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CHOLINE: A LIMITING NUTRIENT FOR TRANSITION DAIRY COWS

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SUMMARY

- The classic symptom for choline deficiency in all animals studied is fatty liver.
- Transition dairy cows experience fatty liver that results from excessive hepatic uptake of mobilized fatty acids, ruminal degradation of dietary choline, and inadequate endogenous synthesis of phosphatidylcholine that is needed for fat export from the liver.
- Supplementation of transition cow diets with choline that is protected from ruminal degradation reduces the severity of fatty liver in transition dairy cows.

- To date, there is no evidence that feeding supplemental methionine can substitute for choline for alleviating fatty liver.
- Feeding rumen-protected choline enhances milk production in early lactation.

INTRODUCTION

Choline has been shown to be a required nutrient for many animals including rats, mice, dogs, pigs, guinea pigs, chickens, and trout. Choline is often referred to as a vitamin, however, it doesn't fit any of the classical definitions for a vitamin. It is not a co-factor in enzymatic reactions, it can be synthesized endogenously as phosphatidylcholine (PC), and it is required in larger amounts than vitamins. The ability to synthesize choline endogenously does not mean it is a dispensable or non-essential nutrient. Deficiency symptoms include suppressed growth rates, renal dysfunction, and development of fatty liver. Choline is crucial for normal function of all cells. The most common form of choline in biological systems is PC, a phospholipid that is a component of all cell membranes and lipoproteins that function to transport lipids through the circulatory system. Choline is a source of methyl groups, therefore, it can spare methionine and have interactions with other nutrients involved in one-carbon metabolism (e.g. folate). Choline is also a component of acetylcholine, an important neurotransmitter.

The NRC (2001) wrote: "The establishment of a choline requirement, either for a lactating dairy cow, or a transition cow in the late dry period and in early lactation, will require more extensive feeding experiments than available at the time of this publication." It has now been 14 years since publication of the last NRC. Since publication of the last NRC, numerous studies have been conducted to examine the effects of feeding ruminally protected choline to dairy cows, particularly as they transition from the dry period to early lactation. In light of new research it seems appropriate to initiate discussion on whether choline should be considered a required nutrient in dairy diets.

TRANSITION COW AND CHOLINE BIOLOGY

Several studies have shown 50 to 60% of transition cows experience moderate to severe fatty liver (Bobe et al., 2004). These studies have been conducted in numerous countries across different genetic lines of cattle, different feedstuffs, and varying management systems and the data were not generated from a population of problem cows or herds . The consistency amongst these studies suggests that development of fatty liver is a "normal" part of the cow's biology. Because fatty liver is a classic deficiency symptom for choline, it is reasonable to question if transition cows are typically deficient in choline.

At calving there are hormonal changes that trigger an intense period of lipid mobilization from adipose tissue and as a result, blood nonesterified fatty acid (NEFA) concentrations typically increase 5- to 10-fold (Grummer, 1993). NEFA remain elevated, although to a lesser extent, during early lactation when cows experience negative energy balance. Blood flow to the liver doubles as a cow transitions from the dry period to lactation (Reynolds et al., 2003). NEFA concentration and blood flow are the two biggest factors affecting how much NEFA is taken up by the liver. As a result, daily fatty acid uptake by the liver increases and estimated 13-fold at

calving, from approximately 100 to 1300 g/day (Overton, unpublished). Not all of the fatty acids taken up by the liver will be stored and contribute to fatty liver. However, Drackely (2001) estimated that during peak blood NEFA concentration, approximately 600 g might be deposited in 24 hours, which would correspond to an increase in liver fat of 6-7% by weight. As a reference, fat above 5% in the liver (wet basis) is considered by the veterinary community to be moderate to severe fatty liver. It is important to understand that this dramatic increase in NEFA uptake by the liver is part of the normal biology of transition cows and is not restricted to fat cows, poorly fed cows, or cows housed in suboptimal environments.

The most desirable fate of fatty acids entering the liver would be complete oxidation to provide energy to the liver or reesterification and export as triglyceride from the liver as part of a very low density lipoprotein (VLDL). Hepatic oxidation increases approximately 20% during the transition period (Drackley et al., 2001). This increase does not represent a strategic move by the cow's liver to cope with the sudden surge of NEFA uptake at calving. It occurs because the liver becomes metabolically more active. Unfortunately, the increase in oxidation is not sufficient to cope with the increased load of fatty acid being presented to the liver. Research conducted nearly 25 years ago at the University of Wisconsin (Kleppe et al., 1988) and Michigan State University (Pullen et al., 1990) revealed that ruminants have a low capacity to export triglyceride from the liver as very low density lipoprotein (VLDL) as compared to nonruminants. This and the inability to markedly increase fatty acid oxidation is why transition dairy cattle develop fatty liver when experiencing elevated blood NEFA.

It is now apparent that choline deficiency is a limiting factor for VLDL triglyceride export from the liver. It has been shown in many species, using a wide variety of experimental approaches, that rate of VLDL export is highly related to the rate of hepatic PC synthesis (Cole et al., 2012). Models include monograstrics fed choline deficient diets, isolated hepatocytes cultured in choline and methionine deficient media, and in knock out mice for genes involved in PC synthesis (Cole et al., 2011). Interestingly, there is no evidence that synthesis of any other phospholipid is required for hepatic VLDL assembly and secretion. In addition to direct PC synthesis from dietary choline, there is endogenous hepatic synthesis of PC via methylation of phosphotidylethanolamine (PE). Sharma and Erdman (1988) demonstrated dietary choline is extensively degraded in the rumen of dairy cows and very little is available to the small intestine for absorption. Choline flow to the duodenum increased less than 2 g/day, even when free choline intake was increased to more than 300 g/d. Therefore, ruminants are more highly dependent than nonruminants on endogenous synthesis of PC from PE. Is endogenous synthesis of PC from PE sufficient during the transition period or do cows require choline supplementation? The high proportion of transition cows developing moderate to severe fatty liver during the transition period suggests that endogenous synthesis is not sufficient in many cows.

EVIDENCE FOR A CHOLINE DEFICIENCY IN TRANSITION DAIRY COWS

The first piece of evidence that transition cows are deficient in choline is the development of fatty liver during the periparturient period (Grummer, 1993; Bobe et al., 2004). More

compelling evidence is the alleviation of fatty liver when supplying cows with choline that is protected from ruminal degradation (Cooke et al., 2007; Zom et al., 2011). Dutch researchers (Goselink et al., 2013) recently demonstrated greater gene expression for microsomal triglyceride transfer protein (MTTP) in liver of transition cows supplemented with rumen-protected choline (RPC). MTTP is an important protein required for hepatic VLDL synthesis. Recently, it was shown that choline, but not methionine, increases VLDL secretion from primary bovine (McCourt et al., 2015). This provided solid evidence that choline limitation is a causative factor for inadequate fat export out of the liver.

The reduction in liver fat content when feeding transition cows RPC is accompanied by improved health and production. Lima et al. (2012) observed reduced incidences of clinical ketosis, mastitis, and morbidity when feeding RPC from 25 days prepartum to 80 days postpartum. It has been known for years that elevated fat in the liver is associated with poor reproductive performance (Bobe et al., 2004). First service conception rate was increased by feeding RPC in one study (Oelrichs et al., 2004) but not another (Lima et al., 2011). We (Grummer and Crump, unpublished) recently completed a meta-analysis for 13 studies that fed RPC to transition cows. Feed stability or evidence of bioavailability of choline source was not a criterion for study selection. Studies were not screened for "soundness" of research. Treatment means and sample size (standard error of the mean) had to be available for the analysis. Ten of the thirteen trials were published in peer-reviewed journals. For studies to be included in this analysis, RPC had to be fed _prior_ to calving. Time when RPC supplementation was started varied between 28 to 7 days prior to expected calving. RPC supplementation was terminated anywhere from the day of calving (one study) to 120 days in milk. Response variables included DMI, milk yield, energy corrected milk yield, fat %, protein %, and fat and protein yield. Insufficient data was available for analysis of liver fat or energy-related blood parameters. Analysis revealed a significant increase of 4.9 lb milk/day and 1.6 lb of dry matter intake/day (Table 2; Figure 1). Milk fat and protein percentage were not significantly affected by treatment but yields were (Table 2). These studies were conducted in several countries under a variety of management conditions and they did not target problem herds or cows. This implies that benefits to supplementing protected choline can be realized by a wide variety of herds. Alleviating a choline deficiency not only reduces liver fat but also improves parameters that are economically important to dairy producers.

Table 1. Studies used in the Meta-Analysis.

Study	Choline Dose, g/d	Product	Duration	Exp.Units	Parity
Hartwell et al., 2000	0,6,12	Capshure	-21 to 120	24	M
Zom et al., 2011	0,15	ReaShure	-21 to 42	19	M
Lima et al., 2007[1]	0,15	ReaShure	-25 to 80	4 (pen)	M, P

| Lima et al., 2007[1] | 0,15 | ReaShure | -22 to 0 | 5 (pen) | P |
Oelrichs et al., 2002[1]	0,15	ReaShure	-28 to 100	32	M, P
Zahra et al., 2006	0,14	ReaShure	-25 to28	91	M, P
Piepenbrink et al., 2003	0,11,15, 19	ReaShure	-21 to 63	12	M
Janovick et al., 2006	0,15	ReaShure	-21 to 21	21	M
Elek et al., 2008	0,25/50 Pre/Post	Norcol-25	-25 to 60	16	M, P
Ardalan et al., 2011	0,14	Col 24	-28 to 70	20	M, P
Pinotte et al., 2003	0,20	Overcholine 45%	-14 to 30	13	M
Xu et al. #1, 2006	0,7.5	Not reported	-7 to 21	7	M
Xu et al. #2, 2006	0,11,22,33	Not reported	-15 to 15	9	M, P

[1]Studies have not been published in a peer-reviewed journal. Standard errors were not reported in abstracts but were obtained from the authors.

Table 2. A Meta-analysis of 13 studies examining the effects of feeding RPC to transition cows on dry matter intake and milk.

	Control	RPC	SEd	P =
DMI, lb/d	39.98	41.60	.46	.0042
Milk, lb/d	70.88	75.75	.75	<.0001
ECM, lb/d	76.87	82.78	1.33	.0038
Fat yield, lb/d	2.788	3.042	.086	.021
Protein yield, lb/d	2.300	2.467	.053	.010

Figure 1. Individual study results from a meta-analysis of 13 transition cow trials that examined the effects of feeding rumen-protected choline (Grummer and Crump, unpublished).

Milk Yield (lb/d) by Study

Average Response = 4.9 lb/d
P < 0.0001

(Y-axis: Milk Yield, lb/d; X-axis: Dose of choline, g/d)
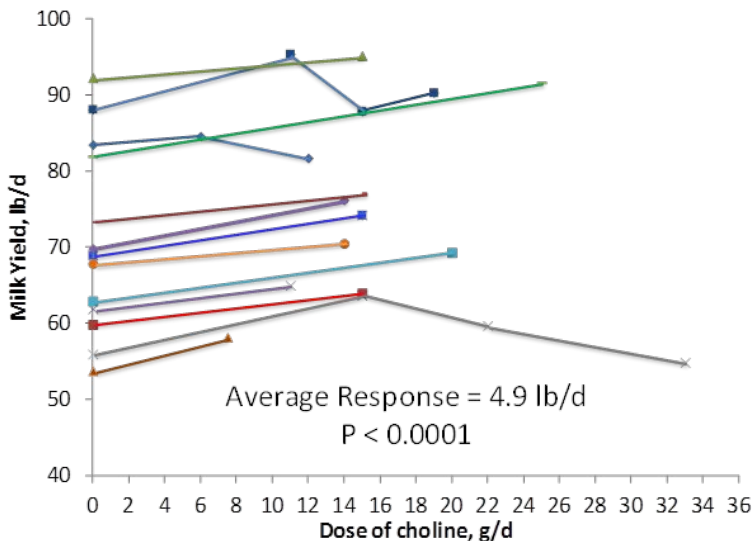
CAN PROTECTED METHIONINE SUBSTITUTE FOR PROTECTED CHOLINE?

Protected methionine has often been suggested as a possible alternative to protected choline for supplementation to transition dairy cows. Methionine and choline both serve as methyl donors. Methionine methyl groups can be used for endogenous synthesis of PC from PE. As an amino acid, methionine is needed for the synthesis of apolipoproteins.   Therefore, there is a conceptual basis for methionine substitution for choline. Five feeding trials have been conducted to examine the effects of methionine on liver total lipid or triglyceride content.

Feeding 13 g/d of 2-hydroxy-4-(methylthio)-butanoic acid (HMB; also referred to methionine-hyrdoxy-analog or MHA) did not reduce triglyceride accumulation in the liver of feed restricted dry cows (Bertics et al., 1997). Feeding 0, .13, .20% of dry matter as HMB from 21 days prepartum to 84 days postpartum did not affect liver triglyceride at 1 day postpartum and resulted in a tendency (P < 0.15) for a quadratic increase in liver triglyceride at 21 day postpartum (Piepenbrink et al., 2004). They also observed a quadratic effect of HMB for increased fat-corrected milk yield indicating that there may have been some improvement in methionine supply to the cows. The amount of HMB absorbed from the gastro-intestinal tract and converted to methionine by the liver has not been well established.

Cows fed 0 or 10.5 g methionine/day as MetaSmart from 14 days precalving to 105 days postcalving had similar liver total lipid postcalving (Socha, 1994). Liver triglyceride was not measured. Milk protein percentage was increased by treatment indicating that supplementation delivered more methionine to the blood stream. Feeding 9 g Mepron/day precalving and 18 g Mepron/day postcalving increased liver triglyceride (P=0.02) but the means were from 4 liver biopsies taken over 16 weeks were small and the increase was small (Preynat et al., 2010). Milk

protein percentage was increased by feeding Mepron which indicated an improved methionine status. Feeding MetaSmart (.18% of DM) or Smartamine (.07% of DM) from 21 days prepartum until 20 days postpartum did not affect total lipid and triglyceride concentrations in the liver (Osorio et al., 2013). Dry matter intake, milk yield, and fat percentage were increased by methionine supplementation indicating that methionine status was improved.

The reason for methionine's failure to prevent fatty liver in transition cows is not known. One explanation may be that the studies cited above employed insufficient doses of protected methionine or methionine analog. Choline contains three methyl groups while methionine only contains one methyl group. When differences in molecular weight between choline and methionine are accounted for, choline by weight is 4.3 times more "potent" than methionine as a methyl donor. Therefore, assuming equal bioavailability of the rumen-protected products being fed, one could speculate that one would need to feed 64.5 g/d of methionine during the transition period to obtain a similar amount of methyl groups as when feeding 15 g/d of choline. As previously mentioned, choline, but not methionine, increases VLDL secretion from primary bovine (McCourt et al., 2015).

CONCLUSIONS

Since the last NRC (2001) publication, a significant body of evidence has accumulated to support choline being a required but limiting nutrient in transition cow diets. There is overwhelming evidence that feeding transition dairy cows 15 g choline/day in a form that is protected from ruminal degradation will alleviate choline's classic deficiency symptom and lead to improvements in health and performance.

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Hepatic Methyl Metabolism: Influencing Success During the Transition to Lactation Period

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SUMMARY

- Adipose tissue mobilization during negative energy balance results in increased hepatic NEFA uptake.
- NEFA can be completely oxidized to energy, incompletely oxidized to ketones, or esterified to triglycerides for storage or export as VLDL.
- VLDL export from ruminant livers is limited, primarily because of limited phosphatidylcholine.
- Use of a cell culture models confirms that increasing choline concentrations can increase VLDL export from hepatocytes. Increasing choline concentrations also tended to reduce oxidative stress associated with a fatty acid challenge.
- Choline can be used to donate a methyl group for methionine regeneration in hepatocytes.
- Increasing concentrations of methionine decreased the need for endogenous regeneration of methionine within hepatocytes. Increasing concentrations of methionine did not change VLDL export or oxidative stress.
- The lack of interaction between methionine and choline in cell culture models supports separate mechanistic roles for methionine and choline within the hepatocyte.

INTRODUCTION

The transition to lactation period is characterized by negative energy balance (NEB) which reflects decreased feed intake and increased energy and glucose demands associated with lactation. During NEB, stored body fat is mobilized in an attempt to compensate for the energy deficit and transported to the liver in the form of nonesterified fatty acids (NEFA) and glycerol. While the mobilized NEFA provide critical fuel sources during the transition to lactation period, inability of the liver to metabolize them can lead to ketosis and fatty liver which have negative effects on productivity and animal health.

HEPATIC UPTAKE AND METABOLISM OF NEFA

During periods of NEB, triglycerides (TG) are mobilized from adipose stores and are transported to the liver to aid in alleviating NEB (Dole, 1956; Gordon and Cherkes, 1956). Hepatic update of NEFA is reflective of blood flow and blood NEFA concentration, both of which are increased after calving. It has been well characterized that blood NEFA concentration increases after calving, reflective of adipose tissue mobilization, and can increase to 1 mmol/L or greater (Grummer et al., 1993; Reynolds et al., 2003). Additionally, blood flow nearly doubles from one week precalving to one and a half weeks postcalving (Reynolds et al., 2003), increasing exposure of the liver to nutrients and metabolites, including NEFA. Glycerol can be used as a gluconeogenic precursor after hepatic uptake. Conversely, NEFA are β-oxidized to acetyl-CoA units with four possible fates: complete oxidation through the TCA cycle, incomplete oxidation through ketogenesis to ketones, TG synthesis and packaging as very-low density lipoprotein for export from the liver (minimal in ruminant animals), or TG synthesis for storage as liver lipids (reviewed by Grummer, 1993). When available acetyl-CoA exceeds the capacity of the TCA cycle, there are increases in production of ketones and deposition of TG, leading to the onset of ketosis and fatty liver syndrome (White, 2015). The progression of these disorders is the response to poor adaptation to the challenges associated with the transition to lactation period.

During this period of NEFA mobilization, the capacity of the liver to completely oxidize fatty acids to energy is only limitedly increased (Grum et al., 1996) and thus, more acetyl-CoA are metabolized through the alternative pathways including ketogenesis and synthesis of TG for storage or export. Capacity of the liver to synthesize TG from acetyl-CoA is increased by 188% at +1 vs. -21 days relative to calving, highlighting the capacity of the liver to store fatty acids that cannot be immediately oxidized (Grum et al., 1996). Accumulation of liver lipids during early lactation can be as high as 500 g/d and it is predicted that 60% of dairy cows have severe or clinical fatty liver, defined as a liver lipid content greater than 10% on wet weight basis (Drackley, 1999; Bobe et al., 2004).

VLDL EXPORT

Just as in nonruminants, export of very low density lipoproteins (VLDL) can prevent accumulation of fat within the liver and can allow for transport of lipid fuel sources to other tissues, including the mammary gland. Although the capacity of the liver to synthesize TG is increased during the transition to lactation, the ability of the ruminant liver to export TG as VLDL is not proportionately high. Components of VLDL includes TG, apolipoproteins (ApoB and ApoE, specifically), cholesterol, and phosphatidylcholine and have been well studied in nonruminant models. Generation of phosphatidylcholine can either be de novo (methylation of phosphotidylethanolamine) or dietary (choline) and depletion of methyl donors from rodent diets significantly increases liver TG accumulation (Rinella et al., 2008; Cole et al., 2012). In ruminants, the component limiting VLDL export is phosphatidylcholine. Supplementation of dairy cows with rumen-protected choline reduces liver TG concentrations during the transition to lactation period (Zom et al., 2011; Goselink et al., 2013). Examination of genes involved in fatty acid transport and VLDL assembly are increased in cows supplemented with rumen-protected choline, suggesting that the decreased liver TG accumulation is due to increased VLDL export (Goselink et al., 2013). Less is know about the interaction of the two pathways to generate phosphatidylcholine in ruminants.

METHYL DONOR METABOLISM

Methyl donors, including choline, methionine, betaine, and folate, are essential for DNA methylation, prevention of oxidative stress, energy metabolism, and protein synthesis; however, because of rumen fermentation, lactating ruminants are deficient in methyl donors (Pinotti et al., 2002). While the role of methyl donors has been extensively studied in nonruminants, less is understood regarding their action and mechanism in ruminants. In order to elucidate the mechanism of methyl donor metabolism, a bovine primary hepatocyte cell culture model was used to examine the role of two methyl donors, choline and methionine, in hepatic metabolism. Cells were exposed to increasing doses of choline and methionine in the absence or presence of a fatty acid cocktail designed to mimic the profile of fatty acids in circulation at calving (Chandler et al., 2015).

Given that methionine is a required amino acid essential for body and milk protein synthesis, regeneration of methionine is a vital role of methyl donors within liver cells. Increasing concentrations of methionine decreased endogenous regeneration of methionine suggesting that endogenous methionine regeneration is a hepatic priority when methionine concentrations are low (Chandler et al., 2015). Increasing choline concentrations increased methionine regeneration, suggesting that choline may serve a role as a methyl donor for methionine regeneration (Chandler et al., 2015).

Quantification of VLDL in ruminants is difficult due to differences in lipid profiles of the VLDL between ruminants and nonruminants. An antibody-based assay was validated and used to quantify VLDL secreted into the cell culture media in cells exposed to choline and methionine in the presence of a fatty acid challenge. Increasing choline concentrations increased VLDL export from the hepatocytes (McCourt et al., 2015). No change in VLDL export was observed as methionine concentrations were increased. This was supported by no differences in PEMT, the enzyme that catalyzes the methylation of phosphatidylethanolamine to phosphatidylcholine (Chandler et al., 2015).

Oxidation of fatty acids is critical for energy production in the liver; however, it also results in oxidative stress within the cells. Given this relationship, accumulation of reactive oxygen species (ROS) were examined in the cell culture model described above. Increasing concentrations of choline, but not methionine, tended to decrease ROS released into the cell culture media (Chandler et al., 2015).

CONCLUSIONS

Negative energy balance and adipose tissue mobilization are well-characterized hallmarks of the transition to lactation period in dairy cows. The ability of the liver to metabolize NEFA and glycerol are not only essential to meeting the demands of lactation, but to avoiding metabolic disorders. Recent attention to methyl donors and their role in maintaining hepatic health and optimizing hepatic function has necessitated a better understanding of their mechanism in the liver. Use of cell culture models aid in understanding specific mechanisms and suggests a biological priority for methyl donor use. The lack of interaction between methionine and choline in cell culture models supports separate roles for methionine and choline within the hepatocyte. It is clear that the requirement for methionine needs to be met, either by dietary sources or by endogenous regeneration. Choline can provide methyl groups for regenerating methionine, but is also involved in increasing VLDL export and may decrease oxidative stress.

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Potential Strategies for Successfully Feeding Choline to Dairy Cows

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SUMMARY

- Choline must be fed in a form that is protected from microbial fermentation in order to supply amounts that can positively influence milk yield and liver health on a consistent basis.
- A positive milk response to choline supplementation with rumen-protected choline (RPC) has not depended upon stage of lactation suggesting that choline may be a limiting nutrient for milk yield regardless of physiological state.
- Based upon limited data, primiparous cows are as likely to produce more milk when supplemented with RPC as are multiparous cows.
- Initiating supplementation of RPC in the last 3 to 4 weeks of gestation has usually improved liver status through reduced concentration of triacylglycerol (TAG) or increased glycogen without apparent changes in plasma BHBA. This may be through improved formation of very low density lipoproteins for lipid export from the liver.
- Although methionine can be used for de novo synthesis of choline, the majority of studies reported a positive response in milk yield to RPC supplementation regardless of methionine status.
- Supplementation of RPC improved milk yield by cows fed diets that were adequate or deficient in crude protein or that contained proportionately high or low amounts of concentrate.
- Incidence of postpartum health disorders (e.g. clinical ketosis and mastitis) can be reduced when RPC is fed in the periparturient period.

INTRODUCTION

Choline is classified as a quasivitamin (Combs, 1998) because it has not been identified as a needed enzyme cofactor and the daily requirement is in much greater amounts than what is recommended as needed for other vitamins. Choline has many known functions including roles in growth including preweaned calves (Johnson et al., 1951), neurotransmission (acetylcholine), release of Ca^+ from storage, blood clotting, uterine implantation of ovum, and prevention of a leg disorder called perosis in heavy-bodied poultry. However, it may be best known for its role in lipid metabolism across species. A deficiency of dietary choline results in a deficiency of the phospholipid lecithin (phosphatidylcholine). Phosphatidylcholine is a very key component of very low density lipoproteins (VLDL, composed of a core of triacylglycerol (TAG) surrounded by polar phospholipids and the apolipoproteins B-100, C, and E) which are primarily synthesized in the liver and required for export of TAG from the liver. The accumulation of fat in the liver may result from a choline deficiency which in turn may limit the synthesis of phosphatidylcholine and thus biosynthesis of VLDL (Cole et al., 2011). When animals are pregnant or lactating, concentrations of choline in the liver decrease dramatically (Zeisel, 2000).

Choline can occur in feeds in a free form but it is mostly stored as phosphatidylcholine as a component of plant cell membranes. Cereals, legumes, and oilseed meals are the best dietary sources for choline. Oilseeds, such as cottonseed meal, soybean meal, and fish meal, contain the greatest concentration of choline among common feedstuffs (0.26 to 0.42%) with corn being about one-third that of barley (0.068 vs. 0.184%; Sharma and Erdman, 1989a). Much of the ingested choline is metabolized by microbes in the rumen (Neill et al., 1978) and small intestine to trimethylamine and methane. Some ingested phosphatidylcholine passes to the lower gut, is absorbed in the upper portion of the small intestine bound to chylomicra, circulates throughout the body as part of the lipoprotein family, and is stored as a vital component in the cell membranes of all tissues. However the appearance of choline in the small intestine of ruminants is low. Feeding 326 g of choline as choline chloride only resulted in 2.5 g of choline flowing to the small intestine which was only a 1.3 g increase above cows consuming 23.5 g/day (Sharma and Erdman, 1988b). Fortunately, phosphatidylcholine can also be synthesized by the body if free methyl groups are available. De novo synthesis of choline involves methionine, folic acid, and vitamin B_{12}. As a result of this microbial metabolism of choline, choline products which are protected in the rumen (RPC) from extensive metabolism have been developed such as ReaShure (Balchem Inc., NY). This allows for substantial delivery of choline to the small intestine for absorption. Feeding choline as choline chloride did not improve milk yield in 5 separate studies (Erdman et al. (1984); Sharma and Erdman (1988a); Sharma and Erdman (1988b)) although concentration of milk fat was increased consistently in these same studies. This lack of effect on milk production was most likely due to lack of delivery of sufficient choline to the small intestine for absorption. Improved milk yield and liver health of dairy cows in early lactation has often been improved when including rumen-protected choline in the diet. Searching for the sweet spots in the life of the dairy cow regarding choline supplementation is the subject of the remaining paper. We always desire that more information is available to make informed feeding decisions but the following recommendations are based upon the published findings that we have.

POSSIBLE SCENARIOS FOR SUCCESSFUL SUPPLEMENTATION OF CHOLINE

Stage of Lactation

Mobilized lipid (nonesterified fatty acids, NEFA) from adipose makes up a greater proportion of the energy needed for milk synthesis during the first 3 weeks of lactation when energy required for lactation and maintenance exceeds that of energy intake. The NEFA travel to the liver where they are absorbed from the blood stream. Once absorbed, the NEFA have 4 fates; namely, storage as TAG, export back into the blood as VLDL, complete oxidation, or incomplete oxidation resulting in release of ketone bodies back into circulation. An over-whelming influx of NEFA during times of significant negative energy balance results in production of ketones and so elevated blood ketones (ketosis defined as >1.2 mol/L of beta-hydroxybutyric acid, BHBA) is most prevalent during this time period. Phosphatidylcholine was almost "completely devoid" in the blood serum of ketotic vs. normal lactating dairy cows (McCarthy et al., 1968) suggesting that phosphatidylcholine may become a limiting factor leading to ketosis. How might this be? Phosphatidylcholine is a very key component of very low density lipoproteins (VLDL) which are primarily synthesized in the liver and required for export of triacylglycerol (TAG) from the liver. If supply of choline is low then phosphatidyl-choline synthesis is low and fat may accumulate in the liver and ketone production enhanced. Using the nonruminant model, a deficiency of dietary choline resulted in accumulation of triglycerides in the liver; restoration of choline sufficiency resulted in movement of triglycerides from the liver (Haines and Mookerja, 1965). Very recently, Heather White's lab at the University of Wisconsin developed an assay for bovine VLDL and reported that incubating increasing concentrations of choline with bovine primary hepatocytes resulted in a linear increase in VLDL in the cell culture media after exposure to a fatty acid cocktail (McCourt et al., 2015). If choline can help export fatty acids from the liver during periods of heavy fatty acid uptake, one might expect that cows supplemented with RPC would have lowered circulating concentrations of BHBA. However, only one study (Elek et al., 2013) reported such an effect and most cows were ketotic at 7 days postpartum (1.46 vs. 1.16 mmol/L for control and RPC-fed cows, respectively). Others have reported no change in plasma concentration of BHBA due to feeding of RPC (Ardalan et al. 2011; Davidson et al., 2010; Janovick Guretzky et al., 2006; Pieperbrink and Overton, 2003; Zahra et al., 2006 (ketotic); Zom et al., 2011).

