



Review

# Inclusion of Probiotics into Fermented Buffalo (*Bubalus bubalis*) Milk: An Overview of Challenges and Opportunities

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**Abstract:** Buffalo-milk-based dairy products provide various health benefits to humans since buffalo milk serves as a rich source of protein, fat, lactose, calcium, iron, phosphorus, vitamin A and natural antioxidants. Dairy products such as *Meekiri*, *Dadih*, *Dadi* and *Lassie*, which are derived from Artisanal fermentation of buffalo milk, have been consumed for many years. Probiotic potentials of indigenous microflora in fermented buffalo milk have been well documented. Incorporation of certain probiotics into the buffalo-milk-based dairy products conferred vital health benefits to the consumers, although is not a common practice. However, several challenges are associated with incorporating probiotics into buffalo-milk-based dairy products. The viability of probiotic bacteria can be reduced due to processing and environmental stress during storage. Further, incompatibility of probiotics with traditional starter cultures and high acidity of fermented dairy products may lead to poor viability of probiotics. The weak acidifying performance of probiotics may affect the organoleptic quality of fermented dairy products. Besides these challenges, several innovative technologies such as the use of microencapsulated probiotics, ultrasonication, the inclusion of prebiotics, use of appropriate packaging and optimal storage conditions have been reported, promising stability and viability of probiotics in buffalo-milk-based fermented dairy products.

**Keywords:** buffalo milk; artisanal fermentation; probiotics; fermented milk; ultrasound; prebiotics; microencapsulation

## 1. Introduction

Buffaloes are currently being farmed in many parts of the world and have been used for many years. In general, there are two types of buffaloes, i.e., African wild buffalo (*Syncerus caffer*) and Asian buffaloes (*Bubalus*) in the world, of which, Asian buffaloes are domesticated. The wild relative of domestic Asian water buffalo, *Bubalus arnee*, is currently classified as being endangered [1]. *Bubalus arnee* contributes to both types of domestic Asian water buffaloes, riverine type (*Bubalus bubalis bubalis*) and swamp type (*Bubalus bubalis carabensis*), which are classified by Macgregor [2] based on the morphological and behavioral criteria. However, the divergence of these two types of buffaloes had occurred well before the domestication [3].

At present, a population of 206.60 million buffalos is distributed in the world, with 97.4% of them being in Asia. [4]. The riverine type is more common in India and Pakistan, while swamp type is more common in China, which is strongly based on the purpose of buffalo rearing. Buffaloes present in Sri Lanka phenotypically represent swamp type, while having 50 chromosomes showing genetic similarities to the riverine type [5]. Irrespective of the type, domestic Asian water buffalo is economically and culturally important livestock, predominantly in developing countries. Buffaloes are responsible for providing draught power for agricultural activities, meat and milk as food commodities, manure as a fertilizer, horn and leather [6–8]. Swamp-type buffaloes are mainly reared for draught power and meat, whereas riverine buffaloes are preliminary important as milk producers [8].

The Asian water buffalo is the second most important livestock in the world, which is responsible for milk production after cows. Buffalo milk provides many health benefits over cow milk and it serves as a crucial protein source for humans, especially in the developing world. Moreover, buffalo milk is rich in calcium, fat and lactose compared to cow milk [7,9]. Hence, buffalo milk provides more energy per unit volume of milk in comparison to cow milk [10]. Buffalo milk also a rich source of iron, phosphorus, vitamin A and natural antioxidant (tocopherol), whereas the content of cholesterol is comparatively lower than cow milk.

However, consumption of raw buffalo milk is not popular compared to that of cow milk and production of value-added buffalo milk products such as butter, ghee, yoghurt and cheese helps to increase the demand for buffalo milk and also to obtain the health benefits associated with buffalo milk [7,11]. Among them, products derived by artisanal fermentation of buffalo milk have been consumed since ancient times. In the global scenario, commonly available artisanal fermented buffalo milk-derived dairy products include *Meekiri* (Sri Lanka), *Dadih* (West Sumatra Indonesia), *Dahi* and related products (South East Asian countries) and *Lassie* (India) [12–14]. These fermented dairy products consist of indigenous microflora, i.e., *Enterococcus faecium*, *Lactobacillus plantarum* and *Lactobacillus fermentum*, and their probiotic potentials have been described previously [15–18]. This reflects the possibility of inclusion of additional probiotics into the fermented buffalo milk products enhancing the health benefits to consumers. Inclusion of probiotics into cow milk is well established and extensively researched. Since artisanal fermentation of buffalo milk is a common practice in Asian rural communities and fermented buffalo milk is an integral part of Asian cuisines, probiotic-enriched fermented buffalo milk products could have a higher potential in ensuring a healthy diet for the people in the developing world. Although it is not a common practice, the inclusion of probiotics into fermented buffalo milk has also been reported to a much lesser extent [12,19,20]. However, research findings on the inclusion of probiotics into artisanal fermentation of buffalo milk are scares; thus, there is a lack of updated knowledge on the potential of developing probiotic-enriched fermented buffalo milk products. Moreover, processing technology for the inclusion of probiotics into the fermented cow milk products is often not suited for buffalo milk processing due to the differences in compositional, physicochemical and functional properties between two types of milk, which may result in undesirable quality parameters in the final product [10,11]. This review focuses on elaborating the potential for inclusion of probiotics into the fermented buffalo milk products and technical prospects to develop probiotic-enriched fermented buffalo milk products.

## 2. Buffalo Milk Production in the World

Global production of buffalo milk is steadily increasing according to (FAO) Food and Agriculture Organization/WHO [21]. Buffalo milk and its processed products are consumed around the world, in diverse forms, and the consumption is increasing with the improved understanding on nutritional benefits of buffalo milk, since it contains high amount of protein, iron, calcium and conjugated linolenic acid. Moreover, there is a potential of using buffalo milk as a cow-milk replacement for people with cow milk allergies [22]. The nutritional composition of buffalo milk depends on several factors, e.g., breed, season, climate, farm management practices, feeding, etc., of which shows a greater level of influences on the composition of buffalo milk than that of cow milk [23,24].

The domestication of buffalos has occurred more recently compared to the domestication of the cow (5000 and 10,000 years, respectively) [25]. Thus, domesticated Asian buffalos (*Bubalus bubalis*) is contributing the global buffalo milk production today, while the African Buffalo (*Syncerus*) remain in the wild [11]. The total buffalo milk production in the world is 127.34 million tons per year. Asia is the dominant buffalo milk producer, which accounts for 98.1% of global production, followed by 0.2% in Europe and 1.7% in Africa [4]. India is the largest and main buffalo milk producer in the world, where it produces more than half of the global produce, followed by Pakistan, China, Egypt, Nepal, Italy, Myanmar, Iran, Sri Lanka and Turkey, which are the other top-ten buffalo-milk producers in the world (Table 1).

**Table 1.** Production statistics of whole fresh buffalo milk (tones) from the top twenty countries over the period of 2010–2018.

