Plasmin activity in Manchega ewe milk: the effect of lactation, parity and health of the udder, and its influence on milk composition and rennet coagulation.

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Abstract

Milk from 40 Manchega ewes was collected monthly and analysed during a complete lactation (5 months). Milk samples were classified by their origin in 3 categories, termed PR (primiparous ewes), M1 (multiparous ewes with no damage of the udder in the previous lactation) and M2 (multiparous ewes with udder damage in the previous lactation). The influence on plasmin of several factors as stage of lactation, parity, somatic cell count and udder health status was studied, as well as the effect of plasmin activity on milk composition and rennet coagulation.

Plasmin activity decreased throughout lactation but was not affected by parity or somatic cell count (P>0.05). A strong negative correlation was found between plasmin activity and protein (especially casein), presumably due to the proteolysis of ß-casein. Plasmin also worsened rennet coagulation, increasing rennet clotting time (RCT) and negatively affecting curd firmness (A_{60}), especially in multiparous ewes. However, the good health condition in the herd may have camouflaged some effects of plasmin over renneting.

Keywords: Dairy sheep; Plasmin; Udder health; Rennet coagulation.

1. Introduction

Proteolysis potentially affects all dairy products (Saint-Denis et al., 2001). This results in a lower quality of the products, the development of bitter flavours in UHT milks, a decrease in cheese yield and a degradation of caseins during storage and ripening (Mara et al., 1998).

Plasmin is an alkaline serine proteinase (Bastian and Brown, 1996). This enzyme is present in milk as its zymogen plasminogen, which activates to plasmin when somatic cell counts exceed 500×10^3 cells/ml. Milk contains the complete plasmin system: plasmin (PL), plasminogen (PG) and a complex structure of plasminogen activators (PA), plasminogen activator inhibitors (PAI) and plasmin inhibitors (PI) (Ismail et al., 2006; Silanikove et al., 2013; White et al., 1995). Both PA and PAI are known to be locally produced by mammary epithelial cells in the mammary gland (Heegard et al., 1994).

The PL system plays an important role in the breakdown of casein, reducing cheese yield and casein content due to the leakage of proteose-peptones into whey (Albenzio et al., 2009). Plasmin mainly attacks β-CN, α_{S2}-CN and α_{S1}-CN (susceptible in that particular order). However, κ-CN seems to resist its action, though some experiments have reported that it can be affected under certain conditions (Groves et al., 1998). An increase of plasmin activity in bovine milk has been described due to udder infections or advanced stages of lactation (Bastian et al., 1991; Politis et al., 1989). On the other hand, results in literature regarding the behavior of plasmin in ovine milk are often controversial (Theodorou et al. 2007). However, most authors have evidenced low levels of plasmin activity in late lactation ewe milk (Albenzio et al., 2009; Koutsouli et al., 2015). REFERENCIA A QUE DEPENDE DE LA RAZA.

In cheese, plasmin-induced proteolysis can contribute to the development of flavour and texture during ripening (Ismail and Nielsen, 2010). Meanwhile, other authors associated an intense plasmin activity as a cause of a development of bitter peptides (Habibi-Najafi and Lee, 1996; Sousa et al., 2001). This seems to be more frequent in high-cooked cheese varieties (Fox and Kelly, 2006).

In milk (whether raw, pasteurized or UHT), to the contrary, proteolysis is the cause of undesirable effects. In severe cases, casein hydrolysis induced by plasmin may greatly affect rennet coagulation (Albenzio et al., 2005; Srinivasan and Lucey, 2002). This represents an important issue concerning the dairy sheep industry, as almost all milk production is intended for cheesemaking. Thus, proteolysis causes a reduction of the processing capacity of milk into cheese, as well as changes in its composition and the development of bitter flavours in processed products

(Guerrero et al., 2003).

Controlling plasmin activity in sheep's milk could lead to an improvement of quality in the dairy industry, perhaps also reducing production costs. Therefore, proteolysis induced by this enzyme has attracted strong interest from researchers, due to its complexity and versatile effects over the quality of milk and dairy products (Ismail and Nielsen, 2010). PÁRRAFO DE MANCHEGA. The aim of this study is to determine the influence of several inherent factors on plasmin activity and to evaluate the impact of plasmin on Manchega milk quality.