Elevated concentration of urinary ketones have been associated with fatty liver (Bobe et al., 2004). Like ketosis, accumulation of fat by the liver is most evident in the first month after lactation and 35 to 50% of our dairy cows have moderate (5 to 10% TAG, wet basis) to severe fatty liver (> 10% TAG, wet basis; Bobe et al., 2004). Despite the lack of consistent evidence that RPC can reduce ketosis in early lactation, evidence is relatively strong that TAG concentration in liver can be reduced when RPC is supplemented. Papers reporting no effect of periparturient feeding of RPC on liver TAG of lactating dairy cows include Hartwell et al. (2000), Pieperbrink and Overton (2003), and Zahra et al. (2006). An equal number of others have reported a reduced concentration of TAG in liver tissue due to periparturient RPC supplementation including Elek et al., 2013 (approximate decreases from 10 to 3% wet basis at 7 days in milk and from 3.8 to 1% at 35 days in milk), Santos and Lima, 2009 (decrease from 10.4 to 6.0%, DM basis at 7-10 days in milk), and Zom et al., 2011 (decrease from ~7.5 to 5% wet basis at week 1 and from ~ 6 to 5% at week 4 postpartum). Although Pinotti et al. (2013)

did not measure TAG in liver, the lower plasma ratio of NEFA to cholesterol indicated a lower risk of fatty liver in lactating dairy cows fed RPC compared with control cows. Lastly, liver concentration of glycogen was increased linearly with increasing intake of RPC in a dose response study with lactating dairy cows in NY (Pieperbrink and Overton, 2003). Ability of high lipid hepatocytes to convert propionate to glucose is reduced so, as a result, glycogen must be metabolized for glucose. However the ability of liver slices to convert propionate to glucose in vitro was not significant (linear effect of increasing intake of choline, $P = 0.21$). Yet the in vitro conversion of palmitate to esterified products in biopsied liver tissue tended to decrease linearly with increasing choline intake ($P < 0.06$) by 82% (Pieperbrink and Overton, 2003). Perhaps the increased dietary supply of choline to the liver resulted in less retention of fat in the liver and allowed for greater replenishment of glycogen.

Feeding RPC also demonstrated its ability to reduce fat accumulation in the liver when pregnant, nonlactating dairy cows were fed diets restricted to consume 30% of their energy requirement (Cooke et al. 2007). Liver TAG was decreased from 16.7 to 9.3 µg/µg of DNA on the 10th day of feed restriction without changing plasma concentrations of BHBA. In summary, TAG accumulation in the postpartum liver can be reduced through RPC supplementation without apparent changes in BHBA. This may be through improved formation of VLDL for lipid export from the liver.

A positive milk response to supplemental choline has been reported using dairy cows in early and midlactation. Many labs have reported a significant improvement in yield of milk or fat-corrected milk yield when RPC supplementation was begun in the last 3 to 4 weeks of gestation and continued postpartum including increases of 2.9 kg/d (Ardalan et al., 2011), 2.4 kg/d (Piepenbrink and Overton, 2003), 4.4 kg/d (Elek et al., 2008), 1.8 kg/d (Lima et al., 2012), 2.9 kg/d (Pinotti et al, (2003), 1.2 kg/d (Zahra et al., 2006), and 1.9 kg/d (Zom et al., 2011). Others reported nonsignificant increases of 2.6 kg/d (Hartwell et al., 2000), 2.3 kg/d (Janovick Guretzky et al., 2006), and 2.0 and 3.0 kg/d (Xu et al., 2006). Waiting to start cows on supplemental choline soon after (5 weeks) or quite a bit after parturition (22 weeks) resulted in a linear increase in yield of 3.5% fat-corrected milk or uncorrected milk, respectively with increasing intake of rumen-protected choline chloride (upper amount of 51 to 58 g/d of choline; Showa Denko, K.K., Tokyo, Japan; Erdman and Sharma, 1991). Cows averaging 150 days in milk at the beginning of the 9-week study produced 3.2 kg/d more milk (24.7 vs. 21.5 kg/d) when abomasally infused with 50 g of choline from choline chloride daily (Sharma and Erdman, 1989b). Davidson et al. (2008) waited until 21 days in milk before feeding 40 g/d of choline (ruminally protected as a Ca salt) and reported a 6.4 kg/d improvement in milk yield (37.7 vs. 44.1 kg/d). In summary, milk response to choline supplementation has not depended upon stage of lactation suggesting that choline may be a limiting nutrient for milk yield in most cow diets regardless of physiological state and intake of other nutrients.

Interaction with other Dietary Ingredients

Methionine. The 1 labile methyl group of methionine can be used to help supply the 3 needed methyl groups for de novo synthesis of choline. Emmanuel and Kennelly (1984) infused carbon 14-labeled methionine into lactating dairy goats and reported that 28% of the methionine was used for synthesis of choline and that 6% of the plasma choline was derived from methionine.

Therefore a portion of the increased milk yield reported from methionine supplementation Osorio et al., 2013) could have resulted from some methionine being converted to choline. On the flip side, it is plausible that increasing the supply of choline to the cow may spare some methionine from being converted into choline, thus allowing more of this key essential amino acid to be used for protein synthesis, milk production, and apolipoproteins. In addition, the 3 methyl groups of choline can only be used to synthesize methionine after the choline is oxidized to betaine. When lactating dairy cows were abomasally infused with choline (30 g/day) or methionine (45.6 g/day), milk yield was similar (28.3 vs. 28.7 kg/day) (Sharma and Erdman, 1988a). However, when the infusate included 2-amino-2-methyl-1-propanol (2AMP), a compound known to inhibit the synthesis of choline from methionine, milk yield was greater for cows infused with choline (27.9 vs. 26.4 kg/day). Investigators concluded that some methionine can be used to help synthesize choline. However, an inordinate amount of methionine would need to be delivered to the small intestine to supply enough methyl groups to synthesize the amount of choline that has improved milk production over several studies. Choline's ability to increase milk fat percentage over methionine infusion held true in this study regardless of delivery of 2AMP (4.08 vs. 3.63% without 2AMP and 4.01 and 3.57% with 2AMP) indicating choline's greater influence on lipid metabolism.

The recommended concentration of methionine for lactating dairy cows is 2.4% of metabolizable protein (MP) and a ratio of methionine to lysine of 1:3 (NRC, 2001). Formulating to these concentrations is difficult. Might choline supplementation be more effective in diets deficient in methionine supply because some choline may be utilized for synthesis of methionine? Choline-feeding studies can be divided into those in which methionine supply was thought to be adequate or deficient. Methionine adequate studies. Providing 14.4 g of RPC (Balchem Inc.) daily to periparturient cows fed methionine-adequate diets increased milk production numerically but not significantly (27.2 vs. 29.1 kg/day, P = 0.29; Zom et al., 2011). Top dressing RPC (11.25, 15, or 18.75 g/d of choline chloride, Balchem Inc.) from -21 to +63 days in milk to multiparous cows fed diets calculated to supply methionine at 2.14% of MP tended to produce more fat-corrected milk compared with control cows (42.8 vs. 45.2 kg/d; Piepenbrink and Overton, 2003). Lastly, primiparous and multiparous cows (n = 369) were fed 15 g/d of rumen protected choline chloride (Balchem Inc.) from 25 days prepartum to 80 days postpartum (Lima et al., 2012). Concentration of dietary methionine was at 2.1 to 2.2% of MP. Yield of fat-corrected milk increased from 42.8 to 44.6 kg/d due to RPC feeding. Methionine deficient studies. Including rumen protected choline (40 g/day of Ca salt of choline; Robt Morgan, Inc.) in a methionine-deficient diet for multiparous cows in early lactation resulted in more milk yield compared to control cows (39.8 vs. 44.1 kg/day) whereas meeting the dietary methionine recommendation (Mepron, Degussa) without choline supplementation did not change milk yield (39.7 vs. 39.8 kg/day; Davidson et al., 2010). In another study in which dietary methionine was calculated to be deficient, Jersey cows produced > 4 kg/d more milk in the first 3 weeks postpartum when fed 15 g of choline chloride/day (Balchem Inc.) starting prepartum whereas milk production by Holstein cows was not affected by feeding RPC (Janovick Guretzky et al., 2006). Ardalan et al. (2011) reported a 2.9 kg/day increase in milk yield by primiparous and multiparous cows in the first 10 weeks of lactation when fed 14.4 g/day of RPC (Col 24, Soda Feed Ingredients, Monaco, France) regardless of whether the diet was deficient or adequate in methionine. These few studies are inadequate for one to conclude

that choline is more effective when methionine intake is below recommendations. However, in most cases, milk yield is increased when RPC is fed regardless of methionine status.

Supplemental methionine has not prevented accumulation of TAG in the bovine liver (Bertics and Grummer, 1999; Osorio et al., 2013; Piepenbrink et al., 2004; Preynat et al., 2010) suggesting that methionine may not be limiting apolipoprotein synthesis for formation of VLDL by the liver.

Concentration of Dietary Crude Protein. It has been proposed that some of the methyl groups from choline can be used to synthesize methionine although Emanuel and Kennelly (1984) reported that none of the carbon 14-labeled choline infused into lactating dairy goats was recovered in circulating methionine. It has been suggested that some of the positive effects of choline supplementation may result from the sparing of methionine for the synthesis of choline, leaving more methionine to serve in its role as an essential amino acid. Feeding increasing amounts of rumen-protected choline chloride (Showa Denko, K.K., Tokyo, Japan) in diets of 13.0 or 16.5% CP increased milk production linearly by 3.1 and 2.0 kg/d, respectively without detection of a choline by CP interaction (Erdman and Sharma, 1991). In separate studies using diets of differing concentrations of CP (13.8 or 16.7%), abomasal infusion of 40 or 50 g of choline daily as choline chloride increased milk production of cows by 1.6 and 3.2 kg/d, respectively (Sharma and Erdman, 1989b). Feeding 7.5 g/day of ruminally protected choline chloride with a 12.5% CP diet to periparturient multiparous cows (n = 14) resulted in a nonsignificant increase of 2.6 kg/d of 4% fat-corrected milk (24.4 vs. 26.4 kg/d; Xu et al., 2006). In the same publication, these investigators used a 12% CP diet for primiparous and multiparous cows (n = 36) and reported milk production of 27.7, 34.0, 29.0, and 29.2 kg/d when periparturient cows (n = 36) were fed 0, 11 22 or 34 g/d of ruminally protected choline chloride. It appears that supplementing with ruminally protected choline has been effective to increase milk yield when dietary intake of CP has been both deficient and adequate.

Monensin. Cows fed monensin usually have a greater proportion of propionate in the rumen which serves as a precursor for synthesis of glucose by the liver. Greater glucose production may reduce the need for mobilization of body fat during the period of negative energy balance in early lactation and so reduce circulating concentrations of BHBA. Monensin given in a controlled-release capsule did not compliment RPC feeding to change milk production in a 182-cow study done in Canada (Zahra et al., 2006).

Parity

In a Latin square study using only 3 primiparous midlactation cows, milk production was increased by 3.2 kg/d when abomasally infused daily with 50 g of choline as choline chloride (Sharma and Erdman, 1989b). In a much larger study, primiparous cows (n = 578) were fed 15 g/d of RPC (Balchem Inc.) only during the last 21 days of gestation and not in the postpartum period. Milk production tended to increase (P = 0.07) from 27.9 to 28.7 kg/d in the first 80 days postpartum (Lima et al., 2012). Primiparous cows did not produce more milk when fed 40 g/d of choline (ruminally protected as a Ca salt) (27.9 vs. 27.5 kg/d) whereas multiparous were responsive (37.7 vs. 44.1 kg/d; Davidson et al., 2008). In those studies in which both primiparous and multiparous cows were used, investigators did not report the effect of feeding

RPC separately by parity (Ardalan et al., 2011; Erdman and Sharma, 1991; Lima et al., 2007; Xu et al., 2006; Zahra et al., 2006). Although the data is limited, but based upon the large number of primiparous cows used in the study of Lima et al., 2007, it appears that primiparous cows should positively respond with more milk to supplemental RPC. According to the review by Bobe et al. (2004), older cows are better candidates for fatty liver. Therefore, the number of fresh primiparous cows that would benefit from reduced fatty liver by supplementation of RPC would be less compared with multiparous cows.

Body Condition

Bobe et al. (2004) stated "The primary nutritional risk factor for fatty liver is obesity." Greater weight gain during the dry period due to supplemental feeding of grain resulted in greater concentration of TAG in liver compared with cows fed less energy prepartum (6.73 vs. 3.05%, wet tissue; Fronk et al., 1980). Therefore, it seems logical that periparturient supplementation with RPC would be most effective for cows that are overconditioned. Hartwell et al. (2000) reported that cows with a BCS of ≥ 3.75 at 28 days prepartum had approximately twice the concentration of TAG in liver at 28 days postpartum compared to those with a BCS of < 3.75. Feeding RPC did not affect TAG content of liver in this study and BCS was not a factor influencing the effect of RPC. Because only 16 cows were scored ≥ 3.75, only 8 cows per treatment were biopsied which reduced the chances of detecting a RPC effect due to the high deviation among cows in TAG concentration. Likewise, other studies have suffered from the same problem of underpower for detecting a RPC by BCS interaction which may have contributed to a failure by authors to report such an analysis. The 32 multiparous cows participating in the study of Elek et al. (2008; 2013) had a mean BCS of 4.0 at 21 days prior to calving. Those cows fed RPC (Norcol-25; Norods Italy, Bussolengo, Italy) during the periparturient period had lower TAG in liver at 7 and 35 days postpartum. Yet RPC has also successfully reduced liver TAG when cows were in moderate body condition to start the study. As a group, the 38 multiparous cows in the Netherlands had a mean BCS of 3.25 (Zom et al., 2011). Concentration of TAG in liver at 1 and 4 weeks postpartum was less due to supplementation with RPC.

When milk response is considered, cows of various body conditions appear to respond positively to RPC supplementation. Multiparous cows of relatively thin body condition (mean of 2.2 between 21 and 91 days in milk) produced more milk (37.7 vs. 44.1 kg/d) when fed 40 g/d of choline which was ruminally protected as a Ca salt (Davidson et al., 2008). Likewise, multiparous cows at < 3.75 BCS at calving (n = 76) tended to produce 2.7 kg/d more milk (43.6 vs. 40.9 kg/d) when fed 15 g/d of rumen protected choline chloride (Balchem Inc.) from 21 days before to 21 days after calving. This positive response of milk yield to RPC was similar to results when all 93 cows (including the 17 heavier cows) were included in the data set (43.5 vs. 41.3 kg/d, mean of 105 days in milk; unpublished, University of Florida). Supplemental choline (25 g/d prepartum and 50 g/d postpartum using fat-encapsulated Norcol-25) was also effective to increase 60-day milk yield (37.2 vs. 41.6 kg/d) of dairy cows (n = 30) that averaged 4.01 BCS at 21 d before calving (Elek et al., 2008). In a 182-cow study from Guelph, only the cows having a BCS ≥ 4.0 at 3 weeks prior to calving produced 4.4 kg/d more milk (27.0 vs. 31.4 kg/d) when top-dressed with 56 g/d of RPC (Balchem) compared with control cows (Zahra et al., 2006). These cows also consumed 1.1 kg/d more DM from 3 weeks prepartum to 4 weeks

postpartum. However, milk production by the 125 cows in more moderate body condition (< 4.0 BCS) at calving did not differ in milk yield (32.0 vs. 32.0 kg/d) due to RPC supplementation. Collectively these studies indicate that RPC supplementation can benefit production and liver health regardless of body condition. There is not enough evidence to date to conclude that cows with greater condition are better candidates for RPC supplementation.

Milk Fat Depression

Concentration of milk fat is seldom changed when RPC is supplemented to lactating dairy cows. Erdman et al. (1984) used unprotected choline chloride to supplement high concentrate TMR diets for dairy cows exceeding 60 DIM. Intake of choline was 15, 35, and 50 g/d in experiment one and 5, 27, 49, and 73 g/d in experiment two. Cows consuming choline at approximately 50 g/d tended to increase milk fat from 3.43 to 3.77% (experiment 1) and from 2.6 to 3.41% (experiment 2) compared to control cows. Milk fat concentration was depressed in experiment 2. Production of FCM tended to increase from 22.5 to 24.5 kg/d (experiment 1) and from 13.1 to 16.8 kg/d (experiment 2) for control cows and those fed ~50 g/d of choline, respectively. More data on the effect of RPC supplementation on milk-fat depressed cows is needed.

Diets Varying in Forage to Concentrate Ratios

Flow of choline to the duodenum may occur primarily via flow of ruminal protozoa (John and Wyatt, 1979) which can readily absorb choline and store it as phosphatidylcholine. Because protozoal numbers are reduced in the rumen of cows fed diets enriched in concentrate feeds and in long chain fatty acids, cows fed these types of diets may be most responsive to supplemental choline. Feeding 20 g/d of rumen protected choline chloride (Overcholine 45% Coated; Ascor Chimici; Forli, Italy) during the periparturient period to multiparous cows (n = 26) fed postpartum diets of > 75% forage resulted in 2.9 kg/d more milk (28.6 vs. 31.5 kg/d) in the first month postpartum (Pinotti et al., 2003). On the flip side, cows consuming diets of 70% concentrate and abomasally infused with 50 g/d of choline produced 3.2 kg/d more milk (21.5 vs. 24.7 kg/d) (Sharma and Erdman, 1989b). In addition, cows fed diets of 60% concentrate also responded positively to increasing supplementation of rumen protected choline (Showa Denko, K.K., Tokyo, Japan) by producing an additional 2.0, 2.0 and 3.1 kg/d more milk or fat-corrected milk across 3 studies (Erdman and Sharma, 1991). Lactating dairy cows fed either high forage or high concentrate diets have benefited from supplementation with RPC.

Role in Postpartum Health

Primiparous and multiparous cows (n = 369) were fed 15 g/d of rumen protected choline chloride (Balchem Inc.) from 25 days prepartum to 80 days postpartum (Lima et al., 2012). Yield of fat-corrected milk increased from 42.8 to 44.6 kg/day due to RPC feeding. Cows fed RPC had less morbidity, especially less clinical ketosis (13.9 vs. 4.7% for primiparous cows and 9.8 vs. 3.5% for multiparous cows) and fewer cases of mastitis (20.0 vs. 17.2% for primiparous cows and 24.1 vs. 13.4% for multiparous cows). However, in a second field study (578 primiparous cows) in which 15 g/d of choline chloride (Balchem Inc.) was fed only during the prepartum period (last 22 days prior to calving), morbidity tended to be greater for cows receiving choline (33.7 vs. 41.2%) largely due to an increased incidence of metritis and fever.

However the number of mastitis cases per cow were reduced when choline was fed. Mean milk yield by primiparous cows fed RPC tended to be greater (27.9 vs. 28.7 kg/d). Authors concluded that RPC supplementation should continue into the postpartum period if health benefits are to be realized. This is when nutrient deficiencies and mobilization of stored lipids and amino acids are most pronounced.

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Dr. Mike Van Amburgh

Mike Van Amburgh is a Professor in the Department of Animal Science at Cornell University where he has a dual appointment in teaching and research. His undergraduate degree is from The Ohio State University and his Ph.D. is from Cornell University. He teaches multiple courses and leads the Cornell Dairy Fellows Program, advises approximately 50 undergraduate students and is the advisor for the Cornell University Dairy Science Club. For the last 17 years, a major focus of his research program has been to describe the nutrient requirements of dairy calves and heifers and aspects of endocrine control of developmental functions such as mammary development. This has evolved into describing and working to understand factors in neonatal life that establish lifetime productivity functions and outcomes.   Mike currently leads the development of the Cornell Net Carbohydrate and Protein System, a nutrition evaluation and formulation model used worldwide and through that effort is focused on enhancing the efficiency of nutrient use by ruminants to improve the environmental impact of animal food production. A significant focus of his current work is to understand whole animal and ruminal nitrogen metabolism and amino acid supply and requirements to enhance the development of the Cornell Net Carbohydrate and Protein System.   Further, his group is active in developing methods to better describe the interaction between forage and feed chemistry, rumen function and nutrient supply to compliment the model. He has authored and co-authored over 70 journal articles and many conference proceedings and is the recipient of several awards including the American Dairy Science Associate Foundation Scholar Award, the Land O'Lakes Teaching and Mentoring Award from ADSA, the AFIA Award for Research, the CALS Professor of Merit Award and the CALS Distinguished Advisor Award.

Development of an Assay to Predict Intestinal Nitrogen Indigestibility and Application of the Assay in High Producing Lactating Cattle: One Step Closer to Feeding a Cow like a Pig?

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SUMMARY

- An up-dated method to estimate intestinal nitrogen indigestibility of feeds for ruminants was developed from a combination of current methods and then refined to reduce particle loss.
- The assay is comprised of a 16 hr in-vitro incubation in rumen fluid and buffer and then a 24 hr in-vitro incubation in a specific intestinal enzyme cocktail in a shaking water bath.

- The assay was developed primarily for non-forage feeds and represents a departure from the detergent system used to fractionate most feeds.
- For most feeds the results from the assay differ significantly from acid detergent insoluble protein demonstrating differences between feed chemistry versus the bio-assay.
- To investigate the accuracy and precision of the assay predictions, a study was conducted with high producing lactating cattle to evaluate the sensitivity to differences in predicted indigestibility o two different blood meal products.
- Milk yield and overall performance of lactating dairy cattle was reduced in cattle fed the lower digestibility blood meal and the difference in the amount of available N supplied was 32 grams, less than 5% of total N intake.

INTRODUCTION

Current cattle diet formulation models rely on library estimates of intestinal digestibility of proteins and carbohydrates to predict metabolizable energy (ME) and protein (MP) supply (NRC, 2001; Fox et al., 2004; Tylutki et al., 2008).   As models become more accurate and precise in the prediction of nutrient supply and evaluation of requirements and nutrient balance, greater scrutiny will be placed on inputs currently relegated to static library values. Although CP is not a functional dietary nutrient for cattle, many diets are still formulated on this metric, creating confusion due to inadequate information provided by the value, especially with regard to MP supply and amino acid availability.   As diets are formulated to be closer to MP requirements and rumen ammonia balance, they will, under most circumstances, be lower in CP, thus, accurate estimates of intestinal digestibility (ID) of protein and amino acids are increasingly important to ensure an adequate supply of those nutrients. Application of outdated feed library values to all feeding conditions can lead to under- and over-estimations of MP and amino acid supply, resulting in variation from expected production. This paper describes the re-development of an in-vitro intestinal digestion (IVID) assay for protein containing feeds used in ruminant nutrition, including intact commercially available feeds designed to resist rumen degradation. The methods used were developed to provide adequate sample size, minimize sample loss, and to allow for standardization of enzyme activity and concentration. The assay contains positive and negative controls to evaluate standardization among and within laboratories.

 The feed library of the Cornell Net Carbohydrate and Protein System (CNCPS) (Tylutki et al., 2008; Higgs et al., 2012) has static values for intestinal protein digestibility values for various protein fractions, and acid detergent insoluble protein (ADIP) is used to define the unavailable protein. The committee that developed the 2001 Dairy NRC adjusted available MP from feed by assigning a digestibility of 5% to the ADIP fraction based on data indicating that some amino acids could be liberated and absorbed from this fraction (NRC, 2001). The results from the assay described in this paper can be compared to both the ADIP and the adjusted ADIP value from the NRC calculation as an unavailable protein fraction.

Further, current cattle diet formulation models rely on library estimates of intestinal digestibility of proteins and carbohydrates to predict metabolizable energy (ME) and protein (MP) supply (NRC, 2001; Fox et al., 2004; Tylutki et al., 2008).   As models become more accurate and precise in the prediction of nutrient supply and nutrient balance, there is a greater need to evaluate and be able to adapt the inputs currently used as static library values. Although CP is not a functional dietary nutrient for cattle, many diets are still formulated on this metric, creating

confusion due to inadequate information provided by the value, especially with regard to MP supply and amino acid availability. As diets are formulated closer to the MP requirements of cattle and subsequently lower in CP, accurate estimates of intestinal digestibility (ID) or indigestibility of protein and amino acids are increasingly important to ensure an adequate supply of those nutrients. Use of outdated feed library values to all feeding conditions can lead to under- and over-estimations of MP and amino acid supply, resulting in variation from expected production.

Since the inception of the Cornell Net Carbohydrate and Protein System (Fox et al., 2004; Tylutki et al., 2008), the detergent system of fractionation has been applied to both the carbohydrate and protein components of feeds (Sniffen et al., 1992). More recent work suggests this approach, especially for feeds not containing NDF, might not be appropriate to accurately characterize how protein is partitioned and digests in the rumen and post-ruminally. Several approaches have been developed to predict the intestinal digestibility of protein in feeds and are a departure from the detergent system of feed chemical composition (Calsamiglia and Stern, 1995; Ross et al., 2013). The N assay was developed to predict N indigestibility, and will be described in that manner throughout the paper. The cattle study described in this paper was conducted by formulating two different diets in high producing cattle using two different blood meals with different predicted intestinal protein indigestibility to test the accuracy and precision of both the assay (Ross et al., 2013) and our ability to apply those values in the CNCPS for diet formulation.

ASSAY DEVELOPMENT CONSIDERATIONS

The following discussion points are provided to highlight potential problems or concerns with current methods and to provide evidence for the need to develop alternative approaches.

Use of Bags:

- Created a microbial barrier to feed access and microbial attachment which artificially prolongs the lag phase of digestion.
- Demonstrated loss of highly soluble components of feeds from the bag prior to digestion and loss of particles as digestion progresses. Measured losses of up to 30% of the initial sample prior to any analyses have been reported.

Use of Enzymes:

- Profiles and activities are not properly described and characterized.
- The digestive process of the ruminant is a continuous flow of digesta with continuous secretion of enzymes and digestive juices (Hill, 1965).

Abomasal Digestion:

- Pepsin, an endopeptidase, hydrolyzes approximately 15-20 % of dietary protein to AA and small peptides (Kutchai, 1998). Bovine pepsin has approximately ~60-70 % of the activity of porcine pepsin with hemoglobin as substrate (Lang and Kassell, 1971). Porcine pepsin is generally used in the first step of *IV* intestinal digestion assays to measure ruminant intestinal digestion (Calsamiglia and Stern, 1995; Gargallo et al., 2006).

- One mg of porcine pepsin contains 200 to 625 units with pH between 1.5 and 2.5, for optimum pepsin activity.
- Lysozymes which aid in digestion of microbes are also secreted in the digestive tract. Bovine digestive lysozyme has a lower optimum pH than chicken lysozyme (7.65 vs. 10.7, respectively) with a pH optimum 5, not 7, making it resistant to pepsin hydrolysis. Furthermore, bovine lysozymes lyse gram-negative *and* gram-positive bacteria, while chicken lysozyme acts only on gram positive bacteria (Dobson et al., 1984; Protection of plants against plant pathogens: http://www.patentstorm.us/patents/5422108/description.html; accessed Nov 1, 2010). However, bovine digestive lysozyme is commercially unavailable.

Small Intestine Digestion:

- Species differences exist in the activities of proteases in the pancreas. In rats, trypsin activity represents ~80 % while in ruminants it represents only 15 % and chymotrypsin makes up 43 % (Keller et al., 1958).
- The calculated activities of trypsin and chymotrypsin in intestinal contents from 5 month old calves (Gorrill et al., 1968) were 19.48 and 15.9 U/ml, respectively using p-toluene-sulfonyl-L-arginine methyl ester (TAME) and benzoyl-L- tyrosine-ethyl ester (BTEE), as substrates.
- In sheep, the activities of trypsin, chymotrypsin and carboxypeptidase A increased from the pylorous to 7 m beyond with maximum specific activities of 24, 150, and 35 µM of respective substrates (benzoyl-L-arginine-ethyl ester (BAEE), acetyl- L-tyrosine-ethyl ester (ATEE), hippuryl-DL-phenyl-lactic acid) per minute per ml digesta, and then decreased (Ben-Ghedalia et al.,1974).
- Sklan and Halevy (1985) found maximal activities of pancreatic enzymes in the proximal segments of the ovine SI at 1 m distal to the pylorous and then relatively constant ratios of enzyme levels (trypsin, chymotrypsin, elastase, carboxypeptidases A & B) to cerium-141, an unabsorbed reference, of 0.065, 0.053, 0.015, 0.05 and 0.045, respectively, 1.5 to 9 m distal to the pylorous. No other *in vivo* activities for bovine pancreatic proteolytic enzymes were measured.
- Units of enzyme activity are dependent upon substrate (a protein or ester) hydrolyzed in addition to the wavelength used. Among the studies reviewed, this data varies considerably and is not standardized.
- The current three step assays (Calsamiglia and Stern, 1995; Gargallo et al., 2006; Borucki Castro et al., 2007; Boucher et al., 2009a,b,c) use 3 g of pancreatin per L after an IV abomasal digestion with 1 g L^{-1} of porcine pepsin in 0.1 N HCl N at pH 1.9 or 2. However, the pancreatin concentration in the assay of Calsamiglia and Stern (1995) was 1.69 mg ml^{-1} based on the conditions described for the assay as published.
- Pancreatin always contains amylase and lipase but over time the proteolytic enzyme has changed from trypsin to many enzymes, including trypsin, ribonuclease and protease (specifications for P7545; (www.sigmaaldrich.com/catalog/product/sigma/p7545?lang=en. accessed, Nov 10, 2010) and specific units of enzymatic activity are not provided.
- Further, lipase activity is essentially nonexistent in bovine pancreatic juice (Keller, 1958) but is high in saliva. Calsamiglia and Stern (1995) attributed the increase in digestion of their proteins over those obtained using the multi-enzyme system of Hsu et al. (1977) to the presence of amylase and lipase in pancreatin.

