Effects of ruminally protected methionine and/or phenylalanine on performance of high producing Holstein cows fed rations with very high levels of canola meal.

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Abstract

Canola meal is the second largest protein feed in the Northern latitudes and inclusion levels in dairy rations are expected to increase due to projected large increases in production of canola seed in Canada. However, a recent study (Swanepoel et al. 2014) showed that even though higher inclusions of canola meal (CM) had a positive effect on production when CM directly substituted for high protein corn based dried distillers grains (DDG), that there was an optimum point at 120 to 135 g/kg of diet dry matter (DM) after which animal performance seemed to decline. Only the amino acids (AA), methionine (Met), phenylalanine (Phe) and leucine (Leu) could have limited production based upon plasma AA concentrations at the highest CM inclusion level. Our objective was to determine if either Met or Phe, or both, was limiting performance of early lactation dairy cows fed a ration containing 180 g/kg of diet DM as CM, by supplementing a calculated target of 7.5 g of intestinally absorbable Phe/cow/d and/or 8.0 g of intestinally absorbable Met/cow/d in ruminally protected (RP) forms to four pens of ~320 early lactation cows/pen in a 4 x 4 Latin square with 28 d experimental periods. Dry matter intake was not affected (avg: 27.6 +/- 0.4 kg/d) by feeding either of the RP AA, or the combination. Phenylalanine supplementation alone had no effect on milk production or composition, and body condition score (BCS) change compared to Control. Supplemental Met alone modestly increased ($P<0.01$) milk protein and fat content, while decreasing ($P<0.01$) milk lactose content and yield, but with no impact on BCS change compared to Control. Combination Met and Phe supplementation decreased milk and lactose yields, as well as lactose content ($P<0.01$), while increasing milk protein content and the BCS change ($P<0.01$). Urine volume (avg: 16.7 +/- 0.31 L/d) and flow of microbial protein (MCP) from the rumen (avg: 2092 +/- 52.7 g CP/d) were not affected by any treatment. Plasma Met levels increased ($P<0.01$) with both Met treatments and plasma tryptophan (Trp) levels decreased ($P<0.01$) with both Phe treatments. However, plasma

Phe levels did not change with any treatment. Results are interpreted to suggest that delivery of Met with RP Met feeding was higher than animal requirements and caused an oversupply of Met. Addition of Phe to the Met supplementation changed the way energy was utilized by the cows, redirecting energy liberated by Met from milk components toward BCS gain. It remains unclear if Phe was limiting in the Control ration or if RP Phe was not fed at high enough levels to have a measurable response on production. However, it is clear that AA limitations, requirements and production responses are governed by much more than plasma AA levels. Results further suggest that AA are bioactive metabolites to the extent that they can change animal performance, even when they are not 'limiting' *per se*, and that their supplementation to practical dairy cattle diets should be approached with extreme caution for this reason.

Keywords: Spot urine purine; Estimated microbial flow; Plasma amino acids; Protein feeding.

Abbreviations: AA, amino acid; ADF, acid detergent fiber; ADICP, AD insoluble CP; ADIN, acid detergent insoluble N; AL, allantoin; aNDF, amylase-treated NDF; aNDFom, aNDF free of residual ash; BCS, body condition score; BCAA, branched-chain AA; BW, body weight; CM, canola meal; CP, crude protein; CR, creatinine; DC305, DairyComp 305 management system; DDG, dried distillers grains; DHIA, Dairy Herd Improvement Association; DIM, days in milk; DM, dry matter; MCP, microbial CP; NDF, neutral detergent fiber; NE_L, net energy for lactation; OM, organic matter; PD, purine derivatives; RDP, rumen degradable CP; RP, rumen protected; SCC, somatic cell count; SG, specific gravity; TMR, total mixed ration; TP, true protein.

1. Introduction

Canola meal (CM) is the second largest protein feed in the Northern latitudes and inclusion levels in dairy rations are expected to increase due to projected increased production of canola seed in Canada (Growing Great, 2015). The goal of the Canola Council of Canada with this

program was to produce 15 million tonnes of canola seed annually by 2015 but, with record breaking seed production in 2013; annual production currently stands at 18 million tonnes. Vegetable oil demand worldwide is expected to rise 60% in the next decade (Canola Council of Canada Annual Report, 2013) and, with the increased crushing capacity in Canada, this will have a cascading effect resulting in increased amounts of CM produced and used in North American dairy rations.

A recent study (Swanepoel et al. 2014) showed that higher inclusions of CM in lactating dairy cow rations had a positive effect on production when CM directly substituted for high protein dried corn distillers grains (DDG), but that there was an optimum point at 120 to 135 g/kg of diet dry matter (DM) after which animal performance started to decline. This agrees with other studies comparing CM to DDG which reported that higher proportions of CM, included at up to 120 g/kg DM, tended to have higher milk and protein yields (Mulrooney et al., 2009). It was clear, however, in Swanepoel et al. (2014) that the high rumen degradable protein (RDP) content of CM, and resultant high rumen ammonia levels, did not limit microbial protein (MCP) production when CM was included in the diet at 200 g/kg DM, suggesting that it may have been the availability of absorbable amino acids (AA), and/or specific AA(s), that limited productive performance of the cows. In Swanepoel et al. (2014), only methionine (Met), phenylalanine (Phe) and leucine (Leu), could have limited production, based upon their declining plasma AA concentrations as the CM inclusion level in the diet increased.

Our objective was to determine if either Met or Phe, or both, was limiting performance of early lactation dairy cows fed a ration containing CM as the sole supplementary crude protein (CP) source, by supplementing Met and/or Phe in ruminally protected (RP) forms.

2. Materials and methods

The experimental design used 4 pens of ~320 early lactation cows/pen in a 4 x 4 Latin square with 28 d experimental periods, utilizing the William's experimental design (Williams, 1949) to balance for potential carryover of treatment effects. The study took place during winter from 28 Dec 2012 to 18 April 2013 with temperature ranging between -3.6 and 28.3^{o}C and humidity between 22.7 and 100.0%. All cows were cared for relative to applicable laws of the state of California and the USA, and were consistent with requirements for "The care and use of animals for scientific purposes", as per the South African National Standard (SANS 10386-2008).

2.1. Farm and management

The same commercial dairy farm (located in Hanford, CA) used in Swanepoel et al. (2014) was selected for this study. Every week cows were randomly allocated to one of four early lactation pens from a single fresh pen and, once confirmed pregnant, cows were moved from these pens to mid lactation pens. At the start of the 1st period, treatments were randomly allocated to each of the four early lactation pens and rotated after each 28 d experimental period consistent with a William's design.

