

# Probiotics in Milk and Milk-Derived Products

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Milk is a source of many valuable nutrients, including minerals, vitamins and proteins, with an important role in adult health. Milk and dairy products naturally containing or with added probiotics have healthy functional food properties. Indeed, probiotic microorganisms, which beneficially affect the host by improving the intestinal microbial balance, are recognized to affect the immune response and other important biological functions.

milk

milk-derived products

probiotics

## 1. Introduction

Due to their content in numerous biologically active components that provide benefits to the host health, milk and milk-derived products can be considered functional foods. Indeed, functional foods are components of the diet that not only provide energy and nutrients but also positively modulate body functions, thus boosting health by reducing the risk of disease and/or by improving a given physiological response <sup>[1]</sup>. Among the nutritional and functional components of milk and its derivatives, probiotics and biologically active peptides (BAPs) play a pivotal role. Probiotics, as declared by the FAO and the WHO and confirmed by Hill et al. <sup>[2]</sup>, are defined as “live microorganisms that, when administered in adequate amounts, confer a health benefit on the host”. Recently, the implications and healthy activities of probiotics against diseases such as irritable bowel syndrome, Parkinson’ disease, and prevention and treatment of allergies have also been recognized <sup>[3][4][5]</sup>. The mechanisms of action include (i) anti-inflammatory effects via suppression of proinflammatory cytokines; (ii) the modulation of gut microbiota through antagonism and inhibition of pathogen adhesion to the intestinal epithelia via production of bacteriocins, biosurfactants and short-chain fatty acids (SCFAs); (iii) enhancement of the gut barrier function of the intestinal mucosa by downregulation of low-grade mucosal immune activation, production of proteins of tight junctions and expansion of the mucus layer; (iv) development and improvement of the immunity system <sup>[3][4][5][6]</sup>.

BAPs are specific protein fragments, mainly consisting of fewer than 50 amino acids, that positively affect body functions or conditions, thus influencing health. Common bioactivities of BAPs comprise several beneficial effects such as antihypertension, antioxidant, antimicrobial, antidiabetic, and anti-inflammation activities; antihypertensive properties are exerted due to the inhibition of angiotensin I-converting enzyme (ACE) and renin activities as well as the induction of vasodilation via upregulation of cyclo-oxygenase (COX), prostaglandin receptor, endothelial nitric oxide synthase expression and L-type Ca<sup>2+</sup> channel blockade <sup>[7]</sup>; the relaxation of the mesenteric artery and the reduction in blood pressure in a cholecystokinin (CCK)-dependent manner has also been demonstrated for the peptide KFWGK released from bovine serum albumin (BSA) after subtilisin digestion <sup>[8]</sup>. In CaCo-2 cells, the antioxidant activity of milk-derived peptides has been attributed to the activation of the Keap1/Nrf2 pathway

responsible for the overexpression of antioxidant enzymes such as glutathione reductase (GR), NADPH quinone oxidoreductase (NQO1), superoxide dismutase (SOD1) and thioredoxin reductase 1 [9]. The main mechanisms of antimicrobial peptides are instead related to changes in the physiological function of membranes and extravasation of cytoplasmic content [10]. Milk proteins can release BAPs during food processing and gastrointestinal digestion through enzymatic hydrolysis and fermentation. BAPs can also be obtained via chemical synthesis or recombinant deoxyribonucleic acid (DNA) technology of predicted active sequences [11][12]; shotgun proteomics and protein-based bioinformatics represent only an example of the current workflow for the identification and characterization of new potential food-derived bioactive peptides [13]. Likewise, the holistic effects of probiotic supplementation on inter- and extra-intestinal diseases are being demonstrated due to multiomics approaches in probiotic studies, coined “pro-biomics” [14].

The growing interest in milk-derived bioactive components, BAPs and probiotics is determined by the rising demand for sustainable nutraceuticals, i.e., produced by processes with high efficiency and low environmental impact, which are also safe, i.e., having a high bioavailability and none or few unwanted side effects. As consequences of this trend, the investigation of minor dairy species (buffalo, goat, sheep, mithun (*Bos frontalis*), yak (*Poephagus grunniens*) camel, donkey, and mare), counting from 11 to 0.2% of worldwide milk production, is increasing in recent years. These latter species show notable differences in composition: ruminant milk (cattle, sheep, and goats) is characterized by a high fat content and more caseins among protein fractions, while non-ruminant milk (mare, donkey) has more lactose and whey proteins content. Also the non-protein nitrogen (NPN) content (free amino acids, peptides, creatine, urea, ammonia, uric acid, orotic acid) is highly variable; for instance, the NPN content in mare milk is approximately 10–15% of the total milk nitrogen content, in cow milk, it is 5%, whereas ruminant milk has approximately 3–5% NPN [15]. Buffalo milk also has higher levels of fats, proteins, lactose, vitamin A, vitamin C and calcium than bovine milk. However, buffalo milk has a lower vitamin E and cholesterol; in addition, buffalo milk exhibits a higher buffering capacity (acidification capacity) than bovine milk. Further differences in the nutritional composition of non-bovine milk are reported by several recent studies [15][16][17].

