Chapter 8
The Enzymology of Non-bovine Milk

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8.1 Introduction

The indigenous enzymes are important milk components that have been studied since 1881, when the peroxidase isolated from milk was given the name lactoperoxidase and was the first enzyme reported to be found in milk (Arnold 1881). Over the years since, the number of enzymes identified and characterized gradually increased, and about 70 indigenous enzymes are reported in normal bovine milk (Fox and Kelly 2006). Although milk from species other than bovine shares wide homology in terms of number and function of enzyme systems, specific studies in small ruminants, buffalo, camel, horse and donkey milk are limited compared to bovine milk; however, this field of research has gained increasing importance through the years, and will be the subject of this chapter.

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A. L. Kelly, L. B. Larsen (eds.), *Agents of Change*, Food Engineering Series,
https://doi.org/10.1007/978-3-030-55482-8_8

8.2 Enzymology of Ovine and Caprine Milk

8.2.1 Proteolytic Enzyme Activities

Ovine and caprine milk are mainly used for cheesemaking, and it is noteworthy that both milk protein and indigenous enzymes impact directly on the ability of milk to be processed into cheese and on the quality of dairy products. The increasing demand for ovine and caprine dairy products, therefore, necessitates increasing knowledge on the role of indigenous proteolytic and lipolytic enzymes on the cheesemaking suitability of milk and on their effects on ripening process.

Several indigenous proteolytic enzymes are associated with somatic cells (SC), which include polymorphonuclear leucocytes (PMN), macrophages, lymphocytes and perhaps mammary epithelial cells. Numerous enzymes are associated with somatic cells in milk: one of the plasminogen activators comprising the complex enzymatic plasmin-plasminogen system is associated with somatic cells. Elastase, cathepsins, and collagenases may be released by the lysosomes of somatic cells (Kelly and McSweeney 2002); alkaline phosphatase originates from myoepithelial cells of the mammary gland (Bingham et al. 1992). Two acid phosphatases are located in skim milk and originate from leucocytes (Andrews and Alichanidis 1975), while catalase and N-acetyl-b-D-glucosaminidase originate from somatic cells and from mammary gland epithelial cells (Kitchen et al. 1970; Kaartinen and Jensen 1988).

Moatsou (2010) reviewed the indigenous enzymatic activities in ovine and caprine milk, focusing on the principal enzymes found in these milks in comparison with bovine milk: plasmin (PL) and plasminogen (PG), cathepsin D, elastase, lipoproteinlipase (LPL), alkaline phosphatase (ALP), acid phosphatase (ACP), ribonuclease (RNase), lactoperoxidase (LPO), catalase, superoxide dismutase (SOD), xanthine oxidoreductase (XOR), γ-glutamyl trasferase (g-GGT), N-acetyl-b-Dglucosaminidase (NAGase), lysozyme and glutathione peroxidase (GPx).

Plasmin is the predominant proteolytic enzyme in all investigated mammalian milks and is also present at a significant level in ovine milk (Albenzio et al. 2005a; Bianchi et al. 2004; Caroprese et al. 2007; Leitner et al. 2004a, b). In caprine milk, the PG:PL ratio, representing the rate of zymogen conversion, is much lower than that observed in other ruminant species, and total PL + PG-derived activities are in general also lower (Moatsou 2010).

Due to the relevance of plasmin activity for protein hydrolysis and milk and dairy product quality, there have been a number of studies of the plasmin system of ovine and caprine milk. As shown in Table 8.1, several studies have shown that the plasmin-plasminogen system varies with SCC, as well as stage of lactation in ovine and caprine milk (Albenzio et al. 2004a, b, 2005a, 2006, 2009a, b, 2012, 2015, 2016; Albenzio and Santillo 2011; Caroprese et al. 2007; Santillo et al. 2009).

Some authors have reported effects of SCC on the activity of the plasmin system in ovine milk, with inconsistent results. Bianchi et al. (2004) observed that high SCC is not correlated with plasminogen-derived plasmin activity, and proposed that

Table 8.1 Indigenous proteolytic enzymes in ovine and caprine milk

Lactating species		Plasmin, U/mL	Cathepsin D, U/mL	Elastase, U/mL	References
Ovine	Stage of lactation				
	Early (<70 DIM)	42.05			Caroprese et al. (2007)
	Mid from (110 < DIM < 130)	31.29			
	Late (>160DIM)	28.19			
Caprine	Early (43 ± 5 DIM)	7.58			Albenzio et al. (2016)
	Mid (120 ± 10 DIM)	10.30			
	Late (205 ± 12 DIM)	18.10			
Ovine	Level of SCC				
	<300,000	6.46	4.98	0.15	Albenzio et al. (2012)
	301,000 < SCC < 500,000	8.42	5.06	0.14	
	501,000 < SCC < 1,000,000	10.44	5.13	0.13	
	1,001,000 < SCC < 2,000,000	11.71	5.61	0.14	
	>2,001,000	10.72	5.01	0.15	
Caprine	<700,000	7.98	0.42	0.10	Albenzio et al. (2015)
	700,000 < SCC < 1,500,000	11.33	0.84	0.19	
	>1,500,000	19.07	0.70	0.14	

the increase in plasminogen level is due to increased quantities of this pro-enzyme by-passing the blood-milk barrier. The same authors proposed that this mechanism of increased plasmin activity in high SCC ovine milk is similar to that found in bovine milk in the final phase of lactation, but opposite from that occurring in mastitic cows, where plasmin and plasminogen both increase with SCC (Politis et al. 1989, 1991). Contrasting results obtained by Leitner et al. (2004a), Rebucci et al. (2005), Theodorou et al. (2007) could be explained by overlapping effect of lactation stage and level of somatic cells that lead to unpredictable behaviour of PL and PG (Albenzio et al. 2004a, b, 2005a).

Albenzio et al. (2004a, b, 2005a) suggested that, in ovine milk, plasminogen activation is related to SCC and that plasmin activity probably depends on plasminogen activators released by macrophages. Indeed, Caroprese et al. (2007) reported that, in bulk ovine milk from healthy flocks, the contribution of macrophage-derived urokinase-type plasminogen activators to the total plasmin activity in milk is minimal. Albenzio and Santillo (2011) reported that plasmin activity was highest in ovine milk samples with SCC higher than 2,000,000 cells/mL, and lowest in milk samples with SCC lower than 300,000 cells/mL. A similar result was reported in caprine milk, where the same authors reported that plasmin activity increased with increasing SCC (from <700,000 cell/mL to >1,500,000 cell/mL), and suggested 700,000 cell/mL as a threshold value to discriminate the health status of the ovine mammary gland (Albenzio et al. 2015).

The study of changes in indigenous proteolytic enzymes in ovine milk during lactation conducted by Albenzio et al. (2009b) suggested that the plasmin and plasminogen activity and PL:PG ratio do not vary significantly throughout lactation;

however, an increase in the content of these enzymes with advancing lactation could be explained by the gradual involution of the mammary gland with all the enzymatic system components passing the disrupted cells *via* para-cellular routes (Caroprese et al. 2007).

In caprine milk, as lactation progresses, plasmin activity significantly increases (Albenzio et al. 2016). In addition, the health of mammary gland in combination with lactation stage magnifies the effects on the PL system at increased SCC in late lactation. In a study conducted by Albenzio et al. (2012), plasmin activity increased with increasing SCC and was always higher in ovine milk samples containing pathogenic bacteria. Udder health also affects the activities of the PL system in caprine milk. Leitner et al. (2004b) reported that PL activity from infected udders was almost double that than found in milk with low SCC, with PA activity being 25% higher (Leitner et al. 2004b). Leitner et al. (2004b) concluded that the PL system works at a lower rate in goats than in sheep and concluded that the enzymology of caprine milk was less responsive to external factors such as infection.

Theodorou et al. (2007) compared the effect of breed on the activities of PL, PG, and PA in milk of sheep of different breeds and, between the tested breeds, PL and PA activities were similar, while differences were detected in the PG:PL ratio, which was attributed to differences in the influx of PG or PL from the blood. Indeed, compared to bovine milk, the PG:PL ratio reported for the milk of various sheep breeds is in general low (from 0.80 to 5.77); for this reason, Theodorou et al. (2007) suggest that PG is converted to PL more efficiently in ovine than in bovine milk.

Housing conditions can affect milk PL activity, as studied by Albenzio et al. (2007); these authors reported that sheep subjected to low ventilation rate had higher PG level, probably due to the negative effect of poor air quality on udder health that results in increased leakage of blood proteins into milk.

Less information is available about the activity of lysosomal proteases, such as cathepsin D and elastase, in ovine and caprine milk in comparison with bovine milk. Cathepsin D and elastase, although less intensely investigated than plasmin, may contribute to the breakdown of casein, impairing coagulation properties of milk before cheese-making (Santillo et al. 2009). Total cathepsin D and elastase concentrations were measured by Santillo et al. (2009) to be 1.14 and 1.81 mg/L, respectively, in milk Garganica goats. Albenzio et al. (2009a, b) found a lower concentration of elastase (0.41 mg/L) and a higher concentration of cathepsin D (2.56 mg/L) in ovine bulk milk, respectively, than those found in caprine milk.

Cathepsin D and elastase activities in ovine milk vary throughout lactation, but their changes do not parallel each other (Albenzio et al. 2009a, b); indeed, elastase activity increases during lactation, whereas cathepsin D activity is highest at mid-lactation. It is known that, in ovine milk, cathepsin D is associated with PMN leucocytes and macrophages, whereas elastase is mainly associated with PMN (Albenzio and Santillo 2011). In caprine milk, the positive correlation between cathepsin D activity and PMN level suggests that PMN may be a main source of this protease in goat milk. Albenzio et al. (2015) reported the variation of cathepsin D and elastase activity during lactation in caprine milk, and the high relative activity of these enzymes observed in medium-SCC milk (from 701,000 to 1,500,000 cells/

mL) could reflect the first activation of the immune response at mammary gland level, consistent with the recruitment of PMN through macrophages, that act as a fast, but nonspecific, defense. In caprine milk with SCC >1,501,000 cells/mL, the same authors explained the minor contribution to the overall indigenous proteolytic activities of cathepsin D and elastase through the activation of specific immune mechanisms that represent the second line of the immune response at mammary gland level. Furthermore, the clotting time of ovine milk is positively correlated both with SCC and with cathepsin D activity (Albenzio et al. 2015). On the other hand, the increase in proportion of PMN parallels elastase content in ovine milk, suggesting that elastase concentration could be a reliable indicator of mammary gland involution in healthy sheep udders (Albenzio et al. 2009b).

8.2.2 Activities of Other Enzymes

Lipoprotein lipase (LPL) is another key enzyme in dairy technology, because it represents almost all lipolytic activity in raw milk. LPL is a glycoprotein which is electrostatically and hydrophobically associated with the casein micelles in bovine milk, and active only at the oil-water interface. In raw bovine milk, about the 80% of lipoprotein lipase is associated with the casein micelles. Chandan et al. (1968) reported that lipase activity in ovine milk is about one-tenth of that in bovine milk. The hydrolysis pattern of ovine milk fat due to ovine LPL is similar to that reported for bovine LPL, with preferential hydrolysis of medium-chain fatty acids over long-chained. In parallel with bovine milk, pasteurization of ovine milk LPL causes 73–95% inactivation of enzyme activity (Chavarri et al. 1998).

In line with the above, Chilliard et al. (2003) reported that the LPL activity also is lower in caprine than in bovine milk, although compared with bovine milk more LPL is bound to the milk fat globules in caprine milk, and the activity is better correlated with spontaneous lipolysis. Two distinct phenomena characterize the hydrolysis of milk triglycerides: lipolysis of the milk fat substrate and on the other hand the LPL activity itself. The first generally takes place at 4 °C, at natural milk pH, in the presence of biochemical factors naturally occurring in milk, and the result of it is generally lower than 1.0 mmol released FA/24 h/L in caprine milk (Chilliard 1982). On the other hand, milk LPL activity is measured as maximal potential activity on an artificial lipid emulsion, at 37 or 39 °C, at alkaline pH, in the presence of apoprotein activators from blood serum, and is generally higher than 20 μmol/h/mL of caprine milk (Chilliard 1982). Thus, potential LPL activity is about 500 times higher than spontaneous lipolysis in caprine milk (Chilliard et al. 2003).