2.2. Diets

Mixing of the total mixed rations (TMR) and all other farm practices were as outlined in Swanepoel et al. (2014). All four of the pens were fed the same base TMR based on alfalfa hay, whole crop winter wheat and corn silages, and corn grain, with a premix containing dry ingredients (*i.e.,* almond hulls, fuzzy and cracked pima cottonseed, wheat straw, liquid molasses, mineral premix, CM), with CM inclusion in all TMR targeted at 180 g/kg of total ration DM (Table 2). Cows were fed *ad libitum* to achieve ~ 3% refusals on an as fed basis, with each pen receiving a total of ~16,000 kg of as-mixed TMR/d in 2 feedings. Cows were fed one full 11,000 kg load of TMR (which contained the RP AA) at the 1st feeding, between 04:30 and 07:30 h, to a

clean bunk as bunks were cleared of all residual feed while the cows were at morning milking. A second ~5,000 kg load of TMR was fed at the 2nd feeding between 11:00 and 12:30 h and weights for each load of TMR fed were recorded on record sheets at the time of feeding and used together with daily refusals to calculate DM intake/cow/pen. The "TMR Tracker" system (Digi-Star LLC, Fort Atkinson, WI, USA) kept a record of the actual ingredient profiles of each load of TMR mixed.

2.3. The rumen protected AA products

The RP Met product (Smartamine M; Adisseo USA Inc., Alpharetta, GA, USA) contains 750 g/kg D,L-Methionine with a 250 g/kg fat encapsulation (stearic acid) and a pH sensitive intestinal release. Using a variety of methods, the average rumen stability of the Met in Smartamine M has been estimated and assumed to be between 750 and 800 g/kg (Schwab, 1995; Rulquin and Kowalczyk, 2003; Schwab, 2007; Chen et al., 2011; Osorio et al., 2013). Specific gravity (SG) is reported by Adisseo to be 0.70 g/cm^3 (http://www.sfm.state.or.us/cr2k_subdb/MSDS/SMARTAMINE.PDF). Since Smartamine M's coating can be damaged and its integrity compromised by physical impact, cutting and abrasion, a physical inspection of the individual product beadlets were conducted twice a week, after the RP Met has been mixed into the TMR and delivered to the feedbunks, to ensure acceptable product delivery. The number of beadlets which were destroyed or physically changed during mixing and feeding was negligible.

The RP Phe product was manufactured by QualiTech Inc. (Chaska, MN, USA) according to the same specifications as their RP Lys product described in Sakkers et al. (2013) except that the Phe product did not contain the Co-EDTA marker. The RP Phe contained 600 g/kg Phe combined with 400 g/kg fat as a matrix after which the pellets were sprayed with another coating of the same fat matrix. The fatty acid profile of the fat matrix, as reported in Wrinkle et al.

(2012), primarily contained rumen stable C14:1 trans, C16:0 and C18:0 fatty acids. Due to reactivity of Lys with its fat coating, it is difficult to incorporate it into a RP form with acceptable rumen stability and intestinal release. Therefore QualiTech adapted its coating to increase the proportion of fat used with its RP Lys product used in Sakkers et al. (2013). Since Phe has not been ruminally protected in the past, this procedure of fat coating was followed to ensure high protection and post ruminal delivery of the Phe. However, since Phe is not as reactive as Lys, it is likely that our RP Phe product had higher rumen stability than the 527 g/kg reported by Sakkers et al. (2013) for the similarly protected Lys product. Therefore, a whole tract stability of 600 g/kg was assumed for our Phe product. The SG, determined to be slightly higher than 1.207 g/cm^3 according to the procedure described by Swanepoel et al. (2010) using different concentrations of a saline solution, is attributed to the long chain fatty acids used in the fat coating (Wrinkle et al., 2012).

Therefore, assuming a total duodenal delivery of 580 g/kg Met (*i.e.,* 750 g/kg Met multiplied by 775 g/kg rumen stability) and 360 g/kg Phe (*i.e.,* 600 g/kg Phe multiplied by 600 g/kg rumen stability) treatments were created by adding either RP Phe alone (PHE), RP Met alone (MET) or both AA together (M/P) by mixing 20.9 g/cow/d of RP Phe (estimated to deliver 7.5 g of intestinally absorbable Phe/cow/d) and/or 13.7 g/cow/d of RP Met (estimated to deliver 8.0 g of intestinally absorbable Met/cow/d) into the base TMR by adding a pre-weighed bag of the RP product(s) to the dry ingredient premix prior to its addition to the TMR mixer.

2.4. Sample collection, preparation and analytical methods

2.4.1. Total mixed rations and ingredients

Individual feed ingredients and TMR were sampled twice during the last 7 d (*i.e.,* the sampling week) of each of the 4 experimental periods. Ten handfuls (of 200 g each) of each TMR were collected according to Robinson and Meyer (2010) at pre-marked posts with evenly

spaced intervals along the bunk-line immediately after feeding and before the cows had access to it. Ingredient samples from all four periods were pooled (*n*=4 samples/ingredient), while TMR samples were pooled within period and pen (*n*=16 TMR samples) for chemical analysis.

All TMR samples, silages and other wet ingredients were weighed, dried at 55°C for 48 h and air equilibrated for 24 h before being sent for chemical analysis to the UC Davis service laboratory. All samples were ground to pass a 1 mm screen on a model 4 Wiley Mill (Thomas Scientific, Swedesboro, NJ, USA). Oven DM was determined as the gravimetric loss when dried at 105°C for 3 h in a forced air oven (NFTA, 2006). Ash determination was based on gravimetric loss by heating samples to 550°C for at least 3 h (Method 942.05, AOAC, 2005). Total N was determined by the Leco method (Method 990.03, AOAC, 2005). Acid detergent fiber (ADF) was determined as the residue after acid detergent extraction with the ADF residue sub-sampled for extraction with sulphuric acid to determine lignin(sa) or analysed for N to determine acid detergent insoluble N (ADIN) according to method 973.18 of AOAC (1997). Neutral detergent fiber (NDF) was determined using neutral detergent and heat (Method 2002.04, AOAC, 2006). Heat-stable amylase was used to remove starch and inactivate enzymes that may degrade the fiber (aNDF). Results for NDF were also reported on an ash-free basis (aNDFom). Starch was determined as total glucose minus free glucose multiplied by a factor of 0.9 as described by Smith (1969). Fat was quantified using the Randall modification of the standard Soxhlet extraction (Method 2003.05, AOAC, 2006).

