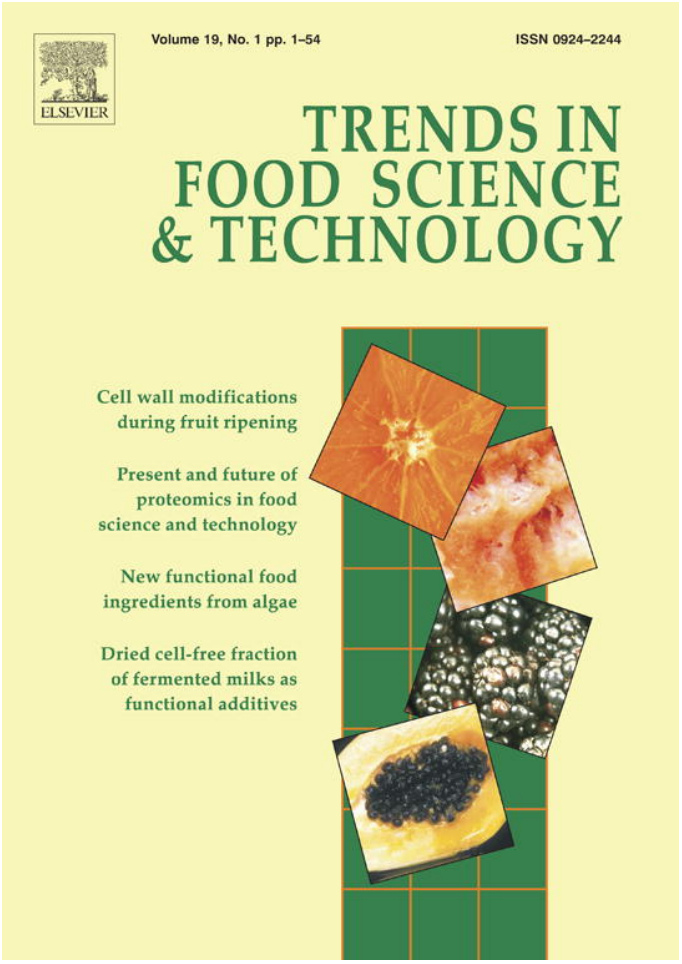

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Trends in Food Science & Technology 19 (2008) 40–46

TRENDS IN FOOD SCIENCE & TECHNOLOGY

Viewpoint

Dried cell-free fraction of fermented milks: new functional additives for the food industry

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The claimed health benefits of fermented functional foods are exerted either directly through ingested live microorganisms (probiotics) or indirectly as a result of ingestion of microbial metabolites produced during the fermentation process. Several reports are emerging concerning the immunomodulating capacity of the cell-free fraction of fermented milks. The soluble metabolites present in the whole non-bacterial fraction could be easily spray-dried and added as dried powders to food matrixes whose chemical composition or technological process of manufacture would seriously threaten the viability of probiotic bacteria. The relative technological easiness for incorporating the same biologically active metabolites present in fermented milks, but as a dried cell-free fraction, into other food matrixes opens the doors to the development of a great new variety of functional foods using food matrixes not suitable nowadays for viable probiotic bacteria. The ability to bring functionality to ordinary food through the incorporation of ingredients from natural sources becomes increasingly attractive to the food industry.

