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Review

Incorporation of bifidobacteria into cheeses: challenges and rewards

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Abstract

As the market for functional foods (i.e. foods that contain health-promoting components beyond traditional nutrients) continues to expand, research in the development of food products containing bifidobacteria and other probiotic bacteria will also continue to grow. Probiotic bacteria and bifidobacteria play an important role in gastro-intestinal therapy, but also provide many additional health-promoting benefits. Bifidobacteria require an anaerobic environment and neutral pH to survive and maintain levels greater than 10^6 cfu g^{-1} that is adjudged as the requirement to provide therapeutic benefits. Thus, not all foods provide the optimal environment for the growth of bifidobacteria. Cheese, however, does provide an environment that would be conducive to the long-term survival of bifidobacteria. Effective incorporation of bifidobacteria into cheeses requires that the bifidobacteria maintain their viability throughout processing, without adversely altering sensory characteristics. This review details technological advances shown to be effective in the incorporation of bifidobacteria into cheeses and techniques for the accurate enumeration of these bacteria.

Keywords: Bifidobacteria; Probiotic bacteria; Enumeration; Cheese

Contents

1. Introduction

Cultured dairy products are an important part of the diet of many societies. These dairy products, initially

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developed as a means to preserve milk also have desirable sensory characteristics. The fermentation by the microbial starter cultures preserves the product through the production of lactic acid from lactose and contributes to the development of characteristic flavour compounds. These products are now recognized for their nutritional benefits. With the growth of the functional foods area, a growing research interest has focused on the incorporation of bifidobacteria and other probiotic bacteria into cultured dairy products to further enhance the nutritional value of these products (Robinson, 1991; Tamime, 2002).

Probiotic bacteria, specifically bifidobacteria and lactobacilli, are normal inhabitants of the human colon. The indigenous bifidobacteria species are dependent on the age of the individual. *Bifidobacterium infantis* and *B. breve* are predominant in infants while *B. adolescentis* is predominant in adults, and *B. longum* is present throughout life (Gomes & Malcata, 1999). These bacteria beneficially affect human health by improving the balance of intestinal microflora and improving mucosal defenses against pathogens. Additional health benefits include enhanced immune response, reduction of serum cholesterol, vitamin synthesis, anti-carcinogenic activity, and anti-bacterial activity (Robinson & Samona, 1992; Blanchette, Roy, & Gauthier, 1995; Gomes & Malcata, 1999; Arunachalam, 1999; Brassert & Schiffrin, 2000; Lourens-Hattingh & Viljoen, 2001).

To perform these functions, the bifidobacteria must be viable at the time of consumption and maintain their viability throughout the gastrointestinal tract (Robinson & Samona, 1992; Blanchette et al., 1995; Arunachalam, 1999; Brassert & Schiffrin, 2000). Recommendations for the minimum suggested level for bifidobacteria in the food to attain this viability are quite variable (Charteris, Kelly, Morelli, & Collins, 1998). Levels of 10^5 cfu g^{-1} (Shah, Lankaputhra, Britz, & Kyle, 1995; Shah, 1997), 10^6 cfu g^{-1} (Robinson & Samona, 1992; Arroyo, Cotton, & Martin, 1994; Rybka & Kailasapathy, 1995; Pagano, 1998) and 10^7 cfu g^{-1} (Samona & Robinson, 1994) have been suggested. In general, the food industry has applied the recommended level of 10^6 cfu g^{-1} at the time of consumption for *Lactobacillus acidophilus* to bifidobacteria and other probiotic bacteria. This standard appears to have been adopted to provide bacterial concentrations that were technologically attainable and cost-effective rather than to achieve a specific health effect in humans (Roy, 2001). More critical than the concentration of the bifidobacteria in the food, however, is the daily intake of the bifidobacteria necessary to attain a therapeutic effect. A daily intake of at least 10^8–10^9 viable cells, which could be achieved with a daily consumption of at least 100 g of a product containing between 10^6 and 10^7 viable cells g^{-1}, has been suggested as the minimum intake to provide a therapeutic effect (Blanchette et al., 1995; Gomes & Malcata, 1999). The ability to enumerate bifidobacteria in cultured dairy products accurately in the presence of lactic acid bacteria is crucial in assessing the health benefits and determining if the dairy products will provide a therapeutic effect (Lim, Huh, Baek, & Kim, 1995; Payne, Morris, & Beers, 1999).

In general, the presumption is that probiotic viability is a reasonable measure of probiotic activity. This is certainly a defensible assumption, considering the definition of probiotics previously mentioned. In most cases, even if viability is not required, it is likely correlated with most effects since it is a useful indicator of the number of cells present, regardless of what cell component may be active. Situations where viability is not required for probiotic activity include digestion of lactose, some immune system modulation activities and anti-hypertensive effects. The fact remains that for the most part, probiotic products are standardized based on viable count, with the assumption that this is the important factor to consider in product functionality (Sanders & Huis in't Veld, 1999).

The growth in the development of foods containing bifidobacteria is expected to continue. To be successful, manufacturers must consider the effects of the environment of the food during processing and storage to ensure that the concentration of bifidobacteria at the time of consumption provides a therapeutic dose to consumers. In addition, the foods with added bifidobacteria must maintain the characteristic sensory attributes of the traditional food. The purpose of this review is to discuss the potential for cheese as an effective vehicle for incorporation of bifidobacteria into the food supply, to discuss the processing conditions necessary to effectively incorporate the bifidobacteria into the cheese and to discuss methods for the enumeration of bifidobacteria in cheese in the presence of other lactic acid bacteria.

2. Characteristics of bifidobacteria

Members of the genus *Bifidobacterium* and *Lactobacillus* are widely used as probiotic microorganisms in functional foods (Corbo, Albenzio, De Angelis, Sevi, & Gobbetti, 2001). Many *Lactobacillus* strains with nutritional benefits characteristic of probiotic bacteria are currently incorporated during the traditional production of cultured dairy products. Therefore, the focus of this review will be on bifidobacteria, which are not commonly used in the production of cultured dairy products.

