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Review

Bifidobacterium spp. and *Lactobacillus acidophilus*: biological, biochemical, technological and therapeutical properties relevant for use as probiotics

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This review focuses on the biological properties and consequent technological roles of intestinal bacteria with potential health-promoting capacities, and provides selected examples available in the literature that are pertinent to the aforementioned concepts. A comprehensive overview pertaining to the taxonomy and ecology, as well as nutritional and health effects of *Bifidobacterium* spp. and *Lactobacillus acidophilus*, is provided; particular attention is paid to their incorporation, and growth and acidification features in fermented dairy products. The typical poor growth of these species in milk is highlighted, and the use of bifidogenic and growth factors, including their nature and function, is discussed. Efforts to establish optimum environmental conditions for their growth are critically reviewed, in addition to the effects of the food and storage conditions on microbial survival. Criteria for selection of effective microbial strains for their probiotic effect are listed, and modifications to improve fermentation efficiency and shelf-life of final dairy products are suggested; among these, the incorporation of *Bifidobacterium* spp. and *L. acidophilus* into a solid matrix, such as cheese, is emphasized.

The use of *Bifidobacterium* spp. and/or *Lactobacillus acidophilus* in fermented or culture-containing milks became popular by the end of the 1970s as a result of the tremendous increase in knowledge encompassing the taxonomy and ecology of bifidobacteria. Their popularity has further increased owing to their reduced acidification during post-processing storage and their relatively high yield of L(+)-lactic acid compared with D(−)-lactic acid. In recent years, much work on bifidobacteria, regarded as microorganisms targeted for technological and therapeutic applications, was performed in Japan, but other countries also became involved (e.g. Denmark, Germany, Poland, Russia, UK and USA) [1]. Such concerted work has led to many publications and patent applications; the literature was reviewed by Rasic and Kurmann [2] in a comprehensive paper that rapidly became a classic reference. The number of patents has meanwhile drastically increased, and pertain to an increasing range of applications including new product development and starter culture production.

Definition

The word 'probiotic', from the Greek 'for life', has over the past few years been used in several different ways. It was originally proposed to describe compounds produced by one protozoan that stimulated the growth of another [3], but in the early seventies, Sperti expanded the term to encompass tissue extracts that stimulated microbial growth. Parker [4] later applied the term

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to describe animal feed supplements that had a beneficial effect on the host by contributing to its intestinal microbial balance. Consequenlty, the word 'probiotic' was applied to ''organisms and substances that contribute to intestinal microbial balance''. This general definition was, however, unsatisfactory because an imprecise word as 'substances' might include a variety of supplements, including antibiotics. Fuller [5] revised the definition to stress the importance of living cells as an essential component of an effective probiotic, and thus defined probiotic as ''a live microbial feed supplement which beneficially affects the host animal by improving its intestinal microbial balance''.

The definition of probiotic was broadened in the current decade [6]. Presently, probiotics are defined as ''viable microorganisms (lactic acid and other bacteria, or yeasts applied as dried cells or in a fermented product) that exhibit a beneficial effect on the health of the host upon ingestion by improving the properties of its indigenous microflora''.

Taxonomy

Genus *Bifidobacterium*

Bifidobacteria were first isolated and described in 1899–1900 by Tissier, who described rod-shaped, non-gas-producing, anaerobic microorganisms with bifid morphology, present in the faeces of breast-fed infants, which he termed *Bacillus bifidus*. Bifidobacteria are generally characterized as gram-positive, non-spore forming, non-motile and catalase-negative anaerobes [7]. They have various shapes including short, curved rods, club-shaped rods and bifurcated Y-shaped rods. Presently, 30 species are included in the genus *Bifidobacterium*, 10 of which are from human sources (dental caries, faeces and vagina), 17 from animal intestinal tracts or rumen, two from wastewater and one from fermented milk (Table 1). Bifidobacteria are phylogenetically grouped in the actinomycete branch of gram-positive bacteria [7], that is characterized by a high guanine plus cytosine (G+C) content, which varies from 54 to 67 mol% (Table 2). In addition, there are notable differences in physiological and biochemical properties, including cell-wall constituents (Table 2). They are saccharolytic organisms that produce acetic and lactic acids without generation of CO_2, except during degradation of gluconate (Fig. 1). Heterofermentation is initiated by splitting fructose-6-phosphate into one C_2 and one C_4 moiety. The conversion of the C_2 moiety to acetate is paralleled by the formation of heptose-7-phosphate from the C_4 moiety concomitant with the formation of a triose moiety derived from an additional molecule of fructose-6-phosphate. The heptose-7-phosphate is subsequently split into two molecules of acetate and one molecule of pyruvate. The second triose moiety left from fructose-6-phosphate is converted into lactate. Therefore, the fermentation of two moles of hexose results in three moles of acetate and two moles of lactate. The key enzyme in such glycolytic fermentation, fructose-6-phosphate phosphoketolase, may be used as a taxonomic character in identification of the genus, but does not enable interspecies differentiation.

Table 1. List of species (by alphabetical order) of the genera *Bifidobacterium* and *Lactobacillus*

Lactobacillus		*Bifidobacterium*
L. acetotolerans	*L. jensenii*[a]	*B. adolescentis*[a]
L. acidophilus[a]	*L. Johnsonii*	*B. angulatum*[a]
L. agilis	*L. kandleri*	*B. animalis*
L. alimentarius	*L. kefir*	*B. asteroides*
L. amylophilus	*L. kefiranofaciens*	*B. bifidum*[a]
L. amylovorus	*L. malefermentans*	*B. boum*
L. avarius	*L. mali*	*B. breva*[a]
L. bifermentans	*L. minor*	*B. catenulatum*[a]
L. brevis[a]	*L. murinus*	*B. choerinum*
L. buchneri[a]	*L. oris*[a]	*B. coryneforme*
L. casei subsp. *casei*[a]	*L. parabuchneri*[a]	*B. cuniculi*
L. collinoides	*L. paracasei*[a]	*B. dentium*[a]
L. confusus	*L. pentosus*	*B. gallicum*
L. coryniformis	*L. pontis*	*B. gallinarum*
L. crispatus[a]	*L. plantarum*[a]	*B. globosum*[a]
L. curvatus	*L. reuteri*[a]	*B. indicum*
L. delbrueckii	*L. rhamnosus*[a]	*B. infantis*[a]
L. farciminis	*L. ruminis*	*B. lactis*
L. fermentum[a]	*L. sake*	*B. longum*[a]
L. fructivorans	*L. salivarius*[a]	*B. magnum*
L. fructosus	*L. sanfrancisco*	*B. merycicum*
L. gallinarum	*L. sharpeae*	*B. minimum*
L. gasseri[a]	*L. suebicus*	*B. pseudocatenulatum*[a]
L. graminis	*L. vaccinostercus*	*B. psudolongum*
L. halotolerans	*L. vaginalis*[a]	*B. pullorum*
L. hamsteri	*L. viridescens*	*B. ruminantium*
L. helveticus		*B. saeculare*
L. hilgardii		*B. subtile*
L. homohiochii		*B. suis*
L. intestinalis		*B. thermophilum*

[a] Species isolated from human sources

Besides glucose, all bifidobacteria from human origin are also able to utilize galactose, lactose and, usually, fructose as carbon sources. The lactose transport system for *B. bifidum* DSM 20082 was identified recently as a proton symport, based on inhibition of lactose uptake by inhibitors of ATP synthesis and by compounds that interfere with proton and metal ionophores [8]. *Bifidobacterium* spp. are, in some instances, also able to ferment complex carbohydrates; a recent study [9], in which 290 strains of 29 species of bifidobacteria from human and animal origin were surveyed for their ability to ferment complex carbohydrates, has confirmed this potential. The substrates fermented by the largest number of species were D-galactosamine, D-glucosamine, amylose and amylopectin. Porcine gastric mucin was fermented only by *B. bifidum*, whereas *B. infantis* was the only species that could ferment D-glucuronic acid. Strains of *B. longum* fermented arabinogalactan and arabic, ghatti and tragacanth gums.

Table 2. Physiological and biochemical characteristics of *Bifidobacterium* spp. and *Lactobacillus acidophilus*[a]

Character	*Bifidobacterium* spp.	*Lactobacillus acidophilus*
Physiology	Anaerobic	Microaerophilic
Cell wall compositon:		
Peptidoglycan type	Variable, basic amino acid in the tetrapeptide is either ornithine or lysine, various types of cross-linkage	Lys-D Asp
Phospholipid composition/Teichoic acid	Polyglycerolphospholipid and its lyso derivatives, alanylphosphatidylglycerol, lyso derivatives of diphosphatidylglycerol	Glycerol
DNA-base composition Mol% G+C (guanine+cytosine)	55–67	34–37
Lactic acid configuration	L	DL
Sugar metabolism	Heterofermentative	Homofermentative

[a] Adapted from Kurmann and Rasic [17] and Mital and Garg [12]

The optimum pH for growth is 6–7, with virtually no growth at pH 4.5–5.0 or below or at pH 8.0–8.5 or above. Optimum growth temperature is 37–41°C, with maximum growth at 43–45°C and virtually no growth at 25–28°C or below. Comprehensive details of their biology are available in the extensive reviews by Rasic and Kurmann [2] and Sgorbati *et al*. [7]. The latter have provided an updated report on the taxonomy status of the genus *Bifidobacterium*, but their list of properly identified bifidobacteria does not include the moderately oxygen-tolerant species *Bifidobacterium lactis*, which was identified recently [10].

