Effects of rumen protected amino acid supplementation on performance of Holstein cows fed rations containing high levels of canola meal

by

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Declaration

I, Nadia Swanepoel, declare that this thesis, which I hereby submit for the PhD Animal Science degree at the University of Pretoria, is my own work, conducted under the supervision of Prof. L.J. Erasmus and Dr. P.H. Robinson and that it has not previously been submitted by me for a degree at this or any other tertiary institution.

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Thank you God for giving me the strength to finish this on time, even with all the delays and other things getting in the way. I hope that I honour You by using my talents to further our knowledge in my field and I pray that I will continue to serve You to the best of my abilities.

Summary

Effects of rumen protected amino acid supplementation on performance of Holstein cows fed rations containing high levels of canola meal

by

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As supplies of canola meal (CM) and dried distiller's grains (DDGS) increase, so does the incentive to use these feeds as protein supplements at higher inclusion levels in dairy cattle rations. However, this could have detrimental effects on animal production due to imbalances of amino acids (AA) or dietary rumen degradable protein (RDP) vs. rumen undegradable protein (RUP) ratios. Few studies have been completed comparing performance of dairy cattle fed CM and DDGS, and little information is available on inclusion levels higher than 120 g/kg dry matter (DM) for either protein source. Overall project objectives were to (1) determine the highest level at which CM and the high protein, low fat DDGS (HPDDG) alternative can be included in dairy rations before adversely affecting production, (2) identify nutritional limitations at high inclusion levels of CM, and (3) identify resolutions for the limitations associated with feeding very high levels of CM to high producing dairy cows. Treatments in Experiment 1 were created by varying

ration inclusion levels of CM and HPDDG: (1) 0 g CM/kg and 200 g HPDDG/kg, (2) 65 g CM/kg and 135 g HPDDG/kg, (3) 135 g CM/kg and 65 g HPDDG/kg, (4) 200 g CM/kg and 0 g HPDDG/kg TMR DM. Results suggest that the optimum level of CM in the ration was in the range of 120 to 135 g/kg DM, and Met and Phe were identified as limiting AA. In Experiment 2 these AA were supplemented in a ruminally protected (RP) form, either alone or in combination, to a Control ration containing 200 g CM/kg DM. Compared to Control, supplemental Met shifted milk energy amongst milk components without affecting milk energy output. Phe alone had no effect on animal performance, but adding it in combination with Met diverted energy away from milk components towards body condition score (BCS) gain. While results suggest that neither Met nor Phe was a 'limiting' AA in this experiment, at least in a classical sense, results suggest that both were metabolically bioactive. Experiment 3 used multiparity cows fed a wide range of contemporary early lactation dairy rations in California (USA), employing sampling practices easily performed on a routine basis on commercial dairy farms, in order to (a) determine normal ranges of microbial crude protein (MCP) flowing from the rumen, and plasma AA concentrations, in early lactation multiparity Holstein cows, to (b) benchmark their high, low and mean levels using sampling methods possible under commercial conditions in order to assist in evaluation of commercial rations formulated with or without the aid of metabolic models, and to (c) create a reference database to help interpret the biological meaning of treatment concentrations of these parameters under commercial and experimental conditions. Since relationships between milk production, total mixed ration (TMR) ingredient profiles and plasma AA concentrations from Experiment 3 confirmed the hypothesis that Phe is important relative to milk production, Experiment 4 was designed to determine if supplementing higher levels of RP Phe would enhance 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. Indeed this was confirmed since increased Phe supplementation regained the animal energy output lost when CM inclusion was increased above the optimal level.

List of 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
AP	absorbable protein
BCAA	branched-chain AA
BCS	body condition score
BUN	blood urea N
BW	body weight
CCC	Canola Council of Canada
CM	canola meal
CP	crude protein
CR	creatinine
CSM	cottonseed meal
DC305	DairyComp 305 management system
DDG	dried distillers grains
DDGS	dried distillers grains with solubles
DHIA	Dairy Herd Improvement Association
DIM	days in milk
DM	dry matter
$dNDFom_{30}$	30 h *in vitro* NDFom digestibility
EAA	essential AA
FA	fatty acid
GSL	glucosinolates
HCM	high CM treatment with RP Met
HCMP	HCM with RP Phe
HPDDG	high protein DDG
LCM	low CM treatment
LSD	least square difference
MCP	microbial CP
MP	metabolizable protein
MUN	milk urea nitrogen
N	nitrogen
NDF	neutral detergent fiber
NE	net energy
NEAA	non-essential AA
NE_L	net energy for lactation
NH_3	ammonia
NSC	non-structural carbohydrates
OM	organic matter
PD	purine derivatives
PDC index	PD to creatinine index
PDV	portally-drained viscera
PUFA	polyunsaturated FA
PUN	plasma urea N
RDP	rumen degradable CP
RFDDGS	reduced fat DDGS
RP	rumen protected
RPM	RP methionine
RPP	RP phenylalanine
RSM	rapeseed meal
RUP	rumen undegradable CP
SBM	soybean meal
SCC	somatic cell count
SG	specific gravity
TMR	total mixed ration
TP	true protein

List of AA:

Ala	Alanine
Arg	Arginine
Asp	Aspartic acid
Glu	Glutamic acid
Gly	Glycine
His	Histidine
Ile	Isoleucine
Leu	Leucine
Met	Methionine
Phe	Phenylalanine
Pro	Proline
Ser	Serine
Thr	Threonine
Trp	Tryptophan
Tyr	Tyrosine
Val	Valine

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Preface

This thesis consist of a number of chapters which includes two papers already published in peer reviewed journals and two submitted for publication and currently under review. Chapters four to seven were therefore originally written in the format required by the Elsevier publishing company, but was adapted to fit the format of this thesis:

1. **Swanepoel, N., Robinson, P.H., Erasmus, L.J.** 2014. Determining the optimal ratio of canola meal and high protein dried distillers grain protein in diets of high producing Holstein dairy cows. Anim. Feed Sci. Technol., 189, 41-53.
2. **Swanepoel, N., Robinson, P.H., Erasmus, L.J.** 2015. Effects of ruminally protected methionine and/or phenylalanine on performance of high producing Holstein cows fed rations with very high levels of canola meal. Anim. Feed Sci. Technol., 205, 10-22.
3. **Swanepoel, N., Robinson, P.H., Erasmus, L.J.** 2015. Rumen microbial protein flow, and plasma amino acid concentration, spectrum in early lactation multiparity Holstein cows fed commercial rations. (Under review. Submitted to Livestock Science in June 2015).
4. **Swanepoel, N., Robinson, P.H., Erasmus, L.J.** 2015. Impacts of ruminally protected phenylalanine supplemented to rations containing high levels of canola meal on performance of high producing Holstein cows. (Under review. Submitted to Anim. Feed Sci. Technol. in August 2015).

Chapters four and five are already published but minor changes have been made to the versions included in this thesis as per examiner suggestions during the thesis review process.

As supplies of canola meal (CM) and corn dried distiller's grains (DDGS) increase, so does the incentive to use these feeds as protein supplements at higher inclusion levels in dairy cattle rations, which is addressed as a general discussion in Chapter one. Few studies have been completed comparing dairy cattle performance between CM and DDGS directly, and little information is available on inclusion levels higher than 120 g/kg DM for either protein source. The history of CM and DDGS and recent developments in amino acid (AA) nutrition are therefore reviewed in Chapter

two. The main project objectives of determining the highest feeding level of CM and DDGS, identifying the nutritional limitations of high inclusion levels of CM, and possible resolutions for these limitations, are outline in Chapter three. Chapter four starts with a study to determine the impacts of different inclusion levels of CM and DDGS protein, in the form of high protein DDG, on lactating dairy cows. Since Met and Phe were identified as potentially limiting AA at high inclusion levels of CM, effects of supplementing Met and/or Phe in ruminally protected (RP) form, to rations with high inclusion levels of CM, were studied in Chapter five. In Chapter six a survey was completed to determine total mixed ration (TMR) ingredient profiles across California, and plasma AA concentrations in early lactation multiparity Holstein cows, to create a reference database to help interpret the biological meaning of these parameters under commercial and experimental conditions. Relationships between milk production, TMR ingredient profiles and plasma Phe concentrations confirmed the hypothesis that Phe is important relative to milk production. In Chapter seven, the effects of supplementing higher levels of RP Phe on performance of early lactation dairy cows were therefore studied. Finally, general conclusions and implications, recommendations for future research and critical evaluation of the experimental work are presented in Chapter eight.

Chapter 1: General Introduction

Protein nutrition is critical to high production efficiency of lactating dairy cows, with the ultimate goal and challenge being optimization of rumen efficiency and milk production with a minimum level of dietary crude protein (CP) in order to reduce costs while minimizing negative environmental impacts. Dietary CP is utilized with a relatively low efficiency of 250-350 g/kg ingested CP expressed as milk and body protein creation. The remainder is excreted in urine and feces, with proportions dependent on the level of dietary CP (Broderick, 2003). Ammonia (NH_3) is considered a major air and water pollutant that can negatively impact water quality in its immediate vicinity and at a considerable distance from the emission source, thereby impairing atmospheric quality to pose considerable risks to human health (Hristov et al., 2011). Since NH_3 starts forming and volatilizing almost instantaneously after urine and feces are excreted, due to combination of urine urea with urease in feces on barn floors and soil (Burgos et al., 2010), farm animals were identified as the biggest contributors of gaseous NH_3 emissions in the United States by the NRC (2003). Even though NH_3 is not a greenhouse gas, it may indirectly contribute to nitrous oxide emissions, which contribute to increased greenhouse gas effects and atmospheric ozone layer depletion. Therefore, in order to improve efficiency of CP use by ruminants, it has been suggested that rations be balanced for specific amino acid (AA) requirements of the cows with assumed resultant improved postruminal AA delivery, which would allow use of lower CP rations.

The major protein sources used in rations of dairy cattle in Western areas of North America include high quality alfalfa hay, whole cottonseed and cottonseed meal (CSM), dried corn distillers grains with solubles (DDGS) and canola meal (CM), with alfalfa being substantial if it serves as the main forage source in rations. However, due to the high price of alfalfa hay, and the presence of secondary compounds (*i.e.*, tannins, gossypol) in cottonseed, their inclusion levels in dairy rations are often limited. Therefore, use of CM and DDGS as major supplemental protein sources are very important in many Western US dairy rations.

The USA is the main market for CM exports from Canada, receiving over 50% of total CM exports with over 90% of this imported CM being utilized by the California dairy industry (USDA, 2011;

Nernberg, 2012). In 2011 the Canola Council of Canada (CCC) developed an initiative (Growing Great 2015) which aimed to double Canada's production of CM by 2015 through increased seed production and crushing capacity. By 2013 canola seed production and processing had exceeded 2015 projections, and by 2015 the production aims were far surpassed. Together with an increasing demand for monounsaturated vegetable oils for human consumption (Canola Council of Canada Annual Reports, 2011-2014) it had a cascading effect, resulting in increased amounts of CM produced and available for use in animal feeds. Since CM trades at a discount relative to the price-setting protein soybean meal (SBM), dairy producers continue to increase CM inclusion levels in North American dairy rations.

Due to steadily increasing crude oil prices, the corn ethanol production industry in the Midwestern USA has been expanding rapidly since 2000, and increased production of DDGS, the major by-product of the corn-starch ethanol industry, is projected to continue in coming years, at least as long as government subsidies persist (Wisner, 2010; Liu and Rosentrater, 2011).

As supplies of CM and DDGS increase, so will pressure and incentive to use these products as major protein supplements, at higher inclusion levels, in dairy cattle rations. However, with as much as 400 g/kg of the CP in contemporary California total mixed rations (TMR) already coming from corn products, inclusion of even more corn DDGS protein could have a detrimental effect on animal production. This is because excessive levels of corn protein, which are known to be limiting for milk protein synthesis in AA, particularly lysine (Schwab et al., 1976; Nichols et al., 1998; Schingoethe, 2008), can cause AA imbalances at the intestinal absorptive site, while adding excessive amounts of corn oil to already corn oil rich diets can cause a depression in milk fat due to the fatty acid profile of corn oil (Bauman and Griinari, 2003). Many studies have documented milk production responses, when comparing CM to other protein supplements, most showing improved milk and protein yields with CM inclusions, although most of these are grass silage, not corn silage, based rations (Huhtanen et al., 2011).

As CM and DDGS have very different CP degradability profiles, CM being primarily a rumen degradable CP (RDP) source (RDP = 600 – 720 g/kg CP) while DDGS is a high rumen undegradable

CP (RUP) source (RUP = 550 – 650 g/kg CP; data summarized by Mulrooney et al., 2009), high inclusion levels of either could lead to an imbalance in the dietary RDP:RUP ratio, thereby negatively affecting rumen function, and/or creating an imbalance in AA available to support milk production.

Few studies have been completed comparing dairy cattle performance when fed CM and DDGS combinations, and even less information is available on inclusion levels higher than 120 g/kg for either protein source alone or in the presence of the other. The main focus of this project was therefore to determine what the highest level is at which CM and DDGS can be included in dairy rations before adversely affecting production, including the two protein sources at levels higher than 120 g/kg DM. The use of a high protein, low fat DDGS (HPDDG) alternative avoids possible detrimental effects associated with high corn oil inclusions in these dairy rations. If cow production is adversely affected, the next step would be to identify what is limiting performance at high inclusion levels of CM, which could related to protein degradability or even limited AA availability. Once the nutritional limitation has been identified, finding possible resolutions for these limitations, such as feeding a higher/lower bypass protein source or supplementing AA that may be limiting cow production and performance, will be investigated.

Chapter 2: Literature Review: Canola meal, corn dried distiller's grains and recent developments in amino acid nutrition of dairy cows

2.1. What is Canola meal?

Rapeseed (*Brassica napus*) is a member of the *Brassicaceae* family (mustard or cabbage) and is generally grown as an animal feed, for vegetable oil for human consumption, and for biodiesel. It produces a bright-yellow flower and contains about 40% oil, which can be used as salad and cooking oil, or in the manufacture of margarine. However, rapeseed oil is high in erucic acid (~50%) and the rapeseed meal (RSM) produced as a byproduct of oil extraction contains high levels of glucosinolates (GSL). Early RSM varieties had GSL levels between 125 – 207 µmol/g dry oil-free meal (Tripathi and Mishra, 2007), depending on variety and origin, thereby limiting its use in animal feeding. Glucosinolates are known to reduce palatability, and therefore animal intake, as well as having detrimental effects on animal growth and production through endocrine disturbances, inducing iodine deficiency with deleterious impacts on the liver, kidney and thyroid function which can lead to death (Tripathi and Mishra, 2007). Varieties have since been bred for reduced GSL concentration and more palatable oil, with "summer rape" (*Brassica napus* L.) being the first cultivar to contain both characteristics (Stefansson and Kondra, 1975).

The name Canola, derived from "Canadian oil" was named as such to distinguish it from traditional rapeseed since Canola contains low levels of erucic acid (<2%) in the oil and low GSL (<30 µmol/g) in the meal, therefore also known as "double-zero rapeseed" (Newkirk, 2009). As levels of these compounds continue to drop due to selection pressure by plant breeders, they are no longer considered to be a problem (Newkirk et al., 2003). Most CM is produced through a process called pre-press solvent extraction during which canola seeds are cleaned, pre-conditioned and flaked, cooked, mechanically pressed to remove some of the oil, followed by solvent extraction to remove most residual oil. The press-cake is then desolventized and toasted at an optimum temperature

(Newkirk, 2009), and can be distributed for animal feeding as a meal, or pelleted to create a more consistent product.

Table 2.1: Chemical composition (g/kg DM) of 16 samples of Canola meal

	Dry matter	Crude Protein	RUP[1]	NDF[2]	ADF[3]	Fat	Ash	
Canola meal	933	440	-	199	129	56	72	a
	924	437	-	220	164	38	91	b
	928	441	395	-	185	11	75	c
	929	410	410	-	121	-	-	d
	976	367	499	-	155	-	-	d
	977	392	498	-	131	-	-	d
	944	422	557	-	143	-	-	d
	945	399	461	-	137	-	-	d
	919	427	440	237	158	-	94	e
	949	440	-	275	208	24	62	f
	879	403	290	-	175	-	75	g
	910	458	274	-	197	-	74	g
	915	383	-	215	175	36	-	h
	928	395	366	272	205	-	-	i
	903	397	-	379	234	43	82	j
	903	378	357	298	205	54	74	k
	-	401	525	319	225	36	80	l
	907	407	243-321[#]	288	185	42	82	m
	-	-	428	-	-	-	-	n
	-	-	395	-	-	-	-	o
	896	406	-	299	182	30	90	p

[1] Rumen undegradable protein (g/kg CP). [2] Neutral detergent fiber. [3] Acid detergent fiber.
Sources: [a] Mulrooney et al., (2009), [b] Christen et al., (2010), [c] Piepenbrink and Schingoethe (1998), [d] Kendall et al., (1991), [e] Brito and Broderick (2007), [f] Maesoomi et al., (2006), [g] Zinn (1993), [h] Bell (1993), [i] Boila and Ingalls (1994), [j] Acharya et al. (2015), [k] NRC (2001), [l] Maxin et al., (2013), [m] Paz et al., (2014), [#]RUP range depending on analytical method used, [n] Jayasinghe et al., (2014), *n*=7, [o] Huang et al., (2015), *n*=5, [p] Broderick et al., (2015).

Table 2.2: Essential amino acid concentration (g/kg DM) of 13 samples of Canola meal

	Arg	His	Ile	Leu	Lys	Met	Phe	Thr	Trp	Val	
Canola meal	28.1	11.9	18.4	33.1	24.3	8.3	18.4	19.6	5.7	24.1	a
	-	14.0	16.5	25.7	20.7	6.9	16.1	16.1	-	19.7	b
	-	12.3	14.1	21.7	17.7	5.3	13.5	14.1	-	16.3	b
	-	13.5	15.7	23.6	18.8	7.4	15.6	16.1	-	17.8	b
	-	14.8	16.4	24.7	18.6	7.6	16.0	16.2	-	19.3	b
	-	13.5	16.4	24.7	19.7	7.8	15.6	16.4	-	18.8	b
	25.0	11.7	16.5	29.5	20.3	6.9	16.9	18.1	-	21.2	c
	28.8	12.7	18.6	32.9	23.2	7.4	18.7	19.1	-	23.8	c
	21.7	10.2	11.3	25.7	20.9	10.6	16.2	16.0	-	14.9	d
	25.4	11.5	16.7	30.4	23.8	8.9	17.4	19.6	4.7	23.3	e
	23.4	12.4	17.3	28.0	23.5	7.7	15.3	17.4	-	21.8	f
	25.9	12.1	16.9	27.6	21.6	8.1	15.0	17.1	-	21.4	f
	26.5	10.6	14.5	25.6	21.2	7.1	15.4	16.7	5.5	17.9	g
	-	10.2	14.9	27.2	19.6	9.3	15.8	17.6	-	17.4	h
	24.1	10.8	15.9	28.8	21.8	7.9	16.4	16.8	5.7	20.9	i

Sources: [a] Piepenbrink and Schingoethe (1998), [b] Kendall et al. (1991), [c] Zinn (1993), [d] Boila and Ingalls (1994), [e] Acharya et al. (2015), [f] Newkirk et al. (2003), [g] NRC (2001), [h] Maxin et al., (2013), [i] Paz et al., (2014).

The basic nutrient composition of CM on a dry matter (DM) basis is in Table 2.1, with essential AA (EAA) contents in Table 2.2. The average CP concentration of CM is 411 g/kg DM with 37 g/kg DM fat and a rumen undegradable protein (RUP) fraction of 404 g/kg CP. Since all GSL share a common base structure, but has a variable side chain that is derived from Met, Trp or Phe (Tripathi and Mishra, 2007), the selection process to reduce total GSL in the CM plant resulted in a relatively low Phe concentration.

2.2. What is corn dried distiller's grain?

The two primary processes used to make ethanol in the United States are wet and dry milling (Murthy et al., 2006). It is the dry milling process that produces the most common form of distiller's grains (DDG) where the solubles are mixed with the residual non-fermentable coarse corn kernel components and sold as either wet or dried corn distiller's grains with solubles (DDGS). Native (unprocessed) corn grain has a starch concentration of ~670 g/kg DM and, since starch is converted to glucose and eventually ethanol through fermentation, all other nutrients increase by about 3x in DDGS compared to corn grain (Table 2.3).

The vast quantities and variability in quality of DDG led to development of new generation ethanol production facilities aiming at improving quality of DDG products to create animal feed DDG with specific purposes through technological advances and procedure adjustments (Robinson et al., 2008). There are currently a number of different DDG products available on the market, differing in nutrient levels, depending on the manufacturing process (Table 2.2).

Research showing the detrimental effects of high levels of DDGS on milk fat production (Bauman and Griinari, 2003; Kleinschmit et al., 2007b) led to development of modified DDGS products with different nutrient profiles and fermentation characteristics *vs.* conventional DDGS to replace conventional protein sources without sacrificing animal performance (Liu and Rosentrater, 2011). These include reduced fat DDGS (RFDDGS), produced by extracting the oil from the final DDGS product through solvent extraction, and high protein DDG (HPDDG), which is produced through a pre-fermentation fractionation process by which the germ and bran is removed from the corn meal

Table 2.3: Chemical composition (g/kg DM) of several samples of corn, dried corn distiller's grains (DDGS), reduced fat DDGS (RFDDGS) and high protein DDG (HPDDG)

	Dry Matter	Crude Protein	RUP[1]	NDF[2]	ADF[3]	Fat	Ash	
Corn, steam flaked	881	94	745	95	34	42	15	v
Corn, rolled	718	92	430	103	36	43	15	v
Dried distiller's grains, corn	905	297	-	388	159	107	53	a
	889	302	-	421	162	109	58	b
	-	314	-	-	168	120	46	c
	-	274	-	-	-	117	44	d
	912	278	-	336	121	113	42	e
	-	308	550	390	161	112	57	f
	908	278	-	297	-	121	-	g
	853	205	-	183	48	107	62	h
	888	321	645	329	160	-	50	i
	877	313	-	312	92	108	45	k
	900	321	-	369	119	101	46	m
	850	309	-	303	120	125	63	n
	869	259	563	339	252	118	63	p
	867	269	332*	302	131	133	76	p
	899	298	-	261	52	96	51	q
	935	375	658	458	181	-	-	r
	935	410	593	414	152	-	-	r
	963	436	635	361	156	-	-	r
	917	282	365*	320	79	114	55	u
	902	297	508	388	197	100	52	v
	885	308	523	315	94	106	44	w
High protein DDGS, corn	939	411	-	230	111	53	19	e
	-	446	-	273	204	42	19	f
	921	400	-	326	-	47	-	g
	914	461	-	264	156	46	25	n
	914	461	561	264	156	46	-	o
	947	454	552	225	66	40	42	p
	904	445	-	287	101	34	19	q
	931	396	-	284	86	58	30	s
	929	424	450^	365	101	39	28	u
	932	415	545	304	105	32	24	w
	-	403	636	262	135	40	35	x
Reduced fat DDGS, corn	903	319	-	339	-	55	60	j
	902	321	-	358	-	53	61	j
	875	340	-	428	125	35	53	k
	860	345	-	450	129	35	52	l
	907	322	-	314	101	64	54	t
	877	340	604	425	124	35	53	w
	892	314	231-488[#]	316	105	61	60	y

[1] Rumen undegradable protein (g/kg CP). [2] Neutral detergent fiber. [3] Acid detergent fiber.
Sources: [a] Cromwell et al., (1993), *n*=9, as cited by Liu and Rosentrater (2011), [b] Spiehs et al., (2002), *n*=118, as cited by Liu and Rosentrater (2011), [c] Belyea et al., (2004), *n*=235, cited by Liu and Rosentrater (2011), [d] Liu (2008), *n*=6, as cited by Liu and Rosentrater (2011), [e] Robinson et al., (2008), *n*=10, [f] Schingoethe (2009), [g] Havlin et al., (2015), [h] Liu et al., (2000), [i] Kleinschmit et al., (2007), *n*=5, [j] Castillo-Lopez et al,. (2014), [k] Mjoun et al., (2010a), [l] Mjoun et al., (2010b), [m] Mulrooney et al., (2009), [n] Kelzer et al., (2009), [o] Hubbart et al., (2009), [p] Kelzer et al., (2010) *Dried distillers product with no heat or cooking before fermentation, [q] Christen et al., (2010), [r] Boila and Ingalls (1994), [s] Acharya et al. (2015), [t] Paz and Kononoff (2014), [u] Williams et al., (2010) ^Calculated for kp = 0.04/h, [v] NRC (2001), [w] Mjoun et al., (2010c), [x] Maxin et al., (2013), [y] Paz et al., (2014), [#]RUP range depending on analytical method used.

before fermentation, and no solubles are added back. While conventional DDGS has an average CP and fat concentration of 309 and 113 g/kg DM respectively (Table 2.3), RFDDGS has a higher CP (329 g/kg DM) and a lower fat (48 g/kg DM) concentration, while HPDDG has an even higher CP concentration (429 g/kg DM) with the same low fat concentration (43 g/kg DM).

Table 2.4: Essential amino acid concentration (g/kg DM) of several samples of corn, dried corn distiller's grains (DDGS), reduced fat DDGS (RFDDGS) and high protein DDG (HPDDG)

	Arg	His	Ile	Leu	Lys	Met	Phe	Thr	Trp	Val	
Corn, steam flaked	4.45	2.94	3.14	10.2	2.87	1.92	4.34	3.44	0.68	4.47	[l]
Corn, rolled	3.54	2.34	3.11	10.7	2.43	1.94	4.20	3.39	0.90	4.51	[l]
Dried distiller's grains, corn	11.8	8.0	11.3	36.9	7.8	5.7	16.1	11.3	2.2	14.9	[a]
	12.0	7.6	11.2	35.5	8.5	5.5	14.7	11.3	2.5	15.0	[b]
	12.9	9.1	10.3	35.0	10.4	7.2	15.0	11.7	-	15.6	[c]
	14.0	8.0	11.0	33.0	10.0	6.0	14.0	11.0	2.0	15.0	[d]
	12.1	6.8	9.3	35.3	7.1	5.8	14.2	10.7	-	13.1	[e]
	14.8	9.4	12.7	36.8	10.9	6.4	14.2	11.8	2.5	16.6	[f]
	14.0	7.8	10.7	28.2	6.9	10.8	16.9	11.1	-	10.0	[i]
	15.7	8.8	11.9	31.1	8.5	7.4	19.1	12.1	-	15.4	[i]
	16.0	8.3	10.5	25.7	8.9	7.6	18.5	11.8	-	14.1	[i]
	7.6	5.0	6.2	23.3	5.6	4.1	9.1	7.8	1.0	8.3	[h]
	5.7	3.5	4.4	18.2	3.7	2.9	7.2	5.6	1.0	6.3	[h]
	12.1	7.4	11.0	28.5	6.7	5.4	14.5	10.2	2.6	14.0	[l]
	14.6	9.2	12.4	36.2	10.7	6.3	13.9	11.6	-	16.3	[m]
High protein DDGS, corn	9.6	6.7	9.9	41.2	6.9	6.6	15.3	10.7	1.9	12.3	[h]
	15.1	10.4	15.4	52.4	12.1	8.0	21.3	15.0	2.3	20.4	[j]
	15.4	11.5	17.4	56.2	12.2	8.5	21.1	15.2	-	21.3	[m]
	-	10.2	15.2	51.7	11.0	9.1	20.8	15.4	-	17.2	[n]
Reduced fat DDGS, corn	16.0	10.4	14.7	42.6	11.0	6.8	16.1	12.9	2.6	18.2	[f]
	13.1	8.4	11.3	38.8	8.8	5.7	15.7	11.9	3.0	15.7	[g]
	13.8	8.1	12.1	35.1	9.6	5.6	14.8	10.7	2.6	16.3	[k]
	16.0	10.4	14.7	42.6	11.0	6.8	10.1	12.9	-	18.2	[m]
	14.6	9.0	12.2	36.1	10.9	6.3	15.5	11.9	2.7	16.7	[o]

Sources: [a] Cromwell et al., (1993), *n*=9, as cited by Liu and Rosentrater (2011), [b] Spiehs et al., (2002), *n*=118, as cited by Liu and Rosentrater (2011), [c] Han and Liu (2010), *n*=3, as cited by Liu and Rosentrater (2011), [d] Kim et al., (2008), *n*=1, as cited by Liu and Rosentrater (2011), [e] Kleinschmit et al., (2007), *n*=5, [f] Mjoun et al., (2010a), [g] Mjoun et al., (2010b), [h] Kelzer et al., (2010), [i] Boila and Ingalls (1994), [j] Acharya et al. (2015), [k] Paz and Kononoff (2014), [l] NRC (2001), [m] Mjoun et al., (2010c), [n] Maxin et al., (2013), [o] Paz et al., (2014).

