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Review

Compositional Characteristics of Mediterranean Buffalo Milk and Whey

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Abstract: The main objective of this review is to summarize the compositional characteristics and the health and functional properties of Mediterranean buffalo milk and whey derived from mozzarella cheese production. Several studies have investigated the composition of buffalo milk and in particular its fat, protein, and carbohydrates contents. These characteristics may change depending on the breed, feeding regime, and rearing system of the animals involved in the study, and also with the seasons. In particular, buffalo milk showed a higher nutritional value and higher levels of proteins, vitamins, and minerals when compared to milks produced by other animal species. Additionally, buffalo milk contains beneficial compounds such as gangliosides that can provide antioxidant protection and neuronal protection, and can improve bone, heart, and gastrointestinal health in humans.

Keywords: nutrition; prebiotics; probiotics; nutraceuticals; ruminant milk

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1. Introduction

The history of milk and dairy products is linked to the history of man since we began to domesticate animals and later to breed them. As early as 8000 years ago, the populations of Mesopotamia tried to domesticate dairy animals and it is likely that even they tried to use and transform milk as a food source.

The first evidence of milk use dates back to the Neolithic period in which humans shifted from nomadic hunting societies to stantial farming ones. With the creation of a permanent dwelling place, humans started to domesticate animals useful as food sources or work the land [1]. This led to the development of the first agricultural practices. Traces of such ancient practices can be found in the Middle East in which goats and sheep began to be domesticated. The choice to domesticate goats and sheep is related to their size, robustness, and adaptability, which are very useful characteristics, as well as their behavior and social nature [2]. For a long time, goats and sheep have been used as sources of food (as providers of both meat and milk) and clothing (wool) [3]. When nomadic migration began, cows, sheep, goats, donkeys, and buffaloes provided milk and meat during the journeys.

The domestic water buffalo, *Bubalus bubalis,* is native to the Asian continent but currently is spread all over the world. This worldwide distribution appears to be both the consequence of historical migrations and recent importations [4]. The two types of water buffalo, namely the river buffalo (*Bubalus bubalis*) with 50 chromosomes and swamp buffalo (*Bubalus carabanesis*) with 48 chromosomes, display not only different karyotypes, morphological, and behavioral traits but also distinct uses and geographical distributions [5]. After the selection of various breeds, the river buffalo was spread from the Indian subcontinent to countries in the eastern Mediterranean (Italy and the Balkans) and in North Africa (Egypt).

The history of buffalo farming in Italy is still not completely understood. In his *Historia Langobardorum* (VIII century), Paolo Diacono reported that buffalo species were introduced

in Italy immediately after its invasion by the Longobardi people. Other historians believe that the animals of Indian origin were first introduced in Sicily by the Arabs and later brought to the rest of the continent by the Normans. Finally, another hypothesis places buffaloes in Italy already in pre-Roman times. In any case, the first documented reports of the presence of buffaloes in Italy dates to the period between the 12th and 13th centuries [6]. In the marshy lands of southern Italy, the presence of buffaloes was not only precious for their milk but also because they were used to work the land. The presence of the buffalo in some areas of southern Italy was linked to the particular adaptability of this species to the local hot-humid climate and swampy soils, and to their ability to feed on the coarse vegetation growing in that region.

Nowadays, there is an ever-increasing interest in foods associated with health benefits and in reducing the risk of disease. A functional food is considered "A food, which beneficially affects one or more target functions in the body, beyond adequate nutritional effects, in a way that is relevant to either an improved state of health and well-being and/or reduction of risk of disease. A functional food can be a natural food or a food to which a component has been added or removed by technological or biotechnological means, and it must demonstrate their effects in amounts that can normally be expected to be consumed in the diet". In Europe, functional foods are regulated by the EFSA. The EFSA also set the criteria for measuring scientific evidence on functional foods and is responsible for verifying the scientific substantiation of the submitted claims. This information serves as a basis for the European Commission and Member States, which then decide whether to authorize the claims [7]. Thus, functional foods constitute a new market segment whose goal is consumer appreciation and satisfaction.

The demand for healthy foods is growing [8,9] and with more and more interest in the quality of the food, consumers are now increasingly more aware of the nutritional characteristics of dairy products.

The aim of this study is to review the evidence reported in literature on the characteristics of buffalo milk from a nutritional point of view, focusing on dairy products and highlighting their characteristics as a functional food.

2. Buffalo Distribution in Italy

The domestic buffalo is also known as the 'water buffalo' because of its keenness to cool itself in waters. In 2000, to protect the characteristics defined through the process of breed isolation that took place during the centuries, the Italian Agricultural Ministry officially recognized the 'Italian Mediterranean Breed'. The distinctiveness of the Mediterranean gene pool is also evident in the blend analysis, as the first split occurring among river buffalo breeds is in the Mediterranean group. With the use of mechanical tools in agriculture, the breed, in the past defined as a triple attitude (milk production, meat production, and working animal), was gradually improved for the production of milk.

As reported in 2019 by the Italian National Association of Breeders of Buffalo Species, buffalo dairy farms in Italy are traditionally relevant to the Campania region in which the Protected Designation of Origin cheese "Mozzarella di Bufala Campana" is produced.

Recently, the demand for buffalo mozzarella has greatly increased, resulting in an increase in the number of animals [10] and a considerable expansion of buffalo farming in the northern regions of Italy [8]. In 2019, the number of Italian buffalo farms was 2711 with a total of 402,796 buffaloes.

In Italy, as emerges from the study of production parameters carried out by Martini et al., (2016), buffaloes are raised almost exclusively to produce milk and 82 billion liters are produced each year, corresponding to 12.5% of the world production [11]. Buffalo milk is the second most produced milk in the world, preceded by cow milk. The amount of milk produced on average by an Italian Mediterranean buffalo is significantly lower than the average amount produced by a cow.

3. Basic Composition of Buffalo Milk

The comparison of different milk compositions from various species has been widely reported in previous studies [11–14]. Milks from different species share common characteristics. Total dry matter, protein, fat, lactose, and minerals are characteristic components in all milks irrespective of the species (Table 1) [13].

Table 1. Milk composition for different species (adapted from [13]).