Genus *Lactobacillus*

In 1990, Moro was the first researcher to isolate facultative anaerobic straight rods from the faeces of breast-fed infants, which he typified as *Bacillus acidophilus*, a generic name for intestinal lactobacilli. Lactobacilli are in general characterized as gram-positive, non-sporeforming and non-flagellated rods or coccobacilli [11]. The G+C content of their DNA is usually between 32 and 51 mol%. They are either aerotolerant or anaerobic and strictly fermentative. Glucose is fermented predominantly to lactic acid in the homofermentative case, or equimolar amounts of lactic acid, CO_2 and ethanol (and/or acetic acid) in the heterofermentative counterpart. At present, 56 species of the genus *Lactobacillus* have been recognized (Table 1). Of these microorganisms, the most commonly suggested for dietary use are *Lactobacillus acidophilus* strains, the definition of which has changed recently.

Comprehensive genetic studies have shown that the original species actually consists of six DNA homology groups, including *L. crispatus, L. gallinarum, L. gasseri, L. amylovorus* and *L. johnsonii*. Although these species are well defined, difficulties are often encountered in allocating newly isolated strains to each of these groups. Investigations based on agglutination tests, cell wall antigen and electrophoretic and antigenic characteristics

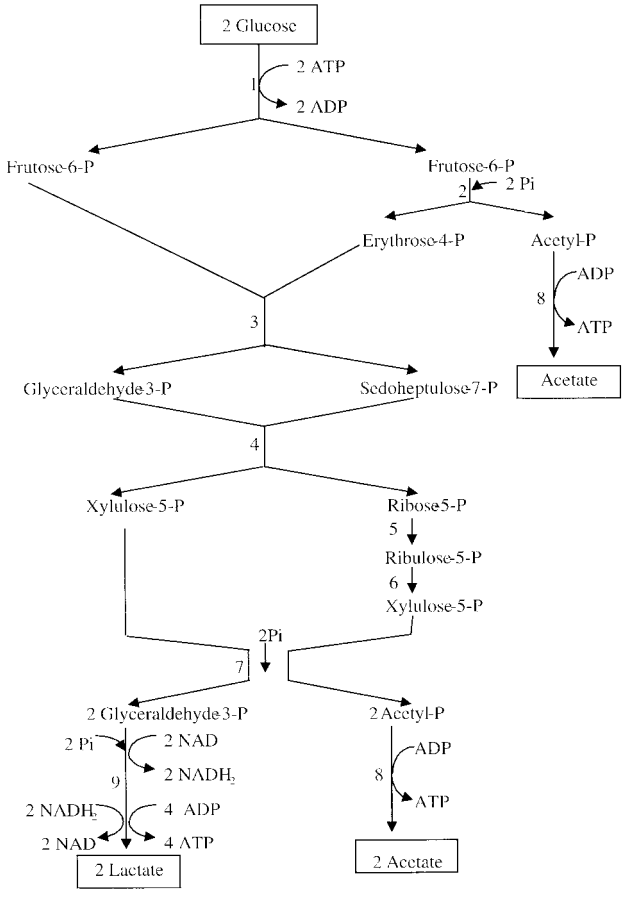

Fig. 1. Formation of acetate and lactate from glucose by the bifidum pathway. 1-hexokinase and fructose-6-phosphate isomerase, 2-fructose-6-phosphate phosphoketolase, 3-transaldolase, 4-transketolase, 5-ribose-5-phosphate isomerase, 6-ribulose-5-phosphate-3-epimerase, 7-xylulose-5-phosphoketolase, 8-acetate kinase, 9-enzymes as in homofermentative pathway (adapted from Rasic and Kurmann [2]).

of D- and L-lactate dehydrogenases (LDH) also point toward heterogeneity of these species [12]. *Lactobacillus acidophilus* is a gram-positive rod with rounded ends that occurs as single cells, as well as in pairs or in short

chains. The typical size is 0.6–0.9 μm in width and 1.5–6.0 μm in length. It is non-flagellated, non-motile and non-sporeforming, and is intolerant to salt. This microorganism does not contain cytochromes and, therefore, is benzidine negative. In addition, it is microaerophilic, so surface growth on solid media is generally enhanced by anaerobiosis or reduced oxygen pressure and 5–10% CO_2. The physiological and biochemical characteristics of this microorganism are given in Table 2. Most strains of *L. acidophilus* can ferment amygdalin, cellobiose, fructose, galactose, glucose, lactose, maltose, mannose, salicin, sucrose, trehalose and aesculine [13]. Lactose is virtually the only sugar present in milk, yet *L. acidophilus* has been reported to utilize sucrose more effectively than lactose [12]; such observations may be ascribed to differences in β-galactosidase (EC 3.2.1.23) and β-fructofuranosidase (EC 3.2.1.26) activities. While β-fructofuranosidase is a constitutive enzyme, β-galactosidase may be induced in *L. acidophilus* [14]. Moreover, both glucose and fructose moieties of sucrose are utilized by *L. acidophilus*, whereas the galactose moiety of lactose cannot be metabolized to an appreciable degree. The glucose moiety is metabolized via the Embden–Meyerhof–Parnas pathway with lactic acid as essentially the sole end product. The yield of lactic acid is 1.8 mol/mol glucose, accompanied by minor amounts of other compounds. Acetaldehyde, a carbonyl flavouring molecule, may also result from metabolism of lactose, although in some instances it may be produced from metabolism of nitrogen-containing substances, e.g. threonine; a very high activity of threonine aldolase has been found in *L. acidophilus* [15].

Growth of *L. acidophilus* may occur at as high a temperature as 45°C, but optimum growth occurs within 35–40°C. Its acid tolerance varies from 0.3% to 1.9% titratable acidity, with an optimum pH lying at 5.5–6.0.

Ecology

Genus *Bifidobacterium*

Bifidobacteria are microorganisms of paramount importance in the active and complex ecosystem of the intestinal tract of humans and other warm-blooded animals, and of honeybees [7]. They are distributed in various ecological niches in the human gastrointestinal and genitourinary tracts, the exact ratio of which is determined mainly by age and diet. The indigenous microflora of infants is dominated by bifidobacteria, which become established shortly after birth. Their proliferation is stimulated by the glycoprotein components of κ-casein in human colostrum and, to a lesser extent, human milk. The number of bifidobacteria decreases with increasing age of the individual and eventually becomes the third most abundant genus (accounting for ca. 25% of the total adult gut flora) after the genera *Bacteroides* and *Eubacterium* [16]. The onset of old age is characterized by significant reductions in the numbers of bifidobacteria, whilst contaminant bacteria such as clostridia and coliforms tend to increase, usually as a result of diminished secretion of gastric juice in this age group [17]. Furthermore, the profile of constituent species changes; *B. infantis* and *B. breve*, typical of infants, are replaced by *B. adolescentis* in adults, whereas *B. longum* persists lifelong [18]. This age profile may obviously be influenced by the dietary intake of bifidogenic factors [19] and by the host physiology [17].

Genus *Lactobacillus*

Lactobacilli are distributed in various ecological niches throughout the gastrointestinal and genital tracts and constitute an important part of the indigenous microflora of man and higher animals. Their distribution is affected by several environmental factors, which include pH, oxygen availability, level of specific substrates, presence of secretions and bacterial interactions. They are rarely associated with cases of gastrointestinal and extraintestinal infection, and strains employed technologically are regarded as non-pathogenic and safe microorganisms. Furthermore, they have the reputation of health promoters, especially in the human gastrointestinal and genitourinary tracts [20].

Growth performance

Effect of substrate on growth

Genus Bifidobacterium

The fact that bifidobacteria can grow in a semi-synthetic medium containing only lactose, three free amino acids (cysteine, glycine and tryptophan), several vitamins and nucleotides, and some minerals contrasts with the generally recognized fastidiousness of lactobacilli with regard to nutritional requirements. Certain strains were found to grow on a simplified medium comprised of lactose (as fermentable carbohydrate), buffers, minerals, ammonium salts, cysteine, and the vitamins biotin and calcium pantothenate [21]. *Bifidobacterium bifidum* var. *pennsylvanicus*, a mutant strain, requires pantothenate and N-acetylglucosamine-containing saccharides for growth and synthesis of its cell wall. The *B. bifidum* strain discovered by Tissier in 1899, as well as some other bifidobacteria, require a nitrogen source (i.e. peptides), yet neither pantothenate nor N-acetylglucosamine-containing saccharides are essential. A striking difference between lactobacilli and some strains of bifidobacteria is the ability of the latter to grow in a medium containing nitrogen in ammonium form; the remaining bifidobacteria strains require nitrogen from organic sources [22]. Evidence indicates that the nutritional properties of bifidobacteria are species-dependent, so any new strain discovered or produced needs to be extensively studied in terms of its minimal nutritional

requirements; this is the case of the newly identified *Bifidobacterium lactis* strain [23].

