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Original article

Feeding milk replacer instead of whole milk affects blood plasma proteome and lipid profile in preruminant calves

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Abstract

The study was undertaken to determine the effect of feeding milk or milk-replacer on the blood plasma proteome and lipid profile in calves during the second week of life. Feeding milk-replacer significantly decreased the expression of plasma apoA-I. Age of calves affected apoA-I expression, which was higher on the 8th than on the 11th and 14th day of life. A significant effect of interaction between diet and age was also observed. The expression of apoA-IV, was significantly affected by diet and was lower in calves fed milk replacer. Expression of this protein was significantly lower at the 8th day of life and was up-regulated in the calves fed milk-replacer at the second week of life. Calves fed milk-replacer had greater expression of haptoglobin, which differed significantly between days of blood sampling, being higher on the 8th than on the11th and 14th day. The interactive effect of diet and age affected haptoglobin expression, which was successively down-regulated in calves fed milk replacer. Diet had a significant effect on the plasma lipid profile. Animals fed milk had a greater concentration of TC, HDLC and LDLC. The composition of milk-replacer, especially fat source, is probably the main factor that affects expression of proteins involved in cholesterol metabolism and level of components of lipid profile in calves fed formula. We claim that the initially increased level of haptoglobin, followed by its decrease during the second week of life in calves fed milk-replacer may indicate the presence of short-term stress induced by changes in the feeding system.

Key words: proteome, calves, milk, milk replacer, blood plasma, two-dimensional electrophoresis, MALDI-TOF mass spectrometry

Introduction

Proper feeding and management is extremely important during the first weeks of a calf's life. First colostrum intake triggers a variety of crucial adaptive changes in the vital organs of newborns, and these changes can be reflected in the changes in body fluid composition (Herosimczyk et al. 2011, 2012). Feeding milk replacers is a common practice on dairy farms. Although currently most of the milk replacers comprise colostrum and milk-derived proteins, these supplements are deprived of essential non-nutritional factors, necessary to maintain homeostasis and proper development of calves. Thus, the absence of these

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biologically active substances may lead to developmental defects of the small intestine morphology. This in turn may result in developmental disorders of absorption, and affects the quantity and activity of digestive enzymes (Blattler et al. 2001, Guilloteau et al. 2009).

Proteomics is considered a sensitive tool for assessment of the physiological status of the organism. To date, proteomic approaches have been successfully applied in many studies aimed at investigating physiological and pathological conditions both in humans and laboratory animals, and have been extensively reviewed by Nair et al. (2004). However, not many proteomic studies have been carried out in farm animals. Recently, domestic cattle (*Bos taurus*) have gained considerable attention as a valuable target for proteomic-based research (Bendixen et al. 2011). Over the last decade, many reference two-dimensional (2-D) maps of bovine body fluids and tissues have been created (Wait et al. 2002, D'Ambrosio et al. 2005, Skrzypczak et al. 2011). There have been numerous studies investigating the effects of different factors on plasma proteome changes in cattle; for example, Yang et al. (2012) found significant changes in plasma acute phase proteins (APPs) expression, using two-dimensional electrophoresis (2-DE), in cows which had developed post-partum subclinical mastitis. An attempt has also been made to evaluate the functional molecular plasma biomarkers for the detection of growth-promoting substances which are illegally used in veal calves (Draisci et al. 2007). In our previous study, the dynamic changes in plasma proteome expression pattern from birth to the seventh day of the life of calves which were fed dams colostrum and milk were observed (Herosimczyk et al. 2013). To our knowledge, the comparison of the effect of milk or milk replacer feeding on blood plasma proteome has never been investigated in neonatal calves. Therefore, this study was undertaken to determine the effect of natural (whole milk) or artificial (milk replacer) feed on blood plasma proteome and lipid profile in dairy calves during the second week of life.

Materials and Methods

Animals: The experiment was carried out on 12 Polish Holstein-Friesian variety black-and-white bull calves during the second week of life. The calves were born from multiparous cows aged between 4 to 6 years with pregnancies of normal length. Immediately after birth the calves were separated from their dams and during the entire experimental period were kept indoors in individual pens under the same environmental conditions.

Animal feeding: Animals of both groups were fed with the colostrum of their own mothers for the first 3 days of life at a rate of 4-5 l/calf/day and then with pooled milk at 6 l/calf/day until the 7th day of postnatal life using a nipple pail (the amount of colostrum and milk was approx. 10% of body weight per day). From birth till the 14th day of life the calves were fed three times per day (6 a.m., noon, and 6 p.m.). Based on the increasing level of the total protein in the plasma of calves during the first 12 hours of life, from a mean value of 7.31 g/dl (immediately after birth) to a level of 9.44 g/dl (12 hour), it can be concluded that the colostrum of all dams had sufficient concentration of IgG, and the passive transfer through the intestines was also sufficient. The calves did not show any disease symptoms during the whole experimental period. On the 7th day the animals were randomly divided into two groups.

The first group (n=6) was fed pooled milk (6-7 kg/head/day) and the second (n=6) milk replacer (6-7 kg/head/day). Avarage concentration of fat and protein in the milk in the second week of lactation was respectively 4.2% and 3.1%. Commercially available milk replacer (Sanolac PREMIUM, Sano) was used in this experiment (21% total protein, 17% crude fat, 0.2% crude fibre, 9.2% crude ash, 1.7% cristal l-lysine, 1.2% calcium, 0.7% phosphorus). Bovine colostrum and milk-derived proteins were used as a main protein source. The fat was a blend of palm and coconut oil. The milk replacer solids (154 g) were reconstitued in water to the total volume of 1 liter. Both feed compositions are compared in Table 1. The use and handling of animals for this experiment was approved by the Local Ethics Committe for the Care and Use of Laboratory Animals (No. 5/2008, 24.01.2008).

Blood was drawn from the jugular vein into K_3EDTA pre-coated tubes once a day three hours after morning feeding from the 8th until the 14th day of life. The blood was centrifuged (10 min, 4°C, 1500 g) and the obtained plasma was stored at (-80°C) until analysis.

Two dimensional electrophoresis was performed on samples collected on the 8th, 11th and 14th day of life. Separation of plasma protein (120 µg for analytical and 1.0 mg for preparative gels) was performed as previously described by Herosimczyk et al. (2013) using 3-10, 17 cm NL ReadyStrip™ IPG Strips (Bio-Rad) and 12% large format (20x25 cm) SDS-PAGE gels. Preparative gels used for protein identification with mass spectrometry were stained with colloidal coomassie dye (G-250) according to Hoving's protocol (Westermeier, 2006). Analytical gels used for the protein expression pattern analysis were stained with silver stain according to Chevallet

Table 1. Gross energy (MJ/kg DM) and composition (g/kg DM) of milk and milk replacer.

	Milk	Milk replacer
Dry Matter (g/kg) of fresh weight	128.5	950
Crude Protein	241.25	221
Crude Fat	326.85	179
Crude Fiber	–	2.1
Ash	54.48	96.8
NFE	377.42	501.1
Gross Energy	25.36	21.20

Table 2. Summary of protein identification from calf blood plasma. Protein names, accession numbers according to Uniprot database, protein sequence coverage, mascot score, theoretical isoelectric points and molecular masses for identified protein spots are shown. Spot numbers from a1 to j2 refer to protein spots marked on Fig 1.

Spot	Protein name	Accession no.	Sequence coverage	Mascot score	Theoretical pI/ Mr [kDa]
a1*	apolipoprotein A-I	P15497	60%	184	5.71/30.26
a2	apolipoprotein A-I precursor	P15497	33%	74	5.71/30.26
b1*	apolipoprotein A-IV	Q32PJ2	48%	165	5.30/42.99
b2*			37%	79	
c1*	haptoglobin	Q2TBU0	25%	71	7.83/45.63
c2*			21%	59	
d1	α1-antitrypsin	P34955	33%	128	6.05/46.42
d2			29%	96	
e1	fibrinogen gamma-B chain	P12799	22%	78	5.54/50.84
e2			26%	99	
e3			37%	136	
e4			44%	155	
e5			52%	177	
e6			22%	77	
e7			28%	102	
e8			36%	133	
f1	fibrinogen alpha chain	P02672	36%	205	6.73/67.48
g1	fibrinogen beta chain	P02676	51%	216	8.45/53.93
h1	serum albumin	P02769	25%	85	5.82/71.24
i1	complement factor B	P81187	8%	46	7.87/86.74
j1	alpha-2-HS-glycoprotein	P12763	30%	49	5.26/39.19
j2			31%	55	

* – indicates protein spots with statistically important altered expression

et al. (2006). Image digitalization was performed using a calibrated GS-800 densitometer (Bio-Rad).

Image analysis: 2-D image analysis was performed using PDQuest Analysis 8.01. Advanced (Bio-Rad) software. Analytical procedures performed on each gel included: spot background substraction, detection and matching. Normalization of each spot was performed using local regression model (LOESS). Coefficient of variation (CV) was calculated for replicate groups and then significance of protein expression changes was then measured.

Mass spectrometry: After manual excision from the gels, protein spots were identified using a Microflex™ MALDI TOF (matrix assisted laser desorption/ionisation – time of flight) mass spectrometer (Bruker Daltonics) as previously described by Herosimczyk et al. (2013).

Total cholesterol (TC), high-density lipoprotein cholesterol (HDLC) and triglycerides (TG) were determined spectrophotometrically (PowerWave™ XA, BioTek) using colorimetric test kits (Biolabo, Aqua-Med, BioMaxima), accordingly to the manufac-

Table 3. Blood plasma concentration of total cholesterol (TC), HDL-cholesterol (HDLC), LDL-cholesterol (LDLC) and triglicerides (TG) in milk or milk-replacer fed calves during the second week of life. The significance of the effect of diet, age and interaction between age x diet is also marked in the table.

Variable	Feeding system		Day of life							P-values		
			8	9	10	11	12	13	14	diet	age	diet x age
TC [mmol/l]	milk (n=6)	$\bar{x}$	3.21	3.33	3.38	3.30	3.28	3.16	3.21	0.001	0.186	0.600
		SD	*0.62*	*0.85*	*0.52*	*0.65*	*0.59*	*0.78*	*1.01*			
	replacer (n=6)	$\bar{x}$	2.40	2.55	2.20	1.98	1.69	1.69	1.77			
		SD	*0.42*	*0.43*	*0.58*	*0.46*	*0.38*	*0.56*	*0.49*			
HDLC [mmol/l]	milk (n=6)	$\bar{x}$	2.46	2.19	2.30	2.29	2.17	2.09	2.11	0.003	0.163	0.892
		SD	*0.51*	*0.48*	*0.52*	*0.48*	*0.39*	*0.57*	*0.60*			
	replacer (n=6)	$\bar{x}$	1.66	1.65	1.58	1.40	1.41	1.23	1.13			
		SD	*0.38*	*0.48*	*0.43*	*0.37*	*0.54*	*0.62*	*0.35*			
LDLC [mmol/l]	milk (n=6)	$\bar{x}$	0.74	0.51	0.78	0.59	0.82	0.73	0.83	0.039	0.737	0.468
		SD	*0.28*	*0.36*	*0.28*	*0.19*	*0.25*	*0.48*	*0.48*			
	replacer (n=6)	$\bar{x}$	0.48	0.32	0.52	0.47	0.42	0.23	0.37			
		SD	*0.25*	*0.16*	*0.38*	*0.15*	*0.21*	*0.14*	*0.21*			
TG [mmol/l]	milk (n=6)	$\bar{x}$	0.64	0.62	0.65	0.73	0.62	0.56	0.59	0.504	0.310	0.955
		SD	*0,39*	*0.35*	*0.39*	*0.47*	*0.34*	*0.21*	*0.33*			
	replacer (n=6)	$\bar{x}$	0.55	0.69	0.54	0.58	0.49	0.50	0.57			
		SD	*0.11*	*0.05*	*0.14*	*0.09*	*0.11*	*0.11*	*0.15*			

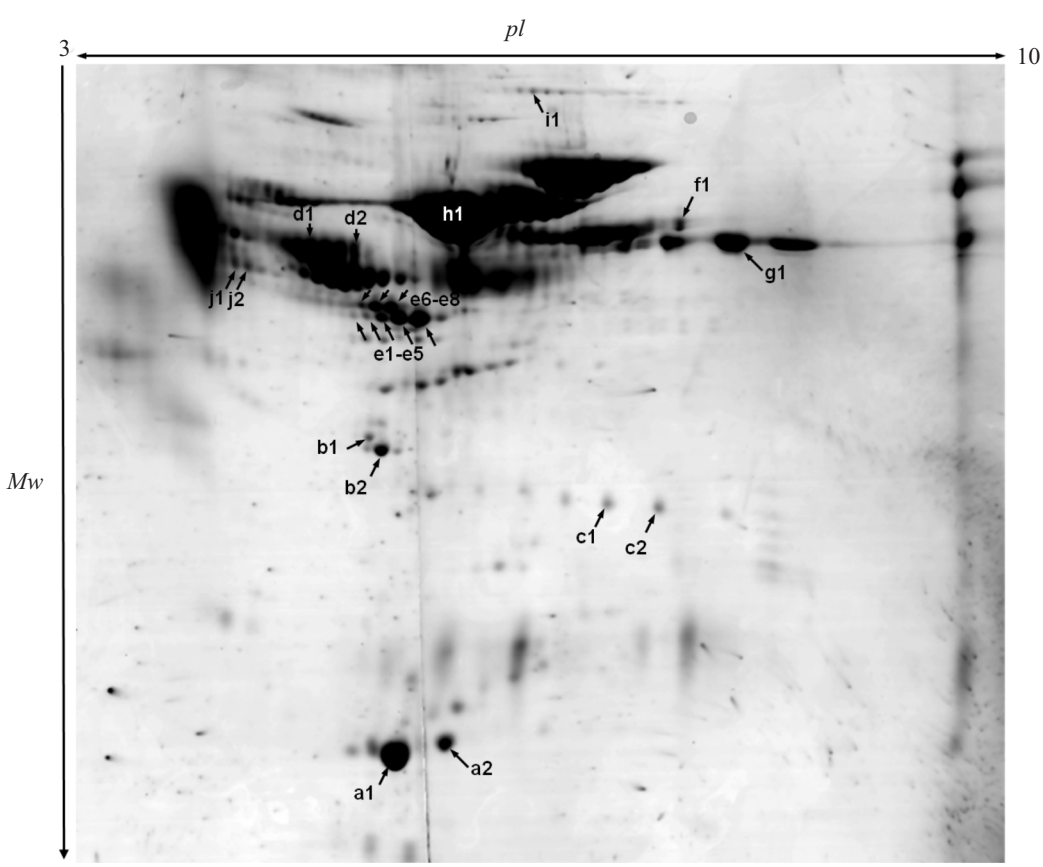

Fig. 1. Two-dimensional map of calf blood plasma proteome. 2-D gel presents coomassie-stained plasma protein pattern (1.0 mg of proteins, 3-10 NL IPG, 12% SDS-PAGE). Spot numbers refer to numbers in Table 1.

turer's specifications. The concentration of low-density lipoprotein cholesterol (LDLC) was calculated on the basis of the Friedewald's formula (LDLC = TC – HDLC – [TG/2.2]).

Statistical analysis: Mean values and standard deviations were calculated for relative abundance of protein spots and biochemical indicators. Data were analyzed by two-way (feeding and time) repeated measures ANOVA using the General Linear Models procedure of the STATGRAPHICS® Centurion XVI ver. 16.1.03 statistical package (Statistical Graphic Corp., 1982-2010). Significant differences between treatments were analyzed *post hoc* by Tukey HSD test. The effects were considered to be significant at $P \leq 0.05$.

Results

Distribution and number of spots on 2-DE profiles of plasma proteins were similar between the two groups of animals and the CV for all estimates did not exceed 50%. Twenty-two protein spots were identified using MALDI-TOF MS, representing 9 species-specific plasma proteins (Table 2, Fig. 1). Five of the identified spots (Figs. 2, 3, 4), representing 3 distinct proteins including: apolipoprotein A-I (spot a1), apolipoprotein A-IV (b1, b2) and haptoglobin (c1, c2) were found to be significantly altered between the experimental groups.

Feeding milk replacer significantly decreased the expression of apolipoprotein A-I (apoA-I) in the calf blood plasma in comparison to the milk-fed group ($P<0.01$). The age of calves also significantly affected apoA-I expression, which was higher on the 8th than on the 11th and 14th day of life ($P<0.01$). Moreover, we have demonstrated a significant effect associated with interaction between diet and age ($P<0.01$), since the expression of apoA-I in milk-fed calves was on a similar level in the second week of life in contrast to calves fed milk replacer, in which the expression of this protein was successively down-regulated.

The intensity of both spots (b1 and b2), representing apolipoprotein A-IV (apoA-IV), was significantly affected by a diet, being lower in calves fed milk replacer than in calves fed whole milk ($P<0.01$). Additionally, the expression of spot b2 was significantly altered both by age ($P<0.05$) and interaction between age and diet ($P<0.05$). The expression of this protein spot was significantly lower on the 8th day of life than on the 11th and 14th day and its level was found to be up-regulated in the blood plasma of calves fed milk replacer in the second week of life.

Calves fed milk replacer had significantly greater expression of both spots (c1 and c2) representing haptoglobin than milk-fed calves, in which expression of this protein was below the detection limit ($P<0.01$). Haptoglobin expression differed significantly between days of blood sampling ($P<0.01$), being higher on the 8th than on the 11th and 14th day. The expression of haptoglobin ($P<0.01$) was significantly influenced by the interaction between diet and age as it was unaltered in milk-fed calves and successively down-regulated in calves fed milk replacer.

Diets had significant effects on blood plasma lipid profile in preruminant calves. Animals fed whole milk had a greater concentration of LDL ($P<0.05$), HDL ($P<0.01$) and total cholesterol ($P<0.01$) than calves fed milk replacer. Plasma triglyceride level did not differ significantly between these two groups. There was no influence of age, and the interaction effect of age and diet, on the indices of lipid metabolism in calves during the second week of life.

Discussion

Using 2-DE and MALDI-TOF MS based proteomic workflow, 9 species-specific plasma proteins were identified. The presence of these proteins in the blood plasma of cattle was previously shown in our study (Skrzypczak et al. 2011) as well as in the works of other authors (Wait et al. 2002, D'Ambrosio et al. 2005, Draisci et al. 2007, Yang et al. 2012).

In the group of animals fed mother's milk, a stable concentration was observed of the tested lipid indicators such as TC, HDLC, LDLC, TG and lipid-binding proteins (apoA-I, apoA-IV). The results obtained by other authors indicate that the concentration of TC, LDLC and HDLC increased rapidly during the first seven days of postnatal life (Rauprich et al. 2000, Brucka-Jastrzębska et al. 2007, Jankowiak et al. 2010, Herosimczyk et al. 2013) and from the second week of life remained relatively stable (Jankowiak et al. 2010). A rapid increase in plasma apoA-I and apoA-IV expression in colostrum and milk fed calves during the first week of life was also reported by Herosimczyk et al. (2013).

The lowered TC, LDLC and HDLC plasma concentration in milk replacer fed calves is consistent with the results of Rauprich et al. (2000) and Hammon and Blum (1998), who showed that calves fed mother's colostrum and milk had a higher plasma concentration of TC in comparison to calves fed milk replacer, even when they were initially fed with colostrum. Van Biervliet et al. (1986) observed in human infants fed milk replacer, a lowered plasma concentration of TC, HDLC and LDL in comparison to infants fed milk. The results of the studies conducted by Hammon and Blum (1998) and Kuhne et al. (2000)

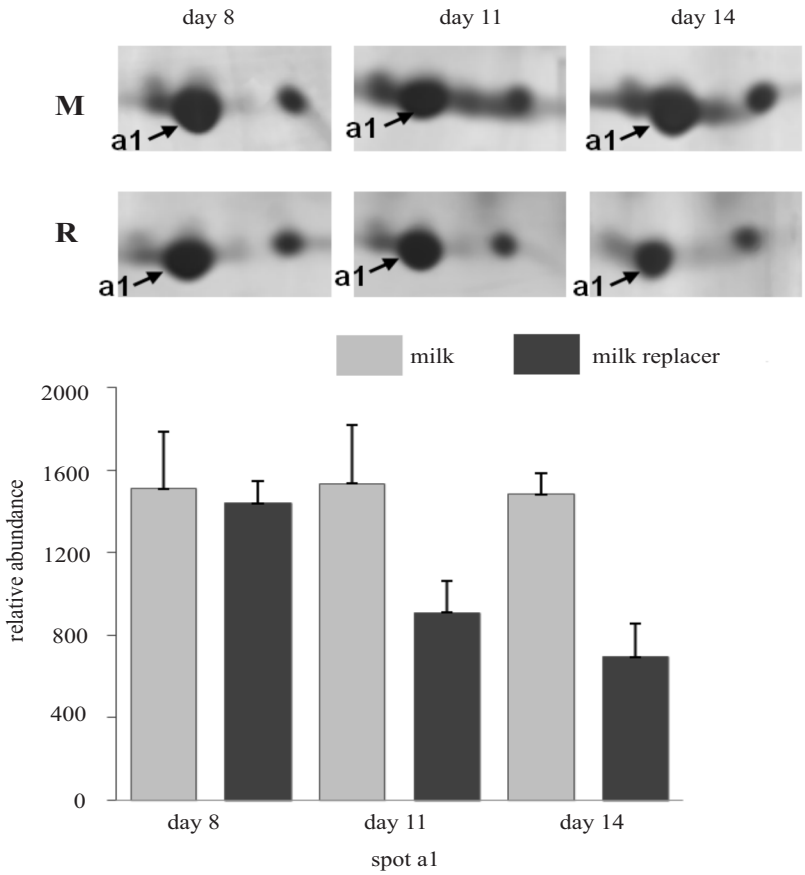

Fig. 2. Comparison of apoA-I expession pattern (spot a1) in calves fed milk (M) and milk replacer (R) during the second week of life (day 8; 11; 14). Relative abundance of apoA-I in calves fed milk or milk replacer during the second week of life. Effects of experimental factors are: diet (P=0.002), age (P<0.001) and diet x age interaction (P<0.001).

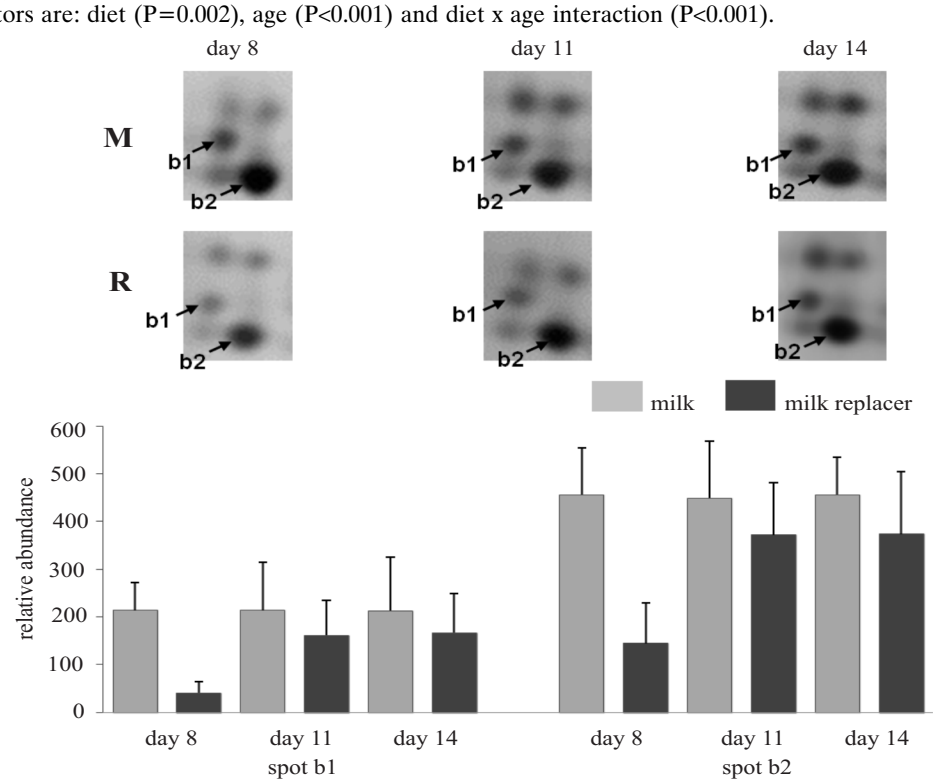

Fig. 3. Comparison of apoA-IV expession pattern (spots b1 and b2); in calves fed milk (M) and milk replacer (R) during the second week of life. Relative abundance of apoA-IV in calves fed milk or milk replacer during the second week of life. Effects of experimental factors are respectively for spot b1 and b2: diet (P=0.002 and P=0.007), age (P=0.142 and P=0.012) and diet x age interaction (P=0.141 and P=0.022).

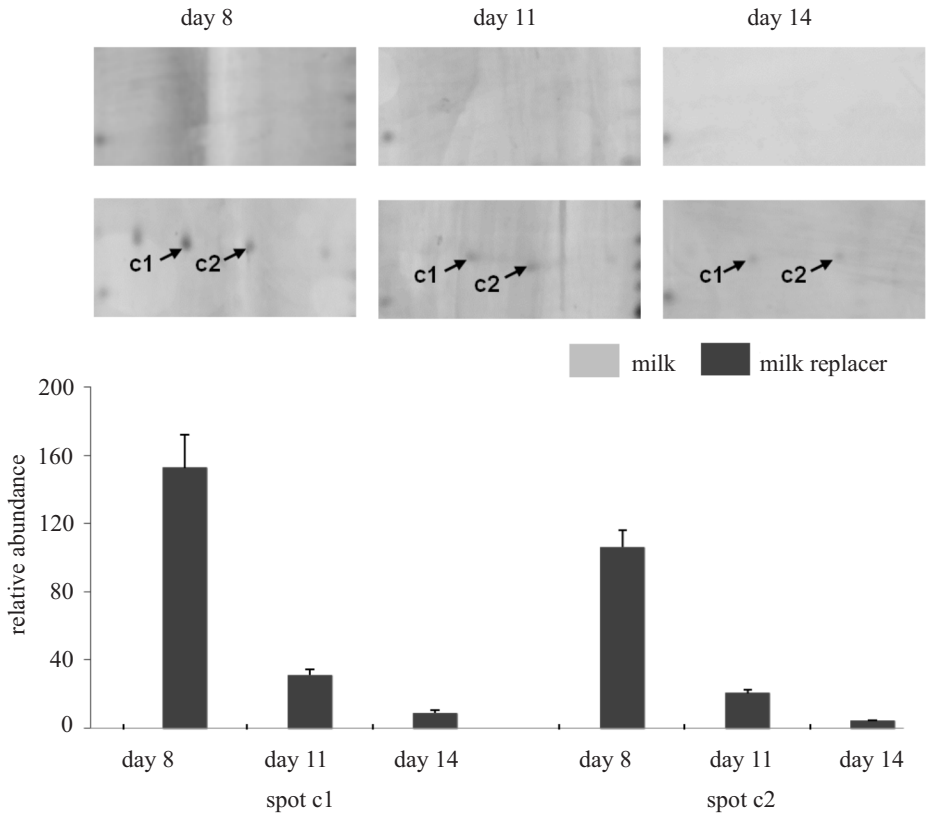

Fig. 4. Comparison of haptoglobin expession pattern (spots c1 and c2); in calves fed milk (M) and milk replacer (R) during the second week of life. Relative abundance of haptoglobin in calves fed milk or milk replacer during the second week of life. Effects of experimental factors for both spots are: diet (P<0.001), age (P<0.001) and diet x age interaction (P<0.001).

indicate that calves fed milk replacer from birth had lower plasma levels of total cholesterol when compared to calves fed mother's colostrum and milk. These authors postulate that this might be the result of higher fat content in colostrum and milk in comparison to milk replacer (Hammon and Blum 1998, Kuhne et al. 2000, Rauprich et al. 2000). This phenomenon as well as lowered HDL and LDL concentration in milk replacer fed calves, can also be attributed to a higher capacity of intestinal lipid absorption resulting from colostral and milk lipase activity and higher pancreatic lipase activity in colostrum- and milk-fed animals (Jandal 1995, Hamosh 1996, Rauprich et al. 2000, Blattler et al. 2001).

The lowered expression of apoA-I in calves fed milk-replacer noted in this study was in accordance with the results obtained by van Biervliet et al. (1986). These authors observed that the concentration of apoA-I was considerably lower in the plasma of human newborns fed milk formula containing palm and coconut oil when compared to newborns fed mother's milk. This may result from the higher lipid content in milk compared to the milk replacer (Hammon and Blum 1998, Kuhne et al. 2000, Rauprich et al. 2000) as well as from the lower digestibility of palm and coconut oil mixture (84.6%) when compared to milk fat (Huuskonen et al. 2005). Carpintero et al. (2005) and Pineiro et al. (2007) claim that the concentration of plasma apoA-I decreased in piglets in response to stress. Thus, it may be hypothesized that a decrease in the expression of plasma apoA-I observed in the present study in calves fed milk replacer was additionaly related to stress-induced feeding changes.

This study demonstrates that feeding calves with milk replacer caused an early and potent decrease in the expression of plasma apoA-IV. The intestinal synthesis of apoA-IV and its plasma concentration are mainly the effect of intestinal absorbtion of long and medium chain fatty acids (Gonzalez-Vallina et al. 1996, Kalogeris et al. 1996). Moreover, Radosavljevic et al. (1992) postulate that dietary carbohydrates such as lactose may also costimulate an increase in the expression of intestinal apoA-IV, and lead to an increase in its concentration in blood plasma. Rafat et al. (2004) confirmed that short-term starvation in humans may markedly lower the concentration of plasma apoA-IV. Given the above, we hypothetise that the lower expression of plasma apoA-IV observed in the present study in calves fed milk replacer may result from the lower digestibility of palm and coconut oil mixture in the milk replacer compared to milk fat (Huuskonen et al. 2005).

The concentration of haptoglobin in the plasma of healthy cattle is relatively low or even below the detection limit (Alsemgeest et al. 1995, Gray et al. 1996). In the group of calves fed milk, protein spots corresponding to haptoglobin were not detected on 2-D gels. This results confirms the statment of Bertoni et al. (2010) that the level of haptoglobin in calf blood plasma after the first three days of life is similar to the concentration observed in adult animals. An initially increased expression of plasma haptoglobin in the group of calves fed milk replacer was partially consistent with the findings of Hammon and Blum (1998). These authors observed an increase in plasma cortisol levels in calves fed milk formula in comparison to the control group fed colostrum and milk, which may indicate that changes in the feeding system may induce stress. In addition, the effect of changes in the feeding pattern on the level of plasma APP's has been proven (Pineiro et al. 2007). This may indicate that stress-induced changes, resulting in the short-term up-regulation of haptoglobin expression, can be attributed to changes in the feed, and particularly to its composition. This acute phase response develops until the gastrointestinal tract will not adapt to food other than milk. Petersen et al. (2004) postulated that haptoglobin is probably involved in lipid metabolism, yet its mechanism of action still remains unknown.

Conclusion

The nutrient composition of milk formula, especially the fat source, is probably the main factor affecting both the quantity and activity of digestive enzymes, and may contribute to the lowered digestibility of milk formulas. This is reflected by down-regulation of proteins involved in cholesterol metabolism (apo A-I and apoA-IV) and lowered level of plasma TC, HDLC and LDLC in calves fed milk replacer. We have also shown that changes in the feeding system may induce short-term stress, resulting in initially increased plasma haptoglobin level followed by its gradual decrease during the second week of life in calves fed milk replacer.

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