	Human	Horse	Donkey	Cow	Sheep	Goat	Buffalo
Total dry matter (g/L)	107–129	93–116	88–117	118–130	181–200	119–163	157–172
Proteins (g/L)	9–19	14–32	14–20	30–39	45–70	30–52	22–47
Casein/whey ratio	0.4–0.5	1.1	1.28	4.7	3.1	3.5	4.6
Fat (g/L)	21–40	3–42	3–18	33–54	50–90	30–72	53–90
Lactose (g/L)	63–70	56–72	58–74	44–56	41–59	32–50	32–49
Ash (g/L)	2–3	3–5	3–5	7–8	8–10	7–9	8–9
Energy (kJ/L)	2843	1936–2050	1607–1803	2709–2843	4038–4439	2802–2894	4244–4479

Lower levels of total dry matter, fat, protein, energy values, and ash are found in the milk of non-ruminant species (horse and donkey) when compared to ruminants. In contrast, higher levels of lactose are reported in the milk of non-ruminants. However, the mean values of TDM, protein, lactose, and ash are similar between non-ruminant and human milk. It should be noted, however, that even within the same species, the composition of the milk can vary. This is closely related to genetic factors (not only at the species level but also at the breed and individual level), physiological factors (parity, lactation phase, and milking interval), factors relating to nutrition (energy value and composition of the feed), and environmental factors (location and season) [15].

The transcriptome description of the somatic cells of buffalo milk through three stages of lactation was first reported by Arora et al. [16]. Throughout lactation, most of the gene changes were involved in biological functions such as protein metabolism, transport, and immune response. The results obtained by Arora et al. showed a significant alteration of the metabolism and immune responses in the first phase of lactation and an increase in the immune response in late lactation. This data reflects what is expected; in fact, the first stage of lactation is characterized by a high metabolic activity functional to the secretion of milk, which stabilizes in the intermediate phase and decreases towards the advanced phase. This is therefore reflected in the transcriptome. During the initial lactation phase, the genes (CSN2, CSN1S1, CSN3, LALBA, SPP1, and TPT1) associated with the synthesis and secretion of milk are activated and their expression is progressively reduced to a minimal in the advanced phase.

Milk fat represents the main fraction of the nutrients and the average fat content in the milk of the Italian Mediterranean buffalo is approximately 8.5%, which in particular favorable conditions can reach 15%. Buffalo and sheep milk showed similar energy values (420–480 kJ/100 g and 410–440 kJ/100 g). In contrast, horse and donkey milk showed the lowest energy value (less than 210 kJ/100 g and 180 kJ/100 g). Milk from goats, cows, and humans showed comparable energy values [10].

3.1. Carbohydrates

From a quantitative point of view, lactose is the main sugar in milk and is present in comparable amounts in horse, donkey, and human milk. Lactose is synthesized from glucose in the mammary gland with the milk protein α-lactalbumin catalyzing the transfer of galactose to glucose. Lactose is a nutrient that plays a fundamental role in promoting the intestinal absorption of calcium, magnesium, and phosphorus, and in the use of vitamin D [17]. Another fundamental function performed by lactose is to maintain the osmotic balance between the blood flow and the alveolar cells in the mammary gland during the synthesis and secretion of milk in both the alveolar lumen and in the duct system of the breast [18].

A minor fraction of oligosaccharides includes galactose, fucose, *N*-actylglucosamine, and *N*-acetyl neuramic acid (sialic acid) found in their free form or bonded to phosphates, lipids, or proteins. In human milk, oligosaccharides characterized by lactose at their reducing end may actively modify the gut microbiota, positively influencing inflammatory processes and providing protection against bacteria and viruses [18,19], although the chemical and structural complexity of these compounds may complicate the study of their functional characteristics [20,21].

When compared to human milk (5–25 g/L), the levels of oligosaccharides in other species' milk are much lower: 0.25–0.30 g/L for goat, 0.02–0.04 g/L for sheep, >0.1 g/L for buffalo, and 0.03–0.09 g/L for cow milk [22].

Oligosaccharides, both free and linked to other macromolecules, perform fundamental functions in living organisms. It has been reported that oligosaccharides bound to sialic acid (but also free sialic acid bound to glycoprotein) are involved in the development of the intestinal flora [23,24]. Furthermore, in the central nervous system, oligosaccharides appear to be involved in the processes determining the level of glycosylation of brain gangliosides [20,21,25]. The comparison of sialic acid levels in human, cow, and horse milk showed 100, 20, and 5 mg/100 mL, respectively [20,25]. However, oligosaccharides are also able to mediate other important physiological functions. For example, in a murine experimental model, a mixture of processed buffalo milk oligosaccharides appears to be able to induce significant antibody stimulation, delay the hypersensitivity response, and stimulate a non-specific immune response [26,27].

3.2. Lipids

Milk is an emulsion in which the lipids are organized into globular milk fat (MFG). Non-polar lipids, mainly triacylglycerols (TAGs) but also cholesterol esters and other minor lipids, are found inside the MFG to form their "core", covered by a membrane made up of amphipathic lipids and proteins [28]. In Table 2, the reported the fat content of milk from different species adapted from Amores et al. and Rodriguez-Alcalà et al. [28,29] is presented.

Table 2. Milk fat content for different species.

Lipid Class	Cow	Sheep	Goat	Buffalo
Triacylglycerol	97.5	98.1	97.3	98.6
Diacylglycerol	0.36	N.M.	N.M.	0.7
Monoacylglycerol	0.027	0.03	0.10	Trace
Cholesteryl esters	Trace	0.02	0.04	0.1
Cholesterol	0.31	N.M.	N.M.	0.3
Free fatty acids	0.027	N.M.	N.M.	0.5
Phospholipids	0.6	0.38	0.65	0.5

Abbreviation: N.M. = not measured.

The milk's fatty acid composition (FA) determines the physicochemical properties and both the sensory and nutritional quality of milk and milk products. For this reason and for the roles of complex lipids in the technological transformations of milk, the determination of the levels of triacylglycerols, free fatty acids, and phospholipids in the milk of various species have been the objective of several studies over time [14,25,30–32].

3.2.1. Triacylglycerols

In the milk of non-ruminant species such as horses and donkeys, the component of triacylglycerols represents 80–85% of the fat, while in the milk fat produced by ruminant species (cow, buffalo, sheep, and goat) and humans, it is much higher (97–98%) [14].

