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Original article

Effects of supplying leucine and methionine to early-lactating cows fed silage-concentrate based diets with a calculated deficiency in leucine and methionine

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Abstract — In a 2 × 2 factorial approach the productive and metabolic response of 24 multiparous Brown Swiss cows fed rations calculated to be deficient in leucine (0.9-fold of requirements) and methionine (0.8-fold) to supplementation either of one or both of these amino acids were investigated. On a dry matter basis the rations consisted of 29% grass silage, 20% maize silage, 6% hay, and 45% concentrate. Blood plasma amino acid data confirmed the intended difference in metabolic supply of leucine and methionine keeping a low variation in the plasma levels of the other essential amino acids, particularly lysine. Live weight, milk yield as well as content and amount of milk fat were not affected by the treatments. Content and amount of milk protein were significantly reduced relative to initial level without additional methionine. Nutrient digestibility and nitrogen balance remained widely unchanged by the supplementations. Except of plasma aspartate amino transferase, cholesterol, creatinine and ornithine, which responded to methionine, hormones, enzyme activities as well as plasma, urine and milk metabolites were not systematically influenced by leucine and methionine supply. The present results gave clearer indications for a deficiency in methionine than in leucine.

amino acids / dairy cow / requirements / leucine / methionine

Résumé — **Effets de l'apport de leucine et méthionine chez des vaches en début de lactation recevant des rations ensilage-concentré à déficit calculé en leucine et méthionine.** Vingt-quatre vaches laitières multipares Brown Swiss ont été affouragées avec des rations calculées pour être déficientes en leucine (0,9 fois du besoin) et méthionine (0,8 fois). Les effets d'une supplémentation de l'un ou des deux acides aminés sur la production et le métabolisme de ces vaches ont été examinés à l'aide d'une approche factorielle 2 × 2. En matière sèche, les rations étaient composées de 29 % d'ensilage d'herbe, 20 % d'ensilage de maïs, 6 % de foin et 45 % de concentrés. Comme

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prévu, les analyses des acides aminés dans le plasma sanguin ont confirmé une disponibilité métabolique différente de la leucine et de la méthionine, alors que les autres acides aminés essentiels n'ont montré que peu de variation, en particulier la lysine. Le poids vif, la production et la composition du lait, ainsi que la quantité de ses matières grasses n'ont pas été affectés par les traitements. Sans méthionine supplémentaire la teneur et la quantité de protéines dans le lait ont diminué significativement par rapport au niveau initial. La digestibilité des nutriments et le bilan azoté sont restés inchangés malgré les supplémentations. Les taux hormonaux et les activités enzymatiques sanguins, ainsi que les métabolites du sang, de l'urine et du lait le plus souvent n'ont pas répondu clairement à l'adjonction de leucine et méthionine. Les résultats suggèrent l'existence d'une déficience en methionine plutôt qu'en leucine.

acides aminés / vache laitière / besoins / leucine / méthionine

1. INTRODUCTION

Most of the commonly applied systems of recommendations for protein supply of dairy cows are based on the protein available in metabolism after small intestinal digestion. Although ruminants, like monogastric animals, have distinct requirements for single amino acids rather than for protein, only very few systems of recommendations, such as the modified French PDI [34] and the Dutch DVE system [8], give the option to consider also the dietary supply of limiting amino acids. Fickler and Heimbeck [11] suggested a factorial approach to estimate the supply of 'metabolisable' amino acids in order to be able to identify limiting amino acids. Knowledge on limitations in individual amino acids allows to supplement these amino acids at minimum additional nitrogen intake thus also avoiding unnecessary environmental pollution by excretion of excessive nitrogen. Methionine and lysine are often regarded as limiting amino acids in dairy cows, particularly with high dietary proportions of maize products rich in leucine [32]. This was recently confirmed by adding lysine and methionine to a forage-based diet calculated to be deficient in these amino acids according to the modified French system [21]. However, under different feeding conditions such as forage-based grass/grass silage rations instead of concentrate-based rations leucine might be the first or second limiting amino acid in dairy cows [4]. Even mixed forage rations containing maize silage fed together with concentrate might be deficient in leucine depending on the proportion of concentrate and the type of concentrate ingredients used. It is not yet completely clear in which order methionine and leucine are required for maximum metabolic protein utilisation, and more reliable information is still needed on the cow's requirements for leucine particularly in relation to methionine and the types of rations in which these amino acids are actually limiting.

The objective of the present study was to determine the degree of limitation by leucine independent from methionine using a mixed basal ration with a calculated deficiency of leucine and methionine but not of lysine. Performance, N balance and various metabolic indicators in blood, urine and milk, including the blood amino acid profile, were determined in order to identify an actual deficiency of leucine and of methionine.

2. MATERIALS AND METHODS

2.1. Feed and treatments

A balance experiment based on a 2×2 factorial design (deficient/sufficient supply of leucine and methionine, respectively) was performed with 24 early-lactating cows,

with a total of six replicates per treatment group. In detail, the experiment was carried out in three subsequent experimental periods with three groups of eight cows each (two cows per treatment in each period). On a dry matter basis, the rations were composed of forage (consisting of 53% early growth-stage, rye grass dominated grass silage, 37% maize silage and 10% late growth-stage meadow hay) and concentrate in a ratio of 1.2:1. Feed amount was individually allocated for each cow according to actual milk yield and live weight. The analysed nutrient and amino acid composition of the feed ingredients is given in Table I.

