Milk proteins as a feed restriction signature indicating the metabolic adaptation of dairy cows

A. Leduc
INRAE, Institut Agro Rennes Angers, PEGASE
S. Le Guillou
Université Paris-Saclay, INRAE, AgroParisTech, GABI
L. Bianchi
Université Paris-Saclay, INRAE, AgroParisTech, GABI
L. Oliveira Correia
AgroParisTech, Micalis Institute, Université Paris-Saclay, INRAE
M. Gelé
Institut de l'Elevage
J. Pires
INRAE, Université Clermont Auvergne, UMRH
P. Martin
Université Paris-Saclay, INRAE, AgroParisTech, GABI
C. Leroux
INRAE, Université Clermont Auvergne, UMRH
F. Le Provost
Université Paris-Saclay, INRAE, AgroParisTech, GABI
M. Boutinaud (marion.boutinaud@inrae.fr)
INRAE, Institut Agro Rennes Angers, PEGASE

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Abstract

Milk production in dairy cows is affected by numerous factors, including diet. Feed restriction is known to have little impact on milk total protein content but its effect on the fine protein composition is still poorly documented. The objective of this study was to describe the effects of two feed restriction trials of different intensities on the milk protein composition of Holstein cows. One restriction trial was short and of high intensity (SH), the second was long and of moderate intensity (LM). Feed restriction decreased the milk protein yield for caseins under the LM trial and of all six major milk proteins under the SH trial. These decreased yields lead to lower concentrations of αs1-, αs2- and β-caseins during the SH trial. The milk proteome was affected as a function of restriction intensity. Among the 345 proteins identified eight varied under the LM trial and 160 under the SH trial. Ontology analyses revealed their implication in carbohydrate, lipid and protein metabolisms as well as in the immune system. These proteins reflected adaptations of the animal and mammary gland physiology to feed restriction and constituted a signature of this change.

Introduction

Milk is a secretory product rich in proteins, lipids, lactose and minerals, rendering it a unique source of nutrient for neonates and adults. Milk yield and composition is influenced by numerous factors such as genetics, environment, health status, lactation stage and nutrition. Undernutrition in dairy cows can rapidly induce a negative energy balance that is known to impact metabolism through body reserve mobilization which might affect health (notably involving an increased risk of ketosis) and also milk production and hence economic outcomes. Such modifications obviously affect milk composition but the extent of feed restriction effects mainly depend on its duration, intensity and the lactation stage at which it occurs[1].

Feed restriction experiments performed on dairy cows have generally shown little or no effect on milk total protein content, and only a few of these studies explored their effect on fine protein composition. Bovine milk contains six major proteins: four caseins (CN) that account for about 80% of total proteins (αs1-, αs2, β and κCN) and two major whey proteins: αlactalbumin (αLA) and βlactoglobulin (βLG). Some feed restriction studies focused their analyses on these six proteins, such as that by Gellrich et al.[2] who did not observe any variations in concentration during a three-day feed restriction period of moderate intensity in early and mid-lactation. Similarly, Vanbergue et al.[3] did not see any variations in major milk protein concentrations after 21 days of feed restriction of moderate intensity (-25% of dry matter intake (DMI)) but showed lower αs2 and βCN concentrations with a conserved grass diet than with a corn silage diet. However, Auldist et al.[4] described decreased concentrations of every CN and βLG during an 8-day feed restriction of high intensity based on pasture allowance (estimated at > 45 *versus* 18 kg DM/day per cow). Based on these data, the intensity of the feed restriction may affect major milk protein concentrations. However, milk does not solely contain these six proteins, and a few proteomic analyses have reported on the effects of feed restriction on global milk protein profiles. Only one studied proteome

variations induced by diet; it involved different ratios of dietary rumen degradable protein to rumen undegradable protein, and the authors did not observe any effects on low-abundance proteins[5]. Furthermore, an aggregation of proteomic data on cow's milk – which included 20 publications – reported a total of 4,654 unique proteins[6]. These proteins varied throughout different lactation stages and originated from various tissues such as the liver, adipose tissue or mammary gland; they may therefore have reflected mammary gland metabolism or even global metabolism at a given time. The authors suggested that some of the milk proteins detected exclusively during early lactation might be biomarkers of a negative energy balance[6].

The aim of the present study was to describe the effects of feed restrictions of different intensity on milk protein composition in dairy cows in order to identify proteins that might characterize this physiological stress. Two feed restriction trials were applied, one of short duration and high intensity (SH) and the other of long duration and moderate intensity (LM). Milk sampled before, during and after these restriction periods was used to explore its major protein profiles and proteomes.

Results

Comparison of trials during pre-restriction periods

Before studying the effects of feed restriction, a comparison of the milk protein composition between both trials was performed before feed restrictions, in order to ensure that the comparison of feed restrictions trials was relevant. Comparison of the milk samples collected before the feed restriction period (d7 and d2 for LM and SH, respectively) showed that the concentrations in each major milk protein did not differ between the trials except for αLA for which concentrations were higher in LM samples than in SH samples ($P < 0.001$, Fig. 2A). The yields of most major protein were higher in LM trial than in SH trial, particularly for αLA, αs1, αs2 and βCN ($P = 0.0004$; 0.006; 0.02 and 0.006, respectively, Fig. 2B).

Proteome analyses performed using LCMS/MS on eight cows from each trial, enabled the identification of 345 different proteins in these milk samples. Of these, only 262 were found during the pre-restriction period, with 90 proteins found in all samples from both trials; 43 proteins exclusive to SH trial and nine exclusive to LM trial. The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium via the PRIDE partner repository with the dataset identifier XXXX[7].

Effects of restrictions on animal performance

The results concerning DMI, energy balance, milk yield and composition and plasma NEFA have been reported previously[8,9]. The two feed restriction protocols were of different intensities as indicated by the reduction in DMI (-20% vs -65%, respectively, in the LM and SH trials; Table 1) and led to a negative energy balance with decreased milk yields (-9% vs -33%, respectively, in the LM and SH trials; Table 1). These protocols induced changes to the milk composition that were similar under both trials, with reduced milk

lactose and protein contents and increased milk fat content (Table 1). These effects were accompanied by body reserve mobilization, as shown by an increase in plasma non-esterified fatty acids[8,9] (Table 1).

Table 1
Effects of feed restriction on dry matter intake (DMI), energy balance (NE_L), milk yield and composition and plasma non-esterified fatty acid (NEFA) levels during the two trials in dairy cows, one of long duration and moderate intensity (LM) and the other of short duration and high intensity (SH).

