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Additional Information

1   **JDR4151 proof to ECN**

2   **Title: Composition, proteolysis indices and coagulating properties of ewe milk as**

3   **affected by bulk tank somatic cell count**

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15   **Shortened version of the title (heading):** BULK TANK SCC and EWE MILK

16   QUALITY

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19   **Summary**

20   The aim of this study was to assess the effect of ovine bulk tank somatic cell count

21   (BTSCC) on composition, proteose-peptone (p-p) content and casein fractions as

22   indicating parameters for proteolysis and coagulating properties of milk. A total of 97

23   samples of bulk tank milk from Manchega breed ewe herds were grouped according to

24   somatic cell count (SCC) into four classes: fewer than 500000 cells/ml, from 500000 to

25   1000000 cells/ml, from 1000000 to 1500000 and more than 1500000 cells/ml. The

26 casein:protein ratio and lactose content decreased with BTSCC. Proteolysis increased

27 with BTSCC, causing a drop in β-casein and an increase in the γ-caseins from a

28 concentration of 500000 cells/ml. Regarding coagulation behaviour, the rennet clotting

29 time (RCT) and firming time (k_{20}) rose from 1000000-1500000 cells/ml milk. The

30 results showed that the impairment of milk quality and milk ability to make cheese as

31 affected by an intramammary infection may be extended to the bulk tank milk of herds

32 with poor udder sanitary condition.

33 **Keywords:** ewe bulk tank milk, somatic cell count, proteolysis, milk coagulating

34 parameters.

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36   In dairy sheep, high somatic cell count (SCC) levels have been shown to be mainly

37 the consequence of an inflammatory process due to the presence of an intramammary

38 infection (IMI) (Gonzalo et al. 2002; Berthelot et al. 2006; Paape et al. 2007), as well as

39 of non-pathological conditions due to physiological or environmental factors (Raynal-

40 Ljutovac et al. 2007; Arias et al. 2012). The variation factors of SCC in bulk tank milk

41 have been studied in dairy ewes flocks and BTSCC has been considered as a useful tool

42 for monitoring udder health in dairy ewe herds and as a basis for milk payment schemes

43 (Gonzalo et al. 2005, 2006, 2010).

44   Previous studies have shown that an increase in ovine SCC is related to important

45 milk yield losses and changes in the composition of milk (Gonzalo et al. 2002; Leitner

46 et al. 2003; Martí-De Olives et al. 2013), higher milk proteolysis activity (Bianchi et al.

47 2004; Leitner et al. 2004; Martí-De Olives et al. 2011), and lower quality of dairy

48 products (Raynal-Ljutovac et al. 2007; Leitner et al. 2008). Regarding changes in

49 protein fraction, high SCC is generally accompanied by an increase in the concentration

50 of proteins from blood because of the higher permeability of the blood–milk barrier

during an IMI, which are not relevant to the dairy industry (Bianchi et al. 2004; Martí-De Olives et al. 2013).

Plasmin (PL) appears to be the major enzyme involved in sheep milk proteolytic phenomena associated with udder inflammation (Bianchi et al. 2004; Leitner et al. 2004). According to Silanikove et al. (2006), β-CN is the preferred substrate for PL and its hydrolysis results in the production of γ-caseins and proteoses-peptones (p-p). Previous research has shown that the higher ovine milk proteolysis activity due to IMI involves a greater content of p-p and higher percentage of γ-caseins, as well as a decrease in β-casein percentage and consequently a higher proteolysis index (PI), defined as the relative proportion of γ-CN to $(\alpha + \beta + \kappa)$-CN (Bianchi et al. 2004). According to Martí-De Olives et al. (2011), this effect of IMI on proteolysis is reflected in a close relationship between individual SCC and the amount of p-p and a group of casein hydrolysis products analogous to bovine γ-caseins. As the cheese-making quality of milk depends, among other factors, on the concentration of intact casein (Bianchi et al. 2004; Leitner et al. 2004; Albenzio et al. 2009), high proteolysis activity by plasmin and other endogenous proteolytic enzymes from somatic cells, such as elastase and cathepsin D, impairs coagulation properties of milk; that is, a longer rennet coagulation time and weak coagulum (Albenzio et al. 2004, 2009). In general, the poor coagulation properties lead to increased curd yield loss (Leitner et al. 2004, 2008).

Bulk tank parameters have been the target of different legal limits or payment-by-quality schemes proposed by different regions, with obvious repercussion on milk marketing (Directive 94/71/EEC; European Union, 1994). However, BTSCC values in ewe milk used for dairy products sold in the European Union has yet to be regulated. Three sanitary herd categories have been proposed relating to the BTSCC in ovine: good (BTSCC<500000), average (BTSCC between 500000 and 1000000) and bad

76 (BTSCC>1000000) (Ariznabarreta, 1999). On the other hand, Sevi et al. (1999)

77 suggested a threshold of 700000 cells/ml for bulk ewe milk of high microbial quality

78 and renneting ability. Research is still needed to study ewe milk quality parameters in

79 relation to SCC at bulk tank level, especially in terms of proteolytic activity and cheese-

80 making ability. BTSCC thresholds would be useful to differentiate ewe milk on the

81 basis of its overall quality.

82 To determine the extent to which impairment of milk quality due to IMI affects bulk

83 tank milk and to contribute to the research of BTSCC thresholds based on overall milk

84 quality, the aim of this study was to assess the effect of different levels of BTSCC on i)

85 composition, ii) p-p content and CN fractions as indicating parameters for proteolysis,

86 and iii) pH and coagulating properties of milk. Correlations were also determined in

87 order to establish the relationship between protein fractions, proteolysis indices and

88 coagulation properties of milk at bulk tank level.

89 **Materials and Methods**

90 *Experimental design*

91 A total of 97 milk samples from bulk tank milk from different flocks (one sample per

92 flock) of Manchega breed ewes were taken over a five week period. Flocks were located

93 in Castilla La Mancha (Spain), and delivered their milk to the Forlasa S.A. cheese

94 company. Herds were bred under identical husbandry systems, each one usually being

95 divided into two groups of animals, with lambing periods distributed throughout the

96 year. Thus, ewes in different lactation stages were always present in the flocks. The

97 herds were selected on the basis of their bacteriological quality (<200000 cfu/ml) and

98 the milk SCC was recorded over the last three months by the quality control laboratory

99 of raw materials and finished products of the Forlasa S.A. cheese company, choosing

100 those that showed BTSCC within the limits of each of the following four classes:

101 BTSCC<500 (<500000 cells/ml), BTSCC 500-1000 (500000-1000000 cells/ml),

102 BTSCC 1000-1500 (1000000-1500000 cells/ml) and BTSCC>1500 (>1500000

103 cells/ml). The BTSCC classes were made up according to previous works (Pirisi et al.

104 1999; Gonzalo et al. 2000), reporting significant differences in milk quality,

105 technological properties and sanitary conditions of herds among them.

106 *Sampling and analysis*

107 Sampling of 500 ml bulk tank milk from selected farms was performed immediately

108 prior to collection of the milk by the Forlasa S.A. cheese company, following the

109 sampling procedure of the International Dairy Federation (ISO-IDF, 2008). Milk

110 samples were kept at 4°C until analysis. From each sample two aliquots were taken and

111 carefully analysed within 24 hours of sampling; one of them was sent to the laboratories

112 of the Institute for Animal Science and Technology of the Polytechnic University of

113 Valencia, where the SCC and the chemicals analysis of milk were carried out; and the

114 other one was sent to the Analysis Service of the Regional Breeding Centre

115 (CERSYRA, Valdepeñas, Ciudad Real, Spain) where pH and rheological properties

116 were determined. SCC was determined for each milk sample with a Fossomatic 90 (A/S

117 N Foss Electric, Hillerød, Denmark) according to the International Dairy Federation

118 (IDF, 1995). Milk samples for SCC determination were preserved with bronopol

119 (0.1%). Milk composition (fat, protein, true protein, casein, whey protein, lactose, and

120 total solids) was determined by midrange infrared spectroscopy using a MilkoScan

121 FT120 (Foss Electric, Hillerød, Denmark), previously calibrated and periodically

122 checked for the ewe milk components. Protein equivalents were calculated from

123 nitrogen data using the factor 6.38.