Consequently, different functional health benefits have been found, e.g., compared to bovine milk, some studies suggest that milks from small ruminants (e.g., goat) cause fewer allergenic reactions due to the protein concentration and polymorphism [16][17]; similarly, conjugated linoleic acid and orotic acid in sheep milk aid the treatment and prevention of type 2 diabetes, cancer, and other diseases [16]. Non-bovine milk products are also good for isolating novel potential probiotics and probiotic carrier candidates [17]. Similarly, novel peptide sequences or peptides with improved stability, bioavailability, and efficiency could be obtained [15].

## 2. Probiotics in Milk and Milk-Derived Products

A growing interest towards healthy foods is emerging worldwide in recent decades, with probiotic foods attracting the highest interest for their beneficial properties exerted on human health. As a result, the research on probiotic microorganisms is growing accordingly. Although probiotics isolated from humans should be more resistant to gastrointestinal conditions, the FAO/WHO [18] reported that the action rather than the source of microorganisms

makes them probiotics. Therefore, probiotics may be found not only in humans but also in other ecological niches. Milk, with its high content of nutritious compounds, is a good medium for both beneficial and detrimental microorganisms [19]. Numerous lactic acid bacteria, which are among the most used probiotic microorganisms, have been isolated from milk and their safety and probiotic potential have been assessed. Sieladie et al. [20] assessed the safety, cholesterol-lowering properties, and antimicrobial activity of 107 lactobacilli isolated from raw milk in the Western highlands of Cameroon. Fifteen isolates were selected for bile and acid tolerance, and all showed the ability to assimilate cholesterol in vitro and bile salt hydrolase activity [20]. Almost all isolates were sensitive to eight of the nine antibiotics tested, while all showed no hemolytic and gelatinase activity [20]. Only one strain, namely isolate 29V, showed antimicrobial activity against the target pathogens. All isolates were identified as *Lactobacillus (Lb.) plantarum* (recently amended to *Lactiplantibacillus plantarum* by phenotypic methods and typed by RAPD-PCR [20]). According to the overall results, the best potential probiotic strains were *Lb. plantarum* strains 1Rm, 11Rm and 29V [20]. Banwo et al. [21] isolated and identified two *Enterococcus faecium* strains from raw milk. The strains were characterized for their technological and probiotic features and a safety assessment was carried out, finding them suitable as starters for the production of fermented foods [21]. Eid et al. [22] isolated several lactobacilli from raw cow, buffalo and goat milk and demonstrated their antimicrobial activity against mastitis pathogens. Bin Masalam et al. [23] isolated 46 lactic acid bacteria strains and assessed their safety and probiotic potential. Two *Lb. casei*, one *Lb. plantarum* and one *E. faecium* strains showed the best probiotic potential [23]. Fourteen *Lactococcus lactis* strains isolated from raw milk and kefir grains were characterized for their technological and probiotic potential, finding that the strains isolated from kefir had a higher probiotic potential than those isolated from milk, which showed the best biochemical and technological features [24]. Reuben et al. [25] assessed the probiotic potential of lactic acid bacteria isolated from indigenous Bangladeshi raw milk, investigating antagonistic activity against pathogenic bacteria, survivability in simulated gastric juice, tolerance to phenol and bile salts, auto- and co-aggregation, adhesion to ileum epithelial cells,  $\alpha$ -glucosidase inhibitory activity, hydrophobicity, and antibiotic susceptibility, finding *Lb. casei* C3, *Lb. plantarum* C16, *Lb. fermentum* G9, and *Lb. paracasei* G10 to be the most promising probiotic bacteria.

Daneshazari et al. [26] carried out a probiotic characterization and safety assessment of *Bacillus* spp. isolated from camel milk. In particular, tolerance to acid, bile salts and artificial gastric juice was assessed, followed by auto-aggregation, cell surface hydrophobicity, antioxidant characteristics, and ability to adhere to HT-29 cells. Hemolytic and lecithinase activities were also evaluated. The *Bacillus subtilis* CM1 and CM2 strains were found to be the most promising probiotics [26].

The probiotic properties of *Bacillus subtilis* GM1, a strain isolated from goat milk, were assessed in vitro [27].

An ancient method to avoid the spoilage of milk, thus preserving it, is fermentation. Raw milk or thermized/boiled milk may be subjected to (i) natural fermentation, (ii) black-slopping or (iii) adjunct of commercial starter or single/multiple autochthonous microbial cultures. Depending on the raw materials used, the production step, the equipment and the manufacturing environment involved, the metabolic activity of the resulting specific microbiota is responsible for the final textural, sensorial, and probiotic features of each fermented milk and dairy product [28][29][30][31][32][33][34][35][36][37][38][39]. Within the microbiota responsible for the transformation of milk into fermented milk

and dairy products, a pivotal role is played by lactic acid bacteria (LABs) while yeasts are arising as important contributors for their technological and probiotic attributes [39].

