Impacts of adding ruminally protected phenylalanine to rations containing high levels of canola meal on performance of high producing Holstein cows

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Highlights

- Supplementation of Phe to early lactation cows receiving 170 g/kg of diet DM as CM.
- Phe changed energy utilization, decreasing milk lactose while increasing BCS gain.
- Phe resulted in an increase in whole tract aNDFom and ADF digestibility.
- Phe support milk production after restoring mobilized peptides to muscle protein.
- Phe regained animal performance lost when CM inclusion was increased.

Abstract

Even though studies supplementing Phe to dairy cattle are rare, it has been identified as limiting in corn silage based rations, after Lys and Met, as well as being important to the mammary gland for overall milk production. Since canola meal (CM) is low in Phe, plasma Phe concentrations decrease as more CM is included in dairy rations. A previous study fed 7.5 g/cow/day of intestinally absorbable Phe, but results suggested that it was insufficient to support increased milk production since it was primarily used to support increased body condition score (BCS; Swanepoel et al., 2015). Our objective was to determine if supplementing 15 g/cow/day of intestinally absorbable Phe in a ruminally protected (RP) form (HCMP) to a ration containing 170 g/kg CM (HCM) would support increased milk production after fulfilling its apparent 1st

priority of restoring previously mobilized peptides to muscle protein synthesis, thereby regaining animal performance possibly lost with higher dietary CM inclusion levels (*i.e.,* 130 g/kg (LCM) to 165 g/kg (HCM)) based upon Swanepoel et al. (2015). Ruminally protected Met (2.0 g/cow/day intestinally absorbable) was added as part of the treatment ration to HCM treatments to avoid a possible Met limitation. The experimental design was a 3 x 3 Latin square using 3 pens of ~315 early lactation cows/pen with three 21 day periods. Dry matter (DM) intake was not affected (avg: 27.5 ± 0.5 kg/day) by feeding RP Phe and there was no impact of treatment on milk and component yields, except a reduced lactose content (P=0.02) with Phe addition. Even though plasma Phe levels only differed numerically between treatments, its supplementation resulted in energy being diverted towards BCS gain as in Swanepoel et al. (2015), but not at the expense of milk components, suggesting that higher Phe supplementation supplied enough Phe to replace mobilized muscle protein while maintaining milk production. The lack in change of plasma Phe concentrations could be due to extensive catabolization by the liver or hepatic conversion of Phe to Tyr, which is supported by the change in plasma Tyr concentrations. Interestingly, addition of Phe to the HCM ration increased whole tract neutral- and acid detergent fiber digestibility. Perhaps Phe released into the rumen when Phe was fed stimulated fibrolytic bacteria through a direct impact on microbes of free Phe, which has previously been shown to enhance growth and/or capabilities of cellulolytic bacteria. Total net energy output decreased with HCM feeding, but was restored to the level of the LCM ration for the HCMP treatment suggesting that further investigation to determine if an even higher Phe supplementation level may have additional benefits on milk production may have merit.

Keywords: Plasma amino acids; Phenylalanine supplementation; Body condition change; Fiber digestibility.

Abbreviations: AA, amino acid; ADF, acid detergent fiber; ADIN, acid detergent insoluble N; AL, allantoin; aNDFom, amylase-treated NDF free of residual ash; BCS, body condition score; BCAA, branched-chain AA; CM, canola meal; CP, crude protein; DC305, DairyComp 305 management system; DDG, dried distillers grains; DHIA, Dairy Herd Improvement Association; DM, dry matter; EAA, essential AA; MCP, microbial CP; MP, metabolizable protein; NDF, neutral detergent fiber; NEAA, non-essential AA; NE_L, net energy for lactation; OM, organic matter; PUN, plasma urea N; RP, rumen protected; SCC, somatic cell count; SG, specific gravity; TMR, total mixed ration. List of AA: Aspartic acid (Asp), Threonine (Thr), Serine (Ser), Glutamic acid (Glu), glycine (Gly), Alanine (Ala), Valine (Val), Methionine (Met), Isoleucine (Ile), Leucine (Leu), Tyrosine (Tyr), Phenylalanine (Phe), Tryptophan (Trp), Histidine (His), Arginine (Arg), Proline (Pro).

1. Introduction

Even though studies supplementing Phe to dairy cattle are rare, it has been identified as potentially limiting in corn silage based rations (Piepenbrink et al., 1998; Mulrooney et al., 2009; Christen et al., 2010), after Lys and Met. However as these limitations are determined using extraction efficiencies across the mammary gland, and since Lys is taken up by the mammary gland regardless of its requirement, it may always appear first limiting (Nichols et al., 1998) regardless of ration fed. This suggests that amino acids (AA) identified as potentially limiting after Lys, such as Met, Phe and Leu, could be alternative limiting AA. As summarized by Schwab et al. (1975), several studies have suggested Phe to be limiting together with Lys (Vik-Mo et al., 1974) or Thr, His and Met (Derrig et al., 1974) with abomasal infusion of casein and/or glucose.

An AA supplementation study reported that the mammary gland has a specific requirement for Phe and Tyr which increases as milk protein production increases (Guinard and Rulquin, 1994), and that supplemented Phe was extracted by the mammary gland in amounts equal to its secretion in milk protein. This indicates that Phe is not extracted in excess and is almost exclusively utilized to support milk production. Indeed, Iroshan et al. (2013) showed that milk protein yield decreased when Phe was absent from AA infusions thereby indicating that limited Phe negatively affected milk and milk protein secretion, thus confirming the importance of Phe in milk production.

Since CM is low in Phe compared to other protein supplements, the plasma concentration of Phe decreases as CM inclusion in the ration increases (Swanepoel et al., 2014). A survey study examining comparisons between milk production, TMR ingredient profiles and plasma AA concentrations within 20 California (USA) dairy farms confirmed that plasma Phe concentrations are negatively correlated to the inclusion level of CM in the ration and that milk yield was negatively correlated with all plasma AA concentrations except Phe (Swanepoel et al., 2015b), supporting the hypothesis that Phe is important relative to milk production. Other studies feeding CM have shown a similar decline in plasma Phe compared to other protein sources (Christen et al., 2010), or a decrease in Phe proportions in metabolizable protein (MP) with higher dietary CM inclusions (Martineau et al., 2014), even though there were no changes in plasma Phe concentrations with different inclusion levels of CM (Mulrooney et al., 2009).

The declining plasma concentrations of Met and Phe with increasing CM inclusion levels (Swanepoel et al., 2014) led us to question whether Met and/or Phe was limiting performance of cows fed diets with high levels of CM (Swanepoel et al., 2015). As results suggested that Met was likely oversupplied at 8 g/cow/day of intestinally absorbable Met, and 7.5 g/cow/day of

intestinally absorbable Phe was insufficient to support increased milk production, it appeared that supplemented Phe was directed towards body condition score (BCS) gain as a 1st priority.

This study was designed to determine if supplementing higher levels of ruminally protected (RP) Phe (than in Swanepoel et al., 2015) would be beneficial to performance of early lactation dairy cows by supplying enough Phe to support increased milk production, after fulfilling its apparent 1st priority of restoring previously mobilized peptides to muscle protein.