Functional foods and probiotic bacteria viability

Diet is a major focus of public health strategies aimed at maintaining optimum health throughout life, preventing the early onset of chronic diseases such as gastrointestinal disorders, cardiovascular disease, cancer, osteoporosis, as well as promoting healthier ageing. The growing demand for 'healthy' foods is stimulating innovation and new product development in the food industry all around the world. The food industry has a central role in facilitating healthier eating practices through the provision and promotion of healthy foods. According to a widely accepted definition, a functional food is any modified food that may provide a health benefit beyond the nutrients it contains (FDA, 2004). These healthy foods include products with reduced fat, sugar or salt, fortified with vitamins, minerals, phytochemicals, probiotic bacteria, bioactive peptides or φ3-polyunsaturated fatty acids. Functional products are a new variety of foods that promise targeted improvement in physiological functions in the body. Continuously increasing consumer health consciousness and expenditure are socio-economic factors responsible for the expanding worldwide interest in functional foods (Mattila-Sandholm *et al*., 2002). Food matrixes such as fermented dairy products containing probiotic microorganisms are one example of functional foods. The joint FAO/WHO working group report on drafting guidelines for the evaluation of probiotics in food recommended the adoption of the definition of probiotics as ''live microorganisms which when administered in adequate amounts confer a health benefit on the host'' (FAO/WHO, 2002). The use of non-viable cells instead of viable microorganisms has been proposed in order to achieve longer shelf-life, easier storage, handling and transportation and reduced requirements for refrigerated storage. Non-viable microorganisms would expand the potential use of probiotics to areas where the strict handling conditions cannot be met, e.g. developing countries (Ouwehand & Salminen, 1998). Good viability and activity of probiotics are considered prerequisites for optimal functionality. However, several studies have shown that non-viable probiotics can also have healthy effects such as immune modulation and carcinogen binding in the host (for a review see Ouwehand & Salminen, 1998; Salminen, Ouwehand, Benno, & Lee, 1999). However, adverse gastrointestinal side effects were associated with the supplementation of infant nutrition with heat-inactivated probiotics. This outcome presents a problem that should be addressed for future studies conducted to evaluate the efficacy and safety of using non-viable probiotics (Kirjavainen, Salminen, & Isolauri, 2003).

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0924-2244/$ - see front matter © 2007 Published by Elsevier Ltd.
doi:10.1016/j.tifs.2007.07.003

Immunomodulation by the cell-free fraction of fermented milks

During milk fermentation, lactic acid bacteria produce a range of secondary metabolites, some of which have been associated with health-promoting properties. The most notable of these are the B vitamins and bioactive peptides released from milk proteins. Bioactive sequences released in fermented milks, together with the capacity of the cell wall of lactic acid bacteria to activate the mucosal immune system (Morata de Ambrosini, Gonzalez, Perdigón, Pesce de Ruiz Holgado, & Oliver, 1996), would explain the beneficial effects observed for fermented milks containing non-viable bacteria. Except for the alleviation of symptoms of lactose maldigestion that are no longer observed in heat-inactivated fermented milks, some of the beneficial effects achieved with fermented milks containing viable bacteria were also reported for the same product containing non-viable cultures, but in many cases to a lesser extent. For example, the oral administration of pasteurised kefir to mice increased the number of IgA producing cells in the gut lamina propria as kefir containing viable cells did, but while pasteurised kefir was administered diluted 1:10 in the drinking water, viable kefir was diluted 1:100 to achieve the same effect (Vinderola, Duarte, *et al*., 2005).