	LM trial[9]				**SH trial[8]**			
Item	Control	Restricted	SEM	*P*-value	Control	Restricted	SEM	*P*-value
DMI (kg/d)	24.1	19.2	0.09	< 0.001	25.1	8.8	0.87	< 0.001
NE_L (MJ/d)	2.2	-21.4	1.15	< 0.001	43.6	-42.3	4.85	< 0.001
Milk yield (kg/d)	38.9	35.4	0.77	< 0.001	28.9	19.3	1.18	< 0.001
Milk fat (%)	3.42	3.63	0.032	< 0.001	3.58	4.06	0.165	0.004
Milk protein (%)	2.93	2.79	0.011	< 0.001	3.06	2.93	0.051	0.009
Milk lactose (%)	5.03	4.93	0.017	< 0.001	5.07	4.89	0.046	0.008
Plasma NEFA (μM)	0.10	0.43	0.028	< 0.001	0.11	0.69	0.056	< 0.001

Variations in major milk proteins induced by feed restriction

Among the cows involved in the LM trial, none of the major protein concentrations was significantly affected by feed restriction except for a tendency towards a lower αs2-CN concentration ($P = 0.06$) in feed restricted *vs* control cows during the restriction period. Nevertheless, the yields of αs1, αs2, β and κCN decreased under feed restriction (P = 0.004; 0.005; 0.002 and 0.05, respectively; Fig. 3). These yields rapidly returned to pre-restriction values after *adlibitum* refeeding.

In the eight cows involved in the SH trial, milk samples were collected for analysis two days before the feed restriction period, on days 2 and 5 after the start of feed restriction and on day 11, after the return to *adlibitum* feeding. αs1, αs2 and βCN concentrations decreased significantly under feed restriction during this trial ($P = 0.004$; 0.0004; 0.01, respectively) and αLA concentrations tended to decrease ($P = 0.06$). The quantities of all six major milk proteins produced per day decreased significantly under feed restriction when compared to pre-restriction values ($P < 0.001$; Fig. 4). These concentrations and yields quickly returned to pre-restriction values after *adlibitum* refeeding.

Proteome variations induced by feed restriction

In the LM trial, proteome analyses led to the identification of eight proteins whose abundance varied during feed restriction ($P < 0.05$). In particular, the abundance of beta lactoglobulin D decreased whereas those of apolipoprotein AIV, alpha1Bglycoprotein, angiotensinogen, serotransferrin and fatty acid synthase increased under feed restriction. In addition, two proteins, Alphaenolase and ceruloplasmin, were only detected in the milk after feed restriction (Fig. 5).

In the SH trial, proteome analysis led to the identification of 160 proteins whose abundance varied during feed restriction ($P < 0.05$), including 92 with a fold change > 2 or < 0.5 (Fig. 6; Supplementary Data S1). Among these 160 proteins, 43 where only present in milk during feed restriction and Transcobalamin2 was only present before restriction. The abundance of 39 proteins decreased and that of 77 increased during feed restriction.

Among these 160 differentially abundant proteins, all those affected by feed restriction during the LM trial were found, except for a fragment of β LG D, a rare genetic variant.

Biological processes affected by feed restriction

Gene Ontology (GO) analyses were only performed on data from the SH trial because too few proteins varied under feed restriction during the LM trial to perform a relevant GO analysis. The GO analyses of the SH trial showed that the major cellular processes affected by feed restriction were related to carbohydrate, protein and lipid metabolisms as well as immune system processes (Table 2).

Groups of GO terms are related classes in an ontology with specific subclasses and parent terms are clustered; the list of all GO terms identified for SH trial is available in Supplementary Data S2. The section on carbohydrate metabolism includes five different families of GO terms and 13 affected proteins. All these proteins except αLA were more abundant in milk during feed restriction than during the pre-restriction period (Table 2A). The lipid metabolism section includes 29 different families of GO terms and 14 affected proteins (Table 2B). Their abundance in milk decreased for seven of these proteins and increased for the seven others during feed restriction. The protein metabolism section groups four different families of GO terms and 33 affected proteins (Table 2C). Most of these affected proteins were more abundant in milk during feed restriction than during the pre-restriction period. The immune system section groups 21 different families of GO terms and 49 affected proteins (Table 2D). Forty-four of these affected proteins were more abundant in milk during feed restriction than during the pre-restriction period, with 17 of them only being detected in milk during feed restriction.

Table 2

Milk proteins affected by feed restriction during the SH trial (short duration and high intensity: -64% of DMI for six days) (n = 8 Holstein dairy cows) and involved in GO terms related to carbohydrate (A), lipid (B), and protein (C) metabolisms or the immune system (D). Fold change (FC) is the ratio between d 5 and d -2. ∞ represent proteins only found in milk during feed restriction and 0 those only found before feed restriction.

A. Carbohydrate metabolism			
Protein name	Gene symbol	FC	adj p.value
6-phosphogluconate dehydrogenase, decarboxylating	PGD	∞	9.62E-05
Fructose-bisphosphate aldolase	ALDOA	∞	1.44E-05
Glucose-6-phosphate isomerase	GPI	∞	1.86E-04
Glyceraldehyde-3-phosphate dehydrogenase	GAPDH	∞	3.14E-07
Hexokinase	HK3	∞	1.32E-03
L-serine dehydratase/L-threonine deaminase	SDS	∞	5.02E-05
Phosphoglycerate kinase 1	PGK1	∞	5.02E-05
Glycogen phosphorylase, liver form	PYGL	∞	9.62E-05
Pyruvate kinase	PKM	43.00	1.01E-11
Alpha-enolase	ENO1	7.17	1.06E-07
Chitinase-3-like protein 1	CHI3L1	3.70	5.29E-08
L-lactate dehydrogenase B chain	LDHB	3.60	6.27E-03
Alpha-lactalbumin	LALBA	0.38	7.44E-03
B. Lipid metabolism			
Annexin A1	ANXA1	∞	1.20E-09
Apolipoprotein A-IV	APOA4	∞	7.61E-06

A. Carbohydrate metabolism			
Myeloperoxidase protein	MPO	∞	1.04E-06
Apolipoprotein E	APOE	9.00	7.93E-03
Perilipin	PLIN3	4.00	2.48E-03
Apolipoprotein A-I	APOA1	2.38	4.28E-05
Complement C3	C3	1.76	6.72E-04
Fatty acid synthase	FASN	0.66	4.13E-02
Platelet glycoprotein 4	CD36	0.62	8.70E-03
CIDE-N domain-containing protein	CIDEA	0.51	2.62E-02
Alpha-S1-casein	CSN1S1	0.47	5.82E-03
NPC intracellular cholesterol transporter 2	NPC2	0.45	1.39E-03
Lipoprotein lipase G	LIPG	0.36	3.65E-03
Lipoprotein lipase	LPL	0.25	2.98E-04
C. Protein metabolism			
Glyceraldehyde-3-phosphate dehydrogenase	GAPDH	∞	3.14E-07
Heat shock protein HSP 90-alpha	HSP90AA1	∞	1.04E-06
Leukocyte elastase inhibitor	SERPINB1	∞	5.03E-08
Myosin heavy chain 9	MYH9	∞	1.44E-05
Protein S100-A8	S100A8	∞	2.70E-05
SERPIN domain-containing protein	LOC786410	∞	9.03E-08