Leucine supply was increased from low supply (Leu-) to higher supply (Leu+) only by changing the ingredient composition of the concentrate. The Leu- concentrate consisted on a dry matter basis of dried sugar beet pulp 20.0%, soy hulls 18.0%, barley 15.0%, citrus pulp 12.0%, wheat bran 10.0%, maize germ 7.0%, soybean meal 6.5%, wheat gluten meal 4.5%, sugar beet molasses 4.0% and mineral premix 3.0%. The Leu+ concentrate was similarly composed except that the 4.5% wheat gluten meal (poor in leucine) were replaced by 6.0% maize gluten meal, which should guarantee a high leucine flow to the small intestine [10], with a corresponding reduction in the proportions of soy hulls to 17.5% and of citrus pulp to 11.0%. The two complete rations had relatively similar contents of net energy, PDI, crude protein and essential amino acids except of leucine (Tab. I). The other two dietary treatments consisted of a supplementation of 1.5 g rumen-protected methionine per kg concentrate supplied as Mepron® M85 (Degussa, Hanau, Germany) both to the Leu- and the Leu+ concentrate, i.e. without changing the ingredient composition of the respective complete rations.

Table II lists the calculated supply of energy, protein and several amino acids. INRA [18] data on feed contents and animal requirements were used for calculation of net energy (NEL), absorbable protein (PDI), digestible lysine and methionine to ensure compatibility of requirement and supply calculations. Accordingly, adequate supply of net energy and PDI was given in all groups. When net energy and absorbable protein contents were calculated from proximate analyses (Tab. I) by the Swiss system [12], in all groups a slight lack of net energy was calculated (not given in the tables) as it is common for early lactating cows. Nitrogen (crude protein) intake was sufficient to ensure maximum rumen fermentation activity [12] although some of the concentrate ingredients contained protein of a relatively low rumen degradability.

Table II also gives the results of two different calculations of metabolic supply of amino acids. One is based on the French concept of digestible amino acids [33, 36] which considers lysine (LysDI) and methionine (MetDI). For calculation of supply by this approach INRA [35] feed tables on the contents of LysDI and MetDI in feedstuffs were used and requirement data of Rulquin et al. [33, 36] were applied. Accordingly, methionine was clearly deficient without supplementation and calculated requirements were fully covered with addition of methionine which increased supply by 23% on average ($p < 0.001$). Requirements for digestible lysine were widely covered in all groups. The second approach to calculate metabolic supply of amino acids was based on a computer program (Degussa, Hanau, Germany). This approach has a factorial structure [11] which takes into consideration estimated feed intake, amino acid contents of feedstuffs either as listed in feed tables or analysed and the assumed metabolisability of these amino acids as opposed to the requirements calculated for maintenance and milk protein secretion. The program includes methionine, leucine, lysine, isoleucine, threonine and valine which are discussed as potentially limiting amino acids in dairy cows [4]. According to this approach, the supply of metabolisable leucine was 7% to 9% below calculated requirements in the Leu- groups and

Table I. Nutrient composition of the feed ingredients and the diets as consumed (means of three determinations ± standard deviation).

	Forages			Concentrates		Diets as consumed	
	Grass silage	Maize silage	Hay	Leu-	Leu+	Leu-	Leu+
DM (g·kg^{-1} original matter)	567 ± 12	334 ± 22	891 ± 9	904 ± 15	907 ± 13	628 ± 10	628 ± 13
Organic matter (g·kg^{-1} DM)	888 ± 12	962 ± 6	937 ± 28	926 ± 1	926 ± 2	923 ± 13	923 ± 11
Crude protein (g·kg^{-1} DM)	158 ± 11	75 ± 1	74 ± 16	180 ± 11	184 ± 4	148 ± 5	149 ± 9
NDF (g·kg^{-1} DM)	484 ± 44	366 ± 24	637 ± 25	319 ± 9	335 ± 12	392 ± 7	401 ± 9
Calculated NEL (MJ·kg^{-1} DM)[1]	5.75 ± 0.12	6.34 ± 0.02	4.67 ± 0.31	6.43 ± 0.06	6.37 ± 0.06	6.12 ± 0.12	6.09 ± 0.10
Calculated PDI (g·kg^{-1} DM)[1]	84 ± 6	74 ± 0	77 ± 6	116 ± 4	117 ± 2	96 ± 3	96 ± 1
Essential amino acids (g·kg^{-1} DM)							
Leucine	11.40 ± 1.41	6.64 ± 0.06	5.80 ± 1.15	12.87 ± 0.87	16.60 ± 0.56	10.84 ± 0.15	12.46 ± 0.09
Methionine	2.13 ± 0.25	1.10 ± 0.10	0.90 ± 0.26	2.80 ± 0.00	3.04 ± 0.21	2.17 ± 0.11	2.26 ± 0.16
Lysine	5.53 ± 0.55	1.64 ± 0.06	3.40 ± 0.66	7.30 ± 0.53	7.40 ± 0.26	5.47 ± 0.20	5.46 ± 0.14
Isoleucine	6.34 ± 0.40	2.54 ± 0.12	3.00 ± 0.71	6.84 ± 0.51	6.87 ± 0.12	5.64 ± 0.11	5.62 ± 0.11
Threonine	6.07 ± 0.80	2.44 ± 0.06	3.10 ± 0.68	6.10 ± 0.46	6.40 ± 0.20	5.20 ± 0.20	5.32 ± 0.16
Valine	8.26 ± 0.65	3.47 ± 0.06	3.90 ± 0.72	8.17 ± 0.45	8.53 ± 0.21	7.03 ± 0.36	7.17 ± 0.19
Arginine	4.67 ± 1.85	1.53 ± 0.12	3.60 ± 0.75	9.33 ± 0.51	9.13 ± 0.21	6.15 ± 0.45	5.98 ± 0.25
Histidine	2.27 ± 0.42	1.30 ± 0.10	1.40 ± 0.46	4.57 ± 0.31	4.63 ± 0.21	3.10 ± 0.22	3.09 ± 0.17
Phenylalanine	7.23 ± 1.16	2.80 ± 0.26	3.77 ± 0.76	8.17 ± 0.35	8.77 ± 0.15	6.60 ± 0.19	6.83 ± 0.14
Non-essential amino acids (g·kg^{-1} DM)							
Alanine	9.43 ± 0.56	5.53 ± 0.25	4.70 ± 0.78	7.77 ± 0.26	10.23 ± 0.26	7.63 ± 0.50	8.74 ± 0.33
Aspargine/aspartic acid	12.93 ± 1.51	4.30 ± 0.51	6.73 ± 1.11	13.23 ± 0.74	14.47 ± 0.50	11.03 ± 0.47	11.53 ± 0.49
Cysteine	1.13 ± 0.19	0.90 ± 0.08	0.93 ± 0.21	3.30 ± 0.16	3.30 ± 0.14	2.08 ± 0.16	2.05 ± 0.11
Glutamine/glutamic acid	11.93 ± 1.81	8.73 ± 0.21	8.13 ± 1.47	33.80 ± 1.66	30.70 ± 0.90	21.24 ± 0.95	19.52 ± 0.81
Glycine	7.33 ± 0.78	3.00 ± 0.00	3.90 ± 0.59	8.27 ± 0.52	8.13 ± 0.21	6.72 ± 0.35	6.62 ± 0.29
Proline	8.53 ± 0.78	4.77 ± 0.05	3.30 ± 0.62	12.93 ± 0.46	12.30 ± 0.64	9.55 ± 0.59	9.17 ± 0.41
Serine	5.73 ± 0.69	2.80 ± 0.08	3.30 ± 0.50	8.20 ± 0.37	8.63 ± 0.12	6.17 ± 0.26	6.31 ± 0.38