Thus, the enzymes used in the assay for the abomasal and intestinal digestion step and their respective activities were based on the data described and were adopted and run in parallel with pancreatin.

ASSAY METHODS

A description of the assay development follows in a sequential manner with statements about sources of variation and decisions made to optimize the assay while minimizing or eliminating irrelevant sample loss.

General Procedures:

- Unless specified otherwise, all analyses were conducted on duplicate samples.
- Dry matter was determined at 105ºC in a forced-air oven overnight.
- Nitrogen (N) content of original feeds and residues was measured by block digestion and steam distillation with automatic titration (Application Note, AN300; AOAC Official method 2001.11; Foss, 2003; Tecator Digestor 20 and Kjeltec 2300 Analyzer, Foss Analytical AB, Höganäs, Sweden; AOAC 2001.11).
 Exposure to rumen microbes:

This step in the assay was evaluated in three stages to evaluate variation and sample loss.

- Three bag materials with different pore sizes (15 μm, mesh; 25 μm, fiber (Ankom) and 50 μm, *in situ* (Ankom)) were evaluated for *in vitro* intestinal digestion following *in vitro* vs. *in situ* fermentation (Ross, et al., 2010). After many attempts at developing conditions that minimized loss of material prior to assay or during the assay, it was difficult to distinguish digestion from bag loss, thus the use of any bags was abandoned.

  From this point forward 16-h fermentation was performed via IV methods in Erlenmeyer flasks.

- Plastic centrifuge tubes were evaluated as a fermentation vessel and found to be unfavorable for rumen bacterial growth and sample size had to be reduced to work appropriately in 50 mL tubes.
- Glass Erlenmeyer flasks provided the greatest digestibility values, and had lower variability and superior repeatability compared to plastic centrifuge tubes. For this reason, flasks were chosen as the vessel for the fermentation step. Commercial protein sources (0.5 g) were included in their un-ground form, while forages, byproducts and non-commercial protein sources were ground through a 2 mm screen in a Wiley Mill (Thomas Scientific, Swedesboro, NJ).

Enzymatic Hydrolysis

- Pepsin: Porcine pepsin used but added at 60 % of previous methods in pH 2 HCl (~0.013 M) to contain ~282 U ml^{-1} in flask.
- Intestinal (ID) enzymes: Initially, enzymes and activities described by Ben-Ghedalia et al. (1974) were used in the enzyme mix until carboxypeptidase A became unavailable.

Different combinations of elastase and carboxypeptidase Y in addition to trypsin and chymotrypsin were then evaluated without duplication of intestinal digestion. Amylase and lipase were added along with trypsin and chymotrypsin (50 and 4; 24 and 20 U ml^{-1}, respectively) which yielded digestion approximately similar to levels observed with carboxypepetidases A & B. Pancreatin at a level similar to Calsamiglia and Stern (1995; 1.72 mg ml^{-1}, difference due to initial dilution so maintained throughout) was also analyzed concurrently with the mixture of individual enzymes.

- Assay termination for both IV fermentation and enzymatic digestion was accomplished by quantitative filtration under vacuum though 9 cm glass microfiber filter (pore size of 1.5 µm; Whatman 934-AH; GE Healthcare Bio-Sciences Corp., Piscataway, NY) using hot water to transfer. Hot water was necessary to help dissolve away viscous residues from the in vitro step.

DISCUSSION

To manufacture feeds that escape ruminal degradation, companies have reduced particles to a size that will flow with rumen fluid and liquid passage rate thus, the IV residues are not always captured on small pore filter paper, despite small pore size (1.5 µm). Consequently, when known water soluble components are present that can pass prior to being fermented or filtered, the filtration eluent has to be captured, the N analyzed and then the freeze dried eluent has to be added back to the filtered residue. This process became necessary when we recognized that several samples provided for analysis had components that solubilized immediately upon addition to water and when immediately filtered could not be recovered on the filter paper. Alternatively, the entire IV fermentation mix can be freeze dried, analyzed for N and corrected for microbial contamination using corn silage ND residue with and without rumen fluid treated in similar fashion. This process makes the rumen escape protein (RUP) estimation a little more ambiguous but it is the only way to capture the soluble component which has been shown to provide MP amino acids to the animal (Reynal et al., 2007; Volden et al. 2002).

Correction of in Vitro Residues for Microbial Contamination

The original objective of the assay was to estimate ID, not RUP, however, it became apparent that in either case, microbial contamination should be accounted for if possible to ensure less bias in the ID determination. Therefore, a series of approaches were evaluated to provide a quantifiable and repeatable indication of microbial contamination:

- ^{15}N was used to label bacteria for estimation of contamination
- Washing with methylcellulose to remove attached bacteria
- Measuring purines as a label for contamination

After much work, none of the previously mentioned approaches were consistent and repeatable among all samples, thus an alternative was needed. The alternative was to develop a substrate that was low in N content, provided adequate substrate for microbial growth, was available to a commercial laboratory, and produced repeatable results. The substrate eventually chosen was neutral detergent residue from corn silage. Bulk volumes of ground corn silage was washed in hot ND solution with amylase, rinsed, washed in ammonium sulfate to remove detergent, and then used both as the fermentation control sample and to estimate microbial contamination. Properly washed corn silage ND residue has very low N content, so any N that is accumulated is assumed to have come from bacteria. Corn silage ND residue can also grow a significant

quantity of bacteria, making it superior to pure cellulose, easy to recover, and easy to measure. Given this, the corrections that are made with the corn silage ND residue are as follows:

- The difference in N content between CS ND residue with and without rumen fluid (after IV incubation), on per g DM basis provides a robust estimate of microbial contamination and is used for ID step.

Use of Positive and Negative Controls to Evaluate IV and Intestinal Digestibility:

Positive and negative controls for both fermentation and intestinal digestibility steps were included. To evaluate the fermentation phase, NDF digestion of corn silage ND residue sample was run concurrently. A heat damaged blood meal with near zero ruminal and intestinal digestibility was included throughout a negative control. A feed with similar digestibility as samples, i.e., a soy product or blood meal, was also included. A blood meal with known high intestinal digestibility was included as a positive control for the ID assay.

Comparison of Modified TSP with Cornell Assay

Digestibility of two blood meals (from Boucher et al., 2011) were evaluated using the new method with the enzyme mix and pancreatin (Table 1) and compared with the modified TSP. Rumen N digestibility of BM4 was 18 % higher using bags but 6 % lower for BM5. The implication from this comparison is that material was solubilized or lost from the bag prior to being analyzed which provided higher rumen degradability in the TSP. Total N digestibility for BM5 was similar between both procedures and the enzyme mix and pancreatin. However pancreatin digestion of BM4 in the modified TSP was lower than either ID digestion using the

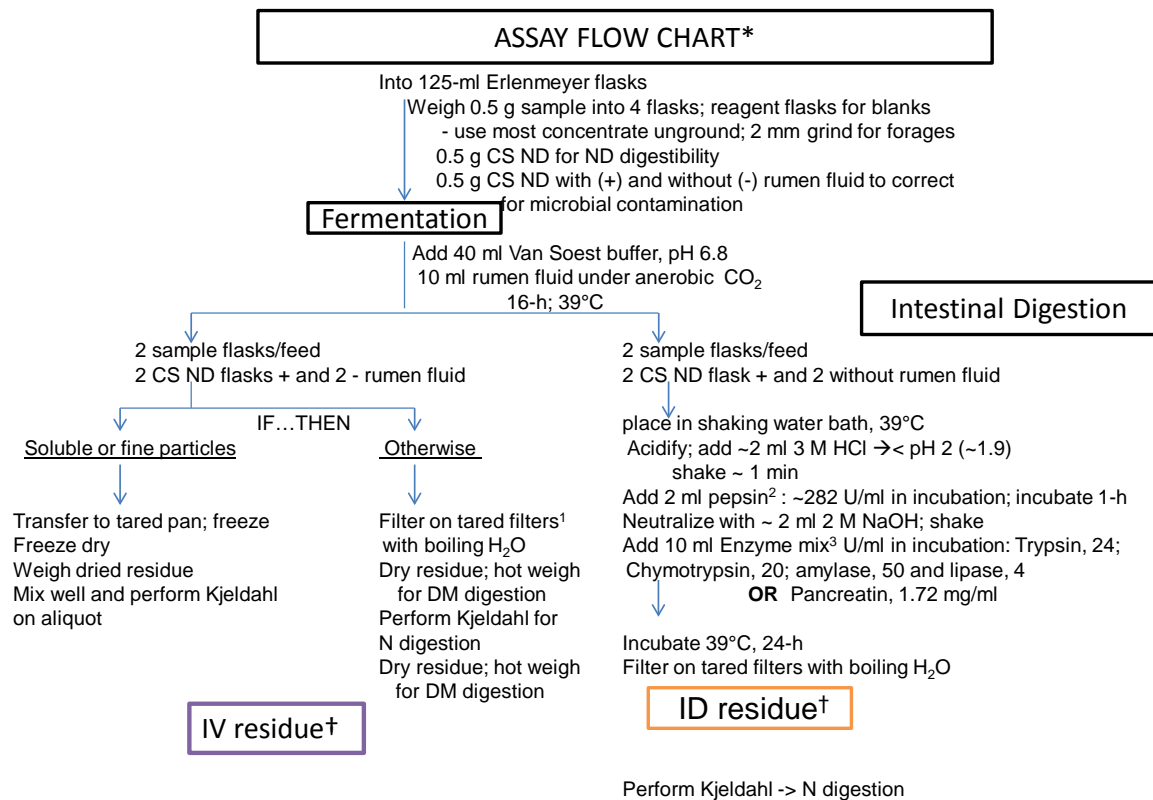Figure 1. Assay flow chart with enzyme activity and specificity descriptions.

ASSAY FLOW CHART*

Into 125-ml Erlenmeyer flasks
Weigh 0.5 g sample into 4 flasks; reagent flasks for blanks
- use most concentrate unground; 2 mm grind for forages
0.5 g CS ND for ND digestibility
0.5 g CS ND with (+) and without (-) rumen fluid to correct
for microbial contamination

Fermentation

Add 40 ml Van Soest buffer, pH 6.8
10 ml rumen fluid under anerobic CO_2
16-h; 39°C

Intestinal Digestion

2 sample flasks/feed
2 CS ND flasks + and 2 - rumen fluid

2 sample flasks/feed
2 CS ND flask + and 2 without rumen fluid

IF…THEN

place in shaking water bath, 39°C
Acidify; add ~2 ml 3 M HCl → < pH 2 (~1.9)
shake ~ 1 min
Add 2 ml pepsin[2] : ~282 U/ml in incubation; incubate 1-h
Neutralize with ~ 2 ml 2 M NaOH; shake
Add 10 ml Enzyme mix[3] U/ml in incubation: Trypsin, 24;
Chymotrypsin, 20; amylase, 50 and lipase, 4
OR Pancreatin, 1.72 mg/ml

Soluble or fine particles

Otherwise

Transfer to tared pan; freeze
Freeze dry
Weigh dried residue
Mix well and perform Kjeldahl
on aliquot

Filter on tared filters[1]
with boiling H_2O
Dry residue; hot weigh
for DM digestion
Perform Kjeldahl for
N digestion
Dry residue; hot weigh
for DM digestion

Incubate 39°C, 24-h
Filter on tared filters with boiling H_2O

IV residue†

ID residue†

Perform Kjeldahl -> N digestion

DAR & MEV, 8/6/13

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Figure 1 continued

[†]Corrections
 - IV residue = 'RUP'
     • original - assay blank and microbial contamination [((cs nd + rumen fluid)/g, DM)-((cs nd + rumen fluid)/g, DM)]*wt, DM)
     • new - assay blank and microbial contamination using the above and adjusting for feed NDIN content by CS NDIN digested
 - ID residue = undigested N
     • original – assay blank
     • new – assay blank and microbial contamination using cs nd +/- rumen fluid carried through entire procedure
[1]Filters: 90 mm; Whatman 934AH, 1.5µm
[2]Pepsin in pH 2 HCl: 16.6 mg/ml
[3]Enzyme mix and Pancreatin prepared daily in 1.8 M KH_2PO_4. Enzyme mix prepared to contain the following U in 10 ml: Trypsin, 1680; chymotrypsin, 1400; amylase, 3500, and lipase, 280. If using pancreatin, prepared to contain 120.4 mg in 10 ml.

Enzyme activity definitions

Pepsin	ΔA_{280nm} of 0.001 per min at pH 2.0,37°C measured as TCA-soluble products using hemoglobin.
Trypsin	ΔA_{253nm} of 0.001 per min at pH 7.6, 25°C equals one unit using Benzoyl-arginine ethyl ester (BAEE).
Chymotrypsin	ΔA_{256nm} of 0.001 per min at pH 7.6, 25°C equals one unit using Benzoyl-tyrosine ethyl ester (BTEE).
Amylase	One unit will liberate 1.0 mg maltose in 3 min at pH 6.9, 37°C.
Lipase	One unit releases 1 uEq of acid from olive oil per min.

*NOTE: Quantitatively transfer all residues

Cornell procedure. Using the Cornell method BM4 had higher intestinal digestion. These differences in intestinal digestibility are partially an artifact of the calculations for intestinal digestibility. If a greater amount of feed protein escapes rumen fermentation, with identical unavailable protein values, the intestinal digestibility is mathematically higher for the feed protein with the greater rumen escape and this aspect of the mathematics is not well appreciated by many that want to employ this concept.

Comparison of Intestinal Digestion with the Acid Detergent Insoluble Protein

Within the current structure of many contemporary nutrition models, acid detergent insoluble nitrogen (ADIN) represents the unavailable N component of feed (NRC, 2001; Tylutki et al., 2008) however, the NRC for Dairy Cattle (2001) provides for 5% digestibility of the ADIN fraction. The implication is that the ADIN fraction is not completely unavailable to the animal. Accordingly, the ID assay as outlined was utilized to ascertain whether ADIN is indigestible (Table 2). The ADIN of solvent extracted soybean meal and Soy1 were very similar to undigested feed N following IV fermentation, abomasal and intestinal digestion with either the enzyme mix or pancreatin; however, the ADIN of heat damaged blood meal was roughly 2 % while undegraded N from both intestinal digestion treatments was 95 %. Undegraded N of corn silage following digestion and after correction for microbial contamination was roughly 3 times higher than ADIN content.

Table 1. Comparison of the percent N digested in two blood meals using the modified three step procedure (from Boucher et al., 2011) with Cornell procedure.

	Modified TSP*		Cornell		
	Rumen	Pancreatin	Rumen	Enzyme Mix	Pancreatin
	----% N digested----		-------% N digested--------------		
BM4	19.9	89	1.0	96.6	97.1
BM5	42.3	94	48.7	97.4	97.0

*Boucher

This approach for determining the unavailable N from feeds departs from the traditional detergent partitioning system established by Van Soest and others, and implementation within nutrition models like the CNCPS will create a fraction that crosses the fractions described by detergent chemistry and has a different behavior. We believe this to be more appropriate approach for describing available protein for cattle. For forages, a longer in vitro step might be necessary to make the assay relevant for estimating protein availability since forage particle

retention is usually great than 16-18 hr and closer to 30 hr so more work needs to be conducted to fully evaluate the assay for those feeds.

Table 2. Comparison of percent feed N and acid detergent insoluble N versus undigested feed N after 16-h IV ruminal fermentation followed by 1-h abomasal digestion with pepsin in HCl and 24-h intestinal digestion using either a mix of trypsin, chymotrypsin, amylase and lipase or pancreatin (n=2).

| | Feed N | ADIN | % Undigested Feed N | |
	% DM	% N	Enzyme Mix*	Pancreatin
Anchovy meal	11.50	1.3	25.5	20.1
Alfalfa silage	3.80	6.1	23.2	21.9
Bakery waste	1.80	3.3	20.6	23.6
Blood meal 1	16.20	4.7	22.9[a]	8.0[b]
Blood meal 285	16.89	1.1	0.0	na
Blood meal 300	16.20	7.5	4.6	na
Blood meal 350	15.13	0.9	23.6	na
Blood meal 800	16.50	1.8	2.8	na
Canola 1	6.50	6.3	16.2	12.5
Canola 2	6.60	5.8	14.0	14.0
Citrus pulp	1.04	15.8	55.0	45.4
Corn germ	4.27	11.2	18.5	9.4
Corn gluten	3.13	16.9	28.7	18.9
Corn gluten feed	3.08	11.2	20.7	16.2
Corn silage 1	1.40	9.2	30.0	25.9
Corn silage 2	1.30	8.6	13.9	21.1
Distillers grains 1	4.90	13.1	11.7	9.5
Distillers grains 2	6.40	32.7	27.9[a]	13.6[b]
Hay silage	2.40	12.5	29.6	31.9
Solv. extract. soybean meal	7.60	6.7	7.8	7.6
Soy product 1	7.70	6.5	9.0	4.3
Soy product 2	7.30	7.9	11.1[a]	6.6[b]
Wheat midds	3.30	3.1	9.3	7.2
Heat damaged blood meal	16.10	1.8	95.0	95.0

[abc]Means with different superscripts in same row differ ($P < 0.05$) using Duncans Multiple Range test. Not all samples were statistically evaluated for this manuscript. NA – not available.

DAIRY CATTLE EVALUATION STUDY

Treatments, Animals and Experimental Design

Treatments were established from a quantity of two blood meals secured through the marketplace that would allow an inclusion level of approximately 1 kg per head per day for the entire experimental period. The two blood meals were analyzed for unavailable N (uN) prior to the start of the study using the in-vitro assay described by Ross et al. (2013). Briefly, 0.5g of sample are placed into a 125ml Erlenmeyer flask. 40ml of rumen buffer and 10ml of rumen fluid are added to each flask. Flasks are incubated in a water bath at 39°C for 16h under continuous CO_2. Samples are then acidified with 3M HCL to bring the pH down to 2. Samples are incubated on a shaking bath for one hour after the addition of 2ml of pepsin and pH 2 HCl. Samples are then neutralized with 2ml of 2M NaOH to stop the pepsin reaction. An enzyme mix containing trypsin, chymotrypsin, lipase and amylase is added to the flask and incubated for 24h in the shaking bath at 39°C. Samples are then filtered with a 1.5 µm glass filter and boiling water. Nitrogen content of the residue is determined by Kjeldahl and expressed as a % of total N in the sample.   The blood meals are characterized by their predicted intestinal N indigestibility (INID) since that is the outcome of the assay. The predicted uN of the low (LOW treatment) INID blood meal was 9%, whereas that of the other treatment (HIGH) was 33.8%. Thus, the two dietary treatments were established by inclusion of these blood meals in two different diets on an iso-N basis. The rest of the diets were formulated to be identical.   The low uN blood meal was 15.04% N and the higher uN blood meal was 14.6% N, thus at approximately 1 kg inclusion level, the maximum difference in intestinal N availability was 38.5g N.   The composition of the two diets fed to cattle is in Table 1.

Due to changes in milk yield in both treatments due to stage of lactation, the protein content of both diets was adjusted down at approximately 5 weeks of treatment by reducing the canola meal inclusion level by 50% to be more consistent with the ME allowable milk and to maintain the N supply to a level the cattle should remain sensitive to the treatment differences in N availability created by the inclusion of the two different blood meals.

Ninety-six multiparous cows (726 ± 14.2 kg BW; 147 ± 64 DIM) and thirty-two primiparous cows (607 kg ± 29.5kg BW; 97 ± 20 DIM) were distributed by DIM and BW into 8 pens of 16 cows (12 multiparous and 4 primiparous). Pens were stratified into four levels of milk production, and each stratum randomly allocated to treatments. Diets were formulated using Cornell Net Carbohydrate and Protein System (CNCPS v6.1; Van Amburgh et al., 2013) using the chemical composition of the ingredients used in the experimental diets (Table 3).

The lactation trial consisted of a two week adaptation period, one week covariate period and 9 week experimental period, between March 30 and June 21, 2014 at Cornell University Ruminant Center (Harford, NY). All cows were fed the LOW uN diet during adaptation and covariate periods. Cows were housed in pens under a four row barn design with one bed and more than one headlock per cow and free access to water. All cows received rBST (Posilac, Elanco Animal Health, Indianapolis, IN) on a 14 day schedule throughout the length of the trial.

Cattle were fed once per day for approximately 5% refusal and milked 3 times per day at 6:00, 14:00 and 22:00 and data from all milkings was recorded using Alpro herd management system (DeLaval International AB, SG). Individual milk samples were collected weekly during three

consecutive milkings, and preserved with 2-bromo-2-nitropane-1, 3-diol at 4°C until analyzed. Milk yield was expressed as 3.5% energy corrected milk (ECM) according to the equation of Tyrell and Reid (1965): ECM (kg) = (12.82 * kg fat) + (7.13 * kg protein) + (0.0323 * kg milk).

Cattle were weighed once per week using a walk scale XR3000 (Tru-test, TX) after the morning milking. Further, BCS on a scale of 1 to 5 was determined every two weeks by the same two evaluators. An average of the two evaluators was used for calculation of the mean BCS.

Table 3. The ingredient content and chemical composition of two diets containing blood meals with Low and High indigestible intestinal N digestibility.

	Treatment	
Ingredient, % DM	LOW uN	HIGH uN
Alfalfa haylage	11.5	11.5
BMR corn silage	49.3	49.3
Bakery	1.8	1.8
Blood meal High	3.7	---
Blood meal Low	---	4.0
Canola meal	3.0	3.0
Corn grain	16.1	16.1
Energy Booster 100	1.8	1.8
Molasses	1.8	1.8
Smartamine M	0.1	0.1
Sodium bicarbonate	0.6	0.5
Soybean hulls	4.6	4.5
Urea	0.2	0.2
Wheat midds	4.6	4.5
Min/vit mix	1.0	1.0
Chemical composition		
DM, % as fed	50.0	50.5
CP, % DM	15.2	15.2
NDF, % DM	31.9	32.3
ADF, % DM	21.3	20.5
Ether extract, % DM	4.3	3.9
Starch, % DM	30.4	31.2
Sugar, % DM	3.6	3.3
Ca, % DM	0.65	0.60
P, % DM	0.43	0.43
ME[1], Mcal/kg DM	1.8	1.7
Lys:Met[1], % MP	3.21	3.19

LOW: low uN diet, HIGH: high uN diet. [1]CNCPS predicted

RESULTS AND DISCUSSION

Animal Performance

Overall DMI and N intake for the treatments were similar and milk yield was significantly different for cattle fed the two treatments (Table 4). Milk yield was 1.6 kg/d lower for cattle fed the HIGH uN diet and energy corrected milk (ECM) was 1.9 kg/d lower on the same diet. Further, cattle fed the HIGH uN diet had significantly lower MUN levels that cattle fed the LOW uN diet (Table 2). From this information, it is apparent that the cattle fed the different blood meals had significantly different MP supply, consistent with the predicted values from the uN assay. The predicted difference described earlier (38.5 g N) is equal to approximately 240 g MP, about the amount required to produce 5 kg of milk under the conditions of this study.

Table 4. Effect of N availability on intake, milk production, milk composition and body weight gain of dairy cows fed diets with low and high unavailable N

Item[1]	Treatment		SEM	P-value
	LOW uN	HIGH uN		
DMI, kg	27.4	27.1	0.61	0.75
N Intake, kg DM	671.1	664.4	14.8	0.77
Milk production				
Milk, kg	42.0	40.4	0.31	<0.01
ECM, kg	41.9	40.0	0.32	<0.01
Fat, kg	1.51	1.42	0.02	<0.01
Protein, kg	1.26	1.23	0.01	0.03
Milk composition				
Fat, %	3.6	3.5	0.03	<0.03
Protein, %	3.03	3.06	0.02	0.20
Lactose, %	4.90	4.86	0.02	0.18
MUN, mg/dl	9.4	8.0	0.18	<0.01
SCC (log1000/ml)	3.9	4.0	0.05	0.13
BW and BCS				
BWinitial, kg	684.1	692.1	10.1	0.58
BWchange, kg	34.7	29.7	2.25	0.12
BCSchange, (1-5)	0.20	0.16	0.03	0.29
Efficiency				
Feed efficiency[2]	1.56	1.50	0.03	0.34
Milk N efficiency[3]	30.0	29.7	0.70	0.76

[1] DMI: dry matter intake, ECM: energy corrected milk yield (Tyrrell and Reid, 1965),
[2] calculated as kg milk / kg DMI
[3] calculated as milk N/N intake*100

However, the observed difference on an ECM basis was 1.9 kg, thus the difference between the absolute levels measured in the assay and the observed ECM yield are either due to differences in digestibility within the cow, the amount of the blood meal arriving at the small intestine or the amount of nutrients partitioned to body reserves, or a combination of all of those factors. Although the change in BW and BCS were not significant, the changes are still biologically relevant given the partitioning of nutrients to reserves and away from milk.

To evaluate the outcome of the study, CNCPS v6.1 with the updated feed library rates and pool sizes was used to evaluate the predictions. The chemical composition of the feeds used in the study was inputted into the model. To evaluate the assay within the structure of the model and against the study data, the blood meal values for the uN and ADIN were the only values changed. For the two blood meals, the uN values were inputted in place of the ADIN value, and intestinal digestibility left at zero. Further, the intestinal digestibility of the NDIN value were set to 100% although after being analyzed for aNDFom, the blood meals do not contain any ND residue, so that pool is zero. With this approach, all of the protein in blood meals is in the A2, B1 and C fractions.

The current intestinal digestibility of the NDIN fraction for all feeds is 80% and it appears that the assay of Ross et al. captures that portion of the indigestible protein, therefore by difference; the remaining fractions should be set at 100% digestibility. Thus, with continued testing and implementation of the uN assay for all feeds, the NDIN fraction ID will be set to 100% because it appears that in NDF containing feeds, the uN assay spans both the ADIN and NDIN fractions.

For the cattle inputs, the expected BW change based on the target growth approach was used and the BCS change was also inputted over the period of the study (9 wks), thus this accounted for the distribution of nutrients to other productive uses and not just milk output. With all of the inputs accounted for, the prediction of ME and MP allowable milk with the uN assay information is in Table 5.

In the CNCPS evaluation in Table 5, it is apparent that the feed chemistry described through the detergent system is not appropriate to allow the model to predict the most limiting nutrient in this comparison using blood meal as the treatment. When the uN data are used to describe the chemistry of the blood meals, the model provides an acceptable and realistic prediction of the most limiting nutrient. It is also important to recognize that an accurate and complete description of the animal characteristics was important to make this evaluation and in the absence of that information, the model would predict over 4 kg of MP allowable milk difference. The sensitivity of the model predictions to complete and accurate animal characterization cannot be overstated and helps explain why literature data to evaluate the model rarely allows for robust predictions of most limiting nutrients due the lack of complete information.

Table 5. The actual and energy corrected milk and the metabolizable energy (ME) and protein (MP) allowable milk for both treatments predicted by the CNCPS using the assay data of Ross et al., (2013) to estimate intestinal digestibility of blood meal, or using the original fractionation approach using acid detergent insoluble nitrogen as the unavailable fraction

| | Treatment | |
Item	LOW uN	HIGH uN
Actual milk, kg	42.0	40.4
Energy corrected milk, kg	41.9	40.0
Using uN assay inputs		
ME allowable milk, kg	45.0	46.0
MP allowable milk, kg	42.6	39.3
Using NDIN and ADIN		
MP allowable milk, kg	44.9	44.6

In summary, the uN assay appears to provide protein indigestibility predictions that are consistent with cattle responses and serves as a platform for modifying the approach to predict protein digestibility within the CNCPS and will improve the model's ability to identify the most limiting nutrient. The data also demonstrate we are ready to move beyond the detergent system of fractionation for protein and move to a system that fractionates proteins based on solubility and indigestibility. This approach should allow us to develop a prediction model to more effectively estimate rates of protein degradation because we now have what appears to be a more robust method to predict the indigestible protein pool, consistent with the approach for NDF (Raffrenato et al., 2009) and this fraction is important for accurate calculations of the rate of digestion of the available protein.