A. Carbohydrate metabolism			
Pyruvate kinase	PKM	43.00	1.01E-11
Apolipoprotein E	APOE	9.00	7.93E-03
Antithrombin-III	SERPINC1	7.80	2.23E-07
Alpha-enolase	ENO1	7.17	1.06E-07
Alpha-1-antiproteinase	SERPINA1	5.50	6.78E-07
Fibronectin	FN1	4.75	1.18E-05
C4a anaphylatoxin	C4A	4.62	1.89E-16
Chitinase-3-like protein 1	CHI3L1	3.70	5.29E-08
Alpha-2-HS-glycoprotein	AHSG	3.33	9.08E-04
Gelsolin	GSN	2.72	1.51E-05
Moesin	MSN	2.63	1.59E-02
Serpin A3-6	SERPINA3-6	2.40	3.62E-03
Apolipoprotein A-I	APOA1	2.38	4.28E-05
Serpin A3-2	SERPINA3-2	2.12	1.89E-03
Serpin A3-7	SERPINA3-7	2.02	1.89E-04
Serpin A3-3	SERPINA3-3	1.89	2.81E-02
Complement C3	C3	1.76	6.72E-04
Clusterin	CLU	1.75	2.67E-02

A. Carbohydrate metabolism			
Factor XIIa inhibitor	281035	1.74	5.84E-03
Alpha-2-macroglobulin	A2M	1.69	7.44E-03
Serpin G1	SERPING1	1.66	8.30E-03
Lactotransferrin	LTF	1.55	1.30E-16
Inter-alpha-trypsin inhibitor heavy chain H4	ITIH4	1.51	2.56E-02
Metalloproteinase inhibitor 3	TIMP3	0.65	3.46E-02
Pigment epithelium-derived factor	SERPINF1	0.63	3.18E-02
Peptidyl-prolyl cis-trans isomerase A	PPIA	0.57	2.17E-03
Lipoprotein lipase G	LIPG	0.36	3.65E-03
D. Immune system			
Alpha-actinin-1	ACTN1	∞	5.02E-05
Annexin A1	ANXA1	∞	1.20E-09
Apolipoprotein A-IV	APOA4	∞	7.61E-06
Cathelicidin-4	CATHL4	∞	6.85E-04
Coronin-1A	CORO1A	∞	3.96E-06
Glucose-6-phosphate isomerase	GPI	∞	1.86E-04
Glyceraldehyde-3-phosphate dehydrogenase	GAPDH	∞	3.14E-07
Glycogen phosphorylase, liver form	PYGL	∞	9.62E-05
Haptoglobin	HP	∞	1.85E-11

A. Carbohydrate metabolism			
Heat shock protein HSP 90-alpha	HSP90AA1	∞	1.04E-06
Ig-like domain-containing protein	ENSBTAG00000048030	∞	1.32E-03
Ig-like domain-containing protein	ENSBTAG00000050586	∞	2.70E-05
LRRCT domain-containing protein	LRG1	∞	9.62E-05
Myeloperoxidase	MPO	∞	1.04E-06
Myosin heavy chain 9	MYH9	∞	1.44E-05
Protein S100-A8	S100A8	∞	2.70E-05
Prothrombin	F2	∞	5.03E-08
Histone H2B type 1	VGNC:83556	11.00	2.40E-03
Apolipoprotein E	APOE	9.00	7.93E-03
Peptidoglycan recognition protein 1	PGLYRP1	9.00	7.83E-06
Complement component C9	C9	7.60	3.65E-07
Cathelicidin-1	CATHL1	7.00	1.73E-06
Fibronectin	FN1	4.75	1.18E-05
C4a anaphylatoxin	C4A	4.62	1.89E-16
Chitinase-3-like protein 1	CHI3L1	3.70	5.29E-08
Alpha-2-HS-glycoprotein	AHSG	3.33	9.08E-04
Serotransferrin	TF	2.87	8.02E-28

A. Carbohydrate metabolism			
Gelsolin	GSN	2.72	1.51E-05
Moesin	MSN	2.63	1.59E-02
Apolipoprotein A-I	APOA1	2.38	4.28E-05
Actin, cytoplasmic 2	ACTG1	2.20	5.04E-06
Lipocln_cytosolic_FA-bd_dom domain-containing protein	LCN2	2.12	1.63E-03
Ig-like domain-containing protein	ENSBTAG00000054702	2.05	7.44E-03
Ig-like domain-containing protein	ENSBTAG00000050373	1.89	2.86E-02
Complement C3	C3	1.76	6.72E-04
Clusterin	CLU	1.75	2.67E-02
Factor XIIa inhibitor	281035	1.74	5.84E-03
Ig-like domain-containing protein	ENSBTAG00000050515	1.72	3.10E-02
SERPIN domain-containing protein	SERPING1	1.66	8.30E-03
Lactoperoxidase	LPO	1.58	2.58E-04
Fibrinogen beta chain	FGB	1.57	7.56E-03
Lactotransferrin	LTF	1.55	1.30E-16
Complement factor B	CFB	1.52	7.44E-03
Inter-alpha-trypsin inhibitor heavy chain H4	ITIH4	1.51	2.56E-02
Gamma-glutamyltransferase 1	GGT1	0.65	7.44E-03

A. Carbohydrate metabolism			
Platelet glycoprotein 4	CD36	0.62	8.70E-03
Peptidyl-prolyl cis-trans isomerase A	PPIA	0.57	2.17E-03
Cytokeratin-1	KRT1	0.39	5.99E-03
Alpha-S2-casein	CSN1S2	0.03	3.63E-02

Discussion

The objective of this study was to investigate variations in milk protein composition induced by feed restriction, as well as the impact of the intensity of feed restriction on these variations. We first compared the milk protein composition in milk samples collected from the two trials before the feed restrictions were applied, in order to ensure that a comparison of both feed restriction trials was relevant. In term of concentrations, the major milk protein profile was similar between the trials, although α-LA was more concentrated under LM than SH trial. This difference in α-LA concentration was most likely due to a difference in the lactation stage, as the cows involved in the LM trial were around 77 DIM and those in SH were around 165 DIM. Similarly, the yields of major protein were higher under LM conditions than under SH, particularly with respect to α-LA, αs1-, αs2- and β-CN, this being linked to higher milk yields in the LM cows that were at peak lactation. Regarding proteomes, 345 proteins were identified during the trials, which was quite consistent with the milk proteomes published previously using LC-MS/MS. Indeed, among the 4654 proteins identified in the aggregation published by Delosière et al.[6], 3288 were specific to colostrum and only 775 and 577 were identified during peak lactation and mid-lactation studies, respectively. Before the restriction period, 43 low-abundance proteins were exclusive to the SH trial and nine to the LM trial. Again, this difference was very likely due to a difference in lactation stage as the proteome changes during lactation, with some proteins being exclusive to each stage[6]. In our trials, these 52 exclusive proteins only accounted for 0.8% of the total protein counts prior to restriction periods. The pre-restriction milk proteomes of both trials were therefore very similar and it was possible to compare their modifications induced by feed restriction.

