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1 **Haptoglobin and serum amyloid A in bulk tank milk in relation to raw milk**

2 **quality**

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4 Maria Åkerstedt [1*], Karin Persson Waller [2,3], Åse Sternesjö[1]

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6 [1] Department of Food Science, Swedish University of Agricultural Sciences, SE-750

7 07 Uppsala, Sweden

8 [2] Department of Animal Health and Antimicrobial Strategies, National Veterinary

9 Institute, SE-751 89 Uppsala, Sweden

10 [3] Department of Clinical Sciences, Swedish University of Agricultural Sciences, SE-

11 750 07 Uppsala, Sweden

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14 Short title: Hp, SAA and bulk tank milk quality

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21 [*] Correspondence to: Maria Åkerstedt, Department of Food Science, Swedish

22 University of Agricultural Sciences, SE-750 07 Uppsala, Sweden.

23 Phone: +46-18-672040; Fax: +46-18-672995; e-mail: Maria.Akerstedt@lmv.slu.se

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25   **Summary**

26   The aim of the present study was to evaluate relationships between the presence of the

27   two major bovine acute phase proteins haptoglobin (Hp) and serum amyloid A (SAA)

28   and raw milk quality parameters in bulk tank milk samples. Hp and SAA have been

29   suggested as specific markers of mastitis but recently also as markers for raw milk

30   quality. Since mastitis has detrimental effects on milk quality, it is important to

31   investigate if the presence of Hp or SAA indicates such changes in the composition

32   and properties of the milk. Bulk tank milk samples (n=91) were analysed for Hp,

33   SAA, total protein, casein, whey protein, proteolysis, fat, lactose, somatic cell count

34   and coagulating properties. Samples with detectable levels of Hp had lower casein

35   content, casein number and lactose content, but higher proteolysis than samples

36   without Hp. Samples with detectable levels of SAA had lower casein number and

37   lactose content, but higher whey protein content than samples without SAA. The

38   presence of APP in bulk tank milk is suggested as an indicator for unfavourable

39   changes in the milk composition, e.g. protein quality, due to udder health

40   disturbances, with economical implications for the dairy industry.

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50   The bulk tank milk composition has a decisive influence on the production of safe,

51   high quality dairy products. One major factor responsible for the deterioration of the

52   quality of the raw milk is mastitis, and its negative effect on the milk composition is

53   well established (Kitchen, 1981; Munro *et al*. 1984). It is the sub-clinical form of

54   mastitis that constitutes problems to the dairies, since these cases often go undetected

55   and the milk is delivered to the dairy (Leitner *et al*. 2008). Studies show that during

56   mastitis the casein content, valuable for the cheese making industry, will decrease

57   while the whey protein content will increase (Barbano *et al*. 1991; Auldist *et al*. 1996;

58   Urech *et al.* 1999). In addition, increased proteolysis is often observed in milk from

59   cows with mastitis (Schaar, 1985; Auldist *et al*. 1996). Proteolysis in milk is one of

60   the major product deteriorating factors with negative impact on the quality and

61   stability of milk and dairy products (Mara *et al*. 1998; Kelly *et al*. 2006). Saeman *et*

62   *al*. (1988) found that after an udder infection the proteolytic activity may sustain even

63   though the SCC has returned to normal levels. Larsen *et al.* (2004) established that

64   casein degradation not only occurred in the infected quarter but also in the

65   neighbouring quarters, even though there was no effect on the SCC. In the bulk tank,

66   milk from healthy udder quarters will be commingled with milk from infected

67   quarters, and thus, the entire bulk tank may be affected by protein degradation. This is

68   a problem, especially for the cheese making industry, since curd formation properties

69   will be impaired and yield reduced (Mara *et al*. 1998, Leitner *et al*. 2008). Likewise,

70   proteolytic activity in UHT-milk may cause off flavours and gelation, and

71   consequently reduced shelf-life of the products (Ma *et al*. 2000; Santos *et al*. 2003;

72   Barbano *et al*. 2006; De Noni *et al*. 2007).

73

74 Milk somatic cell count (SCC) has been used extensively since the 1960s in the

75 diagnosis of mastitis, and the BTMSCC (bulk tank milk somatic cell count) is widely

76 used in the assessment of raw milk quality. In many EU countries milk payment

77 systems favours a low BTMSCC. There is, however, no clear scientific data defining

78 the level of BTMSCC that is associated with additional benefits in terms of milk

79 quality. Several authors have reported that SCC is not a suitable indicator of

80 proteolysis in quarter milk samples (Le Roux *et al*. 1995; Urech *et al*. 1999) and

81 recent studies have also shown that the BTMSCC gives a poor prediction of raw milk

82 quality for cheese production (Leitner *et al*. 2008). Research to find new sensitive and

83 specific markers for disadvantageous changes in raw milk composition due to udder

84 health disturbances is therefore warranted.