The claimed health benefits of fermented functional foods are expressed either directly through the interaction of ingested live microorganisms with the host (probiotic effect) or indirectly as a result of ingestion of microbial metabolites produced during the fermentation process (biogenic effect) (Stanton, Ross, Fitzgerald, & Van Sinderen, 2005), such as bioactive peptides, exopolysaccharides, bacteriocins and organic acids (Clare & Swaisgood, 2000; Cobb & Kasper, 2005; Guarner *et al*., 2005; Vinderola, Perdigón, Duarte, Farnworth, & Matar, 2006a). Some of the effects currently observed after the oral administration of live probiotic bacteria or their fermented dairy products are the production of the regulatory/proinflammatory cytokine IL-6 by intestinal epithelial cells *in vitro* and *ex vivo*, the increase in the number of IgA producing cells in the small and large intestine lamina propia, the increase in the luminal content of polyvalent secretory IgA, the production of proinflammatory and regulatory cytokines in peritoneal macrophages and in adherent cells from Peyer's patches and the enhancement of the phagocytic activity of peritoneal and bronchial macrophages (Maldonado Galdeano, de Moreno de LeBlanc, Vinderola, Bibas Bonet, & Perdigón, 2007; Perdigón, Fuller, & Raya, 2001). This activation of the gut immune response also led, in some cases, to the diminution of the severity of enteric infections caused by enteropathogens such as *Salmonella enteritidis* serovar Typhimurium (Perdigón *et al*., 2001) or enteroinvasive *Escherichia coli* (Medici, Vinderola, Weill, & Perdigón, 2005). Other studies where only the cell-free fraction of fermented milks was administered to mice demonstrated the induction of the same kind of effects described above for fermented milks containing viable bacteria. An *ex vivo* culture model of small intestine epithelial cells (SIEC) from conventional mice was set for the study of the response of enterocytes to the challenge with probiotic bacteria (Vinderola, Matar, & Perdigón, 2005), as an alternative to the use of immortalized cell lines derived from tumoral cells. In SIEC cultured *ex vivo*, the cell-free fraction of kefir induced the production of IL-6 (Vinderola, Perdigón, Duarte, Farnworth, & Matar, 2006b), a cytokine involved in the terminal differentiation of B cells into plasma cells (Christensen, Frokiaer, & Pestka, 2002). The same effect had also been observed after the challenge of SIEC with probiotic bacteria (Vinderola, Matar, *et al*., 2005) or with milk fermented by *Lactobacillus helveticus* R389 (Vinderola, Matar, Palacios, & Perdigón, 2007). The oral administration of kefir supernatant (after rehydrating the dried powder) induced the production of proinflammatory and regulatory cytokines in peritoneal macrophages and in adherent cells from Peyer's patches isolated from the small intestine of mice, which was also observed with kefir microflora (Vinderola, Perdigón, Duarte, Farnworth, & Matar, 2006c). Both kefir and its cell-free fraction were reported (Vinderola, Duarte, *et al*., 2005; Vinderola *et al*., 2006b) to activate the gut mucosal immune response *in vivo*: the oral administration of commercial kefir to mice in the drinking water for 2, 5 or 7 consecutive days lead to an increase in the number of IgA producing cells in the small intestine lamina propria as well as in the number of several cytokine-producing cells. A similar profile of mucosal immune response activation was achieved when mice received, by intragastric intubation, the dried cell-free fraction of the same kefir (Fig. 1). The oral administration of kefir or its cell-free fraction was also reported to be immunologically active in the prevention of a murine breast cancer model. A two-day cyclical administration of both products delayed tumour growth increased the number of IgA+ cells in the mammary gland and IL-10 in blood serum and decreased the number of IL-6+ cells (a cytokine involved in oestrogen synthesis) in mammary gland tissue (de Moreno de LeBlanc, Matar, Farnworth, & Perdigón, 2006; de Moreno de LeBlanc, Matar, Farnworth, & Perdigón, 2007).

Milk fermented by the proteolytic strain *L. helveticus* R389 activated the gut and bronchial immune responses when administered to mice for 3 or 5 days by increasing the number of IgA producing cells in the small intestine lamina propria and in bronchial tissue (Matar, Valdez, Medina, Rachid, & Perdigón, 2001; Vinderola, Matar, Palacios, *et al*., 2007). Similarly, but after 7 days of oral administration, the cell-free fraction of milk fermented by the same strain induced a significant increase in the number of IgA producing cells in the small intestine lamina propria (Vinderola, Matar, Palacios, *et al*., 2007). Moreover, when milk was fermented maintaining the pH at a constant value of 6, the release of peptides was maximized, according to the HPLC analysis of the cell-free fraction, and the activation of the gut mucosal immune response was achieved in

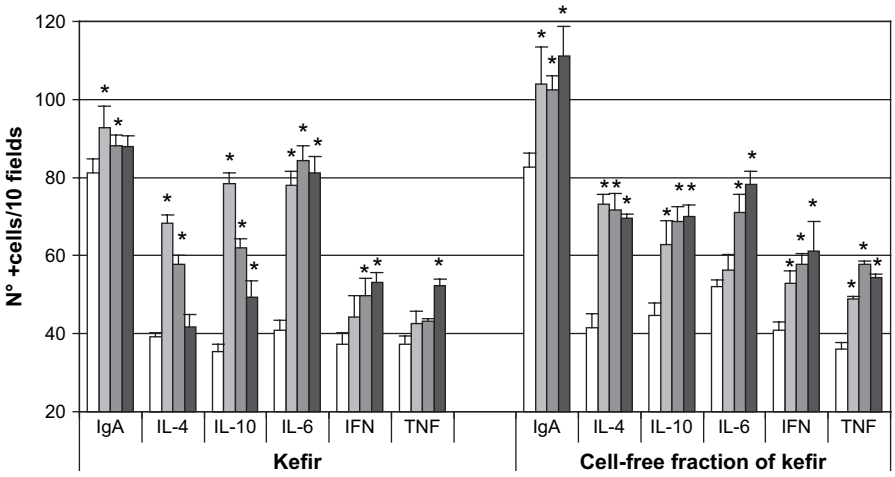

Fig. 1. Effects of the oral administration of kefir or its cell-free fraction for 2 (□), 5 (▩) or 7 (■) consecutive days in the number of IgA- and cytokine-producing cells in the small intestine lamina propria of mice. * Significantly different ($P < 0.05$) from the corresponding control (□) value. Adapted from Vinderola, Duarte, *et al.* (2005) and Vinderola et al. (2006b).