2.4.2. Animal measurements

A group of ~195 cows with the lowest days in milk (DIM, *i.e.,* 10 to 125 DIM) were selected from each pen at the start of the study (*i.e.,* the cows most likely to complete the study due to their low DIM at the start) and coded in DairyComp 305 (DC305 (Valley Agricultural Software, Tulare, CA, USA) is an electronic herd record system) to be used as the base group of cows from

which all representative subgroups were selected for animal samples (*i.e.,* urine, blood) and measurements (*i.e.,* body condition scores; BCS). Milk production and composition data used all cows which remained eligible (*i.e.,* in their originally assigned pen) throughout the study, regardless of their DIM at the start of the study. For a cow to remain eligible *(i.e.,* to be included in any sampling dataset and the resulting statistical analysis), they had to have been in their originally assigned pen for the entire 16 wk. study, which was checked by examination of daily records of cow pen assignment within DC305. In addition, no cow was ever physically observed to be in an incorrect pen.

2.4.2.1. Milk production and composition

Milk data were collected on day 28 of each experimental period by Dairy Herd improvement association (DHIA) personnel. Milk yields were recorded for each cow and milk samples collected by drawing a small representative sub-sample from the sample collection flask (after a short period of mixing) and preserving it with a 2-Bromo-nitropropane-1, 3-diol for subsequent analytical testing. Fat, true protein, lactose and somatic cell count (SCC) were determined with the Bentley Combi using optical infrared analysis at the DHIA laboratory in Hanford (CA, USA).

2.4.2.2. Body condition score

A subgroup of ~140 cows/pen from the base group of ~195 cows/pen (see section 2.4.2.) were body scored throughout the study. This was completed by the same trained scorer on the first day of period 1 and at the end of each experimental period. The 5 point BCS system of Ferguson et al. (1994) was used and adapted as described in Swanepoel et al. (2014) to include intermediate points between the ¼ point scores.

2.4.2.3. Urine

On day 4 of the sampling week of each experimental period, spot urine samples were collected from the first ~35 cows which voluntarily urinated during normal morning lockup (for normal health and reproductive checks; ~ 50 min/pen/d) and immediately placed in ice. Samples were only retained if the cow was a part of the original base group of ~195 cows/pen. The SG of each untreated urine sample was measured using a digital handheld pen refractometer (Atago USA Inc., Bellevue, WA, USA). A small quantity of urine (7 ml) was then combined with 1 ml of 100 ml/L sulphuric acid to reduce the final pH to <2 in order to prevent bacterial destruction of allantoin (AL), diluted with water to a final volume of 35 ml and frozen at -20°C. Urine samples were chemically analyzed for creatinine (CR) and AL as described by Swanepoel et al. (2014). Two inter-run standard urine samples were used in each analytical run to assess variation amongst runs. The average concentration of the inter-run standards over all runs was then used to correct sample concentrations in each run.

2.4.2.4. Blood plasma

A subgroup of 24 cows/pen was selected from the base group of ~195 cows/pen for blood sampling with collection and treatment following the same methods as outlined in Swanepoel et al. (2014).

2.5. Calculations

Final oven DM was calculated as air equilibrated DM (*i.e.,* dried at 55°C and air equilibrated for 24 h) multiplied by the laboratory oven DM (*i.e.,* dried at 105°C) of the air equilibrated sample.

Data backups of the DC305 herd record system were used to determine the number of cows in each pen on each day of the collection week of each period, and used together with the weights of each load of TMR fed and daily refusals to calculate DM intake per cow/pen as:

((Total intake (kg as fed/d) – daily refusals) / cows in pen) x (TMR DM proportion).

Milk energy content (MJ/kg) was calculated using a prediction equation from Tyrrell and Reid (1965), summing the energetic weights of the milk components as:

(((([(4.163 x Fat (g/kg)) + (2.413 x (True protein (g/kg)/0.94)) + (2.16 x Lactose (g/kg))] – 11.72) x 2.204) / 1000) x 4.184,

with the factor 1000 converting kcal to Mcal, 2.204 converting Mcal/lb to Mcal/kg and 4.184 converting Mcal/kg to MJ/kg. True protein (TP) was converted to CP assuming 60 g/kg non-protein N in total milk N (Akers, 2002).

Milk energy output (MJ/d) was calculated by multiplying milk energy content (MJ/kg) by daily milk yield (kg/d).

Body condition score change was calculated as the difference between the BCS at the end and at the beginning of each period and BCS change energy (MJ/d) was calculated as:

((BCS change x 300)/28) x 4.184,

assuming 1 unit BCS change over 28 d = 300 Mcal energy (Chilliard et al., 1991) with the factor 4.184 converting Mcal/d to MJ/d.

Urine volume (L/d) was calculated using an equation derived from data published by Burgos et al. (2005) as:

$332.66*(((SG-1)*1000)^{-0.884})$.

Total daily purine derivative (PD) excretion (mmol/d) is the sum of PD excreted in urine and milk of lactating dairy cows (Chen and Gomes, 1992), and was estimated assuming allantoin is 0.906 of total PD in urine (obtained from values reported by Vagnoni and Broderick, 1997; Valadares et al., 1999; Gonzalez-Ronquillo et al., 2003; Reynal & Broderick, 2005; Moorby et al., 2006) and PD excretion in milk is a constant 0.05 of urine PD excretion (Chen and Gomes, 1992).

Microbial purines absorbed from the intestine (X, mmol/d) were calculated using the equation reported by Chen and Gomes (1992), using a constant assumed body weight (BW) of 673 kg (Swanepoel et al., 2014), as:

$$(\text{Total daily PD} – 0.385 \times (BW^{0.75}))/0.85,$$

assuming that the endogenous contribution of PD is 0.385 mmol/kg $BW^{0.75}$ and that 0.85 represents recovery of absorbed purines as PD in the urine.

Microbial CP production (g CP/d) was then estimated as:

$$[(X \text{ (mmol/d)} \times 70) / (0.116*0.83*1000)] \times 6.25,$$

assuming an N content of 70 mg N/mmol for purines, a ratio of purine N:total N in mixed rumen microbes as 11.6:100, a microbial purine digestibility of 0.83 and the conversion of microbial N to MCP by the factor 6.25.

A partial net energy (NE) output (MJ/d) balance was calculated by summing the milk, BCS change energy and maintenance, with maintenance net energy needs calculated from NRC (2001) and assuming a constant BW of 673 kg, as:

$$(673^{0.75}*0.08) \times 4.184.$$

Net energy for lactation (NE_L) density (MJ/kg DM) of the rations were estimated using the biological responses of the cows, as expressed in the partial NE output, and measured DM intake on a pen basis as:

$$\text{Net energy output (MJ/d) / DM intake (kg/d).}$$

2.6. Statistical analysis

Cows were only included in the statistical analysis if they did not move from their originally assigned pen during the study, for health or any other reason.. Thus the number of cows eligible for statistical analysis of milk production was 608, and for the BCS dataset it was 348. Outlier analysis (completed blind to treatments by excluding values deemed to be not biologically

possible), excluded 12 cows from the milk production dataset (*i.e.,* 8 cows for a milk fat level > 57 g/kg, 3 cows for milk yields below 11.5 kg/d and 1 cow for a milk lactose proportion > 85 g/kg), and 5 cows which were removed from the BCS dataset due to abnormally high BCS changes within an experimental period. A group of 24 (*i.e.,* 6 cows/pen) cows were randomly selected from the 96 eligible blood cows for plasma AA analysis. A total of 529 urine samples were collected from 363 cows, as several cows were sampled in more than one period, but the group of 114 urine samples selected for AL and CR assays only came from the 42 cows which had repeated urine samples between periods.

Animal production, BCS, urine AL, urine CR and plasma AA levels were analysed using the MIXED procedure of SAS (2000) for a 4 x 4 Latin square design, with cow as the experimental unit within pen in the random statement and period, pen and treatment as fixed effects. Dry matter intake (n = 4 pens, calculated on a pen basis with 4 pens/period), TMR components and ingredients and NE balance (n = 4 pens) used pen as the experimental unit in the GLM option of SAS (2000) with period, pen and treatment as fixed effects.

Reported values are least squares means with differences accepted as significant if $P \leq 0.01$ and trends accepted if $P \leq 0.05$.

3. Results

3.1. Ration evaluation and intakes

The chemical composition of the ingredients used in the TMR (Table 1) was similar to ingredients listed in NRC (2001). Only the alfalfa hay had a lower NDF proportion (337 vs. 450 g/kg) and canola pellets a lower CP proportion (380 vs. 420 g/kg).

There was no difference in the chemical profiles of the TMR fed to the four treatment groups (Table 2). At 169 g/kg, the level of CM was slightly lower than the targeted 180 g/kg, but it did

Table 1: Chemical analysis of ingredients used in the total mixed rations (g/kg dry matter) fed to the treatment groups*

	Dry matter	Organic matter	Crude protein	ADF[a]	aNDFom[b]	aNDF[c]	ADIN[d]
Alfalfa, hay	906	897	174	267	322	337	1.5
Alfalfa, fresh chop	253	867	226	277	303	333	1.9
Alfalfa, haylage	398	840	202	323	316	372	2.0
Almond, hulls	973	930	44	233	295	305	1.6
Canola meal, pellets (solvent)	906	931	380	170	219	239	2.8
Corn, flaked grain	876	988	74	27.0	77.0	78.0	IR
Cottonseed, cracked Pima	919	954	216	264	354	367	2.2
Cottonseed, fuzzy linted	920	960	219	317	418	436	2.3
Wheat, straw	932	858	38	440	614	660	1.1
Wheat, silage	359	893	71	352	485	523	0.6
Corn, silage	322	937	66	272	423	437	0.8
Citrus, wet pulp (orange)	162	958	66	173	200	211	<0.5

* $n = 4$; except alfalfa, fresh chop and alfalfa, haylage = 2.
[a] Acid detergent fiber, expressed inclusive of residual ash.
[b] Neutral detergent fiber assayed with heat stable amylase, expressed exclusive of residual ash.
[c] Neutral detergent fiber assayed with heat stable amylase, expressed inclusive of residual ash.
[d] Acid detergent insoluble nitrogen.
IR = Insufficient residue from ADF determination for N analysis.

Table 2: Ingredient profile and chemical composition (g/kg dry matter) of total mixed rations fed to the treatment groups*

	Treatments					*Control vs. (P)*		
	Control	PHE	MET	M/P	SEM	PHE	MET	M/P
Ingredient profile, g/kg DM [a]								
Alfalfa, hay	84	83	84	85	4.6	0.96	0.80	0.90
Premix								
Almond, hulls	155	155	154	154	1.1	1.00	0.85	0.69
Cottonseed, cracked Pima	51.9	51.9	51.8	51.7	0.43	1.00	0.87	0.74
Cottonseed, fuzzy linted	31.3	31.3	31.2	31.2	0.30	0.99	0.89	0.78
Wheat, straw	8.0	8.0	8.0	8.0	0.13	1.00	0.93	0.85
Mineral, premix	16.8	16.8	16.8	16.8	0.09	0.98	0.80	0.62
Canola meal, pellets	169	169	169	169	1.3	1.00	0.85	0.71
(solvent)	14.1	14.1	14.1	14.1	0.06	0.94	0.72	0.46
Molasses, liquid	0.00	1.03	0.00	1.01	0.014	<0.01	1.00	<0.01
RPP Product[b]	0.00	0.00	0.66	0.66	0.008	1.00	<0.01	<0.01
RPM Product[c]	67.1	67.3	67.7	66.5	2.73	0.65	0.90	0.86
Alfalfa, fresh chop/haylage[d]	57.2	58.4	57.0	57.0	2.58	0.71	0.94	0.95
Wheat, silage	181	180	181	181	1.9	0.48	0.79	0.69
Corn, flaked grain	164	164	164	164	5.5	1.00	1.00	0.96
Corn, silage	34.8	34.9	35.5	34.9	3.03	0.97	0.85	0.98
Citrus, wet pulp (orange)								
Nutrient profile, g/kg DM [e]	557	546	554	555	9.7	0.22	0.74	0.84
Dry matter	921	923	921	923	1.0	0.28	0.98	0.28
Organic matter	160	160	161	159	4.1	0.98	0.92	0.86
Crude protein (CP)	64	63	64	63	1.9	0.46	0.94	0.48
ADICP[f]	318	313	317	317	2.8	0.16	0.80	0.70
aNDF[g]	305	299	304	302	2.7	0.12	0.74	0.41
aNDFom[h]	223	216	222	217	2.7	0.08	0.72	0.13
ADF[i]	44	44	45	44	1.0	0.58	0.68	0.88
Fat	172	173	164	175	6.0	0.96	0.29	0.70
Starch								

[*] n = 4 pens.
[a] Based on average ingredient composition during the sampling week for each pen, each period, as reported by TMR tracker system.
[b] Ruminally protected Phe (QualiTech Inc., Chaska, MN, USA). Fed at 13.7 g/cow/d to deliver 8 g intestinally absorbable Phe.
[c] Ruminally protected Met (Smartamine M, Adisseo USA Inc., Alpharetta, GA, USA). Fed at 20.9 g/cow/d to deliver 7.5 g intestinally absorbable Met.
[d] Alfalfa haylage used only in Period 1 & 2. Alfalfa fresh chop used only in Period 3 & 4.
[e] Total mixed ration samples collected twice during sampling week for each pen, each period (*i.e.,* 32 total samples), samples pooled by period and pen (n=2 per period).
[f] Acid detergent insoluble CP (g/kg of CP).
[g] Neutral detergent fiber assayed with heat stable amylase, expressed inclusive of residual ash.
[h] Neutral detergent fiber assayed with heat stable amylase, expressed exclusive of residual ash.
[i] Acid detergent fiber, expressed inclusive of residual ash.

not differ among treatments and was well above the suggested optimum level of 135 g/kg according to Swanepoel et al. (2014).