2. Materials and methods

The experimental design was a 3 x 3 Latin square using 3 pens of ~315 early lactation cows/pen with three 21 day experimental periods. The study took place during winter (22 Jan to 11 March 2014) with temperatures between 2.5 and 26.5^{o}C and humidity between 31 and 100%. 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 commercial dairy selected near Hanford (CA, USA) was the same as used in Swanepoel et al. (2015), and was specifically selected for management and pen structures required to support this type of study. As per normal farm practices, cows were randomly allocated each week to 1 of 4 early lactation pens from a single fresh pen and, once confirmed pregnant, were moved from these pens to mid lactation pens. Only 3 of the 4 early lactation pens were used. At the start of period 1, each treatment was randomly allocated to one of the 3 pens and rotated amongst the pens after each 21 day period.

2.2. Diets

Mixing of the TMR and all other farm practices were as outlined in Swanepoel et al. (2014). Since the optimum level of CM inclusion in the TMR was established to be in the range of 120 – 135 g/kg (Swanepoel et al., 2014) a Low CM treatment (LCM), with CM included at ~130 g/kg dry matter (DM), was the positive control to establish the degree to which performance was negatively impacted when CM inclusion level was increased (Table 1). The other two treatments consisted of the same base TMR based on alfalfa hay, whole crop winter wheat and summer corn silages, and corn grain, with a premix containing most dry ingredients (*i.e.*, almond hulls, fuzzy and cracked pima cottonseed, wheat straw, liquid molasses, mineral premix, CM), with a higher CM inclusion targeted at 170 g/kg of total ration DM (*i.e.*, High CM rations; HCM). Cows were fed *ad libitum* to achieve ~10 g/kg refusals on an as fed basis, with each pen receiving a total of ~16,000 kg of as-mixed TMR/day in 2 feedings. During the 1st feeding of the day, between 04:30 and 07:30 h, cows were fed one full ~11,000 kg load of TMR, which contained the RPAA, to a clean bunk as bunks were cleared of all residual feed while the cows were at morning milking. Between 11:00 and 12:30 h a second ~5,000 kg load of TMR was fed. 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, which was used in statistical analysis to assess if treatment diets differed as per experimental objectives while not creating unexpected collateral differences, as well as weights for each load of TMR mixed and fed, which were used together with daily refusals, and cow numbers in each pen on each day, to calculate DM intake/cow for the final 7 days (*i.e.*, the sampling week) of each experimental period in each pen, as is standard in Latin square design experiments. Of cows in pens during DM intake measurement at the ends of Periods 1, 2 and 3, ~87, ~74 and ~61% (respectively) had been in that pen since the beginning of the study. Thus, on average, the DM intake data is represented ~74% by cows which had been in

Table 1

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

	Dry matter	Organic matter	Crude protein	ADF[2]	aNDFom[3]	aNDF[4]	Lignin(sa)[5]
Alfalfa, hay (High quality)	902	902	198	276	348	364	66.5
Alfalfa, hay (Medium quality)	902	897	192	307	364	379	63.0
Almond, hulls	967	935	47.5	202	260	268	71.0
Canola, pellets (solvent)	903	922	407	191	275	287	80.0
Citrus, wet/pulp	148	946	79.7	183	200	204	11.5
Corn, silage	295	929	73.8	286	473	490	25.0
Corn distillers grains, dry	892	942	306	104	330	331	15.0
Cottonseed, Pima cracked	906	953	236	288	387	406	116
Corn, grain flaked	857	987	74.4	24.0	85.0	85.0	<1.0
Wheat, silage	361	884	89.4	340	452	491	41.0
Wheat, straw	929	871	33.1	461	689	719	48.0

[1] n = 3. One sample/period, all combined for a single analysis.
[2] Acid detergent fiber, expressed inclusive of residual ash.
[3] Neutral detergent fiber assayed with heat stable amylase, expressed exclusive of residual ash.
[4] Neutral detergent fiber assayed with heat stable amylase, expressed inclusive of residual ash.
[5] Lignin treated with sulphuric acid, ash free.

their originally assigned pen since the start of the study. In addition, while all cows measured during each measurement period had been exposed to that diet for the entire experimental period, about 4% had been exposed for 7-14 days and about another 4% for 15-21 days. Thus DM intake values reported overwhelmingly represent cows with a full 21 days of diet consumption.

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. The RP Phe product was manufactured by QualiTech Inc. (Chaska, MN, USA) containing 600 g/kg Phe combined with 400 g/kg fat as a matrix. The products used, as well as methods of evaluation, were fully described in Swanepoel et al. (2015).

Treatments were created by adding RP Met alone (HCM) or in combination with RP Phe (HCMP) to the High CM ration by mixing 41.2 g/cow/day of RP Phe (estimated to deliver 15 g/cow/day of intestinally absorbable Phe) and 3.4 g/cow/day of RP Met (estimated to deliver 2.0 g of intestinally absorbable Met/cow/day) to 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.

Since Swanepoel et al. (2015) reported that Phe supplementation at 7.5 g/cow/day alone had no effect on animal performance and that Phe only became limiting in the combination treatment after requirements for Met were met, Met was added as part of the treatment ration to both our HCM treatments in order to avoid a possible Met limitation from inhibiting the animal response to Phe supplementation. However, since it was concluded that delivery of 8.0 g of intestinally absorbable Met/cow/day in Swanepoel et al. (2015) likely oversupplied Met, thereby leading to a reduction in milk and lactose yields, Met supplementation was reduced in this study to deliver only 2 g/cow/day of absorbable Met to the intestine, a level corresponding to the calculated Met delivery by the optimum CM ration fed in Swanepoel et al. (2014).

2.4. Sample collection, preparation and analytical methods

2.4.1. Total mixed rations and ingredients

The TMR and feed ingredients were sampled twice during the last 7 days (*i.e.*, the sampling week) of each of the 3 experimental periods as described by Swanepoel et al. (2015), resulting in 18 TMR samples for chemical analysis while ingredient samples from all three periods were pooled (n = 3 samples/ingredient). All TMR samples, silages and other wet ingredients were weighed, dried at 55^{o}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) and analysed for DM, ash, N, acid detergent insoluble N (ADIN), neutral detergent fiber (aNDFom), acid detergent fiber (ADF), lignin treated with sulphuric acid (lignin(sa)), starch and fat as described by Swanepoel et al. (2015). Soluble carbohydrates (*i.e.*, free sugars) were determined by high-performance liquid chromatography as described by Johansen et al. (1996). Minerals were determined using the methods of Tracy and Moeller (1990), Meyer and Keliher (1992) and Jones (2001).

2.4.2. Animal measurements

For a cow to be eligible for sampling in any period, and therefore inclusion in statistical analysis, they had to have been in their originally assigned pen for the entire 9 week study. Daily data backups of the electronic herd record system DairyComp 305 (DC305; Valley Agricultural Software, Tulare, CA, USA) were made to crosscheck cow movements. Cow sampling was performed on specified days during normal morning lockup (*i.e.*, ~50 min/pen/day for normal health and reproductive checks immediately after the morning milking).