In both trials, the reduction in milk yield induced by feed restriction was concomitant with a decrease in the major milk protein yield. This effect on major milk proteins increased in line with the intensity of the restriction. During the SH trial, with an important negative energy balance (-42.3 MJ/d) and milk yield loss (-34%), all major milk proteins were quantitatively affected, whereas during the LM trial, with lower negative energy balance (-21.4 MJ/d) and milk yield loss (-9%), only casein quantities were affected. During the high intensity feed restriction, this reduction in yield lowered the concentrations of αs1, αs2- and β-CN. It appeared that the αs2-CN concentration was the most sensitive to feed restriction, as it was

the most significantly affected during the SH trial (-25%) and tended to decrease under the LM trial. When studying corn versus grass diets, Vanbergue et al.[3] only observed variations in milk concentrations of αs2-CN (-22%; p = 0.029) and β-CN (-20%; p = 0.014), which supports the hypothesis that αs2-CN is the most sensitive to feed variation, followed by β- and αs1-CN. Billa et al.[10] also saw a reduction in *CSN1S2* transcripts coding for αs2-CN in the mammary gland during the feed restriction period of the SH trial. This shows that a reduction in the αs2-CN concentration in milk is directly linked to a decrease in *CSN1S2* gene expression in the mammary gland.

Proteomic analyses confirmed the decreased concentrations of α-LA, αs1 and αs2-CN during SH feed restriction, with αs2-CN being the most affected protein. This analysis also showed a significant effect of intense feed restriction on proteins involved in lipid metabolism, with 14 affected proteins involved in this metabolism in the SH trial. Among the seven proteins involved in this metabolism which displayed increased abundance during feed restriction, four involved in lipid transport and storage were found: apolipoproteins (A-I, A-IV and E) and perilipin. Moreover, the decreased abundance of CIDE-N domain-containing protein, a lipolysis inhibitor and storage activator, may have reflected increased lipid mobilization in adipose tissue, which is consistent with the increase in plasma NEFA concentrations observed in both trials. Lower concentrations of fatty acid synthase, which catalyzes the *de novo* biosynthesis of fatty acids, were observed in milk under SH conditions. This finding was in line with the reported decrease of FASN RNA in the cytosolic crescent of milk fat globules during 40% feed restriction over four days[11]. It was also consistent with the decrease in *de novo* synthesized fatty acids during the SH trial, reflected by the reduction in short chain fatty acid concentrations in milk[8]. A rise in the fat content of up to 13% was due to the uptake of long chain fatty acids from lipid mobilization, as indicated by the increase in plasma NEFA concentrations seen during both trials. Such adaptations of lipid metabolism in the context of a negative energy balance have already been well described[1,12,13], and notably involved the downregulation of several mammary lipogenic genes during the first days of short-term feed restriction[11]. However, during our LM trial, concentrations of fatty acid synthase rose slightly after five days of feed restriction, suggesting that intense restriction is necessary for this shift in fatty acid metabolism to occur.

The modifications observed regarding on milk proteins and proteome were consecutive to changes to mammary metabolism, partly because of a reduction in nutrient uptake by the mammary gland during feed restriction, as shown previously by Guinard-Flament et al.[14]. Indeed, these authors showed that feed restriction reduced mammary blood flow alongside reductions in mammary nutrient and dioxygen uptakes during a -30% DMI feed restriction[14]. Nevertheless, under SH conditions in our study, mammary metabolism appeared to partially compensate for decreased nutrient uptake by increasing carbohydrate catabolism and lipid transport. Indeed, among the 12 proteins involved in carbohydrate metabolism that were more abundant in milk during the SH trial, seven are involved in glycolysis (hexokinase, glucose-6-phosphate isomerase, fructose-bisphosphate aldolase, glyceraldehyde-3-phosphate dehydrogenase, phosphoglycerate kinase 1, alpha-enolase and pyruvate kinase), one is involved in the pentose phosphate pathway (6-phosphogluconate dehydrogenase, decarboxylating), which is the parallel pathway to

glycolysis, and glycogen phosphorylase catalyzes the rate-limiting step in glycogenolysis that produces substrate for both the glycolysis and pentose phosphate pathways. This increased abundance of proteins involved in carbohydrate degradation in milk may reflect the high level of energy required to maintain mammary gland metabolism in a lactating cow.

The total protein content decreased during both trials but only by 4% in SH, despite the lower concentrations of some major milk proteins. Lacy-Hulbert et al.[15], who had observed an increased total protein content in milk (+ 8%) during intense feed restriction (-50% of DMI for 26 days) hypothesized that feed restriction tended to concentrate serum-derived proteins in milk. Indeed, 43 proteins identified in the SH milk samples during feed restriction had not been present before restriction, and among the seven proteins with increased concentrations in milk under both the LM and SH conditions, five are normally present in plasma and two were not found in milk before restriction (ceruloplasmin, apolipoprotein A-IV, alpha-1B-glycoprotein, angiotensinogen and serotransferrin). This increase in plasma protein concentrations in milk may reflect a loss of mammary epithelial barrier integrity, which could play a role in reducing milk production during feed restriction. This had already been suggested by Herve et al.[9] who observed an elevated rate of mammary epithelial cell exfoliation under LM trial, as well as an increased Na^+ concentration in milk, and by Stumpf et al.[16] who saw an increased permeability of mammary cell tight junctions during short and intense feed restriction (-50% DMI for seven days). Under our SH conditions, we observed an elevation of lactotransferrin concentrations in milk, an increase that is known to happen during the first days of the dry period[17] when involution starts and the epithelial barrier loses its integrity. This increased permeability of the epithelial barrier is coupled with increased leucocyte infiltration of the mammary gland, as shown by higher milk somatic cell count in the LM trial[9] and during other feed restriction experiments[15,18–20], and an upregulation of immune genes, as observed in the mammary tissue during involution[21]. Moreover, 12 of the 13 proteins involved in positive regulation of immune system processes were more abundant in milk during the feed restriction period under SH conditions, suggesting a similar immune system upregulation. Variations in milk of the immune system related protein were confirmed for five of them, with similar changes to their transcript levels in the mammary gland[10], and in particular C3, which plays a central role in activation of the complement system. However, among the 33 proteins involved in protein metabolism, 12 over-abundant proteins have a protease inhibition function (α-1-antiproteinase, α-2-macroglobulin, antithrombin-III, factor XIIa inhibitor, inter-α-inhibitor heavy chain H4, leukocyte elastase inhibitor and serpins A3-2, A3-3, A3-6, A3-7, B4 and G1) and four are involved in the inhibition of complement activation, inflammation and cell death (chitinase-3-like protein 1, clusterin, factor XIIa inhibitor and heat shock protein HSP 90-α). These results therefore suggest a greater regulation of the immune system in the mammary gland during feed restriction.