Despite lipase activity in caprine milk being about one-third of the activity of bovine milk, caprine milk has a greater tendency to undergo spontaneous lipolysis, which contributes to the characteristic caprine milk flavor (Chandan et al. 1968). In bovine milk, spontaneous lipolysis is not correlated with LPL activity, and lipolysis remains generally low despite the very high LPL activity of this milk, because of hindered access to the substrate by the milk fat globule due to protection by the

MFGM, and maybe also due to presence of some inhibitors in bovine milk (Cartier et al. 1990). Moreover, bovine milk LPL is largely bound to casein micelles, thus diminishing enzyme-fat substrate interactions, except when milk is homogenized (and not pasteurized). Furthermore, in caprine milk, a substantial proportion of the LPL is bound to the cream layer, which could explain the higher level of lipolysis due to LPL in caprine milk.

The soluble nitrogen fraction derived from hydrolysis of β-casein, the proteose peptone fraction, was found to inhibit both spontaneous and induced lipolysis of caprine milk, as similarly observed for bovine milk (Chilliard et al. 2003). The characteristic flavor of caprine milk is related to caprine α_{s1}-casein alleles. Both spontaneous lipolysis and LPL activity are linked to α_{s1}-casein genotype; both lipolytic events are higher in milk from goats with the weak expression of the α_{s1}-casein genotype than in milk from goats with the more strongly expressing genotype. In animals with the weak α_{s1}-casein genotype, the fat content is also lower and furthermore the caprine flavor was found to be stronger (Delacroix-Buchet et al. 1996). This was explained by Chilliard et al. (2003), who reported that lipolysis increased with advancing lactation, and that this was more pronounced in caprine milk with the weak a_{s1}-casein genotypes containing "weak" variants E, F or O.

It is known that heat induces inactivation of indigenous enzymes in milk and this has been well studied in bovine milk. Alkaline phosphatase (ALP) is used as a means of monitoring the accuracy of pasteurization or the addition of raw milk to pasteurized milk (Shakeel-Ur-Rehman et al. 2003; Harding and Garry, 2005; Commission Regulation (EC) No. 1664/2006) (see also Chap. 4). γ-glutamyl transferase (GGT), is more heat-stable than ALP and it may be useful for testing milk heated between 75 and 80 °C (Farkye 2003). Lactoperoxidase (LPO) is one of the most heat-stable enzymes in bovine milk (Seifu et al. 2005) and LPO inactivation has been proposed as an index for monitoring thermal treatments above 78 °C for 15 s (Griffiths 1986).

The effects of high-pressure treatment applied at various temperatures on the activities of some indigenous proteases such as plasmin, plasminogen and cathepsin D in ovine milk were determined by Moatsou et al. (2008). Those authors concluded that high-pressure treatment decreased PL and PA activities in ovine milk and did not affect their distribution between the casein and milk serum fractions.

Raynal-Ljutovac et al. (2007) suggested that values for ALP activity in raw milk are not related to the fat content of milk, but are species-specific. This was explained by the same authors showing that ovine milk contains much more ALP activity than bovine milk, (8000–17,000 vs 1800–4800 μg phenol/mL). ALP activity of ovine milk was negatively correlated with daily milk yield and positively correlated with milk fat content, and its activity was higher in mastitic milk (Anifantakis and Rosakis 1983). The same authors found highest alkaline phosphatase activity at mid-lactation in Greek flocks, while Martini et al. (2013) reported that the maximum activity was detected within 60–120 days of the start of lactation, which was 40 times the activity at day one of lactation.

Caprine milk contains about 2–5 times lower ALP activity than bovine milk, and its activity ranges from 120 to 1300 μg phenol/mL (Merin et al. 1988; Coburn et al.

1992; Roseiro de Bivar and Barbosa 1995; Assis et al. 2000; Raynal-Ljutovac et al. 2007; Sharma et al. 2009). Ying et al. (2002), studying milk from Alpine goats, measured 128 U/L ALP activity in early lactation and 236 U/L in late lactation, and reported that ALP activity in caprine milk is negatively correlated with log SCC. Studies comparing raw milk from cow, sheep and goat breeds showed average ALP activities of 330, 2200 and 95 U/L, respectively (Assis et al. 2000; Barbosa 2005). Vamvakaki et al. (2006), analyzing milk from bovine, ovine and caprine raw milk samples in Greece, reported average ALP activities of 390, 2430 and 134 U/L, respectively. The respective average activities reported by Klotz et al. (2008) were 577, 1216 and 61 U/L or 1562, 3512 and 164 μg phenol/mL.

Medina et al. (1989) reported that LPO activity in individual morning and evening ovine milk samples at mid-lactation ranged from 0.14 to 2.38 U/mL, with a mean value of 0.77 U/mL; however, activity was not significantly correlated with milk production, but significantly varied between individual animals. For this reason, the differences in LPO activity among studies may be attributed to different ovine breeds, as suggested by Chavarri et al. (1998). LPO activity in bulk ovine milk of the Latxa breed has been found to be 4 U/mL on average at mid-lactation, which is about three times higher than that of bovine milk, to increase at mid-lactation, and to be at its lowest value at the end of lactation (Chavarri et al. 1998; Moatsou 2010). Lorenzen et al. (2010) determined that LPO activities were 2015, 2796 and 5190 U/L on average for bovine, ovine, and caprine raw milk, respectively. Values reported by Dumitraşcu et al. (2012) were 970, 1720 and 810 U/L, respectively.

Vamvakaki et al. (2006) reported that heat treatments at 59 °C at various holding times reduced the ALP activity in caprine milk to a lesser extent than in ovine milk. Wilińska et al. (2007) suggested higher stability for bovine milk ALP compared to the caprine enzyme within a temperature range of 54–69 °C. Lorenzen et al. (2010) compared the effects of isochrone (35–85 °C for 90 s) heating with different time-temperature combinations (LTLT- and HTST-heating) on residual activities of alkaline phosphatase (ALP), γ-glutamyltransferase (GGT) and lactoperoxidase (LPO) in bovine (Holstein Friesian Cow), ovine (East Friesian Dairy Sheep) and caprine (German Improved Fawn) milk, and concluded that the investigated enzymes were more heat-stable in ovine and caprine milk compared with bovine milk. Klotz et al. (2008) reported that pasteurization at 72.5 °C for 15 s decreased ALP activity from 61 to 0.223 U/L in caprine milk, from 1459 to 0.181 in ovine milk and from 744 to 0.085 U/L in bovine milk. Dumitraşcu et al. (2012) reported mean z-values of 3.38, 4.11 and 3.58 °C and activation energies of 678, 560 and 641 kJ/mol within the range of temperature from 70 to 77 °C for caprine, ovine and bovine milk LPO, respectively; they concluded that LPO in ovine milk was less heat-stable than its caprine or bovine counterparts.

Chavarri et al. (1998) studied ACP activity in bulk ovine milk from flocks of Latxa ewes and found values higher than those reported for bovine milk, which increased four-fold in the initial phase of lactation, and then remained constant till end of lactation. They also reported that pasteurization did not significantly inactivate the enzyme and that ACP was active at levels of 65–80% in skim milk and 30–34% in whey after renneting.

The activity of GGT in bovine skim milk was, on average, 72% of that found in whole milk, and only limited seasonal variation was noted in either whole or skim raw milk over a period of 300 days (McKellar et al. 1991). Anjos et al. (1998) found, on average, 2880 U/l GGT activity in raw bovine milk. Using two validated analytical procedures, Piga et al. (2009) found GGT activities of 2720–3460 U/l in raw milk of a Sardinian sheep breed (Moatsou 2010).

With regard to RNase activity in ovine and caprine milk, Chandan et al. (1968) found it was much lower than bovine milk, being in ovine milk about 1/4 and in caprine milk about 1/3 the activity in bovine milk. Furthermore, Liu and Williams (1982) report that the RNase activity in caprine milk was 1/10 the activity in bovine milk.

Benboubetra et al. (2004) purified and studied xanthine oxidoreductase (XOR) from ovine milk. The ovine enzyme is similar to the enzyme of bovine milk and was seen as a single band of 150 kDa in SDS-PAGE. The specific XOR activity of ovine milk is about 2.5 times lower than that in bovine milk and about 2.5 times higher than that of the caprine milk enzyme. The low XOR activity is related to a low molybdenum content of ovine milk XOR, which has been estimated as 0.18 atoms Mo per subunit, compared to 0.55 atoms Mo per subunit of the bovine milk enzyme (Moatsou 2010). Sharma et al. (2009) reported the XOR activity in bulk caprine to be 55% of that in bulk bovine milk, and 95% of this activity was found in the cream fraction. However, Atmani et al. (2004, 2005) reported lower activities of XOR in goat milk compared to bovine milk. Caprine milk XOR gave a single band on the SDS-PAGE gels at about 150 kDa, similarly to bovine and ovine XOR (Atmani et al. 2004; Zamora et al. 2009). Cebo et al. (2010) and Martini et al. (2013) studied caprine XOR; the oxidase activity of XOR of ovine milk changed during lactation, being maximum at day 45, and its changes were inversely proportional to the average size of milk fat globules. On the other hand, xanthine dehydrogenase activity of XOR was much lower and constant throughout lactation.

8.2.3 Effect of Enzyme Activity on Milk and Dairy Product Quality

Milk proteins and indigenous enzymes impact directly on the ability of milk to be processed and the quality of dairy products. The characteristics of casein in ovine and caprine milk are of particular interest, due to the high degree of variety that is related to cheese-making properties of milk. Ovine and caprine milk are mainly processed into cheese, which is in increasing demand, and therefore it is of importance in order to understand the role of indigenous proteolytic and lipolytic enzymes on milk quality and cheesemaking abilities, as well as their effects on the ripening process.

Proteolysis in milk occurs in the udder before milking (Saeman et al. 1988) and during milk storage after milking (Albenzio et al. 2004b, 2005b, 2009b; Caroprese et al. 2007). Albenzio et al. (2005a) and Santillo et al. (2009) studied indigenous

proteolytic enzyme activities using natural substrates of ovine sodium caseinate and caprine milk, respectively, to investigate proteolytic activity in small ruminant milk during storage prior to cheese manufacture. In these studies, the authors concluded that the total proteolytic activity was largely dependent on plasmin activity. Several authors have suggested that, if ovine milk has an elevated SCC, its cheese-making properties will deteriorate, i.e., show longer coagulation time and weak coagulums, leading to poor syneresis and increased moisture content, lower cheese yield and lower overall fat contents in the cheeses (Albenzio et al. 2004b, 2005b; Bencini and Pulina 1997; Jaeggi et al. 2003; Pellegrini et al. 1997; Pirisi et al. 1996, 2000).

During cheese ripening, fresh curd is converted to one of the numerous cheese varieties with particular appearance, taste, flavor, texture, and functionality characteristics. All these aspects are involved in the definition of cheese quality and result from the balance and intensity of the ripening process in terms of proteolysis, lipolysis, and glycolysis.

Raw milk compositional and biochemical characteristics, milk technological treatment and renneting process, curd production, and ripening of cheese represent a multiplicity of factors that impact, directly or indirectly, on the quality of the manufactured cheese. Each factor in this complex system is affected by the activity of enzymes originating from milk (i.e., indigenous enzymes and microflora), coagulants, and starter and non-starter microflora.

Regarding indigenous proteolytic enzymes, it has been reported that plasmin acts primarily on β- and α_s-CN, leading to the formation of the polypeptides γ_1-, γ_2 and γ_3-CN and the resulting proteose-peptones, as well as α_s-CN degradation products (Albenzio et al. 2010). However, the hydrolysis of principal caseins proceeds differently along with ripening time, with α_s-CN products being released within the first phase of ripening, whereas the cleavage of β-CN proceeds more slowly as a consequence of the different specificity of proteolytic agents towards caseins (Albenzio et al. 2010; Irigoyen et al. 2000; Revilla et al. 2007).