124 Isoelectric CN for assessing the relative content of each casein fraction was obtained

125 from skimmed milk after centrifugation at 3000 g for 15 min by the addition of acetate

126 buffer according to the procedure of Rowland (1938). The purified caseins were re-

127 dissolved by addition of 4 ml of distilled water and 1 ml of 1 M- NaOH, pH 7.0, and

128 were extended on a Petri plate. The re-dissolved caseins were frozen and freeze-dried

129 for analysis by chromatography. Relative content of each casein fraction was

130 determined by Fast Protein Liquid Chromatography analysis on a Mono Q HR 5/5

131 anion-exchange column (Pharmacia ltd., Milton Keynes, U.K.) according to the

132 procedure of Papoff et al. (1993) for ewe milk. Using this method, α, β, κ and a group

133 of casein hydrolysis products analogous to bovine γ-caseins (hereafter γ-caseins) can be

134 separated. The p-p fraction was extracted using the fractionating scheme recommended

135 by Rowland (1938), as modified by Andrews (1979). The factor used for converting the

136 N content into protein content was 6.54 (Ribadeau-Dumas & Grappin, 1989). The pH

137 was measured on all samples at 20 ºC by a pH-meter (Crison microPH 2001, Spain).

138 Milk renneting characteristics [rennet coagulation time (RCT), min, time to curd

139 firmness of 20 mm (k_{20}), min, and curd firmness 30 min after enzyme addition (a_{30}),

140 mm] were measured using a Formagraph (Foss Electric, Hiller∅d, Denmark) according

141 to the method of McMahon & Brown (1982).

142 *Statistical analysis*

143   Statistical analyses were performed using SAS software (SAS Institute, 2011). The

144 influence of the BTSCC level was analysed with the GLM procedure on milk

145 composition parameters, pH, coagulating properties, p-p fraction, α-, β-, κ- and γ-CN,

146 and the PI**.** The statistical analysis was performed according to the following model:

147         $Y_i = \mu + BTSCC_i + e_i$

148   Where μ is the overall mean; *BTSCC* is the fixed effect of BTSCC category

149 (i=1-4) and *e* is the residual effect.

150   Linear simple correlations were performed among protein components, proteolysis

151   parameters and coagulation properties of bulk tank milk, using the Corr procedure of

152   SAS.

153   **Results and Discussion**

154   Ewe milk is mainly destined for cheese manufacturing and changes in milk quality

155   affect the suitability of milk for processing. It is well known that high SCC in individual

156   and half-udder ewe milk as a consequence of IMI is associated with poor milk quality.

157   However, little is known about the consequences that SCC level has on composition,

158   proteolysis indices and coagulation behaviour of bulk tank milk.

159   The main milk components for the four BTSCC classes are reported in Table 1.

160   Lactose content was the highest in the BTSCC<500 class and declined significantly

161   when BTSCC exceeded 500000 cells/ml. The casein:protein ratio decreased as BTSCC

162   increased, being significantly lower (P<0.05) in BTSCC 1000-1500 and BTSCC>1500

163   categories than in the other categories. In accordance with these results, it has been

164   reported that lactose content and the casein:protein ratio in individual and half-udder

165   ewe milk show significant differences due to IMI, while fat and casein contents remain

166   relatively unchanged (Burriel, 1997; Bianchi et al. 2004; Martí-De Olives et al. 2013).

167   Milk lactose content decreases with IMI mainly because of the reduced synthesis

168   capacity of damaged tissue. In this respect, Auldist et al. (2003) suggest that lactose

169   content can be considered an indicator of the epithelial cells capacity of synthesis being

170   involved in the osmoregulation in milk. The casein concentration in milk frequently

171   does not decrease as a result of IMI due to the reduction in milk volume. However, it

172   has been confirmed that the casein:protein ratio, which is independent of milk volume,

173   decreases as a result of infection. The lower casein:protein ratio found in this study in

174   milk samples with more than 1000000 cells/ml was likely the result of an influx of

175 serum proteins into the milk through the ruptured mammary epithelia and the

176 breakdown of intact casein by endogenous enzymes, and these proteins are not relevant

177 to cheese processing. Accordingly, Auldist et al. (1996) and Klei et al. (1998) report

178 that casein:protein ratio is the parameter related to protein fraction of milk that best

179 explains the cheese yield and protein recovery variations due to SCC.