85

86 The acute phase proteins (APP) have become important diagnostic markers of disease

87 in human medicine and are also being evaluated in veterinary diagnostics (Eckersall,

88 2004). The major bovine APP are haptoglobin (Hp) and serum amyloid A (SAA),

89 which both increase dramatically upon infection, inflammation or trauma. Hp and

90 SAA are mainly produced by the liver but are also produced locally in the mammary

91 gland (McDonald *et al.* 2001; Hiss *et al*. 2004). Some studies have found that Hp and

92 SAA have antibacterial effects (Eaton *et al*. 1982; Hari-Dass *et al*. 2005; Larson *et al*.

93 2005) and considering that they are locally produced, their role in the inflammatory

94 defence is interesting. In several studies Hp and SAA in milk have been evaluated as

95 markers for mastitis (Horadagoda *et al*. 1999; Eckersall *et al*. 2001; Grönlund *et al*.

96 2003; Nielsen *et al*. 2004; Grönlund *et al*. 2005; Eckersall *et al*. 2006; Hiss *et al*.

97 2007), but so far little attention has been paid regarding their potential in predicting

98 changes in milk composition and technological properties of the raw milk. In a

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99   previous study we reported that detectable levels of Hp and SAA could be found in

100   bulk tank milk samples (Åkerstedt *et al*. 2007). In a more recent study we also

101   investigated APP in relation to raw milk quality parameters in cow composite milk

102   samples (Åkerstedt *et al.* 2008). To our knowledge, these papers are the only studies

103   examining APP in bovine bulk tank milk or applying APP research in the field of

104   product quality. However, no studies have been reported on Hp and SAA in relation

105   to the quality of the raw bulk tank milk. Quality programs for milk payment and

106   advisory measures to improve the raw milk quality are mostly based on analysis of

107   bulk tank milk samples. For APP to be a potential candidate as indicator for

108   unfavorable changes in milk composition due to udder health disturbances in the herd,

109   it is important that levels of APP in the bulk tank milk are related to important quality

110   traits of the raw milk.

111

112   The aim of this study was to investigate relationships between the presence of Hp and

113   SAA, and different raw milk quality parameters, i.e. total protein, casein, whey

114   protein, proteolysis, fat, lactose and SCC in bulk tank milk samples. In addition, APP

115   in relation to the coagulating properties of the bulk tank milk samples were evaluated.

116

117   **Materials and methods**

118   *Bulk tank milk samples*

119   The study included 91 bulk tank milk samples collected from different dairy farms in

120   cooperation with the Milko dairy cooperative (Grådö, Hedemora, Sweden). One

121   representative sample from each farm was taken by the tanker driver just before

122   emptying the bulk tank, in connection with the ordinary milk collection, which

123   occurred every second day. At the sampling occasion the farms delivered 90-13,025

124   kg milk (average 1,610 kg), indicating that the herd size of the participating farms

125   varied markedly. The average BTMSCC for the samples was 195,000 cells/ml,

126   ranging from 33,000 to 1,365,000 cells/ml (median 146,000 cells/ml). The bulk tank

127   milk samples were collected at the dairy plant for further transportation to the

128   university laboratory the same day. Sample aliquots for the analyses of Hp, SAA and

129   proteolysis were frozen and stored at $-70^{o}C$ until analysis, whereas the other

130   parameters were analysed using fresh milk samples.

131

132   *Assay of haptoglobin and serum amyloid A*

133   Hp was analysed by an earlier described optical biosensor assay (Åkerstedt *et al.*

134   2006; Åkerstedt *et al.* 2008) with some additional modifications. In this study, the Hp

135   surface was prepared by using a solution of 20 mg/l instead of 500 mg/l Hp in 0.01 M

136   acetate buffer, and the activation of the surface during immobilisation was reduced

137   from 7 minutes to 3 minutes. For regeneration of the sensor surface the concentration

138   of sodium dodecyl sulphate (SDS) was increased from 2 mM to 3 mM. An extra

139   reconditioning step was added, in which 50 mM glycine pH 9.5 was injected over the

140   sensor surface for 30 seconds, after the ordinary regeneration step. Bovine Hp (Life

141   diagnostics, Clarkston, GA, USA) was used for immobilization and standards, and the

142   limit of detection (LOD) of the modified assay was 0.3 mg/l.