2. Materials and methods

2.1. Animal sampling

Milk from 40 Manchega ewes was collected throughout a whole lactation (5 months). All animals belonged to the farm La Nava Del Conejo (Valdepeñas, Castilla-La Mancha, Spain), included in the *Spanish National Association of Manchega Sheep Breeders (AGRAMA)*. Ewes were classified in 3 groups, according to their udder health status in the previous lactation: PR (primiparous ewes), M1 (multiparous ewes with no damage of the udder in the previous lactation) and M2 (multiparous ewes with udder damage in the previous lactation). Samples were divided in 4 aliquots and analysed for milk composition, rennet coagulation, somatic cell count (SCC) and plasmin activity (PL).

2.1. Milk composition and somatic cell count

Major milk components (fat, crude protein, lactose, total solids, casein and urea) were directly measured using a Milko-Scan FT-6000 (*Foss Electric*, Hillerød, Denmark). To determine ash, milk samples (10 ml) were pipetted into crucibles and dried in a laboratory oven for 24 hours to obtain total solids, and then were transferred to a muffle furnace for 2 hours at 550ºC. Finally, crucibles were stored in a desiccator with silica gel for around 30-45 minutes, and weighted to calculate ash content.

SCC was measured using a Fossomatic FC (*Foss Electric*, Hillerød, Denmark) and expressed as cells/ml. A logarithmic transformation was applied to SCC values and milk samples were categorized in 3 groups: low SCC ($< 1.6\times10^3$), mid SCC ($1.6\times10^3 < \text{SCC} < 2\times10^3$) and high SCC ($> 2\times10^3$).

2.3. Rennet coagulation

Samples were preheated at 32ºC and renneting parameters were monitored using a Formagraph viscometer (Foss Electric, Hillerød, Denmark), based on the oscillatory motion of circular pendula immersed in milk during coagulation. The testing time of the analysis was set to 60 minutes and the measured parameters were rennet clotting time (RCT), curd firming time (k_{20}), and curd firmness after 30 and 60 minutes (A_{30} and A_{60}). To measure curd yield, curds were individually placed in centrifuge tubes, cut with a spatula and centrifuged (30 minutes, 2800 × g, 37ºC) to separate the whey.

2.4. Plasmin activity

Plasmin activity was determined according to the procedure described by Richardson and Pearce (1981), with slight modifications. Plasmin cleaves the peptide *N*-Succinyl-L-alanyl-L-phenylalanyl-L-lysyl-7-amino-4-methyl coumarin, and releases 7-amino-4-methyl coumarin (*AMC*), which can be quantified spectrofluorometrically. Milk (3 ml) was added to 1 ml 0.4M trisodium citrate and centrifuged (29,000 × g, 20 min, 4°C). The supernatant and 50 µl of filtrate were added to 825 µl 50mM tris-HCl buffer pH 7.5 and incubated for 5 min at room temperature. Reaction was initiated by adding to the mixture 225 µl substrate (5 mg coumarin peptide in 1.33 ml dimethylsulphoxide and 5.33 ml 50mM tris-HCl buffer, pH 7.5), and fluorescence intensity (380 nm excitation, 460 nm emission) was measured at 5 min intervals over a period of 35 min. Plasmin activity was determined from the linear part of the fluorescence emission versus time curve. One unit of plasmin activity was defined as the activity necessary to release 1 nmol *AMC* min^{-1} ml^{-1} milk under the conditions of the assay.

2.5. Statistical analysis

Data were analysed using the GLM procedure of SAS 9.1 (SAS Institute Inc., Cary, NC). A first statistical analysis of plasmin activity (PL) included stage of lactation (SL), parity (PAR), and their interaction as fixed effects. A second analysis included SCC group, udder health status in the previous lactation (UHS), and their interaction as fixed effects. Contrast analysis was performed to compare least squares means, and statistical significance was declared at P<0.05. In addition, linear correlations were calculated between PL and milk composition, and also between PL and rennet coagulation variables.

3. Results and discussion

3.1. Effect of stage of lactation (SL) and parity (PAR) on plasmin activity

Figure 1 represents the global average of plasmin activity throughout lactation. As lactation advanced, plasmin activity in the flock decreased, and was significantly lower in the last month of lactation than in the beginning.