Country	Year								
	2010	2011	2012	2013	2014	2015	2016	2017	2018
India	62,350.00	65,352.00	67,675.43	70,442.62	74,709.90	76,459.00	81,266.30	86,261.68	91,817.14
Pakistan	22,279.00	22,955.00	23,652.00	24,370.00	25,001.00	25,744.00	26,510.00	27,298.00	28,109.00
China	3050.00	3050.00	3080.00	3050.00	3100.00	2990.67	3005.20	2946.37	3003.32
Egypt	2653.24	2568.14	2564.64	2522.83	2923.03	2394.16	2334.29	2351.12	2120.37
Nepal	1066.87	1109.33	1153.84	1188.43	1167.77	1167.15	1210.44	1245.95	1338.28
Italy	177.46	192.54	192.46	194.89	194.51	195.27	199.16	208.96	247.16
Myanmar	302.97	305.63	171.18	175.53	179.75	184.14	188.49	192.13	193.84
Iran	215.25	222.43	120.22	125.00	128.00	113.91	133.56	145.77	129.90
Sri Lanka	46.99	46.33	61.71	54.06	45.85	36.12	66.13	68.59	85.91
Turkey	35.49	40.37	46.99	51.95	54.80	62.76	63.09	69.40	75.74
Indonesia	129.01	93.37	100.55	82.62	95.83	96.51	96.99	99.17	71.17
Iraq	64.54	27.21	67.62	43.25	30.72	31.59	33.35	33.35	49.89
Bangladesh	36.00	37.20	38.00	39.00	35.17	35.30	35.43	35.56	35.69
Viet Nam	31.65	31.66	32.00	31.00	28.09	28.12	28.10	27.92	27.46
Bulgaria	7.93	8.87	8.08	8.73	8.87	9.47	9.48	10.38	11.75
Malaysia	9.20	9.50	8.90	9.00	8.34	8.22	8.25	8.26	8.19
Syria	6.00	6.00	6.22	6.58	6.31	6.30	6.30	6.30	6.30
Georgia	6.00	6.00	6.00	6.10	6.43	6.43	6.29	6.17	6.19
Greece	0.11	0.15	0.39	0.45	0.27	0.53	3.03	2.89	0.40
Bhutan	0.22	0.39	0.35	0.22	0.30	0.30	0.28	0.29	0.28

Data source: (FAO, 2018 [4]) Values are indicated in tons (×10<sup>3</sup>).

Buffalos play a pivotal role in livestock in countries where primary agricultural production is prominent, mainly because of their good adaptation and suitability for low-input systems (e.g., can thrive well in harsh conditions, consume low-quality roughages and provide draft power) in addition to their extensive uses of milk, meat and manure [9,26,27]. Buffalo milk is used to produce various dairy products. Production of butter from buffalo milk (0.89 million tons in 2014) is mainly (90.1%) reported in Asia (India) and the rest is in Africa (Egypt), whereas the production of Ghee from buffalo milk is only performed in Asia (India and Pakistan). However, the production of buffalo milk cheese (0.28 million tons in 2014) is predominant in Egypt (90%), Italy (5.6%) and China (4.4%) [28]. However, interpretations must be cautious concerning the buffalo milk production and processing in Asia, since often production is underreported due to the prevalence of small-scale rural family-level farms, who produce mainly for their consumption [11].

Two common subspecies of Asian buffalos, namely river and swamp types differ in their number of chromosomes (50 vs. 48, respectively) [27]. River buffalos primarily prefer clear water, while the swamp buffalos usually inhabit marshy lands. Among these two types, river buffalos are primarily reared for milk production, whereas the Swamp buffalos are primarily used for draught power in agricultural works [25]. Several popular buffalo breeds are commonly present in today’s production systems; Murrah, Nili-Ravi, Mehsana, Surti and Jafarabadi are a few common examples [29].

The composition and properties of buffalo milk are varying from the bovine milk, mainly due to the differences in constituents, properties of casein micelles, mineral concentration (Table 2) and therefore, the functionality of buffalo milk is unique. Particularly rennet induced coagulation properties (e.g., coagulation time, gel strength and moisture retention) are significantly different from bovine milk because of differences in composition and properties. However, these rheological properties and curd characteristics of fermented buffalo milk gels are not well defined and studied. In most cases, buffalo milk composition is based on milk of Murrah buffaloes, of which not significantly different from the rest.

**Table 2.** Differences between buffalo and cow milk.

Constituents/Components	Buffalo Milk	Cow Milk	References
Fat (%)	6.7	3.7	[30]
Total protein (%)	4.7	3.5	[30]
Calcium (mg/100 mL)	205.0	115.0	[30]
Water (%)	83.2	87.2	[30]
Total solids (%)	16.3	12.8	[30]
Folate ( $\mu\text{g/L}$ )	60.0	44.0	[31]
Size of the casein micelle (nm)	110–160	70–110	[30]

Buffalo milk is a rich source of lactose, fat, protein, total solids, calcium and folate compared to that of bovine milk [30–32]. Thus, buffalo milk perceived to be thicker than cow milk.

Because of shortage in yellowish pigments carotene, relatively more whiteness can be seen in buffalo milk than that of cow milk, which is often used in visually differentiating these two types of milk. However, the levels of vitamin A is identical in both milk types, despite the absence of precursor, which comes with the feed since buffalo convert carotene to vitamin A [33]. The increased level of folate in buffalo milk is partly explained because buffalo milk contains more lactic acid bacteria (LAB) compared to cow milk [34], which can potentially generate folate. Furthermore, Lin and Young [35] reported that folate levels were higher in milk samples containing LAB and the highest and the lowest amount were reported from *Bifidobacterium longum* and *Streptococcus thermophiles*, respectively. Thus, fermented buffalo milk is likely to be a rich source of folate. Further, an approximately two-fold increase of phosphorus is reported in buffalo milk, compared to cow milk. Due to high peroxidase activity in buffalo milk, it can be preserved for a comparatively longer time than cow milk [36].

Buffalo milk has comparatively larger casein miscalls and higher proportion of  $\beta$ -casein and  $\kappa$ -casein compared to that of the cow milk. Moreover, casein micelles in the buffalo's milk are highly mineralized with higher calcium content and less hydrated than cow milk [37–39]. Because of these differences, fermented buffalo milk shows higher gel strength compared to that of cow milk [40].

The buffering capacity of buffalo milk is comparatively higher than that of its counterpart's, cow milk; thus, there are noteworthy differences in acidification process, and authors have argued that these differences can be attributed to the high casein content present in buffalo milk [37].

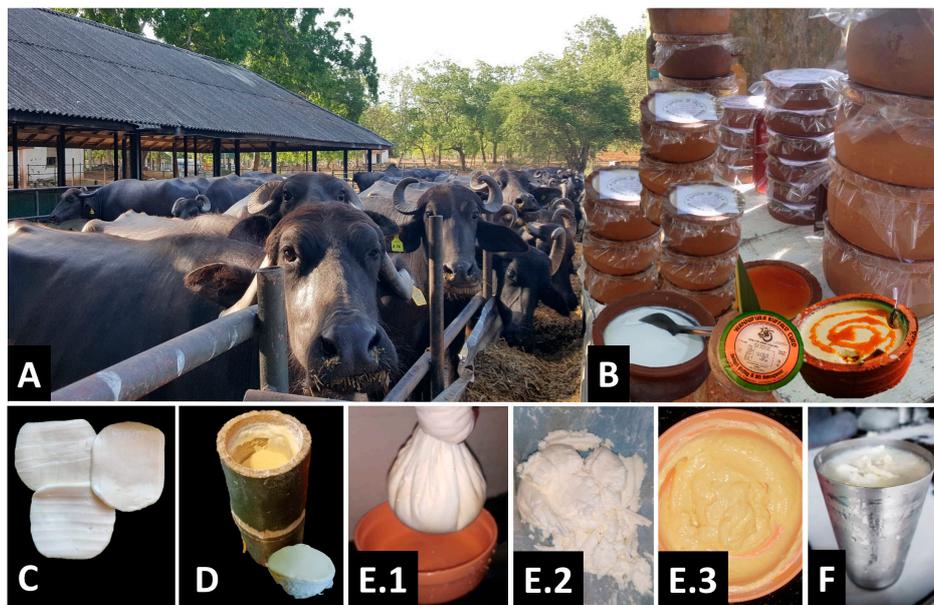
### 3. Artisanal Fermentation of Buffalo Milk

#### 3.1. Fermented Buffalo's Milk Products in the World

Buffalo milk is considered as a suitable raw material in producing certain types of artisanal and modern fermented dairy products and cheeses. The richness of its constituent is superior when compared to the cow milk [41] and thus makes it highly suitable for processing into various dairy products that are widely appreciated across many food cultures. Range of fermented buffalo milk products is available, depending on differences in geographical regions, demographic factors, social cultures and lifestyles. Because of different processing parameters and conditions, coupled with undefined starter cultures and traditional practices, fermented buffalo milk products are further diversified [42]. The unique characteristics of buffalo milk, such as high total solid content, whiteness and

viscosity, provides the opportunities for buffalo milk to process into inimitable dairy product compared to cow milk. Fermented buffalo milk products are commonly consumed in regions where buffaloes have been reared for centuries, as a traditional preservative method (e.g., extending shelf life) for their excess milk yield while adding value to the perishable milk. In addition to fermented milk, there are several other noteworthy popular buffaloes' milk products, which is out of the scope in the present study, such as *Koha* (fresh concentrated traditional Indian buffalo milk product), several types of cheeses including Mozzarella, Domiati, Queso Blanco, etc. [31], paneer (acid-coagulated) [43], butter and ghee (Clarified Butter Fat) [44].