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144   SAA was determined using a commercial ELISA with a LOD of 0.3 mg/l (Phase[TM]

145   Serum Amyloid A Assay, Tridelta Development Ltd, Wicklow, Ireland).

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147   *Measurement of the somatic cell count, total protein, whey protein and casein content,*

148   *casein number, fat and lactose content*

149 SCC in the bulk tank milk samples was measured by an electronic fluorescence based

150 cell counting technique (Fossomatic 5000, Foss, Hillerød, Denmark). Total protein,

151 fat and lactose contents were measured on fresh milk using mid infrared spectroscopy

152 (Fourier Transform Instrument, FT 120, Foss). The casein content was determined by

153 an indirect method which was described earlier (Åkerstedt *et al*. 2008).

154

155 *Measurement of proteolysis*

156 The extent of proteolysis in the milk sample was measured according to a

157 fluorescamine method as previously described (Wiking *et al.* 2002).

158

159 *Measurement of coagulating properties*

160 The coagulating properties of the milk was measured with a Bohlin VOR Rheometer

161 (Malvern Instruments Nordic AB, Uppsala, Sweden) according to Hallén *et al.* (2007)

162 with one minor modification, i.e. Chymax Plus, strength 200 IMCU per gram

163 (Christian Hansen A/S, DK-2970, Hørsholm, Denmark), was used instead of pure

164 chymosin. The coagulation time was measured, i.e. the time (s) elapsed from

165 chymosin addition until a weak coagulum corresponding to 5 Pa was formed. In

166 addition, curd firmness (Pa) was measured 25 min after chymosin addition.

167

168 *Statistical analyses*

169 Parametric t-test using SAS (Version 9.1, SAS Institute Inc., Cary, NC, USA) was

170 used to evaluate the relationships between APP and the different raw milk quality

171 parameters analysed. The bulk tank milk samples were categorised into two groups;

172 detectable or non-detectable levels of Hp or SAA, based on the detection limits of the

173 assays used to determine the proteins in milk. SCC and curd firmness were

174 logarithmically transformed before statistical analyses to obtain normally distributed

175 data. Differences between groups were considered significant if p≤0.05.

176

177 **Results**

178 Descriptive statistics for the milk quality parameters analysed in the study are

179 presented in Table 1. Detectable levels of Hp were found in 19 (21%) of the 91 bulk

180 tank milk samples. The average Hp concentration in these 19 samples was 1.02±0.99

181 mg/l and the average SCC in these samples was 387,000±266,000 cells/ml. Detectable

182 levels of SAA were found in 68 (75%) of the 91 bulk tank milk samples. The average

183 SAA concentration in these 68 samples was 1.12±1.16 mg/l and the average SCC in

184 these samples was 218,000±179,000 cells/ml. Samples not containing detectable

185 levels of Hp had the average SCC of 144,000±78,000 cells/ml while samples not

186 containing detectable levels of SAA had the average SCC of 127,000±116,000

187 cells/ml.

188

189 Table 1 near here.

190

191 Table 2 and 3 present differences in milk composition between samples with and

192 without detectable levels of Hp and SAA, respectively. Bulk tank milk samples in

193 which Hp could be detected had a lower casein number, contained less casein, had

194 increased proteolysis, a lower lactose content and higher SCC compared to samples

195 without detectable levels of Hp. Bulk tank milk samples in which SAA could be

196 detected had a lower casein number, increased whey protein, lower lactose content

197 and higher SCC compared to samples not containing detectable levels of SAA.

198

199   Table 2, 3 and 4 near here.

200

201   In Table 4, results obtained for differences in milk composition between samples

202   containing high BTMSCC (>195,000 cells/ml) and low BTMSCC (<195,000 cells/ml)

203   are presented. Samples with higher BTMSCC had lower casein number than samples

204   with lower BTMSCC. In the high BTMSCC group, 16 of the 29 samples (55%)

205   contained Hp, while 26 of the 29 (90%) samples contained SAA. In the low BTMSCC

206   group, 3 of the 62 samples (5%) contained Hp, while 42 of the 62 (68%) contained

207   SAA.

208

209   **Discussion**

210   The present study is, to our knowledge, the first investigating relationships between

211   the quality of raw bulk tank milk and the presence of APP as indicator of mastitis.