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Updating the Estimation of Protein and Amino Acid Requirements of Dairy Cows According to Biology

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SUMMARY

To improve the current models used to balance dairy rations for metabolizable protein (MP) and amino acids (AA), we propose the following updates to refine the estimation of requirements:
- Milk:
  - AA composition based on true protein
  - updated AA composition (see text)
- No need to include a requirement for duodenal endogenous protein flow because part of this protein will be digested and absorbed (but the digested protein arising from the endogenous protein needs to be removed from digestive flow to yield the net supply)
- Metabolic fecal protein:
  - represents the endogenous protein losses, including the undigested fraction of the duodenal flow of endogenous protein
  - MP requirement of 19 g MP/kg DMI, based on the estimation of ileal endogenous flow
  - updated AA composition based on abomasal isolates in cattle and ileal flow of endogenous in pigs (see text)
- Efficiency:
  - efficiency of utilization of MP and AA should vary with supply of MP and perhaps with energy
  - mammary output:uptake ratio should not represent the efficiency of lactation
  - a combined efficiency of AA utilization for both maintenance and lactation

INTRODUCTION

To the continued challenge of making dairy farming more cost effective is now added the pressure of reducing environmental pollution. Indeed, excretion of N, especially in the urine, is a

potential source of water (e.g. nitrates) and air pollution (e.g. N_2O, a greenhouse gas, or as small particulate polluting aerosols; National Research Council (**NRC**), 2003). Increased cost effectiveness and decreased pollution can be achieved through a lower input of dietary protein, provided productivity is not compromised. To accomplish this without compromising productivity requires that feeds are better balanced to match supply and requirement (**rqt**) of proteins.

But, how is "protein" defined? Current models used to balance dairy rations (referred to as "models" henceforth) recognize that far beyond the sole estimation of crude protein (**CP** = N × 6.25), formulation of dairy rations requires accurate estimation of the real supply of protein to the cow, i.e. the flow of digested protein, also known as metabolizable protein (**MP**). Currently, most of the models used in North America [e.g. Amino Cow (Evonik AG Industries, Hanau, Germany); Cornell Net Carbohydrate and Protein System (**CNCPS**, Fox et al., 2004); the CNCPS-derived Agricultural Modeling and Training Systems (**AMTS**); NRC (2001)] have invested considerable time and effort developing complex rumen sub-models to improve the predictions of the duodenal flow of protein digested in the small intestine and available to the cow, the MP, and associated digestible flows of AA. Although fine-tuning of these models is still needed to improve predictions with variable feed ingredients, their predictions of duodenal flows of protein or AA fit quite well with measured values (Pacheco et al., 2012). Unfortunately, the intensive and regular efforts to improve the predictions of supply over the last decades have not been matched by similar efforts to improve the estimations of nutritional rqt of dairy cows.

Requirements of MP for maintenance in most North American models are still based on work published almost four decades ago (Swanson, 1977) and rqt for both maintenance and milk protein are calculated using a fixed efficiency of utilization, independent of supply of either protein or energy. This simplification of the complex biological event of lactation was necessary as a starting point, because *"the knowledge of metabolism of nutrients is not as advanced as the prediction of ruminal fermentation, because of the almost infinite metabolic routes connecting various tissue and metabolic compartments, the multiple interactions, and the sophisticated metabolic regulations that determine the partitioning of absorbed nutrients"* (Fox and Tedeschi, 2003). The assumed linear relationship between supply and output arising from the use of a fixed efficiency factor is, however, biologically unrealistic. It is well known that MP allowable milk is usually overestimated at high protein intakes and underestimated at low protein intakes with both NRC (2001) and CNCPS (Fox et al., 2004) models. This paper provide a brief review of the current assumptions and then proposes updates on the estimation of the rqt of MP and essential AA for maintenance and milk production based on knowledge gained in the last two decades.

CURRENT ESTIMATIONS OF REQUIREMENTS

This presentation will only deal with rqt for maintenance and lactation, considering cows being mature, non-gestating and not changing body weight and composition. Numerical examples will be given using a 700 kg cow producing 45 kg milk/d at 3.2% CP (3.0% true protein) and with a daily dry matter intake (**DMI**) of 27 kg. Estimation of rqt requires 1) identification of the proteins considered as part of the rqt; 2) either AA composition of these proteins (factorial approach) or estimation of the relationship between AA supply and milk protein yield or concentration (proportional approach); and 3) the efficiency at which the digested protein or AA are used to support the protein secretion.

Proteins

Estimation of proteins to be included in the rqt can easily begin with the protein required for milk protein secretion. This can be directly measured as the amount of protein secreted into milk. To estimate the rqt, NRC (2001) is using true protein whereas CNCPS is using CP minus a fixed assumption of non-protein N (Fox et al., 2004). On the other hand, the estimation of the proteins to cover the rqt for maintenance is not as straightforward as for milk protein production. Due to the inability to feed ruminants a protein-free diet without creating a negative impact on the rumen microflora, the estimation of protein rqt for maintenance in the ruminant has always been a challenge. In 1977, Swanson made a thorough literature review to estimate *"new factors for each of the three loss routes of maintenance nitrogen based only upon appropriate original data from cattle indicative of true maintenance",* these routes being scurf, endogenous urinary and metabolic fecal protein (**MFP**). The propositions he made at that time are those still currently used by NRC (2001) and CNCPS (Fox et al., 2004).

The maintenance rqt for integumental protein includes loss and growth of hair, scurf and scales rubbed from the skin surface, along with some N containing compounds in skin secretions. Export proteins for this overall scurf rqt were estimated at 0.2 g CP/kg $BW^{0.60}$ per day (MP rqt of 0.3 g CP/$kg^{0.60}$, assuming all CP to be true protein and using the efficiency factor of 0.67; Swanson, 1977). Endogenous urinary losses represent the amount of N lost in urine if the animal is consuming a diet adequate in energy but devoid of protein. These losses include creatinine, urea, purine derivatives, nucleic acids, hippuric acid and small quantities of some AA (NRC, 1985). Urinary losses in cattle fed with very low-N diets but adequate energy were used to determine endogenous urinary losses, which were estimated to be 2.75 g CP/kg $BW^{0.50}$ per day (still assuming all CP to be true protein and using an efficiency of utilization of 0.67, this translates to a MP rqt of 4.1 g CP/$kg^{0.50}$; Swanson, 1977).

The last route assessed by Swanson (1977) was MFP, the composition of which is not clearly defined. In Swanson (1977) it was reported to be *"a residue of body secretions and tissue incident to movement of food through the gastrointestinal tract"* (Swanson, 1977), whereas in Swanson (1982) adopted in the last NRC (2001), MFP was defined as *"bacteria and bacterial debris synthesized in the caecum and large intestine, keratinized cells, and a host of other compounds".* The estimation of rqt for MFP used by NRC and CNCPS is based on studies of Swanson (1977, 1982). In these studies, the best estimations of MFP were obtained relative to indigestible DM, but due to uncertainties related to the digestibility of DM, the NRC (2001) chose to use DMI as the basis to determine MFP. In addition, the NRC sub-committee estimated that rumen microbial protein (included in MFP) should not be considered as a metabolic loss and, therefore, 50% of the undigested microbial protein is subtracted from this estimation (the other half is assumed to be digested in the hindgut). Therefore, in NRC (2001), the MP rqt for MFP = (DMI (kg/d) ×30) - 0.50 × ((bacterial MP/0.80) - bacterial MP). The CNCPS currently calculates MFP as 9% of indigestible DM (Fox et al., 2004), as previously estimated by NRC (1989), without any correction for the presence of bacteria. Therefore the CNCPS estimates of MFP are higher than those from the NRC. From these evaluations of excretion, both models assume all CP to be true protein and do not use a coefficient of utilization of digestion protein. In addition, NRC (2001) also assumes that the endogenous protein flow at the entrance of the duodenum should be included in the maintenance rqt. It is calculated as 1.9 g N/kg DMI × 0.50 (ratio of true protein/CP) × 0.80 (digestibility), this being divided by 0.67 to obtain the MP rqt. It should be noted that NRC also includes this endogenous protein flow at the duodenum in MP supply.

Table 1 summarizes MP rqt for our example 700 kg cow producing 45 kg milk/d at 3.2% CP (3.0% true protein) and eating 27 kg DM/d estimated with the NRC (2001) and CNCPS (Fox et al., 2004) ration balancing models.

Amino Acids

Two different approaches are currently used to determine the AA rqt. The proportional approach was the first approach presented in dairy cows, as early as 1993 (Rulquin et al., 1993). It is called proportional because the rqt is expressed in terms of the proportion of AA in the MP. This approach was chosen by NRC (2001) as the sub-committee stated that at that time, "*current knowledge (on AA) is too limited to put forth a model that quantifies AA rqt for dairy cattle*". To determine the proportion of lysine (Lys) and of methionine (Met) needed to maximize milk protein yield (or percentage), a dose-response relationship between the % of individual AA in MP supply estimated by the model and milk protein yield (or percentage) was established, using

Table 1. Estimation of metabolizable protein (MP) requirement (rqt) for maintenance and milk for a 700 kg cow producing 45 kg of milk with 3.2% crude protein, 3.0% true protein.

Function	Variables associated with estimation of MP rqt				
Ration balancing model[1]	CP, g/d	True protein /CP	True protein, g/d	Efficiency	MP rqt, g/d
Milk					
NRC	-		1350	0.67	2015
CNCPS	1440	0.93	1339	0.65	2060
Scurf					
NRC & CNCPS	10	ND[3]	ND	0.67	15
Endogenous urinary					
NRC & CNCPS	73	ND	ND	0.67	109
Metabolic fecal protein					
NRC	631	ND	ND	ND	631
CNCPS	810	ND	ND	ND	810
Duodenal endogenous flow					
NRC[4]	257	0.50	128	0.67	191
CNCPS	ND	ND	ND	ND	ND
Total rqt					
NRC					2961
CNCPS					2994

[1]NRC, 2001 and CNCPS: Fox et al., 2004.
[2]Using a fixed proportion of non-protein N in milk of 7%.
[3]ND: not defined / not used.
[4]Assuming 1436 g of MP from bacterial origin.

a broken stick model. The % observed at the breakpoint represents the proportion of this AA in MP supply required to maximize the targeted output. This approach yielded recommendations for Lys at 7.08% and 7.24% of MP, and for Met at 2.35% and 2.38% of MP, for maximal milk protein yield and milk protein concentration, respectively in NRC (2001). This is also the approach adopted by the INRA ration balancing model (2007), which states fairly similar recommendations.

A second approach, the factorial approach, cumulates the AA needed to cover each individual function (maintenance and lactation in the current presentation). The AA rqt for each function is calculated using the MP rqt excluding the efficiency of utilization, with a defined AA composition and a defined efficiency of transfer for each AA for each function. This is the approach used, for example, by CNCPS (Fox et al., 2004), AMTS and Amino Cow. In addition to the determination of the MP needed to fulfill each function, this approach requires the AA composition of the protein involved in each function as well as the efficiency with which each digested AA will be used to cover that function. In CNCPS (Fox et al., 2004), milk AA composition is derived from a rather old estimation (Jenness, 1974) whereas the assumed AA composition used for endogenous urinary and MFP is based on whole body tissue (Ainslie et al., 1993). For scurf, whole-body tissue AA composition is used (Fox and Tedeschi, 2003), although the AA composition of keratin had been proposed (O'Connor et al., 1993).

Efficiency

In NRC (2001) and CNCPS (Fox et al., 2004), once the "export" protein or AA in the export protein has been determined for maintenance or lactation, they are converted into a rqt for MP supply, using a single transfer coefficient (0.67 and/or 0.65), except for MFP for which no ratio of true protein/CP or efficiency factor is applied.

In CNCPS, a fixed efficiency of utilization of AA, different for each AA but independent of the supply, is associated with maintenance and lactation processes (Table 2). The efficiencies of utilization of AA for maintenance are largely derived from one article (Evans and Patterson, 1985), averaging 0.85 for all essential AA except the branched-chain AA for which it averaged 0.66. Until last year, the efficiency of lactation was based on the output:uptake ratio of individual AA across the mammary gland (Fox et al., 2004). Although it is acknowledged that *"AA absorbed in excess will be used less efficiently, and those absorbed at levels below rqt will be used with a higher efficiency"* (Fox and Tedeschi, 2003), there is no attempt to propose variations in these efficiencies.

Table 2. Coefficients of efficiency for individual amino acids (AA) for maintenance or lactation currently used by CNCPS[1].

Function	version	AA								
		Arg	His	Ile	Leu	Lys	Met	Phe	Thr	Val
Maintenance	6.0	0.85	0.85	0.66	0.66	0.85	0.85	0.85	0.85	0.66
Lactation	6.0	0.35	0.96	0.66	0.72	0.82	1.00	0.98	0.78	0.62
Combined[2]	6.5	0.58	0.76	0.67	0.61	0.69	0.66	0.57	0.66	0.66

[1]Adapted from Fox et al., 2004.
[2] Efficiency combined for both maintenance and lactation: from Van Amburgh et al., 2015; adapted from Doepel et al., 2004 and Lapierre et al., 2007.

PROPOSED AND ADOPTED UPDATES OF REQUIREMENTS

Proteins

Very simplistically, protein rqt should be first based on the quantification of the proteins or N byproducts excreted out of the body of the cow originating from digested AA: these are the proteins (and AA) that the cow's ration needs to replace on a daily basis. These include all

proteins excreted out of the cow (milk, endogenous fecal protein, scurf), plus endogenous urinary N. To be translated in rqt of MP, these exported CP need to be expressed as true protein outputs. Then, the output of true protein must be divided by a factor of efficiency of utilization of the absorbed protein to finally yield the rqt of MP for each function. The sum of all MP rqt for each function would then represent the total MP rqt of that cow. In this way, all the exported proteins would be treated equally, which is not the case in the current models. Naming each of the protein excretions by its own name, rather than under the umbrella "maintenance," could eliminate the ambivalence of a so-called maintenance rqt. For example, currently, the maintenance rqt of a cow 27 kg DM is twice as large as the maintenance rqt of a dry cow consuming 13.5 kg DM, due to MFP rqt. Is this really "maintenance"? In the new French system, they have named these "non-productive functions" (Sauvant et al., 2016).

As mentioned previously, milk protein is probably the easiest export protein to deal with. The estimation of milk protein output should refer to true protein, especially knowing that the proportion of non-protein N can vary with protein supply (e.g. Raggio et al., 2004). In our example cow, the sum of endogenous urinary plus scurf represented less than 5% of the MP rqt: therefore, dairy researchers have had limited interest to look at these assumptions. However, in the next version of the French model, Systali, the endogenous urinary rqt has been rigorously reviewed and updated to a value of 0.05 g N/kg BW, with 100% for the efficiency factor. This estimation is approximately twice as much as that estimated using Swanson (1977) data. Interestingly, Lapierre et al. (2016), taking a totally different approach and identifying the different compounds in urine, derived an estimation of endogenous N, using the database used by Spek et al. (2013), of 0.043 g N/kg BW, a value which is comparable to the Systali estimation.

For the other sources of "maintenance", the NRC (2001) model is the only one that includes a rqt for the flow of endogenous proteins at the duodenum. Based on the definition above, as this flow is not leaving the body of the animal, we propose that this fraction not be included in rqt. Indeed, the fraction that will be digested and reabsorbed does not represent a loss of AA per se for the animal; only the undigested fraction flowing at the ileum and mostly recovered in the feces represents a real loss. This fraction will constitute part of the MFP (see below). Similar to the current proposal, the new Scandinavian model (NorFor, 2011) and the French Systali model (Sauvant el al., 2016) are also not including the endogenous duodenal protein flow in the rqt. However, adequate quantification of the contribution of endogenous protein to the duodenal flow is crucial; this contribution needs to be removed from the total digestive protein flow protein to determine the true net supply because the endogenous proteins, which may represent up to 15-20% of duodenal CP flow, are not a net contributor to the AA supply (Ouellet et al., 2002, 2007 and 2010).

The largest component of the so-called maintenance rqt is MFP. As discussed previously, this component differs substantially between NRC (2001) and CNCPS (Fox et al., 2004, Table 1), due to a different interpretation of Swanson (1977). Another peculiarity of this component of rqt is that neither a ratio of true protein to CP, nor an efficiency of utilization of absorbed protein, is applied to convert the "exported" protein into a rqt. This occurs despite the clear indication given by Swanson (1977): *"Furthermore, whichever portion of the fecal N is designated MFN, it is converted to maintenance rqt for protein only when modified by appropriate factors for utilization efficiency of feed proteins."* An efficiency factor was not applied as this would have yielded maintenance rqt far too high relative to the MP supply. We consider that this is due to an overestimation of MFP due to the methods used in the studies that Swanson (1977) used to obtain that value. For example, included in these estimations of MFP is urea recycled in the

rumen and captured by bacteria, which does not translate into a rqt on digested proteins. Therefore, we propose to use as an estimation of MFP the ileal flow of endogenous proteins estimated by isotopic dilution in dairy cows (Lapierre et al., 2007; Ouellet et al., 2007). As a first step, these estimations were reported based on DMI and average 15.8 g CP/kg DM intake, and using an average proportion of true protein/CP of 0.80 and an efficiency of 0.67, this would yield a rqt of 19 g MP/kg DMI, or 513 g for our example cow. Research is currently underway to update these values and determine if DMI is the major factor affecting the magnitude of MFP. Incidentally, using a totally different approach, the Systali model estimates MFP based on indigestible organic matter (**OM**) which yields a rough average of 19.8 g MP/ kg DMI in their cattle database (Sauvant et al., 2016), i.e. 535 g MP/d for our example cow. The Norfor model uses a fairly complex equation estimating the MFP based on the OM flow out of the rumen and the OM flow out of the small intestine, which would yield, for our example cow, 479 g of MP for MFP rqt.

Amino Acids

When using the factorial approach, the proportions for Lys and Met relative to MP supply proposed by NRC (2001) have been widely used. However, recent analyses conducted by the group of Dr. Schwab clearly indicate that the proportions recommended should be evaluated within each ration balancing model (Whitehouse et al., 2009, 2010a and b). These recommendations can differ substantially between models and also depending of the target, i.e. milk protein yield or milk protein concentration. For example, when assessing the milk protein concentration response, the recommendations for Lys would vary between 6.84% (AMTS) and 7.24% (NRC; Table 3). Similarly, although CNCPS is using a factorial approach, they have estimated recommendations using the proportional approach, based on their updates on feed library (Higgs et al., 2015) and efficiency of utilization, which will be used in version 6.5. Their recommendations for Lys and Met are 7.00 and 6.77 % of MP and 2.60 and 2.85 % of MP for maximal milk protein yield and milk protein concentration, respectively (Van Amburgh et al., 2015).

Table 3. Optimal proportion (%) of lysine and methionine in metabolizable protein supply to maximize milk protein yield or concentration, according to different ration balancing models.

Response to maximize	Ration balancing model	Amino acid	
		Lysine	Methionine
Milk protein yield	NRC, 2001	7.08	2.35
	NRC - expanded database[1]	6.95	2.38
	AMTS[1,2]	6.74	2.31
	CPM[1]	7.36	2.44
	CNCPS, version 6.5[3]	7.00	2.60
Milk protein concentration	NRC, 2001	7.24	2.38
	NRC - expanded database	6.89	2.23
	AMTS	6.84	2.40
	CPM	7.23	2.40
	CNCPS, version 6.5	6.77	2.85

[1]Whitehouse et al., 2010a and b.

[2]Whitehouse et al., 2009 for Met in AMTS ration balancing model.
[3]Van Amburgh et al., 2015.

When using the factorial approach, two factors need to be taken into account to translate the protein exported out of the body into an AA rqt: the AA composition of this protein and the efficiency with which the absorbed AA will be used. Efficiency will be discussed in the following section. For milk AA composition, we propose an update using the AA composition of the reference protein of each protein family (e.g. different caseins, lactalbumin α, different immunoglobulins, etc.) most recently detailed in the Journal of Dairy Science (Farrell et al., 2004; Lapierre et al., 2012). This update includes all the proteins secreted in the milk and not only the proteins synthesized within the mammary gland. As mentioned above, milk AA composition should be determined based on true protein and not CP as the non-protein N fraction of milk can vary with protein supply (e.g. Raggio et al., 2004). Therefore, adopting a constant AA composition relative to CP in milk could be misleading. Also, despite the common use of the factor 6.38 for the conversion of milk N into CP concentration, the factor 6.34 would be more appropriate (Karman and van Boekel, 1986; personal calculations). Table 4 presents the proposed update for milk AA composition.

Table 4. Proposed amino acid (AA) composition of milk and metabolic fecal protein (MFP)[1].

AA	Milk mg AA/g true protein	MFP mg AA/g CP[2]
Arg	37.4	38.4
His	29.0	22.2
Ile	61.3	34.5
Leu	103.6	48.6
Lys	87.6	47.3
Met	29.9	11.8
Phe	52.2	35.3
Thr	47.0	51.1
Trp	16.2	12.0
Val	69.3	46.7
Ala	35.4	43.8
Asn	42.7	59.4
Asp	37.8	-
Cys	9.0	20.8
Gln	96.5	109.5[3]
Glu	128.8	-
Gly	20.0	62.3
Pro	103.8	70.2
Ser	67.4	52.4
Tyr	58.4	31.9

[1]See text for detail.
[2]Assuming 80% of true protein in MFP crude protein (CP).

[3]For MFP, Asn is the sum of Asn+Asp and Gln is the sum of Gln+Glu.

As presented previously, MFP is the other major source of proteins exported out of the cow. Proteins from endogenous origin flowing at the ileum and excreted in the feces originate from very different sources. They are either constitutive proteins (sloughed cells) or proteins exported out of the cells where they have been synthesized (saliva, enzymes, mucins; Tamminga et al., 1995). Therefore, the AA composition of this protein mixture is a challenge to determine, but it is clear that the composition of empty body, which is currently being used, is not the most appropriate composition. In dairy cows, there are very few studies assessing either directly (Ørskov et al., 1986) or indirectly (Larsen et al., 2000) the AA composition of endogenous protein at the duodenal level and virtually no studies defining AA composition of intestinal endogenous proteins. Data from Larsen et al. (2000), however, could not be used because in their studies the duodenal cannula was placed after the biliary duct and therefore, the duodenal digesta included large quantities of Gly, which constitutes 85% of the bile secretion. Some data are available for the AA composition of the endogenous proteins flowing out of the small intestine in pigs (Jansman et al., 2002). As the AA composition of the intestinal secretion in pigs (Jansman et al., 2002) is fairly close to the AA composition of duodenal flow of endogenous proteins reported by Ørskov et al. (1986) in cattle, we propose, for now, an average of these values (Table 4). Although these values need to be improved with future research, they should be closer to the true AA composition of endogenous secretion than those currently used by different models based on whole body empty composition (e.g. CNCPS: Fox et al., 2004). Table 5 summarizes the estimation of MP rqt for the example cow, based on propositions from the current presentation and the latest European models.

Table 5. Estimation of metabolizable protein (MP) requirement (rqt) for maintenance and milk for a 700 kg cow producing 45 kg of milk with 3.0% true protein proposed in the current presentation and in the Scandinavian (NorFor) and French (Systali) new models.

Function	Variables associated with estimation of MP rqt				
Ration balancing model	CP, g/d	True protein/CP	True protein, g/d	Efficiency	MP rqt, g/d
Milk					
as NRC, 2001	-		1350	0.67	2015
Scurf					
as NRC, 2001	10	ND[1]	ND	0.67	15
Endogenous urinary					
Norfor, 2011	73	ND	ND	0.67	109
Systali, 2016[2]	218	1	218	1	219
Lapierre et al., 2016	193	1	193	1	193
Metabolic fecal protein					
Norfor, 2013					479
Systali, 2016[2]	716	0.5	358	0.67	535
Lapierre et al., 2007	427	0.8	341	0.67	509
Duodenal endogenous flow					
Not included					
Total rqt					
Norfor, 2011					2618
Systali, 2016[2]					2783
Current presentation					2732

[2]Systali: Sauvant et al., 2016. Calculations have been made with an efficiency of 0.67 for comparison purposes; however, Systali proposes a system to introduce the variable efficiency concept for all functions excluding endogenous urinary which has an efficiency of 1.

Efficiency

Once the exported proteins have been identified and quantified to estimate the rqt, we need to assess the efficiency with which the absorbed protein or AA will be used for protein synthesis. Two issues deserve consideration at this point. First, the concept of a rqt for maintenance that needs to be fulfilled before milk production occurs does not exist in the lactating dairy cow as the cow will run in negative N balance to support milk production. Second, it is widely recognized that absorbed protein is used with a lower efficiency at higher supply (Hanigan et al., 1998; Raggio et al., 2004; Metcalf et al., 2008), and although it is a real challenge to integrate this variability in models, we need to see how this would come feasible.

Knowledge of biology gives insights on these two former issues. At the individual level, essential AA not used for anabolic functions are removed from blood circulation in specific tissues depending on the presence of the enzymes responsible for their catabolism (Lobley and Lapierre, 2003). For example, essential AA from Group 1 (histidine, methionine, phenylalanine + tyrosine) are mainly removed by the liver and very little extraction of excess AA occurs in the mammary gland or in peripheral tissues other than the amount removed to support milk protein secretion and endogenous secretions. At the opposite, for the essential AA of Group 2 (isoleucine, leucine, lysine and valine), little is removed by the liver whereas oxidation (=inefficiency) occurs in the gut, the peripheral tissues and the mammary gland (Lapierre et al., 2012). Given that removal of excess AA does not occur at the site of protein synthesis and export, but is specifically related to tissues having the enzymes for catabolism, we have proposed that the efficiency for maintenance should not be different from the efficiency for lactation; therefore, we could use a combined efficiency of utilization (Lapierre et al., 2007). In addition, variation of the proportion of absorbed AA removed by the different tissues and recovered in milk greatly depends on AA supply (Raggio et al., 2004). Based on these observations, a meta-analysis was conducted with studies where AA supply was increased through infusions in order to have an assessment of the increased supply independent of any ration balancing model (Doepel et al., 2004). After this first analysis, we calculated a combined efficiency of utilization for AA and MP, relative to MP supply (Table 6; Lapierre et al., 2007). The version 6.5 of CNCPS will adopt the combined efficiencies estimated at 100% of the MP rqt (Table 2; Van Amburgh et al., 2015). Although we can estimate *a posteriori* relationship between efficiency of utilization and MP supply, it is not easy to determine *a priori* the efficiency of utilization. Indeed, observations of the efficiency of utilization at a single point of MP supply varies greatly. Propositions have been made to determine the efficiency using the supply of MP (or AA) relative to energy supply, as done in monogastrics.

Table 6. Combined efficiency (maintenance plus lactation) of utilization of amino acids (AA) and metabolizable protein (MP) in relation to their optimal supply.

	% of optimal supply			
AA	50%	75%	100%	125%
Arg	0.68	0.63	0.58	0.52
His	0.96	0.85	0.76	0.68
Ile	0.77	0.72	0.67	0.60
Leu	0.74	0.67	0.61	0.55
Lys	0.81	0.75	0.69	0.62
Met	0.85	0.74	0.66	0.59
Phe	0.70	0.63	0.57	0.51
Thr	0.68	0.68	0.66	0.60
Val	0.79	0.72	0.66	0.59
MP	**0.72**	**0.67**	**0.62**	**0.56**

[1]From the database of Doepel et al. (2004) and adapted from Lapierre et al., 2007; estimated as AA in milk protein plus the net rqt for maintenance (proposition in this paper) divided by net supply of AA.

CONCLUSION

The suggested recommendations are far from capturing all the complexities of the digestive and lactation processes, but they are based on the most recent knowledge of dairy cow metabolism. They offer an enhanced framework to include this knowledge to improve our estimation of protein and AA rqt using current ration balancing models. Of course, they represent only approximations of complex metabolic pathways operating in dairy cows. Some aspects are not considered, especially interactions such as how changes in amount or type of energy alter outputs and efficiencies. Furthermore, because all the factors are based on empirical observations and equation fits, they do not permit different predictions for cows with different genetic potential, which may alter significantly the response to changes in the ration. Nonetheless, better appreciation of 'true' losses via digestive tract metabolism and the inclusion of a variable coefficient will yield immediate practical benefits. Adoption of such changes should not deter us from developing more mechanistic models, capable of responding to improved genetic selection, animal husbandry and feed processing technology and that can predict both within and between animal responses to changes in nutrient inputs.

