracycline-resistance gene *tet*(*M*) was detected in *Lactobacillus* isolates from fermented dry sausages (Gevers et al. 2002). The 10,877 bp tetracycline-resistance plasmid pMD5057 from *L. plantarum* 5057 was completely sequenced and the sequence revealed that tetracycline-resistant region contains a *tet*(*M*) gene with high homology to sequences of this gene from *Clostridium perfringens* and *Staphylococcus aureus* (Danielsen 2002). Since transfer of antibiotic-resistance genes may occur between phylogenetically distant bacteria, *Lactobacillus* strains that harbour mobile elements carrying resistance genes should not be used either as human or animal probiotics (Saarela et al. 2000).

9.5.2 Technological Properties of Probiotic Bacteria

Potential probiotic strains of *Lactobacillus* and *Bifidobacterium* should fulfil many technological criteria, such as simple large-scale production of a viable culture concentrate, survival during preparation and storage of the career of the food and survival in the intestinal ecosystem of the host (Bhadoria and Mahapatra 2011).

Bifidobacteria are fastidious and noncompetitive organisms. They are very sensitive to environmental parameters and require expensive media for propagation, as well as the addition of growth-promoting factors due to their stringent growth

requirements. Growth of bifidobacteria in milk is often slow or limited compared with lactic acid bacteria, and this appears to be partially due to low proteolytic activities. Bifidobacteria generally have a low survival rate in common processes used to prepare microbial food adjuncts such as freeze-drying or spray-drying. Survival of most bifidobacteria is also low in many dairy products due to acidic pH and exposure to oxygen (Roy 2005). Furthermore, bifidobacteria require strict anaerobiosis in the early phase of growth and long fermentation times due to their weak growth and acid production (de Vuyst 2000). Finally, strains of *Bifidobacterium* differ greatly in their survival in the gastrointestinal tract and in their ability to adhere to epithelial cells (Doleyres and Lacroix 2005). The adhesion of bifidobacteria might be strain specific and depends on the surface properties of bacterial cells (Canzi et al. 2005). The probiotic *Bifidobacterium* species most commonly used in food is *B. animalis* ssp. *lactis*. This species is significantly more robust than human intestinal species *B. longum, B. bifidum, B. breve* and *B. adolescentis* also utilised in probiotics and food (Biavati et al. 2001; Crittenden 2004; Roy 2005).

Lactobacilli are more technologically suitable than bifidobacteria. Lactobacilli can utilise a wide range of carbon substrates, with differences in the carbon substrate profiles occurring between species and strains. They are able to grow and survive in fermented milk and yogurts with pH values between 3.7 and 4.3. Lactobacilli are mostly microaerophilic; thus oxygen levels are rarely an important consideration in maintaining the survival of lactobacilli during manufacturing and storage of probiotics and food products. Lactobacilli are less sensitive than bifidobacteria to acidic conditions of stomach and high concentrations of bile in gut, although this property seems to be strain specific (Hammes and Hertel 2003). There is a wide range of *Lactobacillus* species technologically suitable for application in probiotics and foods. Common examples include *L. acidophilus, L. johnsonii, L. rhamnosus, L. casei, L. paracasei, L. fermentum, L. reuterii* and *L. plantarum* (Bergamini et al. 2005; Gokavi et al. 2005; Phillips et al. 2006; Sameshima et al. 1998).

It is generally believed that probiotics must endure a harsh transit through the intestinal tract with different conditions depending on the location, which affect their viability. Different in vitro and in vivo studies have been performed to determine survival of probiotic lactobacilli and bifidobacteria during GIT transit. In one such study, two strains *Bifidobacterium* sp. were exposed to model stomach conditions for 90 min. A notable 4 log unit decrease of viability was observed for one strain, whereas viability of the another one decreased by only 0.5 log units (Berrada et al. 1991). In another study, 6 *Lactobacillus* and 9 *Bifidobacterium* strains were maintained at pH 1.5–3.0 for 3 h and demonstrated different survival ability depending on the pH, the duration of exposure to acid, and the species and strains used (Pochart et al. 1992).

It is important for probiotic strains to show antagonism against pathogenic and opportunistic microorganisms via antimicrobial substance production and competitive exclusion. Therefore, enormous research efforts have been focused on bacteriocin production. Although probiotic strains of *Lactobacillus* and *Bifidobacterium* may produce bacteriocins, their role in pathogen inhibition in vivo could be very

limited, since traditionally bacteriocins have an inhibitory effect only against closely related species. Low molecular weight metabolites, such as hydrogen peroxide, lactic and acetic acid and secondary metabolites, may be more important since they show wide inhibitory spectrum against many harmful organisms like *Salmonella, Escherichia, Clostridium* and *Helicobacter* (Saarela et al. 2000). Generally, probiotic strains of bifidobacteria demonstrate inhibition of a wide range of pathogenic and opportunistic bacteria, including *Escherichia coli, Klebsiella ozaenae, Listeria monocytogenes, Staphylococcus aureus, Salmonella enteritidis, Enterococcus faecalis, Pseudomonas aeruginosa* and *Gardnerella vaginalis* (Korshunov et al. 1999; Biavati et al. 2001; Bevilacqua et al. 2003; Zinedine and Faid 2007; Vanegas et al. 2010). Strains of *Lactobacillus*, primarily due to the production of organic acids, ethanol, H_2O_2 and bacteriocin-like substances, inhibit growth of certain enteropathogens such as *Salmonella, Listeria, Escherichia, Campylobacter,* as well as *Clostridium difficile* and *Helicobacter pylori*, without interfering with the normal microbiota of the gastrointestinal tract (Chen et al. 2010; Coconnier et al. 2000; Fernandez et al. 2003; Naaber et al. 2004).

9.5.3 Enhancing Stress Resistance of Probiotic Bacteria

Most of currently used *Bifidobacterium* and *Lactobacillus* probiotic strains are fastidious organisms, nutritionally demanding and sensitive to environmental conditions. Therefore, product manufacturing and storage reduce viability of probiotic bacteria, causing an economic burden for manufacturers and compromising the efficacy of probiotic products. The intrinsic stress tolerance of the *Bifidobacterium* and *Lactobacillus* strains seems to be a crucial factor in the overall resistance to manufacture and storage of probiotic products, and enhancing of this property is of great importance. Different strategies are applied to enhance resistance of probiotic *Bifidobacterium* and *Lactobacillus* strains to environmental stresses, including selection of naturally resistant strains, stress adaptation and genetic modification of the strains. The two former approaches used already existing diversity and genetic potential, and the latter one implies genetic manipulations leading to genetically modified organism (Gueimonde and Sanchez 2012).

Different strains of bifidobacteria and lactobacilli demonstrate large differences in their ability to survive different manufacturing and storage conditions. Therefore, the initial screening and selection of the most stress-resistant *Bifidobacterium* and *Lactobacillus* strains is considered the primary target for enhancing their stability in probiotics. In this regard, exopolysaccharide-producing strains may show better stress tolerance and, therefore, could be initially selected (Gueimonde and Sanchez 2012; Stack et al. 2010).

Probiotic strains can be adapted to better tolerate stressful conditions. Three main approaches have been used for this aim: stress pretreatment, mutagenesis and selective pressure. While the first one is limited to physiological changes, the last two usually involved changes in genetic content of the strain.

Stress pretreatment includes the subjecting of strains to the sublethal stress before exposing them to the harsh conditions affecting the viability of microorganisms during product manufacture and storage. Random mutagenesis induced by UV light and chemicals has been successfully used for increasing the stability of *B. animalis* ssp. *animalis* in low pH products (Saarela et al. 2011) and to obtain strains of bifidobacteria producing low amounts of acetic acid (Sánchez and Margolles 2012). Stress-resistant strains of probiotic bacteria can be obtained by exposing sensitive strains to a selective pressure of stress factor. Usually, such derivatives present stable phenotype and cross-resistance to other stresses, which is advantageous in terms of stability in industrial process. This approach has been applied to obtain both *Bifidobacterium* and *Lactobacillus* strains with improved acid, bile, heat and oxygen tolerance (Collado and Sanz 2006; Li et al. 2010; Noriega et al. 2005; Park et al. 1995; Ruas-Madiedo et al. 2005).

An alternative for increasing stability of probiotic bacteria is genetic engineering. However, genetically modified microorganisms are not well accepted by consumers and this strategy has not found wide application (Sánchez and Margolles 2012).

Different approaches used to enhance stability of probiotic bacteria are the subject of several recent reviews (Betoret et al. 2011; Sánchez and Margolles 2012; Sánchez et al. 2012).

9.6 Mechanisms of Probiotic Bacteria Positive Action

The beneficial effects of probiotics may be classified in three modes of action. Modulation of host's defences including integrity of the epithelial border and immunomodulation is most important for the prevention and therapy of infectious diseases, treatment of chronic inflammation of the digestive tract, eradication of neoplastic host cells and treatment of non-intestinal autoimmune disorders. Direct effect of probiotics on other microorganisms, commensal and/or pathogenic ones, is important for the prevention and therapy of infections and restoration of the microbial equilibrium in the gut. Finally, probiotic effects may be based on detoxification of microbial products' host metabolites (e.g. bile salts) and food components in the gut.

In general, the list of health claims made for probiotics is much longer than the list of probiotic effects, for which clinical evidence is available. According to the known review (De Vrese and Schrezenmeir 2008), well-established probiotic effects are as follows: (a) prevention and/or reduction of rotavirus-induced or antibiotic-associated diarrhea as well as alleviation of complaints due to lactose intolerance, (b) beneficial effects on inflammatory diseases of the gastrointestinal tract, (c) normalisation of passing stool and stool consistency in subjects suffering from obstipation or an irritable colon, (d) prevention and alleviation of unspecific and irregular complaints of the gastrointestinal tracts in healthy people, (e) reduction of the concentration of cancer-promoting enzymes and/or putrefactive (bacterial) metabolites

in the gut, (f) prevention or alleviation of allergies and atopic diseases in infants and (g) prevention of respiratory tract infections (common cold, influenza) and other infectious diseases as well as treatment of urogenital infections. The preliminary evidence exists with respect to cancer prevention, a so-called hypocholesterolemic effect, improvement of the mouth flora and caries prevention, prevention or therapy of ischemic heart diseases and amelioration of autoimmune diseases (De Vrese and Schrezenmeir 2008). The molecular processes underlying host–microbe interactions in general and probiotic effects in particular are far from clarifying. Gaining insight into the mechanisms of probiotic action could not only help to improve the credibility of the probiotic concept but also to develop tailor-made strategies for the prevention or treatment of various diseases.

The main constituents of "human part" of a complex ecosystem that include intestinal mucosa, gut-associated immune tissue and resident microbiota are listed briefly as targets of probiotic action. The mucosal surface of the intestinal tract is the largest body surface in contact with the external environment (200–300 m^2). The host is protected from attack by potentially harmful enteric microorganisms by the physical and chemical barriers created by the intestinal epithelium. The intestinal epithelium is composed of four epithelial cell lineages, including the enterocytes, enteroendocrine, goblet and Paneth cells. In addition, M cells that sample bacteria and present them to gut-associated immune tissue are in lymphoid follicle-associated epithelium.

The intestinal epithelium is covered by mucus layer; thickness of the layer is relatively small in the small intestine and gradually increases from the colon to the rectum (Atuma et al. 2001; Matsuo et al. 1997). Intestinal mucus layer secreted by goblet cells consists mainly of compact mesh-like network of viscous, permeable, gel-forming secreted MUC2 mucin, which associates with the secreted mucins (MUC 1, MUC 3A, MUC 3B, etc.) by both covalent and noncovalent bonds. Mucin-type molecules consist of a core protein moiety (apomucin) attached to carbohydrate chains by glycoside bonds. O-linked and N-linked oligosaccharides form up to 80 % of the molecule, and the lengths of the carbohydrate side chains range from 1 to more than 20 residues (Seregni et al. 1997). Main functions of mucins (and especially their oligosaccharide chains) are effect of stoichiometric power that excludes larger molecules and microorganisms, hygroscopic effect that influences the degree of hydration at the epithelial cell surface, ion exchange effect and effect of an area that contains bioactive molecules that are listed below. Additionally, mucin type oligosaccharides provide binding sites for lectins, selectins and adhesion molecules.

Besides mucins, intestinal mucus layer contains other goblet-cell products including trefoil peptides, resistin-like molecule β (Th2 cytokine immune effector molecule, an inhibitor of hemotaxis of parasites and regulator of Muc2 transcription and secretion) and Fc-γ binding protein (substance that binds IgG antibodies and stabilises the mucin network through covalent attachment to MUC2). Other components of mucus layer are Paneth cells products including antimicrobial peptides β-defensins (Ayabe et al. 2000; Bevins 2004), two from six known β-defensins, actually HD-5 and HD-6 (Cunliffe 2003), cathelicidins (Zanetti 2004) and antimicrobial molecules, such as lysozyme. Additionally, mucus layer contains immune

molecules synthesised by gut-associated immune cells and enterocytes (secretory IgA, growth factors, cytokines and chemokines). Thus, intestinal epithelium covered by mucus layer together with resident microbiota provides the front line of defence against pathogenic microorganisms.

Note that the role of mucus layer is controversial because it plays a generally accepted role in cytoprotection (Van Klinken et al. 1995) and simultaneously offers ecological advantages for bacterial growth of both the indigenous enteric microbiota (Lupp and Finlay 2005) and the pathogens that adhere to the mucus (Helander et al. 1997; Lillehoj et al. 2001; Rajkumar et al. 1998; Vimal et al. 2000) through providing of energy source and numerous attachment sites. Consistent with this, bacteria associate with the outer layer of mucus and interact with the diverse oligosaccharides of mucin glycoproteins, whereas an "inner" adherent mucus layer is largely devoid of bacteria.

It is considered that resident intestinal bacteria are able to inhibit the adherence of pathogenic bacteria to intestinal epithelial cells as a result of (a) their ability to increase the production of intestinal mucins and antimicrobial substances and (b) competition for the sites of adhesion; both mechanisms are implicated in probiotic effect known as gut epithelium defence.

9.6.1 Increasing the Production of Intestinal Mucins and Antimicrobial Substances

Studies have shown that germ-free mice can exhibit changes in the number of rectal goblet cells and mucin composition in response to oral administration of microorganisms prepared from the faeces of genetically identical mice (Fukushima et al. 1999). However, the data on the role of the probiotics in the induction of mucin synthesis are very limited. For example, *L. plantarum* strain 299v increases the levels of expression of the mRNA of the secretory mucins MUC2 and MUC3, thus in turn inhibiting the cell attachment of enteropathogenic *Escherichia coli* (EPEC), an effect that can be mimicked by adding purified exogenous MUC2 and MUC3 mucins (Mack et al. 1999, 2003). Note that a spontaneous mutant of *L. plantarum* 299v with reduced adhesion capabilities to such a cell line was unable to induce mucin secretion (Mack et al. 2003). The data suggest that adhesion of probiotic bacteria to host cells could be a mechanism for the induction of mucin secretion through the action of certain bacterial surface proteins. However, the bacteria contained in the VSL#3 probiotic formula which consists of four *Lactobacillus* spp., three *Bifidobacterium* spp. and *Streptococcus thermophilus* and is manufactured by Seaford Pharmaceuticals secrete soluble compounds that are able to induce mucin secretion and muc2 gene expression in murine colonic epithelial cells (Caballero-Franco et al. 2007). How the microbiota can influence antimicrobial peptides production also remains controversial. Some reports suggest that in fact the microbiota has no influence. In contrast, it has been shown that *E. coli* strain Nissle 1917, a human faecal isolate and widely used probiotic, induces the human β-defensin 2

(hBD-2) in Caco-2 cells (Schlee et al. 2007; Wehkamp et al. 2004), and flagellin is the main hBD-2-inducing factor (Schlee et al. 2007). Note that the ability to increase the production of intestinal mucins and antimicrobial substances is common for probiotics, symbionts and intestinal pathogens. For example, the expression of angiogenin-4 (Ang4), a molecule produced by mouse Paneth cells, which is active against a number of gram-negative and gram-positive bacteria can be triggered by lipopolysaccharides from *Salmonella* and unidentified substances of *Bacteroides thetaiotaomicron*, a dominant member of the gut microbiota that currently is not used as probiotic (Hooper et al. 2001, 2003).