Corn DDGS contain high levels of RUP which is generally considered to be a high quality protein source relative to milk protein requirements (Grings et al., 1992), and is not very different from CP in HPDDG. However, due to the relatively low Lys concentration in corn-based products (Table 2.3), milk production may be limited by low intestinally absorbable Lys concentrations with high DDGS

inclusion levels if not supplemented with additional Lys through various dietary means (Nichols 1998, Schingoethe 2009).

2.3. Comparing protein sources

2.3.1. *Animal performance*

2.3.1.1. Corn dried distiller's grains

Data is available on impacts of increasing levels of supplemental DDGS in lactation TMR. A review by Schingoethe et al. (2009) suggests that a nutritionally balanced diet can be formulated with up to 200 g/kg of ration DM as DDGS since studies have reported feeding DDGS up to 300 g/kg TMR DM to lactating dairy cows, often showing positive lactation responses with higher levels of DDGS due to the higher fat concentration leading to more net energy for lactation (NE_L) in the DDGS diets. Other studies also show equal or better performance when DDGS is compared to other protein sources at levels up to 200 g/kg of ration DM (Kelzer et al., 2009; Mjoun et al., 2010a).

Even though there is a consensus amongst commercial dairymen that higher DDGS levels in dairy rations can cause milk fat depression, and some publications have suggested this to be a challenge due to ruminal production of deleterious intermediates from the biohydrogenation of polyunsaturated fatty acids (PUFA) in corn oil (Bauman and Griinari, 2003), Schingoethe et al. (2009) suggested that these claims are not supported by research results and that it is not the feeding levels of DDGS *per se* that causes milk fat depression, but rather poor ration formulation. Also, a meta-analysis of lactational performance responses to DDGS (Hollmann et al., 2011) could neither support nor refute this claim since milk fat concentration response was not related to ration DDGS level, but was directly related to the milk fat concentration of the Control rations. Therefore, no matter the DDGS inclusion level of the ration, if milk fat concentration was higher than 36 g/kg DM, additional DDGS resulted in a drop in milk fat proportions. Hollmann et al. (2011) also suggested that nutritional characteristics of DDGS (*i.e.*, lack of effective fiber, high digestibility of NDF and the high levels of unsaturated C18:2 and C18:3 fatty acids in the corn oil) accentuates dietary factors that may cause milk fat depression, thereby limiting the level of inclusion of conventional DDGS in dairy rations to less than 100 g/kg of

ration DM. This agrees with Schingoethe et al. (2009) in that the temptation to decrease the level of forage in the ration, due to relatively high NDF levels in DDGS, can lead to milk fat depression due to insufficient amounts of physically effective NDF. A short communication by Zanton et al. (2013) reported a linear decrease in milk and milk fat yield with increasing ration inclusion levels of DDGS and attributed this, as did Kleinschmit et al. (2007b), to an interaction between DDGS and corn silage in the corn-silage based rations fed in those studies. Hollmann et al. (2011) also showed a negative influence of the level of corn silage in the ration on the milk fat yield response to DDGS inclusion levels when corn silage levels were above 470 g/kg TMR DM.

A study by Havlin et al. (2015) suggested that the reason for conflicting study results is because most research on DDGS only considers the gross inclusion level of DDGS in the ration and not the effects of its inclusion on the fatty acid (FA) profile of milk and therefore investigated whether the form of supplemental fat could impact animal performance. Havlin concluded that inclusion of DDGS as a fat and energy source in dairy rations should be limited since it does not have a positive impact on animal performance and, even though more fat in the diet generally stimulates production, corn oil is not the best fat source because high concentrations of PUFA in DDGS may have had detrimental effects on rumen microbes while shifting energy away from milk fat synthesis towards increasing adipose stores.

Use of a low corn oil alternative to conventional DDGS was therefore necessary to compare it to CM experimentally, thereby removing the negative interferences from its fat, especially when rations are formulated with high levels of other corn protein sources. Hubbard et al (2009) reported that dairy rations containing HPDDG had a higher predicted dietary NE_L concentration than rations containing SBM due to a higher amount of fat, improved fiber and protein digestibility, which possibly led to their increased milk production when HPDDG was fed. Kelzer et al. (2009) reported similar lactation performance with HPDDG compared to other protein sources at inclusions up to 150 g/kg TMR DM.

By increasing the levels of RFDDGS to 300 g/kg of TMR DM, DM intake, body weight (BW), body condition score (BCS) and milk protein concentration increased linearly, while milk yield and milk fat concentration was unchanged (Castillo-Lopez et al., 2014). Mjoun et al. (2010a) reported that

RFDDGS performs better than SBM in terms of milk protein concentration and yield, but just as well as DDGS, and that levels of RFDDGS of up to 300 g/kg TMR DM can support equivalent lactation performance before production declines (Mjoun et al., 2010b).

2.3.1.2. Canola meal

Due to the relatively high RDP concentration of CM, with an AA profile that may stimulate rumen microbial (MCP) growth, as well as having an AA profile in the RUP protein fraction that is considered to be advantageous for milk protein synthesis, CM has been credited with having one of the highest biological values of all protein supplements (Piepenbrink and Shingoethe 1998).

A review and meta-analysis comparing the feeding value of CM to SBM in grass silage-based lactation rations showed that CM inclusion in the ration resulted in similar or increased milk productions and higher milk protein yields, suggesting that CM can successfully be substituted for SBM without losing animal performance (Huhtanen et al., 2011). These responses were attributed to increased feed intake, possibly due to better AA and energy balance, improved AA supply and CP degradability and improved efficiency of MCP synthesis from CM.

The effects of substituting various protein sources (*e.g.*, SBM and CSM) in lactating dairy rations with CM was summarized in a meta-analysis by Martineau et al. (2013), also concluding that substitution of other protein sources with CM resulted in positive milk and milk protein responses and confirmed that these responses were associated with increased absorption of EAA. More studies completed later report similar performances of CM compared to other protein sources (Chibisa et al., 2012) and that CM improves milk and protein yield as well as N-utilization in dairy cows regardless of the protein content of the rations (Broderick et al., 2015).

2.3.1.3. Comparing CM and DDGS

Even though limited data is available on the optimal ratio of supplemental CM and DDGS protein in lactation rations, a number of studies have reported advantages of combining the two protein sources on overall animal performance (Mulrooney et al., 2009), and comparing CM to DDGS at different inclusion ratios up to 66 and 104 g/kg TMR DM respectively showed a tendency of higher

absolute values for DM intake, milk and protein yields, BW and BCS at higher CM inclusions, especially the $^{2}/_{3}$ CM: $^{1}/_{3}$ DDGS treatment.

However, a study comparing all 4 (*i.e.*, HPDDG, SBM, CM, DDGS) above mentioned protein sources (Christen et al., 2010) concluded that they were equal in terms of animal performance, with no difference in milk production and DM intake at ~120 g/kg DM (210 g/kg for DDGS to make the rations iso-nitrogenous), but that HPDDG outperformed CM as indicated by an improved plasma AA balance over CM, with a more desirable AA profile for milk protein production. Acharya et al. (2015) compared CM and HPDDG at inclusion levels of ~ 90 and 160 g/kg of TMR DM in both a low CP (143 g/kg DM) and high CP (163 g/kg DM) ration and reported no difference in DM intake, BCS or BCS change, milk yield and milk components between CM and HPDDG in either of the CP diets, but that milk urea N (MUN) was lower for the CM ration fed cows at both levels of dietary CP, suggesting that dietary N was utilized more efficiently for milk protein synthesis in cows fed the CM ration. There was also a tendency for milk whey protein to increase with HPDDG feeding, supporting findings of Christen et al. (2010) suggesting that the AA profiles of the two protein sources play an important role in the overall performance of the cows.

2.3.2. *Possible nutritional limitations of canola meal and high protein corn dried distiller's grains*

2.3.2.1. Protein degradability

Even though CM and HPDDG have a very similar CP concentration, these protein sources have different CP degradability profiles which varies considerably between and within protein source depending on the method used to calculate degradability (Table 2.1 & 2.3). With CM being primarily a RDP source while DDGS and HPDDG are high RUP sources, this means that a higher inclusion level of either protein source could lead to an imbalance in the dietary RDP:RUP ratio, thereby negatively affecting rumen function. A lot of research has been dedicated to determining the optimal rumen NH_3 concentrations for MCP synthesis and, even though specific microbial NH_3 requirements are yet to be clearly defined, it has been confirmed that MCP synthesis can be hampered by both a limitation, as well as an excess, of rumen NH_3 and, by extension, RDP levels in the diet (Reynal and Broderick, 2005; Boucher et al., 2007).

2.3.2.2. Amino acid profile

A systemic review of the impacts of supplementation of metabolizable Lys and Met on dairy cow performance (Robinson, 2010) showed that increased levels of corn protein in dairy rations depressed the concentration of Lys in absorbable protein (AP) and that rations with more than 0.35 of total ration CP coming from corn products are responsive to supplemental Lys due to its limitation. This is similar to the finding of Hollmann et al (2011), which showed a negative correlation between the milk fat yield response and DDGS inclusion levels when corn silage levels were above 470 g/kg TMR DM, suggesting that inclusion of excess corn protein in diary diets can lead to detrimental effects, as discussed previously. Most studies evaluating corn products such as DDGS report similar findings; that high levels of DDGS creates an absorbable Lys limitation, but increased delivery of other AA, especially Leu but also Phe and/or Tyr and sometimes Met and Val (Nichols et al., 1998; Liu et al., 2000; Kleinschmit et al., 2007a; Mjoun et al., 2010a; Acharya et al., 2015).

When CM is included in dairy rations, there is a tendency for plasma Leu concentrations to decrease and plasma Lys to increase (Piepenbrink et al., 1998; Mulrooney et al., 2009; Christen et al., 2010; Acharya et al., 2015) compared to DDGS and SBM. Christen et al. (2010) and Acharya et al. (2015) also showed a lower Phe and Tyr concentration for CM *vs.* HPDDG, but not DDGS, in plasma. The meta-analysis by Martineau et al. (2014) confirmed that positive production responses were associated with increased absorption of all EAA due to improved postruminal supply of protein and absorbable AA from the RUP fraction in CM.

Phenylalanine frequently ranks high as a limiting AA and was identified as 3rd limiting after Lys and Met in SBM, CM and DDGS diets with Tyr as 4th limiting in SBM diets (Nichols et al., 1998; Piepenbrink et al., 1998; Christen et al., 2010; Mjoun et al., 2010a) and 5th limiting after Leu in CM diets (Mulrooney et al., 2009). Liu et al. (2000) identified the same sequence of AA limitations in DDGS as well as DDGS, SBM and fish meal blended dairy diets. Acharya et al. (2015) listed Phe and Tyr as 4th and 5th limiting after Arg in CM and HPDDG diets. Mjoun et al. (2010a) showed that substituting DDGS with RFDDGS has no effect on the plasma AA concentrations, except for a higher

Tyr concentration, which suggests that even though there is a difference in total CP levels between these ingredients, their AA profiles are similar.

2.4. Protein and amino acid nutrition of dairy cows

An extensive literature review of this area was in the MSc dissertation of Swanepoel (2009). However, much research has been completed since then and some is discussed here.

2.4.1. *Microbial protein production and estimation*

Estimating MCP synthesis in dairy cows *in vivo* requires accurate measurements of microbial and indigestibility markers entering the small intestine (intestinal cannulation) or passing out of the rumen (omasal cannulation). Thus traditional methods to determine MCP synthesis and AP delivery to the small intestine *in vivo* are invasive, complicated, expensive, time consuming, imprecise and have unknown accuracy (Clark et al., 1992). Therefore the most common method of formulating rations currently involves mathematical and empirical models which simulate ruminal fermentation and predict duodenal protein flow. However, these models inadequately predict MCP synthesis and degradability in the rumen, passage of CP and AA to the intestine (Bateman et al., 2001a,b; Hanigan et al., 2001) and utilization thereof for milk production. Although a recent data analysis reported that metabolic model predicted duodenal EAA flows are sufficiently accurate to balance for EAA in dairy rations under field conditions (Pacheco et al., 2012), it confirmed that the models are less accurate in low concentrate rations and rations not based on corn silage and alfalfa. Also, errors in predicted AA composition of MCP and digestibility of MCP in recently updated models lead to incorrect predictions of duodenal EAA availability and MCP flow (Patton et al., 2015). It was also reported by Lee et al. (2015) that efficiencies of utilization of AA for milk protein are likely overestimated by models due to overestimation of maintenance AA requirements and the continued assumption that efficiency of AA utilization for milk protein synthesis is constant (Doepel et al., 2004), leading to overfeeding of dietary N. Most models still do not allow for additive EAA effects but abide by the single limiting AA theory, which is incorrect, as was demonstrated by Arriola Apelo et al. (2014a, b). Other limitations of current models, such as CNCPS, are that they are CP based, even though analyzed CP

values do not account for all available N, as well as the general failure to acknowledge the contribution of endogenous proteins to the MP supply. However, improvements are being made to newer versions of the models (Tylutki et al., 2008; Tylutki, 2015). Since models cannot yet replace observations from cows, improvement in efficiency of protein utilization by commercial cows requires development of alternative methods to determine and monitor MCP supply, which must be quick and easy for on-farm use (Dewhurst et al., 1996).

As summarized in a review by Shingfield (2000), the correlation between urinary purine derivative (PD) outputs and protein intake in sheep was first documented in the 1930's and research supporting the suggestion that urinary PD could be used to estimate rumen MCP synthesis started in 1965. When nucleic acids are digested in the intestine, the by-product PD are excreted in the urine and milk and can relatively easily be measured. As reviewed by Kanjanapruthipong and Leng (1998) and Tas and Susenbeth (2007), feed purines are not considered to contribute to the PD concentration of the urine since dietary nucleic acids are completely degraded in the rumen. This method therefore assumes that PD metabolites excreted in the urine originate exclusively from microbial nucleic acids degraded in the intestine and therefore reflect rumen MCP synthesis.

Metabolites of PD degradation include allantoin (AL), uric acid, xanthine and hypoxanthine. However only trace amounts of xanthine and hypoxanthine are excreted in cattle, and AL makes up an almost constant molar proportion (0.82 - 0.93) of total PD (summarized by Shingfield, 2000 and Tas and Susenbeth, 2007). Research also indicated that the most important excretory route for PD is urine, accounting for 0.83 to 0.88 of all absorbed PD, with mammary secretion being directly related to that in urine (Susmel et al., 1995), accounting for 0.01 to 0.03 of urinary AL excretion. Indeed Gonzalez-Ronquillo et al. (2003) suggested that excluding PD secretion in milk completely from the calculations would only underestimate total PD excretion by < 7% in dairy cows.

Increasingly, researchers are using urine PD to predict MCP flow from the rumen, thereby avoiding the difficulties associated with traditional *in vivo* methods. However, a review of purine metabolism in ruminants, evaluating use of purine metabolite excretion to estimate MCP supply, documented various sources of error (Shingfield, 2000) associated with the method. These include

the possibility that some feed purines could escape rumen degradation, that rumen microbes vary in their purine concentration, that partitioning of PD between urine and milk can change, and that there is the possibility of endogenous PD metabolism affecting urine PD concentrations. These issues led to studies aimed to overcome these limitations, which were summarized by Firkins et al. (2006). However these errors are mostly associated with caprine and ovine species and, even though urine PD generally estimates a lower duodenal MCP flow compared to direct omasal or duodenal measurements (Tas and Susenbeth, 2007), which themselves may or may not be accurate, it closely reflects changes observed with those measurements and can effectively be used as a non-invasive method to estimate intestinal flow of MCP from the rumen.

In the absence of total urine collection, it has been shown that the PD to creatinine (CR) ratio in spot urine samples is closely correlated to intestinal flow of microbial purines and can be used as a qualitative indicator of rumen MCP supply (Chen et al., 1995; Chizzotti et al., 2008). However, CR is a byproduct of body protein turnover and urine CR concentrations may therefore vary when animals lose or gain BW (Van Niekerk et al., 1963; Susmel et al., 1995). Chen and Ørskov (2004) therefore developed the purine derivative to creatinine (PDC) index which corrects the PD:CR ratio for animal metabolic BW to allow comparisons amongst cows.

Tas and Susenbeth (2007) suggested that total urine volume can be indirectly predicted by using urinary N concentrations or intake and excretion of minerals (*i.e.*, Na, K) but that these methods need further evaluation and validation. Urine specific gravity (SG) has a close relationship with urine volume and can also be used to estimate total urine volume in spot urine samples (Burgos et al., 2005), making it possible to estimate actual MCP synthesis using AL concentrations in the urine (Chen and Gomes, 1995).

Even though a critical review of the spot urine sampling technique suggested that there was considerable diurnal variation associated with these samples (Shingfield and Offer, 1998), among day variation was small, which suggests errors can be overcome by a sampling protocol ensuring comparisons of samples collected at a specific time during the day. Chen and Ørskov (2004) suggested that variability of spot measurements is greater than for total urine collection and that more animals

should be used to reduce errors. Studies designed to evaluate the accuracy of estimating PD output in urine have shown that PD excretion estimated by spot urine sampling was not different from total urine collection (Chizzotti et al., 2008), suggesting that spot urine samples can be used to accurately estimate MCP flow from the rumen under farm conditions. It is the only viable approach currently available for an on-farm diagnostic of MCP supply, and can be used to assess relative differences of total rumen MCP outflow between diets (Shingfield, 2000).

2.4.2. *Predicting amino acid limitations*

Estimating AA requirements of dairy cows can be done by mathematically calculating AA requirements of different body components and incorporating rates at which the nutrients move through digestive and metabolic pools, or by direct- or indirect dose response infusion studies as described in Swanepoel (2009).

However, current research efforts with dairy cows are moving away from trying to define and meet absolute animal AA requirements, due to the lack of success over a 30 year reference period, to examine metabolic AA impacts and changes in cows at different biological stages or fed under different nutritional scenarios. Methods to estimate AA limiting milk protein synthesis are discussed in Swanepoel (2009), but recent research generally focus on three main methods.

2.4.2.1. Uptake to output ratios

$$\text{Uptake to output ratio} = \frac{\text{Arteriovenous difference (g/L) x Mammary blood flow (L/d)}}{\text{AA output in milk (g/d)}}$$

This method describes the efficiency with which AA extracted by the mammary gland are utilized to synthesize milk proteins. Differences between free plasma AA concentrations in coccygeal arterial (before) *vs.* mammary venous (after) (*i.e.*, arteriovenous AA differences before and after mammary utilization), together with estimated mammary blood flow rate, can be used to determine daily mammary uptake of AA from the blood (Nichols et al., 1998). Assuming that the AA composition of milk protein is constant regardless of the ration fed, it is used to calculate daily output of AA in milk using the quantity of milk protein secreted. The AA theoretically limiting milk production can then be identified by comparing uptake of AA by the mammary gland to their calculated output in milk, as

summarized by Clark (1975). If mammary gland extraction of an AA does not meet milk protein requirements, it is considered to be a limiting AA.

2.4.2.2. Amino acid transfer efficiency

$$\text{Transfer efficiency} = \frac{\text{AA output into milk (g/d)} \times 100}{\text{Arterial AA concentration (g/L)} \times \text{Mammary blood flow (L/d)}}$$

This method describes the efficiency with which AA in the blood are transferred to the mammary gland and expressed as milk protein by relating the availability of AA in the arterial (*i.e.*, before mammary utilization) blood plasma to a calculated or theoretical milk protein demand (Vik-Mo et al., 1974). Thus if the concentration of an AA in the blood increases, through supplementation or other means, and the efficiency of transfer of that AA into the mammary gland decreases, this suggests that the supplemented AA is no longer limiting. Therefore transfer coefficients represent utilization for milk protein synthesis of AA that have been extracted from blood by the mammary gland.

2.4.2.3. Amino acid extraction efficiency

$$\text{Extraction efficiency} = \frac{\text{Arteriovenous difference (g/L)} \times 100}{\text{Arterial AA concentration (g/L)}}$$

Assuming that a low arterial concentration of an AA, together with a large extraction percentage, identifies an AA limiting milk production, this method examines arteriovenous differences as a proportion of AA in the plasma of the coccygeal artery to identify limiting AA. It is generally considered to be the most accurate of the three methods since it accounts for all EAA needs in the mammary gland, including intramammary metabolism, protein synthesis and degradation, rather than only AA output which does not differentiate between protein absorbed from the blood and protein synthesized by the mammary gland (Piepenbrink et al., 1998). Indeed it uses only direct numbers without the inaccuracies associated with estimating mammary blood flow (Nichols et al., 1998).

However, since mammary uptake of Lys from the plasma usually exceeds its requirements for milk production (Lapierre et al., 2005; Rulquin and Pisulewski, 2006), being extracted in accordance to its supply regardless of its ‘requirement’ (Guinard and Rulquin, 1994; Metcalf et al., 1994), Lys

may always appear as 1st limiting regardless of the ration and protein supplement fed (Nichols et al., 1998).

2.4.3. *Amino acid supplementation*

Early post-ruminal supplementation of casein consistently resulted in increased milk production in lactating cows (Clark, 1975). However, the elements in casein that caused these increases were not identified and attempts to re-create these improvements by supplementing individual AA have not been successful (Choung & Chamberlain, 1992). Since Met and Lys were widely identified as the most limiting AA for milk production decades ago, most supplementation studies have focused on them, and animal responses to these AA were discussed in detail by Swanepoel (2009). The widespread failure of supplementation of these AA to elicit meaningful lactation responses (Robinson, 2010) may be due to use of inappropriate criteria when limiting AA were identified, and/or that these AA were limiting to such a small extent that their supplementation almost immediately leads to limitations of the 2nd or 3rd limiting AA (Clark, 1975).

A systemic review of the impacts of metabolizable Lys and Met concentrations on cow performance (Robinson, 2010) reported that the extent of benefits in productivity from supplementation of Lys and Met were disappointingly small, even though some differences were statistically significant. Overall, the combined supplementation of Met and Lys provided additional benefits (*e.g.*, increased milk yield, increased milk energy output, increased efficiency of dietary N utilization for milk protein output) over the overall small positive responses to Met alone (*e.g.*, increased milk energy output, increased milk fat and protein %, increased efficiency of N utilization) and overall negative responses to Lys alone (*e.g.*, decreased DM intake). A meta-analysis (Patton, 2010) to investigate effects of supplementing ruminally protected (RP) Met on lactating dairy cattle performance confirmed that responses to supplemental Met and Lys are inconsistent, but that overall Met supplementation increases milk protein concentration and yield while decreasing DM intake and milk fat concentration. It also suggested that a slight increase in milk yield can be expected.

A more recent meta-analysis (Zanton et al., 2014) suggested that results from Met supplementation on milk protein yield was positive regardless of the Met source but that, contrary to

previous meta-analysis, milk fat yields were only increased by certain Met sources. Other feeding studies have also shown that supplementation of Met results in small increases in milk yield, milk protein concentration and/or yield as well as milk fat concentration (Čermáková et al., 2012), with supplementation of Met in the peri-partal period showing similar positive responses (Osorio et al., 2013), while supplementation of Lys had no positive effects on production (Paz and Kononoff, 2014). Wang et al. (2010) confirmed reports reviewed by Robinson (2010) that combinations of Met and Lys increased milk and milk protein yields above that of Met or Lys alone, with Met alone showing additional increases in milk fat concentration while Lys alone showed little improvement over Controls. And again, milk yield and milk protein concentration and yield was increased for the combined supplementation of Met and Lys over either fed alone (Třináctý et al., 2009) while milk protein concentration and yield was increased over Control in Appuhamy et al. (2011). However, this was not the case for Arriola Apelo et al. (2014a), who reported no difference between Control rations and those supplemented with either Met, Lys or a combination. Instead they reported a decrease in milk protein and lactose yields when Lys and Met was supplemented in addition to Leu. Supplementing combinations of AA in order to balance all AA (9 EAA) or provide an 'ideal' AA balance (*i.e.,* Lys, Met, His, Leu) succeeded in increasing milk yield and milk protein concentration and yield above the Control while decreasing milk lactose and fat concentration, but no differences occurred between all EAA and the 'ideal' mixtures of AA (Haque et al., 2012).

Overall results from research supplementing Met and/or Lys show that Met supplementation results in small production benefits regardless of the base ration. This does not seem to support correction of a Met deficiency in the diets since all of the rations differed. However, if one considers AA as metabolically bioactive compounds, an alternative explanation is that Met is bioactive and can elicit small positive lactation responses regardless of the base ration fed due to its diverse metabolic functionality and utilization. In contrast, unsuccessful supplementation of Lys speaks to a biologically inactive AA and utilization of inappropriate criteria to identify limiting AA in AA extraction experiments, since Lys appears to be limiting in many base rations regardless of the ingredient composition, as discussed above.

Even though other AA such as Phe, Ile, Leu, His, Thr and Arg have previously been identified as being potentially limiting depending on the base ration (Derrig et al., 1974; Vik-Mo et al., 1974; Schwab et al., 1976; Fraser et al., 1991; Varvikko et al., 1999), very little research has examined their importance, and effects of dietary supplementation, on lactation responses.

2.4.3.1. Lactational responses to supplementation of other amino acids

Based on balance studies performed on AA in the early 1960's (Mepham and Linzell, 1966), it was concluded that, based on their transfer stoichiometry, AA can be allocated into different groups (Mepham, 1982). Group 1 (*i.e.*, Met, Phe, Tyr, Trp) being AA that are absorbed by the mammary gland in direct proportion to requirements for milk protein synthesis while group 2 (*i.e.*, Val, Ile, Leu, Arg, Lys) are taken up in excess of requirement due to their ability to be oxidized in the mammary gland to synthesize non-essential AA (NEAA). Mepham (1982) also showed that depletion of any EAA reduces mammary cell uptake of group 1 AA considerably, presumably due to the AA limitation reducing their utilization in milk production, but that mammary uptake of group 2 AA were only reduced by a small amount and that oxidation thereof increased markedly.

2.4.3.1.1. Histidine

Early AA infusion studies reported positive milk yield responses to His (Schwab et al., 1976) as its deletion from AA mixture infusates decreased milk and milk protein yield while increasing milk fat concentration in lactating cows (Fraser et al., 1991). Thus His was identified as being 3rd limiting for milk production after Lys and Met, which was confirmed by Lee et al. (2012) and Giallongo et al. (2015), thereby suggesting that His may be limiting milk production in corn- and alfalfa silage based rations after Met and Lys. This would particularly be the case when RUP and metabolizable protein (MP) is deficient, due to increases in milk, milk protein and milk lactose yields when His was supplemented in addition to Lys and Met. Other infusion studies identified His as 1st limiting when grass silage based rations were fed since His supplementation was associated with increases in milk and milk protein yields (Vanhatalo et al., 1999; Korhonen et al., 2000; Huhtanen et al., 2002) and deletion or infusion of His confirmed its importance in milk yield, and milk protein concentration and yield, in lactating goats (Bequette et al., 2000) and lactating dairy cows (Hadrová et al., 2012).

Supplementing His by adding it to drinking water of dairy cows increased milk and milk lactose yields, showing that His can affect milk production if sufficient quantities bypass the rumen (Doelman et al., 2008). Cant et al. (2001) reported a large decrease in milk fat concentration when a mixture of AA were supplemented. However after His was removed, milk fat concentrations returned to normal suggesting that the decrease in milk fat was due to an AA imbalance (*i.e.*, excess His). This was confirmed by Kim et al. (2001) suggesting that supplementation of His beyond its requirements could reduce cow performance since increased supplies of His reduced milk protein concentration and milk yield. Thus the milk fat to protein ratio may be an indicator of a His imbalance.

2.4.3.1.2. Branched-chain amino acids (BCAA)

Catabolism of BCAA (*i.e.*, Ile, Leu and Val) has been studied since the 1970's and their roles as oxidative precursors, in addition to being used in milk protein production, was documented (Mephan, 1982). Substantial amounts of these AA are catabolized, yielding tricarboxylic cycle intermediates to be incorporated into NEAA (Harper et al., 1984). However, their requirement for milk production is uncertain since there was no lactation response when a combination of BCAA was infused, even though it increased plasma concentrations of added BCAA (Mackle et al., 1999; Korhonen et al., 2002). Hopkins et al. (1994) reported that infusion of BCAA plus Arg prevented a milk fat depression when fiber level in the ration was decreased, suggesting their involvement in *de novo* synthesis of milk fatty acids. Even though supplementation of BCAA did not benefit milk production or composition, a decrease in MUN may have suggested a stimulation of body protein synthesis (Appuhamy et al., 2011).

Even though removal of Val from an infusion mixture of His and other BCAA did not affect any milk production parameters in Korhonen et al. (2002), a Val deficiency was suggested to negatively impact milk protein production (Haque et al., 2013) after its removal from the total EAA infusion decreased its plasma concentrations, thereby supporting the hypothesis that Val plays a role in milk protein synthesis. However, supplementing Val by infusing it with Met and Lys had different effects on lactation response in two consecutive experiments in Schwab et al. (1976), one showing no response while the other showed an increase in milk protein concentration.

There was no negative lactation response when Ile was removed from the AA mixture infused by Korhonen et al. (2002) or Haque et al. (2013) even though plasma Ile concentrations declined. However, when Ile was supplemented in addition to Met, Lys and Val via abomasal infusion, it resulted in an increase in milk protein concentration (Schwab et al., 1976). However, upon Ile supplementation with Phe, there were no further milk production responses. This is consistent with a study supplementing Ile in a RP form (Robinson et al., 1999), in which Ile did not impact milk fat or protein production, but unexpectedly tended to stimulate milk lactose synthesis.

Leu was identified as a possible limiting AA together with His in grass silage based rations (Varvikko et al., 1999), but neither its addition or removal from an infusion mixture of His and other BCAA changed plasma Leu concentrations or affected any milk production parameters in Korhonen et al. (2002). However removal of Leu did reduce plasma insulin concentrations, which supports Leu as an insulin secretagogue (*i.e.*, stimulates production of insulin), thereby increasing tissue protein accumulation (Harper et al., 1984). Indeed, no study in which Leu was infused or supplemented has reported any improvement in milk production or contents (Kröber et al., 2001; Huhtanen et al., 2002; Křížová et al., 2008). The only study claiming a tendency for increased milk protein concentration when Leu was infused, in addition to Met, Lys and His, was Richter et al. (2010). It has been suggested that even though Lys and Leu are both taken up by the mammary gland in excess of requirements, Lys is required to maintain milk production while Leu is mainly oxidized to synthesize other AA which promote muscle protein synthesis, but is not required to sustain milk protein yields (Schwab et al., 1976; Bequette et. al., 1996a, 1996b; 1998). Supplementing Leu in addition to Met and Lys decreased milk protein and lactose concentration in Arriola Apelo et al. (2014a), while infusion of increased amounts of Leu in addition to a complete AA mixture linearly decreased milk fat and lactose concentration and yield, without affecting insulin or glucose concentrations, while increasing milk protein concentration (Rulquin and Pisulewski, 2006).

2.4.3.1.3. Phenylalanine and Tyrosine

Phenylalanine was first identified as being potentially limiting for lactating dairy cows when sodium caseinate was infused and estimated uptake of AA were compared to their theoretical

utilization for milk protein synthesis by the mammary gland (Derrig et al., 1974). Vik-Mo et al. (1974) suggested Phe was 3rd limiting after Met and Lys, followed by Tyr. However when efficiency of extraction were taken into account, Phe became 1st limiting followed by Tyr. After reviewing reported data available at the time, Mephan (1982) also suggested that Met and Phe should be considered the most probable AA to limit milk production due to their consistent identification as most limiting for milk production in previous studies. Recent studies feeding different protein sources have identified Phe as limiting after Lys and Met in corn silage based rations (Nichols et al., 1998; Piepenbrink et al., 1998; Mulrooney et al., 2009; Christen et al., 2010) or when casein and/or glucose were infused (Clark, 1975; Clark et al., 1977) while Phe was reported as 4th limiting after His when AA were removed individually or in groups from a synthetic EAA mixture in successive experiments to determine the effects on production responses (Fraser et al., 1991).

Very little research has been completed in which Phe was supplemented to dairy cattle rations to determine its effect on milk production even though Guinard and Rulquin (1994) reported that the mammary gland has a specific requirement for Phe and Tyr which increases as milk protein production increases, and that supplemented Phe was extracted by the mammary gland in amounts equal to its secretion in milk protein. This may indicate that Phe and Tyr are not extracted in excess and are almost exclusively utilized to support milk production (Clark et al., 1977). This was confirmed by Metcalf et al. (1994), who suggested that since free Phe and Tyr uptakes do not meet their outputs in milk, there must be an alternative source of these AA, possibly bound in peptides. Two *in vivo* studies evaluating the ability of the mammary gland to utilize peptide AA confirmed use of Phe and Leu peptides for milk protein synthesis (Backwell et al., 1994; 1996). Studies investigating effects of Phe on gene expression and milk protein synthesis in incubated bovine mammary epithelial cells revealed that peptide-bound Phe enhanced milk protein synthesis, and that they are utilized more efficiently than free Phe up to a level of 7% of total Phe supply (Zhou et al., 2015). This was also the case for peptide-bound Met after supplementation of free Met (Pan et al., 1996 and Wang et al., 1996 as referenced by Bequette and Backwell, 1997). However, Bequette et al. (1998) showed that the normal contribution of peptides to total Phe supply for milk production (~ 8%) decreased when Phe availability was

increased through supplementation, while uptake and utilization of supplemented free Phe from the blood increased. This suggests that increased supplementation of Phe as free AA may displace peptide-bound AA at the risk of decreasing efficiency of milk protein synthesis, indicating that feeding regimes promoting production of peptides in the rumen, or perhaps dietary supplementation of specific peptides, may be the next step in improving milk production and efficiency.

Since Phe is part of a group of AA which are extensively catabolized by the liver, its removal by the liver is directly correlated to total hepatic inflow (*i.e.*, absorbed plus recycled AA), and up to 0.49 could be removed by the liver (Bach et al., 2000; Lapierre et al., 2005). It has therefore been suggested that as more Phe is supplied and absorbed, more will be removed by the liver (Lapierre et al., 2005). However, since almost all (~ 0.97) of the Phe available post-liver is captured by the mammary gland, it is clearly critical to milk protein synthesis. 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, so confirming the importance of Phe in milk production. A study by Schwab et al. (1976) included Phe with Thr in an infusion mixture with Met and Lys, resulting in increased milk and protein yields. However, since a follow-up study showed no milk production response when Phe was included to the same mixture with Ile, another study confirmed that Thr may have been limiting. Infusing Phe with Met and Trp to a low and high CP diet based on grass silage also had no effect on animal production (Choung and Chamerlain, 1992). Studies by Yu et al. (2014) showed that infusion of Phe can increase pancreatic α-amylase secretion, which could in turn increase DM and starch digestibility in the intestine.

Even though Tyr is considered to be a NEAA, its synthesis depends largely on conversion of Phe to Tyr through Phe hydroxylase activity in the liver and lactating bovine mammary gland (Jorgensen and Larson, 1968; Guinard and Rulquin, 1994). Since mammary secretory cells do not require a dietary source of Tyr to synthesize milk protein, as long as adequate Phe is available, classifying Tyr as a ‘conditional EAA’ is perhaps more accurate. Thus, if the diet is deficient in Phe, its conversion to Tyr will decline. Jorgensen and Larson (1968) and Bequette and Backwell (1997) showed that when Tyr is supplemented or provided by the diet, conversion of Phe to Tyr is reduced, but even

though additional Tyr reduces the requirement for Phe, it cannot fully replace Phe (Womack and Rose, 1946) and conversion of Phe to Tyr will continue even at high levels of Tyr supplementation (Grau and Steele, 1953). However, there is a possibility that conversion of Phe to Tyr is not sufficient to supply all Tyr requirements for milk production in high producing dairy cows (Jorgensen and Larson, 1968), suggesting that Tyr itself could become a limiting AA when it, or Phe, is not supplied in adequate quantities in the diet.

Overall, the inconsistency in responses of lactating dairy cattle to AA supplementation in many studies all speak to AA as 'bioactive' metabolites rather than to AA as limiting metabolites. Lactation responses to AA supplementation appears to be highly dependent on circumstances, and how each AA expresses its bioactivity can therefore not yet be predicted or explained. When responses to certain AA supplementations are as expected, the ration fed is usually based on a limited number of ingredients, and the ration was often specifically selected to optimize a production response to the target AA. However, under normal commercial circumstances, with multicomponent lactating dairy cattle rations consisting of many ingredients and by-products, responses to AA supplementation changes to various animal performance responses (*e.g.,* BW, BCS) rather than milk production *per se*. Thus, there is a significant downside risk to supplementing AA at this time due to creation of unexpected AA interactions, even though many researchers do not consider excess availability of AA to be detrimental despite studies demonstrating the deleterious effects of high level supplementation of Lys and/or Met (Robinson et al., 2000; Robinson, 2010). Therefore, supplementation of AA that are not specifically required (*i.e.,* limiting) can change mammary extraction, body AA balances and pools, potentially creating an animal response that is not expected, and not always deemed positive by commercial dairy producers.

Chapter 3: Project objectives

Overall project objectives were to:

1) Determine the highest level at which CM and DDGS can be included in dairy rations before adversely affecting production, using a high protein, low fat DDGS (HPDDG) alternative to avoid possible detrimental effects of corn oil on cow production.
2) Identify the nutritional limitations at high inclusion levels of CM, which could relate to dietary protein degradability and/or limited AA availability, and
3) Find possible resolutions for the limitations associated with feeding very high levels of CM to high producing dairy cows by supplementing potentially limiting AA to high CM rations.

Chapter 4. Experiment 1: Determining the optimal ratio of canola meal and high protein dried distiller's grain in rations of high producing Holstein dairy cows

Abstract

Use of canola meal (CM) and dried corn distillers grains with solubles (DDGS) as major supplemental protein sources are common practice in North American dairy rations and usage of both is projected to increase in the future. Since limited data is available on performance of cows fed rations with different ratios of CM and DDGS, our objective was to determine the optimal ratio of CM to DDGS protein in a contemporary lactation dairy ration by feeding combinations of CM and high protein DDG (HPDDG) to early lactation multiparity dairy cows. The experiment was a 4 x 4 Latin square with 28 d periods using four pens of ~320 high producing cows/pen. Treatments were created by varying the amounts of CM and HPDDG added on a DM basis to be: (1) 0 g CM/kg and 200 g/kg HPDDG, (2) 65 g CM/kg and 135 g/kg HPDDG, (3) 135 g CM/kg and 65 g/kg HPDDG, (4) 200 g CM/kg and 0 g/kg HPDDG. Dry matter intake was not affected by the CM/HPDDG ratio in the ration. Milk and lactose yield, true protein (TP) concentration and yield, milk fat yield as well as milk energy output increased at a decreasing rate with a higher CM/HPDDG ratio. Maximum values for milk and TP yield were at ~135 g CM/kg, while lactose, TP concentration and milk energy were maximized at ~120 g CM/kg inclusion. Milk fat concentration and milk energy density decreased linearly with higher CM inclusion. Body condition score change responded quadratically with the highest gain at ~120 g CM/kg inclusion. The purine derivative to creatinine index increased linearly with higher CM inclusion levels, suggesting that microbial protein production (MCP) was limited in the 0 g CM/kg ration and was progressively stimulated by higher feeding levels of CM. Plasma AA concentrations suggest that the reduction in lysine in dietary protein, together with the decrease in MCP synthesis, resulted in a substantial reduction in lysine available for milk production, thereby limiting performance in the higher HPDDG ration. The only AA which decreased in plasma with higher CM feeding levels were phenylalanine, leucine and methionine. That the concentration of leucine in the plasma was still decreasing linearly, while methionine and phenylalanine responded quadratically at the 200 g CM/kg treatment, was interpreted to suggest that the leucine supply remained higher than its requirement at the highest CM inclusion level, but that phenylalanine and/or methionine was limiting production in the highest CM ration. Overall, results suggest that the optimum ratio of CM to HPDDG in these rations was with 120 to 135 g/kg of ration DM from CM.

Keywords: Milk production; Spot urine purine; Plasma amino acids.

Abbreviations: AA, amino acid; ADF, acid detergent fiber; ADICP, AD insoluble CP; AL, allantoin; aNDF, amylase-treated NDF; aNDFom, aNDF free of residual ash; AP, absorbable protein; BCS, body condition score; BUN, blood urea N; BW, body weight; CM, canola meal; CP, crude protein; CR, creatinine; DC305, DairyComp 305 management system; DDGS, dried distillers grains with solubles; DHIA, Dairy Herd Improvement Association; DIM, days in milk; DM, dry matter; EAA, essential AA; HPDDG, high protein DDG; MCP, microbial CP; NDF, neutral detergent fiber; NE_L, net energy for lactation; OM, organic matter; PD, purine derivatives; PDC index, PD to creatinine index; RDP, rumen degradable CP; RUP, rumen undegradable CP; SCC, somatic cell counts; TMR, total mixed ration; TP, true protein.

4.1. Introduction

Protein nutrition is critical for high production efficiency of lactating dairy cows because it impacts their performance and the environment. Sufficient dietary protein is required to optimize production while an excess has negative effects on the environment, primarily when excreted as urea in urine. The major protein sources used in western areas of North America include high quality alfalfa hay, whole cottonseed or cottonseed meal (CSM), dried distillers grains with solubles (DDGS) and canola meal (CM). Due to the variable quality and high price of alfalfa hay, and the presence of secondary compounds (*i.e.*, tannins and gossypol) in cottonseed, their inclusion levels in dairy rations are limited. Therefore, use of CM and DDGS as major supplemental protein sources is currently very high in many US dairy rations.

The Canola Council of Canada developed an initiative (Growing Great 2015) which aims to double 2011 production of CM by 2015 through increased crushing capacity in Canada (Canola Council of Canada Annual Reports, 2010; 2011). The USA is the main market for CM exports from Canada, receiving over 50% of their total CM exports with over 90% of this imported CM being utilized by the California dairy industry (USDA, 2011; Nernberg, 2012). Due to steadily increasing crude oil prices, the corn ethanol production industry in the Midwestern USA has been expanding rapidly since 2000, and increased production of corn distiller's grains, the major by-product of the corn-starch ethanol industry, is projected to continue in coming years, at least as long as government subsidies persist (Wisner, 2010). As supplies of CM and DDGS increase, so will pressure to use these products as major protein supplements in dairy cattle rations. However, with as much as 400 g/kg of the crude protein (CP) in contemporary California total mixed ration (TMR) already coming from corn products, which is known to be limiting for milk protein synthesis in some amino acids (AA), particularly lysine, inclusion of even more corn DDGS protein could have a detrimental effect on production due to AA imbalances at the intestinal absorptive site, as well as by adding excess corn oil to already corn oil rich diets.

Studies comparing CM to DDGS have reported that higher proportions of CM, included at up to 66 and 104 g/kg DM respectively, tended to have higher absolute values for milk and protein yields

(Mulrooney et al., 2009). However, negative effects of high levels of unsaturated fatty acids in corn oil on milk production, often reducing milk fat concentration and yield (Hollmann et al. 2011; Liu and Rosentrater, 2011), necessitates use of a low oil alternative to conventional DDGS when experimentally comparing dietary protein sources involving corn based DDGS. High protein DDG products (HPDDG) provide the opportunity to do this as they have a very similar proximate nutrient profile to CM (Table 4.1).

Table 4.1: Chemical analysis (± standard errors[a]) of ingredients used in the total mixed rations (g/kg dry matter) fed to cows

	Dry matter	Organic matter	Crude protein	aNDF[b]	aNDFom[c]	Fat
Alfalfa, hay	912	889	195	391	380	20.6
	(1.1)	(3.4)	(2.8)	(10.1)	(10.7)	(0.98)
Almond, hulls	981	928	48.9	332	319	24.3
	(0.8)	(2.4)	(2.74)	(21.4)	(18.5)	(0.61)
Oat, hay	918	890	109	560	542	24.3
	(0.6)	(3.6)	(6.1)	(3.2)	(2.4)	(0.75)
Corn, steam flaked grain	857	986	84.4	85.0	84.5	34.6
	(5.3)	(0.3)	(1.11)	(2.86)	(3.12)	(1.17)
Cottonseed, cracked Pima	915	953	218	403	385	223
	(3.4)	(0.8)	(9.8)	(8.9)	(8.1)	(3.2)
Canola meal, pellets (380	893	924	410	271	237	26.4
g/kg CP, solvent)	(5.1)	(1.0)	(2.3)	(5.3)	(7.6)	(1.54)
Distillers grains, high CP	915	978	395	338	331	54.5
(corn with solubles)	(1.9)	(7.1)	(6.1)	(30.0)	(29.3)	(2.18)
Wheat, silage	321	881	82.2	537	495	29.4
	(6.4)	(1.5)	(6.34)	(8.4)	(6.5)	(0.87)
Corn, silage	331	926	80.0	459	447	24.8
	(5.4)	(4.4)	(1.87)	(5.8)	(4.5)	(1.42)
Citrus, pulp	158	954	72.1	189	185	15.7
	(4.3)	(3.5)	(3.56)	(10.8)	(9.3)	(0.90)
Potatoes, tubers (whole)	197	955	79.8	57.0	55.0	< 2.5
	(3.7)	(2.1)	(4.22)	(2.00)	(1.30)	(-)
Pomegranate, pulp waste	251	955	99.2	301	293	59.5
	(19.0)	(2.3)	(17.97)	(46.5)	(46.3)	(11.13)

[a] Means and (SE) with a 95% confidence level. *n* = 4, except citrus pulp = 3, potatoes = 2, pomegranate = 2.
[b] Neutral detergent fibre assayed with heat stable amylase, expressed inclusive of residual ash.
[c] Neutral detergent fibre assayed with heat stable amylase, expressed exclusive of residual ash.

Christen et al (2010) suggested that HPDDG outperformed CM at 120 g/kg ration DM, and there were indications that cows fed the HPDDG ration had an improved plasma AA balance *versus* CM, with a more desirable AA profile for milk protein production. However, adding HPDDG to diets

which are already high in corn proteins may lead to lysine becoming limiting to milk production. Also, CM and HPDDG have very different CP degradability profiles with CM being primarily a rumen degradable CP (RDP) source while HPDDG is a high rumen undegradable CP (RUP) source (data summarized by Mulrooney et al., 2009). This means that a higher inclusion level of either could lead to an imbalance in the dietary RDP:RUP ratio, thereby negatively affecting rumen function, and/or creating an imbalance in AA available to support milk production. Few studies have been completed comparing dairy cattle performance between CM and HPDDG directly, and little information is available on inclusion levels higher than 120 g/kg for either protein source.

The objective was to determine the optimal ratio of CM to DDGS protein as the sole supplementary dietary protein source in rations which are relatively high in corn proteins, provided as corn grain and corn silage, by feeding combinations of CM and HPDDG to high producing dairy cows, thereby comparing the two protein sources without negative confounding effects from corn oil in conventional DDGS.

4.2. Materials and methods

The experiment was a 4 x 4 Latin square with 28 d experimental periods, and it took place from October 2011 to February 2012. The William's experimental design (Williams, 1949) was used to generate a uniform design balanced for potential carry-over effects between treatments, as every treatment was fed in every period and to each pen, but never in the same sequence among pens.

All cows were cared for relative to applicable laws of the state of California and the USA, consistent with requirements for "The care and use of animals for scientific purposes", as per the South African National Standard (SANS 10386-2008).

4.2.1. *Farm and management*

The commercial dairy farm selected for this study is located near Hanford (CA, USA) and milks ~5000 Holstein cows three times a day starting at 04:00, 12:00 and 20:00 h. Cows were housed in free stall barns, bedded with dried manure solids, with access to an outside dry lot and had fresh water available *ad libitum.* As per normal farm practices, cows were randomly allocated once a week from

a single fresh pen at ~20 days in milk (DIM) to one of four early lactation pens. Each of the four pens housed ~320 multiparity early lactation cows (*i.e.*, those cows which had cleared the fresh pen but were not yet confirmed pregnant) with similar lactation characteristics. Once confirmed pregnant, cows are moved from these pens to mid lactation pens. Normal cow movement in and out of the lactation pens was minimally restricted by the study. Treatments were randomly allocated to one of the four early lactation pens at the start of the 1st period and rotated after each 28 d experimental period as described above for a William's design.

4.2.2. *Diets*

The four rations were formulated by the farm nutritionist to be iso-nutritious for CP and fat, thus allowing comparison of CM and HPDDG as protein sources without confounding treatment effects with other ration nutrient changes, especially dietary fat levels. Treatments were created by varying the ratio of CM and HPDDG added to the ration at 200 g/kg TMR dry matter (DM), while the other 800 g/kg remained the same among treatments. On a DM basis, treatments were designed to be: (1) 0 g CM/kg and 200 g HPDDG/kg, (2) 65 g CM/kg and 135 g HPDDG/kg, (3) 135 g CM/kg and 65 g HPDDG/kg, (4) 200 g CM/kg and 0 g HPDDG/kg TMR DM.

Cows were fed a TMR which was prepared immediately before each feeding by mixing the individual ingredients (*i.e.*, alfalfa hay, wheat and corn silages, CM, HPDDG) and a premix containing the dry ingredients (*i.e.*, almond hulls, oat hay, steam flaked corn grain, cracked pima cottonseed, liquid molasses, mineral premix) in a conventional 2 screw vertical mixer. Cows were fed each morning between 04:30 and 07:30 h, while the cows were at morning milking, and again between 11:00 and 12:30 h for *ad libitum* intake. Each pen received a total of ~15,500 kg of as mixed TMR/d, split into 2 loads (*i.e.*, one full 8,500 kg load of TMR at 1st feeding with a second ~7,000 kg load of TMR at 2nd feeding with the exact amount determined by the feeder). Each 1st feeding of TMR was fed to a clean bunk as bunks were cleared of all residual feed, which was weighed daily by pen, immediately prior to the 1st feeding. 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 per cow/pen. The

"TMR tracker" system (Digi-Star LLC, Fort Atkinson, WI, USA) kept a record of the actual ingredient profiles of each batch of TMR mixed.

4.2.3. *Sample collection, preparation and analytical methods*

4.2.3.1. 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. Ingredients were pooled by period for chemical analysis. Ten handfuls of each TMR were collected at evenly spaced intervals at pre-marked posts along the bunk-line according to Robinson and Meyer (2010) immediately after feeding and before the cows had access to it. All TMR samples, silages and other wet ingredients were weighed, dried at 55°C for 48 h, and allowed to air equilibrate at room temperature for 24 h in order to create moisture stable samples to facilitate determination of their air DM concentration, 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 2 h in a forced air oven (Reuter et al., 1986). Total N and acid detergent insoluble CP (ADICP) was determined by the Leco method (Method 990.03, AOAC, 1997) while acid detergent fiber (ADF) and lignin treated with sulphuric acid (lignin(sa)) was determined according to method 973.18 of AOAC (1997). The neutral detergent fiber (NDF) was determined as described by Van Soest et al. (1991). Heat stable amylase was added to samples with a high starch concentration to prevent filtering difficulties (*i.e.*, aNDF) while aNDFom values do not include residual ash. Ash determination was based on gravimetric loss by heating samples to 550°C for 8 h. Soluble carbohydrates (*i.e.*, free sugars fructose, glucose, sucrose) were determined by high performance liquid chromatography as described by Johansen et al. (1996). Minerals were determined using methods of Johnson and Ulrich (1959), Tracy and Moeller (1990) and Meyer and Keliher (1992). Fat was quantified using a standard Soxhlet extraction (Method 2003.05, AOAC, 2006).

4.2.3.2. Animal measurements

At the start of the study, a group of ~180 cows with the lowest DIM (*i.e.*, 30 to 88 DIM) were selected from each pen and coded in DairyComp 305 (DC305, Valley Agricultural Software, Tulare,

CA, USA), in order to prevent them from being sold or moved unless necessary for health purposes. Due to their low DIM, these cows were the most likely to complete the study in their originally assigned pen. This group of ~180 cows/pen was used as the base group from which all representative subgroups were selected for animal samples (*i.e.*, urine, blood) and measurements (*i.e.*, girth, body scores). Only 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.

Weekly data backups of the DC305 herd record system were made to crosscheck cow movements. 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 week study (i.e., any movement of a cow from their originally assigned pen to another pen, such as the hospital pen, precluded their eligibility. Cows to be sampled or measured (as described in the previous paragraph) were identified by ear tag number during the routine 60 minute 'lockup' which occurred every morning, immediately after milking, for normal pregnancy diagnosis and artificial insemination.

4.2.3.2.1. Milk production and composition

Milk samples were collected, and milk yields recorded, during the first milking (04:00 to 08:00 h) for all four pens on day 28 of each experimental period by Dairy Herd improvement association (DHIA) personnel. Daily milk production was estimated by multiplying the recorded yield by three. A small representative sub-sample of milk was drawn from the sample collection flask (after a short period of mixing) of all cows and preserved with a 2-Bromo-nitropropane-1, 3-diol preservative for subsequent analytical testing. Fat, true protein, lactose and somatic cell counts (SCC) were determined using near infrared spectroscopy at the DHIA laboratory in Hanford (CA, USA).

4.2.3.2.2. Body condition score

A representative subgroup of ~140 cows/pen was selected from the base group of ~180 cows/pen (see section 2.3.2.) at the start of the study for body condition scoring (BCS). This was completed by the same trained scorer on the first day of period 1 and at the end of the sampling week of each experimental period. The BCS system of Ferguson et al. (1994) was used, which works on quarter points based upon several anatomical characteristics of the cows. However, when a cow demonstrated

characteristics which made it difficult to clearly classify her to a specific quarter point (*e.g.*, either 2.00 *versus* 2.25), she was classed as being intermediate (*i.e.*, 2.125). This resulted in addition of an additional 8th point to the system of Ferguson et al. (1994).

4.2.3.2.3. Urine

Spot urine samples were collected on one day during the sampling week of each experimental period from the first ~35 cows from the base group of ~180 cows/pen which voluntarily urinated during morning lockup. Aliquots of urine (7 ml) were transferred into tubes containing 2 ml of 100 ml/L sulphuric acid, reducing the final pH <2 to prevent bacterial destruction of allantoin (AL) and diluted with deionized water (to prevent precipitation of uric acid) to a final volume of 35 ml and frozen at -20^{o}C. Urine samples were chemically analyzed for creatinine (CR) at the Animal Health Diagnostic Center (College of Veterinary Medicine, Cornell University, Ithaca, NY, USA) according to the Jaffé method using a urine creatinine kit (Roche Diagnostics Corporation, Indianapolis, IN, USA) which utilizes a kinetic colorimetric assay during which CR forms a yellow-orange complex with picrate. Analysis for AL was according to Chen and Gomes (1992), which is based on the method of Young and Conway (1942). Standards were prepared to create working concentrations of 20, 40, 60, 80 and 100 mg/L AL. Urine samples were thawed and centrifuged at 1200*xg* for 15 min at 20 to 22^{o}C in order to remove precipitate which could influence the colorimetric reading. Samples were diluted 60 times to fit the standard curve. A duplicate standard curve was included at the start and end of each run in order to calculate the AL concentrations in the urine samples. Two inter-run standard samples were used in each run to assess variation among runs but, as all inter-run standards were within 0.05 of the average over all runs, all runs were accepted without inter-run correction. Each urine sample was analyzed in duplicate with the average used as the final concentration.

4.2.3.2.4. Girth measurements

The group of ~35 cows/pen from which urine had been collected in each period were girth measured the next morning using a weigh tape measure (The Coburn company, Inc., Whitewater, WI, USA), by placing the tape around the girth of each cow, just behind the front legs, making sure it was straight and snug and the cow was relaxed before the reading was made.

4.2.3.2.5. Blood plasma

A smaller subgroup of 24 cows/pen was selected from the base group of ~180/pen for blood sampling. Blood was collected from the tail (coccygeal) vein of each cow using a 10 ml evacuated tube containing K_2 EDTA (Vacutainer, Becton Dickinson, Franklin Lakes, NJ, USA), kept in coolers with ice and centrifuged immediately at 2100*xg* for 15 min at 4°C. Plasma was removed, transferred to duplicate Eppendorf tubes and frozen at -20°C. Samples were sent to the Molecular Structure Facility (University of California, Davis, CA, USA) for physiological AA (*i.e.*, free plasma AA) and ammonia analysis. After samples were acidified with sulfosalicyclic acid to precipitate intact proteins, AA were quantified using a Beckman 6300 AA analyzer (Beckman Coulter, Inc., La Brea, CA, USA) utilizing a lithium citrate buffer system and ion-exchange chromatography to separate AA followed by a "post-column" ninhydrin reaction detection system. Blood urea N (BUN) was measured on the same set at the Animal Health Diagnostic Center (College of Veterinary Medicine, Cornell University, Ithaca, NY, USA), utilizing an automated Roche Modular P Chemistry Analyzer (Roche Diagnostics, Indianapolis, IN, USA).