These adaptations, which are reminiscent of some of those observed during early involution, remained reversible during these restriction trials, as both milk yield and composition recovered after a return to *ad libitum* feeding. Delosière et al.[6] proposed some milk proteins exclusive to early lactation as biomarkers of negative energy balance, and none of these were found in milk during the negative energy balance

induced by feed restriction later in lactation. Nevertheless, some proteins are affected by both moderate and high intensity feed restrictions: alpha-enolase, ceruloplasmin, apolipoprotein A-IV, alpha-1B-glycoprotein, angiotensinogen and serotransferrin.

Alpha-enolase is an enzyme present in all tissues that catalyzes the interconversion of 2-phosphoglycerate to phosphoenolpyruvate; its upregulation indicates an enhancement of glycolysis and has also been observed during ketosis[22], a common metabolic disease induced by a negative energy balance. The five other proteins affected by both moderate and intense feed restriction were mainly found secreted in plasma. Apolipoprotein A-IV is primarily synthesized in the small intestine; this lipid-binding protein is involved in numerous physiological processes such as lipid metabolism and glucose homeostasis[23]. Apolipoprotein A-IV upregulation in the bovine mammary gland has been described during inflammation challenges where its anti-inflammatory activities may balance the immune response[24]. Ceruloplasmin, alpha-1B-glycoprotein, serotransferrin and angiotensinogen are mainly expressed in the liver. Ceruloplasmin is a copper-binding glycoprotein with antioxidant and cytoprotective activities. Increased concentrations of cerulaplasmin in bovine milk have been described during subclinical and clinical mastitis[25] and may indicate inflammation. Alpha-1B-glycoprotein is a glycoprotein of unknown function. In the cow, its serum level seems to increase during various stresses such as tuberculosis[26], high-altitude hypoxia[27] or mastitis[28]. Serotransferrin, an iron binding transport glycoprotein, is seen at high concentrations in milk during early lactation, and then fall rapidly over time. Mastitis events can also increase serotransferrin concentrations in milk through changes to the mammary gland epithelium[29]. Angiotensinogen is the precursor of angiotensin. In dairy cows it has been shown that ketosis may alter the metabolism of angiotensinogen to angiotensin[30].

Conclusion

Feed restriction induced modifications to the milk protein composition even if there was little decrease in the total milk protein content. Feed restriction reduced the yield of major milk proteins, only affecting caseins when the restriction was of moderate intensity. This lower yield could affect the concentrations of major proteins when the restriction was sufficiently intense, probably starting with αs2-, β- and then αs1-CN. Other protein concentrations were also affected by feed restriction and may have reflected mammary gland adaptation to this stress. In fact, proteome modifications suggested metabolic adaptations such as lipid mobilization toward the mammary gland in order to compensate for less *de novo* synthesis or increased carbohydrate catabolism to compensate for reduced mammary nutrient uptake. Proteome modifications also indicated a loss of mammary epithelial barrier integrity and altered immune function, sharing common features with the changes observed during the early phase of mammary gland involution. The six low-abundance proteins that were affected by both moderate and high intensity feed restrictions, as well as αs2-CN, are putative biomarkers of a negative energy balance in dairy cows that are not specific to early lactation.

Methods

Animals, experimental designs and sampling

This article reports on the results of two distinct feed restriction trials: one of short duration and high intensity (SH) and the other of long duration and moderate intensity (LM).

The SH trial was conducted at the INRAE Herbipôle experimental farm (UE Herbipôle, 15190 Marcenat, France; https://doi.org/10.15454/1.5572318050509348E12). All procedures involving animals were approved by the local Ethics Committee of the Auvergne-Rhône-Alpes region and the French Ministry of Higher Education, Research and Innovation (APAFIS #3737–2015043014541577v2).

Eight multiparous mid-lactation (165 ± 21 days in milk (DIM)) Holstein cows were used to study the effects of six days of feed restriction designed to reduce their net energy for lactation (NE_L) by 50%, as described by Billa et al.[8]. The experiment was divided into three periods: pre-restriction (day (d) -3 to -1), restriction (d 1 to 6) and post-restriction (d 7 to 18). During the pre- and post-restriction periods, the cows were fed *ad libitum* with a total mixed ration. During the restriction period, the feed allowance was reduced to 50% of individual NE_L requirements calculated from body weight, DMI and milk yield and composition, as recorded during the pre-restriction period. Milk samples were collected during morning milking, before feed distribution, on d-2, 2, 5 and 11 relative to the initiation of feed restriction (Figure 1).

The LM trial was performed at the INRAE PEGASE experimental farm (IEPL, 35650 Le Rheu, France; https://doi.org/10.15454/yk9q-pf68). All procedures involving animals were approved by the local Ethics Committee in Animal Experiment of Rennes and the French Ministry of Higher Education, Research and Innovation (APAFIS #3063–2015110215066393).

Nineteen peak lactation (77 ± 5 DIM; lactation ranks 1 to 4) Holstein cows were used to study the effects of 29 d of feed restriction designed to reduce their DMI by 20%, as described by Herve et al.[9]. The experiment was divided into three periods: pre-restriction (d -20 to -1), restriction (d 1 to 29) and post-restriction (d 30 to 67). During the pre- and post-restriction periods the cows were fed *ad libitum* with a total mixed ration. After the pre-restriction period, the cows were assigned to either a control group (n = 9) or a feed-restricted group (n = 10) based on pre-restriction DMI, lactation rank, DIM, milk yield and composition. During the restriction period, control cows were fed 100% of their *ad libitum* DMI whereas feed-restricted cows were fed at 80% of their *ad libitum* DMI, as recorded during the pre-restriction period. Milk samples were collected during morning milking, before feed distribution, on d-7, 5, 9, 27 and 37 relative to the initiation of feed restriction (Figure 1).