Lactobacillus acidophilus

Lactobacilli have complex growth requirements. They require low oxygen tension [13,24], fermentable carbohydrates, protein and its breakdown products [25], a number of vitamins of the B-complex [26], nucleic acid derivatives [27], unsaturated free fatty acids [28], and minerals such as magnesium, manganese and iron [29] for their growth. Increased amount of thiol groups present in whey protein-enriched milks favours the growth of *L. acidophilus*, whereas peptone and trypsin stimulate its acid production [22]. Addition of tomato juice (as a source of simple sugars, minerals and vitamins of the B-complex) to skimmed milk has provided evidence for enhancement of both growth of (i.e. higher viable counts and shorter generation times) and activity by (i.e. improved sugar utilization and lower pH) *L. acidophilus* [30]. These essential nutrients should, therefore, be available in the medium for establishment of a predominant microflora of lactobacilli; in their recent study on optimization of cultivation of *L. acidophilus*, Taillandier *et al.* [31] concluded that the optimum conditions are pH 6.0, 30°C, 40 g/L of glucose, 20 g/L of peptone, 20 g/L of yeast extract, 5 g/L of sodium acetate and 3 g/L of sodium citrate.

Milk as an undefined nutrient medium

Bifidobacteria are used in milk fermentations to a limited extent because of their slow growth in that matrix which, in many instances, may be considered as an artificial medium. However, milk is an essentially satisfactory medium because it contains all essential nutrients, except that amino acids and small peptides are present at insufficient concentrations (ca. 0.1 g/L) to support extended growth of bifidobacteria [2,23]. Nevertheless, cases of adaptation to milk upon multiple transfers have been reported [22]. Comparison of various milk types (bovine, ovine and caprine milks) with respect to their available nitrogen fraction has also been exploited in an attempt to further probe the influence of the raw material upon selected probiotic strains, but the higher protein and vitamin contents of ovine milk were not sufficient to sustain growth of *B. lactis* at the high rates required [32]. Moreover, the excess of fatty acid residues present in caprine milk were indicated as responsible for the poor growth of *L. acidophilus*.

In general, bifidobacteria grow better in rich synthetic media, viz. TPY and MRS broths, than in milk; however, said media are complex and costly for large-scale propagation of bifidobacteria. Moreover, unless the cells harvested from such media are extensively washed before incorporation, they may confer off-flavours to the finished dairy products. To manufacture a quality product, both in terms of texture and viability of bifidobacteria, a milk-based medium is usually required because the casein content of milk protein is higher than that of synthetic media (which are generally low in solids). Thus, improvement of growth conditions for the different strains of bifidobacteria in milk, in particular via addition of various easily-available nitrogen sources or redox potential-lowering substances, has provided the impetus for many studies [23,24,32–34], and less fastidious, mildly-acidifying strains can thus be claimed as key candidates for successful bifidobacteria-containing (bio)products.

Bifidogenic and growth factors

Definition

There are clear differences between bifidogenic factors and growth factors for bifidobacteria in terms of nature and function. Bifidogenic factors are defined as compounds, usually of a carbohydrate nature, that survive direct metabolism by the host and reach the large bowel or cecum, where they are preferentially metabolized by bifidobacteria as source of energy. Bifidogenic factors may fall under the new concept of prebiotics, which are defined as non-digestible food ingredients that beneficially affect the host by selectively stimulating the growth and/or activity of one, or a limited number of, bacteria in the colon, and which may thus improve the health of the host [35]. In contrast, growth factors are compounds that promote the growth of bifidobacteria *in vitro* but cannot be delivered to the large bowel or cecum to selectively promote proliferation of bifidobacteria [19]. Studies on the sources and applications for either of these types of compounds have been well documented [19], yet comprehensive reviews of the state of the art in this field are still lacking. Table 3 summarizes established knowledge and more recent developments in the isolation, identification and application of both bifidogenic and growth factors.

Bifidogenic factors

Fructooligosaccharides. The major carbohydrate sources for bacterial growth in the colon are provided by dietary or endogenous means. In this respect, non-digestible oligosaccharides have been used in the diet specifically to increase relative numbers of bifidobacteria in the gut microflora [36–39]. In particular, promising results have been achieved *in vitro* using inulin [37] and especially fructooligosaccharides (FOS), in both batch culture [38] and chemostat culture systems [39]. Among the different types of (linear and branched) FOS studied, oligofructose, a linear chain molecule comprising glucose and fructose at a degree of polymerization of four, exhibited the highest bifidogenic effect. Earlier *in vivo* clinical studies reported a ten-fold increase of the bifidobacterial population in the large bowel upon addition of neosugars (i.e. short-chain FOS) to the human diet at a ratio of 15 g/d [36], which correlated well with the increased

Table 3. Origin and effects of possible bifidogenic and growth factors for *Bifidobacterium* spp

Name	Origin	Strains studied	Effects	Reference
Bifidogenic factors				
Casein bifidus factor (CBF)	Bovine casein submitted to acid and enzyme (papain and pepsin) hydrolysis	*Bifidobacterium bifidum*	Growth promotion, best with acid CBF; CBF supplies both peptides and carbohydrates necessary for growth	Zbikowski and Ziajka [115]
Human κ-casein and glycomacropeptide derived therefrom	Intact κ-casein from human milk and hydrolysed with chymosin and pepsin	*Bifidobacterium infantis* S12	Small growth-promoting activity of intact κ-casein enhanced upon enzymatic hydrolysis Sugar portion and polypeptide portion as important components of bifidus factor	Azuma *et al.* [116]
κ-Casein enzymatic digest	Bovine milk casein digested by trypsin	*Bifidobacterium* spp. (*B. bifidum*, *B. longum*)	Growth promoting activity in fully synthetic medium associated with disulfide/sulfhydryl (cystine) residues and a certain tryptic peptide-unidentified bifidogenic factor	Poch and Bezkorovainy [50]
Casein macropeptide (CMP)	Bovine milk	*Bifidobacterium* spp.	Growth promoting activity	Abd-El-Salam *et al.* [117]
N-acetylneuraminic acid (Neu-Ac)-containing substances (NeuAc, sialyl lactose, glycomacropeptide)	Bovine milk	*Bifidobacterium* spp. and lactobacilli	Growth promoting activity of *B. breve*, *B. bifidum*, *B. infantis*	Idota *et al.* [129]
transgalactosylated oligosaccharides galactosyl galactose, galactosyl glucose	Transgalactosylation enzymatic reaction on lactose	*Bifidobacterium* spp.	Ratio of intestinal bifidobacteria to total bacteria increased from 0.28 to 0.51; decreased levels of toxic short chain fatty acids, faecal pH, faecal ammonia	Ito *et al.* [118]
Fructans	Ash-free white powder from tubers of Jerusalem artichoke	*Bifidobacterium* spp. (*B. infantis* ATCC 15697, *B. adolescentis* ATCC 15703, *B. longum* ATCC 15707)	Growth promoting activity	Yamazaki and Matsumoto [119]
Xylooligosaccharides (xylobiose)	Wheat bran, corn cobs aspen, wheat straw	*Bifidobacterium* spp.	Increase of intestinal bifidobacteria with 1–2 g/d	Okazaki *et al.* [120]
Oligosaccharides	Onion, garlic, chicory root, burdock, asparagus, Jerusalem artichoke, soybeans, wheat bran	*Bifidobacterium* spp.	Proliferation of bifidobacteria and suppression of putrefective bacteria Prevention of constipation and pathogenic diarrhoea	Yamada *et al.* [121] Tomomatsu [41]
Fructo-oligosaccharides (inulin, oligofructose)		*Bifidobacterium spp.*	Growth and acid promoting activity; good carbon and energy source	Gibson and Wang [38,39]
lactulose		*Bifidobacterium* spp.	Growth promoting activity; activation of immune response and infection limitation	Anjana *et al.* [69] Mizotal [47]
2-Amino-3-carboxy-1,4-naphthoquinone	Cell-free filtrate and methanol extraction fraction of cells of *Propionibacterium freudenreichii* 7025	*Bifidobacterium longum* 6001	Growth stimulatory activity at 0.1 ng/ml of the bifidobacterial population in the intestinal microflora	Mori *et al.* [49]
Growth factors				
Depolymerized alginates	Sodium alginate (sea-weed *Lessonia* spp.) depolymerized by bacterial alginate lyase	*Bifidobacterium* spp. (*B. breve*, *B. longum*, *B. adolescentis*, *B. bifidum*, *B. infantis*)	Growth promoting activity in skim-milk	Akiyama *et al.* [122]
Casein hydrolyzates	Alcalase, chymotrypsin and trypsin hydrolysis of casein followed by ultrafiltration (MW cut-off 30 kDa) commercial hydrolyzate (N-Z Case)	*Bifidobacterium* spp. (*B. bifidum* var. *pennsylvanicus*, *B. adolescentis*, *B. breve*, *B. infantis*, *B. longum*)	Growth stimulation of *B. infantis* and *B. breve* in synthetic medium; commercial hydrolyzate promoted better growth of all strains than ultrafiltered hydrolyzates	Proulx *et al.* [53]
Casein hydrolyzates	alcalase, chymotrypsin and trypsin hydrolysis of casein followed by two-step ultrafiltration (MW cut-off 1 kDa)	*Bifidobacterium* spp. (*B. breve*, *B. infantis*, *B. longum*)	Trypsin peptide fraction at 2% in synthetic Garches medium promoted best strain growth; alcalase amino acid fraction at 1–2% repressed growth of, and acid production by *B. breve* and *B. longum*	Proulx *et al.* [54]
Bienzyme hydrolyzed solutions	Soybean and sword jackbean (*Canavalia gladiata*) — China	Bifidobacterium *spp.* for beverage production	Growth stimulation reduction in renneting time	Yang *et al.* [123]
Milk hydrolyzates	Proteinase (MHP) and neutrase (NHN) hydrolysis of UHT milk to different degrees	*Bifidobacterium lactis* and *Lactobacillus acidophilus*	24-h hydrolysis of MHP at 5% promoted best growth of *B. lactis* in bovine, ovine and caprine milks *L. acidophilus* was not affected by either MHP or MHN; free amino acid fraction alone was less efficient than mixture of amino acids and low molecular weight peptides (< 500 Da) of 24-h MHP	Gomes and Malcata [30,22]