Casein degradation and casein breakdown products represent an important index of cheese ripening and involve a complex pattern of events occurring during cheese ripening. However, all these parameters are influenced by several factors and, therefore, they are not easily directly correlated. Albenzio et al. (2004b) found that, in fresh ovine cheese curd, the decrease of PL and PG-derived activities in milk coincided with an increase in non-protein nitrogen (NPN), suggesting that NPN components derived from CN hydrolysis may be involved in the regulation of the plasmin-plasminogen enzyme system. Furthermore, as reported in previous studies (Albenzio et al. 2005b, 2010; Santillo and Albenzio 2008), changes in the formation of γ-CN mainly derived from plasmin activity were not correlated with the changes in PL activity in the cheese during ripening, indicating that factors other than the enzyme activities themselves are important for the resulting breakdown observed.

Furthermore, cheese-making technology is one of the main factors able to affect indigenous enzyme level and activity. In Canestrato Pugliese cheese made from ovine milk, the production protocol includes heating the curd in hot whey, which can promote syneresis and inactivate heat-labile inhibitors of PL and PG activators

(Albenzio et al. 2007). Examples for the effect of cheese-making conditions and cheese physicochemical composition on plasmin and PG-derived activity in various cheese varieties have been presented by Nega and Moatsou (2012).

Salting of fresh curd is a further step able to influence enzymatic activity in cheese during ripening; in fact, salting decreases the activity of most of the enzymes in cheese, including indigenous milk proteinases such as plasmin (Sutherland 2003). Fox (2003) reported that hydrolysis of β-CN is strongly inhibited by a NaCl content in cheese above 5%. In Canestrato Pugliese cheese from ovine milk, a salt concentration of about 4% was able to inhibit β-CN hydrolysis, as indicated by the limited accumulation of small peptides ascribed to γ-CNs over 45 d of ripening (Albenzio et al. 2004b, 2005b). Also, lipolytic activity appeared to be limited at NaCl levels >13 g/kg of cheese in Idiazabal cheese made from raw ovine milk using natural rennet (Nájera et al., 1994).

Flavor of cheese is determined by its taste and aroma and results from the correct balance and concentration of numerous sapid and aromatic compounds perceived during cheese consumption. Proteolysis and lipolysis are of great importance in the development of cheese flavor and are largely due to the activities of residual milk clotting enzyme, milk proteinase and lipase, proteolytic and lipolytic enzymes from starter and non-starter bacteria, and lipases associated with certain coagulants (Collins et al. 2003; Visser 1993). However, excessive proteolysis and lipolysis could result in off-flavors because of high concentrations of bitter peptides and volatile FFA, respectively, and influence cheese flavor either directly or as precursors of other compounds (Pinho et al. 2004). The contribution of indigenous proteinases to the development of cheese flavor has possible negative implications through the accumulation of bitter peptides, which are gradually formed by further degradation of β-CN compounds (Visser 1993).

Revilla et al. (2007) reported a lower overall acceptance of ovine hard cheese made with high SCC milk compared to milk of medium and low SCC, finding that the former was perceived as weakly bonded, very grainy, and crumbly. In contrast, Pirisi et al. (1996, 2000) did not find significant differences in sensory characteristics and lipolysis when comparing ovine cheeses made from milk with low and high SCC. Jaubert et al. (1996) and Morgan and Gaspard (1999) also found a minor effect of SCC of caprine milk on the 'goatish flavor' of cheese, which is instead mainly influenced by cheese-making technology, in particular by the ripening conditions.

Milk contains various components, mainly associated with protein and lipid molecules, that are recognized as biofunctional components. Peptides derived from milk proteins, which are inactive within the sequence of the precursor protein, can be released by enzymatic proteolysis, for instance during cheese ripening (Gómez-Ruiz et al. 2004). Santillo et al. (2009) investigated the effects of indigenous proteolytic enzymes on the release of bioactive peptides from caprine milk using different proteolytic enzyme inhibitors. The authors found that caprine milk incubated with or without inhibitors of acid proteases (e.g., pepstatin, which inhibits cathepsin D activity) showed several peptides with potential bioactivity deriving from β- and α_{s2}-CN, indicating that both aspartic proteases and other protease types were involved in the generation of bioactive peptides.

8.3 Enzymology of Buffalo Milk

In the last few decades, many enzyme activities have been detected in buffalo milk, some of which have been isolated and partially characterized (El-Salam and El-Shibiny 2011). Lombardi et al. (2000) established the levels of ALP, LDH, GGT, and AST in raw buffalo milk, highlighting higher concentrations of enzymes than in bovine milk, which could be related to the different composition of the milk, i.e., higher concentrations of protein, fat, and other solids than bovine milk. The activity of the following enzymes has been reported for buffalo milk: GGT (712 ± 601 IU/L), LDH (386 ± 183 IU/L), ALP (295 ± 164 IU/L) and AST (18 ± 4 IU/L); also, the patterns of heat inactivation of these enzymes suggested that ALP, LDH and GGT are potential markers for heat treatment of buffalo milk.

Concentrations and characteristics of lysozyme, lactoperoxidase, xanthine oxidase, lipase, alkaline phosphatase, ribonuclease, and protease activities in buffalo milk have been reviewed (Ahmad et al. 2013). Priyadarshini and Kansal (2002b) found the molecular weight of buffalo milk lysozyme to be 16 kDa, and determined its antibacterial activity, highlighting that buffalo colostrum contains five times more lysozyme activity than mature milk and higher specific activity than that of bovine milk. Levels of lysozyme in buffalo milk are, however, still controversial, probably due to the different methodologies used for enzyme determination. Kumari and Mathur (1981) reported lower concentration in buffalo milk in comparison to bovine milk (average 15.21 μg/100 mL and 18.00 μg/100 mL in buffalo and bovine milk, respectively). On the other hand, more recently, Priyadarshini and Kansal (2002a) reported a twofold higher lysozyme activity in buffalo milk ($60 \pm 3.9 \times 10^{-3}$ U/mL) than in bovine milk ($29.1 \pm 1.5 \times 10^{-3}$ U/mL), with the enzymatic activity in buffalo milk also being more stable compared with bovine during storage and heat treatment (Priyadarshini and Kansal 2003). Concerning the factors affecting lysozyme activity of buffalo milk, Kumari and Mathur (1981) reported an increase during lactation, while Priyadarshini and Kansal (2002a) found it to be influenced neither by parity nor stage of lactation; however, it increased during extreme weather conditions in winter and summer. The higher lysozyme activity in buffalo milk has been proposed as one of the factors responsible for lesser incidences of udder infections in buffaloes. Furthermore, lysozyme, though mainly from egg-white sources, has found application in food preservation, being already used successfully as an antimicrobial in many foods (Benkerroum 2008).

Lactoperoxidase is abundant in buffalo milk. Its activity has been reported to vary widely, possibly due to different assay methods used, and values from 0.9 U/mL (Hamulv and Kandasamy 1982) to 16.84 U/mL (Tayefi-Nasrabadi and Asdpour 2008) have been reported. Sharma et al. (2009) reported that lactoperoxidase was found free in buffalo milk and exhibited 1.76-fold higher activity (12.02 ± 0.83 U/mL) over that of bovine milk (6.83 U/mL).

The lipase activity of buffalo milk was found to increase gradually during the lactation period from 0.86 U/mL in the first month to 2.48 U/mL in sixth month of lactation (Hofi et al., 1976) The lipase activity was affected by feeding system,

being higher for dry concentrate feeding than during green feeding, and it also decreased with increased number of lactations, although no differences have been reported between morning and afternoon milking (Abd El-Hamid et al. 1977). Feeding protected fat diets containing supplemental tallow had no significant effect on lipase activity of buffalo milk, compared to the control diet (Srivastava et al. 1989).

The activity of XOR measured in Indian buffalo breeds displayed average level of 0.075 ± 0.027 U/mL (Sanhotra and Dutta 1986). The major portion of its activity in buffalo milk was found in the skim milk fraction, with approximately 25% of the total activity located in the cream fraction (El-Gazzar et al. 1999). The same authors also found an increase of about 10% of XOR activity upon heating buffalo milk at 55 °C for 1 min, whereas heating at 70 °C for 1 min and 80 °C for 1 min caused a decrease in activity by 75% and 90%, respectively.

Only a limited number of studies have reported on indigenous proteinase systems and activities in buffalo milk; Manjunath and Bhat (1992) showed 55.0 and 70.2 μg/mL in buffalo and bovine milk of total indigenous proteinase, respectively. Buffalo milk contains the complete plasmin system, cathepsins and elastase, similar to milk of other ruminants, and the presence and activity of plasmin, plasminogen, activators, cathepsin D and elastase have been reported (Madkor and Fox 1991; Fantuz et al. 1998; Santillo et al. 2011). A study of indigenous proteolytic enzyme activity in Mediterranean buffalo milk highlighted, as expected, the main role of the plasmin-plasminogen system, although its activity seemed to be limited compared with milk of other species (Santillo et al. 2011). Moreover, similarly to other ruminants' milk, plasmin activity in buffalo milk was associated primarily with the casein micelles, and completely dissociated at pH <4.6 (Madkor and Fox 1991). Addition of urokinase increased the proteolytic activity by 3.5-fold in milk and fivefold in micellar suspension (Madkor and Fox 1991), while addition of NaCl (at 3–15%) decreased the plasmin activity, and this effect was even more pronounced at high NaCl concentrations (Madkor and Fox 1991).

Heating at 70 °C for 10 min increased the plasmin activity of buffalo milk, while 15% of its activity was lost after heating at 80 °C for 10 min. Most of the plasminogen activators (PA) were associated with casein micelles (Fantuz et al. 1998). Several PAs were identified in buffalo milk, i.e., major fractions with molecular masses of 75 and 120 kDa and two minor fractions of 48 and 38 kDa (Fantuz et al. 1998). The proteins at 38, 75, and 120 kDa were tissue-type PAs, and the 48 kDa fraction was urokinase-type PA (Fantuz et al. 1998).

River buffalo milk is characterized by a peculiar γ-CN defined as β-CN f(69–209) which has no counterpart in bovine milk. This fragment was previously known as "component D" by Trieu-Cuot and Addeo (1981) and was named "protein X" by Angeletti et al. (1998). It was later identified by Di Luccia et al. (2009), who also demonstrated that the formation of this fragment formed earlier with respect to that of γ1-CN, because of conformational exposition of the plasmin cleavage site Lys_{68}-Ser_{69}. For this reason, β-CN f(69–209) and its complementary proteose peptone β-CN f(1–68) have been proposed as candidate molecular markers for the assessment of buffalo milk and curd freshness.

The main indigenous proteolytic enzymes have also been detected in buffalo Mozzarella cheese (Table 8.2, Santillo et al. 2011). The curd heating step has been considered partially responsible for the higher plasmin activity in cheese compared with the corresponding milk (Gobbetti et al. 2002). The measurable activity of acid proteases, such as cathepsin D, have been also found to be higher in Mozzarella than in the cheese milk due to the low pH of the acidified cheese mass before the stretching phase, in line with some activation occurring and leading to elevated measurable cathepsin D activity at lower pH conditions of this cheese matrix. The contribution of the activity of elastase to the proteolysis pattern of buffalo mozzarella was found to be of minor relevance compared to the other enzymes.

8.4 Enzymology of Camel Milk

Raw camel milk is produced by one-humped Dromedary camels in the desert areas of Africa, Asia and Middle East and by two-humped Bactrian camel in more cool areas in Mongolia, China and Russia; therefore, it is consumed in arid and hot areas of Africa and Asia and mainly processed into fermented products (El-Agamy 2017, Al haj and Al Kanhal 2010). The mean gross composition of Dromedary camel milk is similar to that of bovine milk, except for the higher whey protein content of the former. According to El-Agamy (2017), the mean casein content in Dromedary camel milk is 2.40% vs. 2.51% of bovine milk. Bactrian camel milk contains more fat and protein—on average, 5.32 and 4.09%, respectively—and consequently more total solids than bovine milk. The whey protein:casein ratio is 0.34–0.36 in both camel milks, higher than that of 0.24 for bovine milk (El-Agamy 2017).

