

# **BENEFITS OF AMINO ACID BALANCING ON METABOLISM, INFLAMMATION, AND OXIDATIVE STRESS**

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## **KEY AMINO ACIDS AND CHOLINE FOR TRANSITION COWS**

Due to extensive microbial degradation in the rumen, dietary availability of key methyl donors [(e.g., Methionine (MET) and choline (CHOL)] to mammary and liver is limited (Sharma and Erdman, 1989; Girard and Matte, 2005). Consequently, the mobilization of body protein in dairy cows close to calving compensates in part for this shortfall (Komