Dairy Sci. & Technol. (2011) 91:283–308
DOI 10.1007/s13594-011-0005-x

REVIEW PAPER

Viability of probiotic microorganisms in cheese during production and storage: a review

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Received: 18 February 2010 / Accepted: 4 October 2010 /
Published online: 16 February 2011

Abstract Cheese is a dairy product which has a good potential for delivery of probiotic microorganisms into the human intestine due to its specific chemical and physical characteristics compared to fermented milks (higher pH value and lower titrable acidity, higher buffering capacity, greater fat content, higher nutrient availability, lower oxygen content, and denser matrix of the texture). In addition, a large variety of cheese types all over the world, consumption of cheese by everybody in their long-term diet, as well as the nutritional value of cheese have resulted in regular market growth for probiotic cheeses. To be considered to offer probiotic health benefits, probiotics must remain viable in food products above a threshold level (e.g., 10^6 cfu g^{-1}) until the time of consumption, without adversely altering sensory attributes. Therefore, incorporation of probiotic cells into different cheese matrices and studying the influences of different compositional and process factors affecting the viability of probiotics in this product as well as its sensory properties have been the subject of numerous studies. Factor influencing the stability of probiotics in cheese can be categorized into three areas including formulation factors (strains of probiotic bacteria and microbial interactions, pH and titrable acidity, hydrogen peroxide, molecular oxygen, growth promoters and food additives, salt, microencapsulation, and ripening factors), process factors (incubation temperature, heat treatment, types of inoculation, and storage temperature), and packaging materials and systems. This article reviews the viability of probiotic organisms in cheese as well as the main factors influencing their stability during processing and storage.

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摘要 与其他的发酵乳制品相比，干酪具有其独特的物理化学特性（高pH值、较低的滴定酸度、较高的缓冲能力、较高的脂肪含量、高营养利用率、较低的氧气浓度以及浓厚的质地），因此干酪是一种理想的将益生微生物带进人体的载体。世界上有千余种干酪，人类消费干酪具有悠久的历史。由于干酪的高营养价值，使具有益生功能干酪的市场需求量逐年增加。基于益生菌对人体健康的重要性，要求食品中益生菌的活菌数在整个货架期内必须达到一定的数值(如10^6 cfu. g^{-1})，而且产品的感官特性不能发生改变。因此，国内外关于不同干酪底物中益生菌细胞总数对干酪营养成分以及干酪感官特性影响的报道非常多。影响干酪中益生菌稳定性的因素分概括为三个方面，包括组成因素（益生菌菌株和微生物之间的相互作用、pH和可滴定酸度、过氧化氢、分子态氧、生长促进剂和食品添加剂、盐、微胶囊和成熟因素）、加工条件（培养温度、热处理、接种形式和贮藏温度）以及包装材料和包装方式　本文概述了干酪中益生微生物的生存能力以及在加工和贮藏期间影响益生微生物稳定性的因素。

Keywords Cheese · Probiotic · Survival · Viability

关键词 干酪 · 益生菌 · 存活能力 · 生存能力

1 Introduction

Functional foods are defined as “foods that through specific beneficial physiological action, contribute to the health of the consumer” (Corbo et al. 2001). Probiotics are defined as “live microorganisms which when administered in adequate numbers confer a health benefit on the host” (FAO/WHO et al. 2001). Probiotic bacteria, specifically bifidobacteria and lactobacilli, are the normal inhabitants of the human colon. These bacteria beneficially affect human health by improving the balance of intestinal microflora and improving mucosal defenses against pathogens (Juntunen et al. 2001; Wang et al. 2004). Additional health benefits include enhanced immune response, reduction of serum cholesterol, vitamin synthesis, anti-carcinogenic activity, and anti-bacterial activity (Arunachalam 1999; Belviso et al. 2009; Blanchette et al. 1995; Brassert and Schiffrin 2000; Gomes and Malcata 1999; Ibrahim et al. 2010; Lourens-Hattingh and Viljoen 2001; Robinson and Samona 1992; Songisepp et al. 2004).

During the past three decades, significant attention has been paid to fermented dairy products containing probiotic bacteria. As the market for functional foods continues to expand, research in the development of food products containing probiotic bacteria will also continue to grow. With the growth of the functional foods area, a growing research interest has focused on the incorporation of probiotic bacteria into cultured dairy products to further enhance the nutritional value of these products (Robinson 1991; Tamime 2002). Members of the genus *Bifidobacterium* and *Lactobacillus* are widely used as probiotic microorganisms in probiotic foods (Corbo et al. 2001).

To provide health benefits related to probiotic organisms, recommendations for the minimum viable counts of each probiotic strain in gram or milliliter of probiotic products are quite variable. For example, the minimum viable levels of 10^5 cfu g^{-1} (Shah 1997; Shah et al. 1995), 10^6 cfu g^{-1} (Arroyo et al. 1994; Pagano 1998;

Robinson and Samona 1992; Rybka and Kailasapathy 1995), and 10^7 cfu g^{-1} (Samona and Robinson 1994) have been suggested for probiotics in different products. In Japan, microorganisms with potential "probiotic" characteristics must remain viable and survive at 10^7 cfu g^{-1}/cfu mL^{-1} of product in accordance with the standard introduced by the Fermented Milks and Lactic Acid Bacteria Beverage Association in Japan (Ishibashi and Shimamura 1993). However, in general, the food industry has applied the recommended level of 10^6 cfu g^{-1} at the time of consumption for 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). Several scientific papers have proposed a minimum daily dose of 10^8–10^9 cfu g^{-1}/cfu mL^{-1} for consumption of probiotic products, which corresponds to 100 g or milliliters of these products containing 10^6 up to 10^7 cfu g^{-1}/cfu mL^{-1} viable probiotic cells per day (Hoier et al. 1999; Moreno et al. 2006; Talwalkar and Kailasapathy 2004). In terms of the viability of probiotics in cheese, which contains a complex combination of microorganisms that changes with time, it initially contains large numbers of starter lactic acid bacteria (SLAB), and then with maturation (ripening), an increasing number of nonstarter lactic acid bacteria (NSLAB; Ross et al. 2002). Therefore, the viability of probiotic bacteria in fermented cheese is a complex phenomenon. Apart from the viability of probiotics in products until the time of consumption, their survival after exposure to gastrointestinal tract (GIT) conditions is also crucial. Food matrices possess significant effects in successful delivery of probiotics into the intestine (Mattila-Sandholm et al. 2002).

Cheese is a good alternative for the delivery of probiotics into the intestine and as a result has been the subject of various marketing and research studies in recent years (Gardiner et al. 1998; Gobbetti et al. 1998). Cheese has certain advantages as a carrier of probiotics compared with more acidic fermented dairy products such as yogurt. It creates a buffer against the high acidic environment in the GIT and thus creates a more favorable environment for probiotic survival throughout the gastric transit. Furthermore, the dense matrix and relatively high fat content of cheeses such as Cheddar may offer added protection to probiotics in the stomach (Dinakar and Mistry 1994; Gardiner et al. 1999). Most of the cheeses tested succeeded in maintaining the viability of these microorganisms, as well as in achieving appropriate technological and sensorial properties of the final product. However, a prerequisite of probiotic cheese manufacture is that the cultures survive the relatively long cheese ripening times, a factor that should be taken into account when selecting probiotic strains for cheese applications (Tamime et al. 2005). Many researchers and manufacturers have incorporated probiotic bacteria in different types of cheese (Section 2). There are some review articles in which some parts are related to the incorporation of probiotic bacteria in cheeses (Boylston et al. 2004; da Cruz et al. 2009; Grattepanche et al. 2008). However, in none of them, the viability of probiotic bacteria in different cheeses and the main factors affecting their viability have been specifically the subject of review. Therefore, the purpose of this review is to discuss the viability of probiotics in cheese during the production and storage and considering the main factors affecting their viability during the various stages.

2 Viability of probiotic microorganisms in cheese before and after the consumption

Viability of probiotic cells in food products until the time of consumption is the most critical factor of these products. Effective incorporation of probiotic bacteria into cheeses requires that the probiotic bacteria maintain their viability throughout processing, without adversely altering the sensory characteristics (Boylston et al. 2004). Generally, cheese does provide an environment that would be conducive to the long-term survival of probiotic bacteria compared to fermented milks due to higher pH value and lower titrable acidity, higher buffering capacity, greater fat content, lower oxygen content, higher nutrient availability, and denser matrix of the texture (Dinakar and Mistry 1994; Gardiner et al. 1999). One of the main differences among probiotic cheeses and other probiotic dairy products is that in the latter products, probiotic bacteria should maintain their viability during the relatively long ripening-storage period (Tamime et al. 2005).

Different probiotic bacteria have been incorporated into different types of cheese such as Minas fresh cheese (Alegro et al. 2002; Buriti et al. 2005a, b, 2007a; de Souza et al. 2008; Souza and Saad 2008), fresh cheese (Masuda et al. 2005; Suárez-Solís and Cardoso 2002), fresh cream cheese supplemented with inulin (Buriti et al. 2007b), Crescenza (Gobbetti et al. 1997, 1998), Cottage (Blanchette et al. 1996; O'Riordan and Fitzgerald 1998; Roy et al. 1997), soft cheese (Coeuret et al. 2004), Petit Suisse cheese (Cardarelli et al. 2008), Argentinean fresco (Vinderola et al. 2000), Kariesh cheese (Murad et al. 1998), Cremoso cheese (Milesi et al. 2009), Árzúa-Ulloa (Menéndez et al. 2000), Mascarpone cheese (Carminati et al. 2001), Gouda (Gomes et al. 1995), Pategrás cheese (Bergamini et al. 2005, 2009; Milesi et al. 2009), Probiotic goat's cheese (Fernandez et al. 2005; Gomes and Malcata 1998; Khatoon et al. 1990; Martín-Hernández et al. 1992), Festivo cheese (Ryhanen et al. 2001), Canestrato Pugliese hard cheese (Corbo et al. 2001), Tallaga cheese (El-Zayat and Osman 2001), Cheddar (Dinakar and Mistry 1994; Gardiner et al. 1998, 1999; Khatoon et al. 1990; Lynch et al. 1999; Mc Brearty et al. 2001; Mistry and Kasperson 1998; Ong et al. 2007; Phillips et al. 2006; Sharp et al. 2008; Thomas and Crow 1983), Cheddar-like cheese (Daigle et al. 1999), Iranian white-brined cheese (Ghoddusi and Robinson 1996), Turkish white cheese (Kasımoğlu et al. 2004), white-brined cheese (Özer et al. 2008), white cheese (Kasımoğlu et al. 2004), Turkish Beyaz cheese (Kiliç et al. 2009), and cheese-based dips (Tharmaraj and Shah 2004). Numerous strains of probiotic bacteria have been successfully added into different types of cheeses including lactobacilli (*Lactobacillus acidophilus*, *Lactobacillus casei*, *Lactobacillus paracasei*, *Lactobacillus plantarum*, *Lactobacillus rhamnosus*, and *Lactobacillus gasseri*) and *Bifidobacterium* spp. (*Bifidobacterium animalis* ssp. *lactis*, *Bifidobacterium longum*, *Bifidobacterium bifidum*, and *Bifidobacterium infantis*), and to a lesser extent, *Propionibacterium freudenreichii* ssp. *shermanii*. One of the first reports of the addition of *Bifidobacterium* to Cheddar cheese was in 1994 (Dinakar and Mistry 1994). Commercial or immobilized freeze-dried strains of *B. bifidum* were added to the matrix of Cheddar cheese, following cheddaring and salting. Both types of the bifidobacteria remained viable in this cheese (counts higher than 10^7 cfu g^{-1}), but did not exhibit vigorous metabolic activity throughout a 24-week storage period. There were no adverse effects on cheese flavor, texture, or

appearance. Selected publications on the inclusion of probiotic microorganisms in cheese are shown in Table 1.

In general, the viabilities of probiotic bacteria have been reported to be satisfactory in different types of cheeses even at the end of storage periods. According to Table 1, in most studies, the final viable counts of probiotic bacteria at the end of storage periods in cheeses are $>10^6$ cfu g^{-1}, which is generally recognized as the minimum therapeutic level for probiotics. In many investigations, the final viable counts of probiotic bacteria were $>10^7$ cfu g^{-1}, and in many $>10^8$ cfu g^{-1}. These results confirm that cheese is a good probiotic carrier until the consumption. In many studies, probiotic bacteria maintained their viability throughout the storage period, so that the initial and final viable populations were not considerably different (negligible loss or less than one log cycle loss; Coeuret et al. 2004; Gomes and Malcata 1998; Masuda et al. 2005; Tharmaraj and Shah 2004; Vinderola et al. 2000). In some other studies, the viable counts of these bacteria increased significantly during the storage time (near to two log cycles or more; Bergamini et al. 2005; Buriti et al. 2005a, b; Gobbetti et al. 1998; Gomes and Malcata 1998; Kasımoğlu et al. 2004). In a few studies, the viability of probiotics dramatically decreased during the mentioned period (about three log cycles or more; Phillips et al. 2006). According to Table 1, four types of storage period for cheeses are distinguished: <1, 1–3, 3–6, and 6–12 months. As can be seen, different probiotic bacteria maintain their viability satisfactory within all mentioned times. Some cheeses are good or excellent media for maintaining the viability of probiotics, namely, simultaneously added lactobacilli and bifidobacteria (Pategrás Argentino cheese, 60 days storage/White-brined cheese, 90 days/Cheddar cheese, 6 months), bifidobacteria alone (Gouda cheese, 9 weeks), and lactobacilli alone (Cremoso cheese, 60 days). Conversely, some cheeses are fair or unsuitable media for maintaining the viability of probiotics such as Canestrato Pugliese for bifidobacteria (90 days), Tallaga and Brazilian Minas fresh cheeses for bifidobacteria and lactobacilli (21–28 days), and cheese-based French onion dip for bifidobacteria, lactobacilli, and propionibacteria (10 weeks). In some probiotic cheeses, *Streptococcus thermophilus* (Buriti et al. 2007b; Carminati et al. 2001; Milesi et al. 2009; Souza and Saad 2008) and *Lactobacillus delbrueckii* ssp. *bulgaricus* (Corbo et al. 2001; Ghoddusi and Robinson 1996; Jain et al. 2004) have been co-cultured with probiotic bacteria in order to improve the technological and sensory characteristics of the final product (Table 1). The latter case results in the significantly less viability of probiotic bacteria due to inhibitory impacts of *L. delbrueckii* ssp. *bulgaricus* on probiotics (Sections 3.1.3, 3.1.5, and 3.2.1).