In the early 1900s, bifidobacteria were classified as *Lactobacillus* subspecies. However, now these microorganisms are classified as a separate genus, *Bifidobacterium*. Currently, 30 species of bifidobacteria, isolated from human, animal, insect, and environmental sources, have been identified. Of these species, six species from

human origins, *B. adolescentis*, *B. breve*, *B. bifidum*, *B. lactis*, *B. infantis*, and *B. longum*, have been used in dairy products. Bifidobacteria are gram-positive, non-spore-forming, non-motile bacteria. The appearance of the rods is highly variable and may be influenced by nutritional conditions (Scardovi, 1986; Tamime, 2002). The growth characteristics and nutrient requirements of bifidobacteria are unique from most lactic acid bacteria. To successfully develop cheeses and other dairy products containing bifidobacteria, it is important to understand the growth characteristics of the bifidobacteria so that processing conditions can be manipulated to optimize their survival.

Bifidobacteria are classified as strict anaerobes, because they are incapable of respiration using oxygen or growth under aerobic conditions. However, the degree of tolerance of oxygen depends on the species and culture medium (De Vries & Stouthamer, 1969), and even on the morphology of the strains, such as whether they are branched or not (Norris, Flanders, Tomarelli, & Gyorgy, 1950). The use of the term 'air-tolerant anaerobes' for bifidobacteria may come into prominence as more reports appear in the literature (Bezkorovainy, 1989). Certain strains of bifidobacteria, including *B. infantis*, *B. breve* and *B. longum*, may have a mechanism by which they can avoid the toxicity of oxygen, as shown by their limited metabolic activity and production of acid under aerobic conditions (Shimamura et al., 1992).

The initial optimum growth pH for bifidobacteria is between 6.5 and 7.0. Growth of bifidobacteria is inhibited below 5.0 or above 8.0 and is species and strain specific (Scardovi, 1986; Lourens-Hattingh & Viljoen, 2001). In a liquid media with compounds typical of fermented dairy products, negligible losses of bifidobacteria occurred at pH 5, but decreases from 0.1 to 7.6 $\log_{10}$ orders occurred at pH 4 (Vinderola, Costa, Regenhardt, & Reinheimer, 2002a). Under conditions typical of digestion (pH 1.5–3.0), *B. longum* 1941 and *B. pseudolongum* 20097 were more tolerant to the acid conditions than the 7 other strains of bifidobacteria evaluated. Following a 3-h incubation period, the cell counts of these 2 acid-tolerant strains decreased less than 1 $\log_{10}$, while the cell counts of the other bifidobacteria strains evaluated decreased up to 7 $\log_{10}$ (Lankaputhra & Shah, 1995).

The optimum growth temperature for most species of the bifidobacteria of human origin is between 36°C and 38°C, whereas the animal species have growth optima at slightly higher temperatures (about 41–43°C). There is no growth below 20°C and the bifidobacteria have no thermoresistance above 46°C (Rašic & Kurmann, 1983).

Different bifidobacteria species are able to ferment different carbohydrates (Tamime, 2002). The fermentation of glucose by bifidobacteria via the fructose-6-phosphate shunt results in the production of acetic and lactic acid in the molar ratio of 3:2. Carbon dioxide is not produced and ammonia is generally utilized as a source of nitrogen (Dellaglio, 1988). The production of these acids contributes to the bifidobacteria's defence against pathogens through the toxic effect of the undissociated acids on the microorganisms and stimulation of intestinal peristalsis (Robinson & Samona, 1992). The growth of the bifidobacteria is also inhibited by lactic acid and other metabolic products produced by lactic acid bacteria during processing and storage of the cultured dairy products (Blanchette, Roy, Bélanger, & Gauthier, 1996).

3. Bifidobacteria in dairy products

The most popular food delivery systems for probiotic cultures have been freshly fermented or unfermented dairy foods, including milk, yoghurt, ice cream and desserts (Samona & Robinson, 1994; Gomes, Malcata, Klaver, & Grande, 1995; Nighswonger, Brashears, & Gilliland, 1996; Stanton et al., 1998; Corbo et al., 2001). Although cultured dairy products are a logical choice for introduction of probiotic bacteria into the food chain, there are numerous challenges related to the instability of some intestinal strains of probiotic bacteria in fermented milk products (Dinakar & Mistry, 1994; Lourens-Hattingh & Viljoen, 2001). The environment typical of many yoghurt and other cultured dairy products, including the low pH and the aerobic conditions of production and packaging, can result in decreases in the count of bifidobacteria in these dairy products to below the therapeutic minimum (Dinakar & Mistry, 1994; Vinderola, Bailo, & Reinheimer, 2000a; Gobbetti, Corsetti, Smacchi, Zocchetti, & De Angelis, 1998; Shah, 2000; Lourens-Hattingh & Viljoen, 2001). Another major concern is the ability of bifidobacteria to retain their viability in the carrier food until the time of consumption.

One of the major limitations of the incorporation of bifidobacteria into yoghurts is the pH of the product. The pH of most commercial yoghurts is between 3.7 and 4.3 (Hamann & Marth, 1983). However, the pH optimum for bifidobacteria is between 6.5 and 7.0, with growth inhibited at pH values below 5.0 (Scardovi, 1986; Lourens-Hattingh & Viljoen, 2001). Because most strains of bifidobacteria are sensitive to pH values below 4.6, in practical applications, the pH value of the final product must be maintained above 4.6, otherwise the bifidobacterial population will decline rapidly (Tamime & Robinson, 1988; Modler, McKellar, Goff, & Mackie, 1990a; Laroia & Martin, 1990; Vinderola et al., 2002a). For example, laboratory work by Modler (1994) revealed that a bifidobacterial population could decline from 10^9 to 0 in less than a week in low pH yoghurt (pH 3.9–4.0).