4.2.4. *Calculations*

Final oven DM was calculated as the air equilibrated DM (i.e., dried at 55°C) multiplied by the lab oven DM (i.e., dried at 105°C).

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

((([(4.163 x Fat (g/kg)) + (2.413 x (TP (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.

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

Body weight (BW) was calculated using the prediction equation of Mäntysaari and Mäntysaari (2008) in which both heart girth measurements and BCS are considered when estimating BW (kg) as:

93.3 + (230.882 x Girth (m)) – (239.66 x BCS) + (138.318 x (Girth (m) x BCS))

A partial net energy output (MJ/d) balance, used to determine where consumed energy was utilized among the treatments, was calculated by summing the maintenance (NRC, 1989), milk and BCS change energy where maintenance energy (MJ/d) was calculated using BW (kg) as:

$(BW^{0.75} \times 0.08) \times 4.184$

and BCS 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 NE_L (Chilliard et al., 1991) with the factor 4.184 converting Mcal/d to MJ/d.

Net energy for lactation (NE_L) density (MJ/kg DM) of the diets were estimated using the biological responses of the animals, as expressed in the partial net energy output, and measured DM intake as:

Net energy output (MJ/d) / DM intake (kg/d)

4.2.5. *Statistical analysis*

All cows which moved from their originally assigned pen during the study, for health or any other reason, were excluded from statistical analysis, thereby reducing the number of eligible cows in each response parameter subgroup from the starting numbers. This resulted in 533 out of 1282 starting cows being eligible for statistical analysis of milk production and 308 out of 560 (i.e., 140 cows/pen) starting cows being eligible for the BCS dataset. Outlier analysis completed blind to treatments identified 10 cows which were removed from the milk production dataset (*i.e.*, 1 due to missing milk composition values in period 4, 1 cow for a milk fat concentration > 65 g/kg, 4 cows for a milk production < 18 kg/d and 4 cows for SCC > 4,000,000 cfu (which was above the assay range)), and 5 cows which were removed from the BCS dataset due to abnormally high or low values. This resulted in final sets of 523, 303 and 346 cows being included in the statistical analysis for milk production,

BCS and girth measurements respectively. From the group of 77 eligible blood cows, 16 (i.e., 4/pen) were randomly selected for plasma AA and BUN assays and 40 cows (i.e., 10/pen) were randomly selected from the group of 346 eligible urine cows for urine AL and CR assays.

Animal production, BCS, girth measurements, urine AL, urine CR, plasma AA and BUN concentrations 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, which is consistent with guidelines of this journal (Robinson et al. 2006). Orthogonal polynomial contrasts were used in SAS to test linear and quadratic effects of the CM and HPDDG inclusion levels. Second order polynomial regressions were fitted to milk production, milk component, and BCS data in order to depict treatment responses, and the regression equations were used to determine maximum response points.

Dry matter intake (n = 4 pens, calculated on a pen basis with 4 pens/period), TMR components and ingredients and net energy 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 at $P \leq 0.05$.

4.3. Results

4.3.1. *Ration evaluation*

The chemical composition of the ingredients used in the TMR (Table 4.1) was similar to ingredients as listed in NRC (2001). Analysis of HPDDG showed that it had a much higher CP (395 *versus* 300 g/kg) but lower fat (54.5 *versus* 113 g/kg) concentration than conventional DDGS. However, HPDDG was similar to CM for both the CP (395 *versus* 410 g/kg) and fat (55 *versus* 26 g/kg) concentration.

The ingredient profile of the TMR fed (Table 4.2) did not differ among treatments, except for inclusion of CM and HPDDG, which varied among treatments as per the experimental objective. While there were small substitutions of minor byproducts (*i.e.*, pomegranate, whey, citrus, potatoes)

among periods, these changes made up a very small proportion of the total ration (57 g/kg) and were the same among treatments. There were no differences in the DM, CP, fat and starch concentration of the TMR among treatments, confirming that the dietary objective of iso-proximate nutrient rations were achieved. Linear differences in the nutrient composition among treatments, especially in organic matter (OM), sugar, ADICP and some macro- and micro-minerals were consistent with the difference in CM *versus* HPDDG inclusion, but none were judged to be biologically relevant. The NDF level decreased slightly with higher CM inclusion levels, due to the higher relative fiber level of HPDDG. However, these differences were numerically small and not considered to be biologically significant. The TMR met all nutrient requirements of lactating dairy cows producing 45 – 50 liters of milk/d (NRC, 2001).

Table 4.2: Ingredient profile and chemical composition (g/kg dry matter) of total mixed rations fed to cows

	g CM/kg DM in the ration					*P**
	0 g CM	65 g CM	135 g CM	200 g CM	SEM	Linear
Ingredient profile, g/kg DM [a]						
Alfalfa, hay	90.7	89.1	90.0	88.5	2.11	0.50
Premix						
Almond, hulls	96.4	96.9	96.5	97.3	2.37	0.82
Oat, hay	23.5	23.6	23.5	23.7	0.32	0.68
Corn, steam flaked grain	161	162	161	163	7.9	0.91
Mineral, premix	16.4	16.5	16.4	16.6	1.16	0.94
Fat, rumen inert[b]	12.8	12.9	12.9	13.0	0.15	0.65
Cottonseed, cracked Pima[c]	72.2	72.6	72.2	72.8	0.46	0.39
Molasses, liquid	11.4	11.5	11.4	11.5	0.11	0.58
Canola meal, pellets (solvent)	0.00	66.1	135	199	0.86	< 0.01
HPDDG[d]	202	136	67.9	0.00	0.83	< 0.01
Wheat, silage	42.4	43.1	42.5	41.9	1.00	0.59
Corn, silage	214	213	214	216	6.0	0.78
Other[e]	56.7	56.4	56.1	56.7	4.83	0.99
Nutrient profile, g/kg DM [f]						
Dry matter	521	519	518	509	20.6	0.53
Organic matter	927	925	922	918	2.6	< 0.01
Crude protein	170	170	167	170	3.8	0.72
ADICP[g]	96.9	90.2	82.0	71.2	9.44	< 0.01
aNDF[h]	334	321	321	308	7.9	< 0.01
aNDFom[i]	324	311	312	299	7.3	< 0.01
ADF[j]	214	220	221	217	6.7	0.60
Fat	53.8	53.7	53.5	53.0	2.04	0.67
Lignin(sa)[k]	42.8	46.0	47.8	50.0	2.47	< 0.01
Starch	188	192	202	190	7.6	0.47
Sugars	33.8	38.8	40.5	47.5	3.31	< 0.01
Ca	7.84	8.27	8.51	9.25	0.451	< 0.01

	g CM/kg DM in the ration					*P**
	0 g CM	65 g CM	135 g CM	200 g CM	SEM	Linear
P	3.37	3.76	4.17	4.59	0.246	< 0.01
K	14.7	15.6	16.2	16.8	0.55	< 0.01
Mg	2.32	2.58	2.80	3.14	0.084	< 0.01
S	2.47	2.50	2.53	2.62	0.064	0.01
Na	2.50	2.46	2.45	2.37	0.280	0.62
Cl	5.45	5.39	5.55	5.38	0.420	0.96
mg/kg DM						
Zn	75.9	76.4	76.0	79.7	3.04	0.20
Mn	32.3	35.7	39.3	43.7	12.44	< 0.01
Fe	209	195	194	186	16.8	0.14
Cu	14.8	15.0	14.5	15.1	0.81	0.88
Mo	1.11	1.09	1.13	1.14	0.083	0.55
Se	0.29	0.30	0.36	0.40	0.016	< 0.01

* No quadratic effect reached statistical significance (*i.e.*, P>0.08)
[a] Samples pooled by period (*n*=2 per period), based on average ingredient composition during sampling week for each pen, each period (*i.e.*, 16 total samples).
[b] EnerGII. Virtus Nutrition, LLC. 520 Industrial Way, Corcoran, CA, USA.
[c] Fuzzy Upland cottonseed in Period 1.
[d] High protein, low fat, dried distillers grains (see Table 4.1).
[e] Period 1: Pomegranate waste and whey liquid (60:40). Period 2: Citrus pulp and pomegranate waste (40:60). Period 3 & 4: Citrus pulp and potatoes (50:50) & (60:40).
[f] Based on total mixed ration samples collected twice during sampling week for each pen, each period (*i.e.*, 32 total samples).
[g] Acid detergent insoluble crude protein (g/kg of crude protein).
[h] Neutral detergent fiber assayed with heat stable amylase, expressed inclusive of residual ash.
[i] Neutral detergent fiber assayed with heat stable amylase, expressed exclusive of residual ash.
[j] Acid detergent fiber, expressed inclusive of residual ash.
[k] Lignin determined by solubilisation of cellulose with sulphuric acid.

4.3.2. *Animal measurements*

4.3.2.1. Milk production and composition

Milk production (Table 4.3) had a linear and quadratic response, increasing at a decreasing rate with higher CM/HPDDG ratios, reaching a maximum of 47.88 kg/d at 135 g CM/kg inclusion before decreasing slightly. Both milk TP concentration and yield responded quadratically (P<0.01) to the higher CM/HPDDG ratio. However, while TP yield followed the pattern of milk yield with a fitted maximum of 1.4 kg/d at 135 g CM/kg inclusion (Figure 4.1), the fitted maximum TP concentration of 29.4 g/kg was at a level of 120 g CM/kg inclusion (Figure 4.1). Milk fat concentration decreased linearly with higher CM inclusions, even though only to a small extent, while milk fat yield responded quadratically (P<0.01). However, even with this decrease in fat concentration, the fitted maximum fat yield of 1.64 kg/d was still at ~110 g CM/kg (Figure 4.1), mainly due to the higher milk productions at higher CM levels. Milk energy concentration followed fat concentration with a linear decrease as

CM inclusion increased. However, milk energy output had a similar quadratic and linear response (P<0.01) as milk yield with a peak of 136 MJ/day at 120 g CM/kg inclusion (Figure 4.1).

Table 4.3: Production performance and body measurements for cows fed rations with different levels of canola meal and HPDDG[a]

	g CM/kg DM in the ration					*P*	
	0 g CM	65 g CM	135 g CM	200 g CM	SEM	Linear	Quadratic
n = 4 pens							
Dry matter intakes (kg/d)	25.11	25.35	25.84	25.28	0.291	0.63	0.39
n = 523 cows							
Yield (kg/d)							
Milk	44.94	47.41	47.88	47.35	0.335	< 0.01	< 0.01
Fat	1.56	1.64	1.63	1.59	0.015	0.26	< 0.01
True protein	1.30	1.39	1.40	1.38	0.009	< 0.01	< 0.01
Lactose	2.16	2.27	2.27	2.24	0.016	< 0.01	< 0.01
Energy output (MJ/d)	129.1	136.1	135.7	133.2	0.95	< 0.01	< 0.01
Components (g/kg)							
Fat	34.8	34.8	34.1	33.7	0.24	< 0.01	0.23
True protein	29.1	29.4	29.4	29.3	0.10	0.02	< 0.01
Lactose	48.1	47.8	47.3	47.3	0.07	< 0.01	< 0.01
Energy density (MJ/kg)	2.88	2.88	2.84	2.83	0.010	< 0.01	0.19
Somatic cell count (‘000)	171	142	167	149	14.2	0.43	0.64
n = 303 cows	2.36	2.38	2.38	2.36	0.022	0.62	0.06
Body condition score (BCS)	0.011	0.034	0.080	0.029	0.0143	0.11	< 0.01
BCS change (unit/28 d)							
	205.7	205.3	205.8	205.5	0.47	0.98	0.55
n = 346 cows	673	674	675	671	3.5	0.81	0.44
Girth (cm)							
Body weight (kg)							

[a] High protein, low fat, dried distillers grains (see Table 4.1)

4.3.2.2. Body condition score

Body condition score (Table 4.3) was not affected by treatments, but the mean BCS of 2.37 was slightly below the desired range for most efficient milk production of 2.5 to 3.0 (Wildman et al., 1982; Wattiaux 1994). Change in BCS over 28 d was positive for all treatments, which is desirable for cows post peak production, while suggesting that the additional milk at the 135 g CM/kg inclusion level was not produced at the expense of body condition. The best fitted line (Figure 4.1) showed a quadratic response with a fitted maximum BCS gain of 0.063 units/28 d at ~120 g CM/kg inclusion. Energy

used for BCS change (Table 4.6) also had a quadratic response, with the highest energy need of 2.85 MJ/d at ~120 g CM/kg inclusion (Figure 4.1).

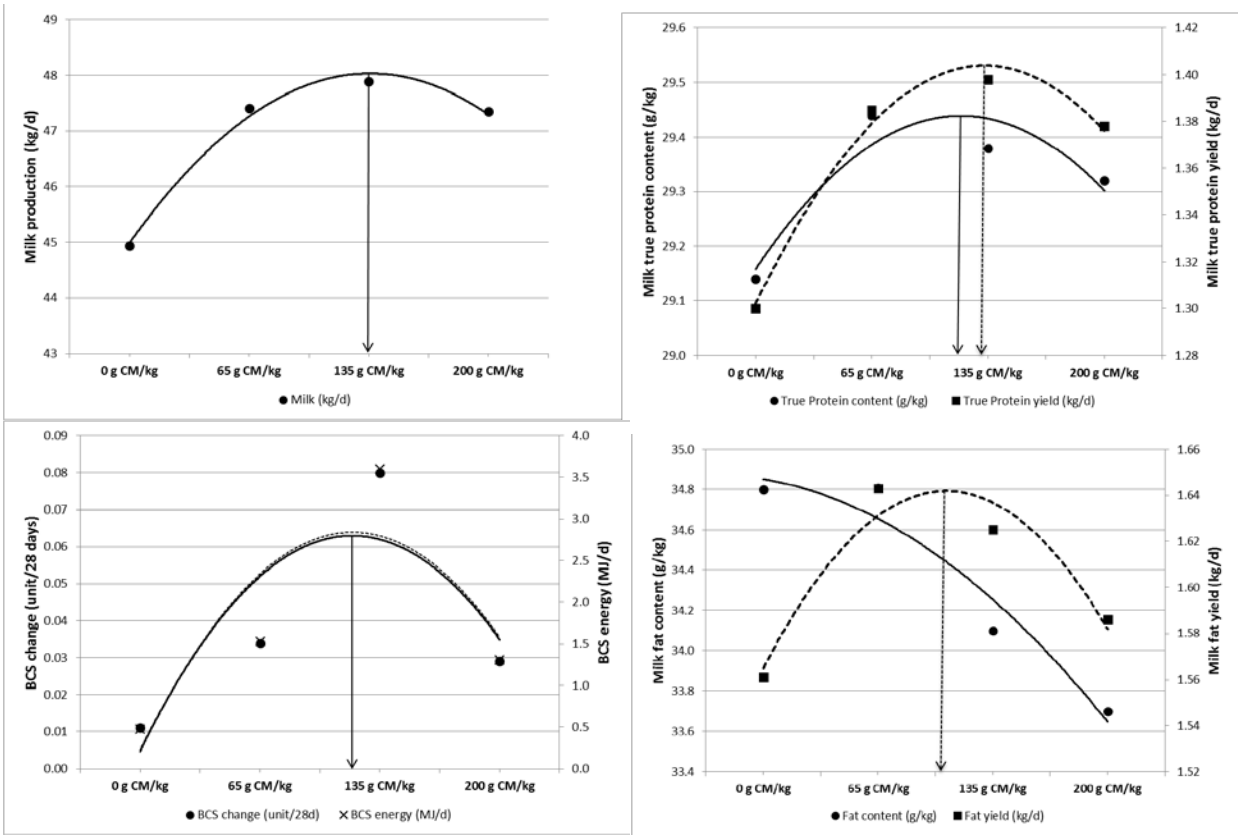

Figure 4.1: Production and body condition data with polynomial regressions (to the 2nd order) fitted to determine the maximum response treatment points.

4.3.2.3. Urine

Urine AL concentrations (Table 4.4) did not differ among treatments while CR concentrations decreased linearly (P=0.01) with higher CM inclusions, which could be due to increased urine volume. However, since total urine was not collected, the ratio of AL to CR was used to estimate the change in rumen microbial growth. The AL:CR ratio increased (P<0.01) with higher CM inclusion levels.

4.3.2.4. Blood plasma

All essential amino acids (EAA) except histidine (P=0.80) responded linearly, with threonine and histidine also responding quadratically, to an increased CM/HPDDG ratio in the ration (Table 4.5; P<0.01). By increasing, or decreasing, in the plasma as the ratio of the two ingredients in the ration changed, the impacts of the differences in the AA profiles of CM and HPDDG were demonstrated.

Table 4.4: Urine allantoin and creatinine concentrations (mg/L) for cows fed rations with different levels of canola meal and HPDDG[a]

	g CM/kg DM in the ration					*P*	
	0 g CM	65 g CM	135 g CM	200 g CM	SEM	Linear	Quadratic
*n = 40 cows**							
Allantoin (AL)	3360	3187	3396	3370	97.9	0.57	0.43
Creatinine (CR)	1082	980	1028	946	35.3	0.01	0.75
AL:CR ratio	3.12	3.29	3.36	3.61	0.088	< 0.01	0.58
PDC index[b]	640	672	690	737	17.1	< 0.01	0.65

[a] High protein, low fat, dried distillers grains (see Table 4.1).
[b] Purine derivative to creatinine index = $(AL_{adjusted}:CR)*(\text{Body weight (kg)})^{0.75}$.
* Only a group of 10 cows/pen were selected from the group of eligible urine cows for urine AL and CR analysis as these were the cows with repeated samples across periods.

Most of the EAA increased slightly between 0 and 65 g CM/kg, increasing at a faster rate from 65 to 135 g CM/kg before plateauing at 200 g CM/kg. Threonine and arginine kept increasing linearly from 65 to 200 g CM/kg. Leucine and phenylalanine decreased linearly with higher CM levels while methionine and histidine decreased from 0 to 65 g CM/kg, remained constant up to 165 g CM/kg before increasing slightly at 200 g CM/kg. The plasma lysine to methionine ratio increased with higher CM levels, with the optimum ratio of 3:1 (NRC, 2001) achieved between 65 and 135 g CM/kg.

Table 4.5: Free amino acid and urea concentrations (µg/ml) in plasma of cows fed rations with different levels of canola meal and HPDDG[a]

	g CM/kg DM in the ration					*P*	
	0 g CM	65 g CM	135 g CM	200 g CM	SEM	Linear	Quadratic
*n = 16 cows**							
Essential amino acids							
Threonine	10.9	10.2	12.1	14.0	0.48	< 0.01	< 0.01
Valine	30.4	30.6	33.3	34.4	1.00	< 0.01	0.42
Methionine	3.89	3.42	3.38	3.48	0.132	0.03	0.03
Isoleucine	14.3	14.2	15.9	16.5	0.51	< 0.01	0.30
Leucine	36.1	31.4	28.5	23.0	1.08	< 0.01	0.64
Phenylalanine	11.4	10.2	9.74	9.46	0.295	< 0.01	0.05
Tryptophan	10.5	10.5	12.2	13.5	0.50	< 0.01	0.06
Lysine	7.91	8.48	10.9	12.7	0.464	< 0.01	0.08
Histidine	8.79	7.85	8.10	8.63	0.239	0.80	< 0.01
Arginine	11.2	11.3	13.4	15.5	0.52	< 0.01	0.03
Lys:Met ratio	2.11	2.59	3.33	3.73	0.146	< 0.01	0.67
Non-essential amino acids							
Homocystine	0.80	0.88	1.07	1.10	0.048	< 0.01	0.60
Aspartic acid	1.13	1.06	1.14	1.19	0.107	0.38	0.41
Tyrosine	13.3	11.3	10.2	9.32	0.479	< 0.01	0.15
Serine	10.1	8.34	8.66	9.29	0.336	0.12	< 0.01

	g CM/kg DM in the ration					*P*	
	0 g CM	65 g CM	135 g CM	200 g CM	SEM	Linear	Quadratic
Glutamic acid	6.83	6.97	6.99	7.22	0.195	0.13	0.78
Glutamine	54.3	51.8	50.5	50.4	2.03	0.15	0.55
Glycine	24.0	21.2	23.9	27.7	1.40	< 0.01	< 0.01
Alanine	21.9	19.6	21.7	23.4	0.76	0.03	< 0.01
3-Methylhistidine	1.10	1.05	0.86	0.87	0.083	< 0.01	0.60
Urea	143	147	152	149	4.3	0.15	0.30
Ammonia	2.40	2.28	2.25	2.19	0.083	0.06	0.68

[a] High protein, low fat, dried distillers grains (see Table 4.1)
* Only a group of 4 cows/pen/period was randomly selected from the group of eligible blood cows and sent for amino acid analysis as this was sufficient to determine significant differences among treatments.

4.3.2.5. Partial net energy balance

Pen averages of response parameters per period were used to calculate the partial NE balance (Table 4.6) for each treatment. Calculated milk and total energy output changed quadratically with increasing CM inclusion levels (*i.e.,* highest values at intermediate CM inclusion levels). However, even though the total NE balance was highest at intermediate CM inclusion levels , the calculated dietary NE_L values did not differ (P=0.6) between the treatments, suggesting that dietary energy was used more efficiently at intermediate CM inclusion levels.

Table 4.6: Partial net energy balance for cows fed rations with different levels of canola meal and HPDDG[a]

	g CM/kg DM in the ration					*P*	
	0 g CM	65 g CM	135 g CM	200 g CM	SEM	Linear	Quadratic
n = 4 pens							
Maintenance energy (MJ/d)	44.3	44.1	44.2	44.4	0.18	0.73	0.57
Milk energy output (MJ/d)	129	136	135	133	1.1	0.16	0.04
BCS [b] energy (MJ/d)	0.6	1.6	3.5	1.2	1.33	0.67	0.43
Total Net Energy (MJ/d)	174	182	183	179	1.5	0.15	0.04
NE_L [c] (MJ/kg DM)	6.93	7.16	7.09	7.12	0.134	0.60	0.61

[a] High protein, low fat, dried distillers grains (see Table 4.1)
[b] Body condition score
[c] Net energy available for lactation

4.4. Discussion

In the current study optimum levels of CM in the ration for BCS change and milk production overlapped in the range of 120 to 135 g/kg DM. This corresponds with Mulrooney et al. (2009) where

numerical values for DM intake, milk yield and composition, BW and BCS were higher with higher inclusions of CM *versus* DDGS, especially at their $^{2}/_{3}$ CM treatment. Increasing or decreasing inclusion levels of CM from 135 g/kg DM in our study resulted in a general decline in cow performance, except milk fat concentration which was higher with higher levels of HPDDG compared to CM, which is also consistent with conclusions of Mulrooney et al (2009) and Christen et al (2010). Concerns about possible milk fat depression at high levels of DDGS, due to excessive dietary corn oil inclusion levels, were avoided by using HPDDG. However, since milk fat concentration decreased linearly with increased CM inclusion, although there was no numerical difference between the 0 and 65 g/kg inclusion levels, it is possible that the highest levels of HPDDG might have prevented further increases in milk fat concentration.

4.4.1. *Potential impacts of differences in dietary CP profile*

The four treatment rations actually fed were evaluated post-experimentally using the metabolic model Shield (Robinson, 2009) which calculates potential over- or undersupply of nutrients and estimates potential nutritional limitations to performance. It was known that, even though the CP concentration is very similar between CM and HPDDG, they have very different rumen degradability and AA profiles, with CM being primarily an RDP source, high in lysine, while HPDDG is an RUP source which is low in lysine. Model evaluations confirmed this by indicating that the rations with the highest HPDDG inclusion were limiting in RDP at only 0.84 of requirement, which would have limited microbial protein (MCP) production for the 0 g CM/kg treatment. In contrast, the 200 g CM/kg treatment was limiting in RUP at 0.62 of requirement, only supplying 0.86 of required absorbable protein (AP).

According to NRC (2001), a drop of dietary RDP below 95 to 105 g/kg DM may depress MCP synthesis. In our study, predicted RDP levels were 84, 89, 95 and 103 g/kg DM for the 0, 65, 135 and 200 g CM/kg treatments respectively. Boucher et al (2007) reported a maximum response of MCP synthesis when RDP was 100 to 108 g/kg DM, while MCP synthesis decreased at 116 g/kg DM, probably due to overproduction of ammonia. At 1330 g/d, soluble CP intake for the 0 g CM/kg treatment according to Shield was only 0.64 of the predicted optimum, and was below the optimum

level of 1200 g/d as suggested in a review by Robinson (1996). Since Robinson (1996) also reported a decline in bacterial N flow when rumen ammonia concentrations fell below 90 mg/L, or exceeded 110 mg/L, either due to negative feedback mechanisms or direct bacterial toxicity, predicted rumen ammonia levels in our study (*i.e.*, 62, 80, 100 and 113 mg/L for the 0, 65, 135 and 200 g CM/kg treatments respectively) suggest that MCP synthesis may have been limited at the 0 g CM/kg treatment. However, rumen ammonia concentrations of 123 and 128 mg/L were reported to be optimal for rumen bacterial growth by Reynal and Broderick (2005) and Boucher et al (2007) respectively. This suggests that RDP and ammonia could have been limiting MCP synthesis in the rumen, thereby reducing performance of cows in the all HPDDG treatments. However, the possibility of an oversupply of RDP, and therefore ammonia toxicity, at 200 g CM/kg does not seem to have occurred.

Previous studies have demonstrated that the urine purine derivative (PD) concentration can be effectively used as a non-invasive method to estimate intestinal flow of MCP from the rumen (Chen and Ørskov, 2003; Gonzalez-Ronquillo et al., 2003). Chen et al (1995) concluded that the PD to CR ratio in spot urine samples correlates well with intestinal flow of microbial purines and can be used as a qualitative indicator of rumen MCP supply, independent of urine volume, thereby obviating the need for total urine collection. Even though it is accepted that CR is excreted at a constant rate on a BW basis, daily CR excretion is related to body protein mass turnover and therefore varies among cows and studies (19 to 29 mg/kg BW; Valadares et al., 1999; Moorby et al., 2006). Thus our estimation of differences in MCP yield from the rumen is limited to relative measurements. Nevertheless, concentrations of AL and CR in our study were 20 to 50% higher than in these previous studies.

In Han et al (1992), Gonzalez-Ronquillo et al (2003) and Moorby et al (2006), lower DM intake, digestibility and milk yield could have been responsible for lower MCP synthesis, and therefore lower AL concentrations, while very high urine volumes diluted AL concentration (mg/L) in Valadares et al (1999). However when DM intake, milk yield and urine volumes similar to ours were reported (Vagnoni and Broderick, 1997; Reynal and Broderick, 2005), AL concentrations are consistent among studies. Our CR concentrations were corrected by a factor of 0.7 (based on our internal laboratory

results) to adjust for loss of CR after acid treatment to stabilize urine samples, which could be one reason why it is higher than in previous studies. The correction factor of 0.7 was obtained by calculating the difference in CR concentration between a urine sample treated with acid and the same urine sample prior to acid treatment. However, Chizzotti et al (2008) reported that heavier animals have lower body protein concentration, and therefore lower urine CR outputs per unit BW. The average BW of the cows in our current study was higher (673 *versus* 627 and 560 kg from Moorby et al (2006) and Gonzalez-Ronquillo et al (2003) respectively), but the BCS was relatively lower than in these studies. Since lean animals with a lower BCS may have a higher urine CR concentration per unit BW, expressing CR values as a function of metabolic BW and BCS converges the CR values among studies.