Considering three stages in lactation (early, mid, and late), a gradual decrease in plasmin activity with the course of lactation was observed in all samples (Table 1). The lowest values of plasmin activity were found in late lactation, although this drop was only significant in multiparous ewes (Table 2).

Although no big differences were found, the high plasmin activity recorded in the beginning of lactation, as well as its decreasing trend, agree with the results obtained in Comisana ewes by Albenzio et al. (2009, 2005, 2004), Caropresе et al. (2007), and in different greek breeds by Koutsouli et al. (2015) and Theodorou et al. (2007), who measured the lowest plasmin values in late lactation milk. Contrastingly, several studies performed on bovine milk (Bastian and Brown, 1996; Ismail and Nielsen, 2010) and milk from other Italian ewe breeds (Bianchi et al., 2004; Sevi et al., 2004) reported a different pattern in plasmin activity, where the highest values were generally recorded in the end of lactation. Politis et al. (1989) suggested that this increase of plasmin activity observed in dairy cows during late lactation is due to an increase of the activation rate of plasminogen to plasmin, rather than to a higher influx of plasmin to the mammary gland. Recent works have discussed that this presence of plasminogen in milk is probably due to a transcellular route of passage from blood (Silanikove et al., 2016). Other authors related the increase of plasmin in late lactation with the involution of the udder (Koutsouli et al., 2015) or an increase of milking interval (Castillo et al., 2008; Kelly et al., 1998). Some experiments performed with goat milk also reported an increase of plasmin activity throughout lactation (Cortellino et al., 2006).

No significant effect of parity was found, although most of the studies performed in different species of domestic ruminants reported that plasmin activity increased with parity and age of the animals (Bastian and Brown, 1996; Battacone et al., 2005; Koutsouli et al., 2015).

3.2. Effect of somatic cell count and udder health status on plasmin activity

Table 3 shows the least square means of plasmin activity as affected by SCC and udder health status category (UHS). Table 4 compares plasmin activity means between different SCC ranges.

Although preliminary results from studies performed with Manchega ewes suggested an increase of casein breakdown due to the amount of somatic cells in milk (Caballero-Villalobos et al., 2015), plasmin activity in the present study was found similar in all UHS categories, despite variations in SCC. This agrees with the results published by Bianchi et al. (2004) for Sardinian ewes and Koutsouli et al. (2015) for Greek sheep breeds, who reported that SCC did not seem to affect plasmin activity. Albenzio et al. (2009) reported that in healthy ewes with SCC < 600×10^3 cells/ml, the plasmin system was not affected, which is consistent with the average values of SCC obtained in the present study ($\approx230\times10^3$ cells/ml). Other authors found that plasmin activity increased with SCC (Battacone et al., 2005; Leitner et al., 2004, Theodorou et al. 2007). Furthermore, experiments performed in Manchega ewes (Castillo et al., 2008) found a positive correlation between plasmin and somatic cells in milk samples with very low SCC (175×10^3 cells/ml), supporting the hypothesis that the influence of SCC over the plasmin-plasminogen system may not follow a specific pattern. Theodorou et al. (2007) suggested that these mixed results may be due to variations between breeds and the different methods used to measure plasmin. However, SCC is not the only variable for predicting PL evolution in milk, as PL activity is affected by a complex network of molecular interactions between enzyme activators and inhibitors (Albenzio et al., 2009).

Results from the present study also suggest that the levels of plasmin in Manchega milk are affected by the sanitary conditions of the udder in the previous lactation. According to UHS categories, in low SCC milk, plasmin activity in M2 was significantly higher than in PR, and also tend to be higher than in M1. In high SCC milk, plasmin activity in M2 was also higher than in PR, but in this case, similar to M1.

3.3. Influence of plasmin on milk composition

Table 5 presents the correlation coefficients for plasmin activity and milk composition variables in PR, M1 and M2.

No correlation was found between plasmin activity and fat concentration, which agrees with Koutsouli et al. (2015), who reported that in greek ewe breeds plasmin did not seem to affect fat content in milk.