release of volatile fatty acids and concomitant decrease in pH (by 0.3 units), together with a reduction in the counts of *Enterobacteriaceae* from 1×10^8 to 4×10^7 cfu/g.

All FOS studied are oligosaccharides that occur in nature, mainly of plant origin (Table 3), and are manufactured industrially from sucrose syrup obtained from sugar beet by an enzymatic process in which β-fructofuranosidase (EC 3.2.1.26) derived from *Aspergillus niger* plays an essential role. These compounds are $\beta(2\rightarrow1)$ linked fructans, typically polydisperse, with degrees of polymerization in the range 2–35; they are resistant to the strong hydrolytic capacities of the human intestinal enzymes and, therefore, reach the colon essentially unaltered, where they behave like soluble dietary fibre and are digested by the colonic flora [40]. Other properties, applications and health aspects closely related to their effect on bifidobacteria growth in the colon have been discussed in several reviews [41,42]. The increased intake of oligofructose-containing foods, or alternatively the increased incorporation of these prebiotics into foods with a significant carbohydrate content, e.g. ice cream, cakes, infant formula feeds and milk-based products [43], offer a rational alternative to the more commonly employed incorporation of viable bifidobacteria into fermented milks. In fact, the main advantage is that survival in the product becomes easier to assure.

Xylooligosaccharides (e.g. xylobiose) and transgalactosylated oligosaccharides (e.g. lactosucrose) are also known as effective promoters of proliferation of intestinal bifidobacteria (Table 3). The former compounds occur naturally, whereas the latter are available only via industrial synthesis. Their importance as bifidogenic factors is reflected in the large number of publications focussed on the preparation of galactooligosaccharides through bioconversion of lactose using various microbial enzymes [44–46]. Oligosaccharides that contain maltose, mannose and soya are also possible bifidogenic factors (Table 3).

Several studies, reviewed by Modler [19] and Mizota [47], reported that the lactose-derivative lactulose, a dissaccharide composed of galactose and fructose (4-*O*-β-D-galactopyranosyl-D-fructose), can exert bifidogenic effects on bifidobacteria resident in the lower intestine and hence can produce many of the desirable effects ascribed to FOS. These effects include improvement of hyperammonaemia and suppression of production of toxic short-chain fatty acids (butyric and valeric acids); in addition, this compound has many other health and medical effects [47]. Lactulose has been used to increase the numbers of viable lactobacilli in the intestine of bottle-fed infants; other candidates, including such sugar alcohols as lactitol and glucosyl-inositol (Table 3), may also be useful in increasing bifidobacteria numbers.

Neosugars, FOS and lactulose are commercially available and widely used in Japan and Europe as bifidogenic compounds. Although safe from a toxicological standpoint and responsible for many positive effects, they are not necessarily ideal bifidogenic factors: to be effective, the bifidogenic factor must promote a selective fermentation in mixed culture so that the profile of the large intestinal microflora is altered towards a potentially healthier community. Unfortunately, both types of compounds can be metabolized by a number of gas-producing bacteria and hence may cause intestinal discomfort.

Quinone compounds. Roland *et al.* [48] reported consistent data *in vitro* (mixed culture of *B. bifidum* and 48 h-old cultures of *Propionibacterium freudenreichii* in MRS broth) and *in vivo* (administration of 5×10^{10} cfu of propionibacteria to 19 males over 2 weeks) in attempts to check the validity of the hypothesis that propionibacterium (a microorganism used in the manufacture of Swiss-type cheese) promotes the growth of bifidobacteria. A parallel study showed that, in a human faecal culture contained in a two-stage, continuous culture system, addition of a quinone compound produced by *P. freudenreichii* cells stimulated the growth of bifidobacteria, thus suggesting a bifidogenic effect regarding regulation of their numbers in the intestine [49] (Table 3). However, more stringent studies are needed to clarify the specific action of this, and other, related quinone compounds upon bifidobacterial growth.

Growth factors

Within the range of compounds investigated, derivatives of human and bovine milks (Table 3) have proven good candidates for the enhancement of growth of bifidobacteria *in vitro*. According to Poch and Bezkorovainy [50], growth-promoting activity of bovine milk κ-casein was ascribed to its conjugated cystine residues, an observation more recently confirmed and refined in a study aimed at comparing the growth-promoting effects of various biological materials on *B. longum* [51] using a synthetic medium. All tentative growth-promoting factors studied failed to exhibit their growth-promoting activity when their dissulfide bonds were reduced or alkylated, thus suggesting that their composition in sulfur-containing peptides might be the key to said effect. The major whey proteins, α-lactalbumin and β-lactoglobulin, were also found to be excellent growth promoters, a trait validated after the studies by Petschow and Talbott [52] on the effectiveness of whey fractions of both human milk and bovine milk. Yeast extract, a commercial product, was found to be an effective growth promoter, and is most often used at concentrations that vary between 0.1 and 0.5%(v/v). A previous study [33] has shown, however, that addition of yeast extract at 0.25%(v/v) did not stimulate growth of *B. infantis*, even though acid production was enhanced; it further demonstrated the good growth-promoting activity of β-glycerophosphate in the presence of cysteine for *B. bifidum* and *B. infantis*, but not for *B. longum* ATCC 15708.

Other biological compounds identified as growth factors for bifidobacteria include threonine [25], cysteine [33], enzyme-treated chlorella, peptone and trypticase [19], as well as dextrin, maltose and extracts from carrots (coenzyme A), potatoes and corn [2], and commercial casein hydrolysates and ultrafiltered hydrolysates [53,54] these hydrolysates are, in general, produced by breaking down casein with proteases, peptidases or strong acids to different yields. Proulx *et al.* [53,54] claimed that peptides obtained from casein hydrolysates might be a preferable source of nitrogen than free amino acids for dairy-related bifidobacteria (Table 3). Apart from that, the development of hydrolysates with better quality at lower cost is essential. Gomes *et al.* [23] expanded on this work using milk (as economical source), hydrolysed to several extents by two alternative proteinases, as nitrogen source to promote growth of, and acidification by *B. lactis* and *L. acidophilus*; these authors established that both the nature of the enzyme and the degree of hydrolysis are important criteria towards attainment of consistent growth of the aforementioned strains, an observation that confirms the growth-limiting characteristics of available nitrogen in the form of low molecular weight peptides (<500 Da) and free amino acids that are more easily assimilated by *B. lactis* (Table 3).

Nutritional and health values of fermented milk products

Historical background

Nutritional and health aspects of functional foods incorporating probiotic bacteria have received considerable attention, and eventually led to numerous purported claims in the literature. These potential advantageous properties, initially summarized by Gurr [55] and more recently reviewed in detail by Gilliland [56], Marteau and Rambaud [57] and Yaeshima [58], are summarized in Table 4 and will be critically described in the following subsections. Despite the many studies on the beneficial nutropharmaceutical aspects of probiotic bacteria, the results obtained are highly variable and sometimes inconsistent with one another; hence, no clear, unequivocal evidence for the actual existence of some of these benefits is yet available, which then renders health claims more difficult to establish. To eliminate these drawbacks, efforts have been pursued worldwide to rationally organize future research using clearer and more reliable bases (via well-designed, randomized and placebo-controlled, double-blind studies) and exploiting statistically validated methods, with special emphasis on intestinal integrity and immune modulation. Increasing numbers of colonization and dose-response studies defining the required doses have been published [59].