As it has been reported that urinary AL makes up an almost constant molar proportion of total PD, uric acid was not analyzed in our study and AL concentrations in urine samples were corrected to total PD using a factor of 0.91 (Vagnoni and Broderick, 1997; Valadares et al., 1999; Gonzalez-Ronquillo et al., 2003; Reynal and Broderick, 2005; Moorby et al., 2006), which was used to determine the purine derivative to creatinine (PDC) index as described by Chen and Ørskov (2003), thereby correcting the PD:CR ratio for metabolic BW to allow comparison among cows. In addition, this study included a correction for BCS to account for differences in lean body mass. The PDC index (Table 4.4) follows the same pattern as the AL:CR ratio, increasing linearly ($P<0.01$) with higher CM inclusions, strongly suggesting that MCP yield was not negatively affected by the increasing level of RDP due to increasing levels of CM in the ration. This suggests that a high level of rumen ammonia did not limit microbial growth on the 200 g CM/kg ration, which corresponds with Reynal and Broderick (2005) and Boucher et al (2007). It seems clear that increased levels of CM, up to 200 g/kg DM, continued to stimulate rumen MCP synthesis.

4.4.2. *Potential impacts of differences in dietary AA profile*

It is generally accepted that lysine is the EAA required in the largest quantities for milk production in high producing dairy cows. It has also been identified, together with methionine, as the 1st or 2nd limiting AA in corn-based dairy rations (NRC, 2001). Originating from corn grain, HPDDG is low in

lysine (NRC, 2001) and, since many contemporary US dairy rations are already high in corn products (Swanepoel et al., 2010), there is a strong possibility that lysine was limiting at the highest HPDDG inclusion level in our study.

Christen et al (2010) concluded that HPDDG delivered a more desirable AA profile for casein production, thereby increasing the TP concentration in milk, with a number of EAA being less limiting in HPDDG compared to CM. However the positive effect that adding HPDDG to the ration had on production only occurred up to 120 g CM/kg inclusion, after which production was reduced. A systematic review of the impacts of metabolizable lysine and methionine concentrations on cow performance (Robinson, 2010) showed that increased levels of corn protein in dairy rations depressed the concentration of lysine in AP and that rations with over 0.35 of total ration CP coming from corn products are responsive to supplemental lysine due to its limitation. In the context of our study, for the two treatments with the highest HPDDG inclusion, the proportions of total CP coming from corn products were 0.51 and 0.66, suggesting the possibility of a lysine deficit.

Plasma AA analysis (Table 4.5) showed that lysine concentrations increased linearly with higher CM inclusions, while plasma methionine decreased with higher CM inclusion, but only to the 135 g/kg level. The sharp initial decline in methionine from 0 to 65 g CM/kg suggests that lysine was the limiting AA in the all HPDDG ration, thereby leaving excess AA unused in plasma at 0 g CM/kg but, as more lysine was supplied with the 65 g CM/kg ration, both methionine and lysine were utilized and their concentrations in plasma declined. All other EAA followed the same general pattern, supporting the hypothesis of lysine being the limiting AA at the highest HPDDG level. Excess AA remained unutilized in blood until lysine was supplied with more CM at the 135 g CM/kg level, after which AA were utilized for production. Alleviation of the AA limitation and the subsequent decrease in concentrations of other AA in plasma corresponds with other studies (*e.g.*, Piepenbrink et al., 1998). The reduction in lysine with the all HPDDG ration, together with the decrease in MCP synthesis, resulted in a substantial reduction in lysine available for milk production, which likely explains the reduced performance for the all HPDDG treatment.

The reduced performance with the 200 g CM/kg ration cannot be attributed to decreased MCP synthesis since there was a linear increase in PD derived MCP flow with increasing CM inclusion levels. Milk protein yield usually increases linearly with increased flow of MCP up to a point at which something other than the total amount of AP limits milk protein production (Vagnoni and Broderick, 1997). Even though MCP provides between 0.40 and 0.93 of total protein reaching the small intestine (Djouvinov and Todorov, 1994; Robinson, 1996), the limited amount of dietary RUP is usually characterized by a limitation of specific AA at the intestinal absorptive site. The only EAA which decreased in plasma with increasing levels of CM were methionine, phenylalanine and leucine. However, in contrast to leucine which decreased linearly, both methionine (P=0.03) and phenylalanine (P=0.05) responded quadratically (*i.e.,* declined less rapidly) with higher CM inclusions, but only plasma methionine tended to increase from the 135 to 200 g CM/kg treatment. That leucine showed a huge quantitative decline in plasma, almost 0.50, with no quadratic effect, suggests that leucine was supplied over its requirement in all rations. Christen et al (2010) reported phenylalanine and leucine as the 3rd and 4th limiting AA in corn silage based rations when HPDDG and CM were fed. When methionine and lysine were added to a CM containing ration by Piepenbrink et al (1998), thereby alleviating their limitation, it increased milk protein concentration while leucine and phenylalanine concentrations in the plasma decreased, suggesting phenylalanine and then leucine as the 3rd and 4th limiting AA. This is also in accordance with Mulrooney et al (2009) who showed the same plasma AA pattern as in our study when CM *versus* DDGS was fed. Most studies comparing protein sources identify lysine as the first limiting AA (Piepenbrink et al., 1998; Mulrooney et al., 2009; Christen et al, 2010). However these studies use extraction efficiencies (*i.e.*, arteriovenous differences of AA concentrations in plasma after (venous) and before (arterial) the mammary gland as a proportion of AA in the plasma of coccygeal artery) to identify limiting AA and, since mammary uptake of lysine from the plasma usually exceeds its requirements for milk production (Lapierre et al., 2005; Rulquin and Pisulewski, 2006), we argue that it will always appear as 1st limiting (Nichols et al., 1998), regardless of ration fed. Thus if lysine is removed from the list of limiting AA in those studies, the only 3 remaining possibilities are methionine, phenylalanine and leucine.

A study in which methionine, lysine and branched-chain AA were infused (Appuhamy et al., 2011) reported that branched-chain AA promoted muscle protein synthesis with no additional milk protein response with infusion of leucine over methionine and lysine. Even though both lysine and leucine are taken up in excess of requirements, mainly to oxidize and synthesize other AA, Lapierre et al (2009) suggested that excess uptake of lysine across the mammary gland was required to maintain milk protein production while leucine oxidation decreased if leucine supply was limited, thereby indicating that excess leucine is not required to sustain milk protein yields. Bequette et al (1996) reported that increasing the supply of leucine to the mammary gland did not enhance milk protein output, but did increase its oxidation in the mammary gland. Lapierre et al (2002) also showed that only 0.16 of the increased supply of leucine available for absorption ended up in milk protein. This suggests that leucine was not the AA which was limiting production in the 200 g CM/kg ration, thereby suggesting that methionine and/or phenylalanine were the limiting AA.

4.5. Conclusions

Overall results under these conditions, which are representative of many contemporary US dairy rations, show that optimum levels for most response parameters overlapped in the range of 120 to 135 g CM/kg inclusion in ration DM. It seems clear that the high HPDDG ration was nutritionally limited by a combination of low MCP flow to the intestine and a low dietary delivery of lysine, resulting in a substantial reduction in lysine available for milk production. That predicted high rumen ammonia levels, due to the high RDP concentration of CM, did not limit MCP synthesis for the 200 g CM/kg ration suggests that total protein delivery to the intestinal absorptive site did not limit productive performance. Thus the limiting factor at the highest inclusion level of CM was likely availability of absorbable AA, with plasma concentrations suggesting methionine and/or phenylalanine as the most likely candidates.

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Chapter 5. Experiment 2: Effects of ruminally protected methionine and/or phenylalanine on performance of high producing Holstein cows fed rations with very high levels of canola meal

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 ration 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 ration 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 concentration, while decreasing (P<0.01) milk lactose concentration 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 concentration (P<0.01), while increasing milk protein concentration 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 concentrations increased (P<0.01) with both Met treatments and plasma tryptophan (Trp) concentrations decreased (P<0.01) with both Phe treatments. However, plasma Phe concentrations 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 expressed by the cows, redirecting the 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 concentrations. 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 rations 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.

5.1. 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 ration 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) concentration of CM, and resultant high rumen ammonia levels, did not limit microbial protein (MCP) production when CM was included in the ration 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 ration 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.

5.2. 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 28.3ºC 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).

5.2.1. *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.

5.2.2. 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 5.2). 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.

5.2.3. 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 degree of protection 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 (degree of protection) 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 a higher estimated degree of protection than the 527 g/kg reported by Sakkers et al. (2013) for the similarly protected Lys product. Therefore, rumen protection 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 protection) and 360 g/kg Phe (*i.e.*, 600 g/kg Phe multiplied by 600 g/kg rumen protection) 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.

5.2.4. *Sample collection, preparation and analytical methods*

5.2.4.1. 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^{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). Oven DM was determined as the gravimetric loss when dried at 105^{o}C for 3 h in a forced air oven (NFTA, 2006). Ash determination was based on gravimetric loss by heating samples to 550^{o}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).

5.2.4.2. 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 the electronic herd record system DairyComp 305 (DC305; Valley Agricultural Software, Tulare, CA, USA) 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.

5.2.4.2.1. 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).

5.2.4.2.2. Body condition score

A subgroup of ~140 cows/pen from the base group of ~195 cows/pen (see section 5.2.4.2.) 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.

5.2.4.2.3. 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 100 ml/L sulphuric acid until the final pH was reduced to <2 (in this case 1 ml) in order to prevent bacterial destruction of allantoin (AL), diluted with water to a final volume of 35 ml and frozen at -20^{o}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.

5.2.4.2.4. 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).

5.2.5. *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 concentration (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 concentration (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 using a coefficient of 0.906 to express AL concentration in total PD of 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 body weight (BW) of 673 kg (Swanepoel et al., 2014), as:

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

with coefficients for the endogenous contribution of PD and recovery of absorbed purines as PD in the urine of 0.385 mmol/kg $BW^{0.75}$ and 0.85 respectively.

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

[(X (mmol/d) x 70) / (0.116*0.83*1000)] x 6.25,

assuming an N concentration of 70 mg N/mmol for purines, and using a ratio of purine N:total N in mixed rumen microbes as 11.6:100. A coefficients for microbial purine digestibility of 0.83 were used while a factor of 6.25 converted microbial N to MCP.

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) x 4.184.

Net energy for lactation (NE_L) density (MJ/kg DM) of the diets 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:

Net energy output (MJ/d) / DM intake (kg/d).

5.2.6. *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 concentration > 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 concentrations 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. Effects were determined as pre-planned contrasts as defined by Steel and Torrie (1980). 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 $P \leq 0.05$.

5.3. Results

5.3.1. *Ration evaluation and intakes*

The chemical composition of the ingredients used in the TMR (Table 5.1) 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 5.2). At 169 g/kg, the level of CM was slightly lower than the targeted 180 g/kg, but it did not differ among treatments and was well above the suggested optimum level of 135 g/kg according to Swanepoel et al. (2014).

Table 5.1: 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.

The only difference in the ingredient profiles of the treatment rations 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).

5.3.2. *Animal measurements*

5.3.2.1. Intake, milk production and its composition

Dry matter intake (Table 5.3) 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).

Table 5.2: 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 (solvent)	169	169	169	169	1.3	1.00	0.85	0.71
Molasses, liquid	14.1	14.1	14.1	14.1	0.06	0.94	0.72	0.46
RPP Product[b]	0.00	1.03	0.00	1.01	0.014	<0.01	1.00	<0.01
RPM Product[c]	0.00	0.00	0.66	0.66	0.008	1.00	<0.01	<0.01
Alfalfa, fresh chop/haylage[d]	67.1	67.3	67.7	66.5	2.73	0.65	0.90	0.86
Wheat, silage	57.2	58.4	57.0	57.0	2.58	0.71	0.94	0.95
Corn, flaked grain	181	180	181	181	1.9	0.48	0.79	0.69
Corn, silage	164	164	164	164	5.5	1.00	1.00	0.96
Citrus, wet pulp (orange)	34.8	34.9	35.5	34.9	3.03	0.97	0.85	0.98
Nutrient profile, g/kg DM [e]								
Dry matter	557	546	554	555	9.7	0.22	0.74	0.84
Organic matter	921	923	921	923	1.0	0.28	0.98	0.28
Crude protein (CP)	160	160	161	159	4.1	0.98	0.92	0.86
ADICP[f]	64	63	64	63	1.9	0.46	0.94	0.48
aNDF[g]	318	313	317	317	2.8	0.16	0.80	0.70
aNDFom[h]	305	299	304	302	2.7	0.12	0.74	0.41
ADF[i]	223	216	222	217	2.7	0.08	0.72	0.13
Fat	44	44	45	44	1.0	0.58	0.68	0.88
Starch	172	173	164	175	6.0	0.96	0.29	0.70

* 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.

The PHE treatment had no effect on milk production or composition vs. Control (Table 5.3), 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) concentration, while decreasing milk lactose concentration (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 concentration (47.8 vs. 47.6 g/kg; $P<0.01$), while increasing the milk true protein concentration (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 concentration (30.6 vs. 30.1 g/kg; $P<0.01$), higher lactose concentration (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 concentration (34.7 vs. 34.1 g/kg) and milk energy density (2.90 vs. 2.88 MJ/kg) compared to M+P.

Table 5.3: 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

5.3.2.2. Body condition score

Body condition score (Table 5.3) 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).

5.3.2.3. Urine

Urine AL and CR concentrations (Table 5.4) 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 5.4: 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.

5.3.2.4. Blood plasma

There were no changes in plasma AA concentrations (Table 5.5) 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 concentrations were not impacted by any treatment. Due to higher plasma Met concentrations, both the MET and M+P treatments decreased the Lys:Met ratio (P<0.01).

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.

Table 5.5: 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.

5.3.2.5. Partial net energy balance

The Partial NE balance (Table 5.6) 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 5.6: 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.

5.4. Discussion

In many feeding situations, supplementation of Met has been shown to increase milk protein concentration (Chen et al., 2011) and/or yield (Čermáková et al., 2012; Osorio et al., 2013) as well as milk fat concentration (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 concentration, but only in a relatively low protein diet. Supplementation of Met in the peri-partal period to decrease the Lys:Met ratio from 3.4 to 2.9 only showed a tendency for milk protein concentration to increase (Osorio et al., 2013). Rulquin and Delaby (1997) also reported an increase in milk protein concentration 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 concentration 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 diet 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 changed energy metabolism, with less energy expressed as milk lactose and more as milk fat and protein, thereby shifting energy output within milk components, addition of Phe to the Met redirected energy utilization 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 concentration. 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 concentrations 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 concentrations 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 concentration 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 ration 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, 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).

Since Jaurena et al. (2005) showed that BCS gain reflects accretion of fat and muscle mass, the increase in BCS may further 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 gain. Also, since more glucose are 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 occured 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 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.

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 concentrations while also increasing milk protein concentration (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 concentration, 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'.

5.5. 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 concentrations. 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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Chapter 6. Experiment 3: Rumen microbial protein flow, and plasma amino acid concentration, spectrum in early lactation multiparity Holstein cows fed commercial rations

Abstract

Formulating and feeding rations that are nutritionally balanced to enhance microbial protein (MCP) production and post-ruminal delivery of absorbable protein, while balancing for specific amino acids (AA), requires accurate prediction of nutrients absorbed from the small intestine. The most common method of ration formulation involves factorial or empirical models which simulate ruminal fermentation and predict protein outflows. However, due to their inability to encompass all animal factors that affect digestion and absorption, metabolic models inadequately predict MCP synthesis in the rumen and passage of nutrients such as crude protein (CP) and AA to the small intestine. Limited attempts have been made to establish ideal concentrations and requirements of AA for ruminants, and interpreting results from experimental treatments using plasma AA concentrations does not relate experimental data to concentrations obtained when contemporary rations are fed to cows under commercial conditions, for which no data exists. A practical and simple on-farm method to obtain “real time” values directly from the cows is required to evaluate commercial rations and establish normal ranges of MCP flowing from the rumen and plasma AA concentrations under commercial circumstances feeding contemporary dairy rations. Urine purine derivative (PD) output has been shown to be an effective index of MCP supply to the intestine and spot urine samples can accurately predict MCP flow from the rumen under farm conditions. Taking blood samples from the tail vein of dairy cows is a practice easily performed on commercial dairy farms and concentrations of free AA in these plasma samples are representative of intestinally absorbed AA, and can be used as an index to predict limiting AA. Cow groups sampled from 20 dairies produced 45 (± 1.2) kg milk/day at 81 (± 2.2) days in milk (DIM). The study successfully documented ranges of urine estimated MCP flowing from the rumen (1703 (± 54.6) g CP/day), and plasma AA concentrations, in early lactation multiparity Holstein cows fed a range of contemporary CA rations under multiple ingredient profile combinations. The dairies selected to create this database were normal well managed operations with animal groups representing a narrow range of milk production and DIM. This database can therefore be used as a benchmark to compare high, low and normal levels for MCP flow and plasma AA concentrations, as well as a real time evaluation of formulated rations in order to pinpoint possible rumen microbial growth and/or absorbable AA problems in commercial groups of dairy cows.

Keywords: plasma amino acids; spot urine allantoin; microbial protein; California total mixed rations

Abbreviations: AA, amino acid; AL, allantoin; aNDF, amylase-treated NDF; aNDFom, aNDF free of residual ash; BCS, body condition score; BW, body weight; CM, canola meal; CP, crude protein; DIM, days in milk; DM, dry matter; $dNDFom_{30}$, 30 h *in vitro* NDFom digestibility; EAA, essential AA; LSD, least square difference; MCP, microbial CP; MP, metabolizable protein; NDF, neutral detergent fiber; NEAA, non-essential AA; NSC, non-structural carbohydrates; OM, organic matter; PD, purine derivatives; PDV, portally-drained viscera; RDP, rumen degradable CP; RUP, rumen undegradable CP; 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).

6.1. Introduction

It is widely acknowledged that dairy cattle must be fed nutritionally balanced rations to enhance microbial protein (MCP) production as well as deliver absorbable protein with appropriate balances of specific amino acids (AA) to the small intestine. However, the required levels of MCP and AA that should be targeted remain unclear.

Traditional methods to determine MCP synthesis and absorbable protein delivery to the small intestine are invasive, complicated, expensive, time consuming, imprecise and have unknown accuracy. Therefore the most common method of formulating rations currently involves factorial or empirical models which simulate ruminal fermentation and predict duodenal protein flow. Although the models can be used as tools to make basic nutritional decisions, by responding to changes in ration composition, they inadequately predict MCP synthesis and degradability of protein in the rumen and passage of crude protein (CP) and AA to the intestine (Bateman et al., 2001a,b; Hanigan et al., 2001) and cannot replace observations from cows. Further development of descriptors to better predict feed interactions and underlying biology of the cows remain.

Since specific AA requirements for ruminants are not known, they are generally estimated based on research with non-ruminants and/or assumed to be similar to the AA concentration of milk protein (Schingoethe, 1996), but little data is available to substantiate these assumptions and limited attempts to establish ideal concentrations of essential AA (EAA) in metabolizable protein (MP) have not been successful (Fraser et al., 1991 as referenced by Rulquin and Verite 1993; Doepel et al., 2004). Interpreting results from experimental treatments by utilizing plasma AA concentrations (as in Swanepoel et al., 2014) amongst different diets can highlight changes and suggest AA limitations amongst treatments, but it does not relate experimental plasma AA concentrations to concentrations that would occur when cows are fed contemporary rations formulated under commercial conditions, often driven by animal production.

In light of the difficulties of using models to predict limitations of MCP and plasma AA concentrations, as well as the lack of available conventional (*i.e.*, non-experimental) plasma data, there is a need for practical and simple on-farm methods of estimating real time MCP flow from the rumen and plasma AA concentrations directly from the animals to successfully evaluate commercial rations balanced for higher intestinal MCP and AA delivery, establish what the normal range would be under conventional commercial circumstances using contemporary rations without experimental alterations, and evaluate model predictions.

This study used multiparity cows fed a wide range of contemporary early lactation dairy rations in California (USA), in order to (a) determine the normal ranges of MCP flowing from the rumen, and plasma AA concentrations, in early lactation multiparity Holstein cows, to (b) benchmark their high, low and mean levels using sampling methods possible under commercial conditions in order to assist in evaluation of commercial rations formulated with or without the aid of metabolic models, and to (c) create a reference database to help interpret the biological meaning of treatment concentrations of these parameters under commercial and experimental conditions.

6.2. Materials and methods

6.2.1. *Farm and management*

A group of commercial dairy farms in California were identified and, from this group, 20 dairy farm co-operators were selected to participate. In order to ensure a representative range of MCP and plasma AA values, the farms represented all major dairy counties in California, fed a wide range of rations and had consulting nutritionists. Other required factors included use of a computerized herd record and management system, a computerized ration mixing and feeding program, monthly milk tests through county or private testing companies, having a pen of at least 150 early lactation multiparity cows, and generally good dairy management. Each farm was visited prior to the day of sampling to determine the location of the high group pen and obtain a backup copy of their herd management files, which were used to create lists of eligible cows prior to sampling (as described below).

6.2.2. *Sample collection, preparation and analytical methods*

6.2.2.1. Total mixed ration

On the day of sampling, a load of total mixed ration (TMR) fed to the target early lactation pen was sampled by taking 10 handfuls at evenly spaced intervals along the bunk-line immediately after feeding, but before the cows had access to it, and immediately sub-sampled to create a representative sample (Robinson and Meyer, 2010). These TMR samples were frozen until chemical analysis. The ingredient profiles of the TMR (g/kg dry matter; DM) for the target pen on each of the 20 farms was obtained from the dairy farmer or nutritionist.

All TMR samples were weighed, dried at 55^{o}C for 48 h, and allowed to air equilibrate at room temperature for 24 h before chemical analysis. 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^{o}C for 3 h in a forced air oven (NFTA, 2006). Ash determination was based on gravimetric loss by heating samples to 550^{o}C for at least 3 h (#942.05; AOAC, 2005). Total N was determined by a Leco method (#990.03; AOAC, 2005). Neutral detergent fiber (NDF) was determined using neutral detergent and heat (#2002.04; AOAC, 2006). Heat-stable amylase was used to remove starch and inactivate enzymes that may degrade the aNDF, and aNDFom does not include residual ash. Fat was quantified using the Randall modification of the standard Soxhlet extraction (#2003.05; AOAC, 2006) and starch was determined as total glucose minus free glucose multiplied by 0.9 (Smith, 1969). Rumen degradable CP analysis, during which all TMR were subjected to a rumen proteolytic simulation for 48 h (chosen to resemble mean retention times in the rumen), in a protease solution containing enzymes from *Streptomyces griseus* was the measure of undegraded CP (Krishnamoorthy et al., 1983). Soluble CP was analysed using the borate-phosphate procedure of Krishnamoorthy et al. (1982) and the 30 h ruminal *in vitro* NDFom digestibility ($dNDFom_{30}$) was measured according to Robinson et al. (1999) with the exception that *in vitro* baths were used instead of the DAISY® Ankom system. The 48 hour *in vitro* gas production procedure was completed at the UC Davis laboratory according to Menke and Steingass (1988) using two dry cows as rumen fluid donors and running duplicate syringes per TMR sample.

6.2.2.2. Animal measurements

Groups of 20 cows within dairy farm were selected from the target pens containing only multiparity cows to average 75 days in milk (DIM) and exclude cows in lactation 5 or higher. Milk yields from the cows were obtained from county or private company milk recordings from each farm's routine monthly milk test which had occurred within 4 days of sampling.

Body condition scoring (BCS) was completed on all cows within each group by the same trained scorer using the 5 point BCS system of Ferguson et al. (1994), which defines quarter points based upon anatomical characteristics of the cows. However, when a cow demonstrated characteristics which made it difficult to clearly classify her to a specific quarter point (*e.g.*, 2.00 *versus* 2.25), she was classed as intermediate.

After removal of the 4 cows with the highest, and the 4 cows with the lowest, BCS values immediately after scoring the 20 cows, blood was collected from the tail (coccygeal) vein of the remaining 12 cows using a 10 ml evacuated tube containing K_2 EDTA (Vacutainer, Becton Dickinson, Franklin Lakes, NJ, USA), kept in coolers with ice and centrifuged within 3 h at 2100*xg* for 15 min at 4^{o}C. Plasma was removed, transferred to duplicate Eppendorf tubes and frozen at -20^{o}C until a set of 7 samples/dairy was sent for analysis of physiological AA (*i.e.*, free plasma AA) and ammonia. Samples were acidified with sulfosalicyclic acid to precipitate intact proteins and AA quantified using a Beckman 6300 AA analyzer (Beckman Coulter, Inc., La Brea, CA, USA) with a lithium citrate buffer system and ion-exchange chromatography to separate AA followed by a "post-column" ninhydrin reaction detection system.

Spot urine samples were collected from any cow in the target pen which voluntarily urinated during the morning lockup for normal health and reproductive checks (~50 min/pen/day) and immediately placed in ice. However samples were only retained from cows adhering to all specifications listed above for blood collection cows, except that the DIM range for urine was expanded to 38 to 151 DIM. This resulted in each dairy having a group of 6 to 12 cows which met the appropriate characteristics. The specific gravity (SG) of each untreated urine sample was measured within 90 min using a digital handheld pen refractometer (Atago USA Inc., Bellevue, WA, USA) to

estimate urine volume (as described below). A small quantity of urine (7 ml) was combined with 1.4 ml of 100 ml/L sulphuric acid so that the final pH was reduced 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 AL according to Chen and Gomes (1992). Standards were prepared to create working concentrations of 20, 40, 60, 80 and 100 mg/L AL. Urine samples were thawed and centrifuged at 1200*xg* for 15 min at 20 to 22°C in order to remove precipitate which could influence the colorimetric reading and diluted 60 times to fit the standard curve. A duplicate standard curve was included at the start and end of each run to calculate AL concentrations in the urine samples which, with estimated urine volume, was used to calculate the flow of MCP from the rumen to the small intestine. Two inter-run standard samples were used in each assay run to assess variation among runs after which the average concentration of the inter-run standards over all runs was used to correct sample concentrations in each run. Each urine sample was analyzed in duplicate with the average used as the final concentration.

6.2.3. *Calculations*

Final oven DM was calculated as the air equilibrated DM (*i.e.*, dried at 55°C) multiplied by the lab oven DM (*i.e.*, dried at 105°C). Gas production (ml/g organic matter; OM) of the TMR samples were determined using the method of Robinson et al. (2004), and calculated as:

((Sample gas production/h since last recording) – (Blank gas production/h since last recording)) /

(TMR DM, g/kg) / (TMR OM, g/kg)

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

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

Total daily purine derivative (PD) excretion (mmol/day) was calculated as the sum of PD excreted in urine and milk of lactating dairy cows (Chen and Gomes, 1992), using a coefficient of 0.906 to express AL concentration in total PD of urine (obtained from values of Vagnoni and Broderick, 1997; Valadares et al., 1999; Gonzalez-Ronquillo et al., 2003; Reynal and Broderick, 2005; Moorby et al., 2006) and PD excretion in milk as a constant 0.05 of urine PD excretion (Chen and Gomes, 1992).

Microbial purines absorbed from the intestine (X, mmol/day) were calculated using the equation of Chen and Gomes (1992), using a constant body weight (BW) of 675 kg, as:

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

with coefficients for the endogenous contribution of PD and recovery of absorbed purines as PD in the urine of 0.385 mmol/kg $BW^{0.75}$ and 0.85 respectively.

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

[(X (mmol/day) x 70) / (0.116*0.83*1000)] x 6.25,

assuming an N concentration of 70 mg N/mmol for purines, and using a ratio of purine N:total N in mixed rumen microbes as 11.6:100. A coefficients for microbial purine digestibility of 0.83 were used while a factor of 6.25 converted microbial N to MCP.

6.2.4. *Statistical analysis*

Correlation analysis using backward elimination through the STEPWISE procedure of SAS (2012) was used to determine predictability of MCP and plasma AA concentrations on milk production and also from single analyte levels and ingredient profiles of TMR. The descriptive statistics function in the data analysis tool pack of Excel 2013 was used to calculate mean and standard errors for dairy characteristics and analytes, as well as to construct box-and-whisker plots with median, minimum, maximum, 20^{th} and 80^{th} percentile calculations for MCP and individual plasma AA concentrations. A least square difference (LSD) analysis was performed on urine MCP and plasma AA data to determine the lowest number of samples at which addition of more samples no longer reduced the LSD for the dataset. This point was used as an indication of the number of samples required to give an accurate representation of the sample group. Fitting a linear trend line within Excel 2013 generated r^2 values to evaluate the magnitude and importance of correlations amongst nutrients and analytes.

6.3. Results

6.3.1. *Dairy farm and total mixed ration characteristics*

Some characteristics of the dairy farms are in Table 6.1. The dairy farms milked 2677 (± 372) cows either 2 or 3 times a day with the target early lactation pens holding 255 (± 20) cows.