180 ----------Table 1 about here----------

181   Table 2 summarises the casein fractions and other milk proteolysis parameters as

182 affected by the BTSCC classes. A significant effect of BTSCC ($P<0.05$) on α-CN was

183 observed, being lower in milk samples with fewer than 500000 cells/ml compared with

184 those milk samples with higher SCC. β-CN displayed a decrease of about 18% passing

185 from BTSCC<500 class to BTSCC 500-1000 class and about 19% passing from

186 BTSCC 500-1000 class to BTSCC>1500 class ($P<0.001$). At the same time, the γ-CN

187 increased about 30-44% when the BTSCC surpassed 500000 cells/ml ($P<0.05$). In the

188 case of k-CN, no significant differences were observed among BTSCC categories. As a

189 consequence of these casein fraction modifications, the PI increased with SCC about

190 34-50% ($P<0.05$) from the BTSCC<500 class to the BTSCC>1500 class. Concerning

191 the p-p content, it had an increasing trend with BTSCC, but the effect was not

192 statistically significant.

193 ----------Table 2 about here----------

194   These results highlight the existence of higher proteolytic activity in milk when

195 BTSCC exceeds 500000 cells/ml. The phenomenon could be partly due to the increased

196 plasmin activity in high SCC milk samples, since this activity is controlled by a

197 complex enzymatic system in which one of the plasminogen activators is associated

198 with somatic cells (Bianchi et al. 2004; Albenzio et al. 2004, 2005, 2011). It is known

199 that β-CN is the primary cleavage substrate of plasmin, producing different γ-CN and

200 proteoses peptones. Furthermore, other enzymes such as elastase and cathepsin, which

201 come from the lysosomes of somatic cells, also act on β-CN, releasing γ-CN and

202 proteoses-peptones (Pinto et al. 2013). Thus the relative proportion of γ-CN to (α+ β +

203 κ)-CN and the p-p fraction are both considered as valid estimation predictors of

204 endogenous proteolysis in individual and half-udder milk with elevated SCC (Le Roux

205 et al. 1995; Martí-De Olives et al. 2011). The absence of significant changes in p-p

206 content as affected by BTSCC in the present study may be due to a lower range of

207 variation of the analysed parameters in bulk tank milk samples than in individual or

208 half-udder milk samples.

209 The above mentioned indigenous enzymes are therefore of significance for milk

210 processing quality through proteolytic disruption of intact casein, since the hydrolysis of

211 caseins reduces the stability of micelles during milk storage leading to the diminution of

212 coagulation properties of milk (Storry et al. 1983). Otherwise, the findings of this study

213 indicated that the increased proteolysis in high SCC milk decreased the ovine β-caseins,

214 but did not diminish α- or κ-caseins, which is in agreement with the results shown in

215 individual and half-udder milk (Bianchi et al. 2004; Martí-de Olives et al. 2011) and

216 bulk tank milk (Revilla et al. 2009). These data are supported by Pinto et al. (2013),

217 who reported that β-CN is more susceptible than α_{s2}- CN, α_{s1}- CN and κ-CN to

218 degradation in high SCC milk in the order β- > α_{s2}- > α_{s1}- >> κ-CN.