9.6.2 Competitive Exclusion of Pathogenic Bacteria Through Probiotics Adhesion to Mucus, Epithelial Cells, Extracellular Matrix Proteins and Plasma Components

It is believed that to be effective, the probiotic bacteria must possess a number of functional characteristics, including the ability to adhere to the epithelium. For example, *L. gasseri* and *L. reuteri* are autochthonous lactobacilli which are able to colonise the mucosal surface of the gastrointestinal tract, but *L. plantarum, L. casei* and *L. rhamnosus* are transient organisms. Despite this, the last three bacteria are used as probiotics mainly due to good technological properties (Reuter 2001).

Adhesion is believed not only to play a role in the persistence of a particular strain in the digestive tract but also to participate in pathogen exclusion by competition and blocking of their binding sites at the mucosa (Collado et al. 2007). Additionally, probiotic adhesion may contribute to immunomodulation (Galdeano et al. 2007). However, some authors have hypothesised that attachment factors in lactic acid bacteria are risk factors that might be indicative of their pathogenic potential (Vesterlund et al. 2007).

Assessment of bacterial adhesion is conventionally performed by using in vitro models, based on tissue-cultured cells and intestinal mucus preparations. Mainly used Caco-2 or HT29 cell lines only mimic enterocytes, thereby underestimating the role of the mucus layer, and mucus-producing cell lines such as HT29-MTX are a more appropriate way of studying the mechanism of adhesion and estimation of binding potential of probiotic bacteria (Turpin et al. 2012). Unfortunately, the known in vitro models do not account for all factors involved in probiotic adhesion to the human intestine and show the data that significantly differ from the data obtained in vivo (Larsen et al. 2009).

It was shown in vitro that probiotics prevent gut colonisation by *B. vulgatus*, *Clostridium difficile*, *Clostridium histolyticum*, *Listeria monocytogenes*, *Salmonella choleraesuis*, *St. aureus* and certain *E. coli* strains (Collado et al. 2007; Lin et al. 2008; Sherman et al. 2005). EcN 1917 protects epithelial cells from the invasion by *Salmonella enterica*, *Yersinia enterocolitica*, *Shigella flexneri*, *Legionella pneumophila*, *L. monocytogenes* and *E. coli* (Boudeau et al. 2003; Altenhoefer et al. 2004).

Competitive exclusion based on binding to the same receptor sites on the intestinal surface (including mucus, epithelial cells and extracellular matrix) by probiotics and pathogenic bacteria appears to be one of the underlying mechanisms explaining these observations (Mukai et al. 2004; Sun et al. 2007). In the last decade, the increasing amount of data dealing with the molecular origin of adhesion has improved our understanding of binding mechanisms.

Though specific mechanisms are not yet well understood, evidence suggests that carbohydrate–protein interactions play a key role in the adhesion of bacterial proteins to mucin-bound oligosaccharides, especially taking into account that numerous mucus-binding proteins contain regions homologous with binding domains of lectins. Besides carbohydrate moiety of mucins, adhesins of commensals are able to interact with the host's extracellular matrix proteins (EMPs), such as fibronectin, collagen and laminin. The latter three substances are shed into the mucus or can be exposed to the intestinal lumen in case of trauma, infection or inflammation. Moreover, some probiotic bacteria can bind to plasminogen just as it was found for pathogens that exploit the proteolytic activity of the plasminogen system to overcome barriers formed by the host's extracellular matrix proteins. Bacterial proteins involved in the adhesion mechanism can be separated into five classes: LPXTG-motif proteins, transporter proteins, surface layer proteins, anchorless housekeeping proteins, and "other" proteins (Ljungh and Wadström 2009). Additionally, adhesion of probiotics to mucus/epithelial surfaces is facilitated by exopolysaccharides and lipoteichoic acid as it was found for lactobacilli (Lebeer et al. 2008; Sánchez et al. 2008). The substances generally play a role in nonspecific interactions of lactobacilli with abiotic surfaces and biotic surfaces by contributing to the bacterial cell surface physicochemical properties. Besides, exopolysaccharides could also act as ligands for host lectins mediating adhesion (Ruas-Madiedo et al. 2006).

The most studied example of mucus-targeting bacterial adhesins is the mucus-binding protein (MUB) produced by *L. reuteri* 1063 (Roos and Jonsson 2002). MUB contains C-terminal sortase recognition motif (LPXTG) for anchoring the protein to peptidoglycan, repeated functional domains and an N-terminal region signalling the protein for secretion. Actually repeated functional domains (referred to as MUB domains) are responsible for the protein adhesive properties and allow including the protein to mucin-binding protein (MucBP) domain family. Numerous MUB homologues and MucBP domain-containing proteins have been found, but almost exclusively in lactobacilli (Van Tassell and Miller 2011). Some of them are listed below: Mub of *L. acidophilus* NCFM (Buck et al. 2005), the mannose lectin (Msa) of *L. plantarum* WCFS1 (Pretzer et al. 2005), the *Lactobacillus* surface protein A (LspA) of *L. salivarius* UCC118 (van Pijkeren et al. 2006) and the mucin adhesion-promoting protein (MapA) of *Lactobacillus fermentum* 104R, recently reclassified as *L. reuteri* 104R (Miyoshi et al. 2006; Rojas et al. 2002). Certain other surface proteins contributed to adhesion of lactobacilli to mucus but are otherwise not well characterised. For instance, a 32 kDa protein associated with adhesion to porcine mucus in *L. fermentum*, named 32-Mmubp, was identified as a homologue of the substrate-binding domains of the OpuAC ABC-transport protein family (Macías-Rodríguez et al. 2009).

A majority of the known EMPs-targeting bacterial adhesins have been identified as surface layer proteins (SLPs) of lactobacilli. Briefly, SLPs of *Lactobacillus* species are highly basic proteins (with computed isoelectric point values ranging from 9.4 to 10.4 and a molecular weights ranging from 25 to 71 kDa), and its only known functional role is adhesion to host tissues (Ävall-Jääskeläinen and Palva 2005). SLPs are non-covalently attached to the cell surface through N-terminal domains, which are responsible for their binding to accessory molecules (such as teichoic acids, lipoteichoic acids and neutral polysaccharides) embedded in the peptidoglycan matrix (Mesnage et al. 2000). Direct experimental evidence of SLPs binding to EMPs and to epithelial cell lines has already been obtained for the protein SlpA of *L. acidophilus* NCFM (protein that additionally carries two mucin-binding domains) (Buck et al. 2005), SlpA of *L. brevis* ATCC 8287 (de Leeuw et al. 2006), CbsA of *L. crispatus* JCM 5810 (Antikainen et al. 2002), SlpB of *L. crispatus* ZJ001 (Chen et al. 2007) and the SlpA of *L. helveticus* R0052 (Johnson-Henry et al. 2007). To date, a number of studies associating SLPs of probiotic bacteria with competitive exclusion of pathogens and pathogen adhesion to mucus have been carried out (Chen et al. 2007; Sánchez et al. 2009; Zhang et al. 2010).

In addition, at least two small surface-associated proteins (with a molecular mass 3 kDa) have been shown to be responsible for the adhesion of *L. fermentum* to Caco-2 cells (Baccigalupi et al. 2005). Other examples of probiotic adhesins are non-covalently surface attached proteins including the fibronectin-binding protein (FbpA) of *L. acidophilus* NCFM (Buck et al. 2005), the collagen-binding protein from *L. reuteri* NCIB 11951 (Roos et al. 1996) and its homologous p29 of *L. fermentum* RC-14 (Heinemann et al. 2000).

It was shown that species of the genus *Lactobacillus* have moonlighting proteins (Jeffery 2009) that carry out the function of adhesion. The term "moonlighting protein" means that the protein performs multiple functions and the additional activity may occur only when the protein is in a different location from that which it normally occupies. Moonlighting proteins are anchorless proteins and they do not possess any export motifs or surface-attachment domains. In particular, glycolytic enzymes of *L. crispatus* strain ST1, namely enolase, glyceraldehyde-3-phosphate dehydrogenase, glutamine synthetase and glucose-6-phosphate isomerase that are known as cytosolic proteins were found in cell wall where they moonlight either as adhesins with affinity for basement membrane and EMP or as plasminogen receptors. The proteins were bound onto the bacterial surface at acidic pH, whereas suspension of the cells to pH 8 caused their release into the buffer (Antikainen et al. 2007a, b; Kainulainen et al. 2012). This could be one of the mechanisms by which probiotic bacteria respond to the physicochemical changes of the gastrointestinal environment. The glyceraldehyde-3-phosphate dehydrogenase of *L. plantarum* LA 318 also acts as an adhesin and is able to bind to human colonic mucin (Kinoshita et al. 2008). Note that the host plasminogen activation by enolase and glyceraldehyde-3-phosphate dehydrogenase secreted by probiotics might interfere in the interaction between plasminogen and gastrointestinal pathogens such as *Helicobacter pylori* and *Salmonella sp*. (Hurmalainen et al. 2007; Jönsson et al. 2004; Lähteenmaki et al. 2005).

Another moonlighting protein known as elongation factor Tu was found on the cell surfaces of *L. johnsonii* NCC 533 and identified as the substance-mediating attachment to intestinal epithelial cells and mucin (Granato et al. 2004). Expression of elongation factor Tu was upregulated in the presence of mucus (Ramiah et al. 2007), and its adhesion to epithelial cells or mucus was pH dependent (Granato et al. 2004). Chaperonin GroEL was detected at the surface of *L. johnsonii* NCC 533 and its moonlighting as an adhesin was proved by detection of attachment of recombinant GroEL expressed in *E. coli* to mucus as well as to the HT29 cell line (Bergonzelli et al. 2006). Both elongation factor Tu and chaperonin GroEL belong to group of anchorless housekeeping proteins implicated in adhesion (Ljungh and Wadström 2009).

It is known that fimbriae, also referred to as pili, are thin proteinaceous extensions from bacterial cells, predominantly in gram-negative bacteria, that promote adhesion (Nakamura et al. 1997). The direct visualisation of pili on cells of *L. rhamnosus* GG (Kankainen et al. 2009) proved for the first time that fimbrial interaction with mucus can mediate adhesion of lactobacilli to host epithelium.

At least 20 genes are reported to be functionally important in the binding of *Lactobacillaceae* to the digestive tract. The genetic screening could have been an ideal tool to assess potential bacterial adhesion, but proved to be inadequate, since there was a gap between the potential identified by screening and the results obtained by functional analysis using tissue-cultured cells (Turpin et al. 2012).

By contrast with lactobacilli, very little is known on the mechanisms of bifidobacterial adhesion. Adhesion of *B. breve* strain 4 to intestinal epithelial cells is mediated by a proteinaceous component present on the cell surface and in spent culture supernatant (Bernet et al. 1993). Binding of human plasminogen in vitro was shown for *B. longum, B. bifidum, B. breve* and *B. lactis*. Chaperone protein DnaK also as the key glycolytic enzyme enolase expressed in cell wall as moonlight proteins was identified as plasminogen receptors implicated in the interaction of the bacteria with host tissues (Candela et al. 2009, 2010). A cell surface lipoprotein named BopA was shown to be involved in adhesion of *B. bifidum* MIMBb75 to Caco-2 and HT-29 cells, and its adhesion strongly depended on the environmental conditions, including the presence of sugars and bile salts and the pH (Guglielmetti et al. 2009). More recently BopA was identified as a *B. bifidum*-specific lipoprotein involved in adhesion to intestinal epithelial cells (Gleinser et al. 2012). Expression of BopA in *B. longum/infantis* E18 allows to enhance adhesion to epithelial cells suggesting possibility to create recombinant bifidobacteria with improved adhesive properties (Gleinser et al. 2012). Another example demonstrating improved probiotic properties of recombinant lactic acid bacteria is a recombinant *L. paracasei* strain expressing the gene coding for the *Listeria* adhesion protein (Lap), which was shown to protect Caco-2 cells from infection with *Listeria monocytogenes* by interaction with host cell receptor Hsp60 (Koo et al. 2012).

While adhesion might play an important role in establishing administered probiotic bacteria in the intestinal tract, the data on correlation between the health-promoting properties of probiotics and their adhesion to intestinal epithelial cells/mucus are limited. For example, in different murine models of intestinal inflammation, it was shown

that animals treated with *B. bifidum* S17, a highly adherent strain, were protected from weight loss, had a normalised colonic weight to length ratio and showed improved histological scores. By contrast, the weakly adherent *B. longum/infantis* E18 had no protective effect (Preising et al. 2010).

9.6.3 Protection and Restoring of Epithelial Integrity

The intestinal epithelial cells are tightly bound together by intercellular junctional complexes that regulate the paracellular permeability and are crucial for the maintenance of barrier integrity. The junctional complexes consist of the tight junctions (TJ), gap junctions, adherens junctions and desmosomes (Farquhar and Palade 1963). Actually TJ are most important because TJ forms a seal between adjacent epithelial cells near the apical surface (Schneeberger and Lynch 2004). TJ are complex structures comprising over 50 proteins. Briefly, transmembrane proteins such as occludin and claudin (tetra-span proteins) and junctional adhesion molecules (JAM) (single-span proteins) form fibrils that cross the plasma membrane and interact with proteins in the adjoining cells (Chiba et al. 2008). Plaque proteins, such as the zonula occludens (ZO) proteins, act as cytoplasmic adaptors that connect transmembrane proteins to several cytoplasmic regulatory proteins and the actin cytoskeleton within the cell (Fanning et al. 1998). TJ are highly dynamic structures that are constantly being remodelled due to interactions with external stimuli, such as food residues and pathogenic and commensal bacteria.

Currently, it is known that probiotics promote intestinal barrier integrity in mouse models of colitis (Madsen et al. 2001) and reduce intestinal permeability in Crohn's disease patients (Gupta et al. 2000) and in rats subjected to psychological stress (Zareie et al. 2006). The known mechanisms of promoting intestinal barrier integrity by probiotics include regulation of TJ structure through (a) changes in TJ protein expression and distribution and (b) changes in activity of the kinases that regulate contraction of the perijunctional actomyosin ring. It is known that treatment of epithelial cells with EcN 1917 leads to increased expression of ZO-2 protein and redistribution of ZO-2 from the cytosol to cell boundaries in vitro (Zyrek et al. 2007). *L. plantarum* regulates human epithelial TJ proteins in vivo and to confer protective effects against chemically induced dislocation of ZO-1 and occludin from Caco-2 monolayers (Karczewski et al. 2010). Furthermore, treatment of Caco-2 cells with the probiotic *L. plantarum* MB452 from the probiotic product VSL#3 results in increased transcription of occludin and cingulin genes, suggesting that bacteria-induced improvements in intestinal barrier integrity may also be regulated at the gene expression level (Anderson et al. 2010).

Interestingly that some probiotics and commensals prevent and even reverse the adverse effects of pathogens on intestinal barrier function. For example, enteroinvasive *E. coli* (EIEC) strain O124:NM induces loss of expression and distribution of TJ-associated proteins in Caco-2 monolayer, but these effects are absent when EIEC and *L. plantarum* strain CGMCC 1258 are incubated with Caco-2 cells simultaneously.

Moreover, the disruption and disorganisation of the actin cytoskeleton induced by EIEC can then be reversed by incubating the epithelial cells with *L. plantarum* (Qin et al. 2009).