The TMR fed to the 20 target pens among dairy farms were relatively consistent in chemical profile (Table 6.1). The CP, starch, fat and aNDFom of the TMR among dairies were 165 (± 2.3), 198 (± 9.2), 47 (± 1.9) and 299 (± 5.1) g/kg respectively. The consistency in the TMR chemical profiles suggests that the nutritional consultants formulated the rations to standards generally consistent with NRC (2001) guidelines, where appropriate. For example, CP and NDF levels in the TMR were within NRC (2001) recommended ranges, while fat concentrations (47.2 g/kg DM) were slightly higher than the NRC (2001) recommendation of 30 to 40 g/kg DM, but slightly lower than the 56.8 g/kg DM previously reported in CA dairy rations (Swanepoel et al., 2010b).

Gas production at 4 h of incubation, indicative of fermentation of non-structural carbohydrates (NSC) immediately available to microorganisms in the rumen (Cone et al., 1997), were 102 (± 1.2) ml/g OM with 24, 30 and 48 h values of 264 (± 3.4), 278 (± 3.4) and 296 (± 3.7) respectively. These values tend to be slightly higher than values from Rauch et al. (2012), but variability among TMR in gas production was generally low.

Table 6.1: Some characteristics of the 20 dairy sites and the chemical composition of the total mixed rations (TMR) fed to the target pen of early lactation cows on the dairy sites

	Mean	SE
Dairy characteristics		
Total milking cows	2677	371.9
Milkings/day	2.5	0.11
Cows in target pen	255	20.4
TMR chemical components (g/kg DM)		
Dry matter (DM; g/kg)	577	12.0
Organic matter (OM)	914	2.3
Crude protein (CP)	165	2.3
Soluble CP[1]	281	10.7
Degradable CP[2]	506	14.6
Starch	198	9.2
Crude Fat	47	1.9
aNDFom[3]	299	5.1
$dNDFom_{30}$[4]	459	9.8

	Mean	SE
Gas Production (ml/g OM) @ hours of incubation:		
4 h	102	1.2
24 h	264	3.4
30 h	278	3.4
48 h	296	3.7

[1] Expressed as g/kg CP as buffer soluble CP (Krishnamoorthy et al., 1982).
[2] Calculated as 100 minus undegraded CP from *Streptomyces griseus* procedure (Krishnamoorthy et al., 1983).
[3] Neutral detergent fiber assayed with heat stable amylase, expressed exclusive of residual ash.
[4] 30 h ruminal *in vitro* aNDFom digestibility (g/kg aNDFom)

Overall, the main ingredients used are very similar to what has been used and fed in CA over the last decade (*e.g.*, Swanepoel et al., 2010b) even though their ingredient profiles and inclusions (Table 6.2) varied considerably amongst farms. Most TMR contained some form of alfalfa in addition to corn silage, almond hulls, corn grain, cottonseed and canola meal (CM), but there were numerous ingredients that were only used in a few rations (*i.e.*, corn earlage, corn gluten pellets, wheat millrun, rice bran, carrot and tomato wet pomace, soybean and cottonseed hulls, pomegranate wet pulp, sunflower meal, bakery waste). This wide variation in the ingredient profiles of the TMR allowed us to meet our objective of a wide range of TMR.

Table 6.2: Ingredient composition (g/kg TMR DM) of the total mixed rations (TMR) fed to the target pens of early lactation cows on the dairy sites

	n[1]	Mean[2]	SE
Forages			
Alfalfa, fresh chop	2	37.4	0.09
Alfalfa, hay	20	160	1.5
Alfalfa, haylage	6	88.4	1.79
Cereal, hay	3	21.1	0.49
Cereal, silage	9	121	3.1
Corn, earlage	2	167	9.0
Corn, silage	16	161	1.2
Sorghum, silage	4	64.3	1.26
Wheat, straw	5	21.0	0.37
Plant by-products			
Almond, hulls	15	101	1.0
Brewers grains, wet	4	37.0	1.14
Carrot, pomace/wet	1	16.0	-
Citrus, pulp/wet	6	13.6	0.43
Corn gluten, pellets	4	60.6	0.65
Corn gluten, wet	2	50.6	0.55
Cottonseed, hulls	1	41.2	-
Corn distillers grains, dry	11	78.9	0.84
Corn distillers grains, wet	4	60.2	1.00
Pomegranate, pulp/wet	1	33.1	-

	n[1]	Mean[2]	SE
Rice, bran	2	59.6	0.38
Soybean, hulls	2	48.4	2.32
Tomato wet, pomace	2	38.1	1.29
Wheat, midds/millrun	6	70.0	1.19
Grains, Whole seeds and Protein meals			
Corn, grain	19	187	1.1
Cottonseed, fuzzy upland	15	73.6	0.54
Cottonseed, pima cracked	5	44.9	0.85
Canola, expeller meal	1	73.3	-
Canola, solvent meal	19	77.8	0.85
Sunflower, expeller meal	1	81.7	-
Minerals and premixes			
Limestone	2	6.15	0.195
Mineral, premix	17	17.6	0.17
Sodium bicarbonate	6	11.0	0.22
Miscellaneous			
Bakery, waste	3	44.5	2.11
Fat, animal	1	13.5	-
Fat, vegetable	1	5.46	-
Fat, rumen inert	7	9.22	0.119
Molasses, liquid	10	23.1	0.30
Whey, liquid	10	30.6	0.34
Unidentified	3	41.9	0.53

[1] Number of dairies for which ingredient appeared in the TMR.
[2] Average inclusion level in the TMR which contained the ingredient.

6.3.2. *Characteristics of cows in the blood and urine sample groups*

The average milk production for the blood and urine sample groups were very similar at 45.1 and 44.4 kg/day respectively with relatively low variation amongst dairies (Table 6.3), as was expected since our cow groups were selected to be high producing cows in a narrow DIM range. The low average lactation number, 2.8, represents current replacement policies/practices applied on a large proportion of California dairy farms due to replacement animal costs being relatively low, increased use of sexed semen yielding higher proportions of heifers with desirable genetic potential in herds with little or no growth potential due to the local regulatory environment, and high beef prices, leading to high herd culling in order to prevent cows becoming sick (*i.e.*, requiring expensive treatments) or dying on dairy farms.

The blood cow group BCS averaged 2.63 with low variation amongst target pens. The difference in DIM between the blood and urine groups, with the urine group having a slightly higher average DIM of 89 compared to the average DIM of 73 in the blood group was due to the method of selection

prior to sampling, as blood cows were pre-selected to average 75 DIM prior to the sampling while urine cows were collected when they voluntarily urinated, and then post-selected to only retain urine from cows that were 38 to 151 DIM. This wider DIM range is reflected in the standard error of 3.9 for the urine group, which is higher than for the blood group of 0.5.

Table 6.3: Characteristics and analysis of the early lactation cows sampled for blood and urine on the dairy sites

	Blood group		Urine group	
	Mean	SE	Mean	SE
Pen characteristics				
Milk production (kg/d)	45.1	1.25	44.4	1.20
Days in milk	73.1	0.52	89.1	3.92
Lactation number	2.8	0.08	2.8	0.09
BCS[1]	2.63	0.036	-	-
Urine analysis				
Specific gravity (g/cm^3)			1.023	0.0007
Volume (L/day)			23.1	1.06
Allantoin concentrations (mg/L)			2445	101.7
Allantoin output (mmol/d)			318.9	8.81
Microbial crude protein (CP; g/d)			1703	54.6
Plasma analysis (µg/ml)				
Essential amino acids (EAA)				
Lysine (Lys)	10.6	0.33		
Methionine (Met)	3.54	0.099		
Histidine (His)	8.08	0.225		
Phenylalanine (Phe)	8.55	0.147		
Leucine (Leu)	21.7	0.67		
Valine (Val)	29.3	0.85		
Isoleucine (Ile)	14.1	0.38		
Arginine (Arg)	14.0	0.39		
Threonine (Thr)	13.1	0.46		
Tryptophan (Trp)	16.1	0.32		
Total EAA	139	3.9		
Non-essential amino acids (NEAA)				
Tyrosine (Tyr)	10.6	0.29		
Glutamine (Gln)	54.6	1.27		
Glutamic acid (Glu)	8.07	0.313		
Alanine (Ala)	23.9	0.56		
Serine (Ser)	9.74	0.159		
Glycine (Gly)	26.9	0.73		
Aspartic acid (Asp)	1.25	0.088		
Proline (Pro)	12.0	0.26		
Total NEAA	147	3.7		
Ammonia	2.8	0.07		

[1] Body condition score; Cows in the urine group were not scored.

6.3.3. *Urine analysis of cows in the urine sample group*

The average urine SG of 1.02 g/cm^3, and estimated volume of 23 L/day (Table 6.3), is similar to ranges reported by Holter and Urban (1992) for early lactation Holstein cows determined by total urine collection, supporting use of SG to estimate urine volume. The average estimated MCP flow from the rumen, 1703 g CP/day is in the range of previous studies in which MCP was directly measured in duodenal samples, as discussed by Swanepoel et al. (2015).

6.3.4. *Plasma amino acid concentrations of cows in the blood sample group*

Plasma AA concentrations (µg/ml), as averages, are in Table 6.3 with all AA having low variation, as indicated by the SE values, except for Asp which is more variable and consistent with our previous studies (Swanepoel et al., 2010a, 2014; Robinson et al., 2011). This is likely because Asp concentrations in dairy cow plasma are typically low, thereby resulting in small peaks during analysis making it difficult for software to separate the Asp peak from contaminating peak "tails".

6.4. Discussion

6.4.1. *Estimating rumen microbial CP production*

Estimating MCP synthesis in dairy cows requires accurate measurement of a marker entering the small intestine (intestinal cannulation) or passing out of the rumen (omasal cannulation). In order to determine the quality and quantity of protein available for digestion and absorption in the intestine, a number of measurements, usually based on microbial markers, or predictions are required. These include ruminal degradation of protein (RDP) and AA in each feedstuff and the conversion into MCP as well as rumen passage of MCP, with its AA profile, to the lower tract, post-ruminal digestibility and absorption of MCP, as well as metabolism of AA across gut tissues, portally-drained viscera (PDV) and liver. While methods to measure most of these parameters *in vivo* exist, they require cannulated animals and measuring procedures are complicated, expensive, time consuming, imprecise and have unknown accuracy (Clark et al., 1992). The urgent need for an on-farm method of estimating real time MCP flow from the rumen has been suggested as an indication of rumen performance and basis for feeding decisions by farmers and advisors (Dewhurst et al., 1996).

When microbial nucleic acids are digested in the intestine, the by-product PD, mainly AL, is excreted in the urine and can be easily measured. Urine PD output has been shown to be an effective index of MCP supply to the intestine (Chen and Ørskov, 2004; González-Ronquillo et al., 2003; Chizzotti, et al., 2008). Even though total urine collection is not feasible under farm conditions, collecting spot urine samples is easily performed on a routine basis. Studies designed to evaluate the accuracy of estimating PD output in urine have shown that PD excretion estimated by spot urine sampling was not different from total urine collection (Chizzotti et al., 2008), suggesting that spot urine samples can be used to accurately estimate MCP flow from the rumen under farm conditions. Chen and Ørskov (2004) suggested that variability of spot measurements is greater than for total collection and that more measurements should be made to reduce errors. However, compared to previous studies in which duodenal samples were collected and MCP directly measured in duodenal chyme (Robinson et al., 1985, 1994, 1996a, 1996b, 1998; Khorasani et al., 1993, 1994; Stensig and Robinson, 1997), our technique of MCP estimation using urine AL analysis correlates (Figure 6.1) with that technique, confirming that spot urine samples can accurately predict MCP flow from the rumen, albeit with unknown precision.

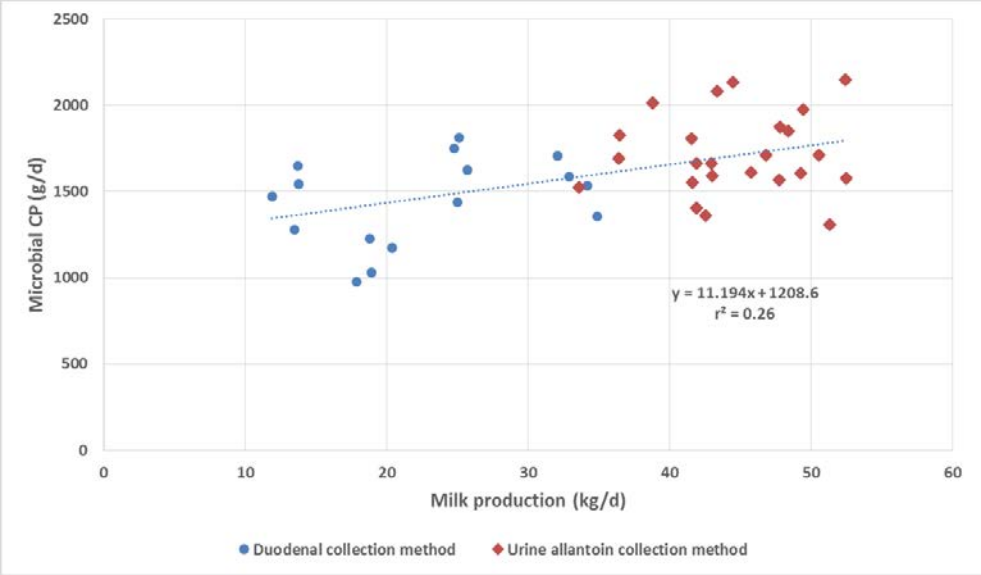

Figure 6.1: Microbial crude protein (MCP) flow from the rumen (g/d) as related to milk production (kg/d) for techniques using duodenally collected samples (Robinson et al., 1985, 1994, 1996a, 1996b, 1998; Khorasani et al., 1993, 1994; Stensig and Robinson, 1997) to analyze MCP (●) compared to the urine allantoin sampling technique (◆) from this study.

6.4.1.1. Ranges of estimated MCP flow from the rumen

Using the LSD analysis previously described, the results suggest that analyzing 8 urine samples/group is required to provide adequate representation of a group of cows. This number of 8 replicates/ration for spot urine samples was also suggested by Dewhurst et al. (1996) and Oetzel (2003) reported that at least 8 cows should be sampled for urine in order to have confidence that the mean values truly represents the entire population of animals.

The normal range of MCP flowing from the rumen when feeding a range of contemporary commercial diary rations is shown in the box-and-whisker plot in Figure 6.2. The average milk production per cow for the dairies in this study is similar to Swanepoel et al. (2015), but average MCP flow was lower (1703 *versus* 2092 g CP/day), which may be indicative of dietary factors affecting MCP. However, it is in the range of studies (763 to 1959 g CP/day) previously reported in the literature when duodenal samples were collected and MCP flow directly measured (Khorasani et al., 1993; Robinson et al., 1994; 1996; 1998; Stensig and Robinson, 1997; Timmermans et al., 2000; González-Ronquillo et al., 2003; Moorby et al., 2006), which suggests that our urine AL estimated MCP flows are biologically sensible.

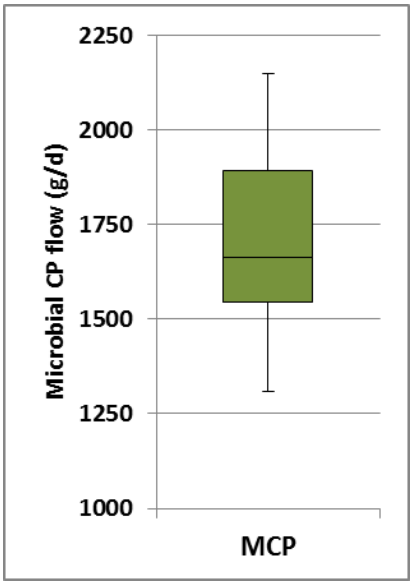

Figure 6.2: Box-and-whisker plot show the distribution of estimated microbial crude protein (MCP) flowing from the rumen (g/kg) with the shaded box indicating the median, upper quartile (80th percentile) and lower quartile (20th percentile) of measured data points and the whiskers representing the maximum and minimum measured data points.

This MCP data can therefore be used as a guideline of normal MCP values for cows fed contemporary CA dairy rations, for use as a benchmark of high vs. low levels in cases where metabolic models are not used to predict MCP outflow, or to evaluate model predictions of MCP flow to the duodenum. Thus values outside this measured normal range (*i.e.*, the shaded box representing 20th to 80th percentile of all data points) might suggest investigation of reasons for the high or low MCP values.

There is currently no commercial laboratory that does AL analysis in urine, even though it would not be difficult to develop since auto analysers and chromatography methods for PD analyses exist. However our colorimetric urine AL analysis technique is performed on a regular basis at the Animal Nutrition lab at the University of California in Davis, CA.

6.4.1.2. Possible drivers of MCP synthesis

Correlations between MCP flow and the TMR variables are in Table 6.4. There were only statistically significant slopes between estimated MCP flow from the rumen and the ash and aNDFom contents of the ration (P<0.01 and P=0.03 respectively), which are illustrated in Figure 6.3. The negative correlation with the ash concentration in the ration is biologically sensible, even though the extent of the change seems high. A review summarizing results from 41 experiments and 161 different rations (Clark et al., 1992) also showed a strong positive correlation (r^2=0.62) between OM intake and MCP flow to the small intestine. The positive correlation of MCP outflow with the NDF concentration of the ration confirms the role of structural carbohydrates in MCP flow from the rumen, as higher levels of structural fiber facilitates increased passage of microbes attached to fibrous particles thereby increasing MCP outflow (Van Soest, 1994). The lack of correlation between MCP flow from the rumen and milk production in this dataset (data not shown; r^2=0.02) is likely due to the narrow range of DIM and milk production of the cows, which was our objective. The NRC (2001) recommends an NDF concentration of 320 g/kg for cows producing 40 kg of milk/day, which is higher than our average of 299 g/kg. Higher levels of NSC in diets can cause rumen acidosis if there is inadequate NDF thereby reducing fiber digestion and MCP synthesis and outflow.

Table 6.4: Microbial protein flow (g/d) from the rumen as influenced by the nutrient and ingredient profiles of the total mixed rations (TMR; g/kg DM)

			P		
	Intercept	Slope	Intercept[1]	Slope[2]	r^2
Nutrient levels of the TMR (g/kg)					
Ash	3061	-158.1	<0.01	<0.01	0.46
aNDFom[3]	102	53.5	0.88	0.03	0.25
Crude protein	2243	-32.7	0.02	0.56	0.02
Fat	1548	32.9	<0.01	0.63	0.01
Starch	1601	5.1	<0.01	0.72	0.01
Gas production of the TMR (g/kg OM)					
4h	3417	-16.8	<0.01	0.11	0.14
24h	1862	-0.6	0.08	0.88	<0.01
30h	1949	-0.9	0.08	0.82	<0.01
48h	1677	0.1	0.12	0.98	<0.01
Ingredient levels of the TMR[4] (g/kg)					
Corn, grain	1405	16.8	<0.01	0.06	0.19
Canola, meal[5]	1909	-26.7	<0.01	0.09	0.16
Cottonseed, fuzzy upland	1577	22.8	<0.01	0.13	0.12
Corn, silage	1573	10.1	<0.01	0.16	0.11
Almond, hulls	1742	-5.0	<0.01	0.62	0.01
Alfalfa, hay	1737	-2.1	<0.01	0.81	<0.01
Corn distillers grains[6]	1701	0.4	<0.01	0.98	<0.01

[1] Probability that the intercept differs from zero.
[2] Probability that the slope differs from 1.
[3] Neutral detergent fiber assayed with heat stable amylase, expressed exclusive of residual ash.
[4] Only ingredients present in more than 50% of TMR were included in the table, except for whey, molasses and mineral premix which were not included due to low inclusion levels in all TMR.
[5] Combined solvent and expeller.
[6] Combined wet and dry.

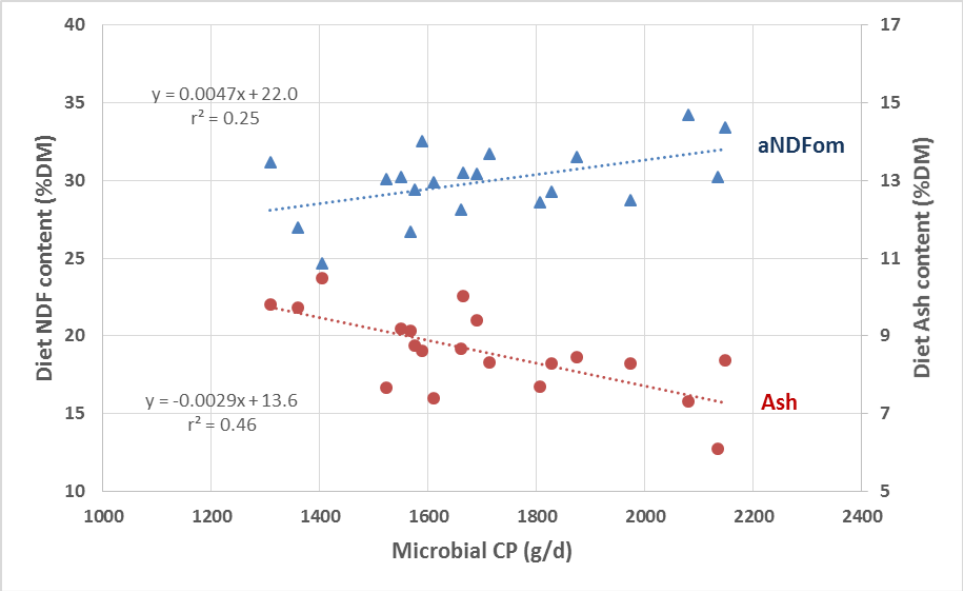

Figure 6.3: Relationships between the ash and neutral detergent fiber (aNDFom) levels of the TMR on a DM basis to microbial crude protein (MCP) flow (g/kg) from the rumen in the 20 groups of cows.

Stokes et al. (1991) reported that NSC contents higher than 240 g/kg of ration DM enhance MCP flow from the rumen. However the tendency for a negative correlation between MCP flow and the 4 h gas production (*P*=0.11) suggests that higher levels of NSC, in the 370 to 450 g/kg DM range in our study, did not support increased MCP flow from the rumen, possibly due to the offsetting lower NDF level in the ration. According to NRC (2001), there is very little benefit to increasing ration NSC above 360 g/kg for cows producing 40 kg milk/day, which could explain why our milk production is negatively correlated (Table 6.5; *P*=0.03) with ration NSC level. This suggests that higher NDF concentration in the ration, enhancing MCP outflow from the rumen, is more important to milk production than MCP synthesis *per se*, unless there is reason to believe that diet energy density is limiting production.

Table 6.5: Milk production (kg/d) as influenced by the nutrient profile of the total mixed ration (g/kg)

			P		
	Intercept	Slope	Intercept[1]	Slope[2]	r^2
Fat	23.92	4.33	<0.01	<0.01	0.47
NSC[3]	84.34	-0.99	<0.01	0.03	0.24
aNDFom[4]	29.66	0.49	0.09	0.38	0.04
Crude protein	28.32	0.97	0.17	0.42	0.04
Organic matter	83.22	-0.45	0.14	0.47	0.03
Starch	44.95	-0.03	<0.01	0.93	<0.01

[1] Probability that the intercept differs from zero.
[2] Probability that the slope differs from 1.
[3] Non structural carbohydrates calculated as 100 minus Ash, Fat, CP, NDF (g/kg DM).
[4] Neutral detergent fiber assayed with heat stable amylase, expressed exclusive of residual ash.

Since NSC increased in the rations at the expense of NDF (data not shown; r^2=0.54), with starch only consisting of 33 to 73% of total NSC, the diets may have supplied an excess of rapidly available energy that could not efficiently be utilized by the microbes (Clark et al., 1992). This could explain the lack of correlation between estimated MCP flow and starch concentration of the ration (Table 6.5), which corresponds with Cameron et al. (1991). In addition to the NSC levels in the ration being higher than NRC (2001) recommendations, lower than recommended RDP contents (84 vs 128 g/kg DM; Table 6.1) of the rations could have limited maximum MCP synthesis for all our rations, which is supported by the literature review by Santos et al. (1998) who reported that high RUP diets resulted in decreased MCP synthesis. No other significant MCP correlations existed with TMR nutrients (*i.e.*,

P>0.1) and there were no significant correlations between MCP flow from the rumen and individual TMR ingredients (*i.e.*, *P*>0.06).

6.4.2. *Determining plasma AA concentrations*

Attempts have been made to establish ideal concentrations of EAA in MP. Methods used to predict limiting AA, and therefore AA requirements, of lactating dairy cows are numerous. These include culturing bovine mammary cells in AA mediums to determine effects on milk protein synthesis (Clark et al., 1978; Arriola Apelo et al., 2014), evaluating changes in plasma AA concentrations and its impacts on milk and milk protein production during intravenous (Fisher, 1972) or post-ruminal supplementation of single or combinations of AA (Clark, 1975; 1978; Schwab et al., 1992a; 1992b; Weekes et al., 2006; Haque et al., 2012; 2013). Other methods using dose-response relationships between animal performance and N inputs (NRC 2001), or calculating the extraction efficiency of the mammary gland by determining arteriovenous differences of AA across the mammary gland (Mulrooney et al., 2009; Christen et al., 2010), as well as uptake of AA from the small intestine (and the mammary gland) relative to their output in milk (Piepenbrink et al., 1998) are also used. However, results are variable and inconsistent, with some methods using metabolic models to predict AA supplied from experimental rations, and data is interpreted differently amongst researchers with overall levels highly dependent on base rations, which varied amongst experiments. Indeed, the procedures used to determine AA requirements based on the AA profile of milk is based on the assumption that 90% of required AA is used for milk production, which is the case when cows produce 45 kg of milk/day (Schingoethe, 1996). However, cows used in the studies designed to determine AA requirements never produced more than 33 kg of milk/day, with DM intakes <20 kg/day, and most rations had a relatively low CP concentration as the rations were formulated for experimental purposes to achieve target limitations/excesses of AA, which are dissimilar to contemporary commercial rations. Also, supplementation of rations with ruminally protected AA or AA infusions could create AA imbalances with unexpected repercussions that are not understood. Animal responses to AA supplementation depends on the status and availability of other AA and nutrients in its surroundings (Liu et al., 2015), while the responsiveness of tissues to AA supplementation depends on postruminal

and surrounding nutrients (Swanson et al., 2003), and unexplored endocrine responses to AA supplementations can alter milk protein responses in the face of AA imbalances (Weekes et al., 2006). This supports the suggestion by Swanepoel et al. (2015) that AA are bioactive molecules that should be considered in total, rather than individually, while considering antagonisms and interactions amongst AA (Haque et al., 2013). These factors may explain the inconsistency of the interpretation of data obtained from these studies.

Interpreting plasma AA concentrations from experimental treatments does not relate treatment plasma AA concentrations to contemporary rations formulated under commercial conditions and, since metabolic models inadequately predict degradability of protein in the rumen and passage of CP and AA to the intestine, there is a requirement for practical and simple on-farm methods to measure real time plasma AA concentrations in the cows. Taking a blood sample from the tail vein of dairy cows is easily performed on commercial dairy farms and concentrations of free AA in plasma from the tail vein are representative of intestinally absorbed AA and can be used as an index to determine AA availability to the mammary gland and surrounding tissue (Clark, 1975; Schingoethe, 1996).

6.4.2.1. Ranges of plasma AA concentrations

The LSD analysis determined that a composite of 6 blood samples/group was adequate to represent a specific group of cows. 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 suggest only 6 samples since plasma AA concentrations have relatively low variability. Even with the large variation in ingredients amongst the 20 TMR, the variation in some of the most important plasma AA amongst dairies was small (see the box-and-whisker plots in Figure 6.4). While it is difficult to compare plasma AA analysis between studies due to variations attributed to laboratory technique, results from our study compare well to other studies reporting plasma AA concentrations obtained from the same laboratory (Swanepoel et al., 2014; 2015).