a shorter period of time than when mice received the cell-free fraction of milk fermented without pH control (Vinderola, Matar, Palacios, *et al.*, 2007). Additionally, for both milk fermented by *L. helveticus* R389 or its cell-free fraction, the protection against *S. enteritidis* serovar Typhimurium infection in mice was also observed (Vinderola, Matar, & Perdigón, 2007). Fig. 2 shows the concentration of secretory IgA in the small intestine lumen of mice that received milk fermented by *L. helveticus* R389 or its cell-free fraction for 2, 5 or 7 consecutive days. After each feeding period, mice were orally challenged with a single infective dose of the enteropathogen *Salmonella* Typhimurium. It

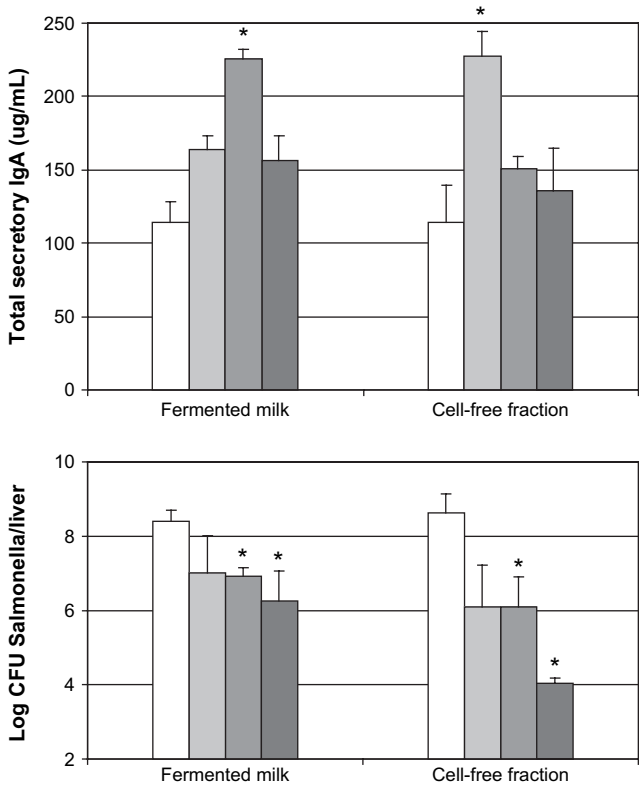

Fig. 2. Effects of the oral administration of milk fermented by *L. helveticus* R389 and its cell-free fraction for 2 (□), 5 (▩) or 7 (■) consecutive days on total secretory IgA in the small intestine lumen of mice (above) and in the number (log CFU) of *Salmonella enteritidis* serovar Typhimurium in the liver after 7 days of the challenge with the enteropathogen (below) in mice that received milk fermented by *L. helveticus* R389 and its cell-free fraction for 2 (□), 5 (▩) or 7 (■) consecutive days. * Significantly different ($P < 0.05$) from the corresponding control (□) value. Adapted from Vinderola, Matar, Palacios, *et al.* (2007).

was observed (7 days post challenge) that mice that had received milk fermented by *L. helveticus* R389, or its cell-free fraction, had a lower liver colonization by the enteropathogen than control animals. Certainly the immunological mechanisms underlying the protection observed were different for the fermented milk or its cell-free fraction (Vinderola, Matar, & Perdigón, 2007).