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Taking and Testing GOOD Samples: A Systematic Approach for Representative Sampling from Field to Test Portion

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SUMMARY

- The following manuscript provides a brief introduction to a systematic approach for representative sampling from field to test portion
- Sampling from field to test portion is a single measurement process
- Error is introduced during each mass reduction stage
- Error propagates as the square root of the sum of squares, so the largest error components have a proportionately greater impact
- A representative sample must be correct (unbiased) and have sufficiently small imprecision
- The systematic approach for developing a sampling protocol is based on two key inputs: sample quality criteria (SQC) and material properties
- This approach requires knowledge of the total error in the measurement process (global estimation error or GEE), from primary sampling through final measurement
- Error is estimated through quality control events
- If the GEE meets the requirements of the SQC, a confident inference can be made
- GOODSamples can be a valuable tool for the forage and feed communities to evaluate current practices and to develop new protocols
- Additional AAFCO resources for feed laboratories are also available

BACKGROUND AND INTRODUCTION

The US FDA awarded a five-year cooperative agreement to the Association of Public Health Laboratories (APHL), Association of Food and Drug Officials (AFDO) and the Association of American Feed Control Officials (AAFCO) to support the implementation of The Food Safety Modernization Act (FSMA). One of the Specific Aims in the cooperative agreement is "Harmonized Policies and Procedures for Equivalency of Data". A task under this Aim is to establish a working group to develop harmonized policies and procedures for sample collection, shipment, analysis, storage and retention of food and feed materials. The Sampling and Sample Handling Working Group effort is led by AAFCO due to its history of recognition of sampling and sample preparation as critically important.

Currently, protocols for sample collection are at least as varied as the number of organizations that collect samples. This wide variety of sample collection techniques does not lend itself to data equivalency among organizations. The goal of the working group is to develop a common sampling strategy for sampling food and feed. With this common sampling strategy, data can be evaluated with respect to "fit for purpose" or, more aptly, "fit for decision" criteria for any organization, project or situation. The first audience for the resulting guidance document is regulatory food and feed programs and their associated laboratories, including management, inspectors, quality assurance officers and laboratory personnel; however it is applicable for all of the related industries, including food, feed, and fertilizer sampling. The guidance document has been titled *Guidance on Obtaining Defensible Samples* or *GOODSamples*.

All of the concepts briefly introduced in this manuscript are dealt with in greater detail in *GOODSamples* and in much greater detail in the resources listed at the end of this manuscript. Please consult them to clarify concepts and provide additional rationale. All of the concepts apply equally to primary sample collection and to mass reduction stages carried out in the laboratory. Comments for primary sampling personnel and laboratory personnel are integrated throughout this manuscript.

TERMINOLOGY

Sampling terminology is problematic! A key aspect of developing the sampling guidance document was to assess sampling terms and come to an agreement on key terms and definitions so that communication could be constructive. Terms were chosen to be consistent with theory of sampling (TOS), ISO standards, and AOAC INTERNATIONAL documents. It is strongly recommended that the terms in *GOODSamples* be adopted. Key terms from *GOODSamples* follow.

- *Decision Unit*: Material from which sample is collected and inference made.
- *Global estimation error* (GEE): Total errors in the entire measurement process, from primary sampling through final measurement.
- *Increments:* Individual portion of material collected by a single operation of a sampling tool and combined with other increments to form a primary sample.
- *Inference*: Estimating a concentration or characteristic about a larger amount of material from data derived from a smaller amount of material.
- *Sample:* A portion of a material selected from a larger quantity of material. The word "sample" should only be used with a modifier as follows:
    - *Primary sample:* The collection of one or more increments taken from a decision unit according to a sampling protocol.
    - *Laboratory sample:* The material received by the laboratory.

- *Analytical sample*: Results from any manipulation of a laboratory sample.
- *Test portion:* The quantity of material taken for measurement.

GLOBAL ESTIMATION ERROR

Sampling is a process of making inference from analytical data through multiple mass reduction stages to a decision unit. There are currently various scenarios for organizational responsibility in the primary sample to test portion pathway. One organization may oversee the entire pathway. More commonly, the sampling activities are carried out by a separate organization from the laboratory activities. In either scenario, it must be recognized that there must be an accounting for the overall process from sampling through analysis. The laboratory itself is involved in a smaller scale "sampling" processes each time it selects a smaller mass from a larger mass (mass reduction). This may happen several times as the material moves through the laboratory workflow, with the final mass reduction stage being the selection of a test portion(s) for an analysis.

Both imprecision and bias errors are introduced in every stage of the measurement process. Generally, only analytical error is estimated while the larger error components associated with primary sampling and with laboratory sample preparation are unknown. Since error does not add directly, but propagates as the square root of the sum of squares, it follows that errors that are largest compared to others will have the greatest contribution to global estimation error (GEE), and mitigating the larger errors will have the most dramatic effect on lowering GEE.

$$Eq.\,1: Global\ Estimation\ Error = \sqrt{(a^2 + b^2 + c^2 + \cdots \ldots + n^2)}$$

where *a, b, c, …., n* are individual imprecision errors for each sampling (mass reduction) stage and analysis.

WHAT IS A REPRESENTATIVE SAMPLE?

A representative sample answers a question about a decision unit with an acceptable level of confidence.
- Imprecision is controlled by collecting an appropriate mass and number of increments to address heterogeneity
- Correctness (bias has been controlled to a negligible level) is achieved when every element in the decision unit has the same probability of being selected (equiprobable)
- Correctness is maintained when additional biases are not introduced during sample preparation and sample handling

A representative sample must
- Be correct
- Have a sufficiently small imprecision
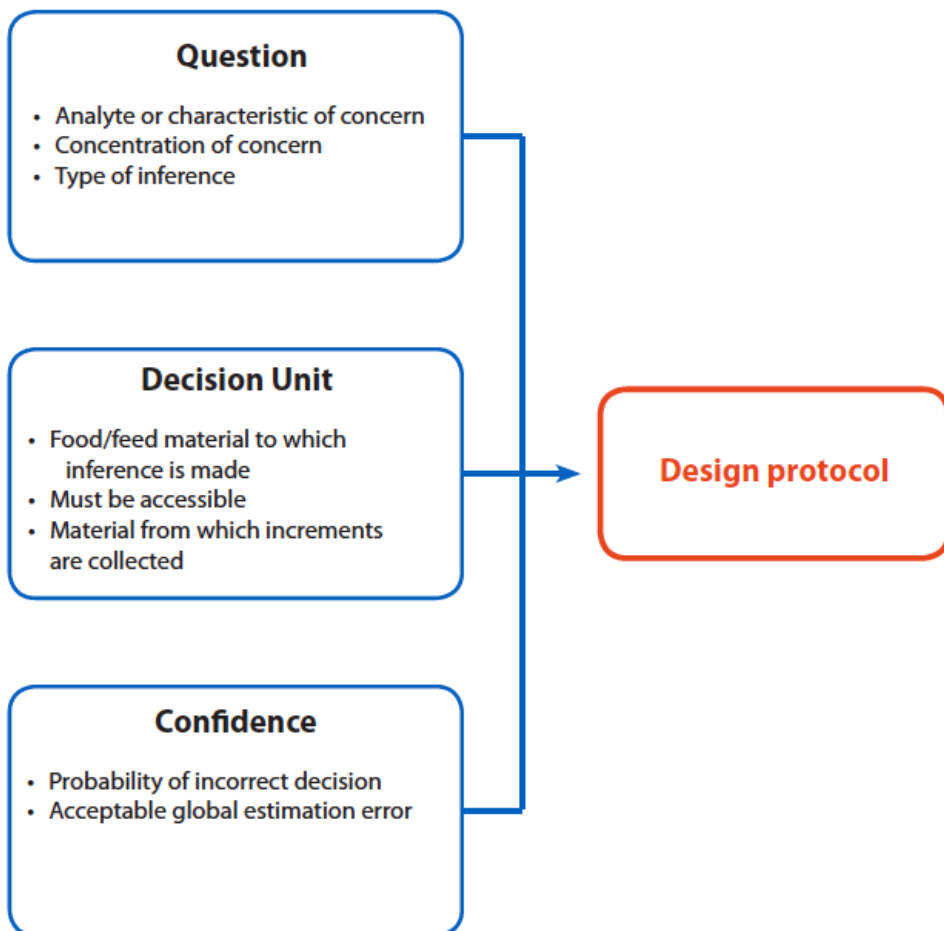
SAMPLE QUALITY CRITERIA (SQC)

The framework for systematic scientific sampling consists of three components: Sample Quality Criteria (SQC), material properties and the theory of sampling (TOS). The first of these three components, sample quality criteria (SQC) is a series of statements that clarify technical and quality needs. The SQC answer the following questions:
1) What is the question to be answered?
   a. What information is sought?

         i. What is the analyte?

        ii. What is the level of concern?

  b. What type of data will be collected?

         i. Is a characteristic of the decision unit being evaluated?

        ii. Is an analyte concentration in the decision unit being sought?

  c. How is the inference going to be made?

         i. Direct inference (from a single result)?

        ii. Probabilistic inference (from a single result)?

       iii. Statistical inference e.g., average of multiple results, confidence interval?

2) What is the decision unit?

  a. The choice of decision unit has a large impact on the sampling protocol. For example, bales from a single alfalfa field are loaded onto 10 trucks, each containing 100 bales. The scale of information drives the decision unit. What information is desired?

         i. The average value of the entire field? If so, all 10 truckloads comprise a single decision unit.

        ii. The average value of each of the 10 trucks. If so, each of the 10 trucks is a decision unit (10 decision units).

       iii. Or must each bale on each truck have a certain characteristic or analyte concentration? If so, each bale is a decision unit (1000 decision units).

3) What is the desired confidence in the inference?

  a. The higher confidence desired, the greater error must be controlled.

  b. The desired confidence is generally related to the risk and consequences associated with an incorrect decision.

Figure 1. Sample quality criteria (SQC)

MATERIAL PROPERTIES

The framework for systemic scientific sampling consists of three components: Sample Quality Criteria (SQC), material properties and the theory of sampling (TOS). The second of these, material properties, refer to the intrinsic properties of the material that comprises the decision unit that must be considered when developing sampling protocols. Material properties include element type and heterogeneity.

Elements are the individual components (e.g., particles or fragments for solid materials, molecules for liquids, particles and molecules for slurries) making up the decision unit. They can be either finite or infinite in nature. Finite element materials are materials composed of elements that can be individually identified and individually selected at random. Infinite element materials are materials composed of elements that cannot be individually identified nor individually selected at random.

Figure 2. Tomatoes representing a finite element material and flour representing an infinite element material.

The second aspect of material properties that must be considered is the heterogeneity. Two types of heterogeneity exist: compositional heterogeneity and distributional heterogeneity. Compositional heterogeneity exists when the individual elements that make up the decision unit exhibit differing concentrations of the analyte of interest (e.g., alfalfa stem vs. leaf tissue, corn vs. added mineral). Compositional heterogeneity always exists to some degree and cannot be altered without comminution.

Distributional heterogeneity results from non-random distribution of elements within the decision unit (e.g., settling of small, dense fines to the bottom of a container). Distributional heterogeneity always exists to some degree and can be altered with physical manipulation of the material (vibration, mixing, etc.).

THEORY OF SAMPLING (TOS)

The framework for systemic scientific sampling consists of three components: Sample Quality Criteria (SQC), material properties and the theory of sampling (TOS). The third component, the theory of sampling (TOS) is a systematic and scientific process for designing sampling protocols that meet the SQC. TOS provides techniques for mitigating and estimating error in sampling. It is most commonly applied to infinite element materials elements since they must be selected in "groups" called increments. TOS describes the final sample mass (combination of all increments), how many increments need to be collected and dictates sample correctness.

Compositional heterogeneity results in an imprecision error, Fundamental Sampling Error (FSE). FSE must be addressed with every mass reduction stage from primary sampling through selection of the test portion. FSE can be controlled to any level by collecting sufficient mass and/or reducing particle size.

$$Equation\ 1:\ FSE^2 \propto \frac{Cd^3}{m_s}$$

C = sampling constant, unique for each material
d = diameter of 95% percentile of largest particles (cm), and
m_s = mass of the sample (g).

Because of the relationship of FSE to particle size, mass and error, mass reduction must take the relationships into consideration. Any type of mass reduction without considering these relationships is unacceptable. The proper mass to collect is based on the heterogeneity of the

material, so it is inappropriate to collect an identical mass as standard practice for all primary sampling situations.

Distributional heterogeneity leads to an imprecision error, Grouping and Segregation Error (GSE). GSE must also be addressed with every mass reduction stage from primary sampling through selection of the test portion. GSE is controlled to any level by collecting sufficient number of random increments. There is no simple calculation to determine the number of increments to collect, but three approaches can be used to reduce the GSE: reduce the FSE, increase the number of increments, and reduce the distributional heterogeneity of the material. The proper number of increments to collect is based on the heterogeneity of the material, so it is inappropriate to collect an identical number of increments as standard practice for all primary sampling situations. Mixing prior to sampling (such as prior to taking a test portion in the laboratory) may be effective, but only if the material particles have a relatively uniform shape, size and density. It is generally unacceptable to select a single, non-random increment as a test portion.

Bias errors are also addressed in TOS as the notion or condition of "correctness". Sample correctness is achieved when selection of elements at increment locations is equiprobable, and it is controlled by proper use of a correctly designed sampling tool. Once sample correctness is achieved with the primary sample, it must be maintained in subsequent mass reduction stages all the way to the test portion.

TOOLS

TOS mandates that sampling tools must be correctly designed and used properly. The correct tool shape is related to the dimensions of the decision unit. Tool shape and respective dimension that are most commonly used for infinite element materials are:
- Slices (cross stream cuts) for a one dimensional flowing stream
- Cylinders (similar to probe) for a two dimensional layer

Sampling tools should not only be correct, they should also:
- Be simple and reliable
- Be easy to decontaminate
- Be inert to the sample/analyte
- Collect increments of equivalent size

EVIDENTIARY AND ANALYTE INTEGRITY

The purpose of evidentiary integrity is (1) to be able to tie a test result to a specific decision unit; (2) to demonstrate that the sample has not been adulterated or compromised during any step of the process from primary sample collection through generation of the analytical data; and (3) to assure that analyte integrity has been maintained. Analyte integrity is the assurance that physical, chemical, biological and/or radiological characteristics of interest in the decision unit have been maintained. Considerations for analyte integrity include preservatives, containers, holding times, sampling techniques and packaging and shipping procedures.

LABORATORY SAMPLING AND PREPARATION

As stated previously, all concepts apply equally for primary sampling and laboratory sampling activities. There are three important responsibilities for laboratories related to sampling:

- To respect the decision unit
- To ensure that analyte integrity is maintained during sample preparation and storage
- To obtain representative test portion(s) of the laboratory sample

Comminution (e.g., grinding) is a technique frequently used in laboratories to control FSE. A single type of particle size reduction equipment cannot handle all types of materials and it is imperative that laboratories have adequate equipment to handle the types of materials they will encounter. When evaluating comminution equipment, it is critical to ensure that:
- it is of sufficient capacity to process the laboratory sample,
- it will reduce the particle size sufficiently to control FSE, and
- it will produce a uniform shape and size to control GSE
- it can be sufficiently cleaned between materials.

When selecting comminution (e.g., grinding) equipment, consider the following:
- physical and chemical properties of the material,
- initial maximum particle size of the material,
- final desired particle size and the range of permissible particle sizes,
- needed capacity and throughput,
- inertness to analyte of interest,
- complete sample recovery,
- ease of cleaning, disinfecting, and sterilization.

A common laboratory preparation practice is splitting of samples for mass reduction. As with any form of mass reduction, minimum mass to control FSE must be a primary consideration and sufficient mass must be available so that the final reduced mass still has acceptable FSE. A second consideration is choosing a technique that provides sufficient increments to control the GSE (more increments results in lower GSE). Common splitting techniques used for this form of mass reduction are: rotary splitting, fractional shoveling, stationary riffle splitting and coning and quartering. Rotary splitting is by far the most accurate because it selects more increments than other techniques followed by fractional shoveling, stationary riffle splitting and lastly, coning and quartering. A third consideration is the correctness of the increment selection. Coning and quartering is a very poor mass reduction method and is strongly discouraged due to the large error it generates. The common practice of arbitrarily splitting an unground laboratory sample for the purpose of analytical efficiency without first reducing particle size to control FSE is very questionable.

Finally, laboratories should be equally concerned with validating sample preparation procedures as with validating analytical methods, especially given that the error associated with sample preparation procedures is greater than error associated with most analytical procedures.

QUALITY CONTROL

Quality control is a tool to assess data quality that is widely implemented in laboratories but seldom implemented in sampling or sample preparation. This absence of quality control from sampling activities is a practice that needs immediate attention. Quality control is used to estimate global estimation error, to determine if a process is in control and to validate a method or protocol.

Quality control checks for bias are blanks. Blanks can be used to check for contamination from containers, the environment or carryover from tools and equipment.

Quality control checks to estimate imprecision are replicates. Replicates can be implemented at multiple points to sort out error contributions form various mass reduction stages. GEE can be estimated from data resulting from replicated primary samples. Replicating test portions from the same analytical sample provides an estimate of the imprecision associated with selection of the test portion and the test (no information about the preceding stages).
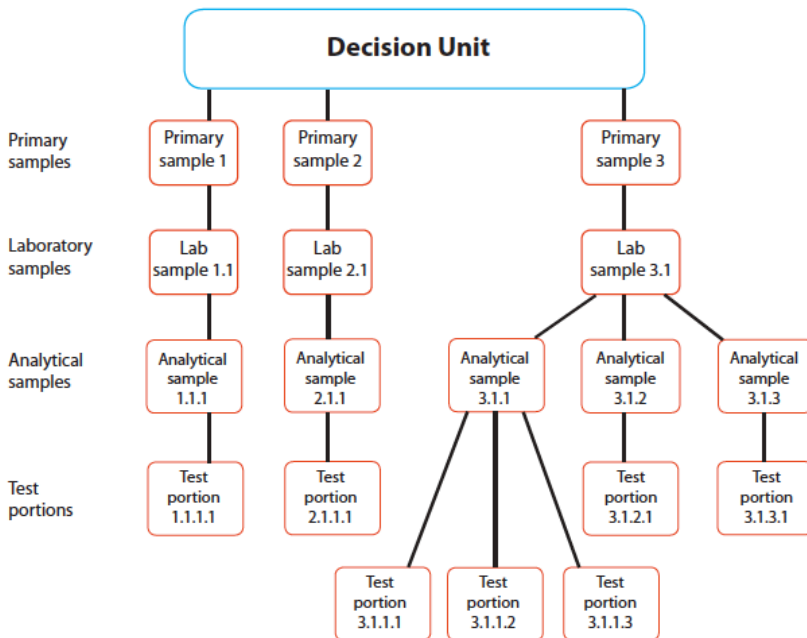
Figure 3. Levels of replication using triplicates.

INFERENCE

Inference is the process of estimating (or inferring) a concentration or characteristic about a decision unit based on a sample(s) collected from that decision unit. Inference is an important aspect of the sampling process often overlooked and misunderstood. Inference occurs at every stage of mass reduction in the primary sample to test portion pathway. Two forms of inference are presented and discussed in *GOODSamples*: estimating the average analyte concentration in a decision unit and estimating a percentage of decision units that have some characteristic or concentration.

DATA QUALITY ASSESSMENT

The final step in the scientific and systematic process is data quality assessment. It includes review of documentation, evaluation of quality control and estimation of the GEE. The documentation should support the premise that the correct protocol(s) was followed. Evaluation of quality control blanks should support the absence of contamination below a critical level. Evaluation of quality control replicates should indicate that they are within an acceptable range. The GEE should be below 35%, and meet SQC. The proximity of the actual concentration and GEE to the specification limit need to be examined to determine if a defensible decision can be made.

CONCLUSION

A shortcoming of current practices is a lack of knowledge of the error in the entire measurement system and in reported data. In addition, there is no systematic process to ensure that sampling protocols meet project objectives. *GOODSamples* address both of these shortcomings. It not only provides a systematic process to develop sampling protocols to meet project objectives, but it also provides a mechanism to evaluate existing protocols to determine if they meet the intended objectives. *GOODSamples* also provides a system for estimating the error in the entire measurement system (GEE), which is critical to data integrity. Implementing *GOODSamples* leads to equivalency of data and defendable and cost effective decisions related to feed nutrition and feed safety. *GOODSamples* is a valuable tool for the forage and feed communities and addresses the limitation of current practice.

ADDITIONAL AAFCO RESOURCES FOR LABORATORIES

AAFCO provides a number of resources for feed laboratories. One of the most visible resources is the AAFCO Check Sample Program, which now offers four proficiency-testing programs

- The monthly AAFCO Check Sample Program consists of monthly shipments of a commercial feed material. The annual series includes a variety of feeds and feed supplements with nutrients, drugs, antibiotics, minerals and vitamins at levels typically encountered in commercial products.
- The AAFCO Pet Food Program consists of quarterly shipments of a pet food ingredient.
- The AAFCO Mycotoxin Program consists of quarterly shipments of an animal feed or pet food that has been contaminated with naturally occurring aflatoxins, fumonisins, deoxynivalenol, zearalenone, ochratoxin A, and T-2 toxin.
- The AAFCO Minerals Program consists of quarterly shipments of animal feed, pet food or food with both naturally occurring and spiked Al, As, B, Cd, Cr, Co, Cu, F, I, Pb, Hg, Mo, Ni, Se, S and V at concentrations of health/toxicological significance.

Questions should be directed to pt@aafco.org and subscriptions can be purchased at http://www.aafco.org/Laboratory/Proficiency-Testing-Programs.

In 2014, AAFCO published revised *Quality Assurance Quality Control Guidelines for Feed Laboratories.* This document serves as a supplement to *ISO/IEC 170254:2005 General Requirements for the Competence of Testing and Calibration Laboratories* and includes a copy of the ISO 17025:2005 Standard. It is available for purchase at:

http://www.aafco.org/Publications/QA-QC-Guidelines-for-Feed-Laboratories. AAFCO also provides free training for labs implementing ISO 217025:2005.

AAFCO has published Best Practices reports for crude fat and phosphorus in animal feed. These are available free of charge at http://www.aafco.org/Laboratory.

AAFCO published *Guidelines for Preparing Laboratory Samples* in 2000 is available for purchase at http:// www.aafco.org/Publications/QA-QC-Guidelines-for-Feed-Laboratories. It was adopted and republished by ISO as ISO 6498:2012. The AAFCO document is currently under revision to ensure compliance with *GOODSamples*, and should be available in late 2016. It will be available free of charge.
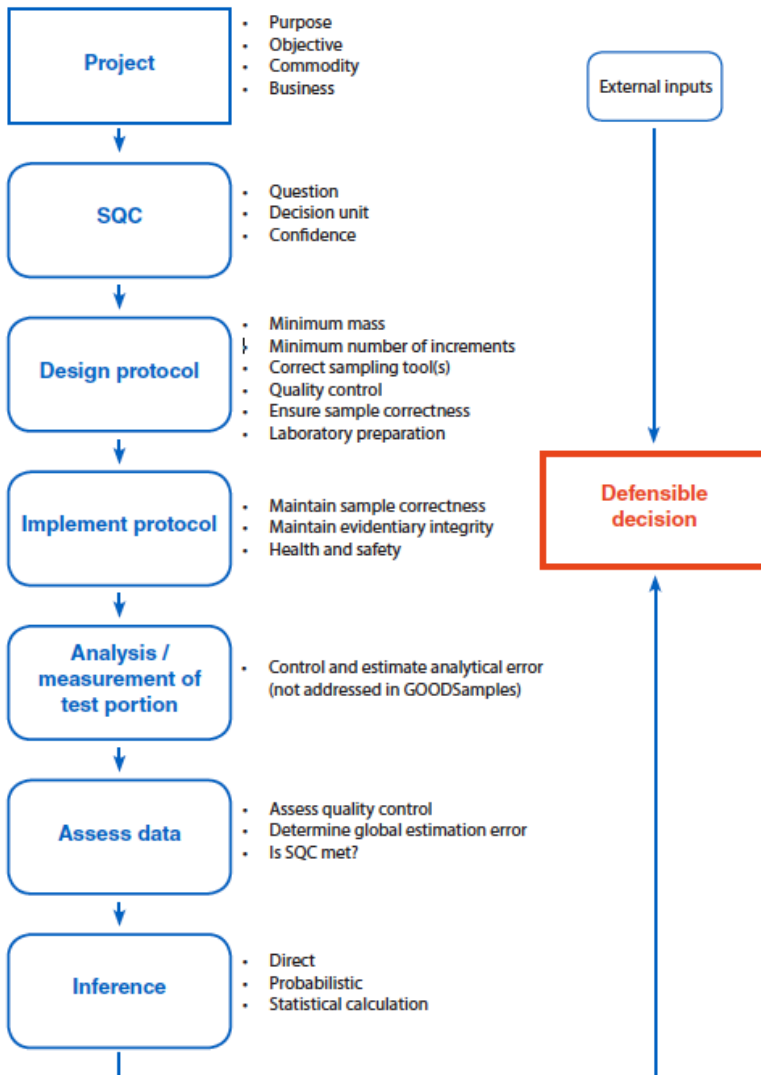
Figure 4. Overview of the *GOODSamples* approach for defensible decisions

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Dr. David Mertens

Dr. Mertens is known nationally and internationally as an expert on the analysis of fiber in feeds, the maximum and minimum fiber requirements by dairy cows, and the mathematical modeling of the intake and digestion of fiber by animals. He developed the physically effective fiber system and the corn silage processing scores used to assess kernel fragmentation. He has authored or co-authored over 300 papers in research journals and conference proceedings and five chapters in books on nutrition and forages. Dr. Mertens has received numerous national and local awards for his teaching and research. He grew up on a dairy farm in central Missouri and received his B.S. and M.S. degrees at the University of Missouri-Columbia and his Ph.D. from Cornell University. He taught at Iowa State University and the University of Georgia before becoming a Dairy Research Scientist at the U.S. Dairy Forage Research Center in Madison, WI in 1984. In 2010, he retired from federal service and started Mertens Innovation & Research LLC to continue his research and educational activities.

Measuring and Using uNDF to Improve Dairy Nutrition[1,2]

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SUMMARY

- Undigested NDF (uNDF) has been used for over 50 years to calculate the digestibility of NDF, typically at maintenance levels of intake or 48 h of in vitro fermentation.
- The concept of indigestible NDF (iNDF) was developed to describe the digestion kinetics of fiber because there is a fraction of total NDF that does not appear to digest in the rumen.
- iNDF is a theoretical parameter defined by two- or three-pool models of digestion and uNDF is the measured undigested NDF residue that is used to estimate iNDF in kinetic models.
- Although we focus on fermentation time as the measurement criterion to define uNDF (e.g., $uNDF_{72}$, $uNDF_{120}$, $uNDF_{240}$), there are numerous factors such as grind size of the sample, in vitro technique, filtration membrane, etc. that affect uNDF and need to be standardized.
- Lignin is a component of uNDF, but uNDF also contains fibrous carbohydrates that are not available for fermentation in the anaerobic ruminal environment. Because it contains these undigested carbohydrates uNDF is a better predictor of NDF digestibility, which alone explains about 70% of the variation in NDFD among forages.
- The most valuable use of uNDF is in deriving kinetic parameters and modeling fiber intake and digestion. However, it can also be used to:
  - benchmark individual feed quality and explain differences in their digestibility,
  - benchmark optimal uNDF in rations for dairy cows,

o   estimate nutrient digestibility on the farm and

o   improve our models of fiber digestion and kinetic parameter estimation.

INTRODUCTION

The undigested neutral detergent fiber (uNDF) in feeds has been measured since the mid 1960s. Van Soest (1965) and Van Soest and Moore (1967) suggested that neutral detergent dissolved the microbial debris and endogenous matter in feces and could be used to determine the "true" digestion of dry matter in feeds. Using this concept he developed a simple summative equation that described DM digestibility as a function of NDF and its digestibility (NDFD) and of neutral detergent solubles (NDS) that had a relatively constant true digestibility and endogenous loss:

Eq. 1 $DMD_{mnt} = NDF*NDFD_{mnt} + .98*(NDS) - 12.9$; where -12.9 is the endogenous loss of DM and insoluble ash. The data used to derive this equation was based on the standard practice of measuring digestibility of feeds at a maintenance level of intake (mnt). Essentially this function describes DMD_{mnt} as a function of digested NDF ($dNDF_{mnt}$) and digested NSD ($dNDS_{mnt}$) in the DM when measured at maintenance levels of intake:

Eq. 2 $DMD_{mnt} = dNDF_{mnt} + dNDS_{mnt}$.

Because $dNDF_{mnt} = NDF - uNDF_{mnt}$, the equation can be written as a function of uNDF:

Eq. 3 $DMD_{mnt} = (NDF - uNDF_{mnt}) + dNDS_{mnt}$.