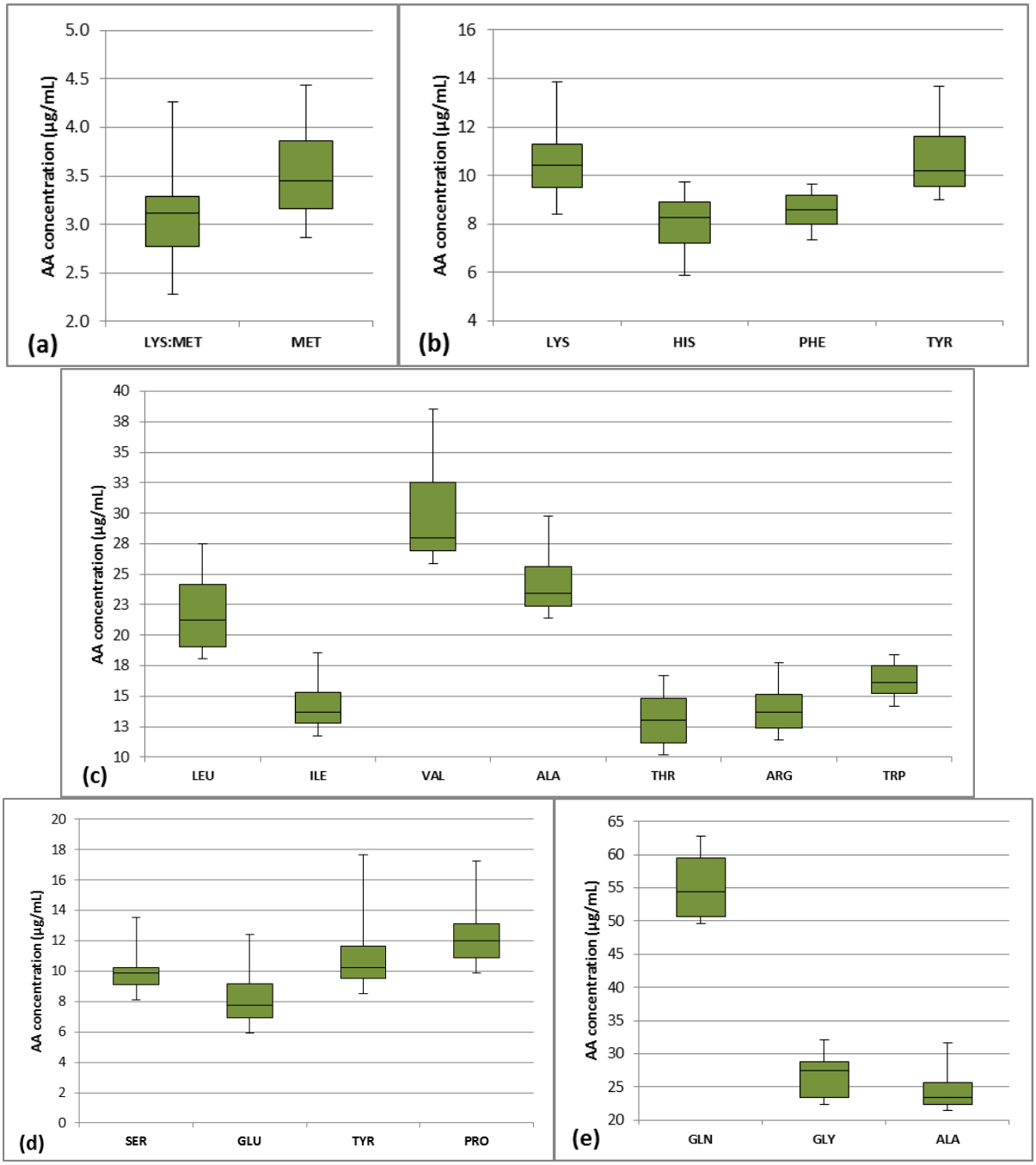

Figure 6.4: Box-and-whisker plots show the distribution of plasma AA concentrations (µg/mL) for EAA (a, b, c) and NEAA (d, e) with the shaded box indicating the median, upper quartile (80th percentile) and lower quartile (20th percentile) of measured data points and the whiskers representing the maximum and minimum measured data points.

These box-and-whisker plots can therefore be used as an index of normal plasma AA values for contemporary dairy rations and as a benchmark of high vs. low levels in cases where metabolic models are not used to formulate to specific AA concentrations, or a confirmation of acceptable ration formulations (*i.e.*, when analyzed plasma AA values fall within the shaded box representing 20th to

80th percentile of all data points). In contrast, investigation of possible ration formulation issues may be suggested when analyzed plasma AA values are outside of their boxed range, especially for potentially limiting EAA.

Methods to analyse plasma AA are readily available at many commercial laboratories, thereby presenting an opportunity to obtain "real time" profiles of AA available for milk production, without analyzing the rations fed or estimating AA digestibility and/or duodenal AA flow data.

6.4.2.2. Possible drivers of plasma AA concentrations

6.4.2.2.1. Correlations of plasma AA with MCP flow from the rumen

Even though it is accepted that 40 to 80% of the AA requirements of a lactating cow is supplied through MCP flowing to the small intestine (Sniffen and Robinson, 1987) and that it is a higher quality protein than from most feeds (Schingoethe, 1996), MCP flow from the rumen did not affect plasma AA concentrations. It could be that the contribution of MCP to the total supply of AA is already so large that the variation in MCP among diets led to changes that were too small to detect, and that it is the AA profile of rumen undegradable CP (RUP) that affected plasma profiles. Due to the relatively narrow CP range of the 20 dairy rations (*i.e.*, 165 ± 2.3 g/kg DM), it is likely that feeding one protein takes away from others (Schingoethe, 1996), which was reported by Clark et al. (1992) when the MCP flow to the intestine was not affected by source of protein in the ration, but passage of AA to the intestine were altered, probably due to difference in RUP among dietary protein sources.

However, this lack of change in plasma AA concentrations do not preclude changes in MCP pools and shifts in microbial AA profiles due to changes in TMR ingredient profiles and/or feed additives, as suggested by Clark et al. (1992), since our method of MCP analysis estimates total microbial N, not specific AA flows.

6.4.2.2.2. Correlations of plasma AA with nutrient profiles of the TMR

Even though there were no correlations of plasma AA concentrations with ration CP, OM, aNDFom or fat levels (r^2<0.16), there were correlations of AA with RUP and starch concentration of the rations (Table 6.6).

The lack of a correlation of plasma AA concentrations with ration CP levels was expected since the protein provided by the ration is fundamentally changed by microorganisms in the rumen, digesting it into MCP, shorter peptide chains and free AA that pass to the lower digestive tract for absorption. While Clark et al. (1992) reported that additional dietary CP increased AA supply to the intestine, it did not affect MCP synthesis. A data review by Patton et al. (2015) also confirmed that, even though plasma concentrations of EAA increased linearly with model predicted duodenal flows (based on the assumption that ration balancing models predict duodenal EAA flows with acceptable accuracy and precision), the nutritional content of the rations did not affect plasma EAA concentrations.

Table 6.6: Correlations of plasma amino acid concentrations (µg/mL) and the nutrient profile of the rations (g/kg DM) of the 20 dairy sites

	RUP[1]		Starch	
	P Slope[2]	r^2	*P* Slope[2]	r^2
Essential amino acids (EAA)				
Thr	0.01	0.31	0.14	0.12
Trp	0.05	0.19	0.66	0.01
Leu	0.19	0.09	0.18	0.10
Arg	0.23	0.08	0.39	0.04
Lys	0.24	0.08	0.65	0.01
Phe	0.26	0.07	0.16	0.11
Ile	0.27	0.07	0.19	0.10
Val	0.44	0.03	0.10	0.14
His	0.47	0.03	0.04	0.22
Met	0.97	<0.01	0.38	0.04
Total EAA	0.39	0.04	0.29	0.06
Ratio Lys:Met	0.28	0.06	0.19	0.09
Non-essential amino acids (NEAA)				
Gly	0.03	0.23	0.14	0.12
Ser	0.04	0.21	0.02	0.25
Ala	0.06	0.18	0.05	0.20
Tyr	0.19	0.09	0.86	<0.01
Gln	0.19	0.09	0.03	0.23
Asp	0.74	0.01	0.87	<0.01
Glu	0.80	<0.01	0.48	0.03
Pro	0.87	<0.01	0.47	0.03
Total NEAA	0.09	0.15	0.02	0.27
Total AA	0.10	0.14	0.56	0.02
Ammonia	0.73	0.01	0.75	<0.01

[1] Rumen undegradable protein. All CP, aNDFom and Crude fat r^2<0.16. Organic matter r^2<0.2 (except for ammonia r^2=0.35) and so are not listed.
[2] Probability that the slope differs from 1.

A correlation of some AA with RUP level in the ration supports previous findings that outflow of RDP in these rations played a larger role in plasma AA profiles than dietary CP or MCP flow from the rumen *per se* (Clark et al., 1992). However, Boucher et al. (2007) showed that even though increased RDP concentrations increase MCP flow to the omasum, it does not change the flow of AA to the omasum. That RUP is negatively correlated to Thr, Trp, Ser and Gly ($P \leq 0.05$) suggests that the higher RUP fractions in some rations, possibly at the expense of MCP, did not supply adequate amounts of these AA. Very little is known about the importance or functions of Ser, but it has been linked to cellular energy metabolism as well as production on Gly, Trp and Thr, all of which were also negatively correlated ($P \leq 0.05$) with RUP levels in the ration. Starch was also correlated with plasma Ser concentrations, together with Gln ($P \leq 0.03$), and both AA are linked to cell energy metabolism, while Ala was correlated to both RUP (negatively; P=0.06) and starch (positively; P=0.05). There was also a negative correlation between the starch level in the ration and plasma His concentrations ($P<0.04$). Since His residues are important components of salivary, and pancreatic, α-amylase with uncertain roles in amyloclastic activity of the amylase enzyme (Ishikawa, et al., 1992; Tseng et al., 1999), this could explain the utilization of His as starch levels in the rations increased.

As with MCP, no correlation existed between RUP levels and milk production, which may be expected since a 12 year literature review reported that higher RUP levels in the diet does not consistently improve lactational performance, even though it changes plasma AA profiles (Santos et al., 1998). However there was a positive relationship ($P<0.01$) between milk production and fat concentration of the TMR (Table 6.5) which suggests that energy available to the animal, rather than protein and AA concentrations, were major drivers of milk production. According to a meta-analysis by Rabiee et al. (2012) of TMR fed systems, and a review by Schroeder et al. (2004) of pasture based systems, fat supplementation to the diet of dairy cows always has a positive effect on milk production regardless of the production system or fat saturation profile.

6.4.2.2.3. Correlations of plasma AA with ingredient profiles of the TMR

As expected, there were correlations between some major TMR ingredients and AA concentrations in the plasma, although most were relatively weak (Table 6.7). The only ingredients

which appeared in all 20 rations were alfalfa hay and CM, and the correlations were positive for increasing inclusion levels of these ingredients and all the AA in the plasma, except Gly, Gln, Ser and His in the case of alfalfa hay and Phe, Tyr and Leu in the case of CM. This corresponds with the lower concentrations of these AA in the two ingredients. It was interesting that the same AA that were negatively correlated with higher levels of RUP in the ration, were also negatively correlated to alfalfa hay inclusion levels in the TMR. Contrary to CM's higher correlation with the total non-essential AA (NEAA; r^2=0.33), alfalfa hay was more correlated with the total EAA (r^2=0.24). All major corn protein sources (*i.e.*, corn grain, corn silage, corn distillers grains) had a negative relationship with plasma Lys (r^2=0.22, 0.30 and 0.25 respectively) when included at higher levels in the TMR.

Table 6.7: Correlations of plasma amino acid concentrations (µg/mL) and the ingredient profile of the rations (g/kg TMR DM) of the 20 dairy sites[1]

	Canola meal[2]	Alfalfa, hay	Corn, grain	Corn, silage	Corn distillers grains[3]
Essential amino acids (EAA)					
Lys	0.14	0.23	0.22	0.30	0.25
Met	0.12	<0.01	0.01	0.05	0.01
His	<0.01	0.02	0.11	0.02	0.04
Phe	0.08	0.08	0.08	0.08	0.09
Leu	0.01	0.02	0.04	0.06	0.28
Val	0.01	0.34	0.25	0.30	0.05
Ile	0.01	0.36	0.21	0.31	0.07
Arg	0.04	0.21	0.19	0.28	0.24
Thr	0.30	0.01	<0.01	0.02	0.19
Trp	0.08	0.28	<0.01	0.02	0.38
Total EAA	0.04	0.24	0.18	0.26	0.04
Ratio Lys:Met	<0.01	0.20	0.31	0.14	0.30
Non-essential amino acids (NEAA)					
Tyr	0.01	<0.01	0.01	0.02	0.29
Gln	0.27	0.11	0.01	<0.01	0.02
Glu	<0.01	0.08	0.09	<0.01	<0.01
Ala	0.20	0.02	0.05	<0.01	0.11
Ser	0.28	0.03	0.05	0.04	0.10
Gly	0.29	0.16	0.04	<0.01	0.12
Asp	0.01	0.31	0.06	0.02	0.01
Pro	0.03	0.13	0.02	0.08	<0.01
Total NEAA	0.33	0.03	0.06	<0.01	0.04
Total AA	0.23	0.05	0.02	0.14	0.07
Ammonia	0.07	0.15	0.05	0.04	<0.01

[1] Values reported are r^2.
[2] Combined solvent and expeller.
[3] Combined wet and dry.

A strategic review suggested that Lys becomes the limiting AA when high levels of corn CP are fed (Robinson, 2010), which is consistent with the negative correlation between the proportion of TMR CP (g/kg) from corn CP and plasma Lys concentrations (r^2=0.51) in the current study (Figure 6.5a). In addition, high corn CP inclusion levels also had a strong negative correlation (r^2=0.56) with plasma Arg concentrations.

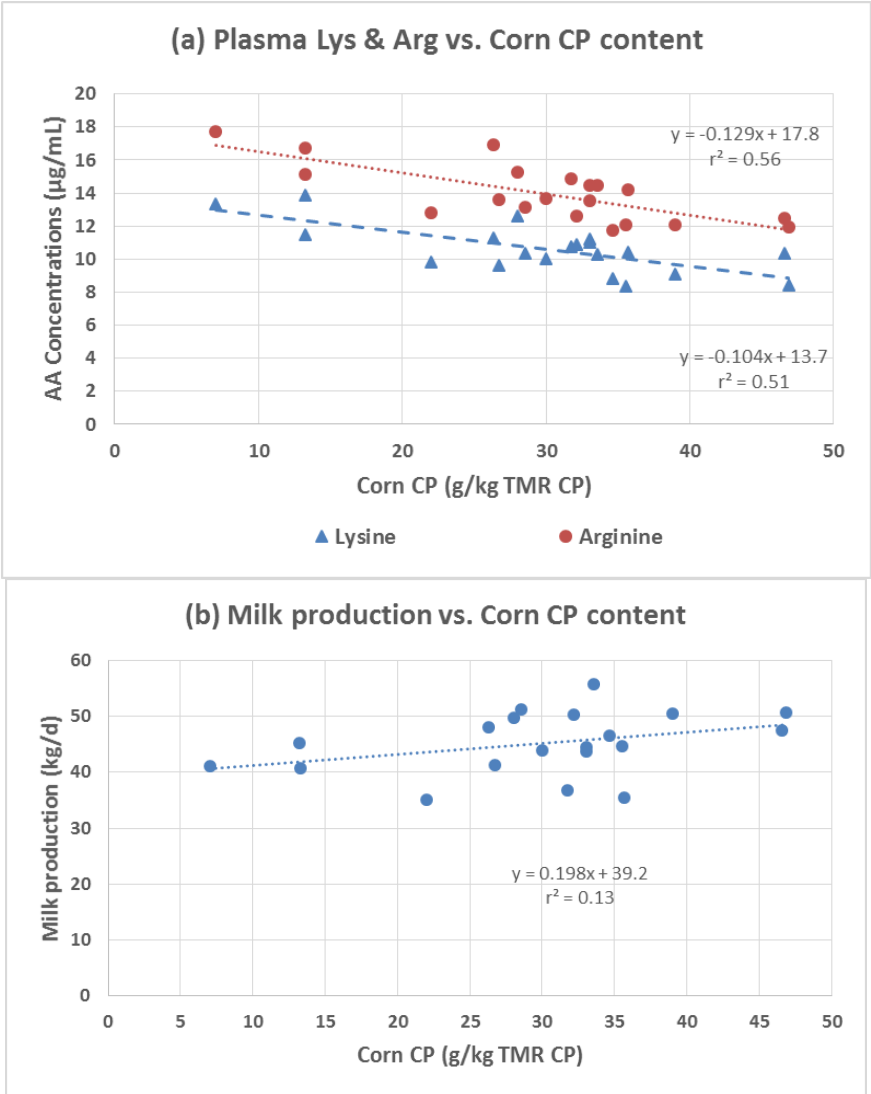

Figure 6.5: Relationship between the contribution of corn crude protein (CP) to total TMR CP (g/kg CP) and plasma AA concentrations (µg/ml) and milk production (kg/d) in the 20 groups of sampled cows.

6.4.2.2.4. Correlations of plasma AA with milk production

There were no correlations between any plasma AA and milk production. Even Lys, Met and the Lys:Met ratio had a poor relationship (*i.e.*, P>0.60) with milk production (Figure 6.6a & 6.6b).

However, contrary to expectation, even with corn CP contributions as high as 470 g/kg of total TMR CP, it had a slight positive correlation (r^2=0.13) with milk production (Figure 6.5b), which was mirrored by estimated MCP flow from the rumen (r^2=0.05).

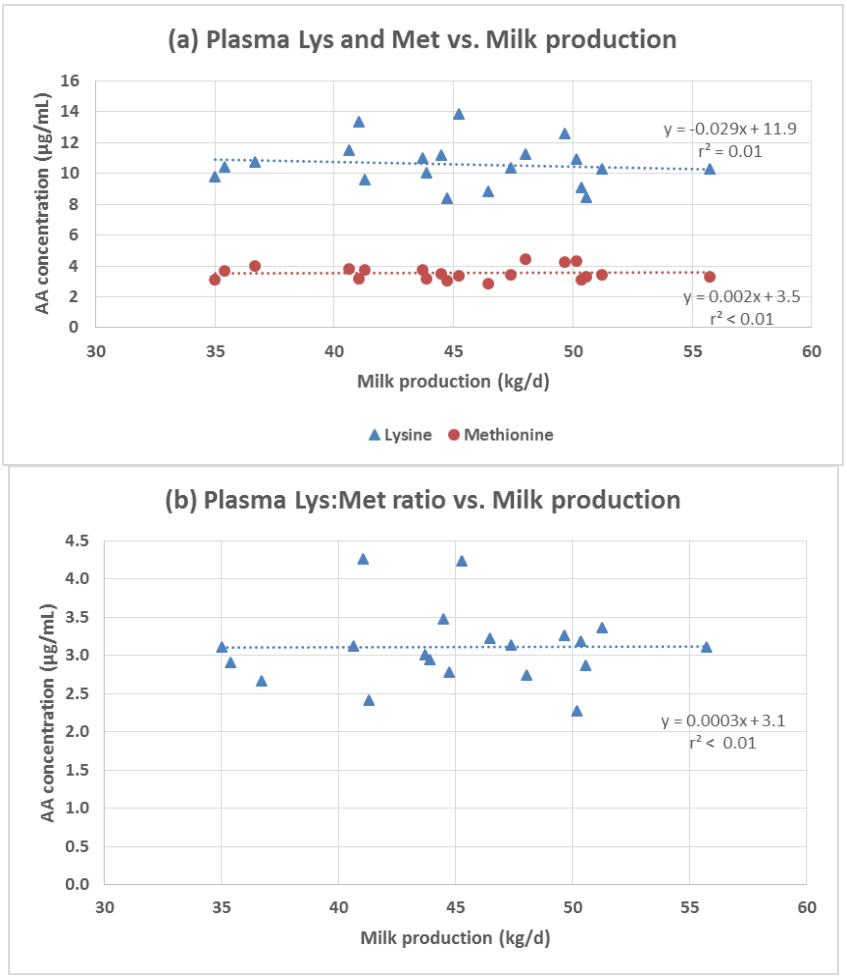

Figure 6.6: Relationship between methionine (Met), lysine (Lys) and their ratio to milk production (kg/d) in the 20 groups of sampled cows.

This concurs with the suggestion that ration formulations by dairy consultants include other complementary CP sources that offset imbalances in AA profiles, and that the lack of a negative impact from low Lys plasma concentrations could be due to an increased contribution from MCP (which is higher in Lys than any dietary protein source) to total protein absorbed by the cows.

6.4.2.2.5. Inter-correlations of plasma AA

The correlations amongst many plasma AA concentrations (Figure 6.7) resembles the network of interactions which have been documented among nutritionally important minerals (Jurgens et al.,

2012). The AA involved in interactions with other AA included the branched-chain AA Ile, Val and Leu, as well as Met and Phe.

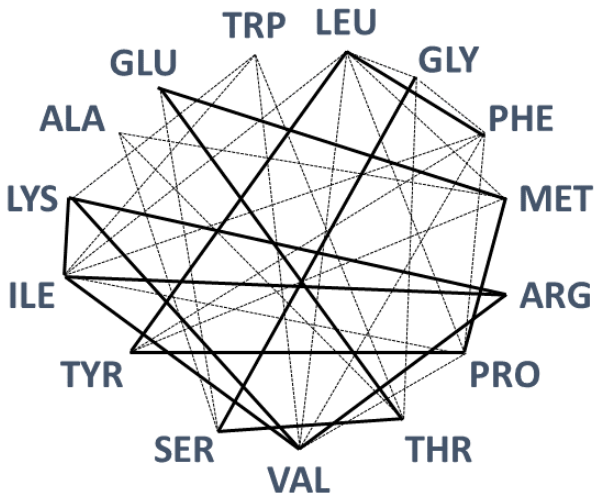

Figure 6.7: Inter-relationships amongst plasma AA concentrations in the 20 groups of sampled cows. Dotted lines represent correlations of r^2=0.20-0.49, solid lines for correlations of r^2>0.50.

Two AA that should be considered in combination are Phe and Tyr since the conversion of Phe to Tyr in the liver is regarded an obligatory step when Phe is catabolized and, since Phe is the primary source of Tyr this makes Tyr a conditionally EAA (Matthews, 2007). Indeed this was confirmed since Phe and Tyr were positively correlated (r^2=0.48; Figure 6.8a), although adding the two AA did not improve their correlation to milk production over that of Phe alone (Figure 6.8b), suggesting that plasma AA concentrations are not predictors of milk production *per se.*

6.5. Conclusions

The average estimated MCP flow from the rumen (*i.e.*, 1703 g CP/day) was slightly lower than reported previously and MCP flow was likely limited by generally lower than optimal NDF (for MCP outflow) and RDP levels in the rations, together with a generally too high supply of rapidly fermentable carbohydrates. The positive correlation of plasma AA with the RUP levels in the ration, and the lack of correlation with estimated MCP flow, suggests that the former played a larger role in plasma AA profiles than MCP, which could support the lack of maximum MCP synthesis.

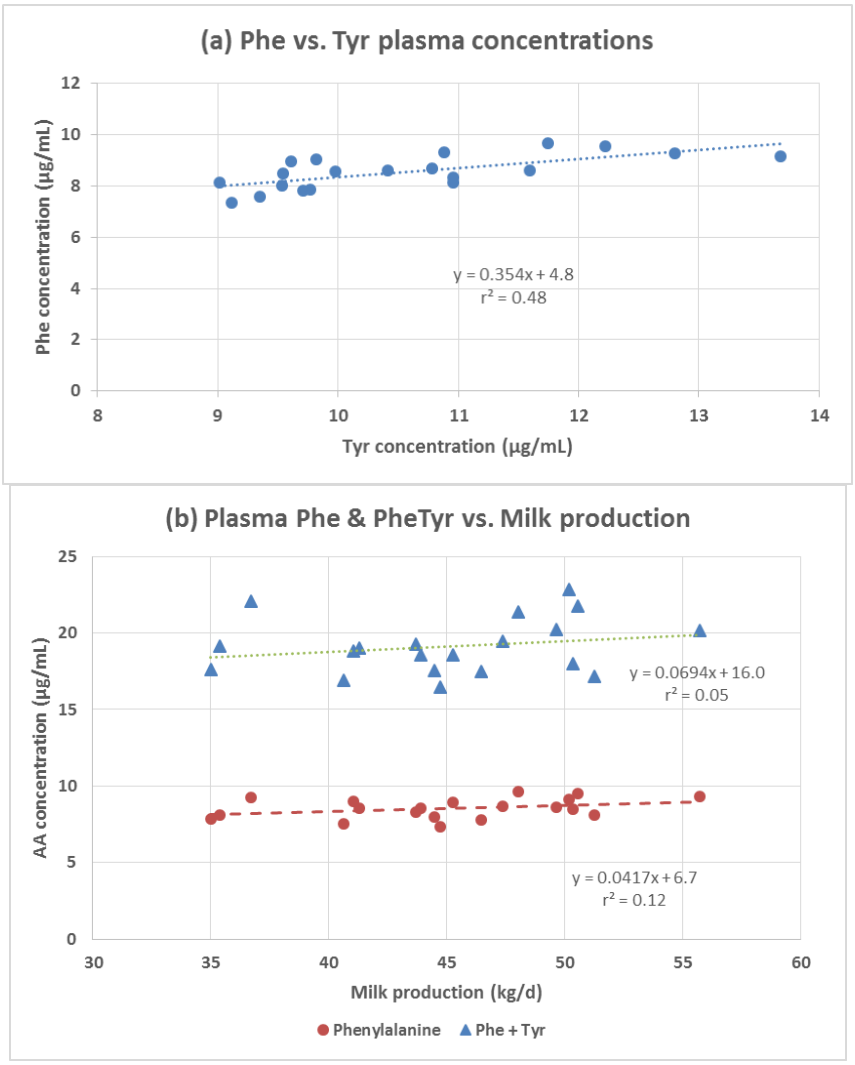

Figure 6.8: Relationship between Phenylalanine (Phe) and Tyrosine (Tyr) and to milk production (kg/d) in the 20 groups of sampled cows.

The lack of correlation between milk production and either MCP, plasma AA or RUP levels, but with a positive correlation with ration fat concentration, suggests that energy available to the cow, rather than protein, was driving milk production.

This study documents normal ranges of urine estimated MCP flowing from the rumen, and plasma AA concentrations, in early lactation multiparity Holstein cows in California fed a wide range of contemporary ration strategies with multiple ingredient profile combinations. The farms selected to create this database were well managed with animal groups representing a narrow range of production and DIM. This database can therefore be used as a benchmark to compare high, low and normal levels for these parameters as a real time guideline for normal MCP and AA values when models are not

used in feed formulation. The data can also be used as confirmation of acceptable ration formulations, or as a suggestion to investigate possible formulation issues, and pinpoint possible rumen microbial growth and/or absorbable AA problems in commercial groups of dairy cows. It also provides a database to interpret experimental study treatment levels of MCP and plasma AA, and to aid in determining the biological sensibility of these values under such conditions.

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Chapter 7. Experiment 4: Impacts of increased levels of ruminally protected phenylalanine, supplemented to rations containing high levels of canola meal, on performance of high producing Holstein cows

Abstract

Even though studies supplementing Phe to dairy cattle are rare, it has been identified as being 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 feeding 7.5 g of intestinally absorbable Phe/cow/day suggested it was not enough to support increased milk production since supplemented Phe was primarily used to support increased body condition score (BCS; Swanepoel et al., 2015). Our objective was to determine if supplementing 15 g of intestinally absorbable Phe/cow/day in a ruminally protected (RP) form (HCMP) to a ration containing 170 g CM/kg (HCM) would support increased milk production after fulfilling its apparent 1st priority of restoring previously mobilized peptides to muscle protein, thereby regaining animal performance expected to be lost with higher CM inclusion levels (*i.e.*, 130 g/kg (LCM) to 165 g/kg (HCM)) based upon (Swanepoel et al., 2015). 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 concentration (P=0.02) with Phe addition. Even though plasma Phe concentrations only differed numerically between treatments, Phe supplementation resulted in energy being diverted towards BCS gain as in Swanepoel et al. (2015), but this time not at the expense of milk components, suggesting that the higher Phe supplementation level was successful in supplying 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 into Tyr, which is supported by a small increase in plasma Tyr concentrations. Interestingly, addition of Phe to the HCM ration resulted in an increase in whole tract aNDFom and ADF digestibility. Phenylalanine released into the rumen when the RP Phe product was fed could have 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 NE output decreased with the HCM treatment, but was corrected to the level of the LCM ration for the HCMP treatment suggesting that an even higher level of Phe supplementation may have additional benefits on milk production.

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).