212   When studying markers for udder health disturbances and effects on milk composition

213   it is important to have in mind that the type of milk sample, i.e. quarter, cow

214   composite or bulk tank milk, may affect the results. Due to the dilution effect from

215   quarter milk to composite and bulk tank milk, significant relationships found at

216   quarter level may not be present at cow or bulk tank level and vice versa. However,

217   the main findings of the present study, i.e. unfavourable changes in protein

218   composition in bulk tank milk samples with detectable levels of APP, are largely in

219   agreement with the results of our earlier study in cow composite milk (Åkerstedt *et al*.

220   2008).

221

222   In this study, bulk tank milk samples with detectable levels of Hp had lower casein

223   content as well as lower casein number. This was most likely due to increased

224 proteolytic activity, since these samples also had significantly higher proteolysis than

225 samples without detectable levels of Hp. Bulk tank milk samples containing

226 detectable levels of SAA also had a lower casein number compared to samples

227 without detectable levels of SAA. In this case, however, the effect was likely due to

228 the observed increased whey protein content in the samples. It is thus likely that

229 decreased synthesis, proteolysis and influx of components from the blood will occur

230 simultaneously but to evaluate to what extent was outside the scope of this paper.

231 In this study, detectable levels of Hp in bulk tank milk was related to increased

232 proteolytic activity, which is in contrast to the results in our previous study on cow

233 composite milk samples (Åkerstedt *et al.* 2008). One possible explanation for not

234 observing proteolysis in cow composite milk but in bulk tank milk may be related to

235 the fact that bulk tank milk consists of commingled milk from different milkings, as

236 well as milk from non-infected and sub-clinically infected glands (Leitner *et al.*

237 2008). Moreover, the storage time before collection and freezing was longer for bulk

238 tank, than for cow composite milk samples, allowing proteolysis to proceed for a

239 longer time. The bulk tank milk samples were collected at the dairy plant and were

240 kept at +4°C during the entire chain from farm to laboratory. Since the milk is

241 collected every second day, the oldest batch of milk in the tank was stored for

242 approximately 2.5 days before it was frozen. Refrigerated storage of raw milk is

243 known to favor the growth of psychrotropic bacteria, which may produce heat-

244 resistant extra-cellular proteases and lipases. Proteases are mainly secreted at the end

245 of the log phase of the bacterial growth, at numbers in the order of 10^7 cfu/ml,

246 indicating that very high numbers of bacteria are required to result in proteolysis

247 (Sørhaug & Stepaniak, 1997). In a study by Haryani *et al.* (2003), such high numbers

248 were reached first after 7 days at 4°C, and proteolysis, as measured by the

249 fluorescamine method, was observed on day 6. Considering the very high numbers of

250 bacteria needed for proteolysis to become a problem, it is unlikely that microbial

251 contamination of milk samples would explain the observed relationships between the

252 presence of Hp and increased proteolysis. An influence of microbial proteases cannot,

253 however, be excluded at this stage.

254

255

256 Bulk tank milk samples with detectable levels of SAA contained less lactose in

257 agreement with earlier studies on udder quarter and cow composite milk samples

258 (Lindmark-Månsson *et al*. 2006; Åkerstedt *et al*. 2008). In the present study, bulk tank

259 milk samples with detectable levels of Hp also contained reduced levels of lactose.

260 The most common explanation to decreased lactose content is reduced synthesis due

261 to damaged epithelial cells. Another explanation, suggested by Silanikove *et al.*

262 (2000), might be that proteolysis of β-casein will result in release of peptides with a

263 regulatory effect on lactose secretion.