A high viable population of probiotic bacteria in food products at the point of consumption does not guarantee the same survival after arrival of the cells into the intestine. The very low pH of the stomach, along with the presence of bile salts in the small intestine, is the main reason for the dramatic decline in viability of delivered cells (Mortazavian et al. 2008b). Therefore, investigating the viability of probiotics after exposure to GIT conditions should be performed as a complementary study to the "in product" work. However, there are limited studies associated with the survival of probiotic cells under GIT in cheeses. In vivo studies regarding survival analysis of probiotics in food products are commonly carried out under simulated gastrointestinal conditions. Dense matrix, high buffering capacity, and relatively high fat content of cheeses offer good protection to probiotic cells during

Table 1 Selected publications on probiotic microorganisms used in cheese

Group of cheese	Cheese type	Viability of probiotics (start/end of storage; cfu g^{-1})	Probiotic microorganisms and co-cultures	Storage conditions	Special remarks	Reference
Fresh cheeses	Minas Fresh cheese	~10^6/>10^6 ~10^5/~10^7	*L. paracasei* ssp. *paracasei* *L. acidophilus* LA-5	5°C, 21 days	Addition of mesophilic homofermentative type O lactic culture R-704 or direct acidification with lactic acid	Buriti et al. 2005a, b
	Minas Fresh cheese	~10^6/~10^6 ~10^6/>10^6	*L. acidophilus* LA-5 solely/ *L. acidophilus* in co-culture with *S. thermophilus*	5°C, 21 days	Addition of lactic acid (0.25 ml l^{-1}), 0.01 gl^{-1} inocula, vacuum packaged	Souza and Saad 2008
	Minas Fresh cheese	~10^6/>10^6	*L. acidophilus* LA-5 *S. thermophilus*	4–5°C, 14 days	–	de Souza et al. 2008
	Fresh cream cheese supplemented with inulin	~10^7/>10^7 ~10^9/~10^9	*L. paracasei* *S. thermophilus*	4±1°C, 21 days	With/without inulin	Buriti et al. 2007b
	Brazilian Minas Fresh cheese	~10^8/>10^8 ~10^8/~10^6	*L. acidophilus* La-5 *B. animalis* Bb-12 *Streptococcus thermophilus*	4±1°C, 21 days	–	Buriti et al. 2007a
	Fresh cheese	>10^8/>10^7 >10^8/>10^7 >10^8/>10^7	*L. acidophilus* JCN 11047 *L. acidophilus* 1132T *L. gasseri* JCM657	7°C, 4 weeks	–	Masuda et al. 2005
	Fresh cheese	Not found/~10^7 Not found/~10^7	*B. bifidum* *L. casei*	15 days	–	Suárez-Solís and Cardoso 2002
	Crescenza cheese	~10^6/>10^8 ~10^6/>10^7 ~10^6/>10^5	*B. bifidum* *B. longum* *B. infantis*	14 days	–	Gobbetti et al. 1998

	Cottage cheese	Not found/~10^6	*B. bifidum*	14 days	–	O'Riordan and Fitzgerald 1998
	Cottage cheese	Not found/~10^6	*B. infantis*	10 days	Combined with cream dressing (14% fat)	Blanchette et al. 1996
Soft cheeses	Pont-L'Evêque cheese	~10^8/>10^7	*L. plantarum* UCMA 3037	75 days	Isolated from unpasteurized Camembert cheese	Coeuret et al. 2004
	Petit-suisse cheese	>10^7/>10^7 >10^6/~10^6	*B. animalis* ssp. *lactis* *L. acidophilus*	4±1°C, 28 days	Symbiotic with inulin, oligofructose and oligosaccharides from honey	Cardarelli et al. 2008
	Fresco cheese	~10^7/>10^6 ~10^7/>10^6 ~10^7/>10^6 ~10^7/~10^7	*B. bifidum* *B. longum* *L. acidophilus* *L. casei*	60 days	–	Vinderola et al. 2000
	Kariesh cheese (Egyptian soft cheese)	~10^{10}/~10^8	Bifidobacteria	10 days	–	Murad et al. 1998
	Vidiago cheese Washed-curd goat's cheese	Not found/>10^8	*L. delbrueckii* ssp. *lactis* UO 004	28 days	–	Fernandez et al. 2005
	Cremoso (soft Argentinian) cheese	~10^9/>10^7 ~10^9/>10^7 ~10^9/>10^8	*L. casei* I90 *L. plantarum* I91 *L. rhamnosus* I73 and I75 *S. thermophilus*	5±0.5°C, 60 days	–	Milesi et al. 2009
Semi-hard cheeses	Gouda semi-hard cheese	>10^8/>10^7 >10^9/>10^8	*L. acidophilus* *B. lactis*	13°C, 9 weeks	Salt-in-dry matter range of 2–4% (*w*/*w*)	Gomes et al. 1995
	Pategrás Argentino cheese (semi-hard)	~10^9/~10^9 ~10^9/~10^8 ~10^9/~10^7	*L. paracasei* *L. acidophilus* *B. lactis*	60 days	Single or mixed cultures (synergistic effects were observed), lyophilized, or after pre-incubation	Bergamini et al. 2009

Table 1 (continued)

Group of cheese	Cheese type	Viability of probiotics (start/end of storage; cfu g^{-1})	Probiotic microorganisms and co-cultures	Storage conditions	Special remarks	Reference
	Pategrás cheese	$\sim10^7/\sim10^8$ $\sim10^8/\sim10^9$	*L. acidophilus* *L. paracasei* ssp. *paracasei*	60 days	Lyophilized culture and pre-incubated in a substrate, respectively	Bergamini et al. 2005
	Pategrás cheese	Not found/$>10^7$ Not found/$>10^7$ Not found/$>10^7$	*L. casei* I90 *L. plantarum* I91 *L. rhamnosus* I73 and I75	12°C, 60 days (80% RH)	–	Milesi et al. 2009
	Queijo de Cabra (Portuguese semi-hard, lightly pressed goat cheese)	$\sim10^7/>10^8$ $>10^6/>10^7$	*B. lactis* *L. acidophilus* Ki *Streptococcus lactis*	6°C, 70 days (92% RH)	–	Gomes and Malcata 1998
	Semi-hard cheese	Not found/$>10^6$	*L. acidophilus* LF221	6 weeks	–	Rogelj et al. 2002
	Semi-hard cheese	$\sim10^9/>10^7$	*L. paracasei* NFBC 338	3 months	–	Gardiner et al. 2002
Hard cheeses	Canestrato Pugliese (hard Italian cheese)	$\sim10^7/\sim10^6$ $\sim10^7/\sim10^5$	*B. bifidum* *B. longum* *S. thermophilus* *L. delbrueckii* ssp. *bulgaricus*	90 days	–	Corbo et al. 2001
	Tallaga cheese	Not found/$>10^6$ Not found/$>10^6$	*B. lactis* Bb-12 *L. acidophilus* La-5	28 days	–	El-Zayat and Osman 2001
	Cheddar cheese	$\sim10^8/>10^8$ $\sim10^7/\sim10^5$	*B. lactis* Bb-12 *B. longum* BB536	8°C, 6 months	–	Mc Brearty et al. 2001
	Cheddar cheese	Not found/$>10^7$	*B. bifidum*	24 weeks	–	Dinakar and Mistry 1994
	Cheddar cheese	$\sim10^8/\sim10^8$	*B. longum* 1941	4 C, 6 months	–	Ong et al. 2007