219 The pH and renneting parameters in milk samples grouped according to SCC levels

220 are reported in Table 3. The highest pH value was found for the category BTSCC>1500,

221 being significantly different (P<0.001) from BTSCC 500-1000 class. Samples

222 belonging to BTSCC>1500 category showed an increase of 30-40% (*P*<0.001) in the

223 RCT and of 40% in the k_{20} compared with BTSCC categories with fewer than 1000000

224 cells/ml. However, the effect on a_{30} was not statistically significant, even though the

225 trend was decreasing with increasing BTSCC. Several authors report an increase in pH

226 with SCC (Pirisi et al. 1996, 1999; Bianchi et al. 2004; Albenzio et al. 2004, 2005,

227 2011). Otherwise, RCT and k_{20} of sheep milk are reported to be significantly increased

228 with high SCC, while a_{30} is not significantly modified (Duranti & Casoli, 1991;

229 Pellegrini et al. 1997; Pirisi et al. 1996, Bianchi et al. 2004; Revilla et al. 2009). In bulk

230 tank milk the changes are noted from 1000000 cells/ml (Pirisi et al. 1999; Albenzio et

231 al. 2004).

232   ----------Table 3 about here----------

233   The negative effect of increased SCC on the coagulation properties of milk is

234 actually a consequence of the impairment of physical and chemical characteristics due

235 to decreased udder health status. It has been demonstrated that the changes in milk

236 composition associated with a high bulk milk cell count can affect the quality of cheese

237 (Auldist et al. 1996). The most important factors in curd structure formation are casein

238 content, pH and calcium content of the milk. At low pH, calcium is progressively

239 dissociated from the casein micelle, and neutralises the negative charges of the casein,

240 favouring extensive aggregation and fusion between the micelles which tend to form a

241 casein network in which the other components of coagulum are entrapped (Park 2007).

242 Thus, casein is the critical component in milk that forms the primary structure of cheese

243 curd. In this study, depressed ratio of casein:protein with increased proteolysis in high

244 BTSCC milk enhanced the extension of the RCT and k_{20} because there are more serum

245 proteins without processing value and the stability of casein micelles are reduced as a

246 result of its hydrolysis. Those changes in turn led to poor syneresis, lower cheese yield,

247 increased moisture content and lower fat and protein content in cheese (Albenzio et al.

248 2005; Revilla et al. 2007).

249　　In accordance with the displayed results, in the present research the pH was

250　positively correlated with RCT (r=0.51; *P*<0.05) and k_{20} (r=0.45; *P*<0.05), whereas it

251　was not significantly correlated with a_{30}. In the literature, the most parameters affected

252　by the pH are the RCT and the k_{20}, which become worse as pH increases, while a_{30} is

253　not correlated with pH (Duranti & Casoli, 1991; Delacroix-Buchet et al. 1994;

254　Pellegrini et al. 1997). These findings highlight the importance of pH to the coagulation

255　behaviour of milk, probably due to an increase in the viscosity of milk (Park 2007).

256　　With respect to the protein fraction, casein:protein ratio and β-CN were significantly

257　correlated with RCT (r=-0.29 and r=-0.25 respectively; *P*<0.05) and k_{20} (r=-0.29 and r=-

258　0.30 respectively; *P*<0.05), but not with　a_{30}. This is in accordance with the known

259　importance of the casein:protein ratio in the technological suitability of ewe milk. The

260　negative correlations found between β-caseins and both RCT and k_{20} confirm that

261　coagulation time is primarily related to the β-CN (Storry et al. 1983).

262　**Conclusions**

263　　A negative effect of elevated SCC on some milk components, casein hydrolysis and

264　milk ability to make cheese has been revealed in bulk tank milk with high SCC. This

265　finding demonstrated that the impairment of milk quality as affected by IMI may be

266　extended to the bulk tank milk of herds with poor udder sanitary condition. Reduction

267　of casein:protein ratio and hydrolysis of β-CN in γ-CN, together with an increase in pH,

268　were probably responsible for the increased RCT and k_{20}. Although the increase in

269　proteolysis began in 500000 cells/ml, the effect of BTSCC on rheological behaviour

270　was noted from 1000000-1500000 cells/ml.