There are several recent reports about the enhanced biofunctionality and therapeutic potential of camel milk, e.g., low or lack of allergy effects due to the absence of β-lg, considerable amounts of bioactive peptides, better digestibility than bovine milk, higher lactoferrin content than in ruminants' milk, and possible hypoglycaemic/antihyperlipidemic effects, due to high concentrations of zinc, vitamin B3, polyunsaturated fatty acids, non-enzymatic antioxidants and an insulin-like protein that mimics the interaction of insulin with its receptor (Al haj and Al Kanhal 2010; El-Agamy 2017; Khalesi et al. 2017; Maqsood et al. 2019; Shori 2015).

Indigenous enzymatic activities have been identified in raw camel milk; Ryskaliyeva et al. (2018) reported that, among the top 70 proteins of skimmed

Table 8.2 Indigenous proteolytic enzymes in buffalo milk and mozzarella cheese produced from Mediterranean buffalos' milk (adapted from Santillo et al. 2011)

Item	Buffalo Milk U/mL	Buffalo mozzarella cheese U/g
Plasmin	3.02 ± 0.1	3.4 ± 0.45
Plasminogen	3.13 ± 0.14	5.33 ± 0.7
Cathepsin D	0.011 ± 0.01	0.22 ± 0.01
Elastase	0.07 ± 0.05	0.02 ± 0.01

camel milk, xanthine oxidoreductase (XOR), lactoperoxidase (LPO) and lipoprotein lipase (LPL) were detected, with molecular masses of 150, 87.7 and 46.5 kDa, respectively.

Lysozyme (LYZ) and lactoperoxidase (LPO) were the focus of early studies. Duhaiman (1988) estimated a level of 0.5 mg lysozyme per 100 mL of camel milk, with a specific activity lower than that of from human milk and egg white, whereas Elagamy et al. (1992) observed that camel milk and egg-white LYZ exhibited a similar antibacterial activity spectrum. Later, Elagamy et al. (1996) estimated a level of 15 μg LYZ per 100 mL camel milk, twice as much as in bovine milk, with higher lytic activity than for bovine milk but lower than egg white LYZ. They report no immunological cross-reactivity between camel and bovine and between camel and egg white LYZ. Lysozyme C (EC: 3.2.1.17) of camel milk consists of 130 AA and has a molecular mass of 14.8 kDa - www.uniprot.org/uniprot/P11376, www.uniprot.org/uniprot/P11375 and www.uniprot.org/uniprot/P37712- and, as reviewed by Hailu et al. (2016), there is about 85% sequence similarity among camel, human and ruminant LYZ. On the other hand, Kappeler (1998) did not found LYZ activity in camel milk, or a cDNA corresponding to LYZ by means of screening with oligonucleotides derived from conserved regions of the already known C-type lysozymes. He suggested either an effect of the stage of lactation or that the literature reported LYZ activity could be related to other enzymes.

Elagamy et al. (1992, 1996) observed that camel milk was bacteriostatic against Gram-positive and bactericidal against Gram-negative bacteria. LPO activity in the presence of H_2O_2 and thiocyanate (SCN^-) similar to that of bovine milk was detected, and molecular masses of 78 and 72.5 kDa for camel and bovine milk LPO, respectively, were estimated and cross-reactivity between them was detected. Similarly, Mahdi et al. (2018) reported that the molecular mass of LPO from camel colostrum was 79 kDa and its concentration was 2.35 mg/mL. Camel LPO consists of 611 amino acids (Hailu et al. 2016). The SCN content of camel milk varies considerably, i.e. from 2.4 to 33 ppm, being higher than in other ruminants' milk (Kamau et al. 2010; Younan et al. 2006).

The enhancement of the LPO system in raw camel milk has been effectively used for the extension of storage of raw camel milk, e.g., resulting in constant total bacterial counts over 6 h at 30 °C (Younan et al. 2006). Addition of 30:30 ppm NaSCN: H_2O_2 was suggested as the most effective supplementation for the extension of raw camel milk shelf-life, which was more effective at 20 and 30 °C than at 10 °C, due to higher activity of LPO at ambient temperatures (Kamau et al. 2010). Similarly, addition of 20:20 ppm SCN: H_2O_2 preserved raw camel milk for up to 18–20 h at 37 °C (Singh et al. 2017). On the other hand, Kamau and Wangoh (2010) noted greater shelf-life extension when pasteurized camel milk supplemented with 8.5 ppm H_2O_2 was stored at 10 °C compared to 20 °C.

Activities of camel LPO other than its preservation effect in milk have been also reported. According to Redwan et al. (2015), the direct interaction of camel milk LPO at a concentration of 1 mg/mLwith hepatitis C virus inhibits virus amplification, while a concentration of 1.5 mg/mL is needed for the bovine milk counterpart. In accordance with the human and bovine counterparts, camel milk LPO exhibits

activity against the herpes simplex virus type 1, which is enhanced in the presence of H_2O_2 (El-Fakharany et al. 2017). LPO from camel colostrum, either alone or in combination with lactoferrin, exhibits significant antibacterial activity *in vivo* and *in vitro* against *Acinetobacter baumannii*, being more effective than the sensitive antibiotic imipenem, due to immunomodulation expressed by the activation of IL-10 and IL-4 levels in lung homogenates (Mahdi et al. 2018). Sheikh et al. (2018, 2019) performed structural studies on camel milk LPO and concluded that common antibiotics can inhibit it, in accordance with the findings of Al-Nazawi and Homeida (2015).

The average alkaline phosphatase activity in camel milk has been estimated as 21.31 ± 1.26 μg/mL, which is lower than for bovine and buffalo milks, i.e., 575.00 ± 18.42 μg/ml and 253.75 ± 8.22 μg/ml, respectively (Yoganandi et al. 2014). Increased ALP activity in milk from camels with sub-clinical mastitis has been observed (Ali et al. 2016).

Indigenous enzymes, mainly alkaline phosphatase (ALP) and lactoperoxidase (LPO), have been also studied in relation to heat treatment efficacy for camel milk. LPO and γ-glutamyltransferase (GGT) of camel milk are destroyed on heating at 74 °C for between 15 s and 5 min, and they have been suggested as suitable markers for the correct pasteurization of camel milk, because ALP exhibits residual activity after treatment at 72 °C for 5 min (Wernery et al. 2006, 2008). Indeed, Lorenzen et al. (2011) estimated mean ALP activities from 15.9 to 23.4 U/L for raw camel milk and from 16 to 10 U/L after pasteurization at 75 ± 1 °C for 15–30 s. According to their findings, mean GGT activity in raw camel milk was 293–343 U/L and residual activity of 0.1–2.2 U/L was detected after pasteurization, implying that it is not suitable as an indicator. Moreover, they estimated too low and highly variable leucine arylamidase (LAP) activity in raw camel milk, in the range of 4–6 U/L, such that, despite its inactivation after pasteurization, it cannot be used as an indicator. In the same study, high residual lipase activity was estimated after pasteurization, i.e., mean lipase activity was in the range of 57–71 U/L in raw and 12–14 U/L in pasteurized camel milk.

Finally, LPO has been proposed for the assessment of pasteurization of camel milk due its high concentration in raw milk (869–1172 U/L) and non-detectable amounts <5 U/L in pasteurized milk. The findings of Tayefi-Nasrabadi et al. (2011) showed that camel milk LPO is less heat-stable than its bovine counterpart within the temperature range 67–73 °C, due to lower values of activation energy and change in enthalpy of denaturation. They report that D-values for camel milk LPO at 71 °C and 67 °C were about 3.1 and 6.7 times lower than for bovine milk, with a higher z-value (6.42 °C vs 4.7 °C), which indicates that camel milk LPO is more sensitive to the extension of heating duration. It has been confirmed that LPO in camel milk is destroyed at 75 °C for 15 s and has been suggested as an indicator for pasteurization of such milk (Wernery et al. 2013).

Studies on other indigenous enzymatic activities in camel milk are limited. Variable plasmin and plasminogen activities were detected in individual camel milks using a fluorogenic substrate by Baer et al. (1994), with proteolytic activity on camel casein similar to the proteolytic activity of bovine plasmin. Ryskaliyeva et al.

(2018) suggested the presence of plasmin activity in camel milk based on the identification of β-casein fragments missing 946 Da, which they hypothesized to result from the cleavage of the first seven N-terminal residues by plasmin.

In the proteomic analysis of Felfoul et al. (2017), lipoprotein lipase isoform 3 was detected in raw camel milk. Xanthine oxidoreductase (XOR) has been also identified in the camel milk fat globule membrane (Saadaoui et al. 2013). The XOR content of camel milk has been found as up to 22.2 mg/L, which is comparable to that obtained for bovine and human milk. Purified camel milk XOR showed no detectable activity towards xanthine or NADH. The mean molybdenum content per XOR monomer was 0.02 ± 0.002, similar to the value of 0.03 ± 0.005 estimated for the human milk enzyme and much lower than the value of 0.56 ± 0.051 for bovine milk (Baghiani et al. 2003). The average catalase activity in camel milk has been found to be higher than in bovine or buffalo milks, being estimated at 6.20 ± 0.18 units, 0.86 ± 0.04 units and 0.62 ± 0.04 units, respectively (Yoganandi et al. 2014).

8.5 Enzymology of Non-ruminant Milk

The milk of members of the Equidae family, such as horses and donkeys, has drawn the attention of dairy and nutrition scientists due to its similarity with human milk in terms of composition, possible low allergy potential, and suggested therapeutic properties. As a result, horse (mare) milk (MM) and donkey milk (DM) have been proposed as more suitable for infant formulas compared to ruminants' milk (Alichanidis et al. 2016; Altomonte et al. 2019; Martini et al. 2018; Massouras et al. 2017; Salimei and Fantuz 2012, 2013; Salimei and Park 2017; Souroullas et al. 2018; Vincenzetti et al. 2017; Uniacke-Lowe et al. 2010).

In brief, MM and DM contain (expressed in g/kg) 110 and 95.3 total solids, 61 and 65.8 lactose, 21.4 and 16.5 N × 6.38, 14.0 and 7.6 fat and 4.5 and 4.1 ash, respectively. The composition of fat of equid milk differs substantially from ruminants' milk. Triglygerides comprise <80% of total lipids, free fatty acids approx. 10% and phospholipids >5%, cholesterol content is low, at 5.0–8.8 mg/dL, and milk fat globules are small with a mean diameter of 2–3 μm. Casein is <50% of crude protein in equid milk and lysozyme is more than 10% of whey proteins. Moreover, the number of somatic cells is low (average SCC is in general <5 log SCC/mL) in these milks, because the mammary gland status is good and mastitis is very rare. The bacterial counts are in general $<10^3$ cfu/mL, the presence of undesirable *Staphylococcus* spp. is rare, and the absence of *Listeria monocytogenes* and *Salmonella* spp. has been reported for raw equid milk produced under hygienic conditions (Doreau and Martin-Rosset 2011; Salimei and Fantuz 2012, 2013; Uniacke-Lowe et al. 2010).

There are a few reports on indigenous enzymatic activities of equid milk, i.e., horse (mare) milk (MM) and donkey milk (DM) and on the effect of treatments on them; the greater part of the recent literature is about DM.

The role of alkaline phosphatase (ALP) as an indicator of proper equid milk pasteurization has been studied. Marchand et al. (2009) reported that mean alkaline ALP activity, determined using the Fluorophos® substrate ranges from 3.1 to 20.8 and from 740 to 1050 U/L for different pools of raw MM and bovine milk respectively, i.e., 35–350 times lower ALP activity for equine milk compared to bovine milk. Also, those authors reported that, in contrast to bovine milk, no specific association of ALP with the fat fraction of MFGM was detected in equine milk. They estimated that the inactivation of ALP in raw MM follows first-order kinetics at 48–64 °C, with a z-value 5.31 °C, and concluded that ALP could not be a useful pasteurization indicator for this milk type, due to the low D-values in the pasteurization range and the low initial ALP activity. On the other hand, Giacometti et al. (2016) reported that ALP can be used as an indicator for proper pasteurization of DM. They estimated 36,047 ± 1009 mU/L ALP activity in raw donkey milk, which after pasteurization at 65 °C for 30 min was 106 ± 10.5, ranging from <100 to 118 mU/L. Moreover, high pressure treatment at 400 MPa at 4–6 °C for 100 s resulted in approximately one tenth of the original residual ALP activity.

The well-known antibacterial activity in equid milk is associated mainly with the rather high levels of lactoferrin and especially of lysozyme (LYZ), which are major components of the whey protein fraction compared to bovine milk. While lactoferrin concentration is intermediate between that of human and bovine milk, lysozyme level is much higher than that of human milk, especially in DM (Uniacke-Lowe et al. 2010; Salimei and Fantuz 2012, 2013). Doreau and Martin-Rosset (2011) reported that MM contains 0.8 g/kg LYZ, while Markiewicz-Kęszycka et al. (2013) found 1.03 ± 0.27 g/L LYZ at late lactation. As reviewed by Altomonte et al. (2019), LYZ concentration in DM ranges from 0.9 to 3 g/L and in human milk ranges from 0.8 to 1.1 g/L. LYZ activity in DM ranges from 4000 to 5000 U/mL and exhibits maximum values at the beginning of the lactation (Pilla et al. 2010; Vincenzetti et al. 2008), being very much higher than the 0.0292 U/mL found in bovine milk and very much lower than the 39,000 U/mL in human milk (Martini et al. 2018). Indicative concentrations or activities of lysozyme in DM milk are shown in Table 8.3.

Equid milk lysozyme binds calcium strongly, which results in a more stable configuration opposite to lysozyme of egg-white, tears and saliva (Brumini et al. 2016; Uniacke-Lowe et al. 2010). MM and DM lysozymes (LYZ), 1,4-beta-N-acetylmuramidase C (EC 3.2.17), contain 129 amino acid residues and bind one Ca^{2+} ion per subunit. Residues Glu35 and Asp52 are related to enzymatic activity and the conserved high-affinity calcium binding site 81–92 similar to that of α-lactalbumin - www.uniprot.org/uniprot/P11376, www.uniprot.org/uniprot/P11375 and www.uniprot.org/uniprot/P37712 -. Two variants of the LYZ of DM differing in three amino acids were identified by Herrouin et al. (2000). Sixty five percent of LYZ of raw and heat treated (95 °C for 1 min) MM remains mainly intact after *in vitro* gastric and duodenal digestions (Inglingstad et al. 2010). Tidona et al. (2011, 2014) reported higher residual intact lysozyme, from 75 to 85%, after *in vitro* digestion of DM with human gastric and duodenal juice.

Table 8.3 Mean lysozyme (LYZ) level or activity in raw donkey milk as reported in various studies

Quantity/activity	Analysis method	Reference
11,531 ± 923 U/mL	Fluorimetric commercial kit	Addo and Ferragut (2015)
4000 mg/L	Microbiological assay[a]	Coppola et al. (2002)
2.03 mg/mL[b]	RP-HPLC	Cosentino et al. (2015)
1090 mg/L	RP-HPLC	Cosentino et al. (2016)
1 mg/mL	RP-HPLC	Polidori and Vincenzetti (2010)
0.95 ± 0.07 mg/mL	RP-HPLC	Ozturkoglu-Budak (2018)
9500 ± 4200 U/mL	Fluorimetric commercial kit	Murgia et al. (2016)
4000–5000 U/mL	Fluorimetric commercial kit	Pilla et al. (2010)
3750 ± 250 mg/L	Microbiological assay[a]	Chiavari et al. (2005)
0.76–1.34 mg/mL[c]	RP-HPLC	Vincenzetti et al. (2008)
3986 ± 278 U/mL	Fluorimetric commercial kit	Ragona et al. (2016)
0.98–3.74 g/L	SDS-PAGE	Šarić et al. (2014)
1.17 ± 0.10 mg/mL	RP-HPLC	Vincenzetti et al. (2018)

[a]According to Lodi et al. (1984)
[b]Under similar conditions, the LYZ content in bovine milk was 0.18 mg/mL
[c]Throughout lactation

Due to its antimicrobial effect, the residual activity of equid milk LYZ after various types of treatments has been studied. Jauregui-Adell (1975) reported that the activity of LYZ in MM was not affected by treatment for 15 min at 71 °C or 2 min at 82 °C. In particular, after 30 min at 62, 71 and 82 °C, 102, 86 and 37% residual activity, respectively, was detected. Residual LYZ in MM treated at 75 °C for 10 min, which is consistent with the treatment of infant formulas, was 85% of the initial activity (Civardi et al. 2007). Under denaturing conditions, LYZ of MM forms a very stable molten globule, which is a compact state with native-like secondary structures, but with little tertiary structure compared to native proteins (Morozova-Roche 2007).

Lysozyme in DM survives heat treatments at 90 °C for 1 min and 100 °C for 5 min, but is destroyed by treatment at 121 °C for 10 min (Coppola et al. 2002), while it does not decrease significantly after pasteurization of DM at 63 °C for 30 min or 72 °C for 15 s (Giribaldi et al. 2017). According to the results of Addo and Ferragut (2015), LYZ activity of DM heat treated at 70 ± 2 and 80 ± 2 °C for 1 min or treated by ultra-high pressure homogenization at 100, 200 and 300 MPa was not significantly decreased compared to raw milk throughout 28 days of storage. Ozturkoglu-Budak (2018) reported that freezing or heating at 75 °C for 2 min did not affect the level of native lysozyme, while heating at 85 °C did.

Chiavari et al. (2005) reported that storage for 30 days did not significantly affect the antimicrobial lysozyme activity in raw and heat treated (63 °C for 30 min) DM. Polidori and Vincenzetti (2010) found that the lysozyme content of fresh, frozen and powdered DM did not differ significantly, although a 30% decrease of activity was observed in the DM powder. Those authors also reported a 50% decrease of activity at 70 °C for 5 min. Concentration of raw or pasteurized (63 °C for 30 min)

DM by rotary vacuum evaporation, to 40% and 20% of the initial value, did not affect either concentration or antimicrobial activity of lysozyme against gram-positive and gram-negative bacteria, with the exception of *Bacillus mojaventis* (Cosentino et al. 2016). Vincenzetti et al. (2018) observed that the level of lysozyme after freeze-drying or spray-drying of DM was significantly reduced compared to control raw milk, being 85% and 82% of the initial, respectively. A more severe reduction was observed for lysozyme activity, i.e., 96% and 58% respectively.

DM has been used in cheese-making as a source of lysozyme against late gas production. According to Cosentino et al. (2015), the addition of DM to bovine cheese milk at levels from 2 to 7.5% decreased the counts of *Clostridium tyrobutyricum* and bacterial spores in semi-hard cheese in a dose-dependent manner. Niro et al. (2017) reported that the supplementation of bovine cheese milk with 2% DM had effects on microbial counts, composition and proteolysis similar to the addition of 1.6 g egg-white lysozyme in cheese milk; no late blowing was observed in either case, although 32 MPN clostridia spores/L were determined in bovine cheese milk.

Information about other indigenous enzymes in equid milk is sporadic. Egito et al. (2002, 2003) suggested that small quantity of PP5-like peptides and 23-kDa γ-like caseins found in MM sodium caseinate could come from the action of indigenous plasmin. They also reported that sodium caseinate prepared from MM exhibited very low, but detectable, plasmin activity. On the other hand, Humbert et al. (2005) found high plasmin activity in fresh milk, along with intense presence of γ-caseins in MM, whereas plasminogen could not be detected following the usual activation protocols.

In contrast to bovine milk, lactoperoxidase (LPO) activity in skimmed DM was found to be very low, 4.83 ± 0.35 mU/mL. Its concentration in DM was estimated to be 0.11 mg/L, seven times lower than that in human milk, and very much lower than the 30–100 mg/L LPO content of bovine milk (Vincenzetti et al. 2017).

Chilliard and Doreau (1985) showed that MM contains high lipase activity that exhibits the characteristics of lipoprotein lipase, i.e., it was activated with 2–8 volumes of equine blood serum, inactivated by 95% after heating at 60 °C for 30 min, was sensitive to sodium chloride, and was not significantly affected by heparin.

Xanthine dehydrogenase/oxidase (XOR) with molecular mass 146 kDa and an electrophoretic profile similar to its bovine and caprine counterparts has been identified among MFGM proteins of pony mares' milk by Cebo et al. (2012). NAGase activity <50 U/mL of has been identified in raw DM for the most part of the lactation period, and it was not affected by the bacteriological status (Pilla et al. 2010).

8.6 Conclusion

Several decades of research has led to significant knowledge of indigenous enzymes in milk from small ruminants, such as buffalo, camel, horse and donkey, and several enzymes have been partially identified and characterized. However, in species with less common use for milk production, significant gaps in understanding the nature,

biochemical properties and technological significance of milk enzymes remain. From this perspective, it is important that the study of the various aspects of all indigenous enzymes continues to be an active area of research in terms of milk of the species covered by this review.

Among the milk indigenous enzymes, the study of proteolytic enzymes has perhaps been most intensive in the milk of small ruminants and buffalo, due to the implications for the quality of the milk protein fraction and therefore on the processing of milk and the quality of dairy products, but deficits in our understanding remain.

As expected, the limited research on camel milk enzymatic activities has focussed on the antimicrobial lysozyme and LPO and on indicators of heat-treatment such as ALP and LPO. The findings for lysozyme are contradictory, but LPO activity in the presence of H_2O_2 and SCN^- similar to that of bovine milk has been detected and interesting antiviral and immunomodulatory behavior has been reported for the enzyme. In contrast to bovine milk, high residual ALP and LPL activities and non-detectable amounts of LPO have been found in pasteurized camel milk, indicating that different approaches to the assessment of heat treatment of this milk kind should be developed.

The profile of indigenous enzymatic activities of the milk of the members of the Equidae family differs from their ruminant counterpart; for example, very high lysozyme–and therefore antimicrobial–activity and low ALP and LPO activity are reported in such milk.

The implementation of ‘omics’ techniques has produced significant new information regarding the presence of indigenous enzymes or enzyme systems in non-bovine milks. However, studies on their behavior under the conditions and treatments applied in raw milk storage and processing are scarce. In particular, this research topic is of great interest for the exploitation of the biological potential of non-ruminants milk in both infant and adult nutrition.