There is also a higher content in terms of free fatty acids (9.5%) and phospholipids (5–10%) in the milk of horses and donkeys compared with the milk of ruminants and female humans (0.5–1.5% of phospholipids and 0.7–1.5% of fatty acids). Based on the scientific data obtained in the last decade, the milk produced by these non-ruminant

species could be classified in the future as a functional food, as phospholipids seems to have anti-carcinogenic properties. In particular, sphingomyelins appears to play a fundamental role in the protective processes against cancer of the colon [33].

Generally, the number of carbon atoms characterizing the lipid milk compounds (i.e., diglycerides and triglycerides) appears to be species-specific. Indeed, in human and non-ruminants milk, the distribution of complex lipid molecules typically follows a unimodal model with a maximum of 50–52 carbon atoms, while a bimodal distribution is observed in the milk of ruminants with two maximum values (the first from 34 to 40 carbon atoms and the second from 42 to 54) [34]. The number of carbon atoms making up the triacylglycerols is of interest but the distribution of fatty acids is also of fundamental importance.

In fact, from a nutritional point of view, the structure of the triacylglycerol is one of the main factors, together with the action of the lipolytic enzymes, which affects the absorption of fats. The exposure on the external side of the triacylglycerol skeleton of one fatty acid rather than another positively or negatively influences the kinetics of the enzymatic reaction of lipolysis and consequently the bioavailability of that particular fatty acid [35]. Palmitic acid (C16:0) in human milk is preferentially esterified at the sn-2 position, which appears to be a position that facilitates the digestion and absorption of this fatty acid in children [36]. In this regard, it is important to highlight that in the milk of horses and donkeys, the (C16:0) fatty acid is also predominantly esterified at the sn2 position. It has been observed that in cow milk, palmitic acid (C16:0) is able to provide beneficial effects both when it is esterified at the sn1 and sn2 positions [35,37]. In cow and other small ruminants' milk, fatty acids with lengths from C4:0 to C10:0 are mainly esterified in the sn-3 position [30,38].

3.2.2. Fatty Acids

Pegolo et al. reported a characterization of the fatty acid composition of Mediterranean buffalo milk [39]. The study analyzed 272 samples of Mediterranean buffalo milk using gas chromatography coupled with a flame ionization detector and determined the profile of 69 fatty acid traits. Table 3 reports the fatty acid profile of buffalo milk compared with cow milk.

Table 3. Fatty acids' profile of buffalo milk compared with cow milk.

Trait	Buffalo (Mean ± SD)	Cow (Mean ± SD)
SFA	70.49 ± 5.14	69.61 ± 4.10
MUFA	25.95 ± 4.76	24.28 ± 3.45
PUFA	3.54 ± 0.65	3.79 ± 0.79
SCFA	9.72 ± 1.82	10.53 ± 1.71
MCFA	53.70 ± 5.44	52.78 ± 5.26
LCFA	32.73 ± 6.59	34.40 ± 5.14
n-6 PUFA	1.77 ± 0.54	2.31 ± 0.65
n-3 PUFA	0.46 ± 0.09	0.69 ± 0.20
Trans fatty acids	2.66 ± 0.72	2.22 ± 0.53

The analysis of the buffalo milk samples revealed the presence of 51 individual fatty acids, of which 24 were saturated fatty acids (SFA) representing about 70.49%, 13 were monounsaturated fatty acids (MUFA) representing 25.95%, and 14 were polyunsaturated fatty acids (PUFA) representing 3.54%. In particular, from a quantitative point of view, the main free fatty acids found in buffalo milk were palmitic acid (C16:0), oleic acid (C18:1 cis-9), myristic acid (C14:0), and stearic acid (C18:0).

Buffaloes and cows seemed to have comparable average milk fatty acid profiles, both characterized by a considerable variability. Conversely, the available comparative studies sometimes reported more pronounced variations or different results [40–42]. Not significant differences seemed to be detected in total SFA and UFA (MUFA + PUFA) proportions in the milk of buffaloes and cows reared in north-east Italy. In contrast, previous data reported significantly higher SFA contents and lower UFA contents in buffalo milk when compared

with the milk of cows reared in the same herd. Buffalo milk also showed lower levels of linoleic acid (18:2 cis-9 and cis-12) and linolenic acid (18:3 cis-9, cis-12, and cis-15) than bovine milk, unlike previous findings [41]. The findings by Pegolo et al. [39] partially agreed with those by Menard et al. [41] who also reported a lower percentage of linoleic acid in buffalo milk when compared with cow milk, but they found a higher percentage of linolenic acid.

Differences in milk fatty acid composition could be due to the size of the MFG [41]. Buffalo milk has larger fat globules than cow milk and because of this, the relative proportion of polar lipids in buffalo MFG is lower [42]. As linoleic acid and linolenic acid are preferentially esterified in polar lipids [43], the differences in the amounts of these acids present in buffalo and cow milk could be linked to the different MFG diameters. Higher amounts of n-6 fatty acids were detected particularly in Holstein cow milk [42,44] compared with buffalo milk (mainly due to the higher percentage of linoleic acid), which consequently affected the n-6/n-3 ratio. Different values of the n-6/n-3 ratio were also reported in other studies, which, however, considered only linoleic acid and linolenic acid as representative for the n-6 and n-3 categories, respectively [40,41]. Pegolo et al. found lower average contents of rumenic acid (RA) proportions in buffalo milk [39], in agreement with Talpur et al. [45], but this was in contrast with the results reported by Menard et al. [41]. Conjugated linoleic acid (CLA) in milk may originate from either endogenous (Δ9-desaturase) or ruminal sources [46]. There appeared to be no great differences between the percentage content of total trans and trans 18:1 fatty acid in buffalo and cow milk, in disagreement with previous studies that found higher percentage contents in buffalo milk [41,42].

According to the US Food and Drug Administration [47] recommendations, our consumption of trans fatty acids must be kept as low as possible by limiting the intake of foods containing trans fatty acids formed during food processing. Indeed, trans fatty acids can be divided into two groups: those that are industrially produced and may increase the risk of coronary heart disease [48], and those that are ruminant-derived and which may actually help in reducing the risk of cardiovascular diseases [49]. The major industrially produced trans fatty acid in the food supply is elaidic acid (18:1 trans-9), whereas the major ruminant-derived trans fatty acid is vaccenic acid (18:1 trans-11), which has been shown to have anti-carcinogenic properties [49].