Various fermented buffalo milk products available in the world are shown in Figure 1 and the product characteristics are described in Table 3.



**Figure 1.** An extensive buffalo dairy farm in Sri Lanka (A), and various fermented buffalo milk products in the world, i.e., Sri Lanka—*Meekiri* (B), Philippines—*Kesong Puti* (C), West Sumatra, Indonesia—*Dadih* (D) and India—*Dahi*, where whey removal of *Dahi* occurs by hanging the fermented coagulum (E.1) to produce *Chakka* (E.2), and then it can further be processed into *Shrikhand* (E.3), by adding optional fruit pulps (e.g., mango). *Dahi* can be prepared in drinkable format, by mixing with water, *Lassi* (F).

**Table 3.** Various fermented buffalo milk products available in the world.

Traditional Name	County of Origin	Product Characteristics	References
<i>Meekiri</i>	Sri Lanka	Traditional buffalo curd fermented in clay pots that resembles a creamy yoghurt.	[45]
<i>Kesong Puti</i>	Philippines	Soft fresh (unripened) cheese made with rennet and natural fermentation process of LAB.	[46]
<i>Dadih</i>	West Sumatra, Indonesia	Sour yoghurt obtained by fermenting raw buffalo milk spontaneously in a bamboo tube, for two days, at ambient temperature, with natural LAB.	[47]
<i>Lassi</i>	India	A refreshing beverage, obtained by blending <i>Dahi</i> with other ingredients such as salt, sugar, spices, fruits and water until it becomes frothy.	[48]
<i>Dahi</i>	India	Fermented buffalo milk yoghurt, made with various naturally occurring strains of LAB, which are introduced into the fresh milk through back-slopping (from the previous fermentation vat).	[49]
<i>Chakka</i>	India	A product obtained by partial separation of whey from <i>Dahi</i> .	[49]
<i>Shrikhand</i>	India	Sweetened concentrated curd produced by further removing whey from <i>Dahi</i> . Served with fruit pulps.	[49]

LAB, lactic acid bacteria.

### 3.2. Indigenous Microflora in the Fermented Buffalo Milk, their Probiotic Potentials and Effect on Product Technological Properties

Researchers are diligently working on the development of fermented buffalo milk to provide probiotic potentials through those products. Because of rich composition and associated health benefits (i.e., low-allergenic potential, beneficial effects for obesity, hypertension, osteoporosis, etc.) consumption of buffalo milk has gained significant popularity [50]. Nevertheless, consumption of buffalo milk with probiotic potentials may confer positive health benefits, since specific strains of bacteria in fermented milk has the potential of surviving and colonizing in the human gastrointestinal tract to deliver a potential probiotic effect [51].

*Meekiri*, also known as curd is a popular and sole buffalo milk-derived product in Sri Lanka. Comparing the syneresis values between *Meekiri* and fermented cow milk (also known as *Deekiri* in Sri Lanka) showed that *Meekiri* had lower syneresis than *Deekiri* [52], and, thus, fermented buffalo milk products are having superior gelling properties compared to fermented cow milk gels. Incorporation of *Bifidobacterium longum* into *Meekiri* showed that  $10^6$  CFU/g of Bifidobacteria was found up to three days of storage, in clay pots, at 29 °C, while after 4 days of storage, an acceptable level of Bifidobacteria count was not reported [12]. Authors of this study confirmed the addition of Bifidobacteria into *Meekiri* did not alter its organoleptic properties and improved the consumer acceptability. However, the use of probiotic starter cultures compared to non-probiotic starter cultures in fermented cow milk gels (regular set-yoghurt) has shown to reduce the overall textural and sensorial properties [53]. Thus, natural probiotic strains containing in buffalo milk or using commercial probiotic starter cultures are not likely to interfere with the product physical characteristics, which harness to maximize the use of buffalo milk as a potential probiotic career. Isolating and purifying *Lactobacillus* strains from fifty different *Meekiri* samples collected from various regions in Sri Lanka reported thirty-nine different isolates, and the majority (80%) was heterofermentative lactobacilli [54]. Moreover, studying 26 *Meekiri* samples by Dekumpitiya et al. [55], concluded that various LAB (i.e., *Lactobacillus delbrueckii* subsp. *lactis*, *L. plantarum*, *L. helveticus*, *L. delbrueckii* subsp. *bulgaricus* and *L. casei* subsp. *casei*, *Streptococcus thermophilus* and *S. lactis*) in addition to *Saccharomyces cerevisiae*, *Micrococcus* spp. and *Bacillus* spp. were present in *Meekiri*. Nine curd samples obtained from Kandy, Sri Lanka to isolate and characterize *Lactobacillus* species by Shuhadha et al. [56] reported that all isolated bacteria could grow at low pH (i.e., pH 3) and able to survive at 0.3% bile salt, while showing antimicrobial activity against *E. coli* and *Pseudomonas aeruginosa*. Further, those isolated *Lactobacillus* species have shown non-hemolysis and no DNase activity; thus, studied *Meekiri* samples were shown to contain probiotic potentials. A recent study conducted by Disanayaka et al. (2018) [57] found that *Lactobacillus fermentum* isolated from Sri Lankan homemade curd (*Meekiri*) can be used to control the *Candida krusei* and *Candida tropicalis* infections.

Buffalo milk is often adulterated with cow milk, because of relatively high availability on a year-round basis and lower farm gate price [58]. Thus, detecting cow milk in buffalo milk or products are of great interest. PCR assays [59] and capillary electrophoresis analysis can be used to detect the adulteration of buffalo curd with cow milk. Randiwela et al. [59] reported that two-thirds of the common market available *Meekiri* brands are adulterated with cow milk in Sri Lanka. This mixing of both types of milk has an impact on the fat%, where it could lower the fat% than the standards set by Sri Lanka Standard Institution [60], of which the minimum level of fat percentage in *Meekiri* should not be lower than 7.5%. In addition to the compositional and organoleptic quality changes of the final product, fraudulent incorporation of non-declared cow milk into buffalo milk will lead to food safety concerns, especially for people with allergies or intolerance for cow milk [22,61].

As per, Sri Lanka Standard Institution (SLSI) standards [60], minimum of 8.5 milk solid non-fat, 4.5 of maximum pH level and absent of *E. coli*, should comply with standards in *Meekiri* available in Sri Lanka. Fermentation of buffalo milk proved to be an effective strategy in eliminating the *Listeria monocytogenes*, in buffalo milk products, where the growth of *Listeria* was not observed below the pH level 5.5. This inactivation of *Listeria* is mainly due to low pH, increasing titratable acidity and production of bacteriocins, i.e., Nisin [62]. Kanthale is one of the popular regions in Sri Lanka for *Meekiri* production [63].