2.4.2.1. Milk production and composition

Milk yields were recorded for each cow on day 21 of each experimental period by Dairy Herd improvement association (DHIA) personnel. Milk samples were 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

The same group of ~120 cows/pen were scored for BCS at the end of every period throughout the study. These cows were pre-determined based upon their DIM being between 20 and 140 at the start of the study. The scoring was completed by the same trained person on the first day of the study 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 when cows could not be clearly allocated to a ¼ point score.

2.4.2.3. Urine

Spot urine samples were collected on day 20 of Period 1 and 2 from the first ~40 cows which had been in their originally assigned period since the start of the study and that voluntarily urinated during normal morning lockup. However in Period 3 spot urine samples were only collected from cows from which urine had been collected in Periods 1 and/or 2 in order to assure that all urine samples were repeated within cow at least once. After collection, urine samples were immediately placed in ice and measured for specific gravity (SG) within 2 h using a digital handheld pen refractometer (Atago USA Inc., Bellevue, WA, USA). Samples were preserved and analyzed as described by Swanepoel et al. (2014), except that only 1.4 ml of 100 ml/L sulfuric acid was required to reduce the pH to <2 immediately after collection. The average concentration of the inter-run standards over all runs were used to correct sample concentrations between runs, as described by Swanepoel et al. (2015).

2.4.2.4. Blood plasma

Blood was collected on day 19 of each experimental period from the tail vein of the same group of 12 cows/pen, pre-determined based upon their DIM being between 20 and 140 at the start of the study. Collection, treatment and analysis for free AA and plasma urea N (PUN) followed Swanepoel et al. (2014).

2.4.2.5. Fecal

Fecal collection was on day 19 of each experimental period by rectal grab sampling the same group of 18 cows/pen as described above. At the end of the 3rd experimental period, the 18 cows/pen collected in each period were combined into a final analytical set by grouping samples (based upon ear tag numbers) from the same 6 cows/pen into 3 groups/pen/period in order to support the assumption that the intake of this group was equal to the overall DM intake. Thus a total of 27 fecal samples were created (*i.e.,* 3/pen/period) that were frozen at -20^{o}C and subsequently analyzed for DM, ash, aNDFom, ADF, lignin(sa) and N after drying and grinding as described earlier for feed and TMR samples.

2.5. Calculations

Final oven DM of TMR, DM intake/cow/pen, milk energy content (MJ/kg) and output (MJ/day), partial net energy (NE) output (MJ/day), NE for lactation (NE_L) density (MJ/kg DM) of the rations, BCS change, urine volume (L/day) as well as microbial crude protein (CP) production (g CP/day) was calculated as described by Swanepoel et al. (2015).

Whole tract apparent digestibility (g/kg DM) was calculated for organic matter (OM) as:

$$1 - [((Lignin(sa)_{TMR} \times 0.95)/OM_{TMR}) / (Lignin(sa)_{Feces} / OM_{Feces})]$$

and apparent nutrient (*i.e.,* CP, aNDFom and ADF) digestibility (g/kg DM) was calculated as:

$$1 - [((Lignin(sa)_{TMR} \times 0.95)/Nutrient_{TMR}) / (Lignin(sa)_{Feces} / Nutrient_{Feces})]$$

assuming that lignin(sa) in the TMR is 950 g/kg indigestible and will be recovered in feces (Stensig and Robinson, 1997).

2.6. Statistical analysis

Cows were excluded from statistical analysis if they moved from their originally assigned pen at any time during the study, for any period of time, for health or any other reason. The final number of eligible cows included in statistical analysis of milk production was 539, with 326 for BCS. Outlier analysis completed blind to treatments by excluding values deemed to be biologically implausible removed 12 cows from the milk production dataset (*i.e.*, 2 cows for a high milk fat level, 7 cows for low milk yields and 3 cows for high SCC), and 1 cow was removed from the BCS dataset due to an abnormally high BCS change between two experimental periods. A group of 171 urine samples were selected for AL analysis, representing 80 cows which had repeated urine samples among periods. A final group of 6 cows/pen were randomly selected from the group of 12 eligible blood cows/pen for AA analysis. A least square difference (LSD) analysis was performed on plasma AA data to determine the lowest number of samples at which addition of more samples no longer reduced the LSD for the dataset (i.e., did not change the residual SE). This point was used as an indication of the number of samples required to give an accurate representation of the sample group. According to Oetzel (2003), a minimum of 8 cows should be sampled for tests with mean outcomes, which applies to most blood samples. However, our results suggested only 6 samples since plasma AA concentrations have relatively low variability. Even though there are a number of different statistical models that may be applied to this dataset, animal production, BCS, urine AL, apparent digestibility and plasma AA levels were analysed using the MIXED procedure of SAS (2000) for a 3 x 3 Latin square design, with cow nested within pen as the random statement (since cows in this study were deemed to be metabolically independent experimental units) and period, pen and treatment

as fixed effects. As outlined above, the dairy farm, and the specific pens used, in this study were selected for specific attributes that support the unique requirements for this kind of study. Differences among treatments were separated using the PDIFF option with SAS (2000).

The DM intake (n = 3 pens calculated on a pen basis with 3 pens/period), TMR component and ingredient composition and total NE balance (n = 3 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 $P \leq 0.05$ and trends accepted if $P \leq 0.10$.

3. Results

3.1. Ration evaluation

The chemical composition of the ingredients used in the TMR (Table 1) was similar to ingredients listed in NRC (2001) as well as ingredients in Swanepoel et al. (2014 and 2015).

Evaluation of the actual rations fed showed that, as per the experimental design, the LCM ration had a lower (P<0.01) inclusion of CM compared to the HCM ration (126 *vs*. 167 g/kg DM; Table 2). However, even with the change in CM inclusion, there was no difference in the chemical profiles of the LCM *vs*. HCM rations (Table 2), thereby indicating that the primary objective of the study, to create desired ingredient differences between rations, were achieved without creating confounding differences which would invalidate the study objectives. This was achieved by balancing the reduced inclusion of CM with an increased inclusion of dried corn distillers grains (DDG; P<0.01), molasses (P<0.01), and cracked pima cottonseed (P=0.02), together with decreased inclusion of steam flaked corn grain (P=0.01). There was no difference in the ingredient or chemical profiles of the two HCM rations.

Table 2

Ingredient profile and chemical composition (g/kg dry matter) of total mixed rations fed to cows.