During early lactation in buffaloes, the effects of the inhibition of de novo fatty acid synthesis (6:0 to 16:0) brought by the uptake of blood long chain fatty acid (LCFA) from the mammary gland are less evident. The only exception is butyric acid (C4:0), which is present at higher levels at parturition and then decreases with the advancing of lactation. This is likely due to a different synthetic pathway that is independent from malonylCoA [50]. The main LCFA in milk (i.e., oleic acid (18:1 cis-9)) may originate not only from the mobilization of the adipose tissue but also from the mammary desaturation of stearic acid and through the diet. At the beginning of lactation, the oleic acid content is higher due to lipid mobilization and then decreases reflecting the pattern of desaturation activity (18:1 index), alongside the decrease in lipid mobilization as well.

In late lactation, oleic acid content increases again as a result of the increase in desaturation activity. This is in line with data reported for buffaloes [51] and cows [52]. Pegolo et al. [40] observed an increase RA as lactation progressed, whereas Tudisco et al. [51] found a different pattern with the highest values in the third month and lowest in the first and fourth months.

All these findings may reflect a shorter period of negative energy balance in buffaloes than in cows due to their lower productivity, resulting in a reduced or null weight losses. Consequently, the fatty acid profile in buffalo milk is probably less influenced by the mobilization of body reserves when compared to that of cow milk. It is also worth mentioning that the lactation period of the Mediterranean buffalo is 270 days [53], compared with a cow's standard period of 305 days. Camargo et al. found no marked variation in the profiles of SCFA and found both 16 carbon chain and all 18-carbon chain UFA in the first 8 weeks of lactation in buffaloes [54].

Role of Pastures on the Fatty Acid Profile of Buffalo Milk

As stated before, the type of diet showed important effects on the general composition of milk. Varricchio et al. reported changes in the percentage of fats in buffalo milk depending on the different management style [55]. Four commercial dairy buffalo farms in southern Italy were used in this study. The herds adopted the total mixed ratio (TMR) system with four different diets. Quantitatively, the five most important fatty acids were C16:0 (mean percentage, 30.6 ± 3.0), C18:1c (21.4 ± 2.0), C18:0 (12.2 ± 2.4), C14:0 (10.6 ± 1.1), and C4 (3.4 ± 0.5), which constituted more than 78% of the total fatty acids. These results were observed in all the herds examined in this study.

Another study by Bergamo et al. investigated the difference between organic and conventional Italian dairy products (from both buffalos and cows) [56]. In terms of the milk fat composition, the amounts of fatty acids were measured to compare the certified organic buffalo milk with that produced in the conventional system (Table 4).

Table 4. Fat percentage and fatty acid composition of buffalo milk relative to organic and conventional management.

Trait	Organic	Conventional
Fat (%)	8.0 ± 0.5	7.9 ± 0.7
Fatty acids (mg/g of fat)		
C14:0	117 ± 3.5	120 ± 6.5
C16:0	344 ± 7.8	357 ± 8.9
C16:1	21.6 ± 1.0	19.5 ± 0.8
C18:0	126 ± 1.5	130 ± 2.6
C18:1	217 ± 2.2	233 ± 3.5
TVA	26.2 ± 1.8	13.3 ± 0.9
LA	18.0 ± 0.9	24.2 ± 1.4
CLA	7.3 ± 0.8	5.5 ± 0.5
LNA	4.6 ± 0.1	3.5 ± 0.2

The fat content was found to be not statistically different for the two types of milk analyzed but the organic milk showed a significantly higher concentration of CLA (conjugated linoleic acid), TVA (trans vaccenic acid), and LNA (linolenic acid). Furthermore, in the organic buffalo milk samples, the concentration of LA (linoleic acid) was found to be significantly lower ($p < 0.05$) and the value of the CLA/LA ratio was almost twice as high in the organic samples compared to the conventional samples. The confirmation analysis of these results was carried out by analyzing organic and conventional dairy products based on buffalo milk. Again, no significant differences in the total fat content were found between the two sample groups.

3.3. Gangliosides

Buffalo milk and whey are known to contain gangliosides of the GM1 class. These compounds were not reported for bovine milk. Ganglioside GM1 (18:1/18:0) is a glycosphingolipid with a ceramide backbone bound to an oligosaccharide with one or more sialic acids (i.e., n-acetyl-neuraminic acid) linked on the sugar chain.

As a functionally qualified constituent of cell plasma membrane receptor sites, GM1 is also involved in the modulation of cellular signal transduction events and shows fundamental properties in immunology.

GM1 levels are about ten times higher in buffalo milk than in cow milk. Significant quantities (45%) of dairy gangliosides were found in the organic phase after the Folch fraction. Specific gangliosides are contained in buffalo milk and whey in quantities comparable to human milk and thus they can be used in the production of infant formula. It is important to highlight that elevated levels of gangliosides have been measured in buffalo whey, a by-product left from the production of the PDO buffalo mozzarella cheese.

In particular, the GM1 species (gangliosides) derived from Italian buffalo milk were found to be able to bind to the Cholera Toxin. Furthermore, the GM3 species derived from buffalo milk can bind rotavirus particles. Gangliosides (GS) from Italian buffalo milk showed anti-inflammatory effects. In addition, clinical studies performed on patients with Alzheimer's disease have provided positive results at high doses of GS (200 mg/day) [57,58]. Further positive effects on human health have been reported by Kidd [59], Blusztajn [60], and Spitsberg [61].

From a health point of view, it should be noted that mature milk from buffaloes from Pakistan and Italy contained 40–100% lipophilic GS species, making it more bioactive than cow milk. All the beneficial effects that characterize buffalo milk make it a precious source of bioactive lipids suitable for the preparation of innovative food products.

By exploiting the beneficial effects associated with the specific gangliosides contained in buffalo milk, skimmed buffalo milk, and buffalo milk whey, we could prepare various functional foods that can be used to reduce symptoms related to cholera and rotavirus infections. They can also enhance the activity of choline acetyltransferase and tyrosine hydroxylase, as well as improve the synthesis of acetylcholine and/or dopamine.

4. Milk Protein: Comparison between Different Breeds

Milk produced by ruminant species is relatively rich in casein, while milk produced by non-ruminant species has a proportionally higher whey protein content as indicated by the lowest casein/whey ratio. Generally speaking, the casein fraction present represents about 80% of the proteins in bovine and ovine milk, about 50% of the proteins in horse milk, and less than 50% of the proteins in human milk [14,20,25,31]. In Table 5, the percentage contents for individual caseins in various milk samples are reported.

Table 5. Casein fraction in cow, buffalo, goat, and sheep milk. Abbreviation: TPC = total protein content [14].

Parameter (g/L)	Cow	Buffalo	Sheep	Goat
TPC	25–28	32–40	42–46	23–47
αS1-casei	8–11	9	15–22	0–13
αS2-casein	3–4	5	0	2–12
β-casein	8–9	12–21	15–18	0–30
κ-casein	2–3	4–6	3–5	2–13

As for other mammalian species, buffalo milk contains casein and whey proteins. A different distribution of αs1, αs2, β, and κ-casein in buffalo milk proteins compared to milk from Holstein cows has already been reported in the literature [62]. The primary structure of αs1-casein appears to be homologous when comparing buffalo and cow milk, consisting of 199 amino acids with a theoretical MW of 22.80 kDa (dephosphorylated form). The same was observed for β-casein, which is made up of 209 amino acids with a theoretical MW of 24.04 kDa [63]. However, there are some protein variants in buffalo milk such as κ casein (X1: Ile135 and X2: Thr135) and αs1 casein (A: Leu178 and B: Ser178), while more than ten variants have been found in cow milk proteins [62,64]. Breed and milk protein polymorphism appear to be connected. In fact, two variants of β-lactoglobulin (A and B) have been found in the milk produced by the Indian and Egyptian buffaloes; however, only one variant (B) has been detected in the Mediterranean water buffalo [65,66].

An HPLC method for the separation and quantification of the most common genetic variants of milk proteins in cow and buffalo milk was developed by Bonfatti et al. [67]. This method was carried out using purified buffalo proteins as calibration standards. The method allowed for the simultaneous separation of all the most common genetic variants of whey proteins. From this study, it emerged that αS1, αS2, βγ, and κ-casein amounted for respectively 32.2%, 15.8%, 36.5%, and 15.5% of the total casein content, while the content of β-lactoglobulin was approximately 1.3 times higher than α-lactalbumin.

In addition, the existence of a κ casein polymorphism in the Mediterranean water buffalo has been demonstrated and genetic variants of αS1 and κ casein have been successfully detected.

In their study, Rafiq et al. 2016 found significant differences ($p < 0.05$) in the protein fractions of milk from different species with variations in crude protein (CP), true protein (TP), caseins and whey proteins, NCN (non-casein nitrogen), and NPN (non-protein nitrogen) contents [68]. Table 6 shows the milk protein fractions in milk from different species. The CP (5.15% ± 0.06%), TP (4.53% ± 0.03%), caseins (3.87% ± 0.04%), and NPN (0.62% ± 0.02%) levels were more abundant in sheep milk followed by buffalo and cow milk. The largest amount of whey proteins was found in camel milk (0.80% ± 0.03%), whereas cow milk contained the least (0.47% ± 0.01%).

Table 6. Milk protein fractions in milk from different species.

Parameter (%)	Cow	Buffalo	Sheep	Goat
Crude protein	3.57 ± 0.03	4.25 ± 0.07	5.15 ± 0.06	3.35 ± 0.02
True protein	3.25 ± 0.03	3.87 ± 0.02	4.53 ± 0.03	2.95 ± 0.02
Casein	2.79 ± 0.02	3.20 ± 0.03	3.87 ± 0.04	2.44 ± 0.03
Whey protein	0.47 ± 0.01	0.68 ± 0.02	0.66 ± 0.02	0.53 ± 0.02
Non-casein nitrogen	0.77 ± 0.02	1.05 ± 0.02	1.28 ± 0.03	0.94 ± 0.01
Non-protein nitrogen	0.33 ± 0.03	0.38 ± 0.02	0.62 ± 0.02	0.39 ± 0.01

Infant formulas enriched with 1 mg/mL of lactoferrin (LF) promoted beneficial microflora such as Bifidobacteria while reducing levels of clostridium in the gut of low weight as well as healthy newborns [69]. Due to its antimicrobial activity, lactoferrin has been studied for its use against the Cronobacter bacteria infections. The Cronobacter (*Enterobacter sakazakii*) is a significant foodborne pathogen that has been associated with meningitis [70], sepsis [71], bacteremia [72], and necrotizing enterocolitis [73] in preterm neonates and immunocompromised adults [74].

Recently, a reclassification of Cronobacter bacteria has been proposed, resulting in the definition of five species: *C. sakazakii*, *C. malonaticus*, *C. turicensis*, *C. muytjensii*, and *C. dublinensis*. Although infections from these bacteria are rare, the mortality rate can reach 80% [75,76]. Most outbreaks caused by *Cronobacter* spp. Have been found to be associated with the use of powdered infant milk formula (PIMF). These bacteria are usually inactivated by the process of pasteurization [77] and therefore its presence in PIMF is mainly due to post-processing environmental contamination, the use of contaminated ingredients when manufacturing the product [78], or the colonization by *C. sakazakii* of tools such as bottles, brushes, and spoons used in the preparation of the final product. *C. sakazakii* can survive in dry environments for periods of ≤2 years [79] and could clearly survive for long periods in containers of PIFM [80].

5. Bioactive Milk Components

Peptides, originating from the enzymatic hydrolysis of milk proteins, are able to perform specific biological activities (i.e., antihypertensive, antimicrobial, antioxidant, and immunomodulatory). Such protein fragments, called bioactive peptides, are formed from the inactive precursor protein during gastrointestinal digestion or can be produced following specific events during food processing [81]. Bioactive peptides with ACE inhibitory properties are of great interest. ACE is a multifunctional enzyme located in several tissues and, as it is responsible for the production of the vasopressor angiotensin II and for the inactivation of the vasodepressor bradykinin, can regulate various systems affecting blood pressure [82].

The isoelectric casein, isolated from buffalo milk, was fractionated to α, β, and K-casein, and each of these fractions were hydrolyzed with trypsin (EC 3.4.21.4). The angiotensin converting enzyme inhibitory activity was measured and the β-casein hydrolysate showed the strongest ACE inhibitory activity. Several multifunctional activities are as-

sociated with cow β-CN (f 193–209): it shows immunomodulatory activity and contains the bioactive sequence of the ACE inhibitor peptide and β-casokinin-10 [83]. Buffalo β-CN (f 193–209) has been isolated in raw buffalo milk [84] and is a potential precursor of bioactive peptides with different functions, including the following.