Profiling of major milk proteins

All milk samples collected before, during and after feed restriction under both trials were used to profile major milk proteins. Milk samples that had been stored at -80°C were thawed for 4h at 4°C and then centrifuged for 20 min at 2600 *g* and 4°C. The fat supernatant was then removed with a spatula. Milk proteins were separated by reverse-phase (RP) HPLC using an Ultimate LC 3000 system (Thermo Fisher Scientific, Waltham, MA) as described by Fang et al.[31]. The relative concentrations of the six major milk

proteins (αS1-CN, αS2-CN, β-CN, κ-CN, α-LA, and β-LG) were estimated by the integration of peaks from UV Absorbance recorded at 214 nm, as a percentage of the total area of peaks, for each individual milk sample. Protein concentrations (g/kg) and yields (g/d) were then calculated from their relative abundance, total protein content and milk yield, all measured at the same sampling date.

Proteomic profiling

Samples from eight restricted cows under each trial, collected on the day before feed restriction and at day 5 during feed restriction were used for proteomic profiling. For the proteome analysis, samples of 15 µL skimmed milk containing around 30 g/L proteins, were loaded into 1D gel electrophoresis (NuPAGE® 4-12% Bis-Tris Gel). After the excision of gel bands, the proteins were reduced (DTT, Sigma), alkylated (iodoacetamide, Sigma) and digested with 1 µg trypsin. The peptides were desalted on a Strata-X-column (33 µm, 30 mg, Phenomenex) , dried under a vacuum and taken up in 30 µL loading buffer (0.08% trifluoroacetic acid, 2% acetonitrile) for LC-MS/MS proteome analysis.

4 µl of each sample were injected into an UltiMate™ 3000 RSLCnano System (Thermo Fisher Scientific) coupled to an Orbitrap Fusion™ Lumos™ Tribrid™ (Thermo Fisher Scientific). Separation was performed at a flow rate of 0.3 µL/min with a linear gradient of 6-30% (0.1% formic acid, 80% acetonitrile) for 110 min, 30-98% for 10 min and 98% for 10 min. A complete run, including regeneration with 99% buffer (0.1% formic acid, 2% acetonitrile) required 147 min. Nanospray ionization was performed by applying 1.6 kV in a positive mode. Capillary transfer was performed at 275°C using a capillary probe SilicaTip Emitter 10 µm.

The mass spectrometer was operated in data dependent acquisition mode. Full MS scans were captured in the Orbitrap (scan range 400-1500 m/z) with a resolution of 120,000. Dynamic exclusion was set at 10 ppm with a duration of 80 s, and the intensity threshold was fixed at 5×10^4. MS2 was performed using High Collision Dissociation (HCD) in the Orbitrap at a resolution of 30,000. Polysilaxolane ions m/z 445.12002, 519.13882, 593.15761, and 667.1764 were used for internal calibration.

Protein identification was performed using X!TandemPipeline C++ 0.4.17[32] and the *Bos taurus* UniProtKB database (version 2019, 46,697 entries). Data filtering was achieved according to a peptide E-value <0.01, protein log (E-value) <−4 and a minimum of two identified peptides per protein. The peptide and protein False Discovery Rates (FDR) were estimated at 0.68% and 0.25%, respectively. MS1 peaks were detected and aligned using MassChroQ 2.2.12[33].

The relative quantification of protein abundances was performed using two complementary methods: spectral counting (SC) defined as the number of MS2 spectra assigned to a protein[34], and extracted ion chromatograms (XIC) defined as the sum of the MS1 intensities of all peptides associated with a protein. The XIC method is suited to detecting subtle differences in protein abundance based on specific peptide data, while SC only enables the detection of larger abundance variations, including that of presence/absence.

Statistical analyses

Statistical analyses were performed using R software v4.0.2 (R Core Team, 2020, http://www.R-project.org) with the lme4 package version 1.1-23. Analyses of variance of the major milk protein data were performed using a mixed model that included day, diet and their interaction as fixed effects, and the cow as a random effect. For the LM trial, pre-restriction values were used as co-factors and feed restriction effects were calculated by comparison with the control group. For the SH trial, the restriction effect was calculated by comparison with pre-restriction values. A trial effect was analyzed for pre-restriction data using a linear model. Analyses of variance of the proteomic data were performed using a mixed model that included diet as a fixed effect and the cow as a random effect. Feed restriction effects were calculated by comparison with pre-restriction values. The threshold for statistical significance was set at $P < 0.05$ and trend-level significance was defined as $0.05 \leq P < 0.10$.

Exploration of *in silico* metabolic pathways

Enrichment analyses were carried out using the PANTHER Overrepresentation Test[35] with the GO Ontology database (DOI: 10.5281/zenodo.5228828; Released 2021-08-18). The *Bos taurus* database (22,798 proteins) was used as the reference list and Fisher's test was performed with a false discovery rate (FDR) cut-off point set at 0.05. Hierarchy sorting was used to identify families of gene ontology (GO) terms.

Declarations

Ethics declaration

All the experimental procedures were carried entirely under animal welfare guidelines (including ARRIVE guidelines) and were approved by the local Ethics Commitees in Animal Experiment and the French Ministry of higher Education, Research and Innovation.

Data Availability

The proteomic data are available on the ProteomeXchange Consortium via the PRIDE partner repository with the dataset identifier XXX[LA1] . Gene Ontology datasets analyzed during the current study are included in the Supplementary Data of this published article, HPLC raw datasets are available from the corresponding author on reasonable request.

Author contributions

MG, FLP, MB, CL and PM conceived the study, SLG, MB, CL and JP supplied the data, AL, LB and LOC analyzed data, AL interpreted the results of the experiments and prepared the Figures, AL, MB and FLP

wrote the paper, and all authors reviewed the manuscript.

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Competing interests

The authors declare no competing interests.