	Treatments				*P*
	LCM[1]	HCM[2]	HCMP[3]	SEM	LCM vs. HCM[4]
Ingredient profile, g/kg DM [5]					
Alfalfa, hay (High quality)	43.5	45.0	44.5	0.48	0.26
Premix					
Almond, hulls	116.0	115.9	116.0	0.03	0.26
Cottonseed, Pima cracked	43.6	43.4	43.4	0.02	0.02
Sodium bicarbonate	7.79	8.16	8.17	0.011	0.19
Humatech[6]	4.31	4.29	4.29	0.098	0.92
Mineral, premix[7]	14.4	14.4	14.4	0.07	0.79
EnerG II[8]	12.9	12.6	12.6	0.06	0.08
Canola, pellets (solvent)	126	167	167	0.3	<0.01
Wheat, straw	15.8	15.7	15.7	0.10	0.75
Molasses, liquid	11.62	6.36	6.36	0.094	<0.01
Corn distillers grains, dry	75.2	28.1	28.1	0.47	<0.01
RPM Product[9]	0.00	0.17	0.17	0.001	<0.01
RPP Product[10]	0.00	0.00	2.12	0.007	<0.01
Alfalfa, hay (Medium quality)	63.1	64.5	62.8	0.43	0.51
Corn, grain flaked	186	196	196	0.4	0.01
Wheat, silage	179	174	175	1.6	0.23
Corn, silage	73.2	78.8	78.7	2.39	0.31
Citrus, wet/pulp	26.7	25.2	25.1	0.60	0.28
Nutrient profile, g/kg DM [11]					
Dry matter	568	563	562	6.4	0.54
Organic matter	909	910	911	0.6	0.28
Crude protein (CP)	164	161	160	1.2	0.03
ADICP[12]	82.2	83.0	87.4	2.00	0.24
aNDF[13]	314	308	308	2.2	0.05
aNDFom[14]	298	293	293	1.9	0.05
ADF[15]	218	219	222	2.4	0.32
Fat	47.5	45.4	45.4	0.87	0.08
Lignin(sa)[16]	45.8	47.7	47.8	0.75	0.17
Starch	171	172	178	2.6	0.05
Sugars	38.6	42.5	42.4	2.04	0.15
Ca	9.03	9.33	9.64	0.206	0.10
P	4.13	4.10	4.10	0.047	0.60
K	15.4	15.3	15.0	0.10	0.05
Mg	3.03	3.07	3.06	0.039	0.49
Cl	5.65	5.53	5.56	0.095	0.39
S	3.01	2.80	2.81	0.029	<0.01
Na	4.89	4.98	4.97	0.083	0.43
mg/kg DM					
Zn	83.4	87.4	85.2	24.05	0.35
Mn	44.3	46.6	45.7	7.49	0.06
Fe	418	433	403	74.3	0.98
Cu	13.8	14.4	14.4	4.91	0.32
Mo	1.36	1.66	1.38	1.958	0.52
Se	0.37	0.34	0.34	0.116	0.08

[1] Low canola meal ration: Canola meal included at 126 g/kg dry matter.
[2] High canola meal ration: Canola meal included at 167 g/kg dry matter, with 2 g of intestinally absorbable Met as part of the base ration.
[3] High canola meal ration and 15 g of intestinally delivered Phe.
[4] P-values for LCM vs. HCM rations. The 2 HCM rations did not differ, except tendencies to be lower in the Phe treatment for K (*P*=0.04) and Fe (*P*=0.01).
[5] Based on average ingredient composition during sampling week for each pen, each period, using TMR tracker system.
[6] DPX 9902. Mixture of humic and fulvic organic acids. Humatch Inc. Houston, Texas, USA.
[7] Premix (988 g/kg DM) contained 15.1 g/kg NDF, 24 g/kg Starch, 27.6 g/kg Fat, 1.6 g/kg N, 205.5 g/kg Ca, 5.2 g/kg P, 36.2 g/kg Mg, 1.1 g/kg K, 1.9 g/kg S, 95.3 g/kg Na, 133.1 g/kg Cl, 0.37 g/kg Fe, 3.84 g/kg Zn, 0.68 g/kg Cu,1.06 g/kg Mn, 437 IU/lb Vit-E on a DM basis.
[8] Nutritech Solutions, Ltd. Abbotsford, BC, Canada.
[9] Ruminally protected Met (Smartamine M, Adisseo USA Inc., Alpharetta, GA, USA). Fed at 3.4 g/cow/d to deliver 2 g intestinally absorbable Met.
[10] Ruminally protected Phe (QualiTech Inc., Chaska, MN, USA). Fed at 41.7 g/cow/d to deliver 15 g intestinally absorbable Phe.
[11] Total mixed ration samples collected twice during sampling week for each pen, each period (*i.e.*, 18 total samples).
[12] Acid detergent insoluble CP (g/kg of CP).
[13] Neutral detergent fiber assayed with heat stable amylase, expressed inclusive of residual ash.
[14] Neutral detergent fiber assayed with heat stable amylase, expressed exclusive of residual ash.
[15] Acid detergent fiber, expressed inclusive of residual ash.
[16] Lignin treated with sulphuric acid.

The only difference in the ingredient profiles amongst treatment diets was the inclusion of 3.4 and 41.2 g/cow/day of RP Met and RP Phe respectively (which was similar to the targeted 3.4 and 41.7 g/cow/day). The TMR met all nutrient requirements of lactating dairy cows producing 45 to 50 liters of milk/day (NRC, 2001).

3.2. Animal measurements

3.2.1. Dry matter intake and apparent whole tract digestibility

The DM intake (Table 3) was not affected (avg: 27.5 +/- 0.46 kg/day) by treatment and were similar to those in Swanepoel et al. (2015). Apparent total tract digestibility of OM and CP was not affected by the treatments, but apparent aNDFom (P=0.01) and ADF (P<0.01) digestibility

Table 3

Dry matter (DM) intakes (kg/d) and apparent total tract digestibility (g/kg DM) of total mixed rations (TMR) and Urine analysis of cows fed TMR with low canola meal (CM), high CM and high CM supplemented with ruminally protected Phe.

	Treatments				*P*	
	LCM[1]	HCM[2]	HCMP[3]	SEM	LCM vs. HCM	HCM vs. HCMP
Dry matter intakes (kg/d)[4]	27.3	27.5	27.7	0.46	0.81	0.84
TMR Digestibility (g/kg DM)[5]						
Organic matter	662	666	672	3.6	0.38	0.10
Crude protein	617	617	605	8.0	0.97	0.32
aNDFom[6]	432	421	443	6.2	0.18	0.01
Acid detergent fiber	395	385	413	6.4	0.17	<0.01
n = 80 cows						
Urine analysis						
Allantoin (AL, mg/L)	3000	2898	2938	80.7	0.27	0.67
Specific gravity	1.026	1.025	1.026	0.0005	0.23	0.28
Urine volume (L/day)	19.3	19.8	19.3	0.44	0.32	0.31
Bacterial CP yield (g/d)	1932	1903	1882	34.2	0.48	0.61

[1] Low canola meal ration: Canola meal included at 126 g/kg dry matter.
[2] High canola meal ration: Canola meal included at 167 g/kg dry matter, with 2 g of intestinally absorbable Met as part of the base ration.
[3] High canola meal ration and 15 g of intestinally delivered Phe.
[4] n=3 Pens
[5] Based on 9 final fecal samples of 6 cows/pen combined into 3 groups/period and 18 final TMR samples, collected twice/pen/period.
[6] Neutral detergent fiber assayed with heat stable amylase, expressed exclusive of residual ash.

was higher for the HCMP *vs*. HCM treatments (443 vs. 421 and 413 vs. 385 g/kg DM respectively).

3.2.2. Milk production and its composition

There was no impact of treatments on milk or component yields (Table 4). Milk component content was not different for LCM *vs*. HCM, but lactose content was lower (*P*=0.02) with Phe addition to the HCM ration (47.2 *vs*. 47.3) compared to HCM alone.

Table 4

Production performance and body scores for cows fed total mixed rations with low canola meal (CM), high CM and high CM supplemented with ruminally protected Phe.