Bifidobacteria are classified as strict anaerobes, because they are incapable of respiration using oxygen or growth under aerobic conditions. However, the degree of tolerance of oxygen depends on the species and culture medium (De Vries & Stouthamer, 1969), and even on the morphology of the strains, e.g. whether they are branched or not (Norris et al., 1950). Shimamura et al. (1992) showed that the pH of the culture medium for *B. infantis, B. breve*, and *B. longum* grown under aerobic conditions after 16 h was lower than the initial pH, indicating that some limited metabolism occurred and suggested that there may be a mechanism by which bifidobacteria can avoid the toxicity of oxygen.

Propagation of bifidobacteria in milk is slow compared with other lactic acid starter bacteria used in cultured dairy products (Gomes & Malcata, 1999). Lactococci, lactobacilli and streptococci are among the starter cultures commonly used in cheese-making, depending on the type of cheese and its characteristics as well as availability, cost and ease of handling and operation. Many of these lactic acid bacteria also produce environments that inhibit the growth of not only pathogens and spoilage microorganisms, but also of probiotic bacteria (Vinderola, Mocchiutti & Reinheimer, 2002b). This inhibitory activity is attributed to several factors, including production of lactic and other organic acids, hydrogen peroxide, and bacteriocins, as well as reduced availability of nutrients (Shah, 2000). The use of higher inocula of bifidobacteria and the addition of growth promoting factors as a nitrogen source should further enhance the growth and viability of the bifidobacteria (Gomes et al., 1995; Gomes & Malcata, 1999). The competition between the bifidobacteria and lactic acid bacteria and the changes in the profiles of these bacteria during processing and maturation of the cheese must be considered to enhance the viability of the bifidobacteria.

Numerous techniques have been adapted to enhance the viability of probiotic bacteria in the harsh conditions characteristic of many cultured dairy products. Selection of oxygen-tolerant, acid-tolerant, and bile-resistant strains, the addition of other lactic acid bacteria that enhance the survival of the probiotic bacteria, and the addition of amino acids, peptides and other micronutrients to supplement the growth of the probiotic bacteria have been beneficial (Gobbetti et al., 1998; Shah, 2000; Takano, Saito, Futami, & Hayakawa, 1988). In yoghurt, the addition of ascorbic acid (Dave & Shah, 1997a) and cysteine (Dave & Shah, 1997b) decreased the redox potential, providing an environment more favourable to the growth of bifidobacteria, but was not effective in increasing the viability of bifidobacteria in the presence of the yoghurt cultures, *L. delbrueckii* ssp. *bulgaricus* and *Streptococcus thermophilus*. Maintaining the viability of probiotic bacteria in dairy products over extended refrigerator storage is also a challenge with most fermented products. Delivery systems are needed that improve the viability of added bacteria and ensure that reasonable numbers of them are delivered to the host upon consumption (Gomes, Vieira, & Malcata, 1998).

4. Unique characteristics of cheeses beneficial to the growth of bifidobacteria

Although considerable marketing and research attention has focused on fermented milks and yoghurts as food carriers for bifidobacteria, these products are not optimal for the maintenance of high concentrations of some strains, as evidenced by poor viability (particularly some strains of *Bifidobacterium*) in commercial yoghurts (Gardiner et al., 1999; Vinderola et al., 2000a). An alternative means to maintain the viability of the bifidobacteria would be to incorporate the organisms into a product like cheese where the pH, lipid content, oxygen level, and storage conditions are more conducive to the long-term survival of bifidobacteria during processing and digestion (Dinakar & Mistry, 1994; Stanton et al., 1998). Cheeses (pH range 4.8–5.6) have a markedly higher pH than fermented milks (pH 3.7–4.3) and provide a more stable medium to support the long-term survival of the acid-sensitive bifidobacteria. The metabolism of the microorganisms within the cheese results in an almost anaerobic environment within a few weeks of ripening, favoring the survival of bifidobacteria and other anaerobic microorganisms (Van den Tempel, Gundersen, & Nielsen, 2002). Furthermore, the matrix of the cheese and its relatively high fat content offers protection to probiotic bacteria during passage through the gastrointestinal tract (Gardiner, Ross, Collins, Fitzgerald, & Stanton, 1998; Vinderola et al., 2002b; Corbo et al., 2001).

The processing of cheese involves the addition of rennet and lactic acid bacteria to milk to facilitate the precipitation of the casein molecules and concentration of milk solids. The precipitated curds are moulded and pressed, and undergo a salting or brining treatment. The characteristic flavours and texture of the cheeses are developed during a ripening period, through the lipolytic and proteolytic activity of the bacteria present in the cheese. The microorganisms used in cheese-making in conjunction with the specific processing treatments contribute to the distinct flavour and texture characteristics of the different types of cheese. Cheeses are categorized, based on texture, from very hard to soft. The flavour of cheese is mild in unripened cheeses and sharp in ripened cheeses (Banks, 1998).

Nutrient availability, presence of growth promoters and inhibitors, pH and the presence of oxygen affect the growth of bifidobacteria in dairy products. The

lactic acid bacteria used to produce the characteristic attributes of different types of cheese also enhance the growth and viability of the bifidobacteria through altering the pH, content of growth promoters and inhibitors, and oxygen content of the cheese. Selected strains of *S. thermophilus* with high oxygen consumption ability have been shown to enhance the viability of the bifidobacteria (Okonogi, Ono, Kudo, Hiramatsu, & Teraguchi, 1984). The unique aspects of cheese-making could potentially enhance the viability of bifidobacteria in cheese. The lack of proteolytic activity in bifidobacteria slows their growth in milk (Shah, 2000). However, para-κ-casein and other casein hydrolysates produced by rennet activity during cheese-making, as well as the proteolytic activity of the starter cultures, have been suggested as growth-promoting factors for bifidobacteria and may decrease the cultivation difficulties of bifidobacteria in cultured dairy products (Kehagias, Jao, Mikolajcik, & Hansen, 1977; Zbikowski & Ziajka, 1986; Poch & Bezkorovainy, 1988, 1991; Otani, 1992; Ballongue, 1993).

Salting of the curd, by immersing it in brine or rubbing salt on the surface, is a common step in the manufacture of cooked varieties of cheeses. Depending on the size of the cheese wheel and the diffusion of the salt into the curd, a salt gradient with a maximum difference of 4- to 5-fold from the periphery to the centre may result initially. During the ripening process, salt diffuses throughout the cheese, so that differences in the salt content at the centre and periphery decrease with ripening time (Mocquot, 1979; De Leon-Gonzalez, Wendorff, Ingham, Jaeggi, & Houck, 2000). The salting contributes not only to the flavour of the cheeses, but also has an impact on the growth of microorganisms, with the viability of bifidobacteria and other bacteria inversely related to salt concentration (Gomes et al., 1998; Vinderola et al., 2002a).

Calcium ions (Misra & Kuila, 1990) and sodium chloride (Modler, McKeller, & Yaguchi, 1990b; Samona & Robinson, 1991) also contribute to morphological changes in bifidobacteria, which, in turn, may alter their acid-producing ability and other growth characteristics (Misra & Kuila, 1990). The effect of the fatty acid components of cows' milk on the growth characteristics of bifidobacteria is variable. Lauric and myristic acids, accounting for 3.6% and 10.5% of the fatty acids in the milk triacylglycerols, inhibit the growth of the bifidobacteria. On the other hand, the more predominant fatty acids, butyric, palmitic, and stearic acids, which account for 8.5%, 23.5% and 10.0% of the fatty acids in the milk triacylglycerols, promote the growth of the bifidobacteria (Rašic & Kurmann, 1983; Walstra, Geurts, Noomen, Jellema, & van Boekel, 1999). The liberation of certain fatty acids during the maturation of cheese and their effect on the growth or survival of bifidobacteria needs to be evaluated.

The successful development of probiotic-containing cheeses would provide cheese industries with a competitive advantage over existing products and contribute to the expansion of the range of dairy products possessing better nutritional and physiological qualities (Stanton et al., 1998). In many parts of the world, cheeses are frequently consumed at least once a day, making cheese an excellent carrier for bifidobacteria because of the high daily consumption. The successful incorporation of bifidobacteria and probiotic bacteria into cheeses requires that the bacteria remain viable throughout the shelf-life of the product and do not adversely affect the composition, flavour, texture, and other sensory characteristics of the traditional cheese (Gomes et al., 1995; Corbo et al., 2001). If the probiotic cheese can be manufactured with little or no alteration of the traditional cheese making technology, this would make the development of probiotic cheeses attractive for commercial production.

5. Incorporation of bifidobacteria into cheeses

Bifidobacteria from human intestinal origin are frequently favoured for incorporation into foods because of their ability to colonize in the human gut. However, bifidobacteria from animal origins are more tolerant of adverse conditions found in cultured dairy products (Gomes & Malcata, 1999). Bifidobacteria have been successfully incorporated into a range of different types of cheeses (Table 1). The success of the incorporation of bifidobacteria into cheeses is dependent on the bifidobacteria strains, the activity of the lactic acid bacteria used in the manufacture of the cheese, the composition of the cheese, and the conditions of processing and ripening.

Bifidobacteria species differ in their nutrient requirements, growth characteristics and metabolic activity. Thus, not all bifidobacteria species will necessarily exhibit the same survivability nor have the same impact on the sensory attributes in dairy products (Gomes & Malcata, 1999; Corbo et al., 2001). Since bifidobacteria are generally anaerobic in nature, oxygen adversely affects the growth of the bacteria. Selection of oxygen-resistant mutants of *B. bifidum* and other bifidobacteria has been shown to be effective in enhancing the survival of the bifidobacteria in foods throughout processing and storage (Mutai, Mada, & Shimada, 1980). *B. infantis*, *B. breve*, and *B. longum* are able to grow under partial aeration, while *B. adolescentis* is suppressed by low oxygen concentrations. The oxygen sensitivity of these bifidobacteria strains is inversely related to their NADH-oxidase and NADH-peroxidase activity, in that these enzymes are able to break down hydrogen peroxide and other activated oxygen compounds toxic to the bifidobacteria (Shimamura et al., 1992). In

Table 1
Characteristics of cheese processed with bifidobacteria

Type of cheese	Storage time	Microorganisms	pH	Moisture (%)	Fat (%)	Salt (%)	Reference
Fresco	60 days	Bifidobacteria, *L. acidophilus* and/or *L. casei*	5.29	58	12	0.9	Vinderola et al. (2000b)
Crescenza	14 days	*B. bifidum* or *B. longum* and *S. thermophilus*	5.27	62	27	0.7	Gobbetti et al. (1998)
Cheddar	24 weeks	*B. bifidum* and mesophilic starter culture		37	33	1.1	Dinakar and Mistry (1994)
Cheddar	84 days	*B. infantis* and *Lc. lactis* ssp. *lactis* and/or *Lc. Lactis* ssp. *cremoris*	5.2	33	30	1.9	Daigle et al. (1999)
Canestrato Pugliese	56 days	*B. bifidum* or *B. longum* and *S. thermophilus and L. delbrueckii* ssp. *bulgaricus*	5.55	39	31	3.0	Corbo et al. (2001)
Gouda	9 weeks	Bifidobacteria ssp. and *L. acidophilus*	5.1	42	29	1.7	Gomes et al. (1995)
Iranian White Brined	60 days	*B. bifidum*, *S. thermophilus*, and *L. delbrueckii* or *Lc. Lactis*	4.85	59.5	20.5	7.15	Ghoddusi and Robinson (1996a)

addition, selection of human strains of bifidobacteria with high resistance against low pH and bile salts is important to ensure the viability of these microorganisms throughout digestion (Lankaputhra & Shah, 1995).