The probiotic lactic acid bacteria most frequently found in fermented milks are strains of the recently amended *Lactobacillaceae* family [40]. Apart from lactobacilli, strains of the *Pediococcus* genus mainly belonging to *P. pentosaceus* and *P. acidilactici* species are arising for their probiotic attributes [41]. Among yeasts, certain strains of *Saccharomyces cerevisiae* have been found to possess probiotic attributes. However, certain clinical and foodborne *S. cerevisiae* strains are arising as opportunistic pathogens so that, as established by the EFSA (who confirmed its Qualified Presumption of Safety, QPS status), the inability to grow above 37 °C and to resist to antimycotics compounds used in human medicine must be demonstrated prior to adding viable cells of strains of this species in the food and feed chain [39].

Once again, lactobacilli are the most prevalent followed by strains of *Enterococcus* spp. However, mainly due to their ability to acquire virulence factors and resistance to several classes of antibiotics as well as their occurrence as opportunistic pathogens, enterococci are not generally recognized as safe (GRAS) microorganism and neither do they have QPS status [42][43]. Thus, their use as probiotics is highly controversial. A similar discussion can be had for yeasts such as *Kluiveromyces marxianus* (whose anamorph name is *Candida kefir*), which was declared as a significant opportunistic pathogen by the EFSA [39] despite the probiotic attributes found in certain strains isolated from dairy products; however, its QPS status has been confirmed [44].

Studies dealing with the probiotic and safety assessment of microorganisms isolated from fermented milk and dairy products are increasing in recent years; and apart from cow milk, non-bovine milks such as buffalo, ewe, goat, yak and camel milk derivatives are important sources of probiotics and promising carriers of probiotics [45][46][47][48][49][50]. Indeed, although bovine milk still dominates the probiotic market worldwide, there is an increasing trend towards the use of milk from species other than cows to deliver probiotics. This is mainly due to the adequate shelf-life viability of probiotics as well as the intrinsic functionality of non-bovine milk. However, given the absence or low amount of kappa casein, which negatively affects their coagulation capability, camel and donkey milk are mainly used as such for probiotic delivery and only seldom for the production of probiotic cheese, whereas ewe, goat, yak and buffalo dairy products are being frequently used as carriers of probiotics.

As for the beneficial effect of probiotics isolated from milk and milk-derived products, it should be highlighted that, although mandatory, only few articles assessed the probiotic features by in vivo studies focusing only on in vitro tests. Moreover, although a thorough safety assessment should target aspects such as antimicrobial susceptibility, metabolic activity, toxin production, side effects in humans, hemolytic activity, adverse outcomes in consumers, and infectivity in immunocompromised animal models [51], too high a number of the studies only carried out the antibiotic susceptibility assessment or did not perform any safety assessment at all. In addition, considering that the beneficial effect of a given probiotic is strain and dose dependent, the species- and strain-level identification of the potential probiotic as well as the enumeration of viable probiotics in a given probiotic food or supplement are mandatory to characterize probiotic microorganisms and authenticate probiotic food/beverage/supplements [52][53][54]. Moreover, the technological features of the potential probiotic should be characterized and their survival in the

processing, storage, distribution, and shelf-life within the probiotic food/beverage/supplement should be assessed [53]. Nevertheless, few studies made these assessments. Moreover, apart from a few articles that carried out in vivo studies in rats or in mice, only one article [55] assessed the beneficial effects of probiotics via double-blind, randomized, controlled study in humans.

### 3. Beneficial Effects of Probiotic Milk and Milk-Derived Products

As have been mentioned in the previous paragraph, no clinical trials in humans but studies using in vitro cell cultures or animal models have so been far carried out for probiotic milk and milk-derived products containing autochthonous potentially probiotic microorganisms. The pivotal role of probiotics in human health is well known [56] but controlled validated clinical trials are mandatory to verify that the health benefits are not altered or lost when the probiotic is incorporated into the food matrix due to the technological stresses it undergoes during manufacturing. The efficacy of probiotic milk and milk-derived products must be demonstrated in controlled validated clinical trials to prove that the probiotic features are not altered or lost passing from in vitro to in vivo studies. But even animal studies maybe not be adequate predictors of human experiences, humans being quite diverse from animals in terms of lifestyles, diet, and gut microbiome. However, clinical trials of probiotic milk and milk-derived products containing commercial probiotics or probiotics isolated from food matrices other than milk-based products have been carried out demonstrating numerous health benefits [57][58][59][60][61][62], leading to hypothesize that milk and dairy food/beverages containing autochthonous probiotic microorganisms would also positively impact human health. However, it should be highlighted that even numerous health claims associated with many probiotic strains already available on the market have been rejected by the EU due to either (i) insufficient characterization, (ii) invalidity of claims/unproven claims, (iii) the absence of beneficial effects on nutrition and or lack of progress on the physiological state of the body, (iv) lack of scientific basis and/or low quality of studies, and (v) the absence of placebo-controlled, double-blind clinical trials.

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