Thus, we have been measuring and using uNDF for nearly fifty years. Goering and Van Soest (1970) used neutral detergent extraction as the second step of in vitro measurements to determine uNDF and to calculate in vitro NDFD. Because 48 h in vitro measurements were related to in vivo digestibilities at maintenance (Tilley and Terry, 1963), $IVuNDF_{48}$ is the appropriate measurement for estimating $uNDF_{mnt}$ for in vivo DMD_{mnt}.

The current interest in uNDF measured at fermentation times >48h is related to the kinetics of fiber digestion. Waldo (1969) postulated that there might be a fraction of cellulose (or fiber) that is not digestible during ruminant digestion. He indicated that, if this fraction was subtracted from the total, the potentially digestible portion of fiber might be described by first-order kinetics. By the late 1960s, many in vitro fermentations had shown that DM and fibrous constituents had a plateau in digestion that was less than 100%. A central tenet of reaction kinetics is that compartments or pools must have homogeneous kinetic properties. If there is a portion of total NDF that is indigestible (iNDF) in the anaerobic ruminal environment (it literally has a rate of digestion (kd) equal to 0), it must be subtracted from total NDF to obtain the potentially digestible NDF (pdNDF) that has a non-zero kd. The kinetic model of NDF fermentation can be described by the familiar equation:

Eq. 4 $NDFRes_{(t)} = D_0*e^{(-kd*[t - lag])} + I_2$; where $NDFRes_{(t)}$ is the NDF residue at any time = t, D_0 is the potentially digestible fraction such that $D_0 = (NDF - I_2)$; kd is the fractional rate constant of digestion, t is time of fermentation, lag is discrete lag time before NDF digestion begins, and I_2 is the iNDF for a two-pool model of fermentation. Equation 4 demonstrates that kd only applies to a potentially digestible portion of NDF and not to total NDF. It can be

rearranged to show that kd can only be determined on potentially digestible NDF by subtracting iNDF from each time-series measurement of $NDFRes_{(t)}$:

Eq. 5 $(NDFRes_{(t)} - I_2) = D_0 * e^{(-kd*[t - lag])}$

Mertens (2002) postulated that the reason NDF is not an ideal nutritive entity with constant digestibility is that it contains two fraction with vastly different fermentation properties. If there is a fraction of NDF that can never be fermented (lignin and fibrous carbohydrates in the cell wall that are associated with lignin and not accessible to bacterial fermentation), it would be an ideal nutritive entity with a digestion of zero. If this fraction were subtracted from total NDF the resulting potentially digestible NDF might have more uniform digestion properties. This concept indicates that estimation of iNDF would improve our ability to understand and estimate the overall digestibility of total NDF in feeds.

The concepts uNDF and iNDF are distinctly different and should not be used interchangeably. Although both terms are expressed as g/kg or % of DM, uNDF is the residue that is *measured* after a time of fermentation (typically using in vitro systems); whereas, iNDF is a theoretical concept that is defined by the digestion model as the NDF that cannot be digested after infinite time. Thus, uNDF is an *estimate* of iNDF when long fermentations (>72 h) are used. Note that uNDF is defined as the 'undigested' (not 'undigestible') NDF. The term 'undigested' implies that the constituent was not digested under a specific set of circumstances (in vivo versus in vitro or level of intake versus time of fermentation). Because fermentation time is a major factor influencing the NDF that is undigested, it should be added as a subscript to uNDF, e.g. $uNDF_{48}$ or $uNDF_{240}$. Literally, 'undigestible' means 'that which cannot be digested', which can never be measured. To eliminate confusion, the term 'indigestible', which also means 'that which cannot be digested' is used to distinguish between $uNDF_{xx}$ and iNDF.

MEASURING uNDF

Since the beginnings of kinetic analysis of NDF fermentation in the 1970s, we have been more focused on the measurement of uNDF as an estimate of iNDF, rather than using uNDF to estimate digestibility. Because NDF fermentation curves plateau sometime after 72 h, it is evident that the time at which uNDF is measured has a significant impact on its accuracy in estimating iNDF. The uNDF in feeds always contains some contamination by the pdNDF that is undigested at the time of measurement (Figure 1).
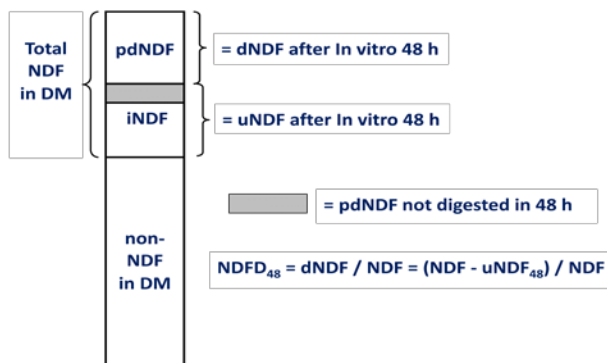

Figure 1. Relationship between indigestible NDF (iNDF) and undigested NDF (uNDF).

The time needed to digest pdNDF is a function of kd and can be calculated easily using the exponential equation for digestion kinetics:

Eq. 6 $D_{(t)} = D_0 * e^{(-kd*t)}$; where $D_{(t)}$ is the pdNDF remaining at time = t. Dividing each side by D_0:

Eq. 7 $D_{(t)} / D_0 = e^{(-kd*t)}$. Taking the natural logarithm (ln) of each side of equation 7.

Eq. 8 $ln[D_{(t)} / D_0] = -kd*t$, which can be solved for t.

The time needed for 99% digestion ($D_{(t)} / D_0 = .01$) of pdNDF can be determined by solving for t with various kd. For kd of .16, .12, .08, .04, .02, and .01/h, the time needed for 99% digestion is 28.8, 38.4, 57.6, 115.1, 230.3, and 460.5 h, respectively. Thus, for a two-pool model of digestion with a single pool of pdNDF and a kd >0.06/h, $uNDF_{72}$ is adequate to estimate iNDF. If we assume a three-pool model contains a slowly digesting pool with a kd of .02/h or less, the iNDF for this model can be estimated by $uNDF_{240}$.

Factors Affecting the Measurement of uNDF Other Than Fermentation Time

The large effect of fermentation time on the amount of pdNDF that is not digested tends to focus our measurement of uNDF only on time (e.g., $uNDF_{72}$, $uNDF_{120}$, or $uNDF_{240}$). However, many other aspects of the techniques used to measure uNDF can have profound effects on results. Factors such as the size of grind of the sample, porosity of the filter membrane used to collect NDF residues, porosity of the bags when used for in vitro or in situ fermentations, in vitro inoculum among runs, anaerobicity of the in vitro system, or blank and ash correction of uNDF will affect results. The interactions among these factors and fermentation time indicate that using time as the sole descriptor of uNDF may not be adequate.

Boyd and Mertens (2011) varied grind size of the sample (cutter mill, 1- or 2-mm; or cyclone mill, 1-mm screen), fermentation type (in situ, in vitro flask, in vitro bag), filtration vessel (crucibles with sand, in situ bag, fiber filtration bag) and fermentation time. They observed that in vitro 144h fermentations of 1-mm cutter or cyclone mill grinds filtered using Gooch crucibles with sand (uNDF = 19.3% of DM) generated results that were not different from in situ 288h fermentations with 2-mm cutter mill grinds using in situ bags (18.6%) or with1-mm cyclone mill grinds using Ankom F-57 fiber filtration bags (19.2%) or from in situ 240h fermentations with 2-mm cutter mill grinds using in situ bags (19.5%). Their results suggest that time alone is not the sole criterion for measuring uNDF. It appears that bags (or larger sample particles) may impede digestion, and that longer times are needed in situ to obtain the same extent of NDF digestion as measured in vitro. European researchers typically use bags with very small pores to reduce the loss of particles during in situ fermentation, but they compensate for the impediment of the bags by using longer fermentation times.

Limitation of Longer Times of Fermentation to Measure uNDF

Regardless of other factors, it seems logical to select longer times of fermentation because the resulting uNDF will be closer to iNDF at an infinite time. This logic has its limits due to the precision of uNDF measurement. For example, if the difference between $uNDF_{120}$ and $uNDF_{240}$ is less than the standard deviation among repeated measures of each uNDF, it is debatable that measuring uNDF at the longer fermentation time is advantageous. Mertens et al. (2012) measured the uNDF organic matter (uNDFOM) after 120 and 262 h of fermentation for two

sources each of wheat straw, corn silage, alfalfa haylage, grass hay and distiller's grains. The uNDFOM were measured using 0.5 g samples and duplicate measurements within each of two in vitro runs (n = 4 for each measurement). For replicated samples, $uNDFOM_{262}$ was smaller than $uNDFOM_{120}$ (22.0 vs 24.1% of DM; SE = 0.26; P < 0.0001) when the residues were corrected for ash and blanks. This result indicated that the difference in uNDF between 262 and 120 h is greater than the variation within each fermentation time. However, if fermentation times longer than 262 h are used, the difference in uNDF between these times should be evaluated to determine if they are statistically relevant.

Mertens et al. (2012) also observed that results differed among in vitro runs and suggested that measurements should be replicated among runs to most accurately measure uNDF. In addition, ash correction of uNDF was essential to remove soil or mineral contamination (uNDFOM). Blank residues averaged 1.6% of DM, and blank correction was necessary, especially when uNDF residues were small and corrected for ash. Mertens et al. (2011) also evaluated the effects on $uNDFOM_{262}$ of removing spent media and re-inoculating with new media after 120 h of fermentation. There was no difference in $uNDFOM_{262}$ with single or double inoculation (22.0 vs 21.6% of DM; P > 0.27). To explain this observation, it is speculated that fiber is digested in a sequential manner by a succession of different microbes. This suggests that the organisms fermenting fiber at 120 h are different from those digesting fiber prior to that time. Thus, re-inoculating bacteria after 120 h adds bacteria that cannot ferment the 120 h NDF residue and therefore does not improve digestion of these residues.

NIR Estimation of uNDF

One of the interesting properties of uNDF residues measured after 36 h of fermentation is that they are highly correlated with NIR spectra (Jung et al, 1998). Dairyland Laboratories, Inc. (2013) reported that NIRS calibration was more highly correlated with measured $uNDF_{240}$ of both corn silage (R^2 =.93; SEC = 0.73) and haylage samples (R^2 =.94; SEC = 1.46) than it was to lignin in corn silage (R^2 =.75; SEC = 0.73) and haylage (R^2 =.77; SEC = 1.20); where SEC is the standard error of calibration. The coefficient of variation for calibration (100*SEC / Average) was also smaller for $uNDF_{240}$ (corn silage = 9.0%; haylage = 7.9%) than for lignin (corn silage = 21.9%; haylage = 19.4%). This suggests that NIRS may estimate uNDF more reliably than lignin for routine analysis.

Most nutritionists would agree that lignin itself is indigestible and that it seems to inhibit the digestion of fibrous carbohydrates in feeds. Smith et al. (1972) used $uNDF_{72}$ as the estimate of iNDF in a two-pool model of NDF digestion kinetics (Equation 4). They reported that up to 75% of the variation in $NDFU_{72}$ (expressed as a percentage of NDF) could be attributed to lignin (% of DM) Traxler et al. (1998) reported R^2 of 0.51, 0.62, and 0.71 for linear relationships of $NDFU_{96 \text{ or } 144}$ and permanganate lignin (% of NDF) for C3 grasses, C4 grasses and legumes, respectively. The regression coefficients for these equations ranged from 3.16 to 3.56. The factor (2.4 X lignin) was used for many versions of the Cornell Net Carbohydrate and Protein System to estimate the iNDF pool (Van Soest et al., 2005). This factor was developed based on undigested residues from 60 to 90 day methane fermentations. Mertens (2015) analyzed a large data set provided by Dairyland Laboratories, Inc. and observed a relationship across samples of legumes, grasses and corn silages of: $uNDFOM_{240} = 2.86*$ lignin; $R^2 = 0.80$ (regression with a zero intercept). Given the precision in measuring $uNDF_{240}$ (Boyd and Mertens, 2011; Mertens et al., 2012) and its ability to be estimated by NIR (Dairyland Laboratories, Inc., 2013) it would seem that estimating iNDF from measured uNDF is preferable to predicting it from lignin.

An alternative method to using long term uNDF measurements is to estimate iNDF using time series measurements with exponential models. When uNDF is measured for fermentation times of ≤96 h, the measurements can be fitted to a two-pool model as described in Equation 4. When uNDF measurements are made at >96 h then a three-pool model is more appropriate:

Eq. 9 $NDFRes_{(t)} = F*e^{(-kf*[t - lag])} + S*e^{(-ks*[t - lag])} + I_3$; where $NDFRes_{(t)}$ is the NDF residue at any time = t, F is the fast potentially digestible fraction and S is the slow potentially digestible fraction such that $(F + S) = (NDF - I_3)$; kf is the fast fractional rate constant of digestion; ks is the slow fractional rate constant of digestion; t is time of fermentation; lag is discrete lag time before NDF digestion begins; and I_3 is the iNDF for a three-pool model of fermentation. Raffrenato and Van Amburgh (2010) indicate that the three pools and two rates of the three-pool model can be estimated using $uNDF_{36}$, $uNDF_{120}$ and $uNDF_{240}$ (or forage group specific ranges for iNDF) using modeling software. This approach appears to estimate the five model parameters with no degrees of freedom for error. Traditional kinetic analysis uses 2 to 3 observations for every parameter to be estimated.

USING uNDF TO IMPROVE DAIRY NUTRITION

Benchmarking Individual Feed Quality

Before we had access to routine in vitro digestibility measurements, lignin was a useful criterion for characterizing the variable digestibility of NDF. Lignin or its ratios to fibrous constituents has been used to predict NDFD (Goering and Van Soest, 1970). The concept of iNDF as a crucial pool that was needed for kinetic analysis of NDF fermentation demonstrated that the amount of iNDF greatly exceeded the lignin concentrations in feeds. This suggests that there are fibrous carbohydrates that are also indigestible in the anaerobic ruminal environment, which may or may not be associated with lignin. Figure 2 (a) and (b) illustrate the relationship of $uNDF_{240}$ (an estimate of iNDF) and lignin to $NDFD_{30}$. Visual comparison indicates that the relationship of $NDFD_{30}$ to $uNDF_{240}$ (Figure 2a) is more uniform across forage types (corn silage, grasses and legumes) than is the relationship to lignin (Figure 2b). Using individual regression equations for each forage type, the pooled R^2 for the relationship of $NDFD_{30}$ to $uNDF_{240}$ is higher than for lignin (0.70 vs 0.60) and the standard error for regression is lower (0.045 vs 0.051). Although the there may be some autocorrelation between $uNDF_{240}$ and pdNDF or kd that magnifies its effect as a sole predictor, the high percentage of variation in $NDFD_{30}$ attributed to $uNDF_{240}$ (70%) suggests that it has a greater impact on digestibility than pdNDF or its rate of digestion.
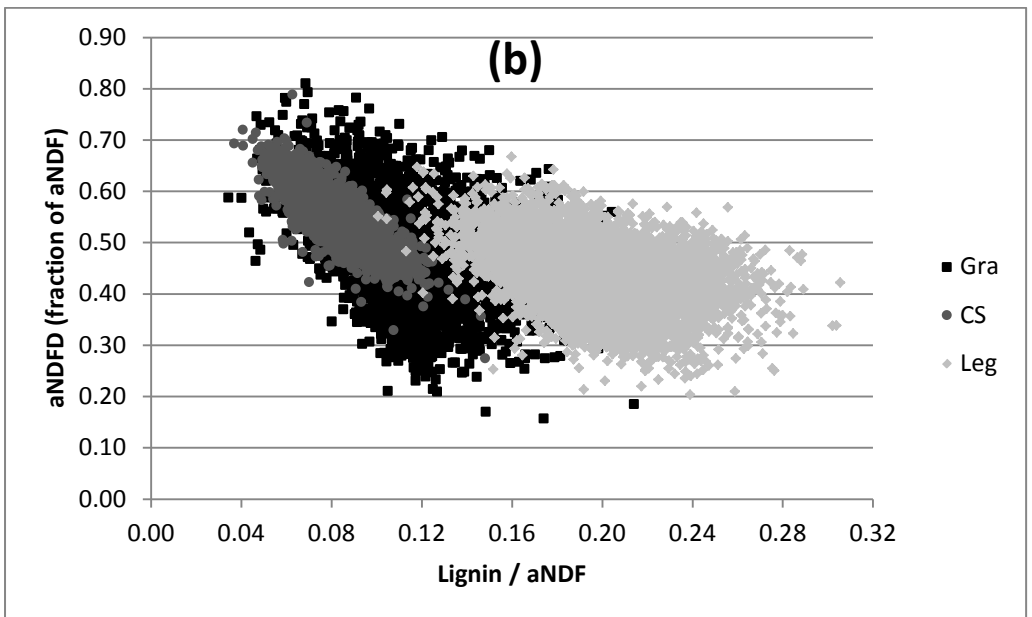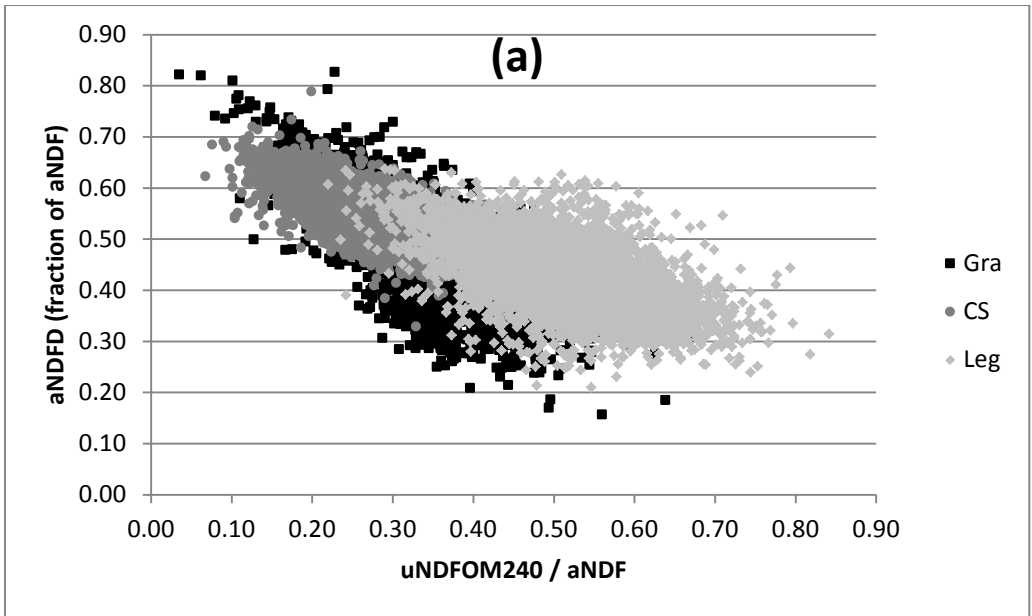
Figure 2. Relationship of undegraded NDF organic matter at 240 h (uNDFOM$_{240}$) of fermentation (a) and lignin (b) to NDF digestibility after 30 h of fermentation for corn silage (CS), grass (Gra) and legume (Leg) forages (n > 24,500) when all variables are expressed as a fraction of total NDF (data courtesy of Dairyland Laboratories, Inc., Arcadia, WI).

Not only is uNDF related to NDFD, but also it is a more consistent index of feed quality. Knowing only the NDFD of a feed has limited value in defining feed quality. For example, if feeds A and B have NDFD$_{48}$ of 45 and 55%, respectively it would be reasonable to conclude

that feed B has higher quality (nutritional value) than feed A. However, if you also knew that feed A contained 50% NDF and feed B contained 60% NDF (DM basis), it becomes much more difficult to determine which feed is higher in quality because the positive effects of higher NDFD may be cancelled by the negative effects of higher NDF. Estimating the digested NDF, which is the product of NDFD and NDF ($dNDF_{48} = NDF \times NDFD_{48}$) would suggest that feed B is better because it has a dNDF of 33% of DM compared to 22.5% dNDF in DM for feed A. Do we really want more dNDF to indicate higher quality given it includes both the positive and negative effects of NDF? Indicating feed quality becomes clearer using uNDF. The $NDFU_{48}$ for feeds A and B are 55 and 45% (NDFU = 100 - NDFD), respectively. The $uNDF_{48}$ for feeds A and B are 27.5 and 33% of DM, respectively. Feed A has better nutritional value because it is lower in undigested DM. The uNDF of feeds is a better indicator of feed quality (lower is better) because it includes both the negative effects of higher NDF and the negative effects of higher NDFU.

Table 1. Chemical composition and fiber digestibilities of forages typically used in dairy rations[1].

Forage	CP	Ash	aNDF[2]	aNDFOM[3]	ADF	Lignin	uNDFOM[4] 240 h	NDFD[5] 30 h
Corn silage	7.2	4.0	35.1	34.1	21.9	2.96	9.75 (6.36-12.67)[6]	.540 (.464-.616)
Corn silage	7.2	4.4	40.0	38.7	24.8	3.22	10.45 (6.90-14.01)	.551 (.472-.631)
Corn silage	7.3	4.8	44.9	43.6	27.7	3.55	11.43 (7.27-15.60)	.558 (.476-.640)
Legume	22.5	11.5	35.1	32.7	29.2	7.00	18.55 (12.82-24.29)	.435 (.310-.560)
Legume	21.1	11.3	40.0	37.5	33.6	7.75	20.63 (14.88-26.38)	.428 (.324-.531)
Legume	19.5	11.2	44.9	42.2	37.4	8.62	23.47 (17.34-29.61)	.409 (.303-.509)
Grass	16.8	10.3	45.0	42.7	31.6	5.66	14.50 (3.88-25.12)	.600 (.427-.774)
Grass	13.9	9.9	50.2	47.5	35.1	6.25	17.50 (7.88-27.11)	.538 (.374-.702)
Grass	12.0	9.4	55.0	52.7	37.8	6.59	19.52 (11.12-29.92)	.509 (.369-.648)
Grass	10.3	9.3	60.0	57.5	40.2	6.79	20.82 (13.26-28.38)	.484 (.357-.612)

[1] Data courtesy of Dairyland Laboratories, Inc., Arcadia, WI. All results except NDFD30 are presented as a percentage of DM. $NDFD_{30}$ is presented as a fraction of aNDF.
[2] amylase-treated NDF.
[3] aNDF organic matter (ash-free aNDF).
[4] undigested aNDFOM after 240 h of in vitro fermentation.
[5] aNDFOM digestibility after 30 h of in vitro fermentaion (fraction of aNDF),
[6] Range of values that includes 95% of all samples.

Information in Table 1 was generated to provide specific targets for benchmarking uNDF and NDFD values for forages typically fed to dairy cows. Analyses provided by Dairyland Laboratories, Inc., Arcadia, WI were edited for correct classification for forage type, and all samples within ±1%-unit of 35, 40 and 45% amylase-treated NDF (aNDF) for corn silages and legumes or 45, 50, 55 or 60% aNDF for grasses were averaged. In most cases, 1000s of samples were averaged. For $uNDFOM_{240}$ and $NDFD_{30}$, the upper and lower limits of the 95% confidence interval were calculated so that a typical range in benchmark values could be generated. Because they were not always identified, bmr and traditional corn silage data were combined.

As expected, the average $uNDFOM_{240}$ of each forage type increased as the aNDF of the forage increased. However, it is interesting that the average $uNDFOM_{240}$ was a consistent percentage of average aNDF within each forage type being 27%, 35% and 56% for corn silage, grasses and legumes, respectively. The high proportion of NDF that is undigested in legumes indicates that the positive production responses associated with legumes is related to their low NDF concentration rather than their fiber digestibility. Although there is a rather consistent average proportion of NDF that is undigested within each forage type, the variation in $uNDFOM_{240}$ within a forage quality (NDF concentration) indicates the potential for large differences in dairy cow responses. Forages that are below the average $uNDFOM_{240}$ benchmark for each forage quality would be expected to have higher intake and digestibility and to generate greater milk production or body weight gain.

Benchmarking Dairy Rations for uNDF

Given that uNDF measured at fermentation times >48 h are good estimates of iNDF and that iNDF can only disappear from the rumen by particle size reduction (chewing) and escape from the rumen, it seems logical that there might be an upper limit on how much $uNDFOM_{240}$ can be consumed by high producing dairy cows. Mike Allen's research group at Michigan State University has done a series of experiments (Dado and Allen, 1996; Oba and Allen, 2000, 2003; Taylor and Allen, 2005; Voelker Linton and Allen, 2007) in which rumens were emptied and NDF and $uNDF_{120}$ intakes were determined. Although many of these experiments were designed to evaluate extremes in forage or starch digestion and not to be optimal diets, they can provide insights into the ranges of uNDF in rations that may be reasonable targets. It appears the intake of $uNDF_{120}$ is lower for high corn silage diets (40% of ration DM with 10% of ration DM from alfalfa silage) with a range of 0.40 to 0.50 % of bodyweight/d compared to high alfalfa silage diets (40% of ration DM with 10% of ration DM from corn silage) with a range in $uNDF_{120}$ intakes of 0.50 to 0.60% of bodyweight/d. These first attempts at defining optimal uNDF intakes should be used with caution until more data can be collected and interpreted. They should be in the ball park of optimal uNDF, but remember that ranges for $uNDF_{120}$ will be slightly larger than those for $uNDF_{240}$. Nutritionists in the field are encouraged to track the uNDF intakes of their optimal rations to see how well these first attempts at guidelines for uNDF fit the production responses from their rations.

The slightly lower intake of uNDF from corn silage compared to alfalfa silage may be related to two factors. Typically optimal rations for high production groups of dairy cows, which contain either corn or alfalfa silage, will have similar ration NDF of 26-28% of DM (these values will be 2-4% units higher when fibrous byproduct feeds are fed). Table 1 shows that, at the same NDF content, corn silage will have less $uNDFOM_{240}$ and thus with similar intakes a high corn silage ration will have lower intakes of uNDF. Also, it is commonly observed that legumes obtain slightly higher intakes than grasses and corn silage, which may be related to the more rapid particle size breakdown of legume fiber resulting in greater rates of passage of uNDF.

Estimating Nutrient Digestibility on the Farm

Mike Allen's research group have successfully used $uNDF_{120}$ as an internal indigestible marker for measuring digestion kinetics and digestibility of nutrients in vivo. With proper sampling of feces from several cows in a group and adequate sampling of the TMR, it is theoretically possible to calculate the DM, OM, CP, NDF and starch digestibility of a specific group of animals fed a particular TMR on a given farm. Currently we have few, if any, laboratory measurements that can be used to predict the numerous interactions between diet composition, cow intake level, and feeding management that can affect the ultimate utilization of diets by dairy cows. Estimating nutrient digestibility on the farm could be a valuable diagnostic tool when dairy cow performance does not meet the target production that is expected.

Estimating Fiber Digestion Kinetics and Modeling Intake and Digestion of Dairy Cows

Although there is opportunity to use uNDF to improve our ability to evaluate feeds and formulate rations, the use of uNDF with greatest reward will be in our ability to improve estimates of kinetic pools and rates, and increase the accuracy of our models of intake and digestion of fiber by dairy cows. We have essentially come full circle in our understanding and use of uNDF. Kinetic analysis led to the observation that total NDF is not nutritionally uniform because there is a fraction of NDF that does not digest in the rumen. Advances in in vitro methodology and modeling have pushed us to estimate iNDF more accurately using long term fermentations with proper technique, grind size, and filtration membranes. Mertens (2015) and Raffrenato and Van Amburgh (2010) have developed the mathematical and modeling frameworks to use uNDF to correctly estimate iNDF. However, it must be kept in mind that the model of digestion also defines the iNDF that is appropriate. The iNDF for a two-pool model is different from that for a three-pool model. Two-pool models of fiber digestion have proven to be useful in the last 40 years and their utility has value today. For these models, a good estimate of $iNDF_{2pool}$ is uNDF measured at 72 to 96 h. Three pool models indicate that fiber digestion is more accurately estimated by the inclusion of a second, slowly digestion pool of fiber. This slow pool digests so slowly that $iNDF_{3pool}$ is best estimated by $uNDF_{240}$. It remains to be determined if the added complexity and cost for estimating a second slowly digesting pool and rate provides insights and utility to justify its use.

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Diet formulation for lactating cows: the good, the bad, and the ugly

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SUMMARY

- Effective diet formulation requires processing of information and relationships to achieve a goal.
- The information is often incomplete or inaccurate.
- Some relationships are quantitative and can be represented in mathematical models, but others are qualitative and difficult to describe with numbers.
- The goal may be to increase production, maintain animal health, enhance fertility, increase efficiency, increase profitability, or some combination of these.
- Which goal is most important varies from farm to farm, for animals or groups within a farm, and over time.
- Philosophies regarding diet formulation vary according to experiences, depth of knowledge, economics, dogma, and paradigms that are shaped by conventional wisdom, tradition, trends, as well as product marketing.
- The complexity of formulation programs varies greatly but it is our view that complexity is not related to success.
- Although formulation programs are helpful tools, the overriding determinant of success in nutritional consulting is the competence of the nutritionist, including their understanding of practical nutrition and ability to communicate effectively.
- Oftentimes we have observed that some inputs and outputs of diet formulation programs receive attention even though they are unimportant while other inputs and outputs that are critical to success are ignored.