		$\sim10^8/\sim10^8$	*B. lactis* LAFTI® B94			
		$\sim10^8/\sim10^8$	*L. casei* 279			
		$\sim10^8/\sim10^8$	*L. paracasei* LAFTI® L26			
		$\sim10^8/\sim10^8$	*L. acidophilus* 4962			
		$\sim10^8/\sim10^8$	*L. acidophilus* LAFTI® L10			
	Cheddar cheese	$\sim10^8/>10^7$	*Bifidobacterium* B94	32 weeks	–	Phillips et al. 2006
		$\sim10^8/\sim10^8$	*Bifidobacterium* Bb-12			
		$>10^8/>10^8$	*Bifidobacterium* DR10			
		$>10^8/>10^3$	*L. acidophilus* LA-5			
		$>10^7/>10^3$	*L. acidophilus* L10			
	Cheddar-like cheese	$\sim10^8/>10^6$	*B. infantis*	4°C, 84 days	–	Daigle et al. 1999
White-brined cheeses	Iranian White-brined cheese	Not found/10^6	*B. bifidum*	60 days	Using full cream pasteurized milk	Ghoddusi and Robinson 1996
			S. thermophilus			
			L. delbruekii ssp. *bulgaricus*			
	Turkish White cheese	$\sim10^4/\sim10^7$	*L. acidophilus* 593N	4°C, 90 days	Ripened in vacuum pack and in brine (vacuum pack is considered better)	Kasımoğlu et al. 2004
	White-brined cheese	$>10^8/\sim10^8$	*B. bifidum* BB-12	90 days	Microencapsulated by an extrusion or an emulsion technique, respectively	Özer et al. 2008
		$>10^9/>10^6$	*L. acidophilus* LA-5			
	Cheese-based French onion dip	$>10^7/>10^6$	*L. acidophilus*	4°C, 10 weeks	Addition NaHCO3 and L-cysteine (NaHCO3 causes more growth)	Tharmaraj and Shah 2004
		$>10^7/>10^5$	*B. animalis*			
		$>10^7/>10^7$	*L. paracasei* ssp. *paracasei*			
		$>10^7/>10^6$	*P. freudenreichii* ssp. *shermanii*			
		$>10^7/>10^7$	*L. rhamnosus* (all in co-culture)			

delivery through the GIT (Dinakar and Mistry 1994; Gardiner et al. 1999). It has been reported that the addition of 5 g of cheese to 10 mL of gastric juice increased the pH from 2.00 to 4.74, whereas 5 g of yogurt increased the pH to only 3.65 (Gardiner et al. 1998).

The *B. bifidum* strains incorporated into 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 et al. 2000). Sharp et al. (2008) used *L. casei* 334e, an erythromycin-resistant derivative of ATCC 334, as a model to evaluate the viability and acid resistance of probiotic strains of *L. casei* in low-fat Cheddar cheese. Low-fat Cheddar cheese, for example, contains 15% more protein and 7.5 g less fat per serving than full-fat Cheddar. Acid challenge studies in 8.7 mmol L^{-1} phosphoric acid (pH 2) at 37°C showed that counts of *L. casei* 334e in cheese samples dropped from 10^7 cfu g^{-1} to about 10^5 cfu g^{-1} after 30 min, and remained near 10^4 cfu g^{-1} after 120 min. Phosphoric acid resists the buffering effect of food. As a whole, they showed that low-fat Cheddar cheese is a viable delivery food for probiotic *L. casei* because it allowed for good survival during storage and helped protect cells against the very low pH that is encountered during stomach transit. Similar trends have been reported by other researchers who used hydrochloric acid (HCl) at pH 2 to evaluate acid resistance of probiotic strains in full-fat Cheddar cheese (Gardiner et al. 1999). However, HCl does not resist the buffering effects of food, so the pH of cheese homogenates in that study fell only to pH 4.74 instead of pH 2 due to the buffering effect of cheese. It has also been reported that the matrix of Minas fresh cheese, its high fat content, and its high buffering capacity offer protection to probiotic bacteria during passage through the GIT (Kailasapathy and Chin 2000; Vinderola et al. 2002a).

3 Main factors affecting the viability of probiotic microorganisms in cheese

The factors influencing viability of probiotic bacteria in cheese until the time of consumption can be divided into intrinsic and extrinsic factors such as pH, organic acids, hydrogen peroxide, dissolved oxygen, ripening and storage temperatures, additives such as sodium chloride, sugar, and anti-microbial preservatives, and volatile compound (Shah 2000). In a more technological approach, the mentioned factor can be categorized into three areas including formulating and processing factors as well as packaging. Figure 1 represents the main factors affecting viability of probiotic bacteria in cheese. These factors are expressed below.

3.1 Formulating factors

The formulating factors which affect the viability of probiotic strains in fermented foods include the specious strain of probiotic bacteria, the inoculation level, pH, acidity, molecular oxygen (especially for bifidobacteria), hydrogen peroxide, salt and sugars, food additives, moisture content, nutrient availability, growth promoters and inhibitors, and microencapsulation of probiotic cells (Roy et al. 1997). These factors are discussed in the following sections.