- Buffalo and cow αs1-CN (f 1–23) differs in two amino acid substitutions (E14 → G14 and H4 → P4). Cow αs1-CN (f 1–23) displayed antimicrobial activity [85]. Consequently, buffalo αs1-CN (f 1–23) may also show potential antibacterial activity, which should be confirmed experimentally.
- The αs2-CN C-terminal fragment (183–207) in cow milk showed antibacterial activity against *Escherichia coli*.
- The reaction between chymosin and both buffalo and cow κ-casein (κ-CN) produced the casein macropeptides (CMPs; f 106–169), which seem to enhance the growth and activity of Bifidobacteria. Further hydrolysis of cow CMP yields the antithrombotic peptide (f 116–127) [86].
- β-lactoglobulin (β-LG) from buffalo and cow milk share a high homology with differences in the N-terminal and C-terminal amino acids [87]. Cow β-LG is a source for bioactive peptides of different functionalities [88]; thus, buffalo β-LG can also be a potential source of bioactive peptides.
- Cow α-lactalbumin (α-LA) has been reported to be a source for several bioactive peptides [89]. The similarity in the amino acid sequences of buffalo and cow α-LA suggests that buffalo α-LA may be a potential source of bioactive peptides similar to cow α-LA.

Petrilli et al. showed that buffalo β-casein, digested with pancreatic juice, did not produce β-casomorphine or morphiceptin peptides [86]. However, to date, not many of the studies carried out on buffalo milk proteins describe the presence of bioactive peptides. From the data reported in a few of these studies, it emerged that an ACE inhibitor peptide is produced by the action of the proteinase *Lactobacillus helveticus* PR4 on the casein of buffalo milk [90].

This peptide was recognized as a buffalo fragment β-CN (f 58–66), which contains the sequence of a part of the peptide ACE (f 58–76) released by the β-CN cow under the same conditions [90]. The β-CN (f 58–76) of the cow presents a very low IC50 (16.2–57.2 μg/mL), while the IC50 of β-CN (f 58–66) produced by the buffalo was slightly higher.

Buffalo β-CN (f 58–66) also shares the sequence with cow β-casomorphine 7 (f 60–66), which exhibits ACE-inhibitory activity in addition to its opioid function. Cationic peptides were separated and partially purified from buffalo peptic digest αs1-CN and αs2-CN by ion exchange membranes. Tests performed using these peptides showed that they possess antimicrobial activity against *Micrococcus leutenus*, *Escherichia coli*, and *Bacillus cerus*.

6. Vitamins

Milk fat is a good source of retinol, tocopherol, and beta-carotene [91]; these lipids exert their antioxidant activity in biological tissues. Both singlet oxygen and lipoperoxides can be eliminated from β-carotene and retinol, thus preventing or limiting the oxidation of fatty acids [92]. B-carotene is one of the carotenoids found in nature and is abundantly available in plants (fruit and vegetables). It is also found in cow milk but not in buffalo, goat, and sheep milk [93,94]. These animals can metabolize carotenoids into vitamin A, which is found in their milk. B-carotene is also known as a pro vitamin A, as it is easily converted into retinol (vitamin A) in the human body. Liver, dairy, and foods of animal origin are rich sources of vitamin A, along with active carotenoids with vitamin A (β-carotenes in plants). Vitamin A belongs to a group of fat-soluble compounds (retinyl esters) [95]. By preventing unwanted oxidative processes in the body, vitamins play a key role in metabolism as part of enzymes/coenzymes and as antioxidants. Vitamin A is an essential nutrient that can be available as retinol, retinal, and retinoic acid, and is involved in critical biological processes such as cell growth and development, reproduction, vision (sight), and in the functioning of the immune system [96,97].

The activity of the proteolytic enzyme plasmin can be effectively inhibited by vitamin E [98]. A-tocopherol is considered the most powerful free radical scavenger among tocopherols and the antioxidant activity of β, γ, and δ-tocopherol is approximately 80–90% lower than α-tocopherol [99]. Γ-tocopherol can trap nitric oxide species and be very useful for the prevention of cardiovascular diseases and cancer. The concentration of vitamin E in cow milk was found to be at about 0.9 mg/mL, although a higher concentration was found in the summer milk compared to the winter milk. In human milk, the concentration of vitamin E (α-tocopherol) ranges from 3 to 13 mg/mL [100]. The addition of 100 mg of α-tocopherol/kg milk fat and 100 mg of ascorbyl palmitate/kg milk fat to UHT milk reduced the concentration of hexanal during a 4-week storage period [101]. The effect of vitamin E on the oxidative stability of sheep butter was studied by Abbas et al. after cream supplementation with 60 IU of vitamin E. The latter effectively inhibited lipid peroxidation and increased shelf stability of sour cream butter made from sheep milk [102]. The antioxidant activity of zinc and selenium in the inhibition of superoxide dismutase is scientifically proven [103]. The functional value and antioxidant capacity of milk are enhanced by glutathione and selenium [104]. The concentrations of vitamins in buffalo and cow milk are given in Table 7.

Table 7. Vitamins content in buffalo and cow milk.

Vitamin	Cow	Buffalo
Vitamin A (IU/mL)	230	340
Thiamine (μg/mL)	0.2	0.2–0.5
Riboflavin (μg/mL)	2.33	1.59
Pyridoxine (μg/mL)	2.6–3.0	3.25
Ascorbic acid (mg/100 g)	1.94	2.2
Tocopherol (μg/g)	312	334

The concentrations of vitamins in buffalo milk are similar to the ones in cow milk, except for vitamin A and tocopherol. However, the total potential level of vitamin A in buffalo milk is lower when compared with cow milk due to the lack of carotenoids and the high fat content in buffalo milk. Similarly, there is a higher concentration of tocopherol in buffalo milk but due to its higher fat content, buffalo milk fat is poorer in total tocopherol (about 26 μg/g) as compared to cow milk fat (35 μg/g) [105].

7. Minerals

The levels of minerals in milk are not constant but are influenced by various factors such as the stage of lactation, the nutritional and health status of the animal, and environmental and genetic factors. Values for the concentration of many minerals and trace elements are provided in literature and these show a wide variation due to these factors [106]. Cow milk was found to contain fewer minerals than buffalo milk. Cashman published the contents of selected macrominerals and trace elements in dairy products [107]. The degree of intestinal absorption and utilization, transport, cell assimilation and conversion into biologically active forms, and therefore the bioavailability of these substances are influenced by the chemical form in which macrominerals and trace elements are found in milk or other foods or supplements.