Nutritional value

The nutritional benefits of probiotics have been mostly studied in milk-based products fermented with lactobacilli and bifidobacteria. These products contain a great many chemical compounds depending on the type of milk used (usually cow's, ewe's or goat's), on the type of microorganisms added (and their specific biochemical activities) and on the manufacturing processes employed. They are characterized by a lower level of residual lactose and higher levels of free amino acids and certain vitamins than non-fermented milks. Furthermore, they preferentially contain L(+)-lactic acid (that is more easily metabolized by human beings than D(-)-lactic acid) produced by bifidobacteria in addition to acetic acid [2], thereby preventing manifestation of metabolic acidosis in infants below one year of age. Moreover, the L(+)-lactic acid absorbed in the intestine

Table 4. Potential health and nutritional benefits of functional foods prepared with probiotic bacteria

Beneficial effect	Possible causes and mechanisms
Improved digestibility	Partial breakdown of proteins, fats and carbohydrates
Improved nutritional value	Higher levels of B vitamins and certain free amino acids, viz. methionine, lysine and tryptophan
Improved lactose utilization	Reduced lactose in product and further availability of lactase
Antagonistic action towards enteric pathogens	Disorders, such as functional diarrhoea, mucous colitis, ulcerated colitis, diverticulitis and antibiotic colitis controlled by acidity, microbial inhibitors and prevention of pathogen adhesion or pathogen activation
Colonisation in gut	Survival in gastric acid, resistance to lysozyme and low surface tension of intestine, adherence to intestinal mucosa, multiplication in the intestinal tract, immune modulation
Anticarcinogenic effect	Conversion of potential pre-carcinogens into less harmful compounds Inhibitory action towards some types of cancer, in particular cancers of the gastrointestinal tract by degradation of pre-carcinogens, reduction of carcinogen-promoting enzymes and stimulation of the immune system
Hypocholesterolemic action	Production of inhibitors of cholesterol synthesis. Use of cholesterol by assimilation and precipitation with deconjugated bile salts
Immune modulation	Enhancement of macrophage formation, stimulation of production of suppressor cells and γ-interferon
Vaccine vehicle	Naturally occurring or rDNA vaccinal epitopes

is used as energy source, with an energy yield of 15 kJ/g, that compares well with 16 kJ/g for lactose [55].

Lactobacillus acidophilus and bifidobacteria have also been reported to synthesize folic acid, niacin, thiamine, riboflavin, pyridoxine and vitamin K, which are slowly absorbed by the body [2,60]. The vitamins of the B-complex are frequently obtained as natural ingredients in foods, so addition of bifidobacteria to the diet will more effectively help meet those requirements. The bioavailability to the host of such minerals as calcium, zinc, iron, manganese, copper and phosphorus may also be enhanced upon consumption of fermented dairy products containing bifidobacteria via lowering the gastric pH (which facilitates ionization of minerals, a requirement for absorption) and improved digestibility of the protein [2].

Therapeutic effects

Effects on intestinal disturbances and intestinal infections

The intact intestinal epithelium, together with the normal intestinal microflora represents a barrier to movement of pathogenic bacteria, antigens and other noxious substances from the gut lumen to the blood. In healthy subjects this barrier is stable, thus protecting the host and assuring normal intestinal function. When either the normal microflora or the epithelial cells are disturbed, as triggered by dietary antigens, pathogens, chemicals or radiation, defects in the barrier mechanisms become apparent; altered permeability facilitates blood invasion by pathogens, foreign antigens and other harmful substances. Several reviews [20,57] have documented the use of probiotic bacteria to treat intestinal disorders, e.g. acute rotavirus diarrhoea in children, as well as food allergy and colonic disorders driven by pelvic radiotherapy and sometimes associated with development of colon cancer. These examples are among those included in Table 5 that illustrate the state of the art in what concerns application of several probiotic strains for therapeutic purposes. In all such disease states, altered intestinal microflora, impaired gut barrier and different types of intestinal inflammation are often present [20], thereby offering a rationale for the effective use of probiotic bacteria, not only for treatment but especially for prevention of such changes. Among the possible mechanisms responsible for favourable clinical responses, one may quote (i) promotion of the immunologic and nonimmunologic defense barriers in the gut [61,62] (ii) capacity to prevent pathogen adherence or pathogen activation via production of inhibitory metabolites, such as organic acids, hydrogen peroxide, bacteriocins and deconjugated bile salts [63–65], or via ferrous iron uptake thus making it unavailable for pathogens [66], and (iii) modification of bacterial enzyme activity and subsequent influence on gut mucosal permeability [20] (see Table 5). Probiotic bacteria are able to survive gastric conditions (characterized by pH 1.5–2.0 during fasting and 4.0–5.0 after a meal), colonize the intestine (at least temporarily) by adhering to the intestinal epithelium in a process mediated by adhesins on the surface of the bacteria (proteins, polysaccharides and cell wall-associated components), and offer new dietary alternatives for the stabilization of the intestinal microflora. In clinical nutrition, for instance, special diets are used for the treatment of intestinal inflammation, radiation enteritis and inflammatory bowel disease: probiotic bacteria do play a role in alleviating these disturbances, so the introduction of such strains into clinical foods and special dietary foods is in order.

Potential antitumor activity

The colonic flora has been shown to be involved in colonic carcinogenesis [20] This effect is mediated by microbial enzymes such as β-glucuronidase, β-glucosidase, nitroreductase and urease, which convert procarcinogens into carcinogens. Experiments performed in animal models showed that a few strains of *L. acidophilus* and *Bifidobacterium* spp. are able to decrease the levels of enzymes responsible for activation of some procarcinogens, and consequently decrease the risk of tumor development [12,17]. Studies in the human body using the same enzymes as end-points demonstrated a general reduction in microbial enzyme activities and concomitant decrease in faecal mutagenicity [57,67] The beneficial effect could be accounted for by a favourable change in the composition of the intestinal flora following introduction of the aforementioned bacteria.

Furthermore, it has been demonstrated, at least for *Lactobacillus casei* (Shirota strain), that there is a potential opportunity in the area of 'dietary prevention' of cancer. Morotomi [68] concluded that the parenteral administration of this strain had antitumor and immunostimulating effects on experimentally implanted tumors; similar effects have been observed in the case of oral administration. The feeding of *B. longum* to rats was recently shown to significantly reduce formation of such preneoplastic markers as azoxymethane-induced colonic aberrant crypt foci; furthermore, the production of glutathione S-transferase, a Phase II enzyme marker, was also increased [69].

The mechanisms that appear to be involved in antitumor activity of systemically administered bifidobacteria include activation of such non-specific cellular factors as polymorphonuclear leucocytes [70], macrophages [71] and natural killer cells via regulation of γ-interferon production [72]; γ-interferon also exhibits antiviral and antiproliferative effects. Orally administered bifidobacteria play a role in increasing, to some extent, the production of IgA antibodies [73] and the functions of Peyer's patch cells [74,75]. It should be noted that closely related strains of *Bifidobacterium* spp. and *L. acidophilus* differ between each other in immunostimulatory properties, so some strains may not have all those properties necessary for activity. Marin et al. [76] have recently

Table 5. Therapeutic applications of *Bifidobacterium* spp. and *Lactobacillus acidophilus* as indigenous microorganisms or dietary adjuncts, and corresponding reported effects