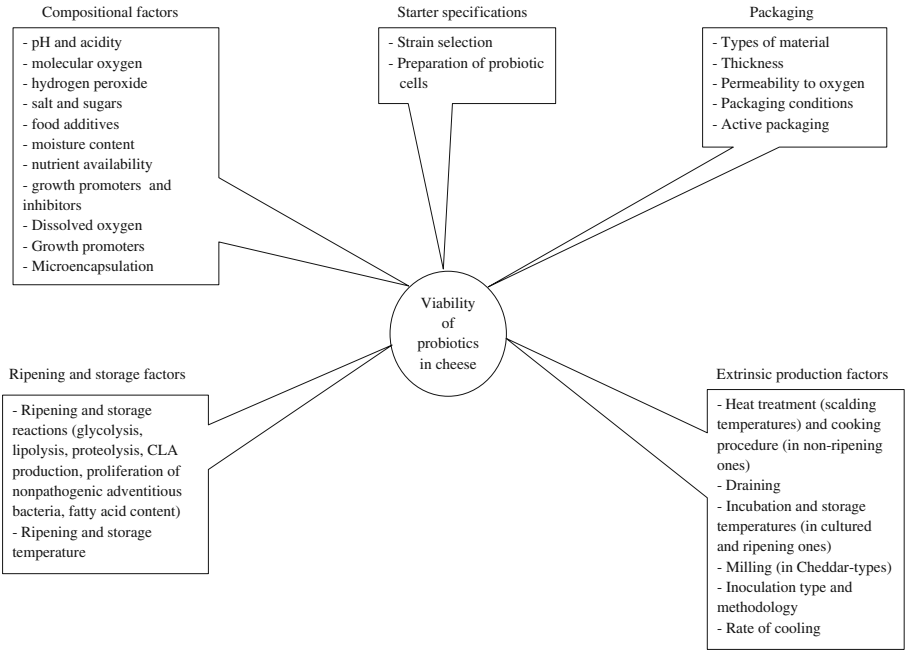

Fig. 1 Main factors affecting viability of probiotic bacteria in cheese

3.1.1 Strains of probiotic bacteria and microbiol interactions

Strains of probiotic bacteria used in different cheeses should be compatible to the specifications of these products. Hard cheeses such as Cheddar have a long ripening period of up to 2 years, and hence, the development of probiotic cheese requires stringent selection of probiotic strains to maintain viability in the cheese throughout manufacture and ripening until the point of consumption. *B. animalis* is often used in fermented products, including cheese, because of its intrinsic tolerance to the product as well as gastrointestinal conditions (Iwana et al. 1993). Also, *B. bifidum* and *B. longum* are among the strains that demonstrate good viability during manufacturing and storage of the cheese. In contrast, *B. infantis* and *Bifidobacterium adolescentis* show poor abilities to survive and would be less preferable for incorporation into fermented dairy products (Boylston et al. 2004). In the study of Ong et al. (2007), six probiotic organisms (*B. longum* 1941, *L. casei* 279, *L. acidophilus* 4962, *B. lactis* LAFTIs B94, *L. paracasei* LAFTIs L26, and *L. acidophilus* LAFTIs L10) were used for the development of probiotic Cheddar cheese. They were shown to maintain high levels of viability at the end of a 6-month ripening period at 4°C. Bifidobacteria require an anaerobic environment and neutral pH (6.5–7.0) to survive and to maintain levels greater than 10^6 cfu g^{-1} (Gomes and Malcata 1999). Therefore, non-fermented cheeses and those which have anaerobic environments (hard cheeses compared to soft cheeses) provide better conditions for growth and maintenance of bifidobacteria.

Lactococci, lactobacilli, and streptococci are among the starter cultures most commonly used in cheese-making. Many of these lactic acid bacteria also produce

environments that inhibit the growth of not only pathogenic and spoilage microorganisms but also of probiotic bacteria (Vinderola et al. 2002a, b). This inhibitory activity is attributed to several factors, including production of lactic and other organic acids, hydrogen peroxide, and bacteriocins or antibiotics, nutrient competition and depletion, alcoholic compounds, diacetyl, and an altered oxidation–reduction potential (Shah 2000). Therefore, the composition of cultures used for probiotic cheeses should be selected in a way that minimizes the antagonistic relationship among the non-probiotic and probiotic starters. It is important to mention that data relating to the coupling of thermophilic starters with probiotic bacteria in cheeses is lacking, as most research studies have been performed in cheese models using mesophilic starters. *B. longum* strains have demonstrated high survival rates in the presence of mesophilic starters and would be acceptable for use in cheese-making. The use of *B. adolescentis* resulted in converse observation (Roy et al. 1995). In a study using Minas fresh cheese, a typical Brazilian fresh cheese, the highest concentrations of *L. acidophilus* during storage were observed when the probiotic microorganism was added in co-culture with *B. lactis* and *S. thermophilus* (ABT culture). This effect was not observed for *L. acidophilus* when it was added only with *B. lactis* (Alegro et al. 2002; Oliveira et al. 2002). Gomes and Malcata (1998) found that a positive relationship between the two strains exited by noticing the increased survival of *B. lactis* throughout ripening of cheese when numbers of *L. acidophilus* were also high. Some other studies have shown the positive contribution of the proteolytic activity of *L. acidophilus* on the growth and maintenance of *B. lactis* (Klaver et al. 1993). Nevertheless, the greater the degree of reduction of total viable counts of *B. lactis* compared to *L. acidophilus* suggested that there was competition between the two strains for nutrients and energy sources within the cheese when maximum densities had been achieved (Gomes and Malcata 1998). Buriti et al. (2005b) incorporated *L. acidophilus* LA-5 in Minas fresh cheese and found that a discrete synergistic interaction between *L. acidophilus* and *Lactococcus lactis* ssp. *lactis* and/or *Lactococcus lactis* ssp. *cremoris* occurred. In the study of Milesi et al. (2009) on Cremoso (soft Argentinian) cheese and Pategrás (semi-hard Argentinian) cheese, *L. casei* I90, *L. plantarum* I91, and *L. rhamnosus* I73 and I75 were incorporated. No interaction was detected between starter and adjunct lactic cultures; streptococcal counts were similar in all the samples. These findings were in agreement with the studies of Bergamini et al. (2006) and Bude-Ugarte et al. (2006).

3.1.2 pH and titrable acidity

The pH is one of the most important factors which restricts the viability of probiotic bacteria. The optimum growth pH for bifidobacteria is between 6.5 and 7.0, and growth of these bacteria is retarded or inhibited below pH 5.0 or above 8.0 and is species- and strain-specific (Lourens-Hattingh and Viljoen 2001; Scardovi 1986). The optimum pH for growth of *L. acidophilus* is 5.5–6.0 (Gomes and Malcata 1998). Bifidobacteria are not as acid tolerant as *L. acidophilus*; the growth of the latter organisms ceases below pH 4.0 (Shah 1997). 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 (Laroia and Martin 1990; Modler et al. 1990a; Tamime and Robinson 1988; Vinderola et al. 2002a).

Cheeses (pH range 4.8–5.6) have a markedly higher pH than fermented milks (pH 3.7–4.5) and provide a more stable environment to support the long-term survival of the acid-sensitive probiotics compared to fermented milk products. In Cottage cheese, 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 to pH 5.0–6.0 (Blanchette et al. 1996). Minas fresh cheese has a high water activity, pH above 5.0, low salt content, and absence of preservatives. Therefore, it offers excellent conditions for survival and growth of probiotic strains (Buriti et al. 2005b). Gomes and Malcata (1998) studied *B. lactis* and *L. acidophilus* in cheese manufactured from goat's milk and applied response surface analysis (RSA) via technological manipulation. In their study, the pH decreased slowly during coagulation and manufacture as a result of the slower acid-producing activities of the starter culture. The pH values for the ripened cheeses reached the range of 5.4 to 5.5, which was similar to those values reported for several studies of goat cheeses that underwent moderate proteolysis during ripening (Khatoon et al. 1990; Martín-Hernández et al. 1992).