Pretreatment with metabolites from probiotic bacteria may also be protective against pathogen-induced changes in intestinal barrier function. The treatment of Caco-2 cells with the cell-free supernatant of *B. lactis* 420 before adding the supernatant of enterohemorrhagic *E. coli* (EHEC) strain O124:H7 increased transepithelial electrical resistance (TEER) which is used as a measure of paracellular ion permeability, whereas adding the supernatant of EHEC alone decreased TEER (Putaala et al. 2008). The increase in TEER was not seen, however, if the supernatant was added with or after pathogen treatment. The data suggests that supernatant of *B. lactis* 420 protects Caco-2 cells against changes induced by EHEC but does not repair TJ integrity after damage. Regulation of TJ structure is achieved via myosin light chain II (MLC) phosphorylation and contraction of the perijunctional actomyosin ring. The enzymes of enterocytes namely protein kinase C (PKC), MLC kinase (MLCK), mitogen-activated protein kinases (MAPK) ERK1/2 and p38 and Rho kinase (ROCK; activated by Rho GTPases) phosphorylate MLC and induce contraction of the actomyosin ring causing increased permeability (Kimura et al. 1996). It was shown that pathogens can alter barrier function by activation of MLCK (*Helicobacter pylori*), inactivation or activation of small Rho GTPases (*Clostridium difficile* and *Salmonella typhimurium*) and enhancement of actin polymerisation by PKC (*Vibrio cholerae*) (Ohland and MacNaughton 2010). The same proteins are possible targets for probiotics that enhance epithelial barrier integrity (Ulluwishewa et al. 2011). For example, the ability of the probiotics *S. thermophilus* and *L. acidophilus* to preserve phosphorylation of occludin in cells infected with EIEC can be reduced by treating the cells with ROCK inhibitors (Trivedi et al. 2003), suggesting that these bacteria employ Rho family GTPases to protect against EIEC-induced TJ disruption. EcN 1917 uses a PKCζ-dependent signalling pathway to reduce epithelial barrier disruption caused by EPEC (Zyrek et al. 2007); activation of PKCζ by the probiotic leads to phosphorylation of ZO-2, thus reducing ZO-2-PKCζ colocalisation and allowing association of ZO-2 with the cytoskeleton. *B. infantis* Y1 secretes metabolites which increase TEER in cultured epithelial monolayer through MAPK-dependent pathways including a transient phosphorylation of ERK1/2 and a decrease in phosphorylation of MARK p38 (Ewaschuk et al. 2008). *B. infantis-induced* TEER increase can be prevented by inhibition of extracellular signal regulated kinases (ERK), a group of MAPK (Ewaschuk et al. 2008). However, it has also been shown that the ability of *S. thermophilus* and *L. acidophilus* to protect against EIEC infection, which is reduced by ROCK inhibitors, does not seem to be affected by inhibition of ERK1/2 or p38 (Trivedi et al. 2003). In general, consumption of live probiotics promotes TJ integrity and prevents pathogenic bacteria and their effectors from entering via the paracellular pathway to cause further damage; different species of probiotics may use multiple pathways to modulate TJ integrity.

Commensals and probiotics are also known to preserve epithelial barrier function by interfering with pro-inflammatory cytokine signalling. Treatment of cell monolayers with the cytokines TNFα and IFNγ leads to a decrease in TEER and an

increase in epithelial permeability (Resta-Lenert and Barrett 2006), and TEER decrease can be prevented by preincubation of the cells with the probiotics *S. thermophilus* ATCC19258 and *L. acidophilus* ATCC4356 or the commensal *Bacteroides thetaiotaomicron* ATCC29184 (Resta-Lenert and Barrett 2006). The reversal of cytokine-induced decrease in TEER was shown to be dependent on activation of kinases ERK and p38, a group of MAPK, and phosphatidylinositol 3-kinase (PI3K) (Resta-Lenert and Barrett 2006). In was shown that following *L. rhamnosus* GG inoculation, IFNγ priming and TNFα stimulation, Caco-2bbe cells maintained TEER and ZO-1 distribution. The signalling interaction between the probiotic and Caco-2bbe cells included suppression of cytokine-induced nuclear factor kappa-light-chain enhancer of activated B cells (NF-κB) inhibition and ERK1/2 response (Donato et al. 2010). DNA from the commensal bacteria *L. rhamnosus* GG and *B. longum* SP 07/3 have also been shown to induce a signal transduction cascade via an epithelial cell surface receptor, which reduces TNFα-induced p38 phosphorylation (Ghadimi et al. 2010).

Note that increased permeability of the epithelial barrier can also be caused by apoptosis via caspase-3 activation (Chin et al. 2002) and probiotics modulate apoptosis initiation by harmful stimuli. Two proteins (p40 and p75) secreted from *L. rhamnosus* inhibited cytokine-induced apoptosis in epithelial cell lines by activating the epidermal growth factor (EGF) receptor and its downstream target serine/threonine kinase Akt (also known as protein kinase B), as well as inhibiting p38 MAPK activation, in vitro and ex vivo (Yan et al. 2007). Akt promotes cell survival by inactivating proapoptotic proteins, including caspases 3 and 9 (Hanada et al. 2004). Expression of p40 and p75 is strain specific because *L. casei*, but not *L. acidophilus*, also produces these proteins (Yan et al. 2007). Additionally, apical or basolateral pretreatment with either p40 or p75 protected several cell lines from hydrogen peroxide-induced disruption of barrier function, as measured by TEER and paracellular permeability. This effect was via inhibition of hydrogen peroxide-induced cytosolic relocalisation of the TJ proteins occludin and ZO-1 and the AJ proteins E-cadherin and β-catenin. These effects were all dependent on activation of PKCε, PKCβI, and the MAP kinases ERK1/2 (Seth et al. 2008). Therefore, bacterial proteins isolated from *L. rhamnosus* cultures effectively block the induction of apoptosis, helping to enhance epithelial barrier function.

Interestingly, that conditioned media from the probiotic *L. rhamnosus* GG induce expression of cytoprotective heat-shock proteins (Hsps) Hsp25 and Hsp72 in intestinal epithelial cells and the effect is mediated by a low-molecular-weight peptide that is acid and heat stable. Inhibitors of MAP kinases block the expression of Hsp72 normally induced by the probiotic (Tao et al. 2006). Similarly, VSL#3 produces soluble factors that induce the expression of cytoprotective Hsps in young adult mouse colonic epithelial cells (Petrof et al. 2004). It is known that Hsps are involved in protein folding, assembly, degradation and intracellular localisation, acting as molecular chaperones, and their overexpression represents a ubiquitous molecular mechanism to cope with stress. Thus, induction of Hsp is another mechanism of probiotic action that provides cellular protection and improves epithelial integrity.

Future investigations into the bacterial factors (such as *Lactobacillus* p40 and p75) involved in improvement of intestinal epithelium integrity would be useful in developing probiotic-derived products for therapy in immunocompromised individuals who cannot consume live probiotics.

9.6.4 Modulation of Host Immune Functions

The gut-associated immune system recognises intestinal microorganisms by pattern-recognition receptors such as the Toll-like receptors (TLRs). The TLRs recognise molecular signatures of different bacteria such as cell wall components or specific DNA motifs (CpG-DNA). Activation of the TLRs results in the induction of complex intracellular signal transduction cascades and finally in the modulation of pro- and anti-inflammatory cytokine expression (Cario 2005). Probiotic bacteria may act through the stimulation of TLRs and it appears that certain effects exerted by some probiotic strains or preparations are mediated through interactions with distinct TLRs. In dextran sodium sulphate ((DSS)-treated mice, γ-irradiated VSL#3 is capable of decreasing the severity of inflammation through TLR9 that is activated by non-methylated bacterial DNA (Rachmilewitz et al. 2004). In contrast, EcN 1917 clearly exerts its effects on DSS-induced colitis in mice through TLR2 and TLR4 (Grabig et al. 2006).

An important aspect of the probiotic immune modulation is the regulation of pro- and anti-inflammatory cytokine production by direct interactions with immune cells. In healthy subjects, *L. rhamnosus* GG triggers the synthesis of the anti-inflammatory interleukin IL-10 and decreases the release of pro-inflammatory IFN-γ, IL-6 and TNF-α from CD4+ T-cells pre-stimulated with intestinal bacteria (Schultz et al. 2003). Co-cultivation of inflamed mucosa explants from celiac disease patients with *L. bulgaricus* LB 10 and *L. casei* DN-114001 reduces the number of TNF-a-secreting CD4+ T-cells and the TNF-a expression by intraepithelial lymphocytes (Borruel et al. 2002). It can be assumed that probiotic bacteria stimulate dendritic cells which in turn produce anti-inflammatory cytokines. This has been demonstrated for *L. reuteri*, *L. casei* and VSL#3, all of which are capable of stimulating IL-10 production by human dendritic cells (Hart et al. 2004; Smits et al. 2005). In addition to the examples given here, probiotics display many other immune modulatory functions, which have extensively been reviewed elsewhere (Shida and Nanno 2008; Vanderpool et al. 2008).