Several factors in milk can affect the expression of genes in the intestinal epithelium, particularly those genes that are associated with enterocyte differentiation (Sanderson, 1998). These factors seems to be also present in the cell-free fraction of milk fermented by *L. helveticus* R389 since its oral administration to mice induced the proliferation of goblet cells and mast cells in the lamina propria of the small intestine of mice and enhanced the expression of TRPV6 calcium channels in the brush borders of enterocytes (Vinderola, Matar, & Perdigón, in press). Beyond the extensive amount of reports dealing with functional and physiological activities of specific peptide sequences derived from milk proteins (Clare & Swaisgood, 2000; Hartmann & Meisel, 2007; Matar, LeBlanc, Martin, & Perdigón, 2003), other immunological and non-immunological effects mediated by the whole cell-free fraction of fermented milks or spent cultures, than the ones previously discussed, were reported. For instance, Ganjam, Thornton, Marshall, and MacDonald (1997) demonstrated antiproliferative effects on cultured intestinal cells by yogurt fractions obtained by membrane dialysis. The spent culture of *Lactobacillus acidophilus* La-5 affected the virulence-related gene expression of *E. coli* O157:H7. When the pathogen was grown in the presence of chromatographically selected fractions of the probiotic cell-free spent medium, a significant reduction of both extracellular concentration and expression of important virulence-related genes was observed (Medellin-Pena, Wang, Johnson, Anand, & Griffiths, 2007). The activity of procarcinogenic enzymes such as nitroreductase and β-glucuronidase significantly decreased in the gut of healthy subjects that received cell-free whey from milk fermented by *Bifidobacterium breve* (Romond *et al*., 1998). The same cell-free whey was also able to beneficially modify the intestinal microflora of mice and humans (Mullié *et al*., 2002; Romond, Ais, Yazourh, & Romond, 1997).

Fate of ingested bioactive molecules *in vivo*: safety aspects

To exert physiological effects *in vivo*, bioactive sequences present in food must escape complete gastrointestinal digestion to aminoacids or they must be released *in situ* from larger protein sequences during intestinal transit to then reach their target sites at the luminal side of the intestinal tract or after absorption in the peripheral organs. Immunoreactive material from the kappa-casein region has been detected in the intestinal lumen and blood after the ingestion of milk products in humans. This suggests that some fragments are resistant to digestion (Fosset *et al*., 2002; Troost, Steijns, Saris, & Brummer, 2001). Evidence for the liberation of bioactive sequences from casein into the intestinal lumen of mammals after the ingestion of milk or a diet containing casein have already been obtained (Pihlanto-Leppala, 2001). In addition to the soluble bioactive peptides released in fermented milks by lactic acid bacteria (Matar *et al*., 2003), many proteins are left – at least partially unbroken – in the cell-free fraction of fermented milks. These proteins could undergo further proteolysis *in vivo* by gastrointestinal enzymes leading to the production of additional bioactive sequences (Hernández-Ledesma, Quirós, Amigo, & Recio, 2007). Mercier, Gauthier, and Fliss (2004) reported that whey proteins contain immunomodulating peptides, which can be released by enzymatic digestion and that they stimulated cell proliferation at much lower concentrations than the ones obtained from total hydrolysates. Kitazawa *et al*. (2007) reported that the digestion of milk proteins by gastric enzymes released potent chemotactic peptides for monocytes and macrophages. Matar, Amiot, Savoie, and Goulet (1996) showed that fermentation of milk by lactic acid bacteria prior to its digestion favored the modification of the protein structure and, consequently, the release of peptides of different hydrophobicities. They reported that the fermentation of milk by *L. helveticus* R389 improved the availability of proline, whose direct liberation from milk proteins during gastric digestion is very slow probably because of the resistance of the imine bond to the activity of pepsin and pancreatin. These authors concluded that the fermentation of milk by proteolytic bacteria may provide a new mechanism leading to the release of novel peptides from milk proteins after digestion. From the wide evidence available in the literature in relation to the *in vivo* digestion of milk proteins, it could be speculated that some of the biologically active sequences present in the dried cell-free fraction will presumably undergo ultimate hydrolysis to amino acids – increasing their availability *in vivo* – while others, depending on their own resistance to proteolysis or on the protective capacity that the new food matrix could confer them, might escape digestion reaching the gut ecosystem as intact sequences with specific physiological or immunological activities. Finally, some sequences and larger proteins still present in the dried cell-free fraction could be subject to additional *in situ* proteolysis by pepsin and pancreatin, leading to the release of novel bioactive sequences *in vivo*.