Roy, Desjardins, and Mondou (1995) studied the growth and acid production of bifidobacteria in milk under conditions typical of cheese-making, and evaluated the survival of the selected strains during storage in the presence of starters used for cheese-making. The bifidobacteria species differed in their ability to grow in different media, including milk, rennetted milk, and acidified milk in the presence of starter cultures and in their ability to produce acetic and lactic acids. *B. longum* strains demonstrated high survival rates in the presence of mesophilic starters and lactic acid bacteria and would be acceptable for use in cheese-making. However, *B. adolescentis* strains were unable to grow in conditions typical of cheese-making. Similarly in cheeses, the viability of different strains of bifidobacteria during cheese-making, ripening, and storage varies greatly. The following discussions will compare the viability of several strains of bifidobacteria in different types of cheeses.

Pure cultures of *B. longum*, *B. breve*, *B. catenulatum*, *B. bifidum*, *B. angulatum*, and *B. infantis* were added to commercial samples of cottage cheese to compare the viability of different bifidobacteria species. After a 14-day refrigerated storage period, viability losses ranged from 0 to almost $3\log_{10}$ cycles and were strain dependent. *B. bifidum* maintained the best survival, showing only negligible losses, while *B. infantis* and *B. breve* demonstrated poor survival rates with a $3\log_{10}$ decrease in counts during the storage period (O'Riordan & Fitzgerald, 1998).

In Crescenza cheese, a soft, rindless, Italian cheese with a short ripening time, the viability of three species of bifidobacteria was compared. The bifidobacteria were added concurrently with the starter cultures, following standard manufacturing procedures for Crescenza cheese. The growth of *B. bifidum* and *B. longum* increased 1–2 $\log_{10}$ cycles while the viable counts of *B. infantis* decreased $1\log_{10}$ cycle during the 14 day ripening period. Rapid acid production by *S. thermophilus* during the fermentation of the milk was hypothesized to contribute to the reduced viability of *B. infantis*. Cheeses with the bifidobacteria had higher concentrations of acetic and lactic acids, but the minor differences in sensory attributes of the cheeses indicated the bifidobacteria did not exhibit extensive metabolic activity. The cheeses in which bifidobacteria had been incorporated were similar to the conventional Crescenza cheese with respect to sensory and physicochemical characteristics, microbial load, and gross composition, but contained higher β-galactosidase specific activity. The higher β-galactosidase activity, attributed to the bifidobacteria or the stimulation of β-galactosidase activity of *S. thermophilus* by the bifidobacteria, increases lactose hydrolysis and provides additional beneficial effects for lactose intolerant individuals (Gobbetti et al., 1998).

A comparison of the viability of *B. bifidum* and *B. longum* in Canestrato Pugliese, an Italian hard cheese, also demonstrated difference in the viability of the two species of bifidobacteria. *B. bifidum* showed a greater survival than *B. longum*. Through 35 days of ripening, the two species showed similar viability with counts of 10^7 through 19 days of ripening, decreasing to 10^6 at 35 days of ripening. However, after 56 days of ripening, the counts for *B. bifidum* were maintained at 10^6, while the

counts for *B. longum* decreased to 10^5 (Corbo et al., 2001).

In the manufacture of Fresco cheese, probiotic concentrated cultures of bifidobacteria, *L. acidophilus*, and *L. paracasei* were added simultaneously with the lactic acid starter bacteria (*S. thermophilus* and *Lactococcus lactis*) to evaluate the interactions between the probiotic and lactic acid bacteria. Three species of bifidobacteria, *B. longum*, *B. bifidum*, and *Bifidobacteria* spp. strain B5 were compared. Throughout the 60-day storage period, the viability of the bifidobacteria species decreased up to 1 $\log_{10}$ cycle, but maintained counts about 10^6 cfu g^{-1}. Viability of the bifidobacteria was affected by the presence of other probiotic bacteria, with the combination of *B. bifidum*, *S. thermophilus*, and *L. casei* resulting in the highest counts of all bacteria evaluated. The *B. bifidum* strains incorporated in Fresco cheese not only maintained good viability during processing and ripening, but also demonstrated good resistance in an acidic environment typical of the stomach (Vinderola, Prosello, Ghiberto, & Reinheimer, 2000b). The in vivo consumption of the Argentinian Fresco cheese containing this mixture of probiotic bacteria also beneficially modulated the immune response in mice (Medici, Vinderola, & Perdigon, 2003).

In another study (Ghoddusi & Robinson, 1996a), batches of typical Iranian white-brined cheese were made using full cream pasteurised milk and inoculated with *B. bifidum* or *B. adolescentis* (1%) and either a mixture of yoghurt cultures [*S. thermophilus* and *L. delbruekii* ssp. *bulgaricus*, (1%)] or a mixture of cheese cultures [*Lc. lactis* ssp. *lactis* and *Lc. lactis* ssp. *cremoris* (1%)] to give four combinations of organisms. With their assumption that the ''therapeutic minimum'' for bifidobacteria is 10^6 cfu g^{-1}, *B. adolescentis* showed poor survival in the presence of both yoghurt and cheese cultures and did not appear to be suited for incorporation into this product, even though the counts remained stable after the initial decline. However, the cheese made with yoghurt culture and *B. bifidum* showed acceptable survival of bifidobacteria up to 60 days after manufacture. *B. bifidum* was more tolerant of the salt and acidity associated with the white-brined cheese than was the *B. adolescentis*.

The consumption of Tallaga cheese in Egypt has been markedly increased during the 1990s as a result of its low sodium chloride content. For the manufacture of Tallaga cheese containing probiotic bacteria, *B. lactis* Bb-12 and *L. acidophilus* La-5 were used as single or mix cultures in a traditional manufacture of Tallaga cheese. Probiotic cultures were added to the standardized and pasteurized milk before the manufacture of the cheese. For both probiotic strains, viable cell counts were higher than 10^6 cfu g^{-1} at the end of the 28-day storage period. Scores for body, texture and appearance were quite similar for all treatments, indicating that Tallaga cheese could be an additional dairy food containing probiotic bacteria (El-Zayat & Osman, 2001).