[1] Calculated by Swiss standard equations to estimate the contents of net energy (NEL) and absorbable protein at the duodenum (PDI) of forage and concentrate from proximate analysis [12].

Table II. Calculated individual supply (% of assumed requirements) of net energy, PDI, crude protein and amino acids[1].

Leucine	–	–	+	+	SEM	Treatment effects (p)		
Methionine	–	+	–	+				
						Leu	Met	Leu × Met
NEL[2]	100	103	107	106	2.7	0.09	0.65	0.37
PDI[2]	103	105	112	111	3.0	0.03	0.75	0.57
Digestible amino acids[3]								
Methionine	76^{c}	100^{ab}	87^{bc}	109^{a}	2.5	0.001	0.0001	0.67
Lysine	95	97	101	101	2.8	0.11	0.74	0.56
Metabolisable amino acids[4]								
Leucine	91	93	104	101	2.6	0.0008	0.80	0.44
Methionine	84^{b}	118^{a}	91^{b}	122^{a}	2.9	0.007	0.0001	0.58
Lysine	98	99	103	100	2.2	0.13	0.70	0.44
Isoleucine	115	116	122	118	2.7	0.09	0.66	0.45
Threonine	114	116	122	119	2.7	0.05	0.74	0.42
Valine	103	104	111	108	2.4	0.02	0.78	0.42

[1] Means without a common superscript are significantly different according to the Tukey procedure ($p < 0.05$).
[2] According to INRA [18].
[3] According to Rulquin et al. [32, 35].
[4] According to Fickler and Heimbeck [11].

was about 10% higher ($p < 0.05$) in the Leu+ groups (Tab. II). Metabolisable methionine was, as in the French approach, calculated to be clearly deficient without additional methionine (9% to 16%) whereas the methionine supplementation elevated supply by some 30% ($p < 0.001$) on average thus exceeding requirements by 20%. The average supply of lysine, isoleucine, threonine and valine was 100%, 118%, 118% and 107% of calculated requirements with mostly low variation between treatment groups.

2.2. Animals

This study was carried out with 24 multiparous Brown Swiss cows in early lactation. At the start of the three weeks of experimental procedure (two weeks of adaptation, one week of data collection) the cows were in lactation for on average 6.5 ± 2.7 weeks. Average live weight was then 632 ± 43 kg. Milk yield was 30.5 ± 5.1 kg·d^{-1} and contents of milk fat and protein were 4.03 ± 0.47% and 3.33 ± 0.21%, respectively. The animals were allocated to one of the four experimental treatments according to data on performance and live weight recorded prior to the start of the experiment. The cows were housed individually in a stall equipped to conduct metabolic trials. The experiment was conducted in accordance with the Swiss guidelines for animal welfare.