The only difference in the ingredient profiles of the treatment diets was inclusion of 13.4 and 20.5 g/cow/d of RP Met and RP Phe respectively (which was equal to the targeted 13.7 and 20.9 g/cow/d). Even though alfalfa haylage was substituted with alfalfa fresh chop in the 3rd and 4th periods, it resulted in no changes in the nutrient profile of the treatment TMR, which were the same among periods. The TMR met all nutrient requirements of lactating dairy cows producing 45 to 50 liters of milk/d (NRC, 2001).

3.2. Animal measurements

3.2.1. Intake, Milk production and its composition

Dry matter intake (Table 3) was not affected (avg: 27.6 +/- 0.40 kg/d) by treatment. Intakes for the Control ration were higher (27.8 vs. 25.28 kg/d) than for the all CM ration in Swanepoel et al. (2014), even with lower milk production (44.1 vs. 47.4 kg/d).

The PHE treatment had no effect on milk production or composition vs. Control (Table 3), but MET increased milk protein (30.2 vs. 30.7 g/kg; $P<0.01$) and fat (34.2 vs. 34.7 g/kg; $P=0.01$) content, while decreasing milk lactose content (47.8 vs. 47.5 g/kg; $P<0.01$) and its yield (2.11 vs. 2.07 kg/d; $P<0.01$). Milk yield tended ($P=0.03$) to decrease with MET, while M/P did decrease ($P<0.01$) milk (44.10 vs. 43.14 kg/d) and lactose (2.11 vs. 2.05 kg/d) yields, as well as milk lactose content (47.8 vs. 47.6 g/kg; $P<0.01$), while increasing the milk true protein content (30.2 vs. 30.6 g/kg; $P<0.01$). The M/P treatment tended to decrease milk fat yield ($P=0.03$) and SCC ($P=0.02$).

Compared to M/P, PHE had a lower milk true protein content (30.6 vs. 30.1 g/kg; $P<0.01$), higher lactose content (47.6 vs. 47.7; $P<0.01$) and yield (2.05 vs. 2.08; $P=0.02$) as well as higher SCC ($P=0.01$), while tending to a higher milk yield ($P=0.05$). For MET, increases ($P<0.01$)

were observed for milk true protein yield (1.33 vs. 1.31 kg/d), milk fat yield (1.50 vs. 1.46 kg/d) and content (34.7 vs. 34.1 g/kg) and milk energy density (2.90 vs. 2.88 MJ/kg) compared to M/P.

3.2.2. Body condition score

Body condition score (Table 3) was not affected by any treatment and the mean of 2.65 is normal for high producing early lactation cows. Cows in all treatments gained BCS, which is expected in cows past peak production, but only M/P increased ($P<0.01$) the change in BCS vs. Control and PHE (0.08 vs. 0.04 unit change/28 d).

Table 3: Production performance and body scores for cows fed rations with different ruminally protected amino acids.

	Treatment					*Control vs. (P)*			*M/P vs. (P)*	
	Control	PHE	MET	M/P	SEM	PHE	MET	M/P	PHE	MET
n = 4 pens										
Dry matter intake (kg/d)	27.8	28.1	28.3	27.7	0.40	0.79	0.62	0.87	0.67	0.52
n = 596 cows										
Yield (kg/d)										
Milk	44.1	43.7	43.5	43.1	0.31	0.10	0.03	<0.01	0.05	0.15
Fat	1.49	1.48	1.50	1.46	0.013	0.41	0.62	0.03	0.17	<0.01
True protein	1.32	1.30	1.33	1.31	0.008	0.06	0.41	0.18	0.57	0.03
Lactose	2.11	2.08	2.07	2.05	0.015	0.06	<0.01	<0.01	0.02	0.29
Components (g/kg)										
Fat	34.2	34.1	34.7	34.1	0.23	0.84	0.01	0.67	0.82	<0.01
True protein	30.2	30.1	30.7	30.6	0.11	0.44	<0.01	<0.01	<0.01	0.10
Lactose	47.8	47.7	47.5	47.6	0.06	0.10	<0.01	<0.01	<0.01	0.08
Energy density (MJ/kg)	2.87	2.87	2.90	2.88	0.011	0.62	<0.01	0.92	0.55	<0.01
Somatic cell count ('000)	127	130	109	98	10.5	0.85	0.16	0.02	0.01	0.38
n = 343 cows										
Body condition score (BCS)	2.65	2.64	2.65	2.66	0.021	0.57	0.69	0.45	0.19	0.25
BCS change (unit/28 d)	0.04	0.04	0.06	0.08	0.011	0.98	0.10	<0.01	<0.01	0.25

3.2.3. Urine

Urine AL and CR concentrations (Table 4) did not differ among treatments. Control AL concentrations were higher (3812 vs. 3370 mg/L) and CR lower (882 vs. 946 mg/L) than for the all CM ration in Swanepoel et al. (2014). Urine volume (avg: 16.7 +/- 0.31 L/d) also did not differ among treatments. As expected, the calculated flow of MCP from the rumen (avg: 2092 +/- 52.7 g CP/d) was also not affected by the treatments. These calculated MCP flow values are higher than the ranges (763 to 1959 g CP/d) previously reported in the literature when duodenal samples were collected and MCP flow directly measured (Khorasani et al., 1993; Robinson et al., 1994; 1996; Stensig and Robinson, 1997; Robinson et al., 1998; Timmermans et al., 2000; González-Ronquillo et al., 2003; Moorby et al., 2006), but this is likely due to the higher DM intakes (27.9 vs. 20.7 kg/d) in our study compared to that literature, which suggests that our MCP flows, estimated using urine AL concentrations, are biologically sensible. However one study (Reynal and Broderick, 2005), with similar milk production (avg. 42.3 kg/d) and DM intakes (avg. 25.5 kg/d) to our study, reported MCP flows that were higher (2683 vs. 2092 g CP/d) using urinary excretion of PD together with N:purine ratios in omasal samples.

Table 4: Urine analysis for cows fed rations with different ruminally protected amino acids.