7.1. 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 to be first limiting (Nichols et al., 1998) regardless of the ration fed. This suggests that amino acids (AA) identified as potentially limiting after Lys, such as Met, Phe and Leu, could be more probable limiting AA. As summarized by Schwab et al. (1975), several studies have suggested Phe to be the limiting AA together with Lys (Vik-Mo et al., 1974) or Thr, His and Met (Derrig et al., 1974) when casein and/or glucose were abomasally infused.

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, and 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 looking at comparisons between milk production, TMR ingredient profiles and plasma AA concentrations within 20 California (CA) 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., *in review*), 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 ration 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 a study to determine if Met and/or Phe was limiting performance of cows when fed high levels of CM (Swanepoel et al., 2015). As results suggested that Met was likely oversupplied at 8 g of intestinally absorbable Met/cow/day, and 7.5 g of intestinally absorbable Phe/cow/day was insufficient to support increased milk production, it appeared that the 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.

7.2. 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 from 22 Jan to 11 March 2014 with temperatures between 2.5 and 26.5°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).

7.2.1. *Farm and management*

The commercial dairy selected (near Hanford, CA, USA) was the same one used in Swanepoel et al. (2015). Cows were randomly allocated to one of four early lactation pens from a single fresh pen on a weekly basis and, once confirmed pregnant, were moved from these pens to mid lactation pens. Only three of the four early lactation pens were used. At the start of period 1, treatments were randomly allocated to the three pens and rotated after each 21 day experimental period.

7.2.2. *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 is negatively impacted when CM inclusion level is increased (Table 7.1). The other two treatments consisted of the same base TMR based on alfalfa hay, whole crop winter wheat and 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).

Table 7.1: 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.

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 ruminally

protected (RP) AA, 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 as well as weights for each load of TMR mixed and fed, which were used together with daily refusals to calculate DM intake/cow/pen.

7.2.3. *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 of intestinally absorbable Phe/cow/day) and 3.4 g/cow/day of RP Met (estimated to deliver 2.0 g of intestinally absorbable Met/cow/day) 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.

Since Swanepoel et al. (2015) reported that Phe supplementation 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 the HCM treatments in this study 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 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).

7.2.4. *Sample collection, preparation and analytical methods*

7.2.4.1. 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).

7.2.4.2. Animal measurements

For a cow to be eligible for sampling in any sampling week in each of the periods, 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).

7.2.4.2.1. 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).

7.2.4.2.2. Body condition score

A group of ~170 cows/pen were scored for BCS throughout the study. This was completed by the same trained scorer on the first day of the study and on day 21 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 scored to a ¼ point.

7.2.4.2.3. Urine

Spot urine samples were collected on day 20 of each experimental period from the first ~40 cows which voluntarily urinated during normal morning lockup and immediately placed in ice. The specific gravity (SG) of each untreated urine sample was measured on site using a digital handheld pen refractometer (Atago USA Inc., Bellevue, WA, USA). Only cows with repeated urine samples (*i.e.*, collected in 2 or 3 periods) were retained for allantoin (AL) analysis and samples were treated and analyzed as described by Swanepoel et al. (2014). Sulfuric acid, 1.4 ml of 100 ml/L, 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).

7.2.4.2.4. Blood plasma

Blood was collected on day 19 of each experimental period from the tail vein of the same group of 18 cows/pen with collection, treatment and analysis for free AA and plasma urea N (PUN) following the same methods as outlined in Swanepoel et al. (2014).

7.2.4.2.5. Fecal

Fecal collection was on day 19 of each experimental period by rectal grab sampling the same group of 18 blood cows/pen 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 from the same 6 cows/pen into 3 groups/period in order to support the assumption that the intake of the

group was equal to the TMR fed. Thus a total of 27 fecal samples were created (*i.e.*, 3/pen/period) that were frozen at -20°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.

7.2.5. *Calculations*

Final oven DM of TMR, DM intake per cow/pen, milk energy concentration (MJ/kg) and output (MJ/day), partial net energy (NE) output (MJ/day), NE for lactation (NE_L) density (MJ/kg DM) of the diets, 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).

7.2.6. *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, and 326 for the BCS dataset. Outlier analysis (completed blind to treatments by excluding values deemed to be biologically implausible), excluded 12 cows from the milk production dataset (*i.e.*, 2 cows for a high milk fat concentration, 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 final group of 18 (*i.e.*, 6 cows/pen) cows were randomly selected from the eligible blood cows for plasma AA analysis. A group of 171 urine samples were selected for AL analysis, representing 80 cows which had repeated urine samples between periods.

Animal production, BCS, urine AL, apparent digestibility and plasma AA concentrations were analysed using the MIXED procedure of SAS (2000) for a 3 x 3 Latin square design, with cow nested

within pen in the random statement and period, pen and treatment as fixed effects. Dry matter intake (n = 3 pens, calculated on a pen basis with 3 pens/period), TMR component and ingredient composition and 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 $P \leq 0.10$.

7.3. Results

7.3.1. *Ration evaluation*

The chemical composition of the ingredients used in the TMR (Table 7.1) was similar to ingredients listed in NRC (2001) as well as the ingredients used in Swanepoel et al., (2014 and 2015).

As per design, the LCM ration had a lower inclusion ($P<0.01$) of CM compared to the HCM ration (126 *vs*. 167 g/kg DM; Table 7.2). However, even with the change in CM inclusion, there was no difference in the chemical profiles of the LCM *vs*. HCM rations (Table 7.2). 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 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.

The only difference in the ingredient profiles amongst the treatment rations was inclusion of 3.4 and 41.2 g/cow/day of RP Met and RP Phe respectively (which was equal 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).

7.3.2. *Animal measurements*

7.3.2.1. Dry matter intake and apparent whole tract digestibility

The DM intake (Table 7.3) was not affected (avg: 27.5 +/- 0.46 kg/day) by treatment and were similar to those in Swanepoel et al. (2015).

Table 7.2: 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.

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

Table 7.3: 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.

7.3.2.2. Milk production and its composition

There was no impact of treatments on milk or component yields (Table 7.4). Milk component concentration was not different for LCM *vs.* HCM, but lactose concentration was lower (*P*=0.02) with Phe addition to the HCM ration (47.2 *vs.* 47.3) compared to HCM alone.

Table 7.4: 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)	47.21	47.52	47.72	0.367	0.32	0.52
Milk	1.58	1.57	1.59	0.014	0.80	0.38
Fat	1.34	1.34	1.34	0.009	0.55	0.90
True protein	2.23	2.25	2.25	0.017	0.33	0.77
Lactose	131.7	131.9	132.5	0.98	0.82	0.57
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.

7.3.2.3. Body condition score

The BCS (Table 7.4) 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. Both HCM treatment cows lost BCS during the study, 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).

7.3.2.4. 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 7.3) did not differ among treatments. However, even though the AL concentrations in this study was 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 two studies and are therefore 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).

7.3.2.5. Blood plasma

There were no changes in plasma AA concentrations (Table 7.5) 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), but that was 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 ration compared to LCM, which corresponds with the decline in plasma Leu concentrations in Swanepoel et al. (2014) with higher inclusion levels of CM in the ration.

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

7.3.2.6. Partial net energy balance

The Partial NE balance (Table 7.6) for each treatment was calculated to determine where energy was utilized. Total milk energy output did not different among treatments. There was a difference in

energy utilized for BCS change for all treatments, with the LCM ration 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 substantially reduced the amount of energy liberated from body condition (-2.73 *vs*. -0.11 MJ/day; $P<0.01$).

Table 7.5: Free amino acid, ammonia (µg/ml) and urea concentration (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
Plasma urea N (mg/dL)	11.61	12.22	11.67	0.418	0.07	0.10

[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 used for amino acid analysis as it was decided that additional samples would not change significance of differences.

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

Table 7.6: 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.

7.4. 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 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 of Phe to the intestine, which is 9.9% higher than the estimated intestinal Phe delivery levels for the HCM ration. However, plasma Phe concentrations did not differ between treatments and were only 5.4% higher for HCMP *vs.* HCM. 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 increased Met, this suggests that 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 necessarily improve milk production, suggesting that it is not the Lys:Met ratio that elicits the milk production response, but rather the concentration of Met relative to its requirement.

Plasma Phe concentrations were only proportionally increased by half 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 be higher (Figure 7.1) in the HCM treatment compared to LCM and HCMP suggests that the HCM ration was limiting in Phe, leaving the other AA unutilized and catabolized, resulting in higher plasma PUN concentrations, while supplementation of Phe to HCM alleviated the 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.

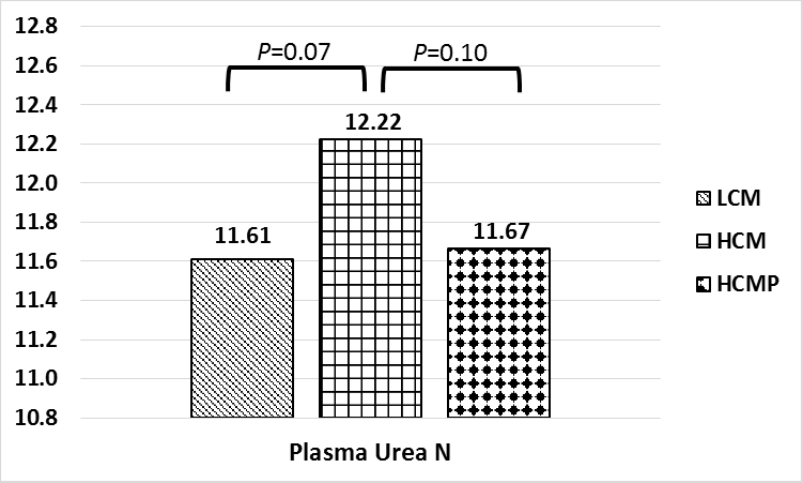

Figure 7.1: Changes in the plasma urea N concentrations (mg/dL) for cows fed the low CM (LCM), high CM (HCM) and high CM ration supplemented with 15 g of intestinally delivered Phe (HCMP).

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 7.2B) with Phe supplementation to the high CM ration in Swanepoel et al. (2015), the small increase in plasma Tyr concentrations in this study (Figure 7.2D) 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 about enhancing or inhibiting effects of Phe on Trp hydroxylation (Kaufman, 1971; Guinard and Rulquin, 1994).

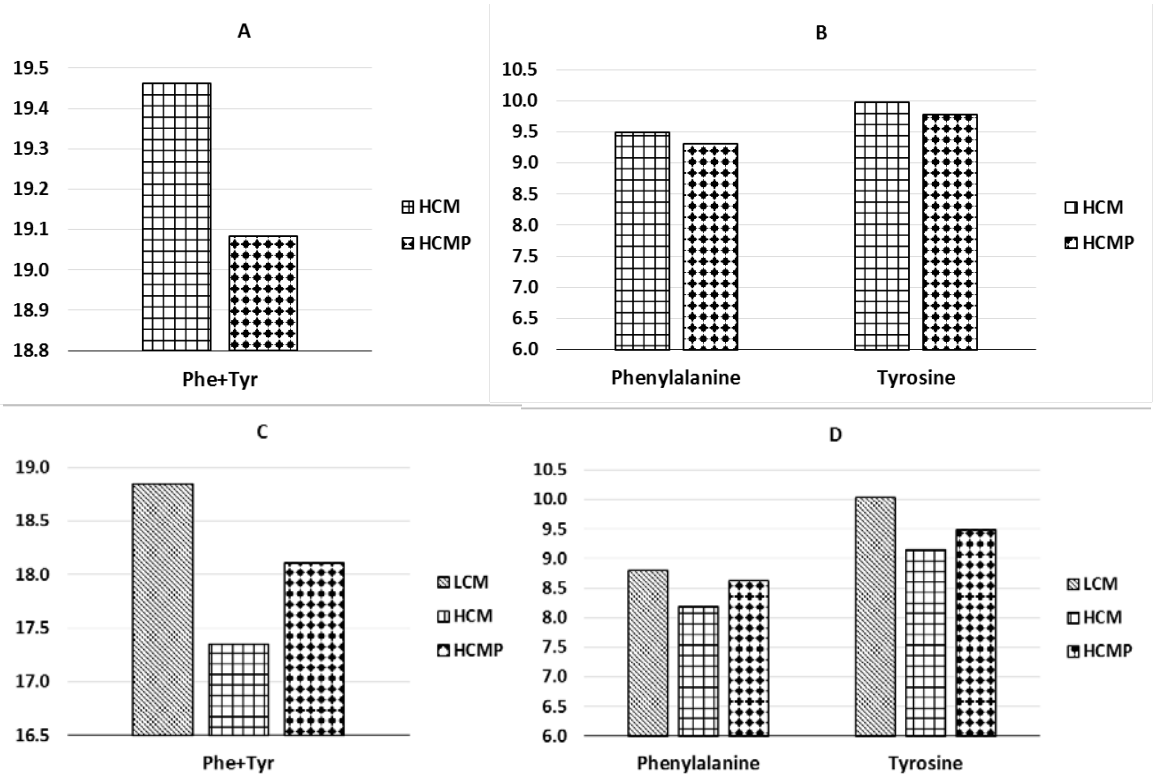

Figure 7.2: Changes in the plasma Phe and Tyr concentrations (µg/mL) for (plots A&B) cows fed the high canola meal (HCM) ration and high CM ration supplemented with 7.5 g of intestinally delivered Phe (HCMP) in Swanepoel et al. (2015) and (plots B&D) cows fed the low CM (LCM), high CM (HCM) and high CM ration supplemented with 15 g of intestinally delivered Phe (HCMP) in the current study.

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 impactful, 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 (~10 g Phe/cow/d), the enhanced fiber digestion could be due to increased concentrations of ammonia, possibly from degradation of the released Phe, stimulating fibrolytic bacteria in the rumen (Van Soest, 1994). However, this seems unlikely due to the low level of 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 7.3A&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, but rather from milk lactose, possibly suggesting that the higher Phe supplementation level was successful in supplying enough Phe to replace mobilized muscle protein while maintaining milk production. Again, Phe could have stimulated release of the peptide ghrelin, which may have prevented fat mobilization from body stores (Vancleef et al., 2015).

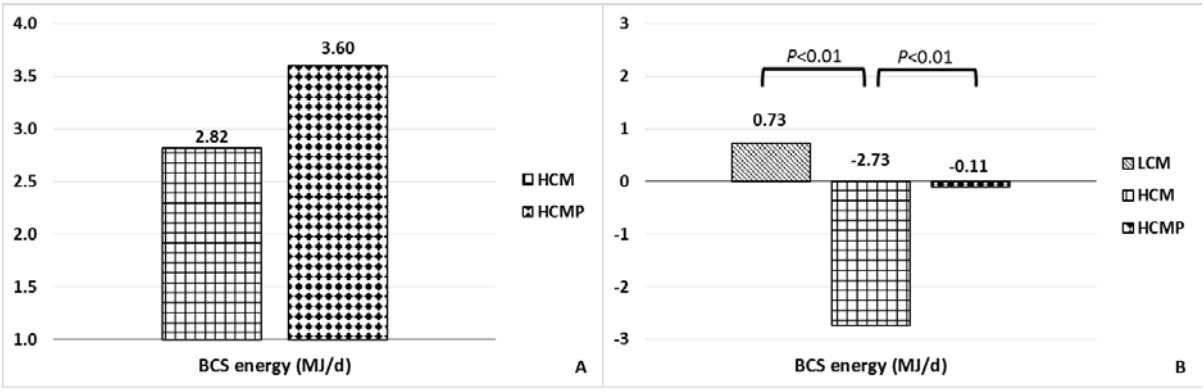

Figure 7.3: Changes in the body condition score (BCS) energy (MJ/d) for (A) cows fed the high canola meal (HCM) ration and high CM ration supplemented with 7.5 g of intestinally delivered Phe (HCMP) in Swanepoel et al. (2015) and (B) cows fed the low CM (LCM), high CM (HCM) and high CM ration supplemented with 15 g of intestinally delivered Phe (HCMP) in the current study.

Due to numerical differences in DM intake between treatments, there was no difference in the calculated dietary NE_L densities of the treatment rations. However, the change in energy utilization among treatments is reflected in differences in total NE output which decreased with the higher CM inclusion (Figure 7.4B). In Swanepoel et al. (2015), total NE output was numerically reduced with Phe supplementation (Figure 7.4A) to the high CM ration but, in the current study, it was increased to the level of the LCM diet, which was determined to contain an optimum CM level in Swanepoel et al. (2014), when Phe was added to the HCM ration thereby suggesting that supplementation of Phe to

HCM regained animal performance that was lost by the HCM diet alone. This suggests that a further increase in the level of Phe supplementation may have additional benefits on milk production.

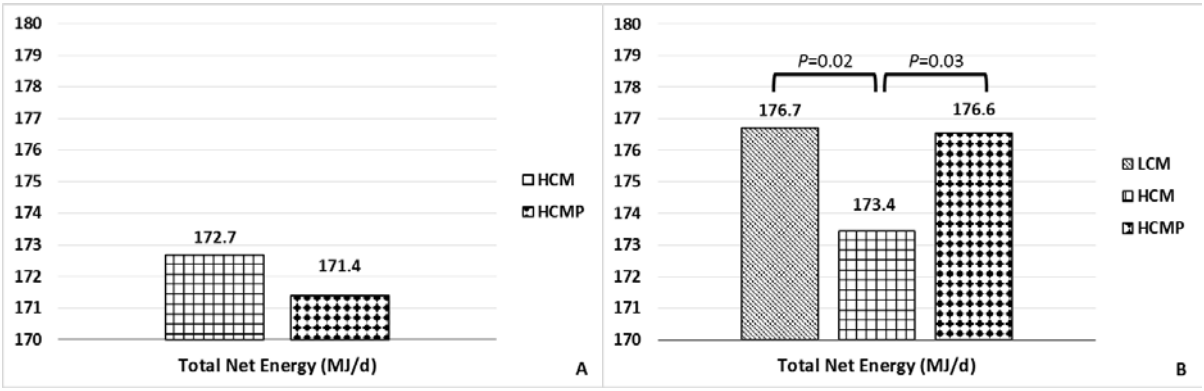

Figure 7.4: Changes in the total net energy (MJ/d) for (A) cows fed the high canola meal (HCM) ration and high CM ration supplemented with 7.5 g of intestinally delivered Phe (HCMP) in Swanepoel et al. (2015) and (B) cows fed the low CM (LCM), high CM (HCM) and high CM ration supplemented with 15 g of intestinally delivered Phe (HCMP) in the current study.

7.5. Conclusions

Even though plasma Phe concentrations did not change between treatments (possibly due to Phe being catabolized by the liver, converted to Tyr or utilized for production responses), Phe supplementation increased BCS gain, probably restoring peptides to the muscle tissue, 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, could have enhanced growth and/or capabilities of cellulolytic bacteria and therefore aNDFom and ADF digestibility in the rumen. The restoration of decreased total NE output with the HCM diet to the same level as the LCM diet with Phe supplementation, together with the positive production responses 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.

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Chapter 8: General Discussion

8.1. Conclusions and implications

Responses to AA supplementation of dairy cattle rations over a period now exceeding 30 years have been inconsistent and unpredictable. 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 concentrations. The perceived correction of a theoretical AA deficiency through its supplementation consistently increases its plasma concentrations while often eliciting small increases in milk protein concentration (Rulquin and Pisulewski, 2006; Weeks et al., 2006; Haque et al., 2012). In contrast, in most cases an estimated imbalance of AA manifests as a small increase in milk fat concentration, or a bit lower 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). Supplementation of AA to diets in which they are not limiting can cause an imbalance, with unexpected effects on animal performance by, for instance, reducing DM intake (Karunanandaa et al., 1994; Robinson et al., 2000; Rulquin and Pisulewski, 2006), sequestration of AA in body protein that exacerbates the AA deficiency (Weekes et al., 2006) or unexpectedly stimulating lactose synthesis (Robinson et al., 1999).

Overall, the inconsistency in responses to AA supplementation in these studies all speak to AA bioactivity, rather than AA limitations. Under this hypothesis, there is a significant downside risk to supplementing AA as AA that are not specifically required can change body AA balances and pools. Thus AA bioactivity, under these changed circumstances, may stimulate a response that is not expected and may not always be deemed positive by commercial dairy producers.

While low level Phe supplementation of a high CM ration had no effect at all on animal performance, the combined Met and Phe treatment diverted energy away from milk components towards BCS gain, suggesting that Phe only expressed its bioactivity in the Met and Phe combination treatment. Results suggest that combined supplementation of Met and Phe may have rectified a Met limitation, by supplying Phe which then became 2nd limiting after Met requirements were met, but that the amount of Phe was not sufficient to support a sustained milk production response in favor of

BCS recovery. Early post-ruminal supplementation of casein has consistently resulted in increased milk production in lactating cows (*e.g.*, Clark, 1975). However, the elements in casein that caused those increases were never identified, and trying to recreate those improvements by supplementing individual AA were not successful. The failure of single AA supplementation to elicit positive production responses similar to casein may be due to a combination of factors that were not supplied by a single AA. As suggested by Clark (1975), AA may be co-limiting with each single AA giving a response which is not experimentally detectable until the total response from several AA are added. Indeed, protein yield is not dependent on only one AA because AA functions are highly interrelated (Doepel et al., 2004).

Varying the inclusion levels of CM and HPDDG resulted in an optimum CM inclusion level of ~126 g/kg DM, and including CM at levels above this resulted in a general decline in cow performance in experiment 1, 2 and 4. As plasma Phe, Leu and Met concentrations decreased with higher CM inclusion in the ration, it suggested that these AA may be limiting. However, supplementing the high CM ration with RP Met and/or Phe did not elicit the response expected in Experiment 2. It has been suggested that it is not blood AA concentrations *per se* that limits milk protein production, but rather metabolic capability which determines maximum velocity of milk protein production (Cant et al., 2001) or gastrointestinal events (Bequette et al., 1996), both of which are under hormonal control. Weekes et al. (2006) suggested it is difficult to know whether an AA supplementation has corrected a deficiency or induced an imbalance, and AA deficiencies and symptoms are poorly diagnosed and inadequately described. Even though Weekes et al. (2006) strived to induce large AA imbalances, changes in metabolic and regulatory pathways dampened expected negative effects on milk protein yields.

Nevertheless, a change in energy utilization with supplementation of Phe was reflected in differences in NE_L output in our Experiments, which decreased when CM inclusion increased. In Experiment 2, the NE_L output was numerically reduced with Phe supplementation. However, when Phe was added in Experiment 4, total NE_L output of the high CM diet was restored to the level of the low CM diet, which contained the optimum CM level as determined in Experiment 1, suggesting that

supplementation of Phe to a high CM ration regained animal performance that was lost when CM inclusion was increased above optimal.

Supplementing Phe in Experiments 2 and 4 resulted in energy being diverted towards BCS gain at the expense of milk protein and fat components at low Phe levels, but at the expense of milk lactose when Phe levels were higher, suggesting that supplemented Phe does not maintain milk production until fulfilling its apparent 1st priority of restoring previously mobilized peptides to muscle protein. Two *in vivo* studies evaluating the ability of the mammary gland to utilize peptide AA have confirmed use of Phe and Leu peptides for milk protein synthesis (Backwell et al., 1994; 1996). However, Bequette et al. (1998) showed that the contribution of peptides to total Phe supply for milk production decreased upon Phe supplementation, while uptake and utilization of supplemented free Phe from the blood increased. Increases in mammary blood flow in response to AA limitations (Bequette et al., 2000) demonstrate the ability of the mammary gland to compensate for deficiencies (Cant et al., 2003). Weekes et al. (2006) also suggested that the lactating cow has great flexibility to maintain milk protein yields under conditions of single AA limitations, with the mammary gland displaying an ability to extract milk precursors from the blood virtually regardless of their concentrations. This ability may abrogate the classic 'limiting AA' theory. Previously reported impacts of independent AA on casein fractional synthesis rate provide additional evidence contradicting the single-limiting AA theory (Arriola Apelo et al., 2014b)

Research has not been able to identify the factors that optimize synthesis and secretion of milk in the mammary gland in the context of AA. Even though many research groups have tirelessly tried to identify 'limiting' AA, answers may be elsewhere (Bequette and Backwell, 1997; Bequette et al. 1998). It is time to reconsider the 'limiting' AA or 'broken stick' concept of AA nutrition of lactating dairy cows in favor of accepting that many AA are bioactive and can change animal performance, even when they are not 'limiting' milk production *per se*, which is likely the normal situation in commercial practice with multi-feed rations. Thus recommendations to use RPAA products must consider the potential for unwanted effects, which could be deemed negative, and are associated with oversupply, or unnecessary supply, of AA to the intestinal absorptive site relative to animal 'needs'.

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.

8.2. Future research and critical evaluation

If SG had been measured on the urine collected in Experiment 1, MCP flow could have been calculated instead of using the PDC index, which is not a quantitative predictor of MCP synthesis, to determine differences in MCP flow amongst treatments. However, it is not known which of the two methods are the best in predicting changes in MCP synthesis. Indeed, the PDC index is not reported in many studies, limiting the ability for a comparison with other research.

The high level of Met supplementation in Experiment 2 was unnecessary and should have been avoided. The original calculated requirement for Met was 2.0 g of intestinally absorbable Met/cow/day, however outside influences insisted that metabolic model predictions specified that 8.0 g of Met was required. This oversupply was corrected in Experiment 4, showing that the lower Met supplementation was adequate to provide required Met.

Experiment 3 documented normal ranges of urine estimated MCP flowing from the rumen, and plasma AA concentrations which could be used as a benchmark to compare high, low and normal levels of these parameters. A possible next step would be to use the dataset to identify a dairy farm feeding a ration with a possible MCP or AA limitation, and validate the procedure by reformulating the ration, or supplementing AA, to rectify the imbalance and determine its effect on production.

The highest CM inclusion level in Experiment 1 was 200 g/kg TMR DM while subsequent experiments were not as high. However, even though the level of 170 g/kg TMR DM in Experiment 2 and 4 was not as high as the first, it was still above the optimum CM inclusion level of ~126 g/kg, and should therefore show similar nutrient limitations as the 200 g/kg ration, even though positive responses to supplementation may have been slightly dampened at the levels used in Experiments 2 and 4.

There was an effect of supplementation of RP Phe on whole tract NDF digestibility in Experiment 4, even though it was unexpected and seemed unlikely. Since this finding cannot be compared with previous experiments, where fecal samples were not obtained, future research supplementing RP AA should determine if our observed effect on digestibility was real and to try and explain how supplementary AA could affect animal digestion.

The largest limitation of doing research on a commercial farm, which seems to draw the attention of many reviewers, is the inability to determine individual cow DM intake. Even though the size of the sampling groups in our experiments should more than make up for the lack of individual intake data, researching a method to determine intakes of individual cows under commercial situations, by way of marker analysis or otherwise, could prove advantageous to researchers and farmers.

Since Experiment 4 showed that supplementation of Phe to a high CM ration regained animal energy output, even though not exactly as anticipated, a further increase in the level of Phe supplementation may have additional benefits on milk production. However, since AA should be considered bioactive, we expect that supplementation of additional Phe may have unpredictable, or even detrimental, effects.

As synthesis of Tyr depends largely on conversion of Phe to Tyr through Phe hydroxylase activity, and since studies showed that an additional supply of Tyr to the mammary gland reduces the requirement for Phe (together with the possibility that conversion of Phe to Tyr is not efficient enough to supply all Tyr requirements for milk production in high producing dairy cows), it would be beneficial to investigate possible lactation responses if Phe supplementation was enhanced with the addition of Tyr, or if part of the Phe is replaced by Tyr. However, since there is some evidence to suggest reduced efficiency of milk protein synthesis when peptide bound Phe is replaced by free Phe, methods to promote production of peptides in the rumen, or perhaps supplementation of peptide bound AA, merits further investigation.

Even though our study results suggested that Phe and Met should be considered as bioactive metabolites, bioactivity of AA is not a new concept. The entire Chapter 2 Literature Review speaks to bioactivity of AA, rather than specific AA limitations, even though it took the compilation of 30

years of research into one chapter to make that clear. Supplementation studies still strive to find the one AA, or combination of AA, that will stimulate milk production as casein did in many early studies, but lactation responses to AA supplementation still cannot be predicted or explained with confidence and hope of such a ‘golden study’ is deteriorating. Indeed, if I had written the Literature Review before any of the studies were completed, I may have approached this project differently. I believe the research currently under way by researchers investigating effects of supplemental AA on molecular pathways and enzyme activity is the correct path to the future.

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