9.6.5 Influence on Host Microbiota and Pathogenic Bacteria

It has been proposed that probiotics exert their effect by modulating gut microbiota composition (Fuller 1989). Indeed, a considerable number of studies support this assumption by demonstrating changes in a number of bacterial groups in response

to the consumption of probiotics. For example, numbers of bifidobacteria and lactobacilli increase in healthy subjects after ingestion of *L. casei* Shirota or *L. johnsonii* La1 while those of enterobacteria or clostridia decrease (Spanhaak et al. 1998; Yamano et al. 2006). In patients suffering from intestinal bowel disease, in particular ulcerative colitis, the intestinal microbiota composition can differ substantially from that of healthy subjects (Sartor 2006, 2008) as reflected by high titers of *Bacteroides vulgatus* and *E. coli* (Fujita et al. 2002; Kotlowski et al. 2007). High proportions of *B. vulgatus* are reduced in the gut of patients suffering from ulcerative colitis by the consumption of fermented milk containing bifidobacteria (Ishikawa et al. 2003). The probiotic preparation VSL#3 is effective in elevating the number of total gut bacteria and in restoring the intestinal microbiota diversity in the patients that have inflammation of the lining of the internal pouch (Kuhbacher et al. 2006). In addition, VSL#3 increases caecal bifidobacteria numbers and modifies the metabolic activity of caecal bacteria in mice with chronic colitis induced by DSS (Gaudier et al. 2005). Thus, one possible mechanism by which probiotics can alleviate the severity of ulcerative colitis is the reduction of bacterial species involved in the pathogenesis.

Proposed mechanisms involved in the modification of the intestinal microbiota composition by probiotics include competition and cooperation for nutrients (Lebeer et al. 2008) and production of antibacterial substances including lactic and acetic acid, hydrogen peroxide and antibacterial peptides. Lactic acid as the end product of LAB metabolism lowers the local pH and thereby inhibits the growth of bacteria sensitive to acidic conditions (Alakomi et al. 2000; De Keersmaecker et al. 2006; Makras et al. 2006). The same effects are typical for acetic acid that is one of the bifidobacterial end products. Hydrogen peroxide production by lactobacilli is an important antimicrobial mechanism, especially in the vagina of healthy women (Servin 2004). Recently it was shown that *L. johnsonii* NCC533 produces up to millimolar quantities of hydrogen peroxide when resting cells are incubated in the presence of oxygen, and the role for hydrogen peroxide in the anti-*Salmonella* activity of the probiotic strain was proved in vitro. The genetic base for this hydrogen peroxide production is not clear, but at least four enzymes are implicated in the effect (Pridmore et al. 2008).

Many LAB produce antibacterial peptides (bacteriocins) that vary in spectrum of activity, mode of action, molecular weight, genetic origin and biochemical properties. According to Klaenhammer (1993), the major classes of bacteriocins produced by LAB include (a) lantibiotics, (b) small heat stable peptides, (c) large heat labile proteins and (d) complex proteins whose activity requires the association of carbohydrate or lipid moieties. The existence of the fourth class was supported mainly by the observation that some bacteriocin activities obtained in cell-free supernatant, exemplified by the activity of *L. plantarum* LPCO 10, were abolished not only by protease treatments but also by glycolytic and lipolytic enzymes (Jimenez-Diaz et al. 1993).

Most recently, bacteriocins were classified mainly into two classes: the lanthionine-containing bacteriocins (lantibiotics) (class I) and the non-lanthionine-containing bacteriocins (class II) (Cotter et al. 2005). The lantibiotics are small

peptides (19–38 amino acids in length) and contain posttranslationally modified amino acids such as lanthionine, beta-methyllanthionine, dehydroalanine and dehydrobutyrine. Covalent bridge formation, as a result of these unusual residues, leads to the formation of internal "rings" which give the lantibiotics their characteristic structural features. The class II bacteriocins are also relatively small (<10 kDa) but unlike the class I bacteriocins are not subject to extensive posttranslational modification. Being a rather heterogeneous group, they have been further classified into the class IIa pediocin-like or *Listeria*-active bacteriocins, the class IIb two-peptide bacteriocins (e.g. lactococcin G (Oppegård et al. 2007)), the class IIc cyclic bacteriocins and the class IId linear non-pediocin-like one-peptide bacteriocins (Nissen-Meyer et al. 2009). Pediocin-like bacteriocins are the most important and well-studied group of class II bacteriocins that includes now more than 20 items, including the "classical" member pediocin AcH from *Pediococcus acidilactici* AcH identified in 1991; plantaricin 423, curvacin A, few sakacins and curvacin A named in accordance with the species of lactobacilli in which they were found; lactococcin MMFII from *Lactococcus lactis*; few enterocines from *Enterococcus faecium*; and bifidocin B from *B. bifidum* (Drider et al. 2006; Nissen-Meyer et al. 2009). They are all cationic and partly amphiphilic and/or hydrophobic and have between 37 and 48 residues, and in the N-terminal region (up to about residue 17), they all contain the conserved Y-G-N-G-V/L "pediocin box" motif and two cysteine residues joined by a disulfide bridge. They also contain more hydrophobic C-terminal region (from about residue 18). Pediocin-like bacteriocins are unstructured in aqueous solution but become structured upon contact with membrane of target bacterial cell. The cationic N-terminal β-sheetlike domain mediates binding to the target cell surface through electrostatic interactions and the hydrophobic C-terminal hairpin-like domain penetrates into the hydrophobic core of target membranes, which induces leakage of ions and leads to cell death.

The two-peptide (class IIb) bacteriocins consist of two very different peptides and optimal activity requires both peptides in about equal amounts. Since the first class IIb bacteriocin (lactococcin G) was identified in 1992, at least 15 two-peptide bacteriocins have been isolated and characterised including thermophilin 13 from *S. thermophilus*, lactococcin G from *Lactococcus lactis*, plantaricin E/F from *L. plantarum C11* (Fimland et al. 2008) and lactacin B from *L. acidophilus* (Tabasco et al. 2009). The individual peptides of two-peptide bacteriocins share characteristics with one-peptide bacteriocins in that they are usually cationic, 30–50 residues long, hydrophobic and/or amphiphilic and are all synthesised with a 15–30 residue N-terminal leader sequence that is cleaved before export of the peptides from cells. Interestingly, the two peptides of class IIb bacteriocins function together as one antimicrobial entity. As a rule, both peptides contain GxxxG motifs which allow forming of membrane-penetrating helix–helix structures interacting with integrated membrane proteins, which induces leakage of ions and leads to cell death.

The cyclic bacteriocins (Maqueda et al. 2008) whose N- and C-termini are covalently linked are placed in class IIc (Nissen-Meyer et al. 2009). At least seven cyclic bacteriocins produced by gram-positive bacteria have been characterised, including gassericin A, reutericin 6, acidocins B and D20079 from *L. gasseri* LA39, *L. reuteri*

LA6 and two strains of *L. acidophilus*, respectively. They are all cationic and relatively hydrophobic, and they range in size from 3,400 to 7,200 Da. All cyclic bacteriocins render the target cell membrane permeable to small molecules, which eventually results in cell death.

The linear non-pediocin-like one-peptide bacteriocins are placed in class IId according to the classification proposed by Cotter et al. (Cotter et al. 2005). The extensive group includes at least 30 items (Nissen-Meyer et al. 2009), in particular, enterocins EJ97, B, L50A, L50B, etc. from the strains of *Enterococcus faecalis* and *E. faecium*; acidocins A, 1B, CH5 from the strains of *L. acidophilus*; aureocins A70 and A53 from the strains of *St. aureus*; and so on. The properties of the bacteriocins belonging to the class IId are heterogeneous and it is difficult to draw their general properties. Interestingly, aureocin A53 functions at micromolar concentrations and acts through the membrane disruption rather than formation of target-mediated pores upon binding with high affinity to specific receptors or docking molecules as is typical for class IIa and class IIb bacteriocins that function at nanomolar concentrations.

Note that the bacteria that synthesize bacteriocins have the so-called immunity proteins that associate with membranes within cells, recognise and bind bacteriocin–permease complex and thus prevent cell self-killing.

Generally, bacteriocins of bifidobacteria are under the initial study and a list of known bacteriocins includes bifidin from *B. bifidum*, bifidocin B from *B. bifidum* NCFB 1454, biflong Bb-46 from *B. longum* Bb-46 and biflact Bb-12 from *B. lactis* Bb-12 (Cheikhyoussef et al. 2008). The most investigated is bifidocin B (Cheikhyoussef et al. 2008) that inhibits the growth of selected species of the genera *Listeria*, *Bacillus*, *Enterococcus*, *Lactobacillus*, *Leuconostoc* and *Pediococcus* but was not active against gram-negative bacteria due to its interaction with teichoic acids that are absent in cell wall of the group of bacteria. In the sensitive gram-positive cells, bifidocin B molecules bind to specific or lethal receptor(s) and form pores leading to cell death with or without lysis. Bifidocin B consists of one polypeptide chain of 36 amino acid residues with a molecular mass of 4432.9 Da and shares significant homology with other class IIa LAB bacteriocins. Production of bifidocin B by *B. bifidum* NCFB 1454 was associated with an 8 kb size plasmid which may be used in the construction of food-grade vectors for improvement of bifidobacteria.