Kariesh cheese is a popular Egyptian soft cheese made from buffalo milk. The Kariesh cheese was processed using traditional techniques with bifidobacterial or yoghurt (control) cultures. Following a 10-day ripening period, the bifidobacterial populations were higher than 10^8 cfu g^{-1}. The Kariesh cheese also received higher sensory scores than the control cheese preparations. In this sense, the introduction of bifidobacteria resulted in cheeses with new dietetic and probiotic characteristics and improved organoleptic properties (Murad, Sadek, & Fathy, 1998).

Careful strain selection is mandatory to warrant the survival of bifidobacteria in the cheese matrix. In the evaluation of the viability of bifidobacteria in several types of cheeses, *B. bifidum* and *B. longum* were among the strains shown to demonstrate good viability through the processing and storage of the cheese. However, *B. infantis* and *B. adolescentis* demonstrated poor survivability and would be less preferable for incorporation into dairy products.

6. Impact of processing parameters on viability of Bifidobacteria

In selection of the type of cheese for incorporation of the bifidobacteria, the effect of processing conditions on the viability of the bacteria is important. The cooking procedure for hard or semi-hard cheeses, the aerobic environment, the impact of lactic acid bacteria starter cultures, and the temperatures of ripening and storage must be evaluated (Gobbetti et al., 1998). Growth conditions must be balanced to promote viability of the bifidobacteria without excessive growth and acid production that could adversely affect sensory attributes of the cultured dairy products (Gomes & Malcata, 1999). The following discussion provides an overview of processing protocols that have been modified or adapted to enhance the viability of the bifidobacteria with compromising the quality of the cheese.

Gomes et al. (1995) adapted the technology of Gouda cheese manufacture for the purpose of producing a probiotic counterpart, containing *Bifidobacterium* ssp. strain Bo and *L. acidophilus*. During manufacture, the metabolism of the bifidobacteria contributed to acetic acid formation without a significant increase in viable cells, while the *L. acidophilus* showed a $\log_{10}$ increase in viable cells. However, during 9-week storage period, the average numbers of *L. acidophilus* decreased by 2 $\log_{10}$ cycles, whereas those of bifidobacteria decreased by less than 1 $\log_{10}$ cycle. In all cases, viable cell counts were higher than 10^7 cfu g^{-1}. Following 9-week ripening period, the survival of the bifidobacteria was dependent on the region of the cheese, the salt concentration, and

the addition of protein hydrolysates. Cheeses with salt contents ranging from 1.90% to 3.90% had a 55–35% survival of the bifidobacteria, with the highest survivability in the centre of the cheese where oxygen and salt levels were lowest. Protein hydrolysates, added during cheese processing to enhance the growth of the bifidobacteria, did not significantly enhance the viability of the bifidobacteria, possibly because of the protein hydrolysis contributed by the rennet activity. However, the protein hydrolysate, even when added at low levels, introduced undesirable flavours to the cheese through the increased free amino acids and peptide contents.

In the manufacture of Canestrato Pugliese, an Italian hard cheese, the viability of the bifidobacteria was maintained through a 90-day ripening period. The composition and sensory attributes of the cheeses with the added bifidobacteria was also characteristic of the traditional cheese. Slight technological modifications were incorporated during processing of Canestrato Pugliese cheese to improve the survival of the bifidobacteria, *B. bifidum* and *B. longum*, added as adjunct cultures with *S. thermophilus* and *L. delbrueckii* ssp. *bulgaricus*. These modifications included reducing the conditions for heating the curd in whey from 80°C for 30 s to 50°C for 2 min and holding the curd at 40°C for about 5 h to limit acidification by the lactic acid starters (*S. thermophilus* and *L. delbrueckii* ssp. *bulgaricus*) and enhance the viability of the bifidobacteria. Canestrato Pugliese cheeses evaluated by sensory panelists after 56-days ripening for appearance, colour, mechanical characteristics, smell, taste and texture indicated all cheeses characterized the traditional Canestrato Pugliese cheese, with no differences evident from the addition of single or mixed cultures of bifidobacteria on the sensory characteristics (Corbo et al., 2001).

Bifidobacteria were incorporated with a mixture of lactic acid bacteria viz; *S. thermophilus*, *L. acidophilus*, *Lc. lactis* ssp. *cremoris*, *Leuconostoc mesenteroides* ssp. *cremoris* to manufacture non-ripening (scalded at 53–55°C for 15 min) and ripening (not scalded) soft cheeses. The heat treatment associated with the non-ripening cheese had an adverse effect on the survival of the bifidobacteria. The *Bifidobacterium* species and four other lactic acid bacteria used for cheese preparation were entirely retained in ripening cheese, whereas in non-ripening cheese, only two species (i.e. *L. acidophilus* and *S. thermophilus*) were recovered (Ariga, Takahashi, Sakmoto & Tsutsui 1989a; Ariga, Hujita, Nakajima & Kanbara, 1989b).

Blanchette et al. (1996) used a dressing fermented by bifidobacteria to produce Cottage cheese enriched with probiotic cultures. The cream dressing was then combined with the cheese curd produced using conventional processing techniques. Pasteurized cream dressing (14% fat) was fermented with a 3% (w/w) inoculum of *B. infantis* at 37°C to pHs ranging from 4.5 to 6.0. During the first 10 days of storage, which corresponds to the normal storage period for Cottage cheese, *B. infantis* survived well with no significant differences in viability due to pH of the dressing. However, the bifidobacterial population dropped rapidly with further storage until no growth was detected at 28 days. The bifidobacteria maintained β-galactosidase activity throughout storage, with storage time causing more rapid decreases in enzyme activity of cottage cheese dressing fermented to pH 4.5, in comparison with cottage cheese dressing fermented to pH 5.0–6.0.