Strain	Experimental parameters	Observations	Effect	Reference
Indigenous microorganism				
Lactobacillus acidophilus RP32, *L. acidophilus* P47	Analysis of cells and spent broth culture in 1% MRS broth containing oxgall	Removal of cholesterol efficient in the presence of oxgall under anaerobic conditions Presence of oxgall caused appearance of less cholesterol in broth and more in cells RP32 and P47 are bile resistant but only RP32 assimilates cholesterol in the presence of bile salt Strain specificity of *L. acidophilus* is suggested	Hypocholesterolemic action (direct action of culture on cholesterol assimilation or co-preciptation with bile salts)	Gilliland [56]
Lactobacillus acidophilus RP32, *L. acidophilus* MUH41, *L. acidophilus* MUH79, *L. acidophilus* MUH117, *L. acidophilus* CH1, *Bifidobacterium bifidum* MUH80	Analysis of cells and spent broth culture in 1% MRS broth containing oxgall	RP32 is effective in the precipitation of cholesterol and bile salts at low pH Absence of bile acids in either supernatant or cells following incubation	Hypocholesterolemic action (co-precipitation of cholesterol in the presence of deconjugated bile salts and low pH)	Klaver and van der Meer [80]
Bifidobacterium longum BB 536 *B. breve* ATCC 15700, *B. animalis* ATCC 25527	Resting cell assays culture in TPY medium containing oxgall	Removal of cholesterol by precipitation and assimilation *B. longum* BB536 is the most efficient in deconjugating bile salts	Hypocholesterolemic action	Tahri *et al.* [81]
Lactobacillus acidophilus 301	Inhibitory activity of *L. acidophilus* against different pathogens (*S. typhi* 83, *P. vulgaris* 204, *S. aureus* C2-T10, *Y. enterocolitica* 03, *E. coli* 6) when grown simultaneously in milk; study of growth-inhibition profiles	Sharp decrease of pathogen populations by 8 h of incubation Inhibition shown by *L. acidophilus* is due to acid production and production of antibiotic-like compounds	Antibacterial activity	Gupta *et al.* [124]
Lactobacillus acidophilus TK8912	Study of mode of action of acidocin 8912, a bacteriocin produced by *L. acidophilus* TK8912	Inhibitory action causes increase in permeability of cytoplasmic membrane of target cells by both dissipation of the proton motive force and pore formation	Bactericidal activity	Tahara *et al.* [64]
Bifidobacterium spp. (*B. breve*, *B. longum*, *B. bifidum*, *B. infantis*)	Enterocyte-like Caco-2 mucus-secreting HT29-MTX cells for adhesion studies; characterization by light microscopy, SEM: type of mechanism involved in interaction with eukaryotic cells	Adhesion to human intestinal epithelial cells; bacterial maintenance in the intestinal tract and inhibition of enteropathogen-cell interactions	Stabilizer of gut mucosal barrier, immune enhancer	Bernet *et al.* [61]
Bifidobacterium spp.	Six different strains; procarcinogens, nitrite and nitrosamines, tested	No growth inhibition at $<50\ \mu mol^{-1}$ nitrite and $<200\ \mu g\ ml^{-1}$ nitrosamine Nitrite elimination by non-enzymic mechanism, possibly via acid production *B. longum* metabolized nitrosamine by intracellular mechanism	Anticarcinogenic effect	Grill *et al.* [125]
Bifidobacterium infantis (NCFB 2205) *Bifidobacterium* spp.	Incubation of strains; with *E. coli* and *C. perfringens* in: batch fermentations, two-vessel chemostat, single-stage continuous culture system, serum tube experiments and plate experiments	Inhibitory effect possibly related to acid production and excretion of antimicrobial substance with broad spectrum of activity (*Salmonella*, *Listeria*, *Campylobacter*, *Shigella*, *V. cholerae*)	Antibacterial activity (possible protection against gastroenteritis)	Gibson and Wang [63]
Lactobacillus acidophilus ATCC 4356 *Bifidobacterium thermophilum* ATCC 25866, *B. breve* ATCC 14917	Cells grown in modified TPY medium, Fe^{2+} accumulation studied in two media at pH 5.0 and pH 6.5 and effect of glucose as energy source	Transport of Fe^{2+} into cell and partial oxidation to Fe^{3+} by intracellular putative ferroxidase Enzyme uses oxygen as substrate to promote Fe^{2+} oxidation and requires glucose as positive effector Extracellular oxidation by *L. acidophilus* via release of H_2O_2 Fe^{2+} unavailable for proliferation of pathogens	Nutritional immunity (antibacterial activity)	Kot *et al.* [62–66]
Bifidobacterium longum SBT 2928 and other *Bifidobacterium* spp.	Competitive binding of *Bifidobacterium* spp. and *E. coli* Pb 176 to ganglio-tetraosylceramide (GA1) examined *in vitro* using TLC overlay binding suppression assay, study of the inhibitory factor(s) in the *Bifidobacterium* culture supernatant fluid	Competitive exclusion of *E. coli* from GA1 by *Bifidobacterium* cells Binding inhibitor produced by *B. longum* SBT 2928 is a proteinaceous molecule Inhibition of pathogenic *E. coli* binding to GA1 on surface of human intestinal mucosa	Antibacterial activity	Fujiwara *et al.* [65]

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Table 5 (*continued*)

Strain	Experimental parameters	Observations	Effect	Reference
Bifidobacterium longum SBT 2928 (BL2928)	Effects on glucose consumption, interleukin (IL-1) production and cytotoxicity of phagocytes studied *in vitro*, BL2928 has high level of mitogenic activity (5 strains selected)	Glucose uptake and cytotoxicity enhanced IL-1 production stimulated even at low doses of BL2928 Stimulatory activity on IL-1 production correlated with mitogenic activity	Immune enhancer	Kado-Oka *et al.* [74]
Bifidobacterium adolescentis M101-4	Isolation, purification and characterization of water-soluble fraction of polysaccharide nature (SHF) SHF was characterized by galactofuranosyl residues *In vitro* assay of murine splenocytes and Peyer's patch cells to test mitogenic activity	Mitogenic activity was exhibited by all fractions of *B. adolescentis* SHF stimulated proliferation of murine splenocytes	Immune enhancer	Hosono *et al.* [75]
Dietary Adjunct				
Lactobacillus acidophilus, Streptococcus thermophilus	Weaning pigs divided into 4 groups, each group given one of 4 diets (basal diet at 2 concentrations of basal + acidophilus yoghurt) for 14 wk Cholesterol, triglycerides and lipoproteins analysed	Reduction of triglycerides and serum cholesterol with 30% yoghurt 15 d-residual effect	Hypocholesterolemic action	Jones *et al.* [78]
Lactobacillus acidophilus LA2	Six healthy subjects administered milk fermented with *L. acidophilus* LA-2 Comparison of faecal mutagenicity before and after administration	Suppression of faecal mutagenicity after ingestion of fermented milk *Lactobacillus* spp. and *Bifidobacterium* spp. populations increased in faeces of all subjects	Antimutagenic effect in human intestine	Hosoda *et al.* [67]
Bifidobacterium longum and *Lactobacillus acidophilus*	Duration of experiment 1 wk, 16 females and 8 males aged 19–59 yr, placebo or mixture *E. faecium* + *B. longum* or mixture *L. acidophilus* + *B. longum* + *L. delbrueckii* subsp. *bulgaricus* + *S. thermophilus* Microbiological evaluation of jejunal aspirates and faecal samples	Reduced anaerobe:aerobe ratio in faeces by 3-fold during treatment Distal intestinal microflora affected	Colonization of intestinal tract	Nielsen *et al.* [126]
Bifidobacterium longum	Duration of experiment of 3 wk, 12 healthy volunteers Yoghurt (500 ml/d) added with *B. longum* (10^9 bacteria/l) and lactulose (5 g/l) or conventional yoghurt	Increased excretion of bifidobacteria after consumption Breath-hydrogen exhalation elevated great Stability/balance of human faecal flora	Colonization of intestinal tract, possible role in colon carcinogenesis	Bartram *et al.* [127]
Lactobacillus acidophilus LA1 *Bifidobacterium bifidum* Bb12	Duration of experiment of 3 wk, two groups of healthy volunteers Each consumed fermented milk product containing one of the two strains, blood samples studied	Faecal colonization persists for 6 wk after ingestion Increases in phagocytosis of *E. coli* coincide Nonspecific, antinfective mechanisms of defense enhanced	Immune enhancer	Schiffrin *et al.* [128]
Bifidobacterium longum (added with lactulose)	61 male Fisher 344 weaning rats divided into 4 groups of 15 rats, each group given one of 4 diets for 13 wk; subcutaneous injection of azoxymethane at 7–8 wk of age; aberrant crypt foci (ACF) in colon and glutathione S-transferase (GST) in colonic mucosa and liver were analyzed	Feeding of lactulose and *B. longum* solely and in combination reduced the number of ACF and total number of aberrant crypts Colonic mucosal GST levels are significantly Higher positive correlation between higher cecal pH and number of ACF	Additive antitumor effect	Anjana *et al.* [69]

indicated, following an *in vitro* study of 14 bifidobacteria strains, that four, used commercially for incorporation in dairy foods (i.e. *Bifidobacterium* Bf-1, Bf-6, Bf-12 and Bf-13) showed a great capacity for cytokine stimulation by macrophages, and could thus potentially modulate the immune response either directly, or indirectly via incorporation as food additives.

Potential hypocholesterolemic actions

Several studies indicated a statistically significant reduction in serum cholesterol during consumption of large doses (680–5000 mL/d) of certain fermented dairy bioproducts [57] and the theory postulated for the hypocholesterolemic effect was the presence of such organic acids as uric, orotic and hydroxymethylglutaric, which actually inhibit cholesterol synthesis [77]. Unfortunately, these data have not permitted extrapolation to more realistic consumption conditions (the doses used are indeed excessively high), and were impoverished by the lack of controls and the fact that in some cases the strains used were ill-defined. Although a few experimental animal studies (Table 5) were successful in positively attributing cholesterol-lowering properties to *L. acidophilus* contained in dairy products administered as acidophilus yoghurt to pigs [78] and mice [79], results cannot be extrapolated to humans since there are differences in the regulation of cholesterol metabolism between animals and humans. Besides the hypocholesterolemic action, Akalin *et al.* [79] also reported a higher number of faecal lactobacilli and fewer coliforms in mice that received acidophilus yoghurt than in those that received plain yoghurt, thus indicating that *L. acidophilus* established itself more effectively in the murine intestinal tract than did *L. bulgaricus* in the yoghurt diet.

The mechanisms by which fermented milk products are able to diminish serum cholesterol have not to date been fully demonstrated. *In vitro* studies by Gilliland [56] showed that bifidobacteria and *L. acidophilus* are able to utilize cholesterol in growth media, both by assimilation and precipitation with deconjugated bile salts under acidic conditions. Their suggestion that the presence of bile salts is a prerequisite for cholesterol assimilation by bifidobacteria was confirmed by the observations of Klaver and van der Meer [80] (Table 5), and more recently by those of Tahri *et al.* [81,82], who went one step further and investigated the nature of the bile salts; according to these authors [82], assimilation of cholesterol was higher in the presence of trihydroxyconjugated bile salts than dihydroxyconjugated bile salts. The aforementioned bacterial assimilation of cholesterol in the intestine may reduce its absorption from the digestive tract into the blood system.