Regarding milk proteins, there was a strong negative correlation between plasmin and casein content, which diminished as plasmin activity increased, especially in multiparous ewes, regardless of their clinical history. Average values for plasmin activity and casein content were 1.05 units/ml and 4.85%, respectively. Despite that some authors did not find an effect of plasmin on levels of casein (Albenzio et al., 2004), and others have found a positive correlation (Baldi et al., 1996; Bianchi et al., 2004), most of the references found in literature have reported similar results to those obtained in the present study (Jaeggi et al., 2003; Leitner et al., 2004; Politis and Ng Kwai Hang, 1989). Numerous authors have related this negative correlation with the proteolytic action of plasmin on β-CN (Leitner et al., 2004; Moatsou, 2010; Nielsen, 2002). Thus, this could also explain the negative correlation between plasmin activity and crude protein observed in PR and M2. In this case, the effect of proteolysis would not be so evident, as the measurement involves total protein and not only casein. This might prove why some recent works have not found a clear decrease in crude protein as plasmin activity increased (Koutsouli et al., 2015). However, the results found in the literature are often controversial, as other authors have revealed a positive correlation between plasmin and crude protein. Proteolysis in low SCC milk seems to be dominated by the action of plasmin. However, as SCC increases, the relative significance of plasmin decreases, while the relative activity of other indigenous and microbial enzymes increases (Albenzio et al., 2005; Kelly et al., 2006; Santillo et al., 2009). Therefore, the further research on other indigenous enzymes should be considered to explain more clearly the effect of proteolysis in milk.

Although some authors have reported a negative correlation between lactose and plasmin activity in Sarda and Assaf breeds (Battacone et al., 2005; Leitner et al., 2004), no correlation has been found in this study for Manchega ewes.

There was a relatively slight correlation between plasmin activity and total solids, probably due to the already mentioned cleavage of casein. Meanwhile, ash content seemed to increase along with plasmin activity, but no references were found in literature to help explain this finding.

Lastly, there seemed to be an increase of pH with plasmin activity in PR ewes. Some authors, such as Battacone et al. (2005) reported a correlation between plasmin activity and pH, although others did not find an effect of plasmin on the native pH of milk (Koutsouli et al., 2015).

3.4. Influence of plasmin on rennet coagulation

Table 6 presents the correlation coefficients for plasmin activity and the renneting parameters of milk in PR, M1 and M2 ewes.

In general, rennet coagulation of milk worsens as plasmin activity increases. This is evident specially in milk from M2, since plasmin activity in this category is significantly higher than in the rest. Therefore, in M2, rennet clotting time increased and curd firmness diminished, which agrees with the results obtained by Albenzio et al. (2004), Battacone et al., (2005) and Mara et al. (1998). According to Battacone et al. (2005), the worsening of rennet coagulation parameters is more related to disorders of permeability in the mammary gland than to casein breakdown by proteolysis. However, as previously mentioned, there were no effects of plasmin over lactose that could imply alterations of the milk-blood barrier. Due to all these reasons, the relationship between casein hydrolysis and rennet coagulation needs to be further investigated in greater detail.

On the other hand, Leiber et al. (2005) and Srinivasan and Lucey (2002) found that, normally, an increase in plasmin activity negatively affected renneting parameters and curd yield. Nevertheless, other authors did not find a clear correlation between plasmin and rennet coagulation properties of milk. However, most of the experiments found in literature concerning plasmin activity describe addition of different plasmin concentrations to milk, to establish different levels or ranges of activity (Mara et al., 1998). Thus, several authors have reported that high levels of plasmin induce casein hydrolysis, affecting milk coagulation. However, in experiments performed on native raw milk, plasmin levels have been reported to be lower. This suggests that, although there was some impact of plasmin on renneting parameters in M2 ewes, in the rest of categories this effect might not be so evident at native concentrations of the enzyme.

In addition, Bastian et al. (1991) found that when SCC did not exceed 300×10^3 cells/ml, as in the animal population studied in this experiment, plasmin levels remained relatively low, and no further correlation between plasmin activity and renneting properties was found. This might certainly explain the low impact of plasmin on milk coagulation in PR and M1.