- This article will address aspects of diet formulation for lactating cows that we believe are most important for success, as well as those that we think should receive little or no attention.

DIET FORMULATION

Diet formulation begins with selection of a representative cow from the group being offered the ration to calculate nutrient requirements (including maintenance, growth, lactation and loss or repletion of body condition). The cow selected doesn't really exist but inputs including yield of milk components, body weight (BW), and expected gain or loss in BW are chosen to be representative of the group as a whole. It is important to note that nutrient requirements of individual cows in the group will differ from this "representative" cow and the extent to which they differ depends upon the amount of variation in the group. Formulation programs in use in the U.S. determine requirements similarly and are generally based on the latest Nutrient Requirements of Dairy Cattle (NRC, 2001). Dry matter intake is then usually predicted from milk yield and BW with an adjustment for decreased DMI in the postpartum period. While equations to predict DMI might vary somewhat across programs, characteristics of the diet known to greatly affect DMI are generally <u>not</u> included; a common deficiency among programs.

Where formulation programs diverge greatly is how they predict supply of energy and protein. Ruminal fermentation greatly affects digestibility of dry matter (DM), especially neutral detergent fiber (NDF), as well as the supply of protein available for absorption in the small intestine. To determine the rumen-undegraded protein (RUP) required, microbial protein supply to the small intestine is predicted from the diet and subtracted from the calculated protein required for absorption in the small intestine. The requirement for rumen-degraded protein (RDP) is estimated to provide adequate protein for microbial growth. Models differ greatly on how they predict digestibility of DM as well as microbial protein and digested RUP supply to the small intestine and RDP supply. Some use empirical predictions and table values, while others include more mechanistic relationships. Most models use equations similar to that of NRC (2001) to estimate the utilization of energy and protein after absorption. Ingredients are then combined so that nutrient supply meets the requirements of the representative cow. Finally, amounts of ingredients and nutrients are divided by DMI to determine concentrations.

MECHANISTIC MODELS

Mechanistic models have been developed and have evolved over the last several decades in an attempt to more accurately predict absorbed energy and protein. While several different groups have developed models, the Cornell Net Protein and Carbohydrate System (CNCPS) and its derivatives (CPM, NTS, AMTS) have been dominant for diet formulation in the US. While these programs have greatly contributed to educating students and nutritionists about the complexities of the rumen, it is our view that they have not increased accuracy of nutrient supply to meet requirements beyond simpler models. Rather, because of the great complexity of the rumen, the many interactions among feeds, the animal, and microbes, as well as lack of knowledge, lack of accurate data, and known faults in model structure, these models likely <u>reduce</u> our ability to accurately supply nutrients to meet requirements compared with empirical models. While models have evolved over time with additions of amino acid (AA) and fatty acid (FA) sub-models, the basic limitations and flaws of the original model persist. Furthermore, it is unrealistic to think that these limitations can be overcome any time soon.

Model Flaws and Limitations

The basic concept of mechanistic rumen models is that digestion/degradation of OM in the rumen is a competition between the rates of digestion and passage. If we knew <u>actual</u> rates of digestion and passage for a specific nutrient fraction (e.g. protein fraction B2) within a feed and if these rates were constant, then the digestibility of the nutrient fraction could be calculated as the rate of digestion as a percent of the total rate of disappearance (rate of digestion plus rate of passage). Because rates of digestion vary greatly among specific feed nutrient fractions, it is critical that each feed fraction have reasonably uniform rates of digestion and passage. Then digestibility of the feed can be calculated as a weighted average of the individual fractions. While this is appealing conceptually, *its application is problematic* for several reasons described in more detail elsewhere (Allen, 2011). The most important problems are as follows. Whereas feeds can be fractionated and rates of digestion of fractions can be measured, the rates obtained do not represent actual rates in vivo because of differences in particle size (surface area), enzyme activity, and pH between measurement conditions and in the rumen of cows. For example, digestion rates are typically measured on ground, dried samples, which are **not** representative of the material consumed by the cow. Also, accurate passage rates for each nutrient fraction that correspond to rates of digestion are non-existent. Moreover, interactions among feeds, microbial populations and the animal greatly affect both rates of digestion and passage and make accurate modeling for ruminal digestion impossible. These problems are intractable and prevent rumen models from <u>ever</u> increasing the accuracy of predicting the supply of absorbed nutrients. Therefore, we view the incorporation of mechanistic rumen models into practical diet formulation software as an exercise in futility. So why are programs with rumen models so widely used despite their failure to improve accuracy? We suggest two general reasons.

1) More complicated models are seductive for those who use them. These models provide acompetitive marketing edge because of the common perception that precision and complication translate into greater accuracy. Of course, as mentioned earlier, this is rarely the case. Complicated models are often promoted by feed test laboratories because more feed analytes are required, which increases revenue, and by some companies because the models show that their products are required (even though direct experiments may demonstrate little effect). Complicated models also provide numerous opportunities for presentations and consulting by academics and others.

Complicated models can lead to self-delusion. Humans tend to notice evidence that supports an opinion but ignore evidence against it. When a model correctly predicts a response to a ration change, the value of the model is reinforced; when the model is incorrect, the error is more likely discounted. Furthermore, a common problem with any complicated model is that the developers and users, over time, will fail to recognize model deficiencies and reject any critical evaluation of alternatives; this phenomenon is called "groupthink" (I. L. Janis, 1971). If you use a complicated model, we exhort you to keep a scorecard! Some say that the model must be "calibrated" to each farm; initially, the model is not accurate, but after tweaking this and that, "Voila, the model is amazing!" But, please consider that, with an almost infinite number of combinations of inputs, you can get any outcome you want. The question is what did you learn? And does it reflect reality? Please ask yourself if tweaking all the inputs does anything to enhance productivity or effectiveness. We believe that mechanistic models can indeed improve the ability of some people to formulate diets, but generally this is because the model stimulates mechanistic thinking, not because it improves accuracy. We strongly promote mechanistic thinking–we just don't think you need a complicated model to do it!

2) The published validation of complicated models is frequently faulty and misleading, thus giving the false impression that they are accurate. For instance, the predicted vs. measured flow of methionine to the small intestine shows remarkable agreement in Figure 5-10 of NRC (2001). However, that figure and others like it are extremely misleading and do not depict actual accuracy of prediction. One problem with Figure 5-10 is that the dataset used to validate the equations was the same as that used to develop them; proper validation, however, requires a different dataset. When we actually formulate diets, we don't use these equations to predict methionine flow in the original database! Another problem is that NRC used actual DMI measured during the experiments while DMI is often predicted during diet formulation. However, the biggest problem with Figure 5-10 and most other validations is that study is included in the validation statistics as a "**random study effect**". Random study effects are included in statistics because we know that the results from one study will differ from another simply because of measurement techniques, base forages and feeds, and cow conditions, for reasons that we really don't know. In any case, the study effect removes most of the variation that we must be able to predict when formulating diets! Proper statistical validations require that the "study effect" be zero for new data not used in developing the equations. It is no surprise to us that the ability of the NRC model to predict when a methionine supplement is needed is very poor compared to the accuracy presented in Figure 5-10.

Diet formulation programs provide much more information than is necessary to formulate diets optimally. It is our view that most, if not all, diet formulation programs can be successfully used to formulate diets as long as the nutritionist knows what information is useful and what is not. The success of the nutrition program is much more dependent upon the nutritionist and interaction and communication between the nutritionist and farm management, as well as personnel feeding, harvesting the crops and purchasing feeds. We view diet formulation programs incorporating mechanistic models as <u>overly complicated</u> and unnecessary for successful nutrition programs.

WHAT <u>IS</u> IMPORTANT?

Diet formulation programs usually focus on supplying adequate nutrient concentrations of consumed DM but completely ignore two of the most important factors for practical feeding: 1) effects of the diet on feed and energy intake and 2) effects of the diet on nutrient partitioning. Most programs require milk yield, BW, and BW change as model inputs, thus determining feed intake and how much energy is partitioned to milk vs body tissues. However, in reality, intake and partitioning cannot be model inputs but instead are determined by the cows and the diet they consume. *This is a major limitation of all diet formulation programs,* and is not likely to be solved anytime soon because of the biological complexity and lack of quantitative relationships for use in models. For instance, while we know many of the factors affecting the filling effect of rations such as the content, digestibility, particle length, and fragility of forage NDF, our measures of these factors are crude and provide only relative differences that cannot be used quantitatively. In addition, these factors interact with each other to affect fill. Furthermore, ruminal distention dominates the control of feed intake differently as cows progress through lactation; feed intake is also affected by metabolic and other physiological mechanisms. The situation is the same for our ability to predict the partitioning of energy between milk and body reserves. We know that energy partitioning is greatly affected by diet as well as many of the other factors involved, but we do not have the ability to quantify the effects of these factors and their interactions on energy partitioning to include in diet formulation programs. For instance, we know that milk fat depression (MFD) decreases energy output in milk and increases body

energy reserves, and we know that MFD is often the result of altered biohydrogenation of long chain fatty acids by rumen microbes which has several risk factors including diet content of polyunsaturated fatty acids, starch, and starch fermentability, among others. However, we cannot even begin to predict these conditions or the extent of MFD accurately with quantitative equations in a computer program.

Although we cannot quantitatively describe all of the important nutritional relationships in a computer program to optimize diets, we do have broad qualitative knowledge that can be used to better formulate diets right now! Successful application requires a paradigm shift for some nutritionists. Instead of focusing on computer programs, optimal diet formulation requires understanding variation in feeds and cows and working to reduce it, understanding and evaluating cow responses to diets, and letting go of the many factors that just don't matter.

Variation in Feeds

Minimizing variation in feeds is imperative to optimize diets, particularly for high-producing cows. Forages and some byproduct feeds can vary widely in nutrient composition while other feeds (e.g. dry corn, high protein soybean meal) are much more consistent. The feed composition table in the Dairy NRC (2001) includes data on variation in nutrient composition for feeds and is a good resource. Each lot of purchased or harvested feed that might be variable should be tested frequently (twice monthly) for at least CP and NDF until extent of variation is understood. Silages and wet feeds should be tested for DM content at least twice weekly. Variation can be reduced in harvested feeds by considering quality differences and then storing feeds of various qualities separately when possible.

Variation Among Cows

Production response of lactating cows to diets varies greatly because of differences in physiological state. In the postpartum period, blood insulin concentration and insulin sensitivity of tissues is low, resulting in mobilization of body reserves, increasing blood NEFA concentration, and suppressing feed intake (Allen and Piantoni, 2013). As milk yield increases, NEFA export as milk fat and DMI increases, control of feed intake becomes dominated by ruminal distention, and low-fill, high-starch rations promote maximum milk. As lactation progresses and milk yield declines, concentrations of glucose and insulin in blood, insulin sensitivity of tissues, and body energy reserves increase. These changes in metabolic priority are difficult to accurately include in a computer program but top-notch nutritionists, who understand cows, can use the qualitative knowledge to optimize nutrition programs.

Grouping

Because of the wide variation in physiological state among cows, we recommend three rations as cows progress through lactation: fresh, high, and maintenance rations. The fresh ration should be moderately filling with ~22-24% forage NDF to maintain adequate rumen fill and buffering and reduce risk of displaced abomasum. Starch content should be ~24-26% to provide the glucose and glucose precursors needed as milk yield increases. Higher ration starch contents can be fed if highly fermentable starch sources such as high moisture corn are limited. Cows should be switched to the high ration after 10 d postpartum if they are healthy and eat aggressively when feed is offered. As feed intake increases, rumen distention begins to limit DMI so the high ration should be less filling, containing 17-20% forage NDF, and contain greater starch content of 28-32% to drive milk yield. Actual concentrations of forage NDF and starch in the high

ration depend on cow responses and space for cows within groups; rations with less fill and higher starch content might result in higher peak milk yield but will also result in faster restoration of body condition and the need to move cows to the maintenance group quicker. Once body condition is restored (BCS ~3.0 on a 5 point scale), cows should be fed a maintenance ration to maintain milk yield and minimize additional gain in body condition. The maintenance ration should have less starch (~18-22%) and somewhat higher forage NDF content; these should be adjusted by evaluating BCS changes of cows at dry-off. Protein should also vary in these diets. The fresh ration ration should contain ~17% CP with the supplemental protein coming from high quality proteins with at least 40% RUP, and the protein sources should vary and have complimentary amino acid profiles. The high group should also be ~17% CP and if the diet has plenty of starch, special RUP sources may provide little benefit. Protein can be reduced in the maintenance group to 15-16% CP. Formulation programs can help determine the types of protein supplements that will be most beneficial but the user must recognize that they are frequently inaccurate. Lower protein rations might be fed to the high and maintenance groups by evaluating cow responses. This grouping strategy will help optimize cow health, production, and efficiency of nutrient utilization compared with feeding one ration to all cows in a herd or compared with feeding rations according to milk yield. For additional details see Allen and Piantoni (2014).

Feed Testing

We think that time and money formulating diets with complicated programs could be better spent on activities that actually help meet the goals in feeding cows. For example, nutritionists could use the extra time to ensure that feeds are tested routinely, variation in feeds is monitored, feed mixing is accurate and uniform, and cows have access to feed most of the day. We recommend more frequent feed testing for fewer nutrients. Variable feeds including forages, many byproducts, and other feeds should be tested for DM, NDF and CP on a regular basis as discussed above to assess true variation. Starch should be included in tests for feeds with variable starch content (such as corn, sorghum and small grain silages). We recommend testing of forages and byproduct feeds for minerals (macro and micro) to better formulate mineral supplements but testing can be less frequent. It is useful to keep a spreadsheet with these (DM, NDF, CP and starch) results for silages and other feeds and retest when deviations occur. This will help identify actual changes in composition and trends over time. Testing forages for in vitro NDF digestibility may help optimize forage allocation to groups and identify reasons for changes in production when forages are switched. Because it is a biological measure with variation from run to run, it is most useful to have all forages compared at the same time.

Diet Formulation

While we recommend testing and quantitatively balancing for only a few nutrients, many qualitative feed characteristics also should be considered when formulating diets. Chief among these are feed characteristics that affect intake and partitioning, something that even complicated computer programs are not able to do. The filling effect of a ration is a function of forage NDF, forage digestibility and forage fragility and is a primary consideration for all groups. Effective NDF should be adequate to form a rumen mat to retain small fibrous particles and increase their digestibility. However, excessive particle length should be avoided because it can reduce feed intake and increase sorting. Starch content and fermentability must be considered. Starch is needed to drive milk yield but rations with excessive ruminal starch digestion can reduce feed intake, ruminal pH, and milk fat yield and partition more energy to body condition at the expense of milk. While starch fermentability can be tested in laboratories,

we think that it isn't necessary for routine diet formulation because it can be estimated reasonably well from inspection of the feeds (e.g. moisture content, hardness, particle size, etc.).

Mechanistic rumen models were developed primarily to improve prediction of absorbed protein and later, amino acids. For this, we think they fail miserably. Some think that success in reducing CP content of diets while maintaining milk yield can be attributed to mechanistic models. While we think that minimizing nitrogen excretion is an important objective, we have little confidence that any success can be attributed to these models because they cannot predict microbial protein production or RUP accurately, never mind specific amino acid flows to the duodenum. We think that simpler models in combination with evaluation of cow response are more likely to be successful in this regard. We test feeds for CP and use this, combined with table values for RUP/RDP, empirical prediction of microbial protein production, knowledge of complementary feeds for amino acid profile, and cow feedback to formulate diets.

Cow Feedback

Evaluation of cow response is a critical component of successful diet formulation. Responses include yield of milk and milk components, milk composition (fat, protein, and MUN), DMI, body condition score, rumen score, fecal consistency, and cow behavior (eating aggressiveness, sorting). Making feed substitutions and monitoring responses can help refine diet formulation, especially when new forages are being offered. The degree to which rumen fill limits DMI can be evaluated by substituting less filling non-forage NDF (e.g. soyhulls) for more filling forage NDF for the high cows. The need for glucose precursors can be evaluated by substituting ground corn for non-forage NDF while protein can be evaluated by substituting soybean meal for ground corn or treated soybean meal for solvent-extracted soybean meal.

Other Important Factors

The success of any diet formulation process is determined by the genetics and environment of the cows. Cow comfort and access to quality air, water, and feed trump all but the most drastic changes in diet formulation. Evaluation of health, reproduction, lactation curves, BCS, and BW records all give important information. In addition, differences in composition among rations formulated, offered and consumed might be substantial. Observations on farm are critical to success. Do feeders always correctly estimate the required amounts called for when mixing the TMR (impossible!) or do they return excess or get additional feeds? Are the ingredients added to the mixer in the correct order? Is the mixer wagon maintained? Evaluation of rations in the feed bunks is sometimes overlooked: Do the cows have feed most of the day (and especially in the middle of the night when the managers are sleeping)? Is the ration over-mixed? Does the ration heat in the feed bunk? Do the feed bunks get cleaned? Are silage faces maintained? Are silages stable? These factors are an integral part of a successful diet formulation process.

CONCLUSION

Diet formulation for lactating cows is a complex process because cows vary in their response to diets as lactation progresses, because of the great variation in nutrient composition of feeds, and because of the complexity of the rumen ecosystem. While complex rumen models have been developed in an attempt to predict changes to the nutrient supply to the cow by ruminal microbes, we believe that they do not improve accuracy and only hinder the process of diet formulation. Much of the information provided and predicted by these programs does not

benefit diet formulation. While most programs can be used to formulate diets, it is beneficial for the nutritionist to understand what information is useful and what should be ignored.

The Good, the Bad, and the Ugly in Diet Formulation

We offer the following based on our assessment of published dairy nutrition research, our observations of successful nutrition programs on farms, and our 50+ collective years of conducting research in dairy nutrition.

The good:
- understanding effects of rations on intake, partitioning, production, and nutrient requirements,
- increasing consistency of rations by reducing variation in feeds,
- grouping to reduce variation among cows being fed the same ration,
- using models only as a guide while considering feedback from the cows,
- forming a management team with training and excellent communication among the nutritionist, feeders, crops and feed purchasing personnel, and veterinarian.

The bad:
- wasting time and resources on overly complicated models with flawed logic.

The ugly:
- lack of understanding of response to diets,
- lack of objectiveness from self-delusion and groupthink,
- selecting feed products based only upon model output and not research or critical evaluation.

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Site of Digestion of NFC – Does it Matter, and Why?

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SUMMARY

- Common methods of processing grain result in both ruminal and post-ruminal digestion, with the relative proportions varying significantly due to difference in starch chemistry and processing.
- Shifting the site of non-fiber carbohydrate (NFC) digestion has potential impacts on energy harvest, which have been widely discussed for many years. However, impacts on microbial protein yield, dry matter intake, splanchnic tissue metabolism, and glucose supply are also important to consider.
- Although some grain sources are poorly digested in the rumen, postruminal digestion largely compensates for this, and processing grain less extensively (i.e. fine grinding vs. ensiling) can sometimes improve productivity by supporting increased feed intake.
- An overall target for ruminally-fermented organic matter supply must take into account fiber as well as the NFC fraction of the diet.

INTRODUCTION

Feeding highly-productive ruminants is a complex challenge. The primary difficulty is the need to meet the nutrient requirements of both ruminal microbes and the host animal without disrupting the rumen environment (e.g. acidosis). Dairy nutritionists have become accustomed to considering the ruminal vs. post-ruminal supply of protein sources in the diet in an effort to meet microbial and animal requirements with minimal waste. However, this type of logic is only beginning to be applied to the carbohydrate fraction of the diet. The objective of this paper is to provide an overview of differences in site of NFC digestion and to consider the implications of changes in site of digestion for lactating dairy cows.

VARIATION IN SITE OF NFC DIGESTION

Dairy nutritionists are often concerned with indigestible starch, because it obviously represents a loss of energy that could potentially be available to the animal. As a result, measuring fecal starch concentration has become popular in some circles, and this has merit for at least 2 reasons. First, fecal starch is well-correlated with total-tract starch digestibility, at least in cows fed typical Midwestern U.S. diets (Fredin et al., 2014). Secondly, elevated fecal starch concentrations are typically a sign of disrupted gastrointestinal function (Wheeler and Noller, 1977). In fact, few individual cows (Fredin et al., 2014) or groups of healthy cattle (Owens, 2005) show fecal starch concentrations in excess of 10% of DM. I will argue that site of digestion has impacts that are far more important for dairy cow productivity than small increases in total-tract starch digestion when the starting point is >90%. By this logic, fecal starch measurement is worthwhile primarily as an indirect measure of poor gastrointestinal health.

The site of starch digestion differs dramatically between types of grains (Patton et al., 2012) and processing methods. Dairy cow diets based on ensiled high-moisture corn (HMC), steam-processed corn (SFC), and dry-rolled corn (DRC) had mean ruminal starch digestibilities of 76%, 54%, and 49%, respectively, in a meta-analysis (Owens, 2005). It should also be noted that many of these diets included highly-fermentable starch from corn silage, meaning that the ruminal digestibility of DRC starch is almost certainly lower than 49%. As implied above, these differences are substantially decreased (though not eliminated) after post-ruminal digestion, with means of 96%, 94%, and 90% for total-tract digestibility. It should also be noted that particle size has an important impact on total-tract digestibility; grinding DRC to < 1,000 μm mean particle size increased mean total-tract starch digestibility to 93% without negatively impacting NDF digestibility (Ferraretto et al., 2013). By changing grain processing methods, it is therefore possible to shift more than 25% of intake starch from ruminal to postruminal digestion while altering total-tract digestibility only 2-3%.

The extent of ruminal starch digestion is a product of degradation rate and residence time, and both digestion and passage rates contribute to variation in site of digestion. As an example of the impact of these factors, let's consider a study in which corn endosperm type (floury vs. vitreous) was varied in lactation diets (Taylor and Allen, 2005). Corn with a relatively vitreous (also known as glassy or flinty) endosperm is high in zein or prolamine proteins. In this study, the vitreous DRC diet had a ruminal starch digestion rate of 13%/hour, compared to 22%/hour for the floury DRC diet. Conversely, passage of starch from the rumen in the vitreous diet was 21%/hour compared to 16%/hour for the floury diet. Therefore, the vitreous endosperm starch was more slowly degraded, but because it also passed from the rumen more quickly than the floury corn, its apparent ruminal digestibility was only 35% compared to 57% for the floury endosperm starch. This is not an extreme case; 7-hour *in vitro* starch digestion analysis of thousands of DRC samples show a range from 45 to 65% digestion (www.dairylandlabs.net), and of course this excludes impacts of particle size and passage rate. The true range of ruminal vs. post-ruminal starch digestion on commercial dairy farms must vary dramatically.

Most lactation diets contain 5 to 15% non-starch NFC, comprised of sugars and the fraction that Van Soest (1991) referred to as nonstarch polysaccharides (NSP). Dietary sugars are generally considered to be completed degraded in the rumen, but the NSP have been largely ignored in the study of site of digestion, even though they can comprise as much as 10% of diet DM. The NSP pool includes a variety of constituents with different *in vitro* digestion kinetics (Lanzas et al., 2007). Determining the digestibility of this composite group would require calculating the

difference of a difference, which would likely lead to highly suspect results. Lack of information on the site of NSP digestion, particularly for diets heavily reliant on byproduct feeds, may limit the precision with which we can balance ruminal and post-ruminal NFC digestion.

PRE-ABSORPTIVE IMPACTS OF SHIFTING SITE OF DIGESTION

As with dietary protein, digestible carbohydrates are needed by both ruminal microbes and by the host, and the first objective in formulating diets for high-producing cows is typically to support adequate microbial fermentation. Sufficient supplies of fermentable carbohydrates are essential to drive rapid microbial growth, which provides the benefits of fibrolytic enzyme synthesis as well as total microbial protein production. On the other hand, excessive fermentation can decrease ruminal pH and disrupt microbial growth and biohydrogenation.

Microbial Protein

Yield of microbial protein is typically modeled as a function of RDP supply and rumen-fermentable organic matter (RFOM) supply, with one factor or the other being the limitation (NRC, 2001). In the U.S., it is rare for dairies to under-feed RDP, and RFOM supply is likely to be the primary constraint on microbial protein yield. Additionally, frequently-observed relationships between RFOM intake and milk protein concentration seem to support the importance of increasing ruminal NFC digestion to promote microbial protein yield (Ferraretto et al., 2013). However, RFOM also promotes insulin secretion, providing an alternative potential explanation for relationships between RFOM supply and milk protein (Winkelman and Overton, 2013).

Measurements of microbial protein yield have not demonstrated consistent relationships with ruminal OM digestion in lactation diets (Firkins et al., 2001, Fredin et al., 2015). In the model of Firkins et al (2001), DMI was the only significant predictor of microbial protein supply, and this may help to explain the lack of an observable relationship between rumen-fermented OM and microbial protein yield when these variables are clearly related biologically. Ruminal fermentation directly impacts DMI (see post-absorptive section) and considering impacts on microbial protein without considering DMI effects may lead to counter-productive approaches to formulation. On the other hand, great differences between labs in measurement of both ruminal OM digestion and microbial protein yield may introduce too much noise in meta-analyses, as some within-study data show reasonably strong relationships between these factors (Oba and Allen, 2003b). There are certainly situations where microbial protein yield is constrained by RFOM supply, particularly when forage quality is very poor, resulting in limited DMI and ruminal NDF digestion.

Ruminal Biohydrogenation

Excessive ruminally-degradable starch supply can contribute to declines in ruminal pH and milk fat yield by altering biohydrogenation of fatty acids in the rumen (Bradford and Allen, 2004, Zebeli et al., 2010). Many factors influence these responses, including physically-effective NDF concentration, animal characteristics, and dietary lipid content and profile. However, RFOM supply and rumen unsaturated fatty acid load are the 2 most critical risk factors for milk fat depression, and this must be kept in mind when altering site of NFC digestion.

Small Intestinal Digestion

There has been much discussion in the literature about whether ruminants have limited capacity to digest starch in the small intestine (McLeod et al., 2006). Duodenal infusion of starch at up to ~1,500 g/d has resulted in ~50% small intestinal disappearance of the starch, whereas infusions of glucose have resulted in >70% disappearance, suggesting that starch degradation may be a limiting factor in cattle (Kreikemeier et al., 1991). However, such results have to be interpreted carefully. To begin with, single nutrient infusions in animals consuming limited diets may lead to responses that are not entirely physiological; for example, co-infusion of amino acids with starch significantly increases small intestinal starch digestion (Brake et al., 2014). Secondly, even "bypass" starch that is consumed in the diet has been affected by the enzymes and chemical environment in the rumen and is likely more digestible than duodenally-infused raw starch (i.e. denaturing and lysis of starch-encasing proteins). These limitations, combined with the fact that much of this work has occurred in young steers, make claims of a limited capacity for starch digestion in lactating cows tenuous at best.

In contrast to suggestions that intestinal starch digestion may be limited to 700 g/d in growing steers (McLeod et al., 2006), apparent postruminal starch digestion has in some cases exceeded 4.7 kg/d in lactating cows (Taylor and Allen, 2005); although some of this would be attributed to large intestinal digestion, it is likely that small intestinal digestion exceeded 3 kg/d. Direct measures of dietary starch disappearance in the small intestine exceeded 2 kg/d in one study with relatively high starch intake (Ali Haïmoud et al., 1995), although another report showed very low small intestinal starch disappearance (Knowlton et al., 1998). A recent meta-analysis of dairy cow data failed to show any apparent ceiling for small intestinal starch digestion, although the fractional digestibility of starch entering the small intestine did decrease as entry rate increased (Moharrery et al., 2014), likely a function of passage rate and/or starch chemistry.

Assuming that the small intestine of dairy cattle is reasonably adept at digesting starch, there are some reasons to believe that the conversion of digestible energy to net energy may be improved via small intestinal vs. ruminal digestion. These factors include potential decreases in methane and heat production and have been nicely summarized by McLeod et al. (2006). However, there has been little empirical evidence to support these theoretical considerations in lactating cows. In perhaps the most direct assessment of the energy efficiency of intestinally-digested starch, Reynolds et al. (2001) duodenally infused starch in lactating cows consuming ~18.5 kg DM/day, irrespective of infusion level. The increase in energy supply was utilized for milk production in mid-lactation and body weight gain in late lactation. Unfortunately, energy utilization was not carefully evaluated in mid lactation. In late lactation, starch infusion did not alter energy lost to methane, but did decrease the proportion of ME that went to heat (from 60.3 to 58.0%). This was due to a dilution of basal heat production by the additional energy infused, but also points to the likelihood of a very low heat increment for intestinally-digested starch. Ultimately, it appears unlikely that small intestinal digestion of starch increases the efficiency of energy utilization by more than a 1 to 2 percentage units.