Many studies confirmed that calcium is certainly one of the most abundant minerals in milk.

Dietary calcium can facilitate the loss of body weight and body fat as it forms soaps in the intestine with fatty acids reducing the amount of fat absorbed, as seen in rats and humans [108–111]. A diet with a high Ca content caused a reduction in the Ca content of basal adipocytes in transgenic mice, regardless of the Ca source [111]. This was not the case with energy restriction alone. A diet rich in calcium reduces the activity of fatty acid synthase (FAS). The release of glycerol indicates that lipolysis was stimulated after a high intake of calcium in the diet, especially if the calcium derived from dairy products. There

was evidence of lower bone mineral density and more frequent bone fractures [112], as well as a higher prevalence of being overweight [113] in children who avoided milk consumption. Furthermore, a significant reduction in body fat, trunk fat, and waist circumference was achieved after 24 weeks with a low calorie diet containing 400–500 mg of calcium in a randomized and controlled study of 32 obese young people [114]. This effect was strongly evidenced when the diet was supplemented with 800 mg of calcium carbonate and even more evident when the calcium derived from dairy products.

The levels of milk minerals in cow and buffalo milk are shown in Table 8.

Table 8. Levels of minerals and trace elements in buffalo and cow milk.

Element (mg/100 mL)	Cow	Buffalo
Calcium	123	184
Magnesium	12	19
Sodium	58	45
Potassium	141	102
Phosphate	95	89
Citrate	160	178
Chloride	119	64
Boron	0.027	0.052–0.145
Cobalt	0.0006	0.00069–0.00161
Copper	0.013	0.007–0.021
Iron	0.045	0.042–0.152
Manganese	0.022	0.0382–0.0658
Sulphur	30	15.7–31.4
Zinc	0.390	0.147–0.728

8. Other Compounds of Interest

Biliverdin IX alpha is a latent blue–green pigment found mainly in fresh buffalo milk [115]. However, it is not found in cow milk and is therefore considered an important component of buffalo milk [116]. In skimmed milk from Murrah and Surati buffaloes, the mean concentration of biliverdin was 51.8 and 65 μg/100 mL. The levels of biliverdin greatly vary with the stage and number of lactations. Biliverdin is mainly associated with the α, β, and γ caseins as well as the proteose-peptone fraction.

Generally, during the storage and acidification of buffalo milk, biliverdin is converted into bilirubin. Another characteristic of this pigment is that it can bind itself to lipids giving the buffalo milk fat and butter, originating through the classic fermentation process, which has the characteristic greenish-yellow appearance.

The production of biliverdin and its conversion to bilirubin, as well as the subsequent glucuronidation, are energetically expensive mechanisms. Bilirubin has an antioxidant effect that is essential during the neonatal period, as at this stage in life, the concentrations of other natural antioxidants in the body are low. A weak but statistically significant inverse relationship between the level of serum bilirubin and the risk of coronary heart disease has been reported in adults [117].

Milk contains significant concentrations of equol, a polyphenolic metabolite of daidzein with antioxidant activity [97]. Such antioxidant systems are able to inhibit the activity of superoxide radicals, hydroxyl radicals, and peroxide radicals. Specifically, buffalo milk contains δ-Valerobetaine (δVB), a compound known for its antioxidant and anti-inflammatory properties [97]. Betaine is produced at the rumen level from Nε-trimethylysine, which is found ubiquitously in plants and is introduced through diet. The δVB added to buffalo milk extracts made these products more effective in reducing reactive oxygen species, lipid peroxidation, and cytokine release during in vitro testing for high glucose-induced endothelial damage compared to milk extracts alone [97].

9. Potential Use of Milk and Dairy Products as Functional Foods

Cow milk proteins were widely investigated to identify and quantify the bioactive peptides present. Numerous peptides with a range of physiological activities have been identified in hydrolysates of various milk proteins and in fermented milk products. For example, some of the bioactive peptides in milk are able to bind metals and opioids, and have immunomodulatory, antibacterial, and antioxidant activities. However, to date, much less attention has been paid to bioactive peptides derived from proteins found in buffalo, camel, yak, and horse milk.

From a practical point of view, new bioactive peptides isolated from different milks can be used as ingredients in the preparation of functional and nutraceutical foods with specific therapeutic properties. Furthermore, the use of specific microorganisms in the fermentation of milks other than cow milk can lead to the production of new functional dairy products.

Furthermore, when fermented cow milk products were compared to those made with buffalo milk, the latter showed better bacterial viability during in vitro simulated gastrointestinal digestion, which suggests a beneficial protective effect on the human microbiome [118].

10. Whey

Whey, the main dairy by-product derived from the production of cheese, is the liquid fraction left after the curdling and filtering of milk, and its use in the food industry worldwide is rapidly increasing. A small percentage of the whey is used to make ricotta cheese, which is obtained from protein precipitation after heating the whey to 85–90 °C for 20–30 min. After the production of ricotta cheese, the liquid fraction of the exhausted whey is called scotta and in southern Europe, its production represents the most abundant dairy by-product. Although whey cheeses are produced all over the world, ricotta cheese is a typical Italian dairy product. Around 30–35% of the whey derived from cheese production is still discarded or used as animal feed. Whey proteins are also used in the food industry as emulsifiers and as gelling and bulking agents. The antioxidant activity of whey proteins is scientifically recognized and, for example, lipid oxidation can be effectively inhibited by whey antioxidants [119]. The chelation of transition metals by lactoferrin and the elimination of free radicals by the sulfur-containing amino acids are responsible for the antioxidant activity of whey proteins [120]. The level of glutathione peroxidase, considered one of the most important water-soluble antioxidant systems, is increased by whey proteins. Oxidative stability is enhanced by the addition of whey proteins to the soybean oil emulsions [121]. The antioxidant properties of salmon oil emulsions are increased as a function of the amount of whey proteins added [119]. Better antioxidant properties can be associated to foods containing whey proteins [122].