References

Abd El-Hamid LB, Mahran GA, Shehata AE, Osman SG (1977) Lipase activity in buffaloes milk 2. Effect of feeding system, animal age and milking phase and meal. Egypt J Dairy Sci 5:7–10

Addo CNA, Ferragut V (2015) Evaluating the ultra-high pressure homogenization (UHPH) and pasteurization effects on the quality and shelf-life of donkey milk. Int J Food Stud 4:104–115

Ahmad S, Anjum FM, Huma N, Sameen A, Zahoor T (2013) Composition and physico-chemical characteristics of buffalo milk with particular emphasis on lipids, proteins, minerals, enzymes and vitamins. J Anim Plant Sci 23:62–74

Al haj OA, Al Kanhal HA (2010) Compositional, technological and nutritional aspects of dromedary camel milk. Int Dairy J 20:811–821

Albenzio M, Santillo A (2011) Biochemical characteristics of ewe and goat milk: effect on the quality of dairy products. Small Rumin Res 101:33–40

Albenzio M, Caroprese M, Santillo A, Marino R, Taibi L, Sevi A (2004a) Effects of somatic cell count and stage of lactation on the plasmin activity and cheese-making properties of ewe milk. J Dairy Sci 87:533–542

Albenzio M, Marino R, Caroprese M, Santillo A, Annicchiarico G, Sevi A (2004b) Quality of milk and Canestrato Pugliese cheese from ewes exposed to different ventilation regimens. J Dairy Res 7:434–443

Albenzio M, Caroprese M, Santillo A, Marino R, Muscio A, Sevi A (2005a) Proteolytic patterns and plasmin activity in ewes' milk as affected by somatic cell count and stage of lactation. J Dairy Sci 72:86–92

Albenzio M, Santillo A, Caroprese M, Marino R, Centoducati P, Sevi A (2005b) Effect of different ventilation regimens on ewe milk and canes-trato Pugliese cheese quality in summer. J Dairy Res 72:447–455

Albenzio M, Caroprese M, Marino R, Muscio A, Santillo A, Sevi A (2006) Characteristics of Garganica goat milk and Cacioricotta cheese. Small Rumin Res 64:35–44

Albenzio M, Santillo A, Caroprese M, Marino R, Annicchiarico G, Sevi A (2007) Effects of ventilation rate and of dietary protein level in an intensive dairy sheep system on the features of Canestrano Pugliese cheese. J Dairy Res 74:26–33

Albenzio M, Santillo A, d'Angelo F, Sevi A (2009a) Focusing on casein gene cluster and protein profile in Garganica goat milk. J Dairy Res 76:83–89

Albenzio M, Santillo A, Caroprese M, d'Angelo F, Marino R, Sevi A (2009b) Role of endogenous enzymes in proteolysis of sheep milk. J Dairy Sci 92:79–86

Albenzio M, Santillo A, Caroprese M, Marino R, Trani A, Faccia M (2010) Biochemical patterns in ovine cheese: influence of probiotic strains. J Dairy Sci 93:3487–3496

Albenzio M, Santillo A, Caroprese M, Ruggieri D, Ciliberti M, Sevi A (2012) Immune competence of the mammary gland as affected by somatic cell and pathogenic bacteria in ewes with subclinical mastitis. J Dairy Sci 95:3877–3887

Albenzio M, Santillo A, Marino R, Della Malva A, Caroprese M, Sevi A (2015) Identification of peptides in functional Scamorza ovine milk cheese. J Dairy Sci 98:8428–8432

Albenzio M, Santillo A, Avondo M, Nudda A, Chessa S, Pirisi A, Banni S (2016) Nutritional properties of small ruminant food products and their role on human health. Small Rumin Res 135:3–12

Ali F, Hussain R, Qayyum A, Gul ST, Iqbal Z, Hassan MF (2016) Milk somatic cell counts and some hemato-biochemical changes in sub-clinical mastitic dromedary she-camels (Camelus dromedarius). Pak Vet J 36:405–408

Alichanidis E, Moatsou G, Polychroniadou A (2016) The composition and the properties of non-cow milks and products. In: Tsakalidou E, Papadimitriou K (eds) Non-bovine milk and milk products. Academic, Cambridge, pp 81–116

Al-Nazawi MH, Homeida AM (2015) Effects of antibacterial drugs on camel milk lysozyme concentration and lactoperoxidase activity. J Camel Pract Res 22:97–99

Altomonte I, Salari F, Licitra R, Martini M (2019) Donkey and human milk: insights into their compositional similarities. Int Dairy J 89:111–118

Andrews AT, Alichanidis E (1975) The acid phosphatases of bovine leucocytes, plasma and the milk of healthy and mastitic cows. J Dairy Res 42:391–400

Angeletti R, Gioacchini AM, Deraglia R, Piro R, Traldi P (1998) The potential of matrix-assisted laser desorption/ionization mass spectrometry in the quality control of water buffalo mozzarella cheese. J Mass Spectrom 33:525–531

Anifantakis EM, Rosakis PS (1983) Alkaline phosphatase activity of sheep's milk and some factors affecting it Egypt. J Dairy Sci 11:173–182

Anjos FD, Machado A, Ferro C, Otto F, Bogin E (1998) Gamma-glutamyltransferase as a marker for the pasteurisation of raw milk. J Food Prot 61:1057–1059

Arnold C (1881) Einige neue Reactionen der Milch. Arch Pharm 219:41–42

Assis G, Roseiro IB, Barbosa M (2000) Determination of alkaline phosphatise activity levels in milk from indigenous Portuguese ewe and goat breeds by the fluorometric method. In: Proceedings of development strategy for the sheep and goat dairy sector, international dairy federation bulletin no 351. International Dairy Federation, Brussels, p 34

Atmani D, Benboubetra M, Harrison R (2004) Goat's milk xanthine oxidoreductase is grossly deficient in molybdenum. J Dairy Res 71:7–13
Atmani D, Banghiani A, Harrison R, Benboubetra M (2005) NADH oxidation and superoxide production by caprine milk oxidoreductase. Int Dairy J 15:1113–1121
Baer A, Ryba I, Farah Z (1994) Plasmin activity in camel milk. LWT Food Sci Technol 27:595–598
Baghiani A, Harrison R, Benboubetra M (2003) Purification and partial characterisation of camel milk xanthine oxidoreductase. Arch Physiol Biochem 111:407–414
Barbosa M (2005) Interest in controlling alkaline phosphatase activity in sheep and goat milks. The future of the sheep, goat dairy sectors (part 3, special issue 0501). IDF-FIL, Brussels, pp 117–127
Benboubetra M, Baghiani A, Atmani D, Harrison R (2004) Physicochemical and kinetic properties of purified sheep's milk xanthine oxidoreductase. J Dairy Sci 87:1580–1584
Bencini R, Pulina G (1997) The quality of sheep milk: a review. Aust J Exp Agric 37:485–504
Benkerroum N (2008) Antimicrobial activity of lysozyme with special relevance to milk. Afr J Biotechnol 7:4856–4867
Bianchi L, Bolla A, Budelli E, Caroli A, Casoli C, Pauselli M, Duranti E (2004) Effect of udder health status and lactation phase on the characteristics of Sardinian ewe milk. J Dairy Sci 87:2401–2408
Bingham EW, Garrer K, Powlem D (1992) Purification and properties of alkaline phosphatase in the lactating mammary gland. J Dairy Sci 75:3394–3401
Brumini D, Criscione A, Bordonaro S, Vegarud GE, Marletta D (2016) Whey proteins and their antimicrobial properties in donkey milk: a brief review. Dairy Sci Technol 96:1–14
Caroprese M, Marzano A, Schena L, Marino R, Santillo A, Albenzio M (2007) Contribution of macro phages to proteolysis and plasmin activity in ewe bulk milk. J Dairy Sci 90:2767–2772
Cartier P, Chillard Y, Paquet D (1990) Inhibiting and activating effects of skim milks and proteose-peptone fractions on spontaneous lipolysis and purified lipoprotein lipase activity in bovine milk. J Dairy Sci 73:1173–1177
Cebo C, Caillat H, Bouvier F, Martin P (2010) Major proteins of the goat milk fat globule membrane. J Dairy Sci 93:868–876
Cebo C, Rebours E, Henry C, Makhzami S, Cosette P, Martin P (2012) Identification of major milk fat globule membrane proteins from pony mare milk highlights the molecular diversity of lactadherin across species. J Dairy Sci 95:1085–1098
Chandan RC, Parry RM, Shahani KM (1968) Lysozyme, lipase and ribonuclease in milk of various species. J Dairy Sci 51:606–607
Chavarri F, Santistebam A, Virto M, de Renobales M (1998) Alkaline phosphatase, acid phosphatase, lactoperoxidase, and lipoprotein lipase activities in industrial ewes' milk and cheese. J Agric Food Chem 46:2926–2932
Chiavari C, Coloretti F, Nanni M, Sorrentino E, Grazia L (2005) Use of donkey's milk for a fermented beverage with lactobacilli. Lait 85:481–490
Chilliard Y (1982) Variations physiologiques desactivite´slipasiques et de la lipolyse spontane´e dans les laits de vache de chevre et de femme: revue bibliographique (suite). Lait 62:126–154
Chilliard Y, Doreau M (1985) Characterization of lipase in mare milk. J Dairy Sci 68:37–39
Chilliard Y, Ferlay A, Rouel J, Lamberet G (2003) A review of nutritional and physiological factors affecting goat milk synthesis and lypolysis. J Dairy Sci 86:1751–1770
Civardi G, Curadi MC, Orlandi M, Cattaneo TMP, Giangiacomo R (2007) Mare's milk: monitoring the effect of thermal treatments on whey proteins stability by SDS-capillary electrophoresis (CE-SDS). Milchwissenschaft 62:32–35
Coburn SP, Mahuren JD, Pauly TA, Ericson KL, Townsend DW (1992) Alkaline phosphatase activity and pyridoxal phosphate concentrations in the milk of various species. J Nutr 122:2348–2353
Collins YF, McSweeney PLH, Wilkinson MG (2003) Lipolysis and free fatty acid catabolism in cheese: a review of current knowledge. Int Dairy J 13:841–866

Coppola R, Salimei E, Succi M, Sorrentino E, Nanni M, Ranieri P, Belli Blanes R, Grazia L (2002) Behaviour of *Lactobacillus rhamnosus* strains in ass's milk. Ann Microbiol 52:55–60

Cosentino C, Paolino R, Valentini V, Musto M, Ricciardi A, Adduci F, D'Adamo C, Pecora G, Freschi P (2015) Effect of jenny milk addition on the inhibition of late blowing in semihard cheese. J Dairy Sci 98:5133–5142

Cosentino C, Labella C, Elshafie HS, Camele I, Musto M, Paolino R, D'Adamo C, Freschi P (2016) Effects of different heat treatments on lysozyme quantity and antimicrobial activity of jenny milk. J Dairy Sci 99:5173–5179

Delacroix-Buchet A, Degas C, Lamembert G, Vassal L (1996) Influence des variants AA et FF de la case´ine a_{s1} caprine sur le rendement fromager et les charact´eristiques des fromages. Lait 76:217–241

Di Luccia A, Picariello G, Trani A, Alviti G, Loizzo P, Faccia M, Addeo F (2009) Occurrence of β-casein fragments in cold-stored and curdled river buffalo (Bubalus bubalis L.) milk. J Dairy Sci 92:1319–1329

Doreau M, Martin-Rosset W (2011) Animals that produce dairy foods. Horse. In: Fuquay JW (ed) Encyclopaedia of dairy sciences. Elsevier, London, pp 358–364

Duhaiman AS (1988) Purification of camel milk lysozyme and its lytic effect on Escherichia coli and *Micrococcus Iysodeikticus*. Comp Biochem Physiol 91B:793–796

Dumitraşcu L, Stănciuc N, Stanciu S, Râpeanu G (2012) Thermal inactivation of lactoperoxidase in goat, sheep and bovine milk – a comparative kinetic and thermodynamic study. J Food Eng 113:47–52

Egito AS, Miclo L, Lopez C, Adam A, Girardet J-M, Gaillard J-L (2002) Separation and characterisation of mares' milk a_{s1}-, b-, k-caseins, g-casein-like, and proteose peptone component 5-like peptides. J Dairy Sci 85:697–706

Egito AS, Girardet J-M, Poirson C, Mollé D, Humbert G, Miclo L, Gaillard J-L (2003) Action of plasmin on equine β-casein. Int Dairy J 13:813–820

El-Agamy EI (2017) Camel milk. In: Park YW, Haenlein GF, Wendorff WL (eds) Handbook of milk of non-bovine mammals. Wiley, Oxford, pp 409–480

Elagamy E, Ruppanner R, Ismail A, Champagne C, Assaf R (1992) Antibacterial and antiviral activity of camel milk protective proteins. J Dairy Res 59:169–175

Elagamy EI, Ruppanner R, Ismail A, Champagne CP, Assaf R (1996) Purification and characterization of lactoferrin, lactoperoxidase, lysozyme and immunoglobulins from camel's milk. Int Dairy J 6:129–145

El-Fakharany EM, Uversky VN, Redwan EM (2017) Comparative analysis of the antiviral activity of camel, bovine, and human lactoperoxidases against herpes simplex virus type 1. Appl Biochem Biotechnol 182:294–310