Safety assessment is an essential step in the development of any new food. In addition to the long history of the safe use of lactic acid bacteria, oral administration of probiotics is well tolerated and has been proven to be safe in hundreds of clinical trials involving thousands of subjects (Gueimonde, Ouwehand, & Salminen, 2004). In the absence of viable active bacteria in a dried cell-free fraction, there would be very low risk of bacterial translocation to extraintestinal sites due to an imbalance in the gut microflora. This issue constitutes an important and desired safety characteristic of these novel functional additives. There has been accumulating scientific evidence of the beneficial effects that

could be drawn from a whole cell-free fraction of fermented milks without the need of the application of sophisticated and high cost laboratory and industrial technologies to identify, separate and purify each bioactive component. However, it is still important to study their technological properties. It is recognized that peptides can be more reactive than proteins, due to their lower molecular weight. Peptides present in cell-free fractions may react with other components such as remaining sugars or exopolysaccharides. The interaction of peptides with carbohydrates and lipids as well as the influence of the processing conditions (especially heating) on peptide activity and bioavailability should also be investigated. In particular, possible formation of toxic, allergenic or carcinogenic substances (biogenic amines), warrants intensive research to evaluate the chemical safety of these new additives (Korhonen & Pihlanto, 2003).

Technological possibilities for the production of a functional dried cell-free fraction from a fermented milk

Spray-drying is a one-step processing operation for turning a liquid feed into a powder product, minimising handling while reducing the bulk weight and size of the powder, and also preserving the product by reducing the water activity required for bacterial degradation (Hayashi, 1989). Commercial drying process such as spray-drying is routinely used to dry protein meals such as blood meal, soy isolates and milk proteins (Fellows, 2000). Although spray-drying has been investigated as a method for the preservation of microbial cultures, it is not widely used for the preparation of probiotics owing to the high loss of culture viability, largely associated with heat exposure (Stanton *et al*., 2005). However, spray-drying could be a low cost accessible tool to produce dried powders from the cell-free fraction of fermented milks, as shown in a schematic diagram in Fig. 3. According to the particular characteristics of the food matrix where the dried cell-free fraction is intended to be used, the presence of lactic acid in the powder could be not desired. In this case, this metabolite could be removed by a nanofiltration step (Bargeman, 2003). The production of a dried cell-free fraction of fermented milks would yield at the same time lactic acid bacteria biomass that could be used in other dairy processes. The application of spray-drying to the cell-free fraction of fermented milks allows nutritional and sensorial qualities to be retained, together with extreme reduction in weight, high solubility, long shelf-life at moderate temperature and the possibility to perform rehydration at any desired level or time or the possibility to add them directly to almost any food matrix, especially those with low water activity where probiotic bacteria viability would be seriously threatened. A study of the influence of the type of drying (spray-drying vs. freeze-drying) on the functional and physicochemical properties of purified ovalbumin showed that the soluble fractions obtained after freeze or spray-drying had a greater

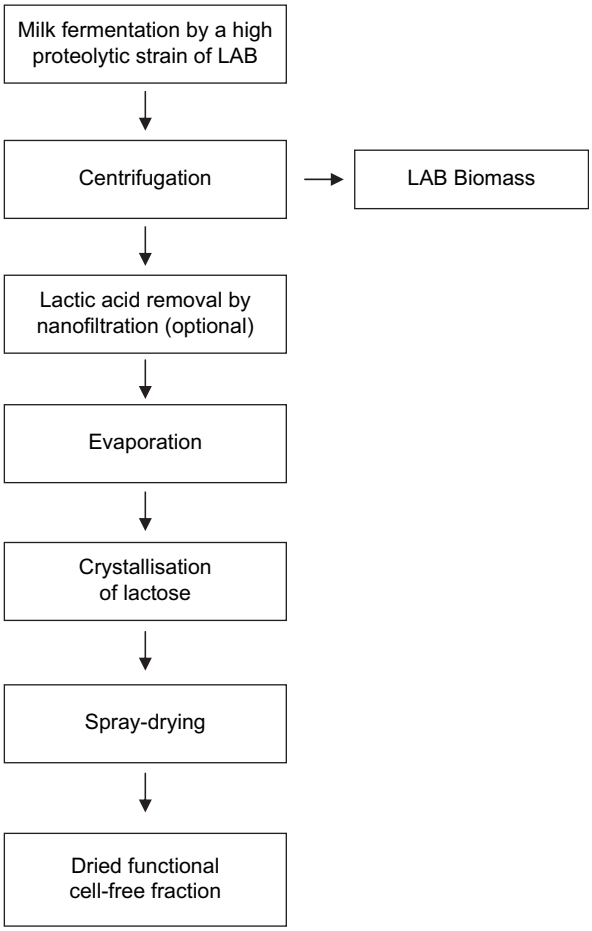

Fig. 3. Schematic diagram for the production of a dried cell-free fraction of fermented milks.