2.3. Data collection and processing of samples

All cows were housed in stall for at least two weeks prior to the start of the experiment and fed uniformly a complete diet consisting of roughage (grass silage, maize silage and hay) and approximately 20% concentrate. This alleviated adaptation to the experimental diet and allowed to get pre-experimental datasets from a similar diet as in the experiment. Live weight was measured before the experiment started and, subsequently, in weekly intervals. Feed

intake was recorded daily in the collection week with a computerised feeding system (Westfalia Landtechnik, Oelde, Germany). In order to achieve the intended forage to concentrate ratio of 1.2:1, refusals of the allocated forage amount were considered by changing the concentrate allowance on the following day accordingly. Feed samples were taken before the experiment started, weekly in the adaptation period and on every second day in the collection week. Milk yield was recorded for every milking with an automatic system (Westfalia Landtechnik, Oelde, Germany). Two milk samples per cow were taken from every milking during the collection week. One was conserved with Bronopol® (BSM2, D&F Control, San Ramon, USA) and the second one was immediately frozen for later pooling within an aliquot sample of the whole collection week. These samples were stored at –20 °C for further analyses.

Two blood samples from the jugular vein were taken per cow within the last four days before the experiment started and two samples in the collection week, one at the beginning and one at the end. These datasets were finally combined to two average values (before and during treatment). Immediately after obtaining blood, the samples were cooled on ice and then centrifuged for 15 min with 1500 *g* at 4 °C. Plasma was then frozen and stored at –20 °C for later hormone and metabolite analyses, except of 1 mL of the heparinised blood plasma which was deproteinised with sulfosalicylic acid (10%; Fluka, Buchs, Switzerland) and stored for 1 h at 0 °C. Subsequently these samples were centrifuged for 10 min with 3000 *g*. The supernatant was stored at –70 °C until the concentration of amino acids were analysed.

During the collection week total faeces of every cow were daily collected in a sliding tray. Faeces were weighed, mixed and a sample was taken and stored in a refrigerator at 4 °C. Finally an aliquot pool sample of the faeces of each cow was created and one part was stored at –20 °C for N analysis while the other part was dried at 60 °C for 48 h for further analysis. Total urine collection was carried out with an urinal fixed on Velcro® tape which was glued onto the clipped skin with special adhesive (Cyanolit, 3M AG, Rueschlikon, Switzerland). An aliquot part of the daily sample was separated by a collection device placed at the end of the urinal and stored at –20 °C. The remainder of the urine was daily collected in a plastic can containing 5 M sulphuric acid to prevent nitrogen loss. Samples of acidified urine were stored at 4 °C. At the end of the collection week aliquot pool samples of acidified and non-acidified urine were created.

2.4. Analysis of samples

Contents of dry matter, organic matter, crude protein and neutral detergent fibre were analysed in feed and faeces using standard procedures [26]. Nitrogen contents of feed, faeces, acidified urine and pooled milk samples were analysed with an automatic C/N-Analyser (Leco-Analyser Type FP-2000, Leco Instruments, St. Joseph, Michigan, USA). In acidified urine creatinine (colorimetric method, Roche diagnostics, Basle, Switzerland) and allantoin (according to Rosskopf et al. [31]) were measured. Urea content of non-acidified urine and of pooled, deproteinised (trichloroacetic acid; 0.3 $mL \cdot L^{-1}$) milk was enzymatically determined (method by Roche diagnostics, Basle, Switzerland). The Bronopol® conserved milk samples were analysed for fat, protein and lactose with infrared technique (Milkoscan 4000, Foss Electric, Hillerød, Denmark). Amino acid analyses in feeds were carried out with a method combining oxidation (16 h) and hydrolysis (24 h) on an amino acid analyser (Biochrom 20, Amersham Pharmacia Biotech, Little Chalfont, Great Britain). In feed analyses methionine was determined as methionine sulfone and cystein as cysteic acid. Asparagine and

aspartic acid as well as glutamine and glutamic acid, respectively, were determined as the acids.

Blood plasma concentrations of amino acids including ornithine were determined with an automatic amino acid analyser (model Alpha plus, Pharmacia LKB, Uppsala, Sweden). All other analyses in blood were carried out according to standard procedures [20]. Most metabolites and hormones were determined in plasma obtained from heparinised samples. For glucose and non-esterified fatty acid (NEFA) analyses, fluoride oxalate (3 mg Na fluoride and 2 mg K oxalate per mL blood) was used to obtain plasma. Blood was deproteinised with 1:1 v/v of 0.7 mol·L^{-1} perchloric acid for the analyses of L-lactate and β-hydroxybutyrate (BHB). Glucose, NEFA, BHB acetoacetate, triglycerides, cholesterol, urea, creatinine, protein and albumin as well as the plasma enzyme activities were photometrically analysed using standard enzymatic methods adapted to the COBAS MIRA autoanalyser (Roche diagnostics, Basle, Switzerland). Commercially available radio immuno assays were used to measure plasma 3,5,3'-triiodothyronine (T_3), thyroxin (T_4), as well as free T_3 and T_4 (Roche diagnostics, Basle, Switzerland) and also immunoreactive insulin (Pharmacia, Uppsala, Sweden).

2.5. Statistical analyses

The experiment was based on a completely balanced design with six different cows per treatment. For all statistical evaluations week means were used. This is also valid for the pre-experimental data on live weight, performance and blood plasma levels. Data of all variables were analysed by ANOVA using version 6.12 of the general linear models procedure of SAS [37]. Leucine and methionine level, the interaction of both and cow group (experimental period) were considered in Model 1, separately so also for the pre-experimental data if available. In Model 1, multiple comparison among means was carried out by the Tukey procedure ($p < 0.05$). In the tables mean values and standard errror of means (SEM) as well as treatment effects (p values) are presented. For comparisons within the same animal between the initial and final collection period data (i.e., live weight, performance and blood plasma concentrations) additionally the mixed procedure statement of SAS as recommended by Littell et al. [22] was applied to consider the repeated measurement character of the data (Model 2). Significant period differences according to Model 2 are indicated in the tables by asterisks.