3.1.3 Hydrogen perioxide (H_2O_2)

The accumulation of hydrogen peroxide in growth media can occur because lactobacilli do not possess a catalase enzyme (Kandler and Weiss 1986). The main reason for the dramatic loss of viability of *L. acidophilus* during fermentation and especially during storage in ABY-type (containing *L. acidophilus*, bifidobacteria, and yogurt bacteria) fermented milks is hydrogen peroxide produced by *L. delbrueckii* ssp. *bulgaricus* (Dave and Shah 1997c; Mortazavian et al. 2006, 2008a; Mortazavian and Sohrabvandi 2006), as in presence of oxygen, *L. delbrueckii* ssp. *bulgaricus* produces hydrogen peroxide. Therefore, lower amounts of dissolved oxygen in milk during fermentation and storage lead to lower amounts of hydrogen peroxide being produced by ABY-type cultures (Mortazavian and Sohrabvandi 2006; Mortazavian et al. 2006). The concentration of hydrogen peroxide produced by starters may not be sufficient to directly affect the cells in the products. Hydrogen peroxide can react with other components to form inhibitory substances (Shimamura et al. 1992).

3.1.4 Molecular oxygen

Bifidobacteria are classified as strict anaerobes because they are incapable respiring of growth in oxygen and under aerobic conditions. However, the degree of tolerance to oxygen depends on the species and culture medium (De Vries and Stouthamer 1969). Certain strains of bifidobacteria, including *B. infantis*, *Bifidobacterium 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. Selection of oxygen-resistant mutants of *B. bifidum* and other bifidobacteria has been shown to be effective in enhancing the survival of strains of

bifidobacteria in foods throughout processing and storage (Mutai et al. 1980). Relatively higher stability of bifidobacteria in cheese could be attributed to the less permeability of cheese matrix to oxygen compared to fermented milks such as yogurt. The cheese core can be considered as an anaerobic environment with very low redox potential of about −250 to −350 mV (Gomes et al. 1998).

Ascorbic acid can act as an oxygen scavenger, and it is permitted in some cheeses as a food additive (Dave and Shah 1997a). Cysteine, a sulfur-containing amino acid, could provide amino nitrogen as a growth factor while reducing the redox potential, both of which might favor the growth of anaerobic bifidobacteria species (Collins and Hall 1984). Collins and Hall (1984) reported improved viability of some bifidobacterial species in reconstituted milk containing 0.05% cysteine. 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 et al. 2002).

3.1.5 Growth promoters and food additives

The addition of growth-promoting factors as a nitrogen source should further enhance the growth and viability of bifidobacteria (Gomes and Malcata 1999; Gomes et al. 1995). Also, the addition of amino acids, peptides, and other micronutrients stimulates the growth of probiotic bacteria (Gobbetti et al. 1998; Shah 2000; Takano et al. 1988). In yoghurt, the addition of ascorbic acid (Dave and Shah 1997a) and cysteine (Dave and Shah 1997b) decreased the redox potential, providing an environment more favorable 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 *S. thermophilus*. In Gouda cheese, protein hydrolyzates added during cheese processing to enhance the growth of the bifidobacteria did not significantly enhance their viability, possibly due to protein hydrolysis by rennet activity. The protein hydrolyzate, even when added at low levels, introduced undesirable flavors to the cheese through increased free amino acids and peptide contents (Gomes et al. 1995). In some cheeses, KNO_3 (0.01%) is added to the cheese milk in order to prevent the growth of clostridia (Thage et al. 2005). Also, cheeses have been sprayed with natamycin (0.3 gL^{-1}) in order to prevent moulds from growing on the surface (Thage et al. 2005). According to the study of Gomes and Malcata (1998), the addition of milk hydrolyzate did not significantly affect the growth of *B. lactis*, while the addition of certain peptides has been claimed to stimulate the growth of bifidobacteria (Modler 1994; Proulx et al. 1992).

Prebiotics are non-digestible dietary components that pass through the colon and selectively stimulate the proliferation and/or activity of populations of desirable bacteria in situ (Mattila-Sandholm et al. 2002). Pre- and probiotics may be combined in a food product, called a symbiotic (Holzapfel and Schillinger 2002). Fructooligosaccharides (FOS) and inulin-type fructans have been those most studied as prebiotics (Fooks et al. 1999; Gilliland 2001; Roberfroid 2005). Fructans are carbohydrates, in which most of the glycosidic bonds are made of fructosyl–fructose bonds and usually have a terminal glucose unit. Inulin is a linear β-(2→1)-linked fructose polymer that occurs in garlic, asparagus root, Jerusalem artichoke, dahlia tubers, or chicory root (Rocha et al. 2006). The degree of polymerization of inulin

typically ranges from 3 to 60 (Kaplan and Hutkins 2000; Murphy 2001), which renders fermentation of this fructan by lactobacilli species difficult. The fructan-type prebiotics inulin and oligofructose may aid survival of probiotic organisms during processing and storage of dairy products, particularly increasing or, at least, retaining the viability of *Bifidobacterium* spp. and of *L. acidophil*us (Bruno et al. 2002; Capela et al. 2006; Özer et al. 2005; Shin et al. 2000). Buriti et al. (2007b) verified that only two strains, *L. acidophilus* IBB 801 and *L. paracasei* subsp. *paracasei* 8700:2, were capable of degrading oligofructose, whereas only the human isolate *L. paracasei* subsp. *paracasei* 8700:2 grew rapidly using both oligofructose and inulin as energy sources at 37°C under anaerobic conditions (atmosphere-controlled containing a mixture of 80% N2, 10% CO2, and 10% H2). Buriti et al. (2007b) also indicated that the metabolism of the starter *S. thermophilus* and of *L. paracasei* did not reduce the fructan content during the storage period at 4±1°C in aerobic conditions (normal atmosphere). Nevertheless, it is important to emphasize that generally, *L. paracasei* strains are mesophilic bacteria with an optimal growth temperature of 37°C (Gardiner et al. 1998; Lynch et al. 1999). Buriti et al. (2007b) in their study applied 7% as a fraction content of synbiotic fresh cream cheese, assuming a daily consumption of 100 g of this cheese. Cardarelli et al. (2008) studied the influence of inulin, oligofructose, and oligosaccharides from honey on probiotic viable count in synbiotic Petit-Suisse cheese. Probiotic populations were $>10^6$ cfu g^{-1} for *B. animalis* ssp. *lactis* after 28 days of refrigerated storage (4±1°C; Cardarelli et al. 2008).