	Treatments				*P*	
	LCM[1]	HCM[2]	HCMP[3]	SEM	LCM vs. HCM	HCM vs. HCMP
n = 527 cows						
Yield (kg/d)						
Milk	47.21	47.52	47.72	0.367	0.32	0.52
Fat	1.58	1.57	1.59	0.014	0.80	0.38
True protein	1.34	1.34	1.34	0.009	0.55	0.90
Lactose	2.23	2.25	2.25	0.017	0.33	0.77
Components (g/kg)						
Fat	33.56	33.30	33.48	0.224	0.24	0.42
True protein	28.49	28.44	28.34	0.105	0.33	0.07
Lactose	47.29	47.31	47.20	0.073	0.68	0.02
Energy density (MJ/kg)	2.80	2.79	2.79	0.010	0.22	0.79
Somatic cell count (‘000)	120	122	128	12.1	0.85	0.62
n = 325 cows						
Body condition score (BCS)	2.61	2.58	2.59	0.019	<0.01	0.28
BCS change (unit/28 d)	0.016	-0.061	-0.002	0.0131	<0.01	<0.01

[1] Low canola meal ration: Canola meal included at 126 g/kg dry matter.
[2] High canola meal ration: Canola meal included at 167 g/kg dry matter, with 2 g of intestinally absorbable Met as part of the base ration.
[3] High canola meal ration and 15 g of intestinally delivered Phe.

3.2.3. Body condition score

The BCS (Table 4) was higher (*P*<0.01) for the LCM treatment compared to HCM and it was also the only treatment with a positive BCS change (*i.e.*, 0.016 units/28 days) which differed (*P*<0.01) from the HCM treatment. Cows in both HCM treatments lost BCS, but the negative

BCS change for HCMP was less (P<0.01) than that of HCM alone (-0.002 vs. -0.061 units/28 days).

3.2.4. Urine

Urine volume (avg: 19.5 +/- 0.44 L/day), urine AL concentrations (avg: 2945 +/- 80.7 mg/L) and calculated microbial CP (MCP) flow (avg: 1906 +/- 34.2 g/day) from the rumen (Table 3) did not differ among treatments. However, even though the AL concentrations in this study were lower (avg: 2945 vs. 3678 mg/L) than Swanepoel et al. (2015), the higher urine volume (avg: 19.5 vs. 16.7 L/day) resulted in a similar estimated MCP yield (avg: 1906 vs. 2093 g CP/day) between the studies and are within ranges (763 to 1959 g CP/day) previously reported in studies collecting and measuring MCP from duodenal samples, as outlined by Swanepoel et al. (2015).

3.2.5. Blood plasma

There were no changes in plasma AA concentrations (Table 5) for HCM *vs*. HCMP, except for the Lys:Met ratio which tended (P=0.05) to be lower in the HCMP treatment (3.12 vs. 3.31), mainly due to a lower plasma Lys concentration rather than an increase in plasma Met which was part of the base TMR in both HCM treatments. However, even with different ingredient compositions, the plasma AA concentrations between the LCM and HCM rations were very similar with the only difference being a lower (P=0.01) plasma Leu concentration (23.0 *vs*. 24.8 μg/mL) for the HCM cows compared to LCM, which corresponds with the decline in plasma Leu levels in Swanepoel et al. (2014) with higher inclusion levels of CM in the ration.

Other differences included a tendency (P=0.04) for LCM cows to have a higher plasma Ser concentration compared to HCM cows (8.95 *vs*. 9.85 μg/mL). Plasma Phe and Tyr levels did not differ between the LCM and HCM cows, but when they are considered in combination the Phe + Tyr concentration tended to be lower (P=0.05) for HCM cows compared to LCM cows (17.4 *vs*. 18.8 μg/mL), which corresponds with the decrease in plasma Phe and Tyr levels as CM inclusion

Table 5

Free amino acid, ammonia concentrations (μg/ml) and urea content (mg/dL) in plasma of cows fed total mixed rations with low canola meal (CM), high CM and high CM supplemented with ruminally protected Phe.

	Treatments				*P*	
	LCM[1]	HCM[2]	HCMP[3]	SEM	LCM vs. HCM	HCM vs. HCMP
n = 18 cows[4]						
Essential amino acids						
Threonine	12.1	12.3	12.9	0.61	0.73	0.36
Valine	33.0	34.1	33.3	0.98	0.29	0.45
Methionine	3.72	3.82	3.89	0.148	0.55	0.70
Isoleucine	15.8	16.0	15.5	0.49	0.71	0.35
Leucine	24.8	23.0	22.5	0.64	0.01	0.48
Phenylalanine	8.80	8.19	8.63	0.376	0.10	0.23
Tryptophan	13.8	14.3	14.3	0.48	0.33	0.94
Lysine	11.7	12.3	11.9	0.48	0.34	0.51
Histidine	7.60	7.71	7.63	0.250	0.72	0.80
Arginine	14.1	14.1	13.9	0.49	0.91	0.78
Lys:Met ratio	3.23	3.31	3.12	0.108	0.37	0.05
Non-essential amino acids						
Homocystine	0.46	0.48	0.52	0.031	0.47	0.08
Aspartic acid	1.13	1.07	1.01	0.073	0.52	0.52
Tyrosine	10.04	9.16	9.48	0.423	0.06	0.48
Serine	9.85	8.95	8.70	0.400	0.04	0.56
Glutamic acid	6.94	7.26	6.83	0.300	0.18	0.08
Glutamine	37.8	35.8	37.3	1.33	0.17	0.30
Glycine	28.9	27.1	26.1	1.50	0.32	0.60
Alanine	23.1	23.5	24.8	0.77	0.64	0.18
3-Methylhistidine	0.57	0.56	0.56	0.032	0.91	0.96
Asparagine	6.45	6.18	6.02	0.305	0.49	0.67
Proline	11.9	11.4	11.1	0.38	0.25	0.58
Phe + Tyr	18.84	17.35	18.11	-	0.05	0.30
Ammonia	2.04	2.00	1.93	0.060	0.27	0.09

[1] Low canola meal ration: Canola meal included at 126 g/kg dry matter.
[2] High canola meal ration: Canola meal included at 167 g/kg dry matter, with 2 g of intestinally absorbable Met as part of the base ration.
[3] High canola meal ration and 15 g of intestinally delivered Phe.
[4] A randomly selected group of 6 cows/pen/period was selected from the available group of 18 and used for amino acid analysis as it was determined using least square difference (LSD) analysis that additional samples do not change significance of differences.

in the ration increases (Swanepoel et al., 2014). All essential AA (EAA) and non-essential AA (NEAA) concentrations were intermediate to the two highest CM inclusion rations from Swanepoel et al. (2014), which was expected since this HCM ration contained CM at a level intermediate to those prior two rations. Most AA concentrations were slightly lower, but still

very similar to, concentrations in Swanepoel et al. (2015), except for Gln concentrations which were lower (avg: 37.0 vs. 48.1 μg/mL).

3.2.6. Partial net energy balance

The partial NE balance (Table 6) for each treatment was calculated to determine where energy was utilized. While total milk energy output did not differ among treatments, there was a difference in energy utilized for BCS change for all treatments with the LCM cows utilizing energy to increase BCS compared to energy being liberated in cows fed the HCM ration (0.73 *vs*. -2.73 MJ/day; $P<0.01$), while addition of Phe to the HCM ration substantially reduced the amount of energy liberated from body condition (-2.73 *vs*. -0.11 MJ/day; $P<0.01$).