3. RESULTS AND DISCUSSION

3.1. Effects of leucine and methionine on blood plasma amino acid concentrations

The alterations in the blood plasma concentrations of the essential amino acids as caused by the dietary treatments are shown in Table III. Initially the plasma levels of leucine and methionine were approximately similar. The variation in supply was clearly reflected in the different ($p < 0.001$) plasma levels of the respective amino acids after feeding the experimental diets for on average 18 days. This agrees with findings of other studies which also described an elevated plasma concentration of amino acids when they were either supplied in a rumen-protected form or directly infused into the abomasum [7, 21, 30, 38]. The calculated supply of additional 10% of metabolisable leucine increased blood plasma leucine by 43%. This indicates that metabolisability of leucine might have been higher than estimated. In contrast, the calculated 33% increase in metabolisable methionine closely matched the elevation found in blood plasma methionine (31%). In the state of subclinical deficiency changes in the plasma concentrations of supplemented amino acids closely

Table III. Concentration of essential amino acids in blood plasma at different leucine and methionine supply as opposed to the values at the start of the experiment (μmol·L^{-1})[1].

Leucine	–	–	+	+	SEM[2]	Treatment effects (p)		
Methionine	–	+	–	+		Leu	Met	Leu × Met
Leucine								
Initial	176.3	181.8	181.3	176.6	13.80	0.98	0.99	0.73
Final	116.7^{b}**	132.0^{b}**	185.9^{a}	169.1^{a}	11.41	0.0002	0.95	0.18
Methionine								
Initial	21.9	23.1	24.7	24.6	1.52	0.17	0.72	0.68
Final	18.0^{b}*	25.5^{a}	20.1^{b}*	24.5^{a}	1.14	0.63	0.0001	0.20
Lysine								
Initial	71.7	69.8	67.7	79.6	4.31	0.11	0.74	0.56
Final	71.9	80.8	66.1	66.5	4.38	0.03	0.31	0.35
Isoleucine								
Initial	141.9	136.2	146.2	137.1	8.89	0.08	0.94	0.16
Final	135.0	148.6	131.3	119.0	10.11	0.80	0.47	0.87
Threonine								
Initial	85.3	79.8	85.6	80.6	7.19	0.94	0.48	0.97
Final	77.3	82.0	71.8*	78.0	5.58	0.41	0.34	0.90
Valine								
Initial	277.6	274.1	290.6	252.7	17.97	0.82	0.27	0.35
Final	252.1	275.0	248.3	225.4	17.83	0.15	0.99	0.22
Arginine								
Initial	49.8	43.4	52.4	42.1	5.51	0.85	0.16	0.78
Final	51.6^{a}	67.4^{b}**	53.0^{a}	55.6^{ab}	4.57	0.27	0.06	0.17
Histidine								
Initial	55.4	60.9	53.6	55.8	5.47	0.54	0.49	0.76
Final	46.8*	51.6*	50.6	48.6	6.60	0.95	0.83	0.61
Phenylalanine								
Initial	56.0	55.1	57.5	60.3	2.64	0.22	0.73	0.49
Final	44.3**	49.6	55.9	55.5	2.80	0.006	0.40	0.32
Total amino acids								
Initial	2 093	2 001	2 060	2 104	85.0	0.69	0.78	0.44
Final	1 859*	1 985	1 810*	1 839*	59.8	0.12	0.21	0.43

[1] Means without a common superscript are significantly different in Model 1 according to the Tukey procedure ($p < 0.05$); significance of period differences as evaluated by Model 2 model considering repeated measurements: * $p < 0.05$, ** $p < 0.01$.
[2] Applying Model 1.

reflect their metabolic availability even when no immediate effects on milk protein synthesis occur. This could be different when the amino acids are supplied in clear excess of requirements for milk protein synthesis or in a not optimally balanced manner [6]. On this basis both leucine and methionine supply could have been deficient in the unsupplemented basal diet. Furthermore the present results indicate that the aim to markedly reduce the supply of leucine and methionine was fully achieved by the combination of ingredients chosen for the basal ration since prior to the experiment the blood

levels of these amino acids had been as high as in the supplemented groups.

Alterations in blood plasma levels of other amino acids would reflect interactions with the supplemented amino acids which, in the case of antagonism, could indicate the necessity to supplement not only the primarily limiting amino acids but also others. There were, however, no significant effects of leucine and methionine supply on plasma levels of lysine, isoleucine, threonine, valine, histidine, and phenylalanine as the other essential amino acids except arginine (Tab. III). The same is valid for the non-essential amino acids (not presented in the table) with the exception of asparagine and tyrosine. Both significantly responded to supplemental methionine by an increase, from 34.0 to 42.2 mmol·L^{-1} (asparagine) and from 43.6 to 67.9 mmol·L^{-1} (tyrosine), respectively. This should have been only of lower biological relevance particularly since, in the case of tyrosine, some of these differences were already present initially. The average final blood plasma concentrations of the other amino acids (mmol·L^{-1}) were: alanine, 247.4 ± 49.1; aspartic acid, 9.4 ± 2.8; cysteine, 17.3 ± 3.9; glutamic acid, 40.1 ± 11.5; glutamine, 242.1 ± 65.3; glycine 287.9 ± 86.0; serine, 71.2 ± 12.7.