foamability and emulsifying ability than the original ovalbumin solution but no important difference was observed between the two drying processes (Kitabatake, Indo, & Doi, 1989). In relation to heat stability, it was reported that the beefy meaty peptide (an octapeptide with desirable sensory properties found in meat) was relatively stable to pasteurisation and sterilization temperatures and less than 10% of BMP was denatured under the sterilization condition (Wang, Maga, & Bechtel, 1995). Abdul-Hamid, Bakar, and Bee (2002) reported that the nutritional quality of spray-dried protein hydrolysate from fish was still high after spray-drying and with high *in vitro* digestibility. Spray-drying of proteins is known to induce more denaturation than freeze-drying. Nevertheless, in certain cases, freeze-dried and spray-dried hydrolysates exhibited similar biochemical properties (Linarès, Larré, & Popineau, 2001). However, the possible interaction of peptides with other food ingredients (especially during heating), in particular, the possible formation of toxic or carcinogenic metabolites makes intensive research necessary in this field before safety can be assured.

A functional powder from the cell-free fraction of fermented milks would create a novelty aspect on the food without necessarily changing the sensory quality of the

product. However, and depending on the functional effect desired, the dose of powder used in a particular food could be the one that influences the organoleptic characteristics of the product due to the aroma compounds released during milk fermentation by lactic acid bacteria (Friedrich & Acree, 1998), transforming then a traditional food product into a new one with novel organoleptic characteristics, expanding the functional product gamma in order to satisfy the heterogeneous consumer's demand.

The use of a dried cell-free fraction of fermented milks opens the possibility to carry some of the functional effects observed for fermented milks to non-dairy foods, broadening the market of functional foods to new matrixes whose physicochemical composition (extreme pH values, high salt or sugar concentration, presence of inhibitory additives, and low water activities) or technological process of manufacture (heating, high pressure, mechanical stress, pasteurisation, and exposure to radiation) are not suitable for maintaining a high level of viable cells during shelf-life until consumption, a characteristic required for live probiotic bacteria in the food chosen as vehicle. Products with an unfavourable water activity are, for example, cereals, chocolate, marmalade, honey and toffees. These products are ''too dry'' for applying live bacteria and ''too wet'' for the application of freeze-dried bacteria (Schmid *et al.*, 2006). Other vehicles not suitable for viable probiotic bacteria but potential food matrixes for dried cell-free fractions would be soups, sweets, muffins, snacks, tea, chewing-gums, high acidic beverages, infant foods, dressings, energy drinks, biscuits, jams, cakes, sweeteners or sports drinks. The development of novel nutraceuticals based on natural fruit fibers and bioactives with spray-drying technology (Chiou & Langrish, 2007) and a functional powder from citrus peel by lyophilization (Kang, Chawla, Jo, Kwon, & Byun, 2006) has recently been reported. Two of the fastest growing processed, functional food markets in the world are the beverage and snack bar markets (Sutton, in press); these products do not constitute at the moment totally suitable vehicles for viable probiotic bacteria. However, they could potentially become carriers of the functional metabolites left in milk by lactic acid bacteria in the form of added dried cell-free fractions.

Concluding remarks

There is an increasing array of functional foods available that are designed to confer health benefits. However, individuals' ''worries'' about new technology and modernity may influence the acceptance of these products. Fermented dairy products are food largely incorporated into the dietary habits of the population. The relative technological easiness of incorporating the same biologically active metabolites present in fermented milks, but as a dried cell-free fraction, into other food matrixes opens the doors to the development of a great new variety of functional foods using food matrixes not suitable nowadays for viable probiotic bacteria.

The knowledge about epithelial signalling implies that the epithelium can modulate the level of immune activity in the mucosal immune system associated to the gut according to the environment of the intestinal lumen. Because gut mucosal immune system plays a large part in the onset or in the successful control of gastrointestinal diseases, opportunity exists then to manipulate the constituents of the lumen of the intestine through dietary means, maybe in the future by any food carrying a functional dried cell-free fraction of fermented milk, thereby opening up a new vista for the maintenance of gastrointestinal health.

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