264

265 Bulk tank milk samples with detectable levels of Hp and SAA had significantly higher

266 SCC compared to samples without Hp and SAA, respectively. In our previous study,

267 bulk tank milk samples with detectable levels of SAA, but not samples with

268 detectable levels of Hp, had higher SCC (Åkerstedt *et al*. 2007). The discrepancy

269 between the studies might be explained by the use of different statistical methods,

270 differences in the categorisation of the samples and the selection of milk samples. To

271 assess the potential of APP as markers for milk quality in comparison with the

272 commonly applied SCC, we also investigated relationships between SCC and the

273 different quality traits in the same samples. In this study, bulk tank milk samples with

274   elevated SCC (>195,000 cells/ml), had a lower casein number than samples with a

275   lower SCC (<195,000 cells/ml). In our previous study (Åkerstedt *et al*. 2008) with

276   cow composite milk, samples with elevated SCC (>83,000 cells/ml) had reduced

277   lactose and increased whey protein content compared to samples with a lower SCC

278   (<83,000 cells/ml). The threshold values used in those studies (195 000 cells/ml and

279   83,000 cells/ml) are median and mean values, respectively. These threshold values are

280   relevant since several studies demonstrate that the composition is deteriorated on

281   quarter and cow level between 50,000-100,000 cells/ml, while the Swedish milk

282   payment system give additional bonus payment when the bulk tank contains less than

283   175,000-200,000 cells/ml. Our studies suggest that the presence of APP in milk is

284   related to disadvantageous changes in several milk quality parameters and that these

285   relationships are valid in both cow composite and bulk tank milk. SCC, on the other

286   hand, is not related to milk quality traits to the same extent as APP and the type of

287   relationships observed differs between cow composite and bulk tank milk.

288

289   No significant relationships between APPs and coagulation properties, i.e. coagulation

290   time and curd firmness, were found in this study. Since there are no other studies

291   published investigating APP in relation to the coagulating properties of milk, it is

292   difficult to evaluate the results obtained. In earlier studies, significant correlations

293   between mastitis and impaired coagulating properties were observed (for review see

294   Munro *et al*. 1984). In contrast, Leitner *et al*. (2008) found no correlation between

295   coagulation time and SCC at bulk tank or silo level. In general, most published

296   research on coagulating properties is based on studies with milk from a small number

297   of animals (Barbano *et al*. 1991; Mazal *et al*. 2007). It is also common that batches of

298   milk with a specific SCC are constructed by pooling milk with very high SCC, often

299   originating from cows with clinical mastitis, and milk with a low SCC. Such a

300   procedure is not ideal, as the composition of milk from cases of clinical mastitis is

301   very deviant from milk originating from cows without clinical signs. Consequently,

302   this type of constructed milk samples are not representative for real bulk tank milk

303   samples, representing commingled milk from a large number of clinically healthy

304   cows.

305

306   The casein content is an important quality parameter in cheese production and

307   decreased casein content implies large losses for the dairy industry. At present, the

308   total protein content is used as a major quality parameter, largely affecting the milk

309   price to the producer. The total protein content, however, also includes the whey

310   proteins, of which those originating from blood, with no interest for the dairies, will

311   increase during mastitis. The extent of proteolysis is another important factor

312   influencing milk quality, although not presently assessed. There are thus no

313   techniques in place allowing reliable, large-scale analyses of the protein quality of the

314   milk, although research and development in this field is ongoing. In a future

315   perspective, the dairies might have the possibility to differentiate raw milk based on

316   quality to be used for different purposes. SCC is a good marker for udder health

317   disturbances at udder quarter level but several studies have demonstrated that SCC is

318   not a strong candidate for predicting the processing quality of the bulk tank milk (Le

319   Roux *et al*. 1995; Urech *et al*. 1999; Leitner *et al*. 2006; Leitner *et al*. 2008). Since

320   many dairy products like cheese and fermented products require high protein quality it

321   should be of great importance to have a sensitive and specific marker for

322   disadvantageous changes in milk composition, e.g. those associated to poor udder

323   health. Changes in levels of such a marker should preferably be associated to the

324   protein composition of the raw milk. In this study, Hp and SAA have shown to be

325   potential candidates for predicting the raw bulk tank milk quality, specifically in

326   relation to protein quality. This study and our previous studies therefore suggest that

327   APP may be used as indicators for changes in milk composition as consequence of

328   udder health disturbances, in quarter or cow composite milk samples at the farm, as

329   well as in bulk tank milk at the dairy plant or milk grading laboratory.

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508 **Table 1.** Milk composition, including contents of haptoglobin (Hp) and serum

509 amyloid A (SAA), and coagulating properties of 91 bulk tank milk samples.