Table 6

Partial net energy balance for cows fed total mixed rations with low canola meal (CM), high CM and high CM supplemented with ruminally protected Phe.

	Treatments				*P*	
	LCM[1]	HCM[2]	HCMP[3]	SEM	LCM vs. HCM	HCM vs. HCMP
Milk energy output (MJ/d)	131.7	131.9	132.5	0.98	0.82	0.57
BCS[4] energy (MJ/d)	0.73	-2.73	-0.11	0.586	<0.01	<0.01
Total Net Energy (MJ/d)[5]	176.7	173.4	176.6	0.26	0.02	0.03
NE_L[6] (MJ/kg DM)	6.48	6.31	6.37	0.095	0.46	0.79

[1] Low canola meal ration: Canola meal included at 126 g/kg dry matter.
[2] High canola meal ration: Canola meal included at 167 g/kg dry matter, with 2 g of intestinally absorbable Met as part of the base ration.
[3] High canola meal ration and 15 g of intestinally delivered Phe.
[4] Body condition score.
[5] Total NE calculated as the sum of maintenance, milk and BCS energy. Maintenance energy (MJ/d) calculated using a constant body weight of 673 kg for all treatments (*i.e.*, 44.23 MJ/d).
[6] Net energy available for lactation. $n = 3$ pens.

While there were no differences in the calculated dietary NE_L densities (avg: 6.4 $\pm$ 0.10 MJ/kg DM) between treatments, the decrease in total NE output with the HCM vs. LCM cows (173.4 *vs*. 176.7 MJ/day; $P=0.02$), was corrected back to the level of the LCM ration when Phe was added to the HCM (176.6 *vs*. 173.4 MJ/day; $P=0.03$).

4. Discussion

The suggestion that Met was oversupplied in our previous study (Swanepoel et al., 2015) was due to a decline in milk and lactose yields when 8 g/cow/day of intestinally absorbable Met was supplemented to the high CM ration. The absence of such a decline in the current study, together with a similar redirection of energy from milk lactose production towards BCS gain, suggests that the level of supplemented Met was enough to prevent a Met limitation, if it existed, but low enough to prevent negative effects due to its oversupply.

This study was designed to deliver 15 g/cow/day of Phe to the intestine, which is ~10% higher than the estimated intestinal Phe delivery levels for the HCM ration. However, plasma Phe levels did not differ between treatments and were only 5.4% higher for HCMP *vs*. HCM cows. This is equal to the previously targeted level (Swanepoel et al., 2015) where a plasma Phe increase of 5.5% was expected to yield a production response. As Phe supplementation reduced the plasma Lys:Met ratio from 3.31 in the HCM ration to 3.12 in the HCMP ration, mainly due to a reduction in Lys rather than an increase in Met, suggesting that the amount of Lys supplied or absorbed from the intestine was somehow reduced or plasma Lys was utilized when the limiting AA, in this case Phe, was fed. This repeats our previous finding that correcting the Lys:Met ratio to the theoretical optimum of 3:1 (Chalupa and Sniffen, 2006) does not improve milk production, suggesting that it is not the Lys:Met ratio that elicits the milk production response, but rather the level of Met relative to its requirement.

Plasma Phe concentrations were proportionally increased by ~50% of what was supplemented, which corresponds with previous findings that Phe is extensively catabolized by the liver and that increased absorption of Phe will result in increased removal (up to 0.49 of portally absorbed Phe) by the liver (Lapierre et al., 2005). However, this was not reflected by an increased PUN concentration in plasma of the HCMP treatment. Indeed, the tendency for PUN to

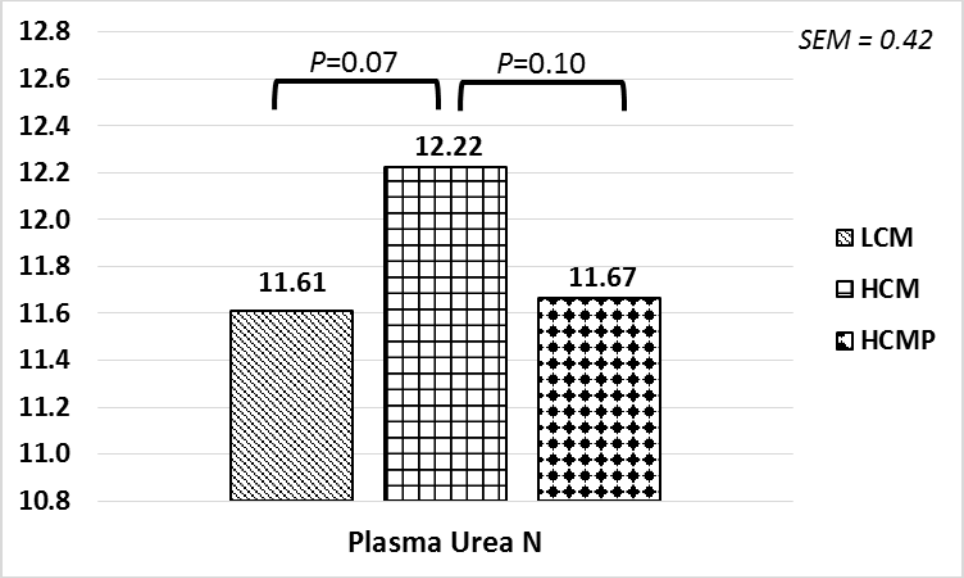

Figure 1. Changes in the plasma urea N concentrations (mg/dL) for cows fed the low CM (LCM), high CM (HCM) and high CM ration supplemented with 15 g of intestinally delivered Phe (HCMP).

be higher (Figure 1) in HCM cows compared to LCM and HCMP cows suggests that the HCM ration was limiting in Phe, leaving the other AA unutilized and catabolized, resulting in higher plasma PUN levels, while supplementation of Phe to HCM alleviated this AA limitation. This corresponds with results by Iroshan et al. (2013) in which a Phe deficiency increased PUN concentrations compared to abomasal infusion of a complete AA mixture, suggesting that excess AA not used for milk protein synthesis was converted to urea.