Although the most common processing stage at which to add the bifidobacteria to the milk would be in conjunction with the other starter cultures, other alternative processing protocols have been successful in incorporating the bifidobacteria into cheese and cultured dairy products. Many of these alternative protocols have been used to develop Cheddar cheese with bifidobacteria. The first report of the addition of *Bifidobacterium* to Cheddar cheese is from 1994. Dinakar and Mistry (1994) added commercial or immobilized freeze-dried strains of *B. bifidum* to the matrix of Cheddar cheese, following cheddaring and salting. Both types of the bifidobacteria remained viable in Cheddar cheese (counts higher than 10^7 cfu g^{-1}), but did not exhibit vigorous metabolic activity through 24 week storage period. The addition of bifidobacteria did not affect the flavour, texture or appearance of the Cheddar cheese.

The application of a two-stage fermentation for cultured dairy products has been shown to be effective in increasing the viability of probiotic bacteria by allowing the probiotic bacteria to become dominant prior to the addition of the starter cultures. Since starter lactic acid bacteria produce inhibitory substances against probiotic bacteria and grow faster than them during fermentation, the viability of probiotic bacteria could be reduced. Fermentation with probiotic bacteria initially for 2 h followed by fermentation with starter cultures may be helpful in improving the viability of the former and result in higher counts, as was reported for *L. acidophilus* 2409 and *B. longum* 1941 (Shah, 2000).

In the production of Cheddar cheese, *B. infantis* was initially cultured in the cream prior to adding to the skim milk and inoculating with the *Lactococcus* starter cultures. The production of acetic and lactic acids in the cream prior to the initiation of cheese-making reduced the time required for curd formation. Through a 12-week storage period, counts of bifidobacteria did not change significantly and were maintained at levels greater than 10^6 cfu g^{-1} of cheese. The bifidobacteria remained metabolically active during the storage period, but did not adversely affect the sensory characteristics and composition of the cheese (Daigle, Roy, Bélanger, & Vuillemard, 1999).

Currently, industrial demand for technologies ensuring probiotic stability in foods remains strong. The encapsulation technology for probiotic or protective cultures provides promising prospects for improved culture performance and protects the bacteria during digestion in the gastrointestinal tract (Rao, Shiwnarain, & Maharaj, 1989; Panyam, 1992; Mattila-Sandholm et al., 2002).

In Crescenza cheese, use of calcium alginate to immobilize a mixed culture of three bifidobacteria strains did not significantly affect the viability of the bifidobacteria in comparison to a non-immobilized mixed culture of the same three strains (Gobbetti et al., 1998). Similarly, Dinakar and Mistry (1994) compared the growth and viability of *B. bifidum*, added either as a commercially available powder or as an immobilized (with κ-carageenan) freeze-dried preparation in Cheddar cheese. With both methods, the number of bifidobacteria in the cheese increased by 1–2 $\log_{10}$ cycles through 24 week storage period. The immobilized preparation protected the bacteria from degradation during the aging period. Maximum bifidobacteria counts occurred at 18 weeks for the commercial preparation and 24 weeks for the immobilized preparation.

Following processing, the selection of packaging materials can further have a significant impact on the survival of the bifidobacteria. Packaging materials with good oxygen barriers, such as PVDC and EVOH, have been shown to be more effective than polyethylene and polystyrene, packaging materials widely used for foods, in maintaining the viability of the bifidobacteria (Ishibashi & Shimamura, 1993).

7. Enumeration of bifidobacteria and lactic acid bacteria in cheese

Not only are there challenges associated with the viability of bifidobacteria, there are similar challenges related to the enumeration of the bifidobacteria. Enumeration of bifidobacteria using a selective medium, where the cultured dairy products contain only bifidobacteria, should not pose many problems. However, most cheeses and other cultured dairy products also contain lactic acid bacteria, which have similar growth characteristics to the bifidobacteria. Currently, the lack of standard methods for monitoring the levels of bifidobacteria in cheeses and other dairy products has caused difficulties in quality control and the establishment and monitoring of official levels of probiotic bacteria (Vinderola & Reinheimer, 1999). Consequently, the introduction of rapid, reliable techniques to enumerate bifidobacteria alone and together with other starter lactic acid bacteria has become essential for the dairy industry (Arroyo et al., 1994).

The traditional phenotypic identification of lactic acid bacteria is rather tedious and not always reliable. Moreover, there are certain species that cannot be readily distinguished by phenotypic characteristics. Phenotypic responses can also be affected by environmental conditions (Schleifer et al., 1995). The simultaneous presence of several species in a cheese matrix (starter cultures and probiotic bacteria) can make it difficult to achieve a differential or a selective colony count of each individual species. When different strains of the same species are added to a cheese, traditional colony count techniques lack the resolution necessary to differentiate them.

Several culture media have been suggested for isolation and differential/selective enumeration of bifidobacteria and *Lactobacillus* species in fermented dairy products, and their performance was discussed in previous reviews (Shah, 1997; Shah, 2000; Roy, 2001). The International Dairy Federation has recommended different culture media for detection and enumeration of bifidobacteria (IDF, 1990; IDF, 1992). The range of different culture media used for the detection and enumeration of probiotic bacteria in fermented foods indicates that there is no standard culture medium (Roy, 2001). The difficulties associated with the detection and enumeration of bifidobacteria is caused by the strain specificity of results, the simultaneous use of different species in the product and differences found in cell recovery or colony differentiation (Roy, 2001).