Lipid peroxidation, the production of peroxide radicals, and oxygen uptake and iron oxide free radicals can be inhibited by lactoferrin and casein [123]. Foods containing whey proteins have better antioxidant properties. As a result of the action of milk coagulating enzymes, milk endogenous proteases, added as a starter and natural microflora, have many peptides that were found in whey from buffalo mozzarella production. Buffalo mozzarella whey peptide extract inhibits cell proliferation by interfering with the cell cycle and exerts a possible proapoptotic activity in Caco-2 cancer cells treated with hydrogen peroxide [124].

Pre-Probiotics

Prebiotics and probiotics are among a number of functional foods that can have beneficial effects on our health and well-being [125–133].

Prebiotics are classified as non-digestible food components [3]; they provide health benefits by modulating the gut microbiota (FAO 2007). Specific groups of bacteria including bifidobacterial and lactobacilli, which live symbiotically within the human gastrointestinal tract, are able to metabolize probiotics. Some components of prebiotic fibers, such as fructo-oligosaccharides (FOS), inulin, and other oligosaccharides, play a significant role

in the large intestine but can also affect bacteria living in the small intestine. In fact, prebiotics can reduce the production of toxins by bacteria such as *Clostridium* spp. And *E. coli*, while showing a physiological effect on intestinal disorders, hyperglycemia, and hypercholesterolemia. Regular consumption of probiotics can bring numerous benefits as they can help in the treatment and prevention of diarrhea, give relief from constipation, contribute to the rapid recolonization of the digestive tract microbiota following antibiotic treatment, and can lead to a reduction in lactose intolerance. Probiotics have also been shown to have positive effects on the immune system and can play a role both in the treatment of inflammatory bowel disease and in the prevention of certain types of cancer; they can also help to increase our resistance to microbial infections and are effective in the treatment for *Helicobacter pylori* infections. Other benefits include the preservation of oral health, reduction in cholesterol levels, and increased mineral absorption. The lactic acid bacteria most used as probiotics in food are *L. acidophilus* and *Bifidobacterium lactis* [134].

The presence of *Lactobacillus acidophilus*, *Lactobacillus rhamnosus*, and *Bifidobacterium longum*, thought to be potential probiotic strains, was reported in buffalo milk by Shafakat-ullah et al. [134]. These species can survive in the stomach and proliferate in the intestine due to their biliary acid and alkaline stability. This will help them to reach the small intestine and colon in which they can contribute to the balance of the intestinal microflora In addition, in possessing significant antibacterial activity, these strains could help relieve diarrhea and intestinal infections. According to Kalhoro et al., buffalo milk is a potential source of probiotic bacteria [135]. *L. plantarum, L. pentosus, L. fermentum*, and *L. reuteri*, obtained from raw buffalo milk, showed promising probiotic characteristics as they possessed resistance to gastrointestinal fluids, particularly acid pH (pH = 2), bile (1%), and lysozyme (100 μg/mL). Probiotic strains showed the following characteristics: high self-aggregation; hydrophobicity; adhesion to the Caco-2 cell line (human colon carcinoma cell lines); heat resistance; inhibition of foodborne pathogens; able to ferment milk; and survive in cold storage conditions for up to 28 days. Therefore, due to their ability to survive in conditions of environmental stress and inhibit the activity of food-borne pathogens, these probiotics can be used as starter cultures for the development of functional foods. In fact, in fermented milks, the addition of prebiotic factors such as inulin has shown greater stability and improved sensory characteristics. In addition, probiotic viability and the physicochemical and sensory characteristics of probiotic dairy products can be enhanced using supplements. The term symbiotic refers to the synergistic effect between prebiotic foods and selective probiotic microorganisms [136]. Symbiotic products are able to improve the survival and deployment of living organisms in the gastrointestinal tract, as well as further promote both the selective growth and activation of the metabolism of bacteria.

Symbiotic products have been developed to overcome the potential difficulties in the survival of probiotics. Several factors, including pH levels, hydrogen peroxide, organic acids, and oxygen and moisture stress, can affect the activity of probiotics, especially in dairy products such as yogurt [137].

11. Infant Formula Milk Based on Buffalo Milk

The protein content in milk is one of the parameters used to evaluate infant formula milks; for this reason, these levels should be finely tailored in terms of the total protein content to take into account the differences in the compositions of the milk obtained from the various species [137].

Breast milk is the gold standard for infants as it contains all the nutrients an infant needs in the right amounts. Artificial or animal milk does not contain the correct amounts of nutrients and thus these foods must be modified and made suitable for infant nutrition. Muzaffar et al. examined the efficacy of buffalo milk in a case study in which an infant was fed with boiled fresh buffalo milk [137]. The protein requirements were adjusted according to the recommended level of 2.46 g/kg B.W. by adding water. The total protein content of the buffalo milk was 4 g/100 mL of fresh milk. The weight-gain with buffalo milk was

steady up to 24 months, showing that buffalo milk is a valid alternative to other milks when the protein content is adjusted properly.

Commercially made infant formulas used to be made by mixing whey in the right proportions according to the nutritional indications. Several studies [138,139] propose formulas with buffalo milk as a baby food.

Infant formulas (IMFs) are designed to mimic the composition of breast milk using a complex combination of macronutrients (proteins, fats, and carbohydrates) and micronutrients (minerals and vitamins). The nutritional needs of infants in the absence of breast milk must be met by using IMF (Codex Alimentarius, 2007, European Commission, 2006).

The international milk market is rapidly expanding and the possibility to take a share of it is attractive to buffalo milk producers, especially in larger countries such as India and Pakistan, despite infant milk formulas based on cow milk being produced and consumed in most parts of the world. In this context, studies on buffalo milk drying techniques should be expanded to facilitate the trade of this milk powder [138] and support the buffalo production chain, as well as to solve the seasonality of buffalo milk production which determines the closure of some dairies for part of the year [139].

12. Conclusions

Nutraceuticals and functional food ingredients that are present in milk and its derivatives have always attracted the interest of the scientific community with the intention to exploit their characteristics in both the nutritional and therapeutic fields. Our focus on buffalo milk and buffalo whey has highlighted the characteristics of the nutritional power of these foods and the possibilities for future development in the food and nutraceutical fields. The nutritional and clinical information collected in our work outlines the profiles of buffalo dairy products, showing that they are rich in fundamental components capable of providing health benefits and economic development. We want to highlight the specific presence of biliverdin and gangliosides (i.e., GM1) in buffalo milk and whey as very important.

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