	Treatment					*Control vs. (P)*			*M/P vs. (P)*	
	Control	PHE	MET	M/P	SEM	PHE	MET	M/P	PHE	MET
n = 42 cows										
Allantoin (AL, mg/L)	3812	3734	3564	3601	112.5	0.59	0.12	0.18	0.37	0.82
Creatinine (CR, mg/L)	882	933	821	894	36.2	0.20	0.17	0.78	0.35	0.11
Specific gravity	1.03	1.03	1.03	1.03	0.001	0.70	0.29	0.22	0.36	0.88
Urine volume (L/day)	16.4	16.6	16.8	16.8	0.31	0.61	0.32	0.20	0.40	0.80
Total PD[a] excreted (mmol/d)	454	446	431	438	9.9	0.56	0.10	0.26	0.55	0.59
MCP[b] yield (g CP/d)	2155	2114	2030	2071	52.7	0.56	0.10	0.26	0.55	0.59

[a] Purine derivatives.
[b] Microbial protein.

3.2.4. Blood plasma

There were no changes in plasma AA levels (Table 5) for MET or M/P vs. Control, except Met which increased with both (*P*<0.01), and Trp tended (*P*=0.04) to decrease with the M/P treatment. In contrast, plasma Trp decreased with PHE vs. Control (*P*<0.01), while plasma Phe levels were not impacted by any treatment. Due to higher plasma Met levels, both the MET and M/P treatments decreased the Lys:Met ratio (*P*<0.01).

Table 5: Free amino acid and ammonia concentrations (µg/ml) in plasma of cows fed rations with different ruminally protected amino acids.

	Treatment					*Control vs. (P)*			*M/P vs. (P)*	
	Control	PHE	MET	M/P	SEM	PHE	MET	M/P	PHE	MET
*n = 24 cows**										
Essential amino acids										
Threonine	14.7	14.9	14.6	14.1	0.50	0.73	0.81	0.27	0.15	0.38
Valine	32.9	32.6	33.4	31.5	0.90	0.67	0.61	0.09	0.19	0.03
Methionine	3.58	3.71	4.97	4.56	0.167	0.47	<0.01	<0.01	<0.01	0.03
Isoleucine	15.0	15.2	15.8	14.8	0.50	0.74	0.15	0.72	0.48	0.07
Leucine	20.2	20.1	20.8	19.7	0.65	0.93	0.34	0.40	0.45	0.07
Phenylalanine	9.21	9.49	9.49	9.31	0.252	0.29	0.30	0.71	0.49	0.50
Tryptophan	16.8	15.2	16.4	15.7	0.46	<0.01	0.40	0.04	0.25	0.23
Lysine	13.2	13.1	13.9	12.9	0.43	0.84	0.12	0.65	0.80	0.05
Histidine	8.81	8.67	8.85	8.46	0.285	0.58	0.89	0.19	0.44	0.15
Arginine	15.5	15.5	16.6	15.2	0.57	0.95	0.06	0.71	0.66	0.02
Lys:Met ratio	3.80	3.60	2.91	2.95	0.086	0.05	<0.01	<0.01	<0.01	0.66
Non-essential amino acids										
Homocystine	1.02	1.02	0.97	0.98	0.108	0.95	0.74	0.74	0.78	1.00
Aspartic acid	1.60	1.56	1.59	1.55	0.083	0.67	0.94	0.58	0.90	0.64
Tyrosine	9.92	9.86	9.98	9.78	0.399	0.88	0.89	0.72	0.83	0.61
Serine	9.83	9.85	9.66	9.48	0.356	0.96	0.62	0.29	0.27	0.57
Glutamic acid	7.60	7.43	7.33	7.35	0.216	0.45	0.22	0.26	0.70	0.93
Glutamine	48.0	48.1	48.9	47.2	2.06	0.94	0.70	0.76	0.70	0.49
Glycine	31.0	30.9	29.2	29.8	1.44	0.95	0.13	0.33	0.36	0.60
Alanine	23.6	23.8	24.7	23.2	0.91	0.79	0.11	0.62	0.44	0.04
3-Methylhistidine	0.71	0.62	0.57	0.67	0.062	0.25	0.07	0.56	0.57	0.21
Ammonia	2.94	3.06	2.91	3.06	0.146	0.48	0.87	0.50	0.97	0.41

* A randomly selected group of 6 cows/pen/period was used for amino acid analysis as it was decided that additional samples would not change significance of differences.

Compared to the M/P treatment, MET tended to increase (*P*<0.05) valine (Val), Met, Lys, alanine (Ala) and arginine (Arg) while PHE decreased Met (*P*<0.01), thereby increasing the Lys:Met ratio from 2.95 to 3.60 (*P*<0.01).

Since it was not measured, when AA changes are discussed it is assumed that the pool sizes (plasma volumes) remained constant between treatments.

3.2.5. Partial net energy balance

The Partial NE balance (Table 6) for each treatment was calculated to determine where energy was utilized. Compared to Control, M/P decreased total energy output in milk (126 vs. 124 MJ/d; *P*<0.01) while increasing energy used for BCS change (1.7 vs. 3.6 MJ/d; *P*<0.01). However, there were no other differences in total NE output, or the calculated dietary NE_L densities, between any treatment and the Control. Therefore, the amount of NE expressed by the cows, and in the diets, did not change among treatments. However, AA supplementation resulted in energy being partitioned differently, especially the M/P treatment, which changed the way energy was utilized by either AA treatment alone compared to Control.

The M/P treatment had a lower milk energy output (124 vs. 126 MJ/d; *P*=0.02) compared to MET but a higher BCS change energy (3.60 vs. 1.71 MJ/d; *P*<0.01) compared to PHE.

Table 6: Partial net energy balance for cows fed rations with different ruminally protected amino acids*.

	Treatment					*Control vs. (P)*			*M/P vs. (P)*	
	Control	PHE	MET	M/P	SEM	PHE	MET	M/P	PHE	MET
Milk energy output (MJ/d)	126	125	126	124	0.8	0.16	0.73	<0.01	0.19	0.02
BCS [a] energy (MJ/d)	1.7	1.7	2.8	3.6	0.48	0.98	0.10	<0.01	<0.01	0.25
Total Net Energy (MJ/d)	172	171	173	172	1.1	0.65	0.74	0.84	0.81	0.59
NE_L [b] (MJ/kg DM)	6.19	6.12	6.12	6.22	0.088	0.72	0.73	0.86	0.60	0.60

* Maintenance energy (MJ/d) calculated using a constant body weight of 673 kg for all treatments.
[a] Body condition score.
[b] Net energy available for lactation. *n* = 4 pens.