In order to better understand our results, we consulted results reported in Swanepoel et al. (2015) and compared them to this study. Phe supplementation did not decrease plasma Trp concentrations in this study as it did in Swanepoel et al. (2015). Indeed, compared to the lower plasma Phe and Tyr concentrations (Figure 2B) with Phe supplementation to the high CM ration in Swanepoel et al. (2015), the small increase in plasma Tyr concentrations in this study (Figure 2D) could be due to hepatic conversion of Phe into Tyr, directing Phe hydroxylase towards Phe hydroxylation, instead of Trp, since there are unknown factors that influence activity of, and therefore the substrate used by, the hydroxylase enzyme, and studies show conflicting results

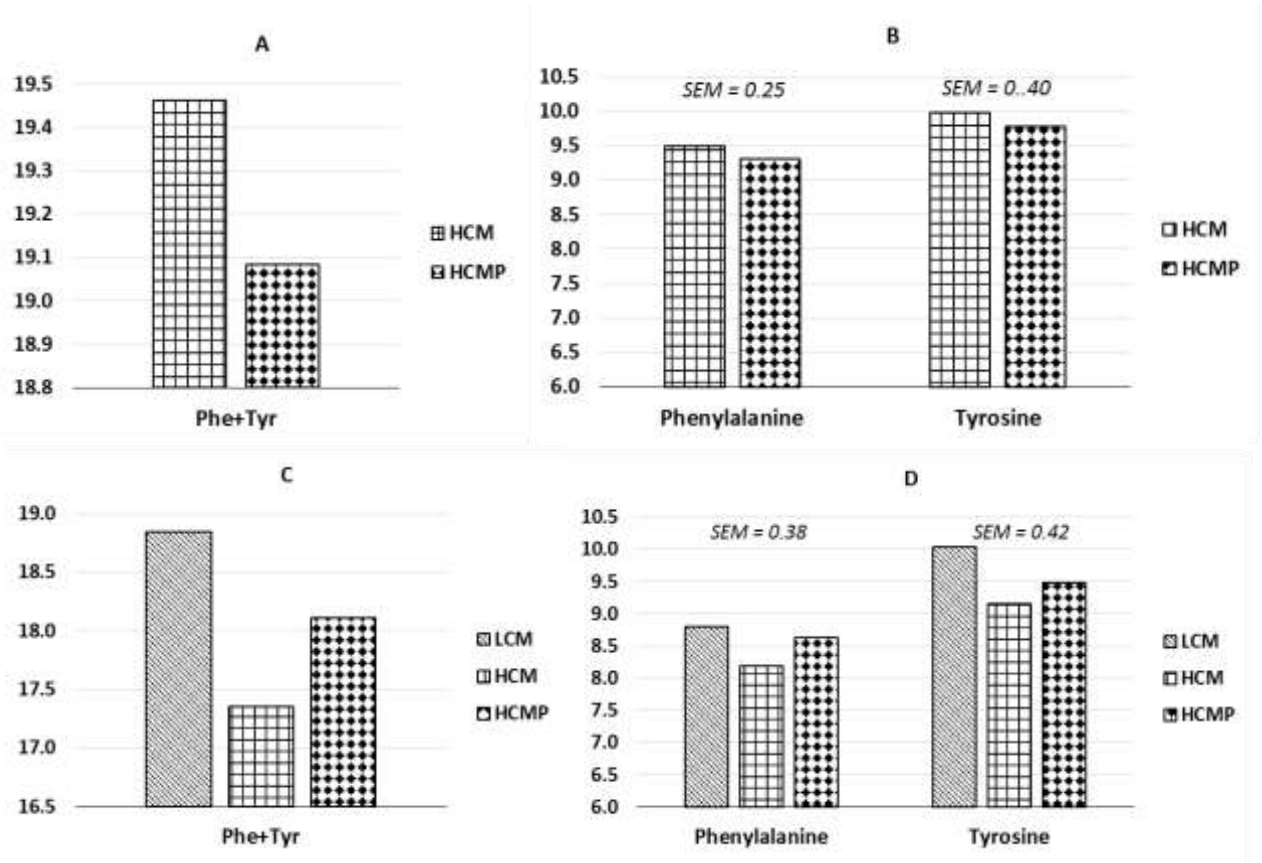

Figure 2. Changes in the plasma Phe and Tyr concentrations (µg/mL) for (A&B) cows fed the high canola meal (HCM) ration and high CM ration supplemented with 7.5 g of intestinally delivered Phe (HCMP) in Swanepoel et al. (2015) and (B&D) cows fed the low CM (LCM), high CM (HCM) and high CM ration supplemented with 15 g of intestinally delivered Phe (HCMP) in the current study.

about enhancing or inhibiting effects of Phe on Trp hydroxylation (Kaufman, 1971; Guinard and Rulquin, 1994).

Addition of Phe to the HCM ration resulted in an increase in whole tract aNDFom and ADF digestibility. Although the change could be due to random variation, since it may not be large enough to be biologically important, it is consistent with the numerically lower MCP flow from the rumen in the HCMP treatment since higher fiber digestibility can result in lower outflows of MCP from the rumen due to a deficiency of fibrous particles for microbes to adhere to (Van Soest, 1994). As it is likely that a portion of the Phe contained in the RP Phe product was released in the rumen, the enhanced fiber digestion could be due to increased concentrations of ammonia, possibly from degradation of released Phe, stimulating fibrolytic bacteria in the rumen (Van Soest, 1994). However, this seems unlikely due to the low level of potential Phe release in the rumen. Even though it has long been accepted that most ruminal bacterial species use ammonia as their sole source of N (Argyle and Baldwin, 1989), other research shows that availability of free AA directly affects, and can stimulate growth rate of, microbes in the rumen (Cotta and Russel, 1982; Argyle and Baldwin, 1989), even though the consensus is that it is a group of AA, rather than specific individual AA that has this stimulatory effect. However, Soto et al. (1994) suggested cellulolytic bacteria growth rate can be stimulated by peptides or specific AA, as long as adequate energy is available, without affecting MCP flow from the rumen or rumen nutrient digestibility. Specific supplementation of branched-chain AA (BCAA) were shown to increase *in vitro* NDF digestibility (Yang, 2002) while Zhang et al. (2013) reported that only Ile affected DM and NDF degradability of wheat straw, in this case negatively, during *in vitro* fermentation.

The requirement of specific AA, specifically Phe, for growth of ruminal organisms has been suggested and studied for decades (Bryant et al., 1959; Allison, 1965). The major role of Phe and

its precursors phenyl-acetic acid and phenylpropionic acid, in ruminal fiber degradation have been previously established (Stack et al., 1983; Morrison et al., 1990) in both continuous-culture and batch culture experiments, suggesting that the affinity of bacteria for cellulose can be improved by changing their adherence capabilities and/or altering enzyme assembly for more efficient substrate conversion with an exogenous supply of Phe and its precursors. Atasoglu et al. (2001) confirmed that Phe synthesis from peptides in cellulolytic bacteria were lower than any other AA and that Phe is essential for growth of cellulolytic bacteria, which agrees with Stack et al. (1983), indicating that cellulose digestion was limited by Phe biosynthesis. Even though these earlier studies used AA concentrations well above what would normally occur *in vivo,* it suggests that delivery of higher levels of free Phe to the rumen, through ruminal release of the RP Phe in our case, could be supplementing an AA that is required by the microbes, thereby enhancing growth and/or capabilities of cellulolytic bacteria and so fiber digestibility in the rumen would increase.