Conclusions

In Manchega sheep, plasmin activity decreased throughout lactation, and its highest values were measured in ewes that suffered udder infections in the previous lactation. Therefore, regardless the health condition of the animals in the beginning of lactation, there seems to be a residual enzymatic activity persisting as a response to a previous infection.

In addition, in ewes with a previous udder infection, plasmin activity had a negative impact on rennet coagulation, probably due to casein breakdown. In the rest of the animals this effect was lighter. However, the good health condition of the herd (reflected by the low somatic cell counts measured) seems to camouflage the possible effects of plasmin on rennet coagulation.

Conflict of interest

The authors declare no conflict of interest.

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Figure 1. Plasmin activity global average during lactation.

(See attached file).

Table 1. Plasmin activity (least squares means) as determined by stage of lactation (SL) and parity (PAR).

	EARLY LACT.		**MID LACT.**		**LATE LACT.**		**SEM**	**P**		
	PRIM	MULTI	PRIM	MULTI	PRIM	MULTI		SL	PAR	SL x PAR
PL (u/ml)	0.76	1.69^{a}	0.71	1.08^{ab}	0.46	0.83^{b}	0.070	0.313	0.076	0.692

Within each PAR group, means without a common superscript are statistically different at $P<0.05$

Table 2. Contrast analysis comparing plasmin activity among different stages of lactation (SL) in primiparous and multiparous ewes.

	Early×Mid	**Early×Late**	**Mid×Late**
Primiparous	0.935	0.640	0.699
Multiparous	0.097	0.036*	0.545

An asterisk indicates significant differences at P<0.05

Table 3. Plasmin activity (least squares means) according to somatic cell count (SCC) and udder health status (UHS).

	LOW SCC			**MID SCC**			**HIGH SCC**			**SEM**	**P**		
	PR	M1	M2	PR	M1	M2	PR	M1	M2		SCC	UHS	SCC x UHS
PL (u/ml)	0.76^{a}	0.90^{ab}	1.44^{b}	0.61	0.94	1.07	0.64^{a}	1.23^{b}	1.29^{b}	0.070	0.562	0.003	0.761

Within each SCC category, means without a common superscript are statistically different at P<0.05

PR = primiparous ewes.

M1 = multiparous ewes with no damage of the udder in the previous lactation.

M2 = multiparous ewes with udder damage in the previous lactation.

Table 4. Contrast analysis comparing plasmin activity throughout different somatic cell count (SCC) ranges in all udder health status (UHS) categories.

	Low×Mid	**Low×High**	**Mid×High**
PR	0.628	0.747	0.925
M1	0.913	0.320	0.430
M2	0.151	0.517	0.376

An asterisk indicates significant differences at $P<0.05$

PR = primiparous ewes.

M1 = multiparous ewes with no damage of the udder in the previous lactation.

M2 = multiparous ewes with udder damage in the previous lactation.

Table 5. Correlation between plasmin activity and milk composition.

	PR	**M1**	**M2**
Fat	-0.1863	-0.0789	-0.1274
Crude protein	-0.2158*	-0.0056	-0.1593*
Total solids	-0.2054	-0.0942	-0.1527*
Lactose	0.1752	0.061	0.0979
Casein	-0.2539*	-0.4333***	-0.4442***
Ash	-0.1127	0.3237**	0.1024***
Urea	-0.0389	0.1674	0.0871
Native pH	0.3845*	0.1974	0.4968

* P<0.05 ; ** P<0.01 ; *** P<0.001

PR = Primiparous ewes.

M1 = multiparous ewes with no damage of the udder in the previous lactation.

M2 = multiparous ewes with udder damage in the previous lactation.

Table 6. Correlation between plasmin activity and rennet coagulation variables.

	PR	**M1**	**M2**
RCT	0.0914	0.0296	0.1685*
k_{20}	-0.0857	-0.0073	0.137
RCT + k_{20}	0.0587	0.0223	0.1928*
A_{30}	-0.1907	-0.0037	-0.1731*
A_{60}	-0.0136	-0.3006**	-0.214**
Curd yield	-0.2785**	0.26*	0.0429

* P<0.05 ; ** P<0.01 ; *** P<0.001

PR = Primiparous ewes.

M1 = multiparous ewes with no damage of the udder in the previous lactation.

M2 = multiparous ewes with udder damage in the previous lactation.