Not all proposed culture media give good results when selective or differential enumeration of bifidobacteria is required in the presence of cheese starter cultures, especially when the media are applied to perform a colony count of strains that are different from those employed to design the methodology (Samona & Robinson, 1991; Ghoddusi & Robinson, 1996b; Dave & Shah, 1996). The culture media proposed for a differential or selective enumeration of bifidobacteria for routine analysis should not be complex or time-consuming to prepare and should offer a good cell recovery for the microorganisms (Lim et al., 1995). Many of the culture media developed to enumerate several strains of different species fail because of the lack of recovery of one or more species (Lankaputhra & Shah, 1996), lack of selectivity (Lim et al., 1995; Pacher & Kneifel, 1996) or differentiation among colonies (Kneifel & Pacher, 1993; Ghoddusi & Robinson, 1996b; Nighswonger et al., 1996).

MRS agar is frequently used for the enumeration of lactic acid and probiotic bacteria in cultured dairy products, and is effective when *Bifidobacterium* are the only live culture present (Blanchette et al., 1995). More frequently however, inhibitors or supplements are used in combination with other compounds to enhance selectivity of the bifidobacteria and inhibit the growth of the lactic acid bacteria. The required selectivity and

Table 2
Microbiological media used for the detection and enumeration of bifidobacteria in cheese

Media	Cheese	Reference
AMC Agar	Canestratro Pugliese cheese	Corbo et al. (2001)
MRS Agar	Kariesh cheese	Murad et al. (1998)
	Cottage cheese	Blanchette et al. (1996)
MRS Agar with NPNL solution (neomycin sulfate, paromomycin sulfate, nalidixic acid, penicillin G, lithium chloride)	Cheddar cheese	Dinakar and Mistry (1994)
	Crescenza cheese	Gobbetti et al. (1998)
MRS Agar with cysteine and Bactogar		
	Cottage cheese	Blanchette et al. (1996)
MRS Agar with cysteine, sheep's blood, lithium chloride, and sodium propionate	Cottage cheese	O'Riordan and Fitzgerald (1998)
MRS Agar with cysteine, sheep's blood, lithium chloride, sodium propionate, and bile salts	Gouda	Gomes et al. (1995, 1998), Gomes and Malcata, (1999)
MRS Agar with bile salts, lithium chloride, and sodium propionate	Fresco cheese	Vinderola et al. (2000b)
MRS Agar with NNL solution (neomycin sulfate, nalidixic acid, and lithium chloride)	Tallaga cheese	El-Zayat and Osman (2001)
Columbia agar with raffinose, cysteine, lithium chloride, and sodium propionate	Cheddar-like cheese	Daigle et al. (1999)

ease of preparation of the agar must be considered in the selection of the appropriate media for the enumeration of the bifidobacteria, especially in the presence of lactic acid bacteria. Table 2 summarizes several selective or differential techniques frequently used for the enumeration of the bifidobacteria added to cheeses. Differences in the appearance of the lactic acid bacteria and bifidobacteria may also be used to differentiate colonies (Vinderola et al., 2000b). The lactic acid bacteria may also be enumerated and differentiated from the bifidobacteria using media specific for lactic acid bacteria, such as TGV (tryptone, glucose and meat extract) agar (Gomes et al., 1995, 1998), or incubated modified aerobic condition (5% CO_2) to prevent the growth of the bifidobacteria (El-Zayat & Osman, 2001; Vinderola et al., 2000b).

Due to challenges associated with the traditional phenotypic identification of bifidobacteria in the presence of lactic acid bacteria, other methods must be explored. Molecular biology methods for the study of lactic acid starter and probiotic bacteria have been efficiently applied to the identification and study of the genetic diversity of species of the intestinal microbial complex (Matsuki, Watanabe, Tanaka, Fukuda, & Oyaizu, 1999; Xanthopoulos, Ztaliou, Gaier, Tzanetakis, & Litopoulou-Tzanetaki, 1999; Satokari, Vaughan, Akkermans, Saarela, & de Vos, 2001), cheeses and cheese starters (Cibik, Lepage, & Tailliez, 2000; Giraffa, De Vecchi, Rossi, Nicastro, & Fortina, 2000) and fermented milks (Vincent, Roy, Mondou, & Déry, 1998; Roy & Sirois, 2000) as well as to the rearrangement of microbial collections (Tailliez, Quénée, & Chopin 1996; Daud Khaled, Neilan, Henriksson, & Conway, 1997). PCR techniques using fluorescent agents can also be used for species identification and enumeration without isolating the bacteria from the food (Tailliez, 1998). Currently, however, there is still a lack of routine methodologies to allow the use of these techniques to quantify lactic acid bacteria in dairy products. However, the development of the PCR-based molecular techniques will provide a more rapid, reliable and accurate method for the identification of bacterial species and will be critical in the development of products with bifidobacteria and other probiotic bacteria (Mannu, Riu, Comunian, Rozzi, & Scintu, 2002). The application of these and other molecular biology techniques will not only improve the ability to enumerate the bifidobacteria, but also improve our understanding of the effects of processing conditions and interactions with lactic acid bacteria on the survival of bifidobacteria and the successful incorporation of bifidobacteria into cultured dairy products.

8. Conclusions

Limited research on the incorporation bifidobacteria into different types of cheeses has shown that cheese is a viable carrier for bifidobacteria. However, to reach the target viable cell count of 10^6 cfu g^{-1} for the bifidobacteria in the final product may require some technological changes both in selection of the appropriate species and strains, and in supporting the cells to maintain their viability throughout storage before consumption. Therefore, research on improving the survival of bifidobacterial cultures in the final product is of significant importance. This review has presented several techniques that have been shown to be effective in increasing the viability of the bifidobacteria in cheeses. Among these techniques are the selection of bifidobacteria

strains with high resistance against lower pH, bile salts, and oxygen; the application of mixed cultures of bifidobacteria together with selected strains of starter cultures to enhance the viability of the bifidobacteria; encapsulating the bifidobacterial cultures; and modifying the cheese processing to promote an environment suitable for the growth of the bifidobacteria. The successful development of cheeses containing bifidobacteria will contribute to the evolution of healthier foods with desirable sensory characteristics in today's food industry.

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