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Figures

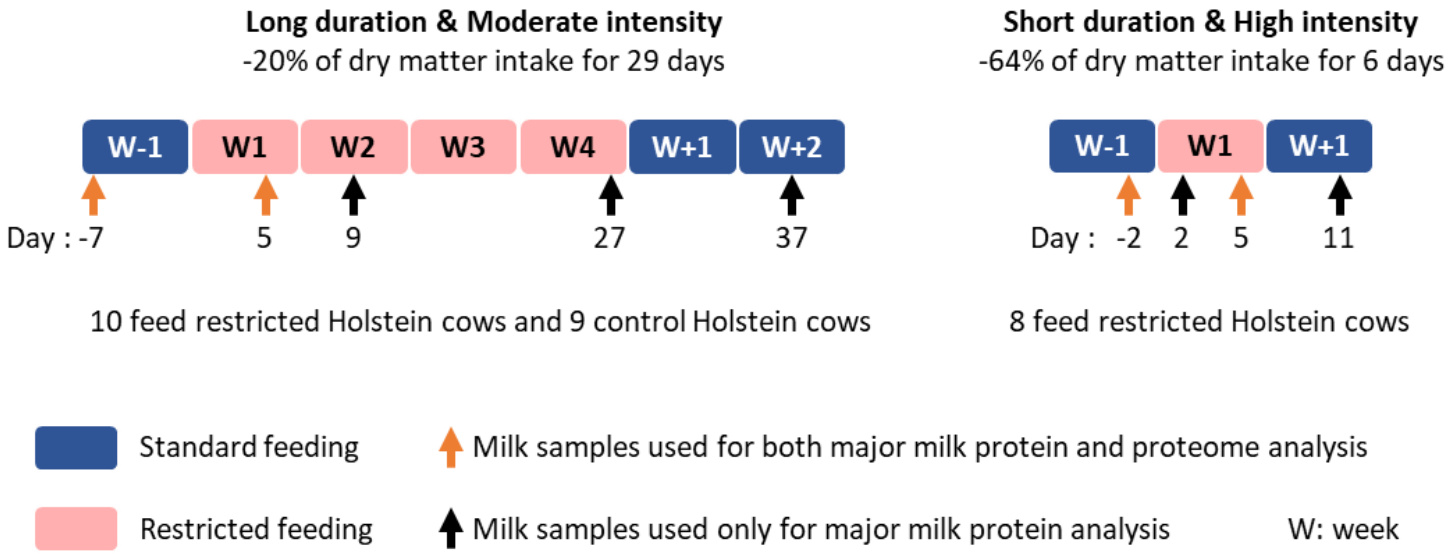

Figure 1

Experimental design of the two feed restriction trials: one of long duration and moderate intensity (LM) and one of short duration and high intensity (SH) in dairy cows. Before and after the feed restriction periods, all cows received 100% of their *ad libitum* dry matter intake. During feed restriction, rations were reduced relative to the pre-experimental period, except for control cows in the LM trial.

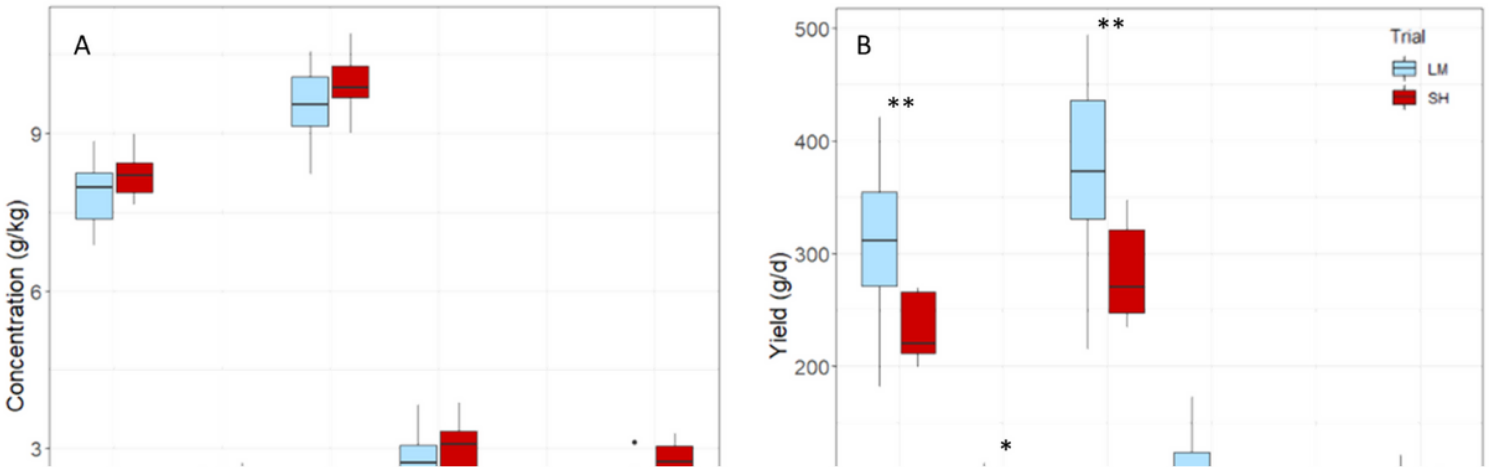

Figure 2

Boxplots showing the comparison of the concentrations (A) and yields (B) of the six major milk proteins analyzed using RP-HPLC during the pre-restriction period of both trials. In light blue the trial of long duration and moderate intensity (LM) (19 cows, -20% dry matter intake for 29 days), and in red the feed restriction trial of short duration and high intensity (SH) (8 cows, -64% dry matter intake for six days). Analyses of variance between the trials: * $P \leq 0.05$; ** $P \leq 0.01$; *** $P \leq 0.001$.