Currently bacteriocin-like substances from bifidobacteria are under investigation. For example, six selected *Bifidobacterium* strains produce bacteriocin-like substances that are active against gram-positive and gram-negative bacteria and yeasts (Collado et al. 2005). The substances are active at pH values between 3 and 10, stable at 100 °C for 10 min, resistant to alpha-amylase and lipase A, sensitive to proteinases (trypsin, proteinase K, protease A, pepsin and cathepsin B) and have molecular weighs less than 30 kDa (Collado et al. 2005).

The potential of bacteriocin-producing gut isolates as bioprotective agents against pathogenic bacteria both in vitro and in vivo has been well documented in the literature (Gillor et al. 2008). For example, the ability of a five-strain *Lactobacillus/Pediococcus* combination from porcine intestines has been shown to

protect against *Salmonella* infection in a porcine model (Casey et al. 2007). In a mouse model, production of the bacteriocin Abp118 was shown to be responsible for inhibition of *L. monocytogenes* infection (Corr et al. 2007). In a similar type of study, human isolates of *Pediococcus acidilactici* producing pediocin PA-1 and *L. lactis* producing nisin Z were shown to reduce vancomycin-resistant enterococci intestinal colonisation in a mouse model (Millette et al. 2008). In view of the widespread resistance to currently available antibiotic treatments, bacteriocins which inhibit pathogenic bacteria offer a highly favourable alternative. Moreover, bacteriocin-producing microorganisms in the gut may provide a viable mechanism for therapeutic delivery to the site of infection, an approach likely to be more effective than the bacteriocins themselves, which would undoubtedly be broken down during passage through the gastrointestinal tract (Gardiner et al. 2007).

In recent years, interest in the bacteriocins has grown substantially due to their potential usefulness as friendly food biopreservatives either in the form of protective cultures and as additives (Ross et al. 2010; Settanni and Corsetti 2008). Note that fermentation of various foods by LAB is one of the oldest forms of biopreservation practised by mankind. Currently, nisin is the only bacteriocin that is applied as a food additive in European countries and the USA (Delves-Broughton et al. 1996; FAO/WHO 2007; Vandenberg 1993). Nisin prevents clostridial spoilage of processed and natural cheeses, inhibits the growth of some psychrotrophic bacteria in cottage cheese, extends the shelf life of milk, prevents the growth of spoilage lactobacilli in beer and wine fermentations and provides additional protection against spores of *Bacillus* and *Clostridia* in canned foods.

Besides bacteriocins probiotics produce also certain antibiotics. The production of the antibiotic reuterin (3-hydroxypropionaldehyde) by *L. reuteri* strain ATCC55730 has been reported. Reuterin is a broad-spectrum antibiotic active not only against gram-positive and gram-negative bacteria but also against yeast, fungi, protozoa and viruses (Cleusix et al. 2008). Additionally, probiotic bacteria are able to produce so-called deconjugated bile acids that are derivatives of bile salts synthesised by the host, show a stronger antimicrobial activity compared to the bile salts (Kurdi et al. 2006) and are interesting in the context of cholesterol-lowering effects of the bacteria.

9.7 Supercritical Carbon Dioxide ($scCO_2$) for the Extraction of Polar Lipids from Probiotic Bacteria

Extensive investigations of biologically active compounds from probiotic bacteria are primarily directed at the development of immunostimulants (Sekine et al. 1985). Among these substances glycolipids and phospholipids are the most abundant components in bacterial cell (Poupard et al. 1973). We described a novel isolation procedure for polar lipids from probiotic bacteria with the aid of supercritical fluid extraction (Novik et al. 2006). The extraction of polar lipids from biomass was performed by supercritical carbon dioxide using SFE-2X100F system (Fig. 9.4).

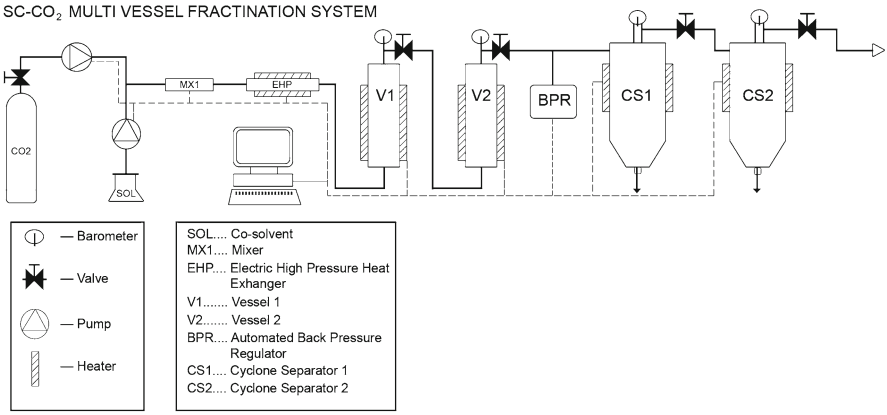

Fig. 9.4 Schematic diagram of the $scCO_2$ extraction system from Thar supercritical fluid extraction system Manual, April 2003, Thar Technologies, INC. 100 Beta Dr. Pittsburgh, PA 15238, USA, section "System Schematic" (Adapted by Estera Szwajcer Dey)

Temperature and flow rate of carbon dioxide are the major factors effecting the polar lipids extraction from probiotic bacteria (Novik et al. 2006). Conditions such as pressure 250 bar, temperature 45 °C, flow rate of CO_2 5 g/min and concentration of co-solvent (methanol/water 9:1) 10 % make possible isolation of major and minor glycolipids, as well as significant amounts of phospholipids (Fig. 9.5). Double or triple amounts of glycolipids and phospholipids in comparison with classical methods were found in the lipid extracts from bifidobacteria and lactobacteria (Izhyk et al. 2012; Novik et al. 2006; Rakhuba et al. 2009). ELISA of SFE lipid fractions from probiotic bacteria showed that the glycolipids are more immunoreactive compared with phospholipids. By employing the modification of $scCO_2$ settings, the high purity polar lipids of probiotic bacteria can be effectively extracted. The $scCO_2$ isolation technology can be combined with metabolic engineering and immunological studies in biotechnologies.

9.8 Brewing Waste as Media for Growth of Probiotic Bacteria

The brewing process involves specially prepared raw materials and yeast. The classical starting raw material is barley which has to be malted. The malting process consists of steeping, germination and drying (kilning). The outcome is malt, which further undergoes mashing to produce wort used for brewing of beer. The leftover solid material after mashing is referred to as "brewers' spent grains (BSG)." The BSG is a waste enriched in proteins and fibres (Ishiwaki et al. 2000; Lasztity 1984).

Modern technologies of probiotic production require not only active strains but low-cost media for their cultivation. Media with protein and carbohydrate