3.2. Effects of leucine and methionine on performance

The average feed intake of 19.5 kg DM·d^{-1} was similar in all treatment groups (Tab. IV) because feed was offered restrictedly, but nevertheless was at a level reaching almost the assumed ad libitum intake. The forage-concentrate ratio was 1.18:1. Obviously the dietary differences in leucine and methionine which are assumed to be regulatory factors of feed intake did not affect feed intake in the present study. Also live weights and live weight alterations occurring during the experiment were not significantly influenced by amino acid supply.

Effects of additional leucine and methionine on milk yield were minor particularly when the initial differences in yield are taken into consideration (Tab. IV). This contradicts findings of an elevated milk yield with supplementary methionine [29], whereas in other studies also no performance effect occurred [2, 21]. Rulquin and Vérité [32], however, mentioned in a review the high variability in the lactational response of dairy cows to additional rumen-protected methionine. In the present study groups receiving different methionine levels did not significantly differ in content and yield of milk fat, but milk fat content significantly increased relative to initial in the Leu-/Met+ group. In some other studies additional methionine increased milk fat content [15, 28] whereas some authors did not find a response to methionine [6, 14]. Effects would be caused by the role of methionine in milk fat formation due to its ability to activate high-energy methyl groups for choline synthesis [9]. A positive effect on milk fat percentage was also shown when dairy cows received an intraperitoneal infusion of leucine together with isoleucine, valine and arginine using a low fibre diet [17]. The results suggest that the amino acids infused may have increased de novo synthesis of C_{16} fatty acids in milk. Without any supplementation or when only leucine was added, content and amount of milk protein decreased relative to initial values whereas methionine supplementation prevented this decrease. Group differences in the final collection period were, however, not significant, possibly because of certain unintended intial group differences. The trend from initial to final values nevertheless suggests that methionine was clearly limiting protein synthesis with the basal diet [1, 14, 27]. In this study the lower realised group differences in leucine supply (appr. +0.1-fold of basic supply) as compared to methionine (appr. +0.3-fold) may have masked a clearer effect of leucine on performance. No effect on milk protein concentration occurred when leucine was

Table IV. Feed intake and performance at different leucine and methionine supply[1].

Leucine	–	–	+	+	SEM[2]	Treatment effects (p)		
Methionine	–	+	–	+				
						Leu	Met	Leu × Met
Feed intake and live weight development								
Dry matter intake (kg·d^{-1})	19.7	19.5	19.1	19.5	0.88	0.41	0.71	0.82
Live weight (kg)								
Initial	635	633	628	631	17.9	0.77	0.97	0.90
Final	624	635	629	634	17.2	0.91	0.64	0.85
Milk yield and composition								
Yield (kg·d^{-1})								
Initial	33.4	29.9	29.2	29.4	2.01	0.25	0.43	0.37
Final	32.2	29.6	28.1	28.7	2.03	0.23	0.64	0.45
Fat content (%)								
Initial	4.26	3.85	3.87	4.14	0.151	0.66	0.04	0.006
Final	4.17	4.15*	4.03	4.05	0.153	0.45	0.97	0.90
Fat yield (kg·d^{-1})								
Initial	1.40	1.15	1.13	1.22	0.092	0.27	0.40	0.08
Final	1.33	1.23	1.13	1.16	0.091	0.16	0.68	0.49
Protein content (%)								
Initial	3.29	3.30	3.38	3.35	0.071	0.35	0.86	0.76
Final	3.13**	3.27	3.26*	3.37	0.098	0.26	0.22	0.85
Protein yield (kg·d^{-1})								
Initial	1.09	0.98	0.98	0.98	0.057	0.34	0.36	0.33
Final	0.99**	0.96	0.90*	0.96	0.051	0.66	0.97	0.81
Lactose (%)								
Initial	5.23	5.26	5.21	5.19	0.066	0.59	0.98	0.72
Final	5.21	5.25	5.16	5.09**	0.058	0.10	0.76	0.35

[1] Significance of period differences as evaluated by Model 2 considering repeated measurements: * $p < 0.05$, ** $p < 0.01$.
[2] Applying Model 1.

abomasally infused to well-fed cows receiving a diet consisting of alfalfa hay, cracked corn and soybean meal as main components [25]. This indicates the importance of proportion and type of concentrate used for supplementation for adequate amino acid supply. Milk lactose content was significantly decreased in the Leu+/Met+ group and tended to be lower in the Leu+/Met- group at the end of the experiment compared with the initial content. In contrast, lactose yield increased at a constant lactose concentration in another study [16] when cows received more intestinally digestible protein as compared with a supply of a calculated deficiency.

3.3. Effects of leucine and methionine on digestion and nitrogen balance

Coefficients of digestibility of organic matter, neutral detergent fibre and crude protein were similar in all treatment groups (Tab. V). Also nitrogen balance was not significantly affected by the supplemented amino acids. This agrees with findings of Donkin et al. [7] in cows who also did not

Table V. Nutrient digestibilities and nitrogen turnover at different leucine and methionine supply.

