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Effect of genetic potential and level of feeding on milk protein composition

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Summary. Two groups of 15 multiparous cows in mid-lactation were used in a Latin square design experiment with 4-week experimental periods. The genetic milk protein concentration level was high in the first group and low in the second. Each group of cows was given in a random order three feeding levels that covered 85, 100 and 115% of energy requirements and 90, 110 and 125% of nitrogen requirements, respectively. In both groups, increasing level of feeding induced a significant increase in milk yield (+2·4 kg/d between lowest and highest levels) and in protein concentration (+1·7 g/kg). The proportion of paracasein in total proteins was not altered by either genetics or nutrition. The proportion of casein in total proteins was slightly increased by 0·5 percentage points ($P < 0{\cdot}05$) with the intermediate level of feeding. Plasmin and plasminogen activities were not significantly modified by the genetic milk concentration level. Plasmin activity significantly increased with nutrient supplementation, but only in animals of low genetic potential (+21% between low and high levels, $P < 0{\cdot}01$). Casein composition was not significantly altered by the genetics or level of nutrition. Over the whole range of individual measurements taken ($n = 90$), the relationships between casein or paracasein and total protein concentrations were linear and very narrow ($R^2 = 0{\cdot}92$ and 0·95, respectively). The proportion of casein or paracasein in total proteins significantly decreased as plasmin activity increased.

Keywords: Milk proteins, dairy cow, nutrition, plasmin.

Milk fat and protein contents are predominant factors affecting cheesemaking potential. In the last few years, French cheesemakers have noted a stagnation of their cheese yield despite increasing concentrations of milk protein. This raises the issue of the value of those proteins for cheesemaking, especially during periods when milk protein concentrations are very high. In late lactation, low casein/protein ratio values have sometimes been observed in herd milk (Auldist *et al.* 1996), but only

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when the quality of the diet was low (Kefford *et al.* 1995). In individual cows, the casein/protein ratio can be affected in very early lactation (colostrum period), in late pregnancy (Rémond & Bonnefoy, 1997) or during mastitis (Munro *et al.* 1984), but is relatively independent of dietary factors and protein concentration (Coulon *et al.* 1998*a*), except perhaps in extreme cases of undernutrition. Conversely, that ratio is highly dependent on β-lactoglobulin (β-lg) variants and to a lesser extent on those of κ-casein (Grosclaude 1988; Rahali & Ménard 1991; Coulon *et al.* 1998*a*). Furthermore, in recent experimental cheesemaking studies, a very close and linear relationship was noted between milk true protein concentration and cheese yield (Verdier-Metz *et al.* 2002). A number of hypotheses may explain this discrepancy between experimental results and field observations. Cheese yield is the result of complex and interactive phenomena linked to milk quality and processing. Hence, on the individual animal scale, it is necessary to ensure that problem milks are neither due to mastitis nor presence of colostrum and to confirm that high protein concentrations (linked to advanced lactation stage, genetic potential or nutrition) do not modify the casein/protein ratio. It is also necessary to verify that the casein/protein ratio is a good indicator of the suitability of milk for cheesemaking. In blended milk, it is necessary to monitor the change of paracasein concentration with time during storage, because of possible proteolysis. Lastly, at the cheesemaker's level, it should be ascertained whether the technology applied makes use of all fat and proteins when they are present in high concentration (serum losses), and whether other milk components (e.g. urea) or certain indigenous enzyme activities (plasmin, in particular) can disrupt that process.

This study aimed to analyse the effects of dietary and genetic factors on the characteristics of milk proteins in mid-lactation milk from mastitis-free cows.

Materials and methods

Cows and feeding

Thirty Montbéliarde cows (27 multiparous and 3 primiparous, mean liveweight 613 kg) in mid lactation (between weeks 9 and 19, on average 15 at the beginning of the trial) were included in a 3×3 Latin square design with 4-week periods. Fifteen of these cows exhibited high genetic protein level (as assessed from their genetic index for protein content and the value recorded in the early stages of the current lactation) and the other fifteen cows exhibited a low genetic protein level. The expected mean deviation between these two groups of cows was around 2 g/kg. Both groups were made up so as to balance the genetic variants of β-lg and κ-casein, in order not to bias the possible impact of the genetic component of the protein level by the known effects of those variants. At the beginning of lactation all animals were fed according to INRA (1989) recommendations. One month before the beginning of the trial they received a diet of fixed composition (70% hay and 30% barley) supplying 0·89 UFL[1]/kg DM, 87 g PDIN[2]/kg DM and 92 g PDIE[3]/kg DM and fed at a level to cover their energy and nitrogen requirements. In the course of the trial,the cows were given the same diet but in variable amounts according to treatment. The

[1] UFL, feed unit for lactation (1 UFL = 11·3 MJ metabolizable energy; INRA, 1989).

[2] PDIN, true protein truly digestible in the small intestine when energy in the rumen is not limiting (INRA, 1989).

[3] PDIE, true protein truly digestible in the small intestine when degraded N in the rumen is not limiting (INRA, 1989).

three experimental treatments supplied 85 and 90 % (low treatment), 100 and 110 % (medium treatment) and 115 and 125 % (high treatment) of the cows' energy and nitrogen requirements, respectively. Cows were randomly allocated to the different treatment sequences of the Latin square design. Transition between treatments took 4 d. Cows chosen were mastitis free: no signs of clinical mastitis were observed at the start of the lactation or during the course of the trial.

Sampling and measurements

Milk yield was measured daily for individual cows and protein and fat concentrations were assessed twice a week. During the last week of each of the three experimental periods, individual samples (500 ml) were collected at morning milking on 2 consecutive days. These samples were divided and distributed into several subsamples to measure fat concentration (infrared method), total nitrogen and non-protein nitrogen concentration (International Dairy Federation, 1993), soluble nitrogen and total casein (International Dairy Federation, 1964), serum nitrogen (International Dairy Federation, 1993) and urea (Tondu, 1986) and to determine plasmin activity and that induced by plasminogen activation. Plasmin-plasminogen activities were determined using a variant of the technique described by Rollema *et al.* (1983). The milk sample (3 ml) was clarified by addition of 1 ml 0·4 M-tri-sodium citrate and centrifugation at 27000 ***g*** at 4 °C for 20 min. Supernatant (20 μl) was deposited in each of two wells of a 96-well microtitration plate (Nunc F96 Maxisorp, Nunc Kamstrup, Roskilde, Denmark). Urokinase (5 μl), a plasminogen activator, was added to one of the two wells and the plate was left to incubate for 15 min at 37 °C. Urokinase (Sanofi Winthrop, Gentilly, France) induces the activation of inactive plasminogen to active plasmin and thus provides the measure of total plasmin + plasminogen activity. Lastly, 200 μl of a solution containing 0·6 mM-D-valyl-L-leucyl-L-lysine-4-nitroanilide (Serva, COGER, Paris, France), 160 mM-Tris-HCl-40 mM-KCl-100 mM-EDTA-25 mM-ϵ-aminocaproic acid, pH 7·0, were added to each well. The plate was left to incubate at 37 °C and the absorbance at 405 nm was determined for each well every 30 min for 3 h. Plasmin activity was obtained by computing the slope of absorbance of the urokinase-free well. Total plasmin + plasminogen activity was obtained by computing the slope of absorbance of the well containing urokinase. The activity linked to plasminogen was therefore obtained by subtracting plasmin activity from total activity. Plasmin-plasminogen activities were expressed in arbitrary units (1 unit = 1 dA405/dt × 10000). To determine the paracasein concentration, rennet was added to one of the samples. Paracasein concentration is the difference between milk total nitrogen and serum total nitrogen, as obtained by enzyme-induced milk clotting. Coagulation was obtained by adding 1 ml rennet (52 mg chymosin/l) to 100 ml milk that had previously been warmed to 37 °C in a hot water bath. The rennet-added milk sample was kept at 37 °C to permit coagulation. Ten minutes after rennet addition, the curd was firm enough to be grossly sliced to promote serum exudation. The sliced coagulum was left to rest at 37 °C for 30 min, then the soluble phase was separated from the solid phase by filtration with a Whatman 41 filter. The protein content was determined from the serum so obtained (International Dairy Federation 1993).

On a day of the last week of each of the three experimental periods, the milk from two cows representative of the entire population of each group (according to β-lg and κ-casein genetic variants) was sampled at the morning milking to analyse casein fractions (precipitation at pH 4·6) by PAGE electrophoresis in urea mode (Andrews, 1983). Curds were fixed, stained (Coomassie G250) and discoloured as outlined by

Blakesley & Boezi (1977). The density of each band was measured by absorption using a scanner fitted with a red filter (Bio-Rad G670, 92430 Marnes-la-Coquette, France) and the results were expressed as percentages or areas identified within the same pattern. Care was taken to analyse each sample three times, each time with different curds from different eluates. The samples to be compared could be deposited on the same curd and the sample arrangement inside each curd was randomized for each repetition. Somatic cell count (Somacount; Bentley Instruments, Chaska, MN 55318, USA) and total flora (Association Française de Normalisation, 1992) were measured on all the samples collected.

The genetic variants of lactoproteins were identified by isoelectric focusing of a milk sample (Seibert *et al*. 1985).

Statistical analysis

Data were processed by analysis of variance (SAS, 1987). The period, feeding level, genetic level of protein concentration, cow nested by genetic level of protein concentration and the interaction genetic level of protein concentration × feeding level were introduced as factors in the Latin square experimental model. The statistical effect of genetic level of protein concentration was tested using cow nested by genetic level of protein concentration as the error term (Winer *et al*. 1991).

RESULTS

The increase in feeding level increased milk yield and milk protein concentration by 2·4 kg/d and 1·7 g/kg, respectively, between L and H levels ($P < 0{\cdot}01$). That increase did not differ with the cow's genetic potential. Paracasein changed in proportion to the protein concentration, so that the ratio between paracasein and total proteins (0·784 on average) was not affected by feeding level. The casein/total protein ratio was slightly higher at the intermediate (normal) level of feeding, compared with the high or low levels (+0·5 percentage point, $P < 0{\cdot}01$).

Mean difference in protein concentration between the two genetic levels was 3·3 g/kg, 80 % of which was due to casein (+2·5 g/kg). Casein proportion in total proteins therefore did not differ with the genetic potential of the cows (0·806 and 0·803 in groups H and L, respectively, $P > 0{\cdot}05$). The same result was noted for the proportion of paracasein in total proteins. Relationships between casein or paracasein concentrations and protein concentration were linear and very close in all individual measurements ($R^2 = 0{\cdot}92$ and 0·95, respectively; Fig. 1). Slopes of each of those two regression curves were identical regardless of the genetic potential or feeding level. Whatever the values of those factors, the differences between casein and paracasein concentrations remained, on average, stable and close to 0·6 g/kg. That difference was less marked as plasmin activity was higher ($R^2 = 0{\cdot}31$, $P < 0{\cdot}01$). It was unrelated to the proportion of κ-casein in total proteins.

Plasmin and plasminogen activities were not significantly modified by the genetic potential of the cows, though there was a trend towards higher plasmin activity in cows of high genetic potential (Table 1). In contrast, plasmin activity increased significantly with level of feeding ($P < 0{\cdot}01$), but only in animals of low genetic potential. Considering all samples, overall, the proportion of casein in total proteins was markedly and significantly decreased ($R^2 = 0{\cdot}45$, $P < 0{\cdot}01$) as plasmin activity increased. When allowance was made for the effect of β-lg variant in the regression, plasmin activity accounted for 74 % of the overall variability in casein proportion

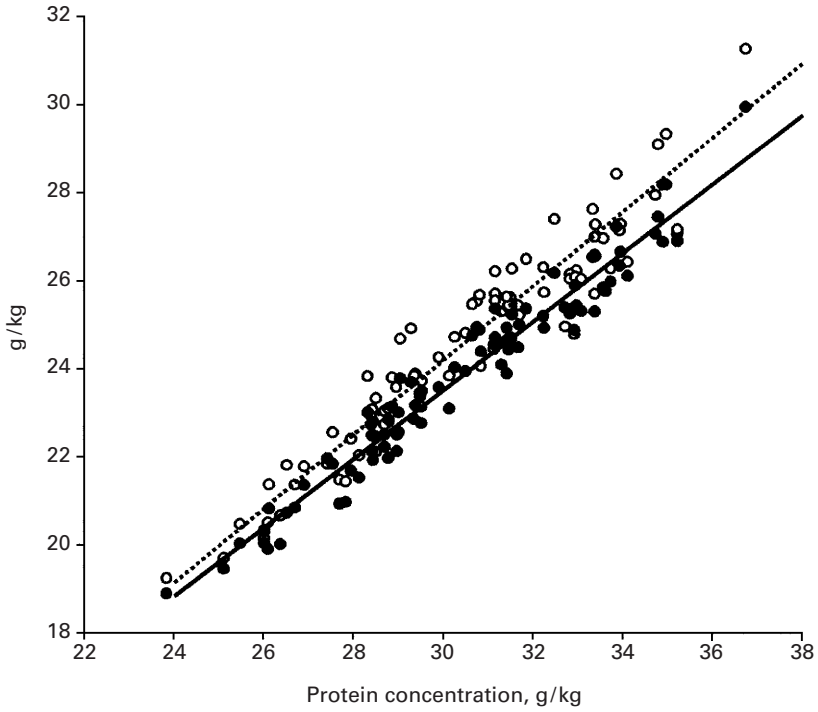

Fig. 1. Relationship between casein concentration (○ · · ·) or paracasein concentration (●—) and milk protein concentration.

(Fig. 2). The same change was noted, although with a twice-weaker slope, with the proportion of paracasein in total proteins. Over the wide range of plasminogen activity observed (14 to 51), no significant relationship was noted with the proportion of casein in total proteins or the proportion of paracasein in total proteins.

Analysing electrophoretic profiles identified about ten bands in the following order of elution: X1, X2, κ_B-CN and κ_A-CN, X3, β_B-CN, β_{A2}-CN, β_{A1}-CN, α_{S2D}-CN, α_{S2A}-CN, α_{S1C}-CN, α_{S1B}-CN, X4 and X5. The identity of the bands marked X has not been ascertained. The bands marked X2 and X5 very probably correspond to degradation products of β-casein under the effect of plasmin, the activity of which they are strongly correlated to ($R^2 = 0{\cdot}67$ and $0{\cdot}49$, respectively). The band marked X5 probably corresponds to proteose-peptones and the band marked X2 to fragment β-CN(106-209)(γ2). The other fragments produced by plasmin from β-CN, β-CN (29-209) and β-CN (108-209), migrate to the same location as the κ-casein they contaminate and could not be measured.

Dietary and genetic levels did not significantly influence the electrophoretic composition of casein, except the X5 band whose proportion (about 2·5% of the curve) appeared to be slightly but significantly reduced (by $\sim 0{\cdot}2$ percentage point) when dietary supplementation was high ($P < 0{\cdot}05$). There was also a trend towards an increase in the proportion of κ-casein when the protein concentration increased under the effect of either genetics or nutrition: a positive relationship was thus noted on all available points ($n = 36$) between the proportion of κ-casein and the milk protein concentration ($R^2 = 0{\cdot}28$, $P < 0{\cdot}01$).

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Table 1. *Effect of genetics and feeding level on cow's milk characteristics*

	Genetic level of protein concentration (PC)								
	Low			High			Significance		
Feeding level (FL)	Low	Medium	High	Low	Medium	High	PC	FL	RSD
Energy supply, UFL/d	10·1	12·0	13·8	9·8	11·4	13·3	ns	**	0·4
Nitrogen supply, PDIE/d	1112	1304	1496	1074	1242	1444	ns	**	41
Energy balance, UFL/d	−0·8	0·2	1·8	−0·8	0·3	1·7	ns	**	0·6
Nitrogen balance, PDIE/d	77	173	334	76	195	334	ns	**	62
Milk, kg/d	14·5	16·5	17·0	12·7	13·6	14·9	ns	**	1·5
Fat conc. g/kg	35·6	35·8	36·4	38·3	39·1	38·4	ns	ns	2·0
Protein conc. g/kg	28·0	28·8	29·8	31·2	32·5	32·8	**	**	0·8
Casein conc. g/kg	22·6	23·3	23·9	25·0	26·2	26·3	**	**	0·8
Paracasein conc. g/kg	22·0	22·6	23·4	24·4	25·5	25·7	**	**	0·7
Rennet serum protein, g/kg	6·0	6·1	6·4	6·8	7·1	7·2	**	**	0·3
Acid serum protein, g/kg†	5·4	5·5	5·9	6·2	6·3	6·6	**	**	0·3
Casein : protein ratio, %	80·7	80·9	80·2	80·2	80·6	80·0	ns	**	0·8
Paracasein : protein ratio, %	78·4	78·7	78·4	78·3	78·3	78·2	ns	ns	0·7
Cells, 1000/ml	282	210	188	88	157	116	ns	ns	155
Urea, mgN/100 ml	12·3	10·9	11·4	12·4	11·2	12·1	ns	**	1·5
Plasmin activity‡	3·68	3·98	4·44	5·19	4·94	5·21	ns	**	0·53
Plasminogen activity‡	30·06	30·54	30·15	30·92	32·01	32·14	ns	ns	3·1
Casein fractions, %§									
$\alpha_{s2}+\alpha_{s1}$Casein	48·2	47·4	47·5	48·3	48·6	48·9	ns	ns	0·7
β-Casein	36·6	36·3	36·6	35·3	35·5	35·2	ns	ns	2·1
κ-Casein	6·28	6·76	7·15	7·70	7·75	8·10	ns	ns	0·6
X2	1·59	1·55	1·57	1·72	1·78	1·43	ns	ns	0·29
X5	2·58	2·52	2·23	2·66	2·57	2·29	ns	*	0·27

† Protein concentration – casein concentration.
‡ Arbitrary unit, see text.
§ Analyses performed on two cows per group.
* $P < 0·05$, ** $P < 0·01$.

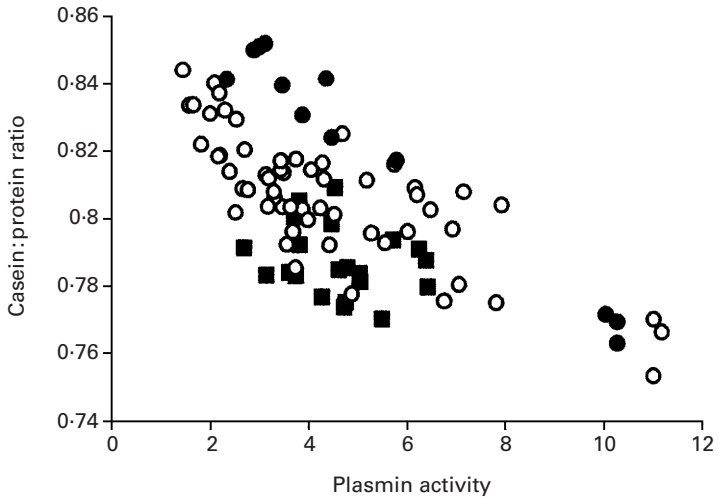

Fig. 2. Relationship between casein : protein ratio and plasmin activity, according to the β-lactoglobulin genetic variant (■, AA variant; ○, AB variant; ●, BB variant).

DISCUSSION

This trial confirmed the marked effect of the level of energy nutrition on milk protein concentration (Sutton, 1989; Spörndly, 1989). The overall average response in protein content was 0·5 g/kg per additional UFL (0·04 g/kg per additional MJ

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metabolizable energy), i.e., a value slightly lower than that previously suggested (Coulon & Rémond, 1991) from literature results. It shows that such a response was not linked to the cows' genetic potential.

The proportions of paracasein and casein in total proteins were not markedly altered by level of nutrition. These results confirmed most literature reports (Colin *et al.* 1993; Murphy & O'Mara, 1993; Malossini *et al.* 1996; Moorby *et al.* 1996; Rulquin & Delaby 1997; Coulon *et al.* 1998*a*). Some authors, however, noted a sharp reduction of the casein/protein ratio during severe dietary restrictions at the end of lactation (Kefford *et al.* 1995; Lacy-Hulbert *et al.* 1999): it would appear in that case that the effect of dietary restriction accelerates the normal mammary involution process at the end of lactation by reducing milk production. Cellular permeability is increased and the concentrations of a number of milk constituents from blood increase (somatic cells, plasmin, IgG, BSA) (Dupont *et al.* 1998). Such was not the case in this study, where milk production, although significantly reduced by treatment (2·4 kg/d between H and L groups, i.e. 16 %), sustained a much higher level than that reported by Kefford *et al.* (1995) and Lacy-Hulbert *et al.* (1999). Likewise, somatic cell count was not modified by dietary treatment and plasmin activity was not increased by a reduction in feeding level, quite the opposite. Using late-pregnancy, individual milk samples, Rémond *et al.* (1992) showed that the reduction of the casein/protein ratio depended more on the level of milk production than on the stage of lactation.

The proportion of paracasein and casein in total proteins was not modified either by the genetic potential of the cow or the protein concentration in the milk. That result complements and refines our previous findings (Coulon *et al.* 1998*b*) whereby we did not find evidence of any marked modifications of that ratio when proteins increased under the joint effect of physiological condition and individual genetic variability. When comparing milk samples from cows of different breeds with different protein concentrations, sensitive variations of that ratio can sometimes be observed between breeds, to the advantage of high-protein breeds (Blake *et al.* 1980; Malossini *et al.* 1996). These differences are essentially due to different inter-breed distribution of variants B of β-lg and of κ-casein, which strongly influence the casein/protein ratio (Grosclaude, 1988; Rahali & Ménard 1991; Coulon *et al.* 1998*a*).

In this trial, the relationship between paracasein or casein and the protein concentration was linear over the whole range of protein concentration studied (24–37 g/kg). The mean difference between casein and paracasein concentration was restricted (0·6 g/kg) and smaller than that described by Karman *et al.* (1987) for individual milk samples (0·9 g/kg) or by Grappin & Lefier (1993) for herd milk (1·2 g/kg). That difference was due to the caseino-macropeptide produced by the action of rennet on κ-casein, less the proportion (about 20 %) of the proteose-peptones resulting from β-casein degradation (Karman *et al.* 1987). The fact that the difference noted in this trial was unrelated to the proportion of κ-casein but decreased as plasmin activity increased suggests that its variation is linked more to variation of milk proteose peptone content than to caseino-macropeptide.

To conclude, this study shows that increasing milk protein concentration by genetic selection or by feeding, or by a combination of both, has little effect on the composition of the milk proteins. In particular, the proportion of paracasein in total proteins was not modified. Also, the relationship between paracasein or casein concentration and protein concentration remained linear and narrow over the very wide range of protein concentrations studied. However, although we used fresh milk from mastitis-free cows, we noted a wide individual variability of the cheesemaking

properties (as assessed from the proportion of casein or paracasein in total proteins). Three-fourths of that variability can be explained by taking joint account of the genetic variant of β-lg and milk plasmin concentration. This finding is important for cheesemaking quality, especially if milk is kept in refrigerated storage for several days before processing. During storage, plasmin may induce milk casein hydrolysis into soluble peptides that will be eliminated during cheese exudation. In a recent trial (Coulon *et al.* unpublished results), we noted a slight reduction of the casein/protein ratio in milk kept in refrigerated storage for 96 h.

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