4. Discussion

In many feeding situations, supplementation of Met has been shown to increase milk protein content (Chen et al., 2011) and/or yield (Čermáková et al., 2012; Osorio et al., 2013) as well as milk fat content (Wang et al., 2010). This has also been confirmed in a recent systemic review of the literature (Robinson, 2010) and meta-analysis (Patton, 2010), and it agrees with our results in which milk protein and fat proportions increased modestly with the MET treatment. Patton (2010) suggests that a slight increase in milk yield can also be expected, and, even though this is consistent with the increases in milk yield reported earlier (Wang et al., 2010; Čermáková et al., 2012; Osorio et al., 2013), it does not agree with the reduction in milk lactose yield, or the tendency for milk yield to decrease with MET, in our study. A study by Robinson et al. (2000), to determine effects of a Met oversupply, showed that abomasal Met infusion to increase its intestinal delivery by 34 to 39% markedly reduced animal performance. Indeed Robinson et al. (2000) reported a sharp decline in milk and lactose yields, which is similar to our results in relative terms, although not quantitatively as all changes in our study were modest even though supplementation with RP Met increased estimated intestinally available Met by ~ 38% in our study, suggesting that an oversupply of Met was possible.

It is generally suggested that Lys and Met should be fed in the ratio of 3:1 (at the intestinal absorptive site) in order to obtain beneficial responses in milk production and composition (Chalupa and Sniffen, 2006). Since our two Met supplementation treatments reduced the plasma Lys:Met ratio (which should be reflective of the ratio of absorbable Lys and Met at the intestinal absorptive site) from 3.80 in the Control ration to 2.91 and 2.95 respectively, this theoretically near perfect ratio should have resulted in a positive production response. However, this was not the case. Even though the Lys:Met ratio concept seems clear when looking at the data published in NRC (2001), other studies in the literature do not always agree. For instance, Chen et al.

(2011) reduced this Lys:Met ratio from 3.6 to 3.0, resulting in an increase in milk protein content, but only in a relatively low protein ration. Supplementation of Met in the peripartal period to decrease the Lys:Met ratio from 3.4 to 2.9 only showed a tendency for milk protein content to increase (Osorio et al., 2013). Rulquin and Delaby (1997) also reported an increase in milk protein content when the Lys:Met ratio was decreased from 3.8 to 3.0 through Met supplementation, although their plasma AA analysis showed that the ratio was actually reduced from 4.8 to 2.2. As the predicted Lys:Met ratios may not reflect the actual ratios, interpretation of the Lys:Met ratio concept in studies outside the NRC (2001) is difficult. In the study of Robinson et al. (2000), creating an imbalance in the suggested optimum Lys:Met ratio by supplementing Lys (*i.e.*, changing the ratio from 3.0 to 3.9) had no effect on production, but creating a theoretical imbalance by supplementing Met (*i.e.*, changing the ratio from 3.0 to 2.3), reduced DM intake, milk production and lactose yields. While restoring the ratio to 3:1 by supplementing Lys and Met together did not impact production, the negative effects of high Met supplementation remained, even at the 3.0 Lys:Met ratio. This seems to suggest that Met supplementation to some point results in positive production responses, regardless of the theoretical Lys:Met ratio, but that production responses became more and more negative at higher and higher Met supplementation levels, regardless of the Lys:Met ratio. This strongly suggests that it is the level of Met relative to its requirement that elicits the response rather than its ratio with Lys *per se*. Indeed this hypothesis agrees with results of a study where the control ration had the lowest production, even with a calculated ratio close to 3.0, while positive production responses were seen for both Met and Lys supplementation, even when the supplementation changed the ratios to 1.3 and 4.6 respectively (Wang et al., 2010).

Robinson et al. (2000) reported that oversupplying both Met and Lys simultaneously changed partitioning of energy, with their combined infusion improving energetic efficiency of the cows

without affecting general animal performance, thereby increasing the NE density of the ration to prevent a negative energy balance in the cows. This corresponds to our study where combined supplementation of Phe and Met changed the way energy was utilized vs. when Met was supplemented alone. While it is clear that Met supplementation alone directed energy from milk lactose to milk fat and protein production, thereby shifting energy output within milk components, addition of Phe to the Met redirected energy from lactose production towards BCS gain. As explained by Swanepoel et al. (2010), it is possible that an oversupply of Met resulted in increased fat synthesis but that supplementation of the limiting AA, in this case Phe, rectified the AA imbalance, thereby restoring the fat content. This suggests that Phe was limiting in our study, but that its supplementation level was not high enough to allow for an effect on production. In contrast, it is clear that supplementation of Phe alone had no effect on animal performance, or at least any response parameters measured in our study, suggesting that Phe only became limiting in the combination treatment after Met requirements were met, but that a possible oversupply of Met, and undersupply of Phe, in the M/P treatment did not allow for a production response. Arginine (Arg) metabolism in the liver is mainly directed towards the urea cycle in order to dispose of AA in excess relative to the limiting AA (Bach et al., 2000). The tendency of Arg to increase in the plasma for the Met treatment in this study (P=0.06) support the hypothesis that Met was probably limiting and that it's supplementation allowed utilization of other AA, reducing the amount of surplus AA catabolized by the liver and therefore the requirement for Arg in the urea cycle.

Our study was designed to deliver 7.5 g of Phe to the intestine, which is 5.5% higher than the estimated intestinal Phe delivery levels for the Control ration from Swanepoel et al. (2014). However, plasma Phe levels did not differ significantly between treatments and were only 3.0% higher for PHE vs. Control, possibly suggesting that RP-Phe failed and that little or no Phe

reached the intestinal absorptive site. However this seems unlikely since the manufacturing technology of this RP-Phe was the same as the RP-Lys product evaluated in Sakkers et al. (2013) which was measured *in vivo* to have a rumen escape of 52% of consumed Lys. Haque et al. (2013) demonstrated that the separate infusion of Arg, Isoleucine (Ile) and Val to the duodenum resulted in increased concentrations of Ile and Val but not Arg, showing that plasma AA concentrations are not always directly associated with its supplementation or removal. In this case the failure of plasma Phe concentrations to increase in response to its duodenal supplementation can be interpreted to suggest that Phe was utilized by the cow, instead of building up in the plasma, suggesting a more likely hypothesis, which is supported by the data, that Phe was under-delivered relative to its needs. Indeed it has been suggested that Phe enhances the rate of Trp hydroxylation (Kaufman 1971), the first step in catabolizing Trp to synthesize serotonin and, since Trp levels in the plasma of both Phe supplemented treatments decreased vs. Control, this supports delivery of Phe to the intestinal absorptive site, as well as its absorption and availability in high enough quantities pre-liver to have elicited this enhanced effect on Trp hydroxylase in the liver.

Phenylalanine is part of a group of AA which are extensively catabolized by the liver and removal of these AA are directly correlated to their hepatic inflow (Lapierre et al., 2005; Bach et al., 2000). Thus as more of these AA are absorbed, more will be removed by the liver (up to 0.49 of portal absorption). Bach et al. (2000) showed that even though supplementation of the limiting AA reduced extraction and deamination of most AA by the liver, due to their utilization elsewhere, the rate of catabolism of Phe by the liver remained constant. The liver also has the capability to export AA bound to peptides or proteins in the blood, rather than free AA, which would not be reflected in the plasma data since blood AA concentrations were not analysed in this study due to the difficulties associated with it (Bach et al., 2000). These phenomena could

account for the lack of an increased post-liver plasma Phe level in both Phe treatments which is consistent with Lapierre et al. (2005), suggesting that the reason for increased removal of AA by the liver is to remove excess AA, thereby equalizing post-liver supply with both mammary uptake and milk output requirements. This suggests that Phe was not limiting production in the Control diet and that its supplementation in an RP form was unnecessary.

Another possible explanation for the lack of a production response to supplemental Phe alone could be that when AA which are usually extracted at levels lower than milk protein requirement (*e.g.,* Phe, Tyr) are supplemented, extraction rates and efficiencies considerably increase (Guinard and Rulquin, 1994), substituting mammary utilization of peptide-derived Phe with that of added free Phe. This suggests that supplemented Phe did not provide an additional supply of AA, but rather replaced peptides as the source of Phe to support milk protein synthesis (Bequette and Backwell, 1997). It is also possible that, due to the relatively high MCP flow in our study and the large contribution of MCP to total absorbable protein, any benefit that resulted from supplementation of Phe alone was too small to be measured (Robinson, 2010).

The shift in energy utilization with M/P vs. MET supports the hypothesis that Phe was absorbed into blood and that enough was delivered to the mammary gland to elicit a response. If the additional free Phe replaced use of peptide-derived Phe in the mammary gland, it is possible that more Met, which liberated energy from milk fat and lactose, provided the energy required to incorporate the peptides back into muscle protein, resulting in some of the increased BCS gain. Jaurena et al. (2005) showed that BCS gain reflects accretion of both fat and muscle mass, which was not the case with Phe supplementation alone. This is supported by the tendency for a number of AA (*i.e.,* Met, Lys, Arg, Ala, all branched-chain AA (BCAA)) to decrease in the M/P vs. MET treatments. Since these AA were not incorporated into milk *per se*, it is possible that they were utilized to support synthesis of muscle protein, as the increase in BCS change may attest.

This is also supported by the decrease in concentrations of Alanine (P=0.04) and Lys (P=0.05), these being the AA which are most abundant in muscle tissue (Bach et al., 2000).

Responses to AA (mainly Lys and Met) supplementation in general have been inconsistent, relatively small and, in many cases, not what was expected in a literature which now dates back over 30 yrs. Some authors have ascribed a lack of response to other dietary factors and/or other AA being more limiting than those supplemented (Karunanandaa et al., 1994; Robinson et al., 1998; Liu et al., 2000), although this often seems to be 'form' reasoning. It seems more plausible that supplemented AA are used in biological processes, resulting in effects on animal performance which differ depending on whether they were actually limiting milk production, or not, with actual limitations being rare. As suggested by Robinson et al. (1998), Met supplementation consistently enhances milk component yields, albeit to a small degree, beyond that expected from its role as a limiting AA *per se*, and seemingly without connection to the expected basal Met flows to the intestinal absorptive site. The perceived correction of a theoretical AA deficiency through its supplementation consistently increases its plasma levels while also increasing milk protein content (Rulquin and Pisulewski, 2006; Weeks et al., 2006; Haque et al., 2012). However, the same cannot be said for calculated AA 'imbalances' at the intestinal absorptive site. In most cases an estimated imbalance of AA manifests as increased milk fat content, or a low ratio of protein:fat in milk (Chamberlain et al., 1992; Varvikko et al., 1999; Robinson et al., 2000; Cant et al., 2001; Kim et al., 2001; Weekes et al., 2006) possibly through mechanisms in the mammary gland, such as increased blood flow to maintain milk protein yield, thereby supplying more milk fat precursors to stimulate milk fat yield (Cant et al., 2001; Weekes et al., 2006). It has been suggested that endocrine responses to total AA imbalances can override imperfections in AA profiles in order to maintain milk protein yields (Weekes et al., 2006), although not all of the resulting changes may be deemed positive by dairy

farmers. In addition, supplementation of AA to rations in which they are not limiting are known to negatively affect animal performance through, for instance, reducing DM intake (Karunanandaa et al., 1994; Robinson et al., 2000; Rulquin and Pisulewski, 2006) and sequestration of AA in body protein that exacerbate the AA deficiency (Weekes et al., 2006).

In some cases, as in our study, supplementing a second AA (or group of AA) may result in the opposite, or a reversal, of the response from only one AA (Polan et al., 1991; Kim et al., 2001; Wang et al., 2010), or simply a completely different response as was seen when supplementation of BCAA in addition to Met and Lys, which were deemed limiting, had no additional effect on milk production but stimulated muscle protein synthesis (Appuhamy et al., 2011). It has been suggested that it is not blood AA concentrations *per se* that limits milk protein production, but rather the metabolic machinery which determines maximum velocity of milk protein production (Cant et al., 2001) or a gastrointestinal event (Bequette et al., 1996), both of which are under hormonal control.

It may be time to reconsider the 'limiting AA' or 'broken stick' concept of AA nutrition of lactating dairy cows in favor of accepting that most AA are bioactive and can change animal performance, even when they are not 'limiting' milk production *per se*, which is likely the normal situation. Thus recommendations to use RPAA products must consider the potential for unwanted effects, which could be deemed negative, which are associated with oversupply, or unnecessary supply, of AA to the intestinal absorptive site relative to animal 'needs'.

5. Conclusions

Phenylalanine supplementation alone in an RP form caused no animal response since, even though the results suggest that it was delivered and absorbed, it likely increased Phe catabolism in the liver since Met was limiting its use for production, thereby lowering the amount of Phe

which reached the mammary gland. In contrast, Met was likely oversupplied with Met supplementation alone, which showed as increased milk fat and protein proportions. However, supplementation of the combination of both AA possibly rectified the Met limitation and supplied Phe which became 2nd limiting after the Met requirements were met. However it seems that we did not supply enough Phe to support a sustained milk production response. Instead, as a first priority, free Phe may have replaced peptides which were previously mobilized from the muscle to rectify the Phe limitation, directing those back to muscle protein synthesis with no surplus Phe remaining available to increase milk production.

Responses to AA supplementation in dairy cows in a research time frame now exceeding 30 yrs. have been inconsistent and unpredictable and, although authors provide many reasons to justify the seemingly random (but generally low or no) responses to AA supplementation, it is clear that AA limitations, requirements and production responses are governed by much more than their plasma AA levels. Indeed our results strongly suggest that AA should be viewed as bioactive metabolites to the extent that they can change animal performance characteristics, even when they are not 'limiting' *per se*, and that their supplementation to practical dairy cattle rations should be approached with extreme caution for this reason.

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