Large Intestinal Fermentation

One impact of shifting starch digestion post-ruminally is that a greater amount of starch will reach the large intestine and stimulate fermentation there. Although relevant data are limited, measurements of large intestinal digestion of NFC range from <1% to approximately 12% of intake (Gressley et al., 2011), with the relative proportion strongly related to illeal flow of NFC (Moharrery et al., 2014). Hindgut fermentation is generally assumed to offer similar energy

yield as ruminal fermentation, although some VFA are lost in feces. Little microbial protein produced in the hindgut is captured.

High rates of NFC fermentation in the hind-gut result in rapid VFA production, and can significantly decrease fecal pH, termed hindgut acidosis (Wheeler and Noller, 1977, Gressley et al., 2011). Hindgut acidosis is a concern among many nutritionists, and there is at least some experimental evidence from induction models that associate this condition with poor health outcomes in a subset of animals (Gressley et al., 2011). These models, though, have not isolated hindgut acidosis from other potential impacts of the treatments employed. It is widely believed that high-energy diets are a risk factor for hemorrhagic bowel syndrome (Berghaus et al., 2005) presumably through impacts on hindgut microbiota, but such links remain speculative.

POST-ABSORPTIVE PHYSIOLOGICAL IMPACTS

Considering the impacts of site of NFC digestion on energy and metabolizable protein yield is certainly important, but it is clearly not the end of the story. Digestion of NFC via fermentation vs. small intestinal digestion results in very different end-products being absorbed. With what we know today about the signaling effects of specific nutrients (Bradford et al., 2016), this too must be factored in.

Ruminal fermentation of NFC leads to the production of VFA, with the profile varying by source of NFC; starch fermentation generally promotes propionate production (critical for gluconeogenesis), whereas sugars promote butyrate production. Small intestinal digestion of starch leads to uptake of glucose by enterocytes, although net portal flux of glucose for cows fed typical lactation diets was reported to be negative (Reynolds et al., 1988). This negative net flux likely reflects utilization of arterial glucose as a fuel source by splanchnic tissues. What happens to absorbed glucose? Much of it is likely metabolized by intestinal tissue, either oxidized to CO_2 or converted to lactate through anaerobic glycolysis. Net portal flux of lactate is indeed substantial (Reynolds et al., 1988), and can contribute significantly to gluconeogenesis.

Starch can therefore provide substrate for gluconeogenesis wherever it is digested. In addition, as starch delivery to the small intestine increases, net portal appearance of glucose can become positive. In steers abomasally infused with up to 60 g glucose / hour, net portal glucose appearance increased by 40 g/h, accounting for about 2/3 of the infusate (Kreikemeier et al., 1991). Other likely effects (not measured) may have been increases in lactate flux as well as fluxes of glutamine, glutamate, and aspartate, three fuels preferentially utilized by enterocytes. More recent work in lactating dairy cows directly compared equicaloric infusions of propionate (ruminal) and glucose (duodenal) to assess whole-body glucose turnover rate (Lemosquet et al., 2009). In the context of the diet fed in this study, both types of infusions increased plasma glucose rate of appearance, but the glucose infusion caused a greater increase than propionate. Through a combination of displacing utilization of arterial glucose, supplying lactate for gluconeogenesis, and directly entering the portal bloodstream, intestinal glucose absorption appears to be a more potent means to supply glucose to the cow than propionate, on an equicaloric basis. Of course, this comparison implies intestinal digestion that is adequate to liberate the glucose for absorption. *If* intestinal starch digestion is a real barrier to using bypass starch efficiently, products designed to deliver sugars post-ruminally may offer a novel solution (Russi et al., 2015).

The first and most important factor determining nutrient supply is DMI. While feed efficiency is always a goal, dairy cows in early to mid-lactation will generally respond to increased DMI with

greater milk yield; when considering the resource costs of maintaining a cow (both biological and facility costs), incremental increases in milk production are generally beneficial if they do not come at the expense of health or fertility. This is relevant because shifting the site of NFC digestion has clear impacts on DMI. Multiple studies have demonstrated that directly replacing HMC with DRC in high-starch diets can increase DMI of lactating dairy cows by 1 to 2 kg/d, with similar increases in solids-corrected milk yield (Oba and Allen, 2003a, Bradford and Allen, 2007, Ferraretto et al., 2013).

High ruminal fermentability of starch leads to rapid production and absorption of propionate, which can contribute to suppression of feeding (Allen et al., 2009). Ruminal vs. postruminal degradation of starch can also trigger larger post-meal spikes in plasma insulin concentrations (Oba and Allen, 2003a), which are likely related to DMI responses to these diets (Bradford and Allen, 2007) and may promote a shift in energy partitioning from milk to BCS. Formulation decisions that alter site of starch digestion may therefore have metabolite and endocrine effects that alter both feed intake and nutrient utilization.

NET IMPACTS ON DIET FORMULATION

There are examples in the literature of starch sources that were insufficiently processed, leading to total-tract digestibility of less than 90%, but adequate grinding (mean particle size <1,000 µm for DRC) can easily prevent such mistakes (Ferraretto et al., 2013). Key differences between diets then become the composition of NFC and its site of digestion. Note that rate of digestion is not necessarily the only factor influencing digestion dynamics in the rumen. Sugars, for example, are very rapidly degraded in the rumen, but can counter-intuitively stabilize ruminal pH and fatty acid biohydrogenation (Martel et al., 2011, Oba, 2011), via unknown mechanisms.

Bearing cost in mind, the NFC fraction of a lactating dairy cow diet should be formulated to support adequate microbial protein production without depressing milk fat yield, while supplying sufficient energy (a product of energy concentration and DMI) to drive high milk protein and lactose production. In cows near peak lactation, cows appear to handle very high loads of RFOM and respond with greater milk yield (Bradford and Allen, 2004, Weiss and Pinos-Rodriguez, 2009), whereas similar ruminal loads in late lactation can lead to subclinical acidosis, milk fat depression, and excessive body weight gain. Varying both NFC load and site of digestion, ideally by level of production, can help to manage these responses.

Novel processing methods to either enhance (Holt and Garner, 2013) or limit (Deckardt et al., 2013, Russi, 2013) ruminal availability of NFC may provide options for low-inclusion supplements to tweak ruminal vs. post-ruminal NFC supply, similar to the way that urea and bypass protein sources are used for balancing protein supply. Ultimately, site of NFC digestion cannot be fine-tuned without consideration of NDF digestibility. Site of NDF digestion varies more than is commonly recognized (Gressley et al., 2011), and total-tract NDF digestibility varies much more than total-tract NFC digestibility. The gold-standard lactation diet will continue to be built around a palatable, highly-digestible forage. Nevertheless, choices that influence the site of NFC digestion can make the differences between average and highly-productive cows.

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A Multidisciplinary Approach to Prevention, Assessment, and Mitigation of Mycotoxicosis in the Dairy Industry

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SUMMARY

- Mold growth is an inevitable consequence of feed production, as a result their harmful metabolites "The Mycotoxins" are commonly found in livestock diets.
- In the last 40 years great advances in the field of mycotoxins have increased our knowledge on the detrimental effects of these toxins on animal production.
- Climate change and agronomic practices play an important role in the unpredictability of mycotoxin contamination of feedstuffs.
- The primary classes of mycotoxins are aflatoxins, zearalenone (ZEA), trichothecenes, fumonisins, ochratoxins (OTA) and the ergot alkaloids.
- Due to the high variety of feedstuff utilized in dairy operations and the high production stress typically associated with modern dairying mycotoxins are important anti-nutritional factors in dairy nutrition programs.
- In order to maximize dairy performance and health, mycotoxins analysis and mycotoxins prevention strategies must be part of the all dairy nutritional and health programs.

INTRODUCTION

Dairy profitability is highly dependent on proper nutrition and health. It is therefore important to consider the negative role of anti-nutritional compounds naturally present in feedstuffs

commonly utilized to feed these animals. Among these compounds "the mycotoxins", which are toxic secondary metabolites produced by fungi (molds), should be closely monitored to minimize their impact on animal health and productivity. There are hundreds of mycotoxins known, but few have been extensively researched and even fewer have good methods of analysis that are commercially available. The primary classes of mycotoxins are aflatoxins of which aflatoxin B1 (AFB1) is the most prevalent, zearalenone (ZEA), trichothecenes - primarily deoxynivalenol (DON) and T-2 toxin (T-2) - fumonisins, ochratoxins (OTA) and the ergot alkaloids.

A practical definition of a mycotoxin is a fungal metabolite that causes an undesirable effect when animals or humans are exposed. Usually, exposure is through consumption of contaminated feedstuffs or foods. Mycotoxicoses are diseases caused by exposure to foods or feeds contaminated with mycotoxins (Nelson et al., 1993). Mycotoxins exhibit a variety of biological effects in animals: liver and kidney toxicity, central nervous system effects and estrogenic effects, to name a few. Some mycotoxins, i.e., aflatoxin, fumonisin and ochratoxin, are carcinogenic.

MOLDS, PLANTS, CLIMATE INTERACTIONS

The primary mycotoxin-producing fungal genera are *Aspergillus*, *Fusarium* and *Penicillium*. Many species of these fungi produce mycotoxins in feedstuffs. Molds can grow and mycotoxins can be produced pre-harvest or during storage, transport, processing or feeding. Mold growth and mycotoxin production are related to plant stress caused by weather extremes, to insect damage, to inadequate storage practices and to faulty feeding conditions. In general, environmental conditions — heat, water and insect damage — cause stress and predispose plants in the field or feed in transit or storage to mold growth and mycotoxin contamination (Coulumbe, 1993). Computer models to predict mycotoxin concentrations in corn prior to harvest are based on temperature, rainfall and insect pressure (Dowd, 2004) and similarly for DON in wheat (Prandini et al., 2009). Molds grow over a temperature range of 10-40°C (50-104°F), a pH range of 4-8, aw (water activity) above 0.7 and moisture content >13-15%. Most molds are aerobic, and therefore high-moisture concentrations that exclude adequate oxygen can prevent mold growth. However, in practical situations, molds will grow in wet feeds such as silage or wet byproducts, when oxygen is available.

Worldwide, approximately 25% of crops are affected by mycotoxins annually (CAST, 1989), which could lead to billions of dollars of losses. The annual economic cost of mycotoxins to the U.S. agricultural economy is estimated to average $1.4 billion (CAST, 2003). Economic losses are due to effects on livestock productivity, crop losses and the costs of regulatory programs directed toward mycotoxins. The implications of mycotoxins on agricultural trade have been reviewed (Dohlman, 2003).

Occurrence and concentrations of mycotoxins are variable by year and associated with variation in weather conditions and plant stresses known to affect mycotoxin formation (Coulumbe, 1993). In the 2009-10 crop year, several regions of the U.S. experienced higher concentrations and incidence of mycotoxins primarily due to a wet and delayed harvest season. These weather/climate trends have been more and more frequent in recent years (Figure 1 and 2(. Climate change and agronomic practices play a critical role in the plant/mold interactions necessary for mycotoxin outbreaks. A recent study by a group of subject matter experts (Wu et al. 2011) hypothesized that climate change (and the overall temperature increase) would play a

significant role in increasing aflatoxin and fumonisin contamination in maize, while DON concentrations would see a reduction related to the ambient temperature/mold relationship. However, these researchers postulated that DON concentrations in maize could also increase in relation to climate change related cropping practices and other agronomic changes. One of the most significant and potentially detrimental changes could be the trend to reduce or even eliminate tilling practices. Mansfield et al. (2005) looked at the effect of tilling on DON content in maize and concluded that although tillage type (no-till vs moldboard till) had no effect on DON incidence, no tilling resulted in significantly higher DON concentrations than moldboard tilling (figure 3.)

Although mycotoxins occur frequently in a variety of feedstuffs and are routinely fed to animals, it is less frequent that mycotoxins occur at concentrations high enough to cause immediate and dramatic losses in animal health and performance. However, mycotoxins at low levels interact with other stressors to cause subclinical losses in performance, increases in incidence of disease and reduced reproductive performance. To the animal producer, these subclinical losses are of greater economic importance than losses from acute effects and even more difficult to diagnose.

MYCOTOXICOSIS

The study of mycotoxins began in early 1960's with the outbreak of Turkey-X disease in the U.K. This outbreak was linked to peanut meal imported from Brazil (Sargeant et al., 1961). Because of an intensive multidisciplinary research effort, a blue-fluorescent toxin was isolated and mycelia of *A. flavus* were observed. *A. flavus* was shown to produce the same toxic compound(s) found in the toxic peanut meal. The toxin was characterized chemically and biologically and was given the trivial name aflatoxin. Aflatoxin was shown to be very toxic and carcinogenic in some of the test animal species used, and it resulted in a toxic metabolite in milk of dairy cows (Allcroft and Carnaghan, 1962; 1963).

The discovery of aflatoxin and elucidation of some of its effects led to research on other livestock health and production problems linked with moldy feedstuffs. This research led to the discovery of additional mycotoxins produced by other fungi. In dairy cattle, swine and poultry, mycotoxin contamination of feeds affects growth, milk production, egg production, reproduction and immunity (Diekman and Green, 1992). Mycotoxins have also been involved in outbreaks of human diseases (CAST, 1989).

Animals experiencing a mycotoxicosis may exhibit a few or many of a variety of symptoms, including: digestive disorders, reduced feed consumption, unthriftiness, rough hair coat or abnormal feathering, undernourished appearance, low production, poor production efficiency, impaired reproduction and/or a mixed infectious disease profile. Mycotoxins can increase incidence of disease and reduce production efficiency. Some of the symptoms observed with a mycotoxicosis may therefore be secondary, resulting from an opportunistic disease, present because of mycotoxin-induced immune suppression. Immunotoxic effects of mycotoxins have been reviewed (Oswald et al., 200; Bondy and Pestka, 2008). The progression and diversity of symptoms in a mycotoxicosis can be confusing, making diagnosis difficult (Schiefer, 1990). Diagnosis is further complicated by limited research, lack of feed analyses, nonspecific symptoms, few definitive biomarkers and interactions with other stress factors.

With few exceptions, a definitive diagnosis of a mycotoxicosis cannot be made directly from

symptoms, specific tissue damage or even feed analyses. However, experience with mycotoxin-affected herds increases the probability of recognizing a mycotoxicosis. A process of elimination of other factors, coupled with feed analyses and responses to treatments can help identify a mycotoxicosis. More definitive diagnoses can be made for specific mycotoxins by detecting aflatoxin in milk or for fumonisin by induced changes in sphingolipid concentrations (Riley and Pestka, 2005). Regardless of the difficulty of diagnosis, mycotoxins should be considered as a possible cause of production and health problems when appropriate symptoms exist and problems are not attributable to other typical causes (Schiefer, 1990).

SAFE LEVELS OF MYCOTOXINS

Some of the same factors that make diagnosis difficult also contribute to the difficulty of establishing levels of safety. These include lack of research, sensitivity differences of animal species, imprecision in sampling and analysis, the large number of potential mycotoxins, interactions among mycotoxins and interactions with stress factors (Schaeffer and Hamilton, 1991). Field toxicities appear to be more severe than predicted from laboratory research.

Naturally contaminated feeds are more toxic than feeds with the same level of a pure mycotoxin supplemented into the diet. Aflatoxin produced from culture was more toxic to dairy cattle than pure aflatoxin added to diets (Applebaum et al., 1982). In swine, Foster et al. (1986) demonstrated that a diet containing pure added DON was less toxic than diets with similar concentrations of DON supplied from naturally contaminated feeds. Smith and MacDonald (1991) have suggested that fusaric acid, produced by many species of *Fusarium*, occurs along with DON to produce more severe symptoms. Lillehoj and Ceigler (1975) gave an example where penicillic acid and citrinin were innocuous in laboratory animals when administered alone, but were 100% lethal when given in combination. These studies strongly suggest the presence of other unidentified mycotoxins in naturally contaminated feeds and that mycotoxin interactions are extremely important. It is well documented that several mycotoxins may be found in the same feed (Hagler et al., 1984). Abbas et al. (1989) demonstrated *Fusarium* species isolated from Minnesota corn produced multiple mycotoxins. Because animals are fed a blend of feedstuffs and because molds produce an array of mycotoxins, many mycotoxin interactions are possible. Speijers and Speijers (2004) discussed the combined toxicity of mycotoxins and, therefore, suggest daily tolerable intake limits for groups of mycotoxins.

Mycotoxin interactions with other factors also make it difficult to determine safe levels of individual mycotoxins. Animals under environmental or production stress may show the more pronounced symptoms. For example, there is a clear temperature interaction with fescue (ergot) toxicity, such that more pronounced symptoms are expressed during heat stress (Bacon, 1995). Jones et al. (1982) demonstrated that productivity losses in commercial broiler operations occurred when aflatoxin concentrations were below concern levels determined by controlled research in laboratory situations. The researchers hypothesized that general production stress had a significant contribution to the animal's susceptibility to the low concentrations of the toxins. The known dietary factors that interact with mycotoxins include nutrients such as fat, protein, fiber, vitamins and minerals (Brucato et al., 1986; Galvano et al., 2001). Thus, many factors and interactions make it difficult to relate field observations to those from controlled research. Mycotoxin effects vary by species and are also moderated by factors such as sex, age, duration of exposure and stresses of the environment and production.

Overall health and immune status also affect the animal's capability to cope with a specific

concentration of a toxin or a combination of toxins. This is primarily due the many mycotoxins with immunosuppressive properties and their interaction with animal health (Schiefer, 1990). Diagnostic therefore is quite difficult since disease outbreaks may be secondary, resulting from an opportunistic disease, due to a mycotoxin-induced immune suppression. Immunotoxic effects of mycotoxins are reviewed (Oswald et al., 2005; Bondy and Pestka, 2008).

MYCOTOXIN TESTING

The accurate determination of mycotoxin concentrations in grain and feeds depends on accuracy from sampling to analytical techniques. A statistically valid sample must be drawn from the lot, which is not simple because mycotoxins are distributed unevenly in grains and other feedstuffs. Most of the error in a single analysis is due to sampling — as much as 90% of the error is associated with the taking of the initial sample (Whittaker, 2003). Once collected, samples should be handled to prevent further mold growth. Wet samples may be frozen or dried before shipment, and transit time should be minimized.

The second-largest source of error is inaccurate grinding and subsampling of the original sample. Finally, the subsample is extracted, the extract purified using one of several techniques, and then the toxin is measured. Toxin determination may be by thin-layer chromatography plates, high- performance liquid chromatography, gas-liquid chromatography, enzyme-linked immunosorbent assays, spectrophotometer or by other techniques. New technologies are progressing rapidly.

Mold spore counts may not be very useful and are only a gross indication of the potential for toxicity, but mold identification can be useful to suggest which mycotoxins may be present. Blacklighting for bright-greenish-yellow flourescence (BGYF) is often used as a screening technique for aflatoxin in corn, but it is very inaccurate. Newer and better methods should be used.

Generally, laboratories provide analysis for only a limited number of mycotoxins, perhaps including aflatoxin, OTA, DON, ZEA, fumonisin and T-2 toxin. Laboratory analysis may be directed toward detection of high levels of mycotoxins associated with acute toxicity and serious animal disease rather than low levels associated with chronic effects such as production losses, impaired immunity and significant economic losses. Therefore, minimum detection limits set by a laboratory may inhibit the diagnosis of a chronic mycotoxicosis.

Analytical techniques for mycotoxins are improving, costs are decreasing and several commercial laboratories are available that provide screens for an array of mycotoxins. The Federal Grain Inspection Service (USDA-GIPSA) provides a list on the Internet of approved mycotoxin tests for grains and provides excellent background materials for the feed industry (at www.usda.gov/gipsa/pubs/mycobook.pdf). Laboratory methods can be found in "Official methods of analysis of AOAC International". Krska et al. (2008) provided an update on mycotoxin analysis focusing on recent developments including multi-mycotoxin methods and quick tests. Maragos and Busman (2010) reviewed the rapid and advanced tools for mycotoxin analysis.

Because analytical methods can be either qualitative or quantitative, done by inexpensive kits or by sophisticated analytical instruments and can be quick or time fairly time consuming, it may be difficult to determine and select the right method for the right need (Scudamore, 2005).

CONCLUSIONS

More information is needed about why mycotoxins occur, when to expect them, how to prevent their occurrence and how to deal with their presence. More data are needed about animal toxicity and about interactions with other mycotoxins, nutrients and stress factors such as disease organisms or environmental stress and about the role of mycotoxins in immunosuppression. Improved screening techniques are needed for monitoring mycotoxin occurrence, including the detection of multiple toxins, diagnosing toxicities and prevention and treatment (CAST, 2003).

Practical Guidelines for dealing with a mycotoxicosis:

- Observe for general symptoms

- Rule out other possible causes (nutrition, disease, poor management etc.)

- Follow a mycotoxin control program (scientifically tested)

- Test feeds for common mycotoxins (aflatoxin, fumonisin, DON, T-2 toxin, ZEA, OTA)

- Remove or dilute contaminated feeds (observe effects)

- Follow recommended practices to increase the animals intake

- Increase nutrients (Protein, fat, effective fiber, antioxidants like vitamin E, Cu, Zn, Mn, Se, etc.)

- Avoid feeding contaminated feeds to more susceptible animals (young, pregnant and transition)

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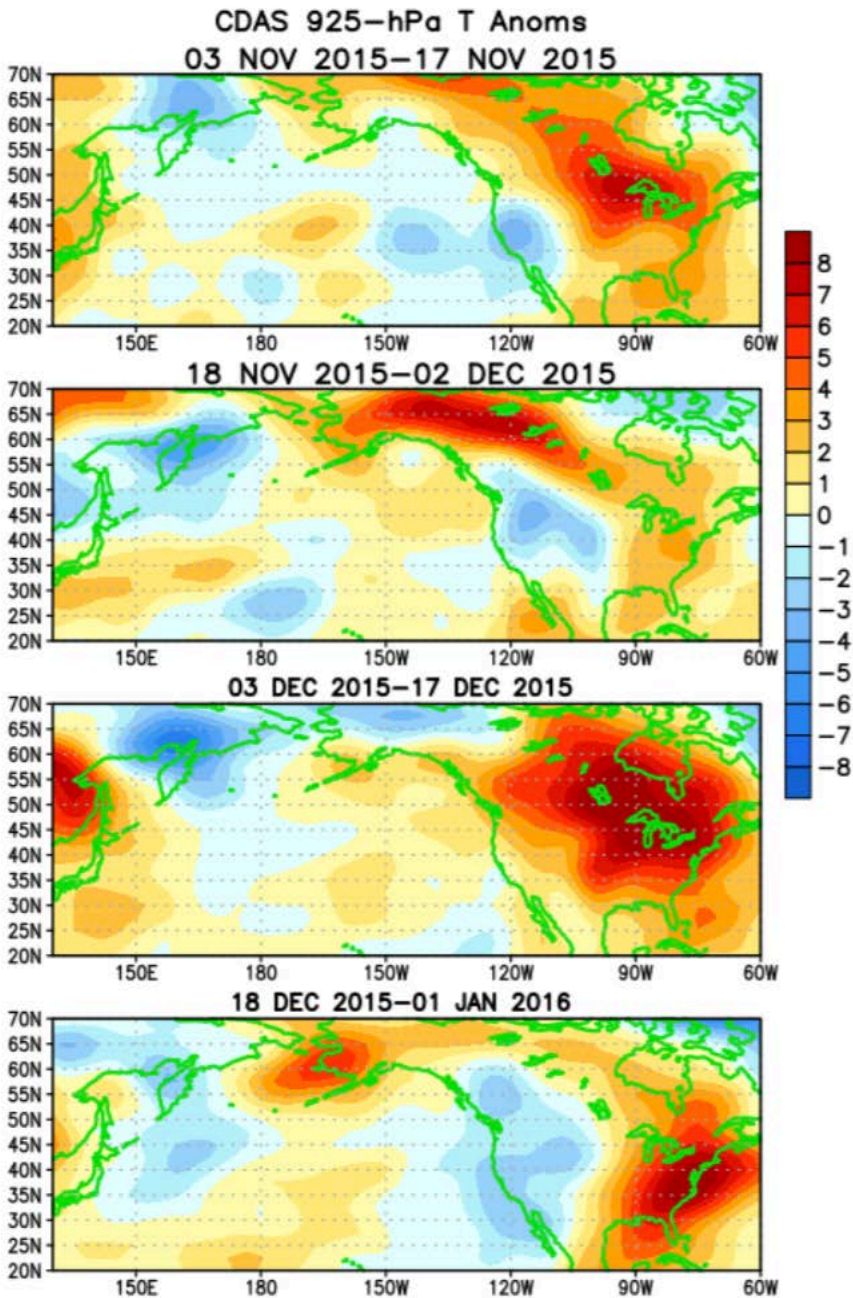
Figure 1. Atmospheric anomalies over North America from November to December 2015. Above-average heights/temperatures dominated over the East and near-to-below average heights/temperatures were observed over the West. From NOAA's El Niño Portal (http://www.elnino.noaa.gov/)
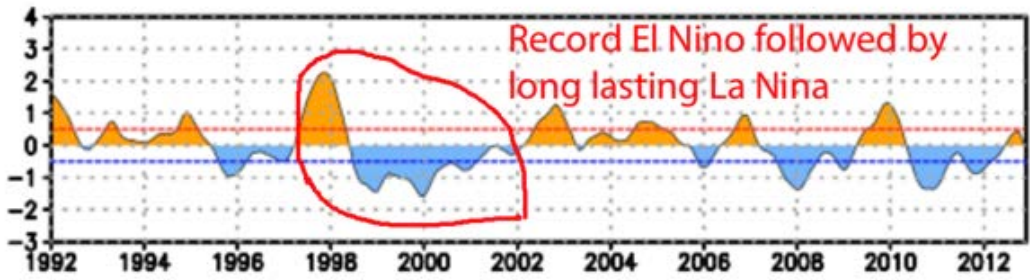
Figure 2. Occurrence of La Niña events following strong El Niño years. From NOAA's El Niño Portal (http://www.elnino.noaa.gov/)
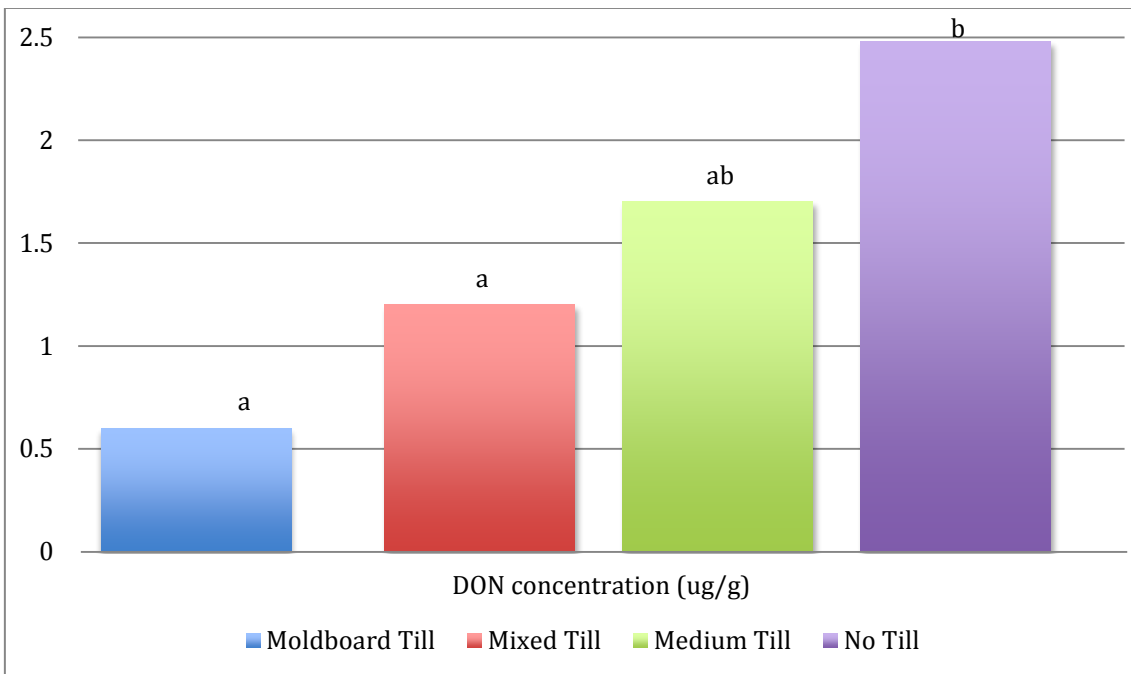

Figure 3. Average deoxynivalenol (DON) concentration in 2001 and 2002 silage samples managed under different tillage systems (adapted from Mansfield et al. 2005)