Thirty-six *Lactobacillus* strains were found in samples collected from spontaneously fermented traditional buffalo curd in clay pots from the Kanthale region [64]. Wickramaratne and Gunasena [64] reported strain-specific antimicrobial properties of those isolates against several pathogenic bacteria, while they observed the highest antibacterial activity against *Listeria monocytogeneus*. Therefore, it can be argued that the natural microflora used in traditional *Meekiri* production in Kanthale have antibacterial activity, which could protect the curd from bacterial spoilage to enhance the shelf-life while increasing the food safety from food-borne bacterial diseases. Analyzing shelf available *Meekiri* in Sri Lanka to identify probiotic *Lactobacillus* species by Rajapakse et al. [65] confirmed the availability of *Lactobacillus acidophilus* (LA-5). The microbial count of *L. acidophilus* was higher than the minimum required level ( $10^7$  CFU/g) up to 12 days of shelf-life and survived up to low pH (decrease by acidification up to pH 1.5) and tolerance to bile salt (0.15%–0.3%) compared to other *Lactobacillus* species. However, they did not find *Lactobacillus casei* from the isolates.

Raw buffalo milk analyzed for probiotic strains in Karnataka, India reported containing *Lactobacillus acidophilus*, *Lactobacillus rhamnosus* and *Bifidobacterium longum* [66]. In addition to those reported in Karnataka area, India *Lactobacillus casei* and *Lactobacillus fermentum* have been isolated from buffalo milk collected from Khulna division of Bangladesh, which demonstrated to exhibit probiotic potentials [67]. Packaging materials are of importance for optimal probiotic delivery [45], where shelf-life of *Lassi* was affected by the oxygen permeation rate. *Lassi* stored in ethylene-vinyl-alcohol copolymer and glass bottles could store up to 25 days at  $5 \pm 1$  °C without deteriorating the organoleptic properties and probiotic survivability [68]. This observation applies to Sri Lankan *Meekiri*, where the survival of bifidobacteria was affected by the type of packaging materials. Jayamanne and Adams [12] investigate the effect of three packaging materials (i.e., clay pots, plastic cups and glass bottles) on the survival of *Bifidobacterium longum* NCTC11818 in buffalo curd and reported bifidobacteria survived better in curd stored in plastic cups and glass bottles than in clay pots. This difference in Bifidobacteria count was attributed to the differences in permeability of packages for oxygen and storage temperature since Bifidobacteria are obligate anaerobes [69].

*Dahi*, a popular fermented milk and the Indian version of Sri Lankan *Meekiri* is made with lactic acid fermentation of milk sugars. *Dahi* made with individual starter strain of *Lactococcus*, has demonstrated antidiabetic effects (e.g., lowered fasting blood glucose, glycosylated hemoglobin, insulin, free fatty acids and triglyceride levels) on rats fed with a *Dahi*-based diet [70]. Feeding *Dahi* made with starter culture consisting *Leuconostoc citrovorum*, *Lactococcus lactis*, *Lactococcus diacetylactis* and *Lactococcus cremoris* to experimental mice report to protect against the enteric infections caused by *Shigella dysenteriae* and confirm that *Dahi* activates the non-specific immune systems in mice [71]. However, further clinical research is warranted to confirm these findings concerning human health. Sensory properties were not altered by adding *L. acidophilus* and *Bifidobacterium* to regular starter cultures in preparation of *Dahi* and therefore, technological properties of *Dahi* is usually well preserved while providing the advantages of delivering health benefits associated to the probiotic potentials [72] also as previously discussed for *Meekiri*.

Probiotic potential of bacterial strains isolated from *Dadih* samples sourced from three geographical regions in west Sumatera demonstrated varying levels of probiotic capacities [47], which emphasize the importance of preserving the traditional microflora in indigenous fermented products. Swamp buffalo milk screened for probiotic bacterial strains in west Sumatera by Melia et al. [18] reported that it possessed *Lactobacillus fermentum* strain L23, which shows probiotic potentials. This bacterial strain presents naturally in western Sumatera's buffalo milk could confer probiotic effects, even when the milk is fermented to *Dadih*. Moreover, studying the microbial content of buffalo milk in west Sumatera reported containing higher levels of lactic acid bacteria ( $>1 \times 10^6$  CFU/mL), which indicates that milk could contribute as a potential source of probiotic bacteria. However, due to the higher LAB count, cautious should be made during the handling of raw milk [34]. LAB isolated from buffalo milk (e.g., *Lactobacillus fermentum* strain L 23 (A 3.3), *Lactobacillus fermentum* strain 6704 (TD 7.2) and *Lactobacillus oris* strain J-1 (A 3.2)) in the same region showed antimicrobial activity against *Listeria*

*monocytogenes* [73]. *Lactobacillus plantarum* strain 8m-21 isolated from *Dadih* in West Sumatera reported containing probiotic properties because they inhibit the *E. coli* due to its antimicrobial properties [74]. Analyzing *Dadih*, we saw that it showed that its naturally occurring LAB can produce antimicrobial substances (i.e., class II bacteriosins) with the molecular weight of 10 kDA [75].

#### 4. Inclusion of Probiotics into the Artisanal Fermentation of Buffalo's Milk

##### 4.1. Probiotics

Probiotics are living cultures of a single microbial strain or a mixture of different strains that beneficially affect the well-being of the host animal, directly or indirectly, by improving its intestinal microbial balance when administered in sufficient quantity. It was at the beginning of the 20th century that Nobel laureate Elie Metchnikoff (1845–1916) first proposed the concept of probiotics as it is known today [76]. However, it was later, in the 1960s, that Lilly and Stillwell proposed the term “probiotics”, meaning “for life” in Greek [77].

The definition of probiotics has evolved over the years and one of the most used definition was proposed by Fuller [78] which is “probiotics are the live microbial food/feed supplements that exert beneficial effects for the host by improving its intestinal microbial balance”. Besides, in 1992, Fuller extended the definition of probiotics to “live microbial food ingredients that beneficially affect the health of consumers by improving their intestinal microflora balance when ingested live in sufficient numbers” [79]. Furthermore, various scientists have given a definition of probiotics considering their activities and their survival in the digestive tract. According to Salminen et al. [80], probiotics are “microbial cell preparations or components of microbial cells that have a beneficial effect on the health and wellbeing of the host”. Tannock et al. [81] described probiotics as “microbes which transit the gastrointestinal tract and which, in doing so, benefits the health of the consumer”. In 2001, the current and most widely used definition was proposed by experts from the FAO (Food and Agriculture Organization of the United Nations) and WHO (World Health Organization) working group and they defined probiotics as “live microorganisms which when administered in adequate amounts confer health benefits on the host” [21]. This definition was endorsed in 2014 by the International Scientific Association for Probiotics and Prebiotics [82].

##### 4.2. Commonly Used Probiotics

There is a rapid increase in the production of products containing probiotic bacteria, due to a better understanding of the role of these bacteria in maintaining the health of the host. Different strains of probiotics can be incorporated in the form of cell suspensions or lyophilized form, depending on the food product. In the context of dairy products, there are many popular delivery systems for probiotics, such as raw and fermented milk, yoghurt, ice cream, desserts, cheese, various milk-based drinks, cookies and powdered milk (Boylston et al., 2004 [83]; Dagmar et al., 2011 [84]). Bacteria mainly from the genera *Lactobacillus* and *Bifidobacterium* are mainly considered as probiotics with GRAS status (generally recognized as safe); however, some other bacteria and yeast species have been recommended as probiotics (Table 4). Some strains of *Propionibacterium*, *Bacillus*, *Enterococcus* and *Escherichia* have also been shown to have probiotic characteristics. The yeast *Saccharomyces* has also been shown to be probiotic in some food products [85].

**Table 4.** Commonly used probiotic microorganisms.

Genus	Species
<i>Lactobacillus</i>	<i>L. acidophilus</i> , <i>L. casei</i> , <i>L. crispatus</i> , <i>L. delbrueckii</i> subsp. <i>bulgaricus</i> , <i>L. fermentum</i> , <i>L. gasseri</i> , <i>L. johnsonii</i> , <i>L. paracasei</i> , <i>L. plantarum</i> , <i>L. reuteri</i> , <i>L. rhamnosus</i> , <i>L. helveticus</i> , <i>L. lactis</i> , <i>L. sporogenes</i> , <i>L. amylovorus</i> , <i>L. brevis</i> , <i>L. salivarius</i>
<i>Bifidobacterium</i>	<i>B. bifidum</i> , <i>B. breve</i> , <i>B. infantis</i> , <i>B. longum</i>
<i>Propionibacterium</i>	<i>B. lactis</i> , <i>B. animalis</i> , <i>B. adolescentis</i> , <i>B. essensis</i> , <i>B. laterosporus</i>
<i>Bacillus</i>	<i>P. acidipropionici</i> , <i>P. freudenreichii</i> , <i>P. jensenii</i> , <i>P. thoenii</i>
<i>Enterococcus</i>	<i>B. alcalophilus</i> , <i>B. cereus</i> , <i>B. clausii</i> , <i>B. coagulans</i> , <i>B. subtilis</i>
<i>Escherichia</i>	<i>E. faecium</i>
<i>Saccharomyces</i>	<i>E. coli</i> Nissle <i>S. boulardii</i> , <i>S. cerevisiae</i>

Adapted from Gorbach [86]; Senok et al. [87]; Caplan and Frost (2011) [88]; Ranadheera (2012) [89]; Saad et al. (2013) [90]; Stefan et al. (2013) [91].

### 4.3. Incorporation of Probiotics into Buffalo Milk Fermentation for Product Development

Having higher fat and casein content, buffalo milk could be used to produce a range of quality products with good consistency and creaminess that are different from those traditionally made with cow milk [92]. Buffalo milk is an effective food matrix for transporting probiotics. There are different types of probiotic buffalo milk products. These products range from liquid products, such as beverages, to semi-solid products, such as yoghurt and *Dahi*. Furthermore, there are findings that probiotics have been successfully incorporated into a different type of cheeses [93–95].

#### 4.3.1. Probiotic Buffalo Milk-Based Beverages

Buffalo milk has been successfully used to produce acidophilus milk. In the production process, buffalo milk is standardized to around 3.5% fat and 8.5% SNF and followed by pasteurization. The incubation is carried out using a pure culture of *Lactobacillus acidophilus* at around 40 °C until pH reaches 4.4 [96]. Recently, probiotic dairy beverages were developed from buffalo milk with different level of whey (0, 25 and 50%) using *Streptococcus thermophilus*, *Lactobacillus bulgaricus* and *Lactobacillus acidophilus*. The researches demonstrated that the bacteria used in the production of beverages had higher survival in buffalo milk beverage during in vitro stimulation of gastrointestinal stress compared to fermented cow milk products [92]. In another study, Macedo, Freitas, Pandey and Soccol [97] showed that a probiotic beverage could be successfully produced with a mixture of 35% skimmed cow milk, 35% buffalo cheese whey and 30% soymilk using co-cultures of *Lactobacillus casei* subsp. *shirota* and *Bifidobacterium adolescentis*. Kefir is a creamy and sour fermented probiotic dairy drink originating from the Caucasus Mountains and is produced using milk from various species such as cow, goat, sheep, camel and buffalo. Kefir production is done according to the traditional method using kefir grains containing a complex mixture of lactic acid bacteria, acetic acid bacteria and yeasts or on an industrial scale using kefir starter cultures [98]. There are many published articles on the successful use of buffalo milk in manufacturing kefir [99–103]. Kefir made from buffalo milk was reported to have improved water holding capacity, firmness, viscosity, storage and loss modulus values and consistency index when compared to those of kefir made with cow milk [104].

#### 4.3.2. Probiotic Buffalo Yoghurt

Yoghurt is a semisolid fermented dairy product which is used as a popular carrier for probiotics [105]. Traditional yoghurt production involves a mixture of thermophilic starter cultures consisting of *Lactobacillus delbrueckii* subsp. *bulgaricus* and *Streptococcus thermophilus*, which are different from the cultures traditionally used for the fermentation of buffalo milk. However, the new trend is to use probiotic bacteria such as *Lactobacillus acidophilus* and *Bifidobacterium* spp. in the product for their potential health benefits [106]. Homogenization of milk is not typically practiced in the manufacturing of buffalo milk yoghurt. Moreover, milk solid fortification and the incorporation of thickeners are

not practiced in the manufacturing process due to the higher solid content of buffalo milk [107,108]. Different types of probiotic buffalo yoghurt have been reported such as plain [108], low fat [109], symbiotic [110] and fiber incorporated [111].

#### 4.3.3. Probiotic Buffalo Curd

Buffalo curd is an acidic fermented dairy product with a texture similar to that of yoghurt. It is mainly produced on a small scale using a traditional blend of starter cultures. The culture blend includes *Lactococcus lactis* subsp. *lactis*, *Lactococcus lactis* subsp. *lactis* biovar *diacetylactis*, *Lactococcus lactis* subsp. *cremoris* singly or in combination with *Leuconostoc* spp. [12]. The production process includes pasteurization or boiling of milk which is followed by inoculation with the Meekiri/Dahi from the previous day as the starter. Incubation is normally carried out at room temperature (~30–35 °C) for overnight (~10–12 h). Various probiotic species, such as *Lactobacillus acidophilus* [112], *Lactobacillus casei* [113], *Lactobacillus plantarum* [114] and *Bifidobacterium bifidum* [112,115], have been used in producing buffalo Meekiri/Dahi.

#### 4.3.4. Cheese

Cheese is a popular milk-based food product consumed around the world as an integral part of the diet of the population. Consumption of cheese containing probiotic bacteria has been linked to various human health benefits, such as improvements in the immune system, oral health in the elderly and gastrointestinal health [116]. Cheese is a good carrier for the target delivery of probiotics in the gastrointestinal tract compared to other fermented dairy products because cheese has a dense matrix, high pH, low oxygen levels, buffering capacity and high-fat content which could protect them during the entire shelf life and their transit in the gastrointestinal tract [117]. However, high-salt varieties of cheese are not suitable for incorporating probiotics as they can retard their growth [118]. Buffalo milk cheese has proven to be an effective carrier for various probiotics. Cheddar cheese from buffalo milk was produced using *Lactobacillus acidophilus* LA-5, *Bifidobacterium bifidum* Bb-11 and *Bifidobacterium longum* BB536 and shown to have good probiotic viability during the storage [119]. *Lactobacillus casei*, *Lactobacillus plantarum* and *Lactobacillus brevis* were used in producing Himalayan cheese (Kalari) using buffalo milk and incorporation of probiotics improved quality characteristics of the cheese [118]. In another study, buffalo Minas Frescal cheese produced with *Bifidobacterium animalis* subsp. *lactis* was shown to have acceptable quality parameters [120]. *Lactobacillus casei*, *Lactobacillus rhamnosus* and *Bifidobacterium bifidum* were used to improve conjugated linoleic acid content in buffalo cheese [121]. Ricotta cheese made from buffalo milk is an appropriate carrier for the probiotic *Lactobacillus acidophilus* La-05, without affecting its sensory attributes and consumer acceptability [122].

#### 4.4. Challenges of Probiotic Inclusions into Fermented Buffalo Milk Products

The probiotic bacterial strains have already been used for years in fermented milk products, they have inferior behavioral characteristics than those of the traditional lactic acid bacteria used in fermented milk products, which limits their possible applications. More precisely, they grow weakly in cow milk and require long fermentation times, anaerobic conditions and a low redox potential for their growth [123,124]. Their poor acidifying performance has a direct effect on the textural properties of fermented dairy products, which results in poor rheological properties of probiotic fermented dairy products. The rheological properties of fermented milk products are very important at the industrial level because they are considered as quality parameters of the end-up products [125].

Moreover, the viability of probiotics can be suppressed and reduced by environmental stress parameters, such as the presence of oxygen, mechanical damage, high processing temperatures and interaction with the foods in which they are added. Furthermore, poor compatibility with the traditional starter of fermented milk during fermentation and high acidity of dairy products or milk-based products with high acidity could lead to poor viability starter organisms [126]. Probiotics incorporated into

dairy products should not lead to poor sensory properties or texture. Therefore, sensory evaluation of the newly developed product must be carried out before it is introduced into the market [127].

### 5. Technological Prospects to Develop Probiotic Fermented Products Using Buffalo Milk

Several innovative technologies have been reported to increase the stability and viability of probiotic bacteria in fermented products. Some of these methods have been successfully adapted to overcome technological challenges associated with the probiotics such as the presence of the toxic by-products (organic acids and hydrogen peroxide) during the propagation of probiotics, mechanical stress caused by extreme temperature conditions employed during the preservation of probiotics such as freeze-drying or spray-drying, high rate of acidification of the buffalo set-yoghurts during storage and the presence of acidic conditions and the bile salts in the gastrointestinal tract of the consumer, which resulted in poor viability and survival of probiotic microorganisms [128]. Use of power ultrasound, application of encapsulation techniques and microbead coating are some of such innovative techniques attempted by many researchers to increase the viability of probiotics. Moreover, the selection of desirable probiotics for different carrier foods, determination of required doses of probiotics, use of appropriate packaging conditions, the addition of prebiotics and growth-promoting factors are some of the other potential measures, which enhances the survival of probiotics in bio-yoghurts.

#### 5.1. Selection of Probiotic Strains for the Fermentation of Buffalo’s Milk

Type of the probiotic strains used for the development of probiotic-based functional food is one of the major factors affecting the viability of probiotics and the organoleptic, rheological and texture properties of yoghurts. A significant number of potential probiotic microorganisms have been isolated and identified using the traditional fermented buffalo milk. It was revealed that the probiotic characteristics were mainly depended on the strain of the bacteria. Most of the bacterial strains which were screened for the potential probiotic properties, using in vitro studies, showed promising characteristics, such as acid and bile tolerance, survival through the gastrointestinal tract, the ability to adhere to the intestinal mucosa, antimicrobial activity against potentially pathogenic bacteria, etc. [129], as summarized in Table 5.

**Table 5.** Health benefits of probiotic bacteria isolated from fermented buffalo milk.

Type of Microorganisms Isolated/Investigated	Beneficial Characteristics Revealed in the Study	Products Associated	References
<i>Enterococcus faecium</i> IS-27526	Stimulating the total salivary serum IgA level in underweight preschool children increase of secretory IgA level	Traditional fermented buffalo milk in Indonesia ( <i>Dadiah</i> )	[16]
<i>Lactobacillus plantarum</i> IS-10506	Inhibitory, competitive and displacing properties against pathogens and reduced pathogen adhesion to mucus	Traditional fermented buffalo milk in Indonesia ( <i>Dadiah</i> )	[130]
<i>Enterococcus faecium</i> , <i>Lactobacillus plantarum</i>	Ability to auto aggregate, together with cell surface hydrophobicity and co-aggregation abilities with pathogen strains	Traditional fermented buffalo milk in Indonesia ( <i>Dadiah</i> )	[15]
<i>Lc. Lactis</i> subsp. <i>Lactis</i> IS-10285, IS-7386, IS-16183, IS-11857, IS-29862, <i>L. brevis</i> IS-27560, IS-26958 and IS-23427, <i>Leu. mesenteroides</i> IS-27526 and <i>L. casei</i> IS-7257	Possessed a good survival rate at low pH (PH 2 and 3), bile tolerance and lysozyme tolerance	Traditional fermented buffalo milk in West Sumatra ( <i>Dadiah</i> )	[131]
<i>Lactobacillus casei</i> subsp. <i>casei</i> R-68, <i>Lactobacillus casei</i> strain <i>Shirota</i>	Inhibited the growth of <i>Staphylococcus aureus</i> FNCC-15, <i>Listeria monocytogenes</i> FNCC-0156 and <i>Escherichia coli</i> FNCC-19 Antibacterial activity was resistant to heat treatment (60–95 °C), amylase, various proteolytic enzymes and various types of antibiotics.	Traditional fermented buffalo milk in Indonesia ( <i>Dadiah</i> )	[132]

Table 5. Cont.

Type of Microorganisms Isolated/Investigated	Beneficial Characteristics Revealed in the Study	Products Associated	References
<i>Lactobacillus fermentum</i> (L23).	Resistance to low pH and 0.3% and 0.5% bile salts, antimicrobial activity against pathogenic bacteria ( <i>Escherichia coli</i> O157: H7 and <i>Staphylococcus aureus</i> ATCC 25923), antibiotic resistance and hydrophobicity	Traditional fermented buffalo milk in West Sumatra ( <i>Dadih</i> )	[18]
<i>Lactobacillus delbrueckii</i> subsp. <i>bulgaricus</i> M3 40-3 <i>Streptococcus bovis</i> J2 40-2	High acid production and tolerance at high acidic medium. High angiotensin I-converting enzyme (ACE) inhibitory activity	Traditional fermented buffalo's milk in Bangladesh ( <i>Dahi</i> )	[133]
<i>L. plantarum</i> strain S1.30 and SL2.7 <i>L. plantarum</i> strain S1.30	Tolerated low pH, survived bile salt concentrations and resistant to vancomycin Possessed antimicrobial activity against the selected pathogens and able to adhere to Caco-2 colon cancer cells	Naturally fermented buffalo's milk namely <i>Dangke</i> and <i>Dadih</i> of Indonesia	[134]
<i>L. plantarum</i> IS-20506	Significantly lowered fecal mutagenicity of rats	Indigenous <i>Dadih</i> originated from Bukit Tinggi, West Sumatra	[131]
<i>Lactobacillus fermentum</i> I-11 and <i>Leuconostoc lactis</i> subsp. <i>lactis</i> I-2775	Tolerant to acid and oxgall (bile) and deconjugated sodium taurocholate, bound cholesterol	Traditional fermented buffalo's milk ( <i>Dadih</i> )	[135]
<i>Lactococcus lactis</i> IS-16183 and <i>Lactobacillus rhamnosus</i> IS-7257	Significantly inhibited the adhesion of <i>Escherichia coli</i> O157:H7	Traditional fermented buffalo milk in Indonesia ( <i>Dadih</i> )	[134]
<i>Pediococcus pentosaceus</i>	Reduced the stool frequency, lower TNF- $\alpha$ and improve the gut microflora balance in diarrhea-induced mice using enteropathogenic <i>Escherichia coli</i>	Traditional fermented buffalo milk in Indonesia ( <i>Dadih</i> )	[136]
<i>Weisella mesenteroides</i>	Reduced the stool frequency, lower tumor necrosis factor- $\alpha$ and improve the gut microflora balance in diarrhea-induced mice using enteropathogenic <i>Escherichia coli</i>	Traditional fermented buffalo milk in West Sumatra ( <i>Dadih</i> )	[137]
<i>Lb. reuteri</i> IS-27560, <i>Lc. lactis</i> IS-16183, <i>Lb. rhamnosus</i> IS-7257, <i>Enterococcus faecium</i> IS-275	Adherence to mucus layer and Caco-2 cells, IS 16,183 and IS7257 inhibit the adhesion of <i>Escherichia coli</i> O157:H7 to the human intestinal mucosal surface	Traditional fermented buffalo milk in Indonesia ( <i>Dadih</i> )	[138]

Numerous in vitro studies and animal studies, together with the limited number of clinical studies, reported the probiotic properties of bacteria isolated from fermented buffalo milk. It was observed that *Lactobacillus reuteri* IS-27560, *Lactococcus lactis* IS-16183 and *L. rhamnosus* IS-7257 that were isolated from “*Dadih*” (fermented buffalo milk product popular in Indonesia) showed adhesion to the human intestinal mucosal surface and Caco-2 cells, which were used as the model for human intestinal cells. Moreover, *L. lactis* IS-16183 and *L. rhamnosus* IS-7257 significantly inhibited the adhesion of *Escherichia coli* 157:H7 to the human mucosa [138]. It was revealed that the adhesion properties of IS-16183 and IS-7257 were similar to the *L. casei* *Shirota* and *L. rhamnosus* GG, which are used as commercial probiotic strains. A similar study of Collado et al. [130], using *L. plantarum* IS-10506 and IS-20506, and *Enterococcus faecium* IS-27526, IS-23427 and IS-16183, which were isolated from *Dadih*, showed competitive exclusion of pathogens in the mucosa.

However, all of the selected strains with promising probiotic properties are not suitable for the production under the industrial scale because of low yields in the growth medium or poor survival during downstream processing [139]. Therefore, these selected bacterial strains with potential probiotic properties should be further investigated for their technological properties, such as the capability for mass production and ability to incorporate into the target food without reducing cell viability and undesirable flavor or texture [128]. Furthermore, such selected probiotic strains should be evaluated for high survival rates in downstream processing (such as freeze-drying or spray-drying) before commercialization.

### 5.2. Addition of Prebiotics

Prebiotics are indigestible nutrients having health-promoting benefits for the host through promoting growth or activity of one or more probiotic bacteria [110]. Prebiotics should not be hydrolyzed or absorbed in the upper part of the gastrointestinal tract; they should be a selective substrate to stimulate the growth of one or a limited number of beneficial bacteria in the colon and capable of altering the composition of colonic microflora in a healthy manner [140]. The most commonly used prebiotics for the production of functional dairy products are fructans and resistant starches. Several authors have reported the use of prebiotics in the fermentation of buffalo milk incorporated with probiotic bacteria.

Ehsani et al. [110] observed a higher population of *Lactobacillus acidophilus* and *Bifidobacterium bifidum* (above 7 logs CFU/mL) in buffalo set-yoghurts incorporated with lactulose, oligofructose and inulin, under refrigerated storage (4 °C), for 21 days. Furthermore, incorporation of prebiotics reduced the syneresis and post acidification of buffalo set-yoghurts. A similar study on the effects of mannan extracts from yeast cell walls on the probiotic buffalo milk yoghurts containing *Lactobacillus acidophilus*, *Bifidobacterium* sp. and *Streptococcus thermophilus* increased the viability of probiotic bacteria in between  $10^7$  and  $10^9$  CFU/mL after fermentation [141,142], which is the recommended dose of viable probiotic bacteria at the time of consumption of a probiotic product [110]. Moreover, post-processing acidification of buffalo milk yoghurts reduced with the addition of prebiotics. Karki, Shrestha, Bohara and Jyakhwa [143] reported similar findings, that addition of lactulose, galactomannan (Sunfibre®) and inulin into buffalo milk yoghurts increased the viable cell counts of *L. rhamnosus* up to 8 logs CFU/ML, until 15 days of storage (4 °C). Similarly, incorporation of prebiotics reduced the titratable acidity and pH of buffalo yoghurts.

Buffalo milk contains a comparatively high amount of carbohydrates than cow milk, resulting in high titratable acidity [144]. The concentration of undissociated organic acids in fermented products enhances the bactericidal effect; therefore, the survival of probiotics during storage is considerably reduced by the low pH and high titratable acidity of the products [145]. Inclusion of prebiotics into buffalo milk may be an effective method to reduce the post-fermentation acidification of buffalo yoghurt and enhance the viability of probiotics, which is one of the major constraints associated with the production of functional buffalo milk products.

### 5.3. Microencapsulation

Microencapsulation is a process of enclosing the probiotic cells by covering them with an appropriate material, which leads to protecting the cells during digestion and releases into the intestinal medium [145]. Different materials have been used to encapsulate probiotics such as sodium alginate [146–148], calcium alginate [148,149], xanthan gum [150], chitosan [151], starch,  $\kappa$ -carrageenan [152,153], cellulose acetate phthalate, gelatin [154,155], caseinate [156] and whey proteins [157]. These encapsulating materials were acting as physical barriers to protect the sensitive probiotic cells against a harsh gastrointestinal environment, thus increasing their survival in the colon [158]. Additionally, encapsulation helps to protect probiotic bacteria during processing and storage, such as high oxygen stress.

Several encapsulation techniques have been reported such as emulsification [159], extrusion [160] and spray-drying [161], fluid-bed method [162] and freeze-drying [163]. Selection of the appropriate encapsulation method depends on several factors such as the required size of the capsules in the target product, the viability of the probiotic bacteria under the processing conditions used for the encapsulation, cost of the operation, etc. [142].

Microencapsulation has been extensively investigated to increase the viability of probiotic bacteria in fermented cow milk [164–167]. Encapsulation resulted in higher probiotics survival during both refrigerated storage and simulated gastrointestinal conditions compared to that of the free cells. Similarly, it reduced the post-acidification of cow milk yoghurts.

Therefore, it can be assumed that microencapsulation may be a successful technique to increase the viability of probiotics in buffalo milk set-yoghurts by protecting the cells from detrimental factors such as higher post-acidification compared to cow milk yoghurts during refrigerated storage. Shoji et al. [168] incorporated *Lactobacillus acidophilus* into buffalo milk yoghurts, which were microencapsulated with pectin and casein (1:1). They observed an increase in the survival of *L. acidophilus* ( $10^7$  CFU/g) at refrigerated conditions (4 °C). Similarly, buffalo set-yoghurts prepared with microencapsulated cultures resulted in lower post-acidification when compared to the product prepared with free culture. However, it was revealed that the encapsulation was not a successful technique to protect the *L. acidophilus* against the low pH conditions, which were similar to the human stomach.

Application of coating has been reported to enhance the protective ability of probiotic microcapsules. Therefore, the coating may be a useful technique to increase the resistance of microencapsulated probiotics under harsh gastrointestinal environment. This can be done using different materials such as alginate, chitosan, poly-l-lysine, whey proteins, etc. These coatings can form an additional layer on microcapsules, preventing the exposure of probiotics to oxygen during storage and, thus, enhancing their stability at low-pH and high-temperature conditions [169]. Several techniques have been used to apply coatings on probiotics microcapsules, such as layer-by-layer assembly (immersion of microcapsules in the polymer solution) and coacervation (forming a coacervate between microcapsules' surface and a polymer coating) [142]. Hence, the application of an appropriate coating technique to enhance the viability of microencapsulated probiotics in buffalo milk warranted further studies.

#### 5.4. Ultrasonication

Ultrasound refers to sound waves above a frequency of 20,000 Hz, which cannot be detected by humans [170]. High-intensity ultrasound (20–100 kHz) is a potential tool to accelerate the functionality and viability of probiotic bacteria through a process called “sonoporation”, which changes the cellular structure of probiotics. Sonoporation defines the progressive opening of the cell membrane due to micro-bubble cavitation upon the exposure of cells to ultrasound. This resulted in some cellular damages such as micro-cracks, micro-voids and ruptures in the cell membrane, causing enhanced permeability [120]. Consequently, sonoporation enhances mass transfer of substrates across the microbial cell membrane, which eventually improves microbial growth.