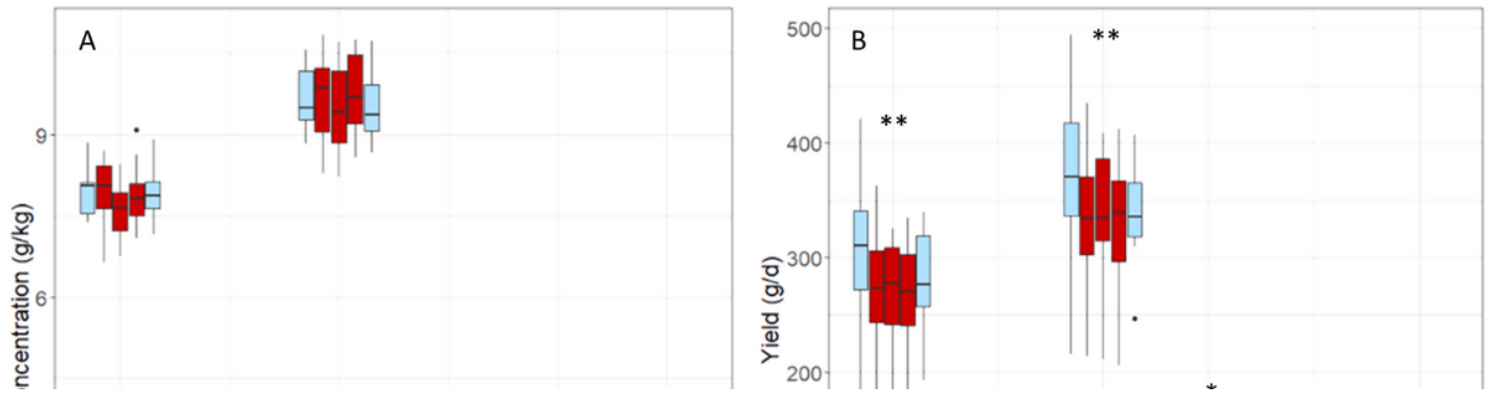

Figure 3

Variations in the concentrations (A) and yields (B) of the six major milk proteins analyzed using RP-HPLC during the feed restriction trial of long duration and moderate intensity (LM) (-20% dry matter intake for 29 days) in the 10 feed restricted cows. The five boxplots represent each day of sampling for each protein: in light blue during *ad libitum* feeding (d -7 and 37) and in red during the restriction period (d 5, 9 and 27). Analyses of variance between diets: *t*: $P \leq 0.1$; * $P \leq 0.05$; ** $P \leq 0.01$; *** $P \leq 0.001$.

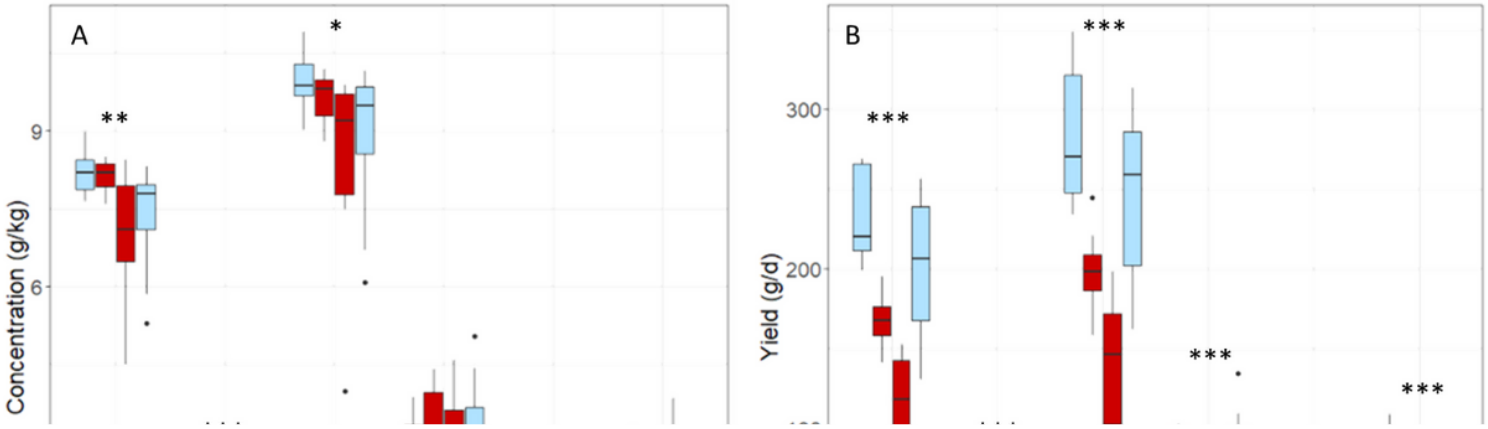

Figure 4

Variations in the concentrations (A) and yields (B) of the six major milk proteins analyzed using RP-HPLC during the feed restriction trial of short duration and high intensity (SH) (-64% dry matter intake for six days) in the eight feed restricted cows. The four boxplots represent each day of sampling for each protein: in light blue during *ad libitum* feeding (d-2 and 11) and in red during the restriction period (d2 and 5). Analyses of variance between pre-restriction and restriction periods: *t*: $P \leq 0.1$; * $P \leq 0.05$; ** $P \leq 0.01$; *** $P \leq 0.001$.

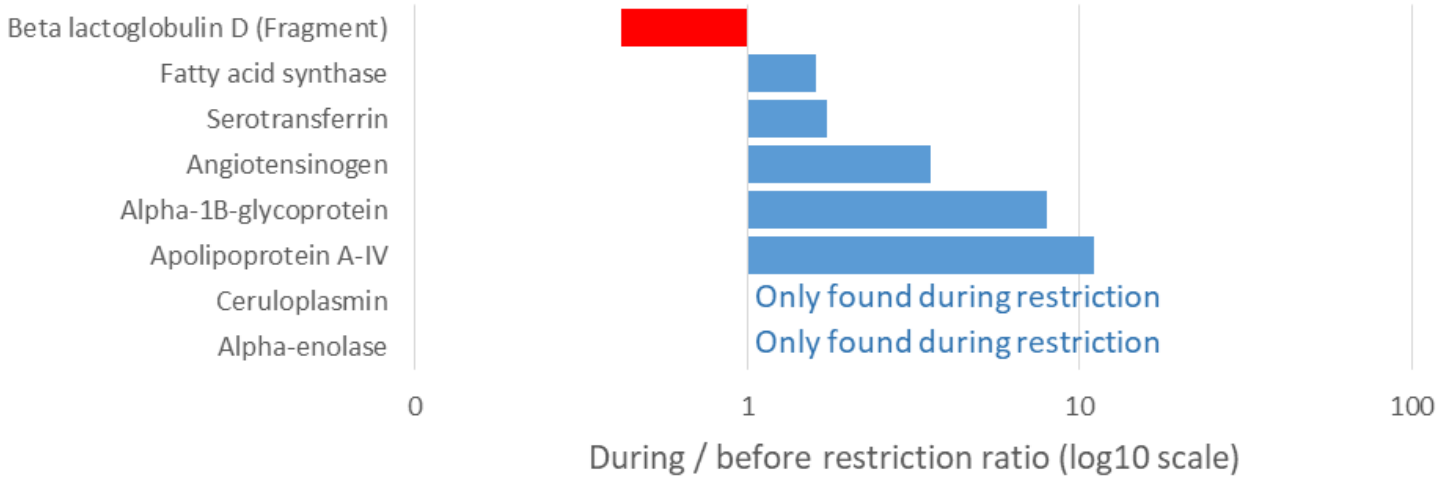

Figure 5

Variations in protein abundance in milk from eight dairy cows after five days of moderate feed restriction (LM trial: -20% of dry matter intake). Analyses of variance between the pre-restriction and restriction periods: $P \leq 0.05$.

Figure 6

Proteins identified in milk from eight cows subjected to high intensity feed restriction (SH trial: -64% dry matter intake) with a fold change in abundance >2 or <0.5 after five days of feed restriction during the SH trial. Analyses of variance between pre-restriction and restriction periods using a P value ≤ 0.05.

Supplementary Files

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