Supplementation of Phe resulted in energy being diverted towards BCS gain (Figure 3A and B), as in Swanepoel et al. (2015), but this time the change was statistically significant and not at the expense of milk protein and fat components, possibly suggesting that the higher Phe supplementation level was successful in supplying enough Phe to replace mobilized muscle protein while maintaining milk production. Since Jaurena et al. (2005) showed that BCS gain reflects accretion of fat and muscle mass, the increase in BCS may also be explained by involvement, or activity, of Phe in lipogenesis. Research investigating treatment of type 2 diabetes have shown that use of a Phe-based GPR142 agonist successfully increased serum insulin while decreasing blood glucose concentrations (Du et al., 2012). Since insulin is a peptide hormone produced by the pancreas to metabolize blood glucose to fatty acids, increased insulin concentrations, and therefore increased lipogenesis, may be partly responsible for increased BCS

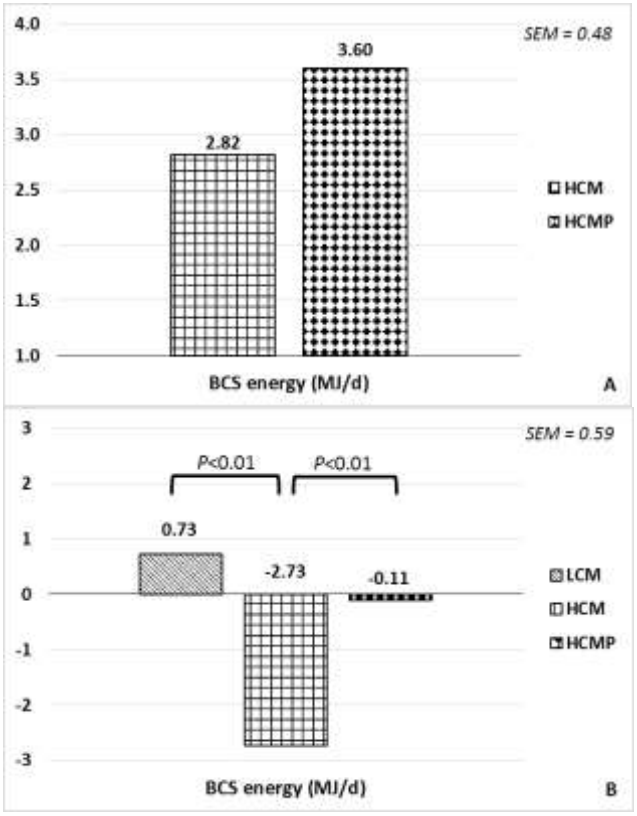

Figure 3. Changes in the body condition score (BCS) energy (MJ/d) for (A) cows fed the high canola meal (HCM) ration and high CM ration supplemented with 7.5 g of intestinally delivered Phe (HCMP) in Swanepoel et al. (2015) and (B) cows fed the low CM (LCM), high CM (HCM) and high CM ration supplemented with 15 g of intestinally delivered Phe (HCMP) in the current study.

gain. Also, since more glucose is metabolized to fatty acids, less would be available for lactose synthesis, resulting in reduced milk lactose content. It has previously been suggested that the mammary gland has a high metabolic flexibility, maintaining milk synthesis by utilizing various nutrients in different pathways, with milk component yields changing depending on how their precursors are partitioned (Lemosquet et al., 2010). However, this does not explain why the increase in BCS only occurred in the combined M+P treatment and not with Phe supplementation alone. Phenylalanine has also been identified as one of the AA, together with Met, that stimulates ghrelin secretion/release in the stomach and small intestine (Vancleef et al., 2015). Ghrelin is a peptide hormone which has been shown to stimulate intake, prevent fat utilization/mobilization and increase body weight by influencing glucose and lipid metabolism

(Romero et al., 2010), which could also contribute to the possible higher adiposity and BCS of M+P supplemented cows. Due to numerical differences in DM intake between treatments, there was no difference in the calculated dietary NE_L densities of the treatment diets. However, the change in energy utilization among treatments is reflected in differences in total NE output which decreased with the higher CM inclusion (Figure 4B).

In Swanepoel et al. (2015), total NE output was numerically reduced with Phe supplementation (Figure 4A) to the high CM ration but, in the current study, it was increased to the level of the LCM ration, which was determined to contain an optimum CM level in Swanepoel et al. (2014), when Phe was added to the HCM thereby suggesting that supplementation of Phe to HCM regained animal performance that was lost by the HCM ration alone. This suggests that a further increase in the level of Phe supplementation may have additional benefits on milk production.

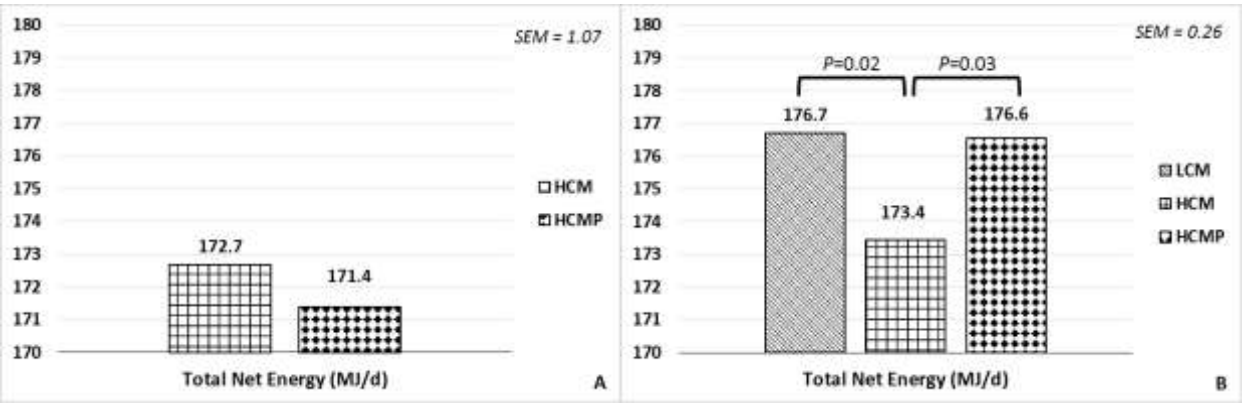

Figure 4. Changes in the Total Net energy (MJ/d) for (A) cows fed the high canola meal (HCM) ration and high CM ration supplemented with 7.5 g of intestinally delivered Phe (HCMP) in Swanepoel et al. (2015) and (B) cows fed the low CM (LCM), high CM (HCM) and high CM ration supplemented with 15 g of intestinally delivered Phe (HCMP) in the current study.

5. Conclusions

Even though plasma Phe levels did not change between treatments (possibly due to Phe being catabolized by the liver, converted to Tyr or utilized to support production responses), Phe feeding increased BCS gain, probably restoring peptides to support the muscle tissue synthesis while reducing fat mobilization through stimulation of ghrelin peptides, but not at the expense of milk production and components. Results suggest that delivery of free Phe to the rumen, through ruminal release of the RP Phe product, may have enhanced growth and/or capabilities of cellulolytic bacteria and therefore aNDFom and ADF digestibility in the rumen. Restoration of decreased total NE output with the HCM fed cows to the same level as with the LCM ration with Phe supplementation, together with the regained animal performance with only a small increase in plasma Phe, merits further investigation with even higher levels of Phe to determine if it supports an increase in milk production and/or components after fulfilling its apparent 1st priority of restoring peptides to muscle tissue.

Acknowledgements

The authors thank Mr William van Die and the personnel (*i.e.*, George, Nacho, Pedro, Junior, José) at Cloverdale Dairy for their interest, assistance and patience during the completion of the study. The senior author also thanks fellow students Kristan Reed, Noe Gomez and Casey Dykier for help during sample collection and processing. Very special thanks go to Grace Cun for going above and beyond in both assistance and support.

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