Ultrasound has been applied to numerous probiotic dairy products and results revealed shorter fermentation time, high probiotic survival and less requirement of ingredients such as prebiotics [171] and are outlined in Table 6.

**Table 6.** Major effects of employing ultrasound into probiotic bacteria.

Ultrasonic Conditions	Types of Probiotic Bacteria	Major Effects Observed	References
100 mL of inoculated milk was sonicated before fermentation at 100 W and 20 kHz for 7, 15 and 30 min, using an ice bath, energy density 420, 900 and 1800 J mL <sup>-1</sup>	<i>B. breve</i> ATCC 15700, <i>B. infantis</i> , <i>B. longum</i> (BB-46) and <i>B. animalis</i> subsp. <i>lactis</i> (BB-12) in skim milk	Reduced fermentation time for <i>B. breve</i> , <i>B. infantis</i> and BB-12, Promoted growth of bifidobacteria	[172]
150 mL of inoculated milk sonicated before fermentation at 20 kHz and 450 W, 225 W and 90 W for 1, 6 and 10 min, using a 13 mm diameter probe; energy density 36–1800 Jm L <sup>-1</sup>	<i>Bifidobacterium</i> and <i>Lb. acidophilus</i> in cow milk	Faster acid development Decreased fermentation time	[173]
Sonication of cultures before inoculation at 84 and 102 W for 75 and 150 s with a 12 mm diameter probe and frequency of 20 kHz. Sonication temperature: 37 °C.	<i>Lb. acidophilus</i> (La-5) in thermosonicated whey (480 W, 8 min, 55 °C)	Shorter time of fermentations, Increased viable cell count	[174]
28 kHz, pulsed US (100 s on and 10 s off), 100 WL <sup>-1</sup> for 1 h before fermentation (≈360 J mL <sup>-1</sup> ) and 30 min during fermentation (≈180 J mL <sup>-1</sup> ).	<i>L. paracasei</i> CICC 20241	Increase of 49.5% in the peptide content and 43.5% of viable cells in the fermented skim milk compared to untreated samples	[175]

Reducing the fermentation time (interval between the time of addition of cultures and the time at which the pH of the yoghurt reaches pH 4.7) [176] and increasing the viability of probiotic bacteria using high-intensity ultrasound is one of the most promising approaches. Reduction of fermentation time resulted in shorter production cycles and, thus, lower costs. It was reported that the sonication of reconstituted skimmed milk (15%, w/v) inoculated with *Bifidobacterium* sp. at 20 kHz and 100 W, for 15 min, followed by the fermentation at 37 °C, reduced the fermentation time by 11–26% while increasing the cell viability [172]. Similarly, the fermentation of reconstituted sweet whey (6% of the dry matter) by the ultrasound treated culture of *Lactobacillus acidophilus* with 84 W for 150 s was reported to reduce fermentation time by 30 min [174]. However, studies on employing ultrasound on probiotic buffalo milk product are scarce in the literature and warranted further investigations.

Moreover, it was reported that the application of high-intensity ultrasound on cow-milk set-yoghurts promoted the gel texture and viscosity of set-yoghurts, while reducing the syneresis and whey separation [120]. A more recent study reported similar observations for buffalo-milk set-yoghurts, as the gel hardness was increased by 98% and syneresis was reduced with the ultrasonic treatment (20 kHz and with 1188 J/mL energy density) [177]. Therefore, it could be assumed that ultrasound may be a potential tool to promote the viability of probiotics in buffalo milk yoghurt, while improving the gel properties.

### 5.5. Use of Appropriate Packaging and Storage Conditions

The presence of viable probiotic cultures in dairy products at the time of consumption is an essential legal requirement. However, probiotic bacteria often have insufficient viability in marketable products including fermented buffalo milk and thereafter the harsh gastrointestinal conditions [168]. Therefore, the selection of proper packaging conditions and storage conditions is vital. Viability and survival of probiotic bacteria are significantly influenced by several factors such as pH, titratable acidity, oxygen level, processing conditions and starter production conditions [139,144]. The ideal pH ranges for the growth of *bifidobacteria* and *lactobacillus* species are recognized as 6.00–7.00 and 5.50–6.09, respectively [145]. Therefore, low pH conditions associated with fermented buffalo milk is the major reason for the decline of the viability of probiotic bacteria during the storage. Similarly, the presence of oxygen can cause the generation of toxic metabolites, such as hydrogen peroxide, in probiotic bacterial cells, leading to cell death. It is reported that the presence of oxygen can induce the production of toxic peroxides and free radicals, which have the potential to damage DNA in probiotic bacteria [178]. Therefore, it is important to minimize the processing steps such as agitation and mixing, which facilitate air incorporation into the product. Moreover, appropriate packaging materials containing oxygen barriers and oxygen scavengers (active and intelligent packaging), which prevent the introduction of high levels of oxygen into the final product, should be used during storage and product distribution.

Storage temperature is another important factor, which affects the probiotic survival in yoghurts [145]. It is reported that the viable counts of probiotics reduced during refrigerated storage (4 °C) [89]. Mortazavian, Ehsani, Mousavi, Sohrabvandi and Reinheimer [179] investigated the effect of cold storage temperatures (2, 5 and 8 °C) on the survival of probiotics in yoghurts. They found that the highest survival rate of *L. acidophilus* and *Bifidobacteria* was 8 and 2 °C for a 20-day period, respectively. Moreover, low storage temperature causes reduced rates of metabolic activities and less generation of harmful metabolites, which enhances the survival of probiotics. Similar results were obtained by Celik and O'Sullivan [180] during storage of freeze-dried *B. animalis* subsp. *lactis* Bb-12 up to 21 °C, which showed the optimum survival rate. However, stress-sensitive probiotic strains such as *B. longum* DJO10A showed the highest probiotic survival under frozen storage (–20 to 80 °C). Survival of probiotics were further promoted by immobilization techniques during refrigerated storage [165]. Loss of viability of microencapsulated *L. plantarum* in 3% alginate coated with chitosan in cow milk yoghurt was approximately four times higher as compared to cells kept in saline suspension during 38 days of storage (4 °C). However, it is a common practice of cottage and medium scale

industries to store and distribute buffalo set-yoghurts under ambient temperature (30–35 °C). This may be another challenge to the inclusion of probiotics into fermented buffalo milk.

## 6. Conclusions

Research findings of buffalo-milk-based dairy products (e.g., “Meekiri”, “Dahi” or “Dadih”), which resulted in artisanal fermentation, revealed various probiotic potentials through its microbial consortium. Buffalo milk has the potential of being a probiotics carrier food, to confer health benefits, since it is the second most-consumed type of milk after cow milk, is a potential carrier food for probiotics and confers health benefits to the consumers. A comprehensive review of such products and their challenges and strategies to overcome the limitations of incorporating probiotics into buffalo milk products was presented in this paper. It was found that the proper selection of probiotic strains that can withstand the typical high acidic conditions associated with buffalo milk helps to overcome a major challenge of manufacturing probiotic-enriched fermented buffalo milk products. However, the addition of probiotic strains with specific health benefits into buffalo milk products can be achieved through several interventions, such as applying specific stresses to the cultures (i.e., ultrasound), the addition of growth-promoting factors (i.e., prebiotics) or protecting the cells through microencapsulation and/or coating techniques. Overall, probiotic-enriched buffalo milk products play a significant role in delivering probiotics with potential health benefits to humans. Further advances in processing, packaging and storage of probiotic-enriched fermented buffalo milk-based products may provide a competitive advantage of harnessing buffalo milk’s full probiotic potentials.

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