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428   **TABLES**

429   **Table 1.** Means (±SE) of pH and milk components as affected by the BTSCC class

	BTSCC classes (x 10^3 cells/ml)				
Parameter	BTSCC<500	BTSCC 500-1000	BTSCC 1000-1500	BTSCC>1500	SL[†]
Obs., no.	20	35	25	20	
Fat, %	7.90 ± 0.18	7.85 ± 0.13	7.49 ± 0.17	7.46 ± 0.18	ns
Protein, %	6.04 ± 0.08	6.06 ± 0.06	5.99 ± 0.08	6.00 ± 0.08	ns
True protein, %	5.70 ± 0.08	5.75 ± 0.06	5.69 ± 0.07	5.66 ± 0.08	ns
Casein, %	4.75 ± 0.06	4.77 ± 0.05	4.68 ± 0.06	4.69 ± 0.07	ns
Casein:protein, %	78.74 ± 0.17[a]	78.63 ± 0.13 [a]	78.22 ± 0.17 [b]	78.17 ± 0.18 [b]	*
Whey protein, %	0.95 ± 0.02	0.99 ± 0.02	0.97 ± 0.02	0.99 ± 0.02	ns
Lactose, %	5.61 ± 0.05 [a]	5.47 ± 0.04 [b]	5.48 ± 0.05 [b]	5.40 ± 0.06 [b]	*
Total solids, %	19.40 ± 0.24	19.29 ± 0.17	18.74 ± 0.22	18.77 ± 0.24	ns

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431   [a,b]Means within a row with different superscripts differ

432   [†]Significance level : ***P < 0.001; *P < 0.05; ns not significant

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447 **Table 2.** Means (±SE) of the milk proteolysis parameters as affected by the BTSCC class

	BTSCC classes (x 10^{-3} cells/ml)				
Parameter	BTSCC<500	BTSCC 500-1000	BTSCC 1000-1500	BTSCC>1500	SL[‡]
Obs., no.	20	35	25	20	
α-CN, %	28.74 ± 2.13 [a]	34.80 ± 1.67 [b]	34.67 ± 2.19 [b]	39.23 ± 2.25 [b]	*
β-CN, %	52.44 ± 1.77 [a]	42.88 ± 1.38 [b]	41.88 ± 1.81 [b]	34.84 ± 1.87 [c]	***
κ-CN, %	11.32 ± 1.21	12.58 ± 0.95	13.38 ± 1.25	15.08 ± 1.28	ns
γ-CN, %	7.51 ± 0.86 [a]	9.74 ± 0.67 [b]	10.07 ± 0.88 [b]	10.85 ± 0.91 [b]	*
PI[†], %	8.20 ± 1.07 [a]	10.98 ± 0.84 [b]	11.53 ± 1.10 [b]	12.29 ± 1.13 [b]	*
p-p content, g/l	1.61 ± 0.10	1.61 ± 0.07	1.75 ± 0.10	1.77 ± 0.10	ns

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449 [a,b,c]Means within a row with different superscripts differ

450 [†]Proteolysis Index, calculated as the ratio of γ-CN to (α + β + κ)-CN.

451 [‡]Significance level : ***$P < 0.001$; *$P < 0.05$; ns not significant

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469   **Table 3.** Means (±SE) of the milk coagulation properties as affected by the BTSCC class

	BTSCC classes (x 10^{-3} cells/ml)				
Parameter	BTSCC<500	BTSCC 500-1000	BTSCC 1000-1500	BTSCC>1500	SL[¶]
Obs., no.	20	35	25	17	
pH	6.63 ± 0.18 [ab]	6.56 ± 0.18 [a]	6.63 ± 0.18 [ab]	6.66 ± 0.18 [b]	***
RCT[†], min	12.07 ± 1.25 [a]	12.93 ± 0.92 [a]	14.71 ± 1.22 [ab]	16.84 ± 1.30 [b]	***
K_{20}[‡], min	2.42 ± 0.16 [a]	2.36 ± 0.11 [a]	2.77 ± 0.15 [ab]	3.14 ± 0.16 [b]	***
A_{30}[§], mm	62.85 ± 1.90	62.05 ± 1.40	61.25 ± 1.85	57.66 ± 1.97	ns

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471   [a,b,c]Means within a row with different superscripts differ

472   [†]Rennet coagulation time

473   [‡]Time to curd firmness of 20 mm

474   [§]Curd firmness 30 min after enzyme addition

475   [¶] Significance level : ***P < 0.001; ns not significant

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