El-Gazzar H, Rafaie MOI, El-Aziz MA (1999) Activity of xanthine oxidase in milk and its products and effect of heat and folic acid upon its activity. Ann Agric Sci Ain Shams Univ Cairo 44:631–639

El-Salam MA, El-Shibiny S (2011) A comprehensive review on the composition and properties of buffalo milk. Dairy Sci Technol 91:663–699

Fantuz F, Baldi A, Dellorto V, Polidori F, Rossi CS, Pollitis I, Heegaard CW (1998) Distribution of plasminogen activator forms in different fractions of buffalo milk. J Dairy Res 65:521–527

Farkye NY (2003) Other enzymes. In: Fox PF, McSweeney PLH (eds) Advanced dairy chemistry. Elsevier Applied Science, London, pp 571–603

Felfoul I, Jardin J, Gaucheron F, Attia H, Ayadi MA (2017) Proteomic profiling of camel and cow milk proteins under heat treatment. Food Chem 216:161–169

Fox PF (2003) Significance of indigenous enzymes in milk and dairy products. In: Whitaker JR, Voragen AGJ, Wong DWS (eds) Handbook of food enzymology. Marcel Dekker, New York, pp 255–277

Fox PF, Kelly AL (2006) Review indigenous enzymes in milk: overview and historical aspects-part 2. Int Dairy J 16:517–532

Giacometti F, Bardasi L, Merialdi G, Morbarigazzi M, Federici S, Piva S, Serraino A (2016) Shelf life of donkey milk subjected to different treatment and storage conditions. J Dairy Sci 99:4291–4299

Giribaldi M, Antoniazzi S, Gariglio GM, Coscia A, Bertino E, Cavallarin L (2017) A preliminary assessment of HTST processing on donkey milk. Vet Sci 4:50

Gobbetti M, Morea M, Baruzzi F, Corbo MR, Matarante A, Considine T, Di Cagno R, Guinee T, Fox PF (2002) Microbiological, compositional, biochemical and textural characterisation of Caciocavallo Pugliese cheese during ripening. Int Dairy J 12:511–523

Gómez-Ruiz JA, Ramos M, Recio I (2004) Identification and formation of angiotensin-converting enzymes-inhibitory peptides in Manchego cheese by high-performance liquid chromatography-tandem mass spectrometry. J Chromotogr A 1054:269–277

Griffiths MW (1986) Use of milk enzymes as indices of heat treatment. J Food Prot 49:696–705

Hailu Y, Hansen EB, Seifu E, Eshetu M, Ipsen R, Kappeler S (2016) Functional and technological properties of camel milk proteins: a review. J Dairy Res 83:422–429

Hamulv G, Kandasamy C (1982) Increasing the keeping quality of milk by activation of its lactoperoxidase system: results from Sri Lanka. Milchwissenschaft 37:454–457

Harding F, Garry E (2005) Collaborative evaluation of a fluorometric method for measuring alkaline phosphatase activity in cow's, sheep's, and goat's milk. J Food Prot 68:1047–1053

Herrouin M, Mollé D, Fauquant J, Ballestra F, Maubois JL, Léonil J (2000) New genetic variants identified in donkey's milk whey proteins. J Protein Chem 19:105–115

Hofi AA, Mahran GA, Abd El-Hamid LB, Osman SG (1976) Lipase activity in buffalo's milk. 1. Effect of lactation period and individuality. Egypt J Dairy Sci 4:111–114

Humbert G, Chang O, Gaillard JL (2005) Plasmin activity and plasminogen activation in equine milk. Milchwissenschaft 60:134–137

Inglingstad RA, Devold TG, Eriksen EK, Holm H, Jacobsen M, Liland KH, Rucke OE, Vegarud GE (2010) Comparison of the digestion of caseins and whey proteins in equine, bovine, caprine and human milks by human gastrointestinal enzymes. Dairy Sci Technol 90:549–563

Irigoyen A, Izco JM, Ibánez FC, Torre P (2000) Evaluation of the effect of rennet type on casein proteolysis in an ovine milk cheese by means of capillary electrophoresis. J Chromatogr A 881:59–67

Jaeggi JJ, Govindasamy-Lucey S, Berger YM, Johnson ME, McKusick BC, Thomas DL, Wendorff WL (2003) Hard ewes milk cheese manufactured from milk of three different groups of somatic cell count. J Dairy Sci 86:3082–3089

Jaubert G, Bodin JP, Jaubert A (1996) Flavour of goat farm bulk milk. In: Proceedings of the sixth international conference on goats, vol 1. International Academic Publishers, Beijing, pp 66–69

Jauregui-Adell J (1975) Heat stability and reactivation of mare milk lysozyme. J Dairy Sci 58:835–838

Kumari V, Mathur MP (1981) Buffalo milk lysozyme. Indian J Dairy Sci 34:385–390

Kaartinen L, Jensen NE (1988) Use of N-acetyl-beta-glu-cosaminidase to detect teat can inflammations. J Dairy Sci 55:603–607

Kamau PNM, Wangoh J (2010) Use of the lactoperoxidase system to enhance keeping quality of pasteurised camel milk. FoodReview 4:61–63

Kamau PNM, Lamuka P, Wangoh J (2010) Effect of lactoperoxidase-thiocyanate-hydrogen peroxide system and storage temperature on keeping quality of raw camel milk. Afr J Food Agric Nutr Dev 10:4186–4201

Kappeler S (1998) Compositional and structural analysis of camel milk proteins with emphasis on protective proteins. Zurich. Dissertation for the degree of Doctor of Technical Sciences, Swiss Federal Institute of Technology. Diss. ETH No. 12947

Kelly AL, McSweeney PLH (2002) Indigenous proteinases in milk. Adv Dairy Chem 1:494–519

Khalesi M, Salami M, Moslehishad M, Winterburn J, Moosavi-Movahedi AA (2017) Biomolecular content of camel milk: a traditional superfood towards future healthcare industry. Trends Food Sci Technol 62:49–58

Kitchen BJ, Taylor GC, White IC (1970) Milk enzymes – their distribution and activity. J Dairy Res 37:279–288

Klotz V, Hill A, Warriner K, Griffiths M, Odumeru J (2008) Assessment of the colorimetric and fluorometric assays for alkaline phosphatase activity in cow's, goat's, and sheep's milk. J Food Prot 71:1884–1888

Leitner G, Chaffer M, Shamay A, Shapiro F, Merin U, Erza E, Saran A, Silanikove N (2004a) Changes in milk composition as affected by subclinical mastitis in sheep. J Dairy Sci 87:46–52

Leitner G, Merin U, Silanikove N (2004b) Changes in milk composition as affected by subclinical mastitis in goats. J Dairy Sci 87:1719–1726

Liu DK, Williams GH (1982) Species differences in ribonuclease activity of milk and mammary gland. Comp Biochem Physiol Part B Comp Biochem 71:535–538

Lodi R, Todesco R, Carini S (1984) Preliminary study on the use of lysozyme in provolone cheese production. Ind Latte 20:37–50

Lombardi P, Avallone L, D'Angelo A, Mor T, Bogin E (2000) Buffalo-milk enzyme levels, their sensitivity to heat inactivation, and their possible use as markers for pasteurization. J Food Prot 63:970–973

Lorenzen PC, Martin D, Clawin-Rädecker I, Barth K, Knappstein K (2010) Activities of alkaline phosphatase, glutamyltransferase and lactoperoxidase in cow, sheep and goat's milk in relation to heat treatment. Small Rumin Res 89:18–23

Lorenzen PC, Wernery R, Johnson B, Jose S, Wernery U (2011) Evaluation of indigenous enzyme activities in raw and pasteurised camel milk. Small Rumin Res 97:79–82

Madkor SA, Fox PF (1991) Plasmin activity in buffalo milk. Food Chem 39:139–156

Mahdi L, Mahdi N, Al-kakei S, Musafer H, Al-Joofy I, Essa R, Zwain L, Salman I, Mater H, Al-Alak S, Al-Oqaili R (2018) Treatment strategy by lactoperoxidase and lactoferrin combination: Immunomodulatory and antibacterial activity against multidrug-resistant *Acinetobacter baumannii*. Microb Pathog 114:147–152

Maqsood S, Al-Dowaila A, Mudgil P, Kamal H, Jobe B, Hassan HM (2019) Comparative characterization of protein and lipid fractions from camel and cow milk, their functionality, antioxidant and antihypertensive properties upon simulated gastro-intestinal digestion. Food Chem 279:328–338

Marchand S, Merchiers M, Messens W, Coudijzer K, De Block J (2009) Thermal inactivation kinetics of alkaline phosphatase in equine milk. Int Dairy J 19:763–767

Markiewicz-Kęszycka M, Wójtowski J, Kuczyńska B, Puppel K, Czyżak-Runowska G, Bagnicka E, Strzałkowska N, Jóźwik A, Krzyżewski J (2013) Chemical composition and whey protein fraction of late lactation mares' milk. Int Dairy J 31:62–64

Martini M, Altomonte I, Pesi R, Tozzi MG, Salari F (2013) Fat globule membranes in ewes' milk: the main enzyme activities during lactation. Int Dairy J 28:36–39

Martini M, Salari F, Altomonte I, Ragona G, Piazza A, Gori R, Casati D, Brajon G (2018) Effects of pasteurization and storage conditions on donkey milk nutritional and hygienic characteristics. J Dairy Res 85:445–448

Massouras T, Triantaphyllopoulos KA, Theodossiou I (2017) Chemical composition, protein fraction and fatty acid profile of donkey milk during lactation. Int Dairy J 75:83–90

McKellar RC, Emmons DB, Farber J (1991) Gamma-glutamyl transpep-tidase in milk and butter as indicator of heat treatment. Int Dairy J 1:241–251

Medina M, Gaya P, Nunez M (1989) The lactoperoxidase system in ewe's milk: levels of lactoperoxidase and thiocyanate. Lett Appl Microbiol 8:147–149

Merin U, Rosental I, Maltz E (1988) The composition of goat milk as affected by nutritional parameters. Milchwissenschaft 43:363–365

Moatsou G (2010) Indigenous enzymatic activities in ovine and caprine milks. Int J Dairy Technol 63:16–31

Moatsou G, Katsaros G, Bakopanos C, Kandarakis I, Taoukis P, Politis I (2008) Effects of high-pressure treatment at various temperatures on activity of indigenous proteolytic enzymes and denaturation of whey proteins in ovine milk. Int Dairy J 18:1119–1125

Morgan F, Gaspard CE (1999) Influence des cellules somatiques sur les qualities technologiques du lait de chèvre et sur les caractéristiques des fromages de chèvre (Influence of somatic cells

on technological properties of goat milk and on characteristics of goat cheese). Renc Rech Rumin 6:317
Morozova-Roche L (2007) Equine lysozyme: the molecular basis of folding, self-assembly and innate amyloid toxicity. FEBS Lett 581:2587–2592
Murgia A, Scano P, Contu M, Ibba I, Altea M, Bussu M, Demuru M, Porcu A, Caboni P (2016) Characterization of donkey milk and metabolite profile comparison with human milk and formula milk. LWT Food Sci Technol 74:427–433
Manjunath GM, Bhat GS (1992) Effect of processing on native proteinases in milk. J Food Sci Technol 29:195–196
Nájera AI, Barron LJR, Barcina Y (1994) Changes in free fatty acids during the ripening of Idiazabal cheese: influence of brining time and smoking. J Dairy Res 61:281–288
Nega A, Moatsou G (2012) Proteolysis and related enzymatic activities in ten Greek cheese varieties. Dairy Sci Technol 92:57–73
Niro S, Fratianni A, Colavita G, Galassi L, Zanazzi M, Salimei E (2017) Technological use of donkey milk in cheesemaking. Int J Dairy Technol 70:439–442
Ozturkoglu-Budak S (2018) Effect of different treatments on the stability of lysozyme, lactoferrin and-lactoglobulin in donkey's milk. Int J Dairy Technol 71:36–45
Pellegrini O, Remeuf F, Rivemale M, Barillet F (1997) Renneting properties of milk from individual ewes: influence of genetic and non-genetic variables, and relationship with physicochemical characteristics. J Dairy Res 64:355–366
Piga C, Urgeghe PP, Piredda G, Scintu M, Sanna G (2009) Asssessment and validation of methods for the determination of glutamyltransferase activity in sheep milk. Food Chem 115:1519–1523
Pilla R, Daprà V, Zecconi A, Piccinini R (2010) Hygienic and health characteristics of donkey milk during a follow-up study. J Dairy Res 77:392–397
Pinho O, Mendes E, Alves MM, Ferreira IM (2004) Chemical, physical, and sensorial characteristics of "Terrincho" ewe cheese: changes during ripening and intravarietal comparison. J Dairy Sci 87:249–257
Pirisi A, Piredda G, Podda F, Pintus S (1996) Effect of somatic cell count on sheep milk composition and cheesemaking properties. In: Somatic cells and Milk of small ruminants. EAAP publication no. 77. Wageningen Press, Wageningen, pp 245–251
Pirisi A, Piredda G, Corona M, Pes M, Pintus S, Ledda A (2000) Influence of somatic cell count on ewe's milk composition, cheese yield and cheese quality. In: 6th proceedings of Great Lakes dairy sheep symposium, Guelph, Canada. Ontario Dairy Sheep Association, Shelburne, ON, pp 47–59
Polidori P, Vincenzetti S (2010) Differences of protein fractions among fresh, frozen and powdered donkey milk. Recent Pat Food Nutr Agric 2:56–60
Politis I, Lachance E, Block E, Turner JD (1989) Plasmin and plasminogen in bovine milk: a relationship with involution. J Dairy Sci 72:900–906
Politis I, Zhao X, McBraid BW, Burton JH, Turner JD (1991) Plasminogen activator production by bovine milk macrophages and blood monocytes. Am J Vet Res 52:1208–1213
Priyadarshini S, Kansal VK (2002a) Lysozyme activity in buffalo milk: effect of lactation period, parity, mastitis, season in India, pH and milk processing heat treatment. Asian-Aust J Animal Sci 15:895–899
Priyadarshini S, Kansal VK (2002b) Purification, characterization, antibacterial activity and Nterminal sequencing of buffalo-milk lysozyme. J Dairy Res 69:419–431
Priyadarshini S, Kansal VK (2003) Biochemical characterization of buffalo (Bubalusbubalis)milk lysozyme. J Dairy Res 70:467–472
Ragona G, Corrias F, Benedetti M, Paladini I, Salari F, Altomonte I, Martini M (2016) Amiata donkey milk chain: animal health evaluation and milk quality. Italian J Food Saf 5:173–178
Raynal-Ljutovac K, Park YW, Gaucheron F, Bouhallab S (2007) Heat stability and enzymatic modifications of goat and sheep milk. Small Rumin Res 68:207–220
Rebucci R, Fusi E, Pecorini L, Pinotti L, Cheli F, Baldi A (2005) Evaluation of the biological activation of plasmin plasminogen system in sheep and goat milk. Italian J Anim Sci 4:330–332

Redwan EM, Almehdar HA, El-Fakharany EM, Baig A-WK, Uversky VN (2015) Potential antiviral activities of camel, bovine, and human lactoperoxidases against hepatitis C virus genotype 4. RSC Adv 5:60441–60452

Revilla I, Rodríguez-Nogales GM, Vivar-Quintana AM (2007) Proteolysis and texture of hard ewe's milk cheese during ripening as affected by somatic cell count. J Dairy Res 74:127–136

Roseiro de Bivar ML, Barbosa M (1995) Phosphatase activity levels in pasteurized goat's milk. J Soc Dairy Technol 48:9–12

Ryskaliyeva A, Henry C, Miranda G, Faye B, Konuspayeva G, Martin P (2018) Combining different proteomic approaches to resolve complexity of the milk protein fraction of dromedary, Bactrian camels and hybrids, from different regions of Kazakhstan. PLoS One 13(5):e0197026

Saadaoui B, Henry C, Khorchani T, Mars M, Martin P, Cebo C (2013) Proteomics of the milk fat globule membrane from Camelus dromedarius. Proteomics 13:1180–1184

Saeman AI, Verdi RJ, Galton DM, Barbano DM (1988) Effect of mastitis on proteolytic activity in bovine milk. J Dairy Sci 71:505–512

Salimei E, Fantuz F (2012) Equid milk for human consumption. Int Dairy J 24:130–142

Salimei E, Fantuz F (2013) Horse and donkey milk. In: Park YW, Haenlein GFW (eds) Milk and dairy products in human nutrition. Wiley-Blackwell, Oxford, pp 594–612

Sanhotra M, Dutta SM (1986) Effect of cold storage, heating and homogenization on xanthine oxidase activity in buffalo milk. Indian J Dairy Sci 39:423–425

Santillo A, Albenzio M (2008) Influence of lamb rennet paste containing probiotic on proteolysis and rheological properties of pecorino cheese. J Dairy Sci 91:1733–1742

Santillo A, Kelly AL, Palermo C, Sevi A, Albenzio M (2009) Role of indigenous enzymes in proteolysis of casein in caprine milk. Int Dairy J 19:655–660

Santillo A, Ruggieri D, Albenzio M (2011) Caratterizzazione chimica, enzimatica, e microbiologica del latte e della mozzarella di bufalo prodotti in provincia di Foggia. Sci Tec Latt Casearia 62:377–386

Šarić LC, Šarić BM, Mandić AI, Kevrešan ZS, Ikonić BB, Kravić SZ, Jambrec DJ (2014) Role of calcium content in antibacterial activity of donkeys' milk toward *E. coli*. Eur Food Res Technol 239:1031–1039

Seifu E, Buys EM, Donkin EF (2005) Significance of the lactoperoxidase system in the dairy industry and its potential applications: a review. Trends Food Sci Technol 16:137–154

Shakeel-Ur-Rehman, Fleming CM, Farkye NY, Fox PF (2003) Indigenous phosphatases in milk. In: Fox PF, McSweeney PLH (eds) Advanced dairy chemistry. Elsevier Applied Science, London, pp 523–543

Sharma R, Kaur S, Rajput YS, Kumar R (2009) Activity and thermal stability of indigenous enzymes in cow, buffalo and goat milk. Milchwissenschaft 64:173–175

Sheikh IA, Jiffri EH, Ashraf GM, Kamal MA, Beg MA (2018) Structural studies on inhibitory mechanisms of antibiotic, corticosteroid and catecholamine molecules on lactoperoxidase. Life Sci 207:412–419

Sheikh IA, Jiffri EH, Ashraf GM, Kamal MA (2019) Structural insights into the camel milk lactoperoxidase: homology modeling and molecular dynamics simulation studies. J Mol Graph Model 86:43–51

Shori AB (2015) Camel milk as a potential therapy for controlling diabetes and its complications: a review of in vivo studies. J Food Drug Anal 23:609–618

Singh R, Mal G, Kumar D, Patil NV, Pathak KML (2017) Camel milk: An important natural adjuvant. Agric Res 6:327–340

Souroullas K, Aspri M, Papademas P (2018) Donkey milk as a supplement in infant formula: benefits and technological challenges. Food Res Int 109:416–425

Srivastava A, Mathur MP, Mudgel VD (1989) Influence of feeding protected fat to buffaloes on some enzymes in milk. Indian J Dairy Sci 42:354–355

Sutherland BJ (2003) Salting of cheese. In: Roginski H, Fuquay JW, Fox PF (eds) Encyclopedia of dairy sciences. MPG Books, Bodmin

Salimei E, Park YW (2017) Mare milk. In: Park YW, Haenlein GFW, Wendorff WL (eds) Handbook of Milk of Non-Bovine Mammals: Second Edition. John Wiley & Sons Ltd, Oxford, pp 369-408

Tayefi-Nasrabadi H, Asdpour R (2008) Effect of heat treatment on buffalo (Bubalus bubalis) lactoperoxidase activity in raw milk. J Biol Sci 8:1310–1315

Tayefi-Nasrabadi H, Hoseinpour-fayzi MA, Mohasseli M (2011) Effect of heat treatment on lactoperoxidase activity in camel milk: a comparison with bovine lactoperoxidase. Small Rumin Res 99:187–190

Theodorou G, Kominakis A, Rogdakis E, Politis I (2007) Factors affecting the plasmin-plasminogen system in milk obtained from three Greek dairy sheep breeds with major differences in milk production capacity. J Dairy Sci 90:3263–3269

Tidona F, Sekse C, Criscione A, Jacobsen M, Bordonaro S, Marletta D, Vegarud GE (2011) Antimicrobial effect of donkeys' milk digested in vitro with human gastrointestinal enzymes. Int Dairy J 21:158–165

Tidona F, Criscione A, Devold TG, Bordonaro S, Marletta D, Vegarud GE (2014) Protein composition and micelle size of donkey milk with different protein patterns: effects on digestibility. Int Dairy J 35:57–62

Trieu-Cuot P, Addeo F (1981) Occurrence of gamma casein in buffalo milk. J Dairy Res 48:311–317

Uniacke-Lowe T, Huppertz T, Fox PF (2010) Equine milk proteins: chemistry, structure and nutritional significance. Int Dairy J 20:609–629

Vamvakaki AN, Zoidou E, Moatsou G, Bokari M, Anifantakis E (2006) Residual alkaline phosphatase activity after heat treatment of ovine and caprine milk. Small Rumin Res 65:237–241

Vincenzetti S, Polidori P, Mariani P, Cammertoni N, Fantuz F, Vita A (2008) Donkey's milk protein fractions characterization. Food Chem 106:640–649

Vincenzetti S, Pucciarelli S, Polzonetti V, Polidori P (2017) Role of proteins and of some bioactive peptides on the nutritional quality of donkey milk and their impact on human health. Beverages 3:34

Vincenzetti S, Cecchi T, Perinelli DR, Pucciarelli S, Polzonetti V, Bonacucina G, Ariani A, Parrocchia L, Spera DM, Ferretti E, Vallesi P, Polidori P (2018) Effects of freeze-drying and spray-drying on donkey milk volatile compounds and whey proteins stability. LWT Food Sci Technol 88:189–195

Visser S (1993) Proteolytic enzymes and their relation to cheese ripening and flavour: an overview symposium: proteolytic enzymes and cheese ripening. J Dairy Sci 76:329–350

Wernery U, Maier U, Johnson B, George RM, Braun F (2006) Comparative study on different enzymes evaluating heat treatment of dromedary milk. Milchwissenschaft 61:281–285

Wernery U, Fischbach S, Johnson B, Jose S (2008) Evaluation of alkaline phosphatase (ALP), γ-glutamyl transferase (GGT) and lactoperoxidase (LPO) activities for their suitability as markers of camel milk heat inactivation. Milchwissenschaft 63:265–267

Wernery U, Wernery R, Masko O, Johnson B, Gnanaraj B, Jose S, Nagy P, Lorenzen PC (2013) Lactoperoxidase: a suitable enzymatic marker of camel milk pasteurization. J Camel Pract Res 20:35–38

Wilińska A, Bryjak J, Illeová V, Polakovič M (2007) Kinetics of thermal inactivation of alkaline phosphatase in bovine and caprine milk and buffer. Int Dairy J 17:579–586

Ying C, Wang HT, Hsu JT (2002) Relationships of somatic cell count, physical, chemical and enzymatic properties to the bacterial standard plate count in dairy goat milk. Livestock Prod Sci 74:63–77

Yoganandi J, Jain AK, Mehta BM, Wadhwani KN, Aparnathi KD (2014) Comparative studies on selected enzymes activities of camel, cow and buffalo milk. J Camel Pract Res 21:249–252

Younan M, Farah Z, Galett V (2006) Application of the activated lactoperoxidase-thiocyanate-hydrogen peroxide system to improve the storage of raw camel milk. Milchwissenschaft 61:391–394

Zamora A, Guamis B, Trujillo AJ (2009) Protein composition of caprine milk fat globule membrane. Small Rumin Res 82:122–129