Despite the existing and ever increasing body of knowledge, considerable research is still warranted to fully elucidate causal relationships between the consumption of probiotic bacteria and reduced serum cholesterol levels in humans.

Dairy products supplemented with *Bifidobacterium* spp. and/or *Lactobacillus acidophilus*

General characteristics

The species most frequently employed in the production of probiotic dairy products are of human intestinal origin because it is generally accepted that they are better suited to the physiological needs of the host and can more easily colonize his/her intestine than wild strains, or strains that exist in the colon of other animals. These human-borne strains include *Bifidobacterium adolescentis*, *B. bifidum*, *B. breve*, *B. infantis*, *B. longum*, *Lactobacillus acidophilus*, *L. casei* subsp. *rhamnosus* and *Enterococcus faecium* [22,83]. Results of recent studies support the use of selected strains of *B. longum* as dietary adjuncts in dairy products, with *B. adolescentis* and *B. infantis* as adequate alternatives [84]. It is again worth noting that each strain within these species exhibits unique properties, viz. growth rate, metabolic rate, proteolytic activity and flavour promotion. Consequently, careful management of such factors via tailored manufacturing technologies will enable these species to meet with varying degrees of success in multiple industrial applications. Cases of milk products fermented by bifidobacteria of animal origin have also been reported: these strains are much easier to cultivate and can withstand the adverse conditions encountered during industrial production, viz. low pH and presence of detectable levels of oxygen. In addition to a beneficial role after consumption [85,86], the recently identified *B. lactis* strain is also a promising candidate due to its good acid and oxygen tolerance [23,87].

The most frequent functional food products in the market are of dairy origin (Table 6); Japan, a leading country in their manufacture, produces and markets more than 50 different dairy products containing viable cells. Similar trends are also observed in such developed European countries as France, Germany and Sweden (where probiotic products account for ~25% of all fermented milk products). It is estimated that there are ~80 bifido-containing products available in the world market, and more than 45 dairy plants in Europe currently produce bifido-acidophilus products [83]. Besides the commercial products, research studies have been more and more focused on fermented milks [12,88], yoghurt [1], frozen yoghurt and desserts [89,90], ice cream [91,92], cheese [93–95,106], fermented soya milk [96] and soya yoghurt [97]. The designation of new products is normally obtained via adding the prefix 'Bio' to its traditional (sometimes partially simplified) name (e.g. Biogurt, Biodrink, Biokys). The probiotic strains of bifidobacteria and *L. acidophilus* may be added, either alone or following combination with other lactic acid bacteria during fermentation, to the final fermented

Table 6. Commercial products containing *Bifidobacterium* spp. and *Lactobacillus acidophilus*[a]

Product	Country of origin	Microorganisms
A-38	Denmark	*Lactobacillus acidophilus*, *Bifidobacterium bifidum*, *Leuconostoc mesenteroides* spp. *cremoris*, mesophilic lactococci
Acidophilus buttermilk	USA	*Lactobacillus acidophilus*, *Leuconostoc mesenteroides* spp. *cremoris*, mesophilic lactococci
Progurt		*Bifidobacterium bifidum*, *Lactobacillus acidophilus*, mesophilic lactococci
Acidophilus milk	Several countries	*Lactobacillus acidophilus*
Acidophilus yeast milk	Former USSR	*Lactobacillus acidophilus*, *Saccharomyces fragilis*, *S. cerevisiae*
A-B Yoghurt	France	*Bifidobacterium bifidum*, *Lactobacillus acidophilus*
Cultura	Denmark	Ibidem
Milky	Italy	Ibidem
Nu-Trish A/B Milk	USA	Ibidem
Biomild	Several countries	*Bifidobacterium* spp., *Lactobacillus acidophilus*
Acidophilus yoghurt (ACO-yoghurt)	Several countries	*Lactobacillus acidophilus*, *L. delbrueckii* subsp. *bulgaricus*, *Streptococcus thermophilus*
B-Active	France	*Lactobacillus acidophilus*, *Bifidobacterium bifidum*, *L. delbrueckii* subsp. *bulgaricus*, *Streptococcus thermophilus*
Fresh BA	UK	Ibidem
Kyr	Italy	Ibidem
Yoplus	Australia	Ibidem
Biogarde	Germany	*Lactobacillus acidophilus*, *Bifidobacterium bifidum*, *Streptococcus thermophilus*
Ofilus	France	Ibidem
Philus	Norway	Ibidem
Bifidus milk	Several countries	*Bifidobacterium bifidum*, *B. longum*
Bifighurt	Germany	*Bifidobacterium bifidum*, *Streptococcus thermophilus*
Biogurt	Germany	*Lactobacillus acidophilus*, *Streptococcus thermophilus*
Biokys	Czech Republic	*Bifidobacterium bifidum*, *Lactobacillus acidophilus*, *Pediococcus acidilactici*
Mil-Mil	Japan	*Lactobacillus acidophilus*, *Bifidobacterium bifidum*, *B. breve*
Akult	Japan	*Lactobacillus acidophilus*, *Bifidobacterium bifidum*, *B. breve*, *L. casei* subsp. *casei*

[a] Adapted from Kurmann [22] and Hoier [83]

product, or to the fresh product before distribution. General accounts of the physicochemical and technological aspects of commercial fermented milk products containing *Bifidobacterium* spp. and *L. acidophilus* have been provided by Kurmann and Rasic [17], Mital and Garg [12] and Tamine *et al*. [60].

In addition to food products containing probiotic bacteria, there are various health products as well as pharmaceutical preparations containing probiotics, available in the market. In general, these consist in encapsulated freeze-dried bacterial populations that are used in the treatment of gastrointestinal disturbances (diarrhoea, including side effects of antibiotic therapy), constipation and certain hepatic diseases.

Technological characteristics

Production of high-quality fermented milk products containing *Bifidobacterium* spp. and *Lactobacillus acidophilus* is a major challenge to dairy plants owing to the sensitive character of the microorganisms in these bioproducts, which adds to the usual difficulties encountered in novel food products (i.e. poor palatability and consequent limited consumer acceptability). In particular, bifidobacteria tend to exhibit weak growth and acid production in milk, which invariably requires long fermentation times and conditions of anaerobiosis, low redox potential at least in the early phase of growth [24,98] and, often, addition of growth-promoting factors to the milk [19,23,32,53,54], as previously discussed. In addition, bifidobacteria produce, during fermentation, acetic and lactic acids at the ratio 3:2, so excessive growth may yield products with vinegar-like taste and aroma, which are obviously not acceptable to consumers [83]. Careful selection of the strains employed and good monitoring throughout the manufacturing process are therefore compulsory in attempts to control efficiently the metabolic products and hence the final pH. The use of combined cultures of bifidobacteria and *L. acidophilus* or other lactic acid bacteria, viz. *Lactobacillus delbrueckii* subsp. *bulgaricus* and *Streptococcus thermophilus, S. thermophilus* alone or mesophilic aromatic cultures, has also been advocated as a solution for many such problems [23,83,94,99]; increased growth rates and reduction of fermentation time, absence of certain sensory and texture defects and further improvement of nutritional value of 'bifidus' products are advantages brought about by the latter possibility. Adverse effects with respect to viability have, however, been reported for some strains of *Bifidobacterium* [99] and *L. acidophilus* [98].

A large number of studies has been published pertaining to characteristics of good probiotics [5,83] and

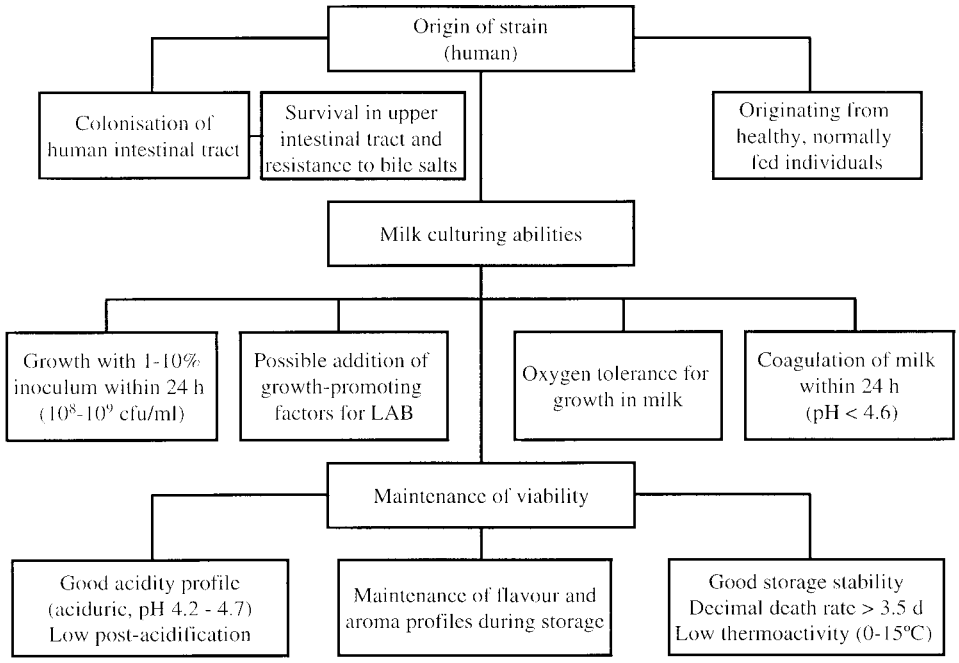

Fig. 2. Criteria used in selection of *Bifidobacterium* spp. for application as dietary adjuncts.

criteria for selection thereof [17,20,84,100]; these are summarized in Fig. 2. The most important properties include: (i) acid and bile tolerances, which are essential to maintain high viable cell numbers during storage and during passage through the digestive tract following oral uptake [24,101–103]; (ii) adherence to the human intestinal mucosa, which is needed for temporary colonization of the human gastrointestinal tract; and (iii) production of antimicrobial substances, with concomitant inhibition of pathogens [20]. Furthermore, the strains selected should produce a final product possessing good taste and acceptable body and texture, a selection step that cannot be achieved unless the product is actually manufactured.