3.1.6 Salt

The salt in cheese influences cheese ripening through its effect on water activity. The salt concentration influences microbial growth, various enzyme activities, and proteolysis of cheeses. Lower levels of salt in moisture (S/M) have been correlated with higher microbial growth, increased acid production, increased proteolysis, and increased bitterness (Mistry and Kasperson 1998). 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 four- to fivefold from the periphery to the center may result initially (De Leon-Gonzalez et al. 2000). During the ripening process, salt diffuses throughout the cheese so that differences in the salt content at the center and periphery decrease with ripening time (Mocquot 1979). The salting contributes not only to the flavor of the cheeses but also has an impact on the growth and activity of starter microorganisms. The viability of starter bacteria and probiotics is inversely related to the salt concentration (Gomes and Malcata 1998; Vinderola et al. 2002a). Calcium ions (Misra and Kuila 1990) and sodium chloride (Modler et al. 1990b; Samona and Robinson 1991) also contribute to morphological changes in bifidobacteria, which, in turn, may alter their acid-producing ability and other growth characteristics (Misra and Kuila 1990). Three methods are available for cheese salting including dry salting, surface dry salting, and brine salting/brine immersion (da Cruz et al. 2009). In Gouda cheese, following a 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 hydrolyzates. Cheeses with salt contents ranging from 1.90% to 3.90% had a 55–35% survival of the bifidobacteria,

with the highest ability to survival being in the center of the cheese where oxygen and salt levels were lowest (Gomes et al. 1995). A level of S/M >4.5% is necessary to prevent the development of bitterness in cheese (Mistry and Kasperson 1998). These ratios reflect the amount of free water available for microbial growth. A lower level of S/M and high water activities allow excessive bacterial growth, promote excessive proteolysis and lipolysis, and in turn lead to defective body, texture (open, soft, or greasy), flavor (unclean or bitter), and consumer unacceptability (Beresford and Williams 2004). Gobbetti et al. (1998) claimed that the viability of probiotic strains is hindered considerably when the salt level in cheese exceeds the upper limit of 4% of cheese. Salt has been reported as the major limiting factor for the growth of probiotics in white-brined cheeses (Özer et al. 2008).

3.1.7 Microencapsulation of probiotics

Microencapsulation of probiotic cells can be defined as the process of entrapment of cells by coating them with proper hydrocolloid(s) in order to segregate the cells from surrounding detrimental environment, in a way that result in appropriate cell release in the intestine medium (Mortazavian et al. 2007, 2008a, b; Mortazavian and Sohrabvandi 2006). Microencapsulation seems to be the most promising technique for bacterial protection (Krasaekoopt et al. 2003). Entrapment of living microbial cells in calcium alginate is simple and low cost. Furthermore, alginate is nontoxic so that it may be safely used in foods. Alginate gels can be solubilized by sequestering calcium ions thus releasing the entrapped cells (Rao et al. 1989; Ravula and Shah 1999). In Crescenza cheese, the 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 nonimmobilized mixed culture of the same three strains (Gobbetti et al. 1998). The growth and viability of *B. bifidum*, added either as a commercially available powder or as an immobilized (with *k*-carageenan) freeze-dried preparation in Cheddar cheese, were compared (Dinakar and Mistry 1994). With both methods, the number of bifidobacteria in the cheese increased by one to two log cycles over a 24-week storage period. Maximum bifidobacteria counts occurred at 18 weeks for the commercial preparation and 24 weeks for the immobilized preparation.

Encapsulation by extrusion and emulsion techniques has been used for the protection of probiotic bacteria against adverse environmental conditions in cheeses (Doleyres and Lacroix 2005; Jankowski et al. 1997; Kebary et al. 1998). In addition to the increased protection of microbial viability after microencapsulation, the chemical and physical properties of the cheeses containing probiotic bacteria in the encapsulated form may be affected as well. Özer et al. (2008) demonstrated that microencapsulation did not affect the basic composition of Kasar cheese; however, in other studies, it has been noted that the development of proteolysis was more pronounced in the cheeses containing probiotic bacteria in the encapsulated form. It was demonstrated that the addition of probiotic cultures, either in the free or encapsulated states, did not seem to significantly affect textural parameters such as springiness and cohesiveness of 7-week-old Feta cheese (Kailasapathy and Masondole 2005). It may be possible that exchanging sodium ions with calcium ions binding alginate capsules together led to the disintegration of the capsules, thus

releasing the probiotic bacteria into the medium. During the time-dependent release of probiotic bacteria, a slight salt adaptation might have developed in the probiotic cells, and hence, cell death remained relatively limited. The role of sodium chloride in the disintegration mechanism of *k*-carragenan capsules is not clear and needs further investigation (Özer et al. 2008). Similar results were reported by Kailasapathy and Masondole (2005) who demonstrated that microcapsules formed by calcium alginate polymers containing starch were susceptible to disintegration in Feta cheese ripened in brine. On the other hand, apart from slow disintegration of microcapsules during ripening, the salt might penetrate into the beads and affect the viability of probiotic bacteria. Özer et al. (2008) studied the viability of *B. bifidum* BB-12 and *L. acidophilus* LA-5 microencapsulated by either an extrusion or an emulsion technique in white-brined cheese. The numbers of *B. bifidum* BB-12 microencapsulated reached $>10^8$ cfu g^{-1} at day 90. The numbers of *L. acidophilus* LA-5 microencapsulated by an extrusion or an emulsion technique reached $\sim 10^9$ after 90 days of storage. In this study, both microencapsulation techniques were efficient enough to keep the number of probiotic bacteria above the threshold level for therapeutic minimum (10^7 log cfu g^{-1} of cheese; Özer et al. 2008). However, in contrast, in other studies, it has been claimed that microencapsulation of probiotic cells in Feta cheese caused higher cell loss, either by preventing encapsulated bacteria from interacting with the environment for survival or inhibiting disposal of cell metabolites that may be accumulating inside the encapsulated capsules causing death (Kailasapathy and Masondole 2005).

3.1.8 Ripening factors

A series of chemical and biochemical changes occur during cheese ripening including glycolysis, lipolysis, and most importantly, proteolysis (Fox et al. 1993). During this period, various factors can affect the viability of probiotic bacteria. Bifidobacteria were incorporated with a mixture of lactic acid bacteria (*S. thermophilus*, *L. acidophilus*, *L. lactis* ssp. *cremoris*, and *Leuconostoc mesenteroides* ssp. *cremoris*) to manufacture unripened (scalded at 53–55°C for 15 min) and ripened (not scalded) soft cheeses. The heat treatment associated with the unripened 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 ripened cheese, whereas in unripened cheese, only two species (*L. acidophilus* and *S. thermophilus*) were recovered (Ariga et al. 1989a). Hayes et al. (2006) observed that CLA-producing *B. breve* NFBC 2258 did not retain sufficient viable cell numbers during cheese ripening. However, a method for the manufacture of a Swiss-type cheese incorporating a CLA-producing *Propionibacterium* has been developed in their laboratory. In their experiment, cheese was manufactured using *S. thermophilus* 1842, *Lactobacillus helveticus* 4571, and *P. freudenreichii* 9093, and the viability of the *Propionibacterium* was assessed during ripening. After 16 weeks of ripening, the *Propionibacterium* strain was present in the cheese at a level of $>10^7$ cfu mL^{-1}, demonstrating that this strain remains viable at high numbers during ripening (Hayes et al. 2006).

A potential problem associated with fermented cheese is that during ripening, a population of nonpathogenic adventitious bacteria, usually lactobacilli and

pediococci, can proliferate and often become the dominant micro flora in the cheese. The role of these NSLAB still remains unclear; some NSLAB contribute to flavor development by positively affecting the maturation process, whereas others have been found to be associated with defects in the cheese such as slit formation and calcium lactate crystal formation (Thomas and Crow 1983).

Para-*k*-casein and other casein hydrolyzates 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 (Ballongue 1993; Kehagias et al. 1977; Otani 1992; Poch and Bezkorovainy 1991, 1988; Zbikowski and Ziajka 1986). These factors can positively affect the viability of bifidobacteria during cheese ripening.

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, inhibited the growth of the bifidobacteria. 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 and Kurmann 1983; Walstra et al. 1999). Hayes et al. (2006) reported that when a CLA-producing *B. breve* was used as an adjunct culture during cheddar cheese manufacture, it survived product manufacture, but viability decreased significantly during ripening.

3.2 Process factors

Manufacturers must consider the effects of the environment of the cheese during processing and storage to ensure suitable retention of probiotic viability in products at the time of consumption. For example, the cooking procedure for hard or semi-hard cheeses, the aerobic environment, and the temperatures of ripening and storage must be evaluated (Gobbetti et al. 1998). Some important factors are mentioned below.

3.2.1 Incubation temperature, heat treatment, and storage temperature

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 40–43°C). There is no growth below 20°C, and the bifidobacteria have no thermoresistance above 46°C (Rašic and Kurmann 1983). In the manufacture of Canestrato Pugliese, 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 limited acidification by the lactic acid starters (*S. thermophilus* and *L. delbrueckii* ssp. *bulgaricus*) and enhanced the viability of the bifidobacteria (Corbo et al. 2001). Bifidobacteria were incorporated with a mixture of lactic acid bacteria (*S. thermophilus*, *L. acidophilus*, *L. lactis* ssp. *cremoris*, and *L. mesenteroides* ssp. *cremoris*) to the manufacture of 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 (Ariga et al. 1989a, b).

Different ripening temperatures are used for various cheeses such as 13°C for Gouda cheese, 8°C for Cheddar cheese, 6°C for Portuguese goat's cheese, and 4±1°C for other types of cheese (Table 1). Ripening temperature significantly affects viability of probiotics during cheese ripening. In the study of Ong et al. (2007) on Cheddar cheese, the pH of the cheeses with *B. longum* 1941, *B. animalis* B94, *L. casei* 279, or *L. acidophilus* L10 ripened at 8°C was significantly lower than that ripened at 4°C after 24 weeks reasonably due to adverse effect of higher temperatures on viability of cells as well as sharper drop in pH.

3.2.2 Types of inoculation

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 SLAB 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. This allowed the probiotic bacteria to be in their final stage of lag phase or early stage of log phase, and thus could dominate the flora, resulting in higher counts. The initial counts of probiotic bacteria have been found to increase by four to five times in the product made by the two-step fermentation process (Shah 2000). The probiotic bacteria might be also totally added at the end of fermentation (da Cruz et al. 2009).

Bergamini et al. (2005, 2009) compared two types of inoculation method; in one type of experimental cheese, probiotic bacteria were added directly to the cheese milk as a lyophilized culture, while in the other, they were pre-incubated in a substrate composed by milk and milk fat, then added to the cheese milk. As a result, the direct addition of probiotics as a lyophilized culture was considered more efficient, as direct addition was easier, more rapid, and less vulnerable to contamination. Although pre-incubation in the substrate increased the probiotic population in the inoculum almost by one log cycle, which can contribute to reducing the costs of probiotic cultures for the dairy industry, the addition of probiotics after pre-incubation in the substrate did not improve their survival during cheese ripening. The substrate did not only enhance the protection of probiotic bacteria but also was a more complex methodology than direct addition of lyophilized culture. First, it was more time consuming, and in the second place, pre-incubation could be a sensitive step taking into account issues with contamination and phage attack.

Probiotics have been introduced into cheese in ways that vary slightly from industrial protocols. Immobilized cells and powder preparations of bifidobacteria have been added to Cheddar cheese at the milling stage, while large inocula of bifidobacteria have been added to Gouda cheese at the time of inoculation, and cells immobilized in calcium alginate gels have been added to Crescenza cheese, all in an effort to improve the survival of bifidobacteria in the final product (Dinakar and Mistry 1994; Gobbetti et al. 1998; Gomes et al. 1995).

3.3 Packaging

The selection of packaging materials can further have a significant impact on the survival of the bifidobacteria especially. 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 and Shimamura 1993). To exclude oxygen during the production of bifidus milk products, special equipment is required to provide an anaerobic environment. Oxygen can also enter the product through packaging materials during storage. Dave and Shah (1997c) studied the survival of yogurt and probiotic bacteria in yogurt made in plastic containers and glass bottles. The increase in numbers and survival of *L. acidophilus* during storage were directly affected by the dissolved oxygen content, which was shown to be higher in yogurts made in plastic containers than glass. The initial counts of the bifidobacteria population were 1.6-fold higher in yogurt prepared in glass bottles than in plastic cups. Although, the acid contents were similar in products stored in glass bottles and plastic cups at 4°C, the survival rate was 30% to 70% higher in products fermented and stored in glass bottles than in plastic cups. Thus, it may be important to store the products in glass containers or to increase the thickness of the packaging materials used for AB or ABC products (Dave and Shah 1997c; Klaver et al. 1993). Better survival and viability of bifidobacteria in deaerated milk has been observed by Klaver et al. (1993).

Using active packaging is another way to increase the viability of bifidobacteria. In active packaging, oxygen scavengers are used in sachets suspended under the lids or incorporated into packaging polymers (such as photo-scavenging dyes; da Cruz et al. 2007; Miller et al. 2003). The use of oxygen-scavenging active packaging as an adjunct to the oxygen-barrier packaging efficiently decreases the oxygen permeability of packaging materials.

4 Conclusion

Cheese is a promising carrier for maintaining the stability of probiotics until the time of consumption compared to fermented milks such as yogurt. Also, it is a good protective carrier against harsh gastrointestinal conditions. The reasons are a relatively higher pH, lower titrable acidity, and oxygen content compared to fermented milks such as yogurt, good buffering capacity, higher nutrient availability, and a dense solid matrix, as well as a relatively high fat content which protects probiotic cells against detrimental factors. In addition, a very wide variety of cheese types as well as its consumption by everybody in their long-term diet increases the attraction of consuming this product. The probiotic cells must remain viable in cheese above a standard threshold level (e.g., 10^6 cfu g^{-1}) until the time of consumption, without adversely altering their sensory attributes. Factors affecting the viability of probiotic bacteria was discussed in the present article in three categories including formulation factors (strains of probiotic bacteria and microbial interactions, pH and titrable acidity, hydrogen peroxide, molecular oxygen, growth promoters and food additives, salt, microencapsulation, and ripening factors),

process factors (incubation temperature, heat treatment, types of inoculation, and storage temperature), and packaging. Future research could be focused on aspects such as considering the suitability of other types of cheeses as a vehicle for the delivery of probiotics, the use of more resistant strains of probiotics, improving formulation and processing factors, as well as packaging systems and conditions. However, industrialization of these factors in cheese-making would encounter the producers with different technological challenges due to adaptation of existing protocols for each type of cheese (among a wide variety of cheeses in markets) and having the feedback of markets (from sensory and price standpoints), as well as adopting suitable test methods for probiotic cheeses from quality control point of view.

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