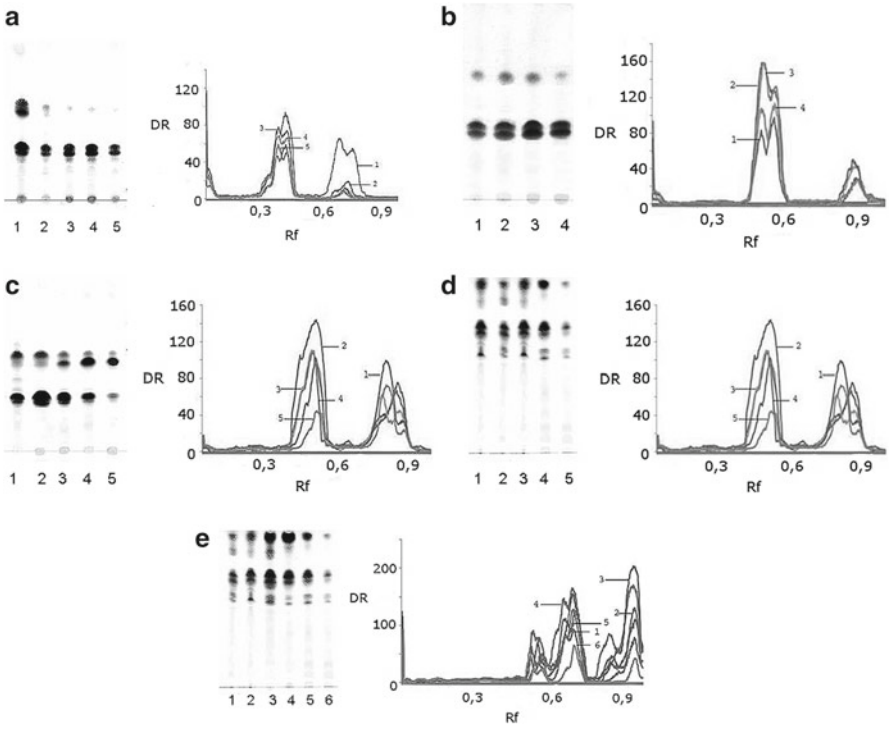

Fig. 9.5 Examples of HPTLC chromatograms of glycolipids and densitometric spectra thereof (*right*) showing lanes from chromatograms. *Bifidobacterium longum* (**a**) *lane 1*—classical method fraction, *2–5 lanes*—SFE fractions, (**b**) *1–4 lanes*—SFE fractions, (**c**) *1–5 lanes*—SFE fractions; *Bifidobacterium angulatum* (**d**) *lane 1*—classical method fraction, *2–5 lanes*—SFE fractions and (**e**) *1–6 lanes*—SFE fractions. *Rf* relative flow, *DR* density response (Izhyk et al. 2012)

components readily utilised by bifidobacteria and LAB allow recycling of a waste product and cultivation of probiotic biomass. Components of growth media act as sources of carbon, nitrogen and phosphorus and can also have beneficial effects on human health. The products of grain processing are known to be efficient substrates for cultivation of LAB and bifidobacteria; they provide for high growth potential, metabolite production, and cell viability during a long-term storage (Bamba et al. 2002; Charalampopoulos et al. 2002b; Kanauchi et al. 2003). These products can act as prebiotics which selectively stimulate growth of LAB and bifidobacteria in the intestines. Cereal grains contain water-soluble carbohydrate polymers (beta-glucan and arabinoxylan), oligosaccharides (galacto- and fructooligosaccharides) and water-insoluble polysaccharides (xylan, cellulose and starch), which presumably act as prebiotics. Moreover, these alimentary fibres can be included in the diet as sources of carbohydrates with multiple beneficial physiological effects (Charalampopoulos et al. 2002a). Recently, food additives containing the species of Lactobacillus and Bifidobacterium and the products of cereal grain processing, which serve as prebiotic components, have appeared in the market.

Germinated barley products and their polysaccharide fractions are known to prevent diarrhoea and enteritis and can be used for prophylaxis of colitis (Bamba et al. 2002). The components of BSG have traditionally been used as additives to ruminant fodder and ingredients of bakery products. The BSG protein and polysaccharide fractions are of growing interest as dietary supplements for treatment of dyspepsia and as alternative sources of protein and carbohydrates (Dongowski et al. 2002; Schrezenmeir and de Vrese 2001). Protein content of BSG varies from 8 to 55 % depending on the initial protein level in the barley and subsequent processes of malting and wort preparation (Crittenden et al. 2001; Das and Singh 2004; Hosono et al. 1997; Ishiwaki et al. 2000; Janer et al. 2005; Kanauchi et al. 1999; Kleyn and Hough 1971; Lasztity 1984; Lauer and Kandler 1976; Shukla 1998; Szponar et al. 2003; Szwajcer Dey et al. 1992). Earlier, we studied application of protein and polysaccharide fractions of BSG as a basis of growth media for probiotic bacteria (Novik et al. 2007). The protein fraction is mainly composed of hydrophobic peptides and proteins showing poor water solubility. It is known that some species of Bifidobacterium and Lactobacillus are able to produce extracellular proteinases, allowing the cells to utilise casein, albumin and some immunoglobulins (Novik et al. 2001). Probiotic bacteria were shown to decompose hardly hydrolysable protein compounds (Janer et al. 2005). These bacteria were characterised by synthesis of proteolytic enzymes which break peptide bonds between an amino acid and proline or glutamic acid in the P1 position. Our results demonstrated production of similar enzymes by the studied LAB and bifidobacteria (Szwajcer Dey et al. 1992). The findings indicated that protein and polysaccharide fractions of BSG can be used as components of media for cultivation of probiotic bacteria (Novik et al. 2007). High values of biomass yield, cell viability and organic acid production were observed in the variants of media containing BSG supplemented with lactose, ascorbic acid, yeast extract and mineral salts. Cells of LAB and bifidobacteria showed the typical rod-shaped morphology. We also recommended protein fraction from BSG as the main component of the media for isolation and cultivation of actinobacteria, production of biologically active substances and intense sporulation (Szponar et al. 2003). These results agree well with the data on the use of media containing protein fraction of BSG for isolation of Xanthomonas sp., which produced proline-specific endopeptidase (Szwajcer Dey et al. 1992). Since both fractions are suitable for bacterial growth, upgraded BSG can also be applied for probiotic production. The fraction containing poorly soluble polysaccharides (alimentary fibres) supplemented with protein from yeast extract may be used as a food additive for prophylaxis of diarrhoea and colitis (Bamba et al. 2002). The BSG media supported active bacterial growth, high biomass accumulation and formation of organic acids, which opens new prospects for the brewing waste in optimising technologies of low-cost probiotics production. Biomass of probiotic bacteria can serve as a promising source of immunostimulating compounds, such as polysaccharides and glycolipids used in vaccine production. A method for isolation of immunostimulating substances using fluidised carbon dioxide has been described (Novik et al. 2002, 2006). The obtained results can be used for development of low-cost manufacturing of an array of biopreparations, including probiotics, prebiotics

and vaccines. Moreover, brewing wastes may be an integral part of industrial bioresource recycling scheme (Crittenden et al. 2001; Das and Singh 2004; Hosono et al. 1997; Kanauchi et al. 1999; Kleyn and Hough 1971; Shukla 1998).

9.9 Conclusion

Currently, probiotics are becoming an increasingly important part in the diet of industrialised countries, as their general and gastrointestinal beneficial effects are being gradually proven. Many people over the world have started to take probiotics, and various probiotics have been used in a wide variety of pharmaceutical forms similar to medicines, which are known as nutraceuticals, and food products such as dietary supplements, yogurt and infant formulas. Therefore, now consumers can choose what kind and form of probiotics they prefer. However, different probiotics have distinct properties and effects found in one species or strain of probiotics do not necessarily hold true for others. Thus, it is very important to select the appropriate probiotic strain. It has become necessary to harmonise marketing criteria, evaluate the efficacy of probiotics and correctly define the effective doses.

The safe use of probiotics is an absolutely crucial point. *Lactobacilli* and *Bifidobacteria* are considered as GRAS, although certain doubts have been raised regarding their use at massive doses in immunodepressed patients or in those who undergo intestinal resection due to benign or malignant disease. Therefore, there is a great need for controlled studies in humans to further document the health benefits of probiotics as part of the human diet. Important target groups for such studies include healthy people, elevated disease risk and people for developing a disease and people searching for dietary-management techniques to control symptoms. All these groups would benefit from publicly funded research of probiotics as foods or supplements. Strains of the same probiotic species can be different, which has been demonstrated both in vitro and in animals, although similar data in humans are rare. Thus, clinical results from one study are applicable only to the strain or strains being evaluated.

Taking into account that effects of probiotics are strain specific, strain identity is important to link a strain to a specific health effect as well as to enable accurate surveillance and epidemiological studies. Both phenotypic and genotypic tests using validated standard methodology should be conducted for accurate identification of probiotic bacteria at species and strain level. Nomenclature of the bacteria must conform to the current, scientifically recognised names.

Technological efficiency of probiotics must also be determined, such as the strains ability to be grown to high yields and concentrations, to be stable, both physiologically and genetically, through the end of the shelf life of the product and at the active site in the host.

While there have been numerous health benefits attributed to probiotic lactobacilli and bifidobacteria, some of which have been discussed above, the precise mechanisms by which these bacteria function as a probiotic are yet to be understood.

Additional research is therefore required to confirm a number of these health benefits credited to probiotic bacteria. The recent technological advances in the area of genomics and proteomics are now beginning to provide one important avenue of research along which the role of probiotic bacteria and the molecular mechanisms of probiotic action can be investigated.

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