It is also important that the strains selected can be produced in large scale; in fact, owing to a slow propagation in milk, they will hardly be competitive in the presence of other microorganisms and will, thus, be easily outnumbered. Therefore, aseptic working conditions and special growth-promoting factors (as discussed above) are pre-requisites to ensure high initial viable cell counts thereof. Voelki and Spillmann [104] optimized the process of preparation of starters for posterior manufacture of bifidus milk and concluded that active starters can be prepared with two specific strains, *Bifidobacterium breve* ATCC 15700 and *B. longum* ATCC 15707, under non-strictly anaerobic conditions, by using milk initially supplemented with readily available N-compounds (e.g. lactalbumin hydrolysate). A wide range of studies have made it clear that the production of starters of bifidobacteria and *L. acidophilus*, in individual and highly concentrated freeze-dried or deep frozen forms, for direct inoculation of processed milk, offers great flexibility in control of the desired sensory and microbiological qualities (in terms of ratio of bifidobacteria to lactobacilli) [83,105]. More recently, a ratio of 1:1 of frozen concentrates of *B. lactis* to *L. acidophilus* has been considered adequate for optimum growth of, and acidification by *B. lactis* when in coculture in plain milk, and the concomitant enhanced growth of *L. acidophilus* has also suggested a certain degree of symbiosis between these strains [23]. Furthermore, it is important that strains selected for Direct-Vat-Set (DVS) starters can undergo concentration of up to 10^{10}–10^{11} cfu/g in order to permit the desired performance in commercial manufacture of fermented milk products. Apart from this requirement, the starter produced should also display acceptable stability (usually guaranteed for 3–12 mo) throughout processing, and subsequent storage and distribution. Direct inoculation of a dairy product with a frozen concentrated culture (ca. $5–10\times10^{10}$ cfu/g) is recommended at a rate of 0.05–0.1 mg per L of milk [23,83,94,106]

Production of functional fermented milk products also requires safety assessment of the probiotic strains; a tentative strain must obviously be non-pathogenic for technological uses. There is a large consensus that lactic acid bacteria are benign microorganisms, with a well-established record of safety and no record of food poisoning outbreaks. Several studies document the safety of dairy strains [107], and an updated review on safety of probiotic bacteria has been made available by Donohue and Salminen [108]. Doses of probiotic strains as high as 10^{12} cfu/g have failed to exhibit toxicity, and

among the different species of the genus *Bifidobacterium* only *B. dentium* was considered to actually be pathogenic to man in view of its role in dental caries [7].

Growth and survival characteristics

Strain viability in the carrier food by the time of consumption is closely related to the aforementioned technological requirements. Strain survival depends on pH (and its buffering capacity), presence of competing microorganisms, storage temperature and presence of microbial inhibitors (e.g. NaCl and H_2O_2) in the food matrix [17]. Studies examining the viability of *B. lactis* and *L. acidophilus*, particularly in milk containing a range of experimental NaCl concentrations between 0 and 6%(w/w) and stored at different temperatures within the range of 5–15°C, indicated that pure cultures of *L. acidophilus* were more susceptible to higher NaCl concentrations than their *B. lactis* counterpart [32]. In addition, the mechanistic model made available by these authors considers the behaviour of the pure and mixed microbial populations to be described by specific death rates that vary with temperature (following Arrhenius relationships) and NaCl levels in the milk medium (following simple inhibition kinetics), and is therefore useful in efforts to rationalize the dynamics of cell population survival under a variety of combinations of easily manipulated environmental conditions.

Moreover, it is essential that fermented bioproducts contain a satisfactory number of active cells at the moment of consumption, i.e. at least 10^6 cfu/mL, because the minimum therapeutic daily dose is usually considered as 10^8–10^9 viable cells, realizable through an intake of, say 100 g of fermented bioproduct containing 10^6–10^7 viable cells/mL [2]. However, it is necessary that such fermented milks are consumed regularly in order to maintain the effect of these special microorganisms on the composition of the intestinal microflora. Cases of marked loss of viability in dairy products have been reported for both *Bifidobacterium* spp. and *L. acidophilus*, more often during refrigerated storage at low pH [24,89,109], and reflect, as stressed previously, the need for careful strain selection. These studies urge processors to more deeply understand the implications of adding acid-sensitive bacteria to highly acidic products like yoghurt. In contrast, bifidobacteria survive well in low-acid products such as frozen yoghurt [89], ice-cream [92] or cheese when added exclusively as dietary adjuncts [93]. New methods for enhancing the long-term survival of probiotic strains, and hence ensure that reasonable numbers of bacteria are delivered to the host, have been investigated either by replacing the carrier food [92–94,106] or by improving the protection of acid-sensitive strains via microencapsulation with cellulose acetate phthalate [110], κ-carrageenan [93] or Ca-alginate [111]; the first method appears to be technologically and commercially the most feasible. For example, a starter composed solely of a mixture of *L. acidophilus* and *B. lactis* has been successfully used in the manufacture of cheeses following modified technologies of the well-known Gouda cheese [94] and of Cabra cheese, a cheese with a large social and economic importance in the Mediterranean basin [106]. Growth behaviours of either strain were dependent on the physicochemical characteristics prevailing in both cheese-types, yet acid production was not affected. Survival of the probiotic strains was monitored during ripening, and the final viable numbers were still above the accepted threshold. Both strains contributed significantly to ripening of both cheese-types, especially in terms of formation of low molecular mass peptides and free amino acids, but lipolysis was not greatly affected. The best compromise between sensorial, physicochemical and probiotic attributes of the final cheese was obtained for 3×10^7 and 7×10^6 cfu/mL of *B. lactis* and *L. acidophilus*, respectively, 3.5%(w/w) salt, addition of 0.3%(v/w) milk hydrolysate and ripening for 70 d.

The selection of oxygen-tolerant species may also be a solution toward minimization of the adverse effect of oxygen in fermented bioproducts containing *Bifidobacterium* spp. and *Lactobacillus acidophilus*. The mechanisms of susceptiblity of *Bifidobacterium* to molecular oxygen have interested researchers at early stages [112], who estimated the oxygen sensitivity of twelve strains by measuring the extent of growth inhibition when they were grown in deep agar cultures and the extent of growth in aerated cultures; three categories of *Bifidobacterium* species with regard to their oxygen sensitivity were accordingly suggested. These researchers were not, however, able to establish a correlation between oxygen sensitivity and enzyme activities relating to oxygen metabolism, and it was only in the present decade that studies provided a clearer insight into a more fundamental relationship. The initial study by Shimamura *et al.* [113] has elucidated oxygen uptake by several bifidobacterial species when carbohydrates are metabolized, and reported that this mechanism is effective in diminishing the impact of environmental oxygen even when faced with an insufficient energy source, e.g. glucose; involvement of NADH oxidase for oxygen uptake activity in *Bifidobacterium* was thus demonstrated. In their follow-up study, Shimamura *et al.* [114] reported that all four *Bifidobacterium* spp. strains assayed expressed both NADH oxidase and NADH peroxidase activites, but to different degrees; a strong inverse correlation between enzyme activites and degree of oxygen sensitivity was consequently established. *Bifidobacterium infantis*, *B. breve* and *B. longum* were much more tolerant to oxygen (resulting from higher activities of the aforementioned enzymes) than *B. adolescentis* was (arising from very low activities thereof); therefore, the former are better suited for industrial applications. These results permitted refinement of their initial

hypothesis [113] in that the pathway involving the two NADH oxidative enzymes apparently operates as a defense mechanism to reduce oxygen toxicity in *Bifidobacterium* spp.

Finally, a probiotic strain must show good survivability, both in the product and after digestion. It is clear from the above lines that certain strains are able to survive well when exposed to gastric acids, or certain secretions of the small intestine.

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