Leucine	–	–	+	+	SEM	Treatment effects (p)		
Methionine	–	+	–	+				
						Leu	Met	Leu × Met
Nutrient digestibility coefficients								
Organic matter	0.74	0.73	0.73	0.73	0.007	0.48	0.56	0.40
NDF	0.58	0.60	0.60	0.61	0.013	0.20	0.36	0.91
Crude protein	0.58	0.59	0.61	0.58	0.011	0.63	0.52	0.12
Nitrogen balance								
Intake ($g \cdot d^{-1}$)	473	462	458	466	16.7	0.74	0.94	0.60
Faeces ($g \cdot d^{-1}$)	197	188	179	194	6.9	0.38	0.72	0.10
Urine ($g \cdot d^{-1}$)	102	101	91	90	6.1	0.088	0.90	0.95
Milk ($g \cdot d^{-1}$)	156	153	147	155	7.6	0.63	0.79	0.51
Balance ($g \cdot d^{-1}$)	17	20	40	28	20.3	0.92	0.79	0.69
Milk N (% of N intake)	33.1	33.3	32.3	33.5	0.81	0.74	0.40	0.54

report effects of additional rumen-protected methionine on nutrient digestibility and data of nitrogen balance despite clear effects on milk protein percentage. It seems, however, that the combined supply of additional leucine and methionine could have reduced the proportion of nitrogen lost via urine (19.3% vs. 21.6%, 21.9% and 19.9% with Leu-/Met-, Leu-/Met+ and Leu+/Met-, respectively) thus indicating a slightly improved metabolic nitrogen utilisation. Nevertheless, overall utilisation of dietary nitrogen was neither improved by leucine nor by methionine. In the state of a distinct deficiency the selective supplementation of the respective limiting amino acid should have increased nitrogen utilisation by a higher incorporation into milk protein and a correspondingly lower excretion provided N intake remained approximately constant. Lynch et al. [23] found in lactating ewes a clearly increased efficiency of nitrogen utilisation with low intake of nitrogen (crude protein) and a simultaneous supply of protected methionine. However, these results were more likely a result of the decreased content of diet protein than of the simultaneously supplemented methionine. From experimental evidence obtained by duodenal infusions, Guinard and Rulquin [14] assumed a higher efficiency of the mammary gland with additional methionine.

3.4. Effects of leucine and methionine on metabolic indicators

Table VI illustrates the effects of leucine and methionine on selected hormones and metabolites as analysed in blood plasma during the collection week as well as the initial average levels measured in advance of the experiment. These did not significantly differ among the later experimental groups. Some of the hormones responded to the dietary alterations carried out. The concentration of immuno reactive insulin increased in all treatment groups relative to the initial level. T_4 was significantly elevated by an additional supply of leucine and methionine but not when these amino acids were given alone or not added at all. The concentration of free T_3 significantly declined in the Leu- groups relative to the initial value. In contrast, the concentration of free T_4 was increased in all groups, significantly so without any and with both amino acids supplemented. An increase of T_4 and free T_4 by supplementary amino acids might have resulted from an indirect effect on energy turnover.

Table VI. Metabolic characteristics at different leucine and methionine supply[1].

Leucine		–	–	+	+	SEM[2]	Treatment effects (p)		
Methionine		–	+	–	+				
	Initial overall means						Leu	Met	Leu × Met
Blood plasma hormones									
Immuno reactive insulin (pmol·L^{-1})	42.7	59.1*	65.0**	76.7***	68.2**	8.76	0.25	0.88	0.42
T_3 (nmol·L^{-1})	2.40	2.39	2.26	2.45	2.52	0.161	0.34	0.86	0.52
T_4 (nmol·L^{-1})	61.0	66.3	59.6	62.0	65.2**	3.48	0.85	0.62	0.17
Free T_3 (nmol·L^{-1})	6.43	5.83*	5.87*	5.64	6.09	0.204	0.97	0.24	0.32
Free T_4 (nmol·L^{-1})	15.5	16.6**	16.4	16.3	16.9**	0.82	0.90	0.87	0.64
Blood plasma enzyme activities									
γ-glutamyl transferase (U·L^{-1})	22.4	23.2*	23.0	24.1	24.0	2.01	0.64	0.93	0.96
Aspartate amino transferase (U·L^{-1})	88.3	80.0*	71.5**	85.4	72.1*	4.29	0.24	0.07	0.29
Alkaline phosphatase (U·L^{-1})	43.8	44.1	33.8	49.3	42.3	6.82	0.26	0.17	0.93
Creatine kinase (U·L^{-1})	157	159	131	152	141	12.1	0.87	0.12	0.51
Blood plasma metabolites									
Glucose (mmol·L^{-1})	3.18	3.04	3.02	3.14	3.29	0.096	0.07	0.47	0.41
L-lactate (mmol·L^{-1})	0.33	0.42	0.51**	0.38	0.42**	0.062	0.29	0.32	0.74
Triglycerides (mmol·L^{-1})	0.19	0.20	0.45*	0.23	0.20	0.125	0.38	0.40	0.28
Non-esterified fatty acids (mmol·L^{-1})	0.17	0.14	0.08	0.13	0.07	0.004	0.77	0.10	0.97
β-hydroxybutyrate (µmol·L^{-1})	743	890	1 039***	745	907	73.7	0.07	0.05	0.93
Acetoacetate (µmol·L^{-1})	48.3	45.5	62.9	56.4	48.6	6.25	0.70	0.52	0.77
Cholesterol (mmol·L^{-1})	4.84	5.91**	4.94	6.04	5.15	0.392	0.68	0.03	0.93
Protein (g·L^{-1})	78.0	78.0	78.4	77.7	77.4	0.14	0.66	0.97	0.81
Albumine (g·L^{-1})	33.5	34.0	33.5	34.5	32.8	0.60	0.86	0.09	0.33

Table VI. (Continued).