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Parameter	Unit	Mean (SD)	Minimum	Maximum
Hp	mg/l	ND[†]	<0.3	4.70
SAA	mg/l	ND	<0.3	8.79
Total protein	%	3.54 (0.18)	3.12	4.21
Casein	%	2.58 (0.14)	2.20	3.07
Casein number		0.73 (0.01)	0.71	0.75
Whey protein	%	0.91 (0.05)	0.82	1.09
Proteolysis	eq leu[‡]	1.11 (0.11)	0.89	1.71
Fat	%	4.48 (0.43)	3.54	6.25
Lactose	%	4.61 (0.11)	4.30	4.91
Somatic cell count	cells/ml	195,000 (169,600)	33,000	1,365,000
Coagulation time	s	120 (42)	32	278
Curd firmness	Pa	350 (118)	162	798

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513 [†]ND= not determined. Since many of the samples did not contain detectable levels of

514 Hp or SAA, i.e levels were below 0.3 mg/l, it was not considered relevant to calculate

515 a mean value.

516 [‡]eq leu = equivalent mM leucine.

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Table 2. Differences in milk composition between bulk tank milk samples with (Hp+)

and without (Hp-) detectable levels of Hp. Differences between Hp+ and Hp- samples

were evaluated by parametric t-test and were considered significant if p≤0.05.

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	Hp+ (n=19)	SE [†]	Hp- (n=72)	SE	p-value
Total protein (%)	3.493	0.025	3.549	0.023	NS [‡]
Casein (%)	2.532	0.018	2.593	0.017	0.016
Casein number	0.725	0.001	0.731	0.001	<0.001
Whey protein (%)	0.913	0.008	0.908	0.006	NS
Proteolysis (eq leu) [§]	1.159	0.019	1.100	0.014	0.038
Fat (%)	4.455	0.049	4.491	0.055	NS
Lactose (%)	4.579	0.014	4.618	0.014	0.05
Log SCC (cells/ml)	5.528	0.049	5.104	0.027	<0.001
Coagulation time (s)	110	9.862	123	4.963	NS
Log curd firmness (Pa)	2.547	0.039	2.517	0.015	NS

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526 [†] SE = Standard Error

527 [‡] NS = not significant

528 [§] eq leu = equivalent mM leucine.

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544 **Table 3.** Differences in milk composition between bulk tank milk samples with

545 (SAA+) and without (SAA-) detectable levels of SAA. Differences between SAA+

546 and SAA- samples were evaluated by parametric t-test and were considered

547 significant if p≤0.05.

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	SAA+ (n=68)	SE[†]	SAA- (n=23)	SE	p-value
Total protein (%)	3.553	0.021	3.488	0.042	NS[‡]
Casein (%)	2.588	0.016	2.559	0.032	NS
Casein number	0.728	0.001	0.734	0.001	<0.001
Whey protein (%)	0.918	0.006	0.883	0.011	0.004
Proteolysis (eq leu)[§]	1.122	0.014	1.082	0.023	NS
Fat (%)	4.477	0.049	4.504	0.102	NS
Lactose (%)	4.583	0.012	4.692	0.018	<0.001
Log SCC (cells/ml)	5.261	0.029	4.989	0.063	<0.001
Coagulation time (s)	122	5.055	113	9.276	NS
Log curd firmness (Pa)	2.521	0.016	2.533	0.030	NS

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550 [†] SE = Standard Error

551 [‡] NS = not significant

552 [§] eq leu = equivalent mM leucine.

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567 **Table 4.** Differences in milk composition between bulk tank milk samples with high

568 SCC (>195,000 cells/ml) and low SCC (<195,000 cells/ml). Differences between high

569 SCC and low SCC samples were evaluated by parametric t-test and were considered

570 significant if p≤0.05.

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	high SCC (n=29)	SE [†]	low SCC (n=62)	SE	p-value
Total protein (%)	3.498	0.027	3.555	0.025	NS
Casein (%)	2.541	0.021	2.599	0.018	NS
Casein number	0.726	0.001	0.731	0.001	0.002
Whey protein (%)	0.909	0.008	0.909	0.007	NS
Proteolysis (eq leu) [§]	1.126	0.020	1.105	0.014	NS
Fat (%)	4.453	0.059	4.498	0.060	NS
Lactose (%)	4.585	0.014	4.622	0.015	NS
Coagulation time (s)	118	8.053	121	5.359	NS
Log curd firmness (Pa)	2.507	0.030	2.531	0.015	NS

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573 [†] SE = Standard Error

574 [‡] NS = not significant

575 [§] eq leu = equivalent mM leucine.

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