Leucine		–	–	+	+	SEM[2]	Treatment effects (p)		
Methionine		–	+	–	+				
	Initial overall means						Leu	Met	Leu × Met
Urea (mmol·L^{-1})	3.36	5.05**	4.87***	4.70***	4.86***	0.229	0.44	0.98	0.47
Creatinine (µmol·L^{-1})	90.0	94.6	88.8	95.5	84.2	3.74	0.62	0.03	0.46
Ornithine (mmol·L^{-1})	39.8	32.6	38.3	35.1	40.3	1.89	0.25	0.01	0.90
Urinary metabolites									
Urea (mmol·L^{-1})	–[3]	141	137	143	133	8.9	0.93	0.43	0.68
Urea (mol·d^{-1})	–	2.56	2.60	2.30	2.34	0.282	0.12	0.65	0.02
Creatinine (mmol·L^{-1})	–	4.43	4.17	2.94	3.17	0.689	0.09	0.98	0.73
Creatinine (mmol·d^{-1})	–	83.6	78.2	47.5	53.2	16.20	0.03	0.85	0.44
Allantoin (mmol·L^{-1})	–	13.8	13.5	14.5	12.8	0.77	0.98	0.20	0.38
Allantoin (mmol·d^{-1})	–	250	314	292	191	24.2	0.11	0.45	0.03
Milk metabolites									
Urea (mmol·L^{-1})	–	6.44	6.10	6.20	5.88	0.255	0.38	0.21	0.96
Urea (mmol·d^{-1})	–	201	174	174	164	14.3	0.22	0.21	0.57

[1] Significance of period differences as evaluated by a model considering repeated measurements: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.
[2] SEM applying Model 1.
[3] No initial values determined.

Response in enzyme activities to amino acid supply remained low with the exception of plasma aspartate amino transferase activity which declined to a lower level with supplementary methionine but not with leucine supplementation. This enzyme mediates the biosynthesis of glutamate from aspartate and an increase in plasma concentration usually reflects a strain of hepatocytes [5]. In turn, the slight decrease in the plasma activity of the aspartate amino transferase may indicate that methionine supplementation reduced the metabolic stress on liver cells related to lactation.

Energy-related plasma metabolites such as glucose, lactate, triglycerides, non-esterified fatty acids, ketone bodies and cholesterol widely differed in their response to the amino acids supplied (Tab. VI). L-lactate and β-hydroxybutyrate were significantly increased in relation to the initial level by the groups receiving additional methionine but not with leucine or without amino acid supplementation. This contradicts results of Lynch et al. [23] in lactating ewes who found no effects of methionine supplementation on lactate. Similar to the present study, they also did not observe an influence of an increased methionine supply on insulin, glucose, triglycerides and non-esterified fatty acids. There was a general increase in plasma cholesterol in our investigation, but final levels were significantly lower ($p < 0.05$) with the addition of methionine.

In the metabolites related to body protein and nitrogen turnover some minor differences in treatment responses occurred. Plasma protein and albumin remained largely unaffected whereas plasma and urinary creatinine were significantly reduced ($p < 0.05$) by methionine and leucine supplementation, respectively. However, individual variation was extremely high in urinary concentration as well as in excretion of creatinine. Elevated creatinine values indicate an increased body protein mobilisation which would occur in a state of protein deficiency [19]. Blood plasma urea was elevated similarly in all groups by the experimental rations. This increase from prior to the experiment could be explained by the slightly different types of rations fed before (lower proportion of concentrate which had a higher protein content as the forage mixture) and in the experiment. Treatment differences on urine and milk urea were not significant, but values were generally numerically highest without any amino acid supplementation. This further supports the assumption that the amino acids slightly improved metabolic nitrogen utilisation. Plasma level of ornithine, an amino acid occurring in the urea cycle and an important precursor of proline, glutamate and aspartate for the milk protein synthesis [24], was increased by supplementation of leucine (not significant) and methionine ($p < 0.05$). For methionine this confirms previous results [21]. Urinary allantoin excretion as an indicator to estimate microbial protein reaching the duodenum [3, 13], i.e. the microbial part of PDI, was not significantly affected by the dietary treatments (Tab. VI). This coincides with the lack of effects on digestion and nitrogen turnover. Since at least methionine was provided in a rumen-protected form, greater effects on rumen microbial protein synthesis seem basically unlikely.

4. CONCLUSIONS

The results of this experiment suggest that, under the conditions described, methionine was first limiting, as can be seen from the significantly reduced milk protein synthesis without supplementation relative to initial value, whereas the effect of additional leucine supply was marginal. However, it has to be kept in mind that, despite the lower numerical response in plasma level, with methionine compared to leucine both calculated deficiency (0.2 vs. 0.1-fold of requirements) and additional supply (+0.3 vs. +0.1-fold of basic supply) were much more pronounced. Furthermore, if methionine would have been not totally sufficient

even at the high level of supplementation applied, this amino acid still could have been first limiting thus preventing any leucine effects. The response of plasma amino acid concentration to the increased methionine and leucine supply confirmed its use as a reliable indicator to assess an additional metabolic amino acid supply. This allows to test the efficacy of dietary modifications which are designed to alleviate subclinical amino acid deficiency. The few variables apart from plasma amino acids responding to the amino acids supplied indicate an additive rather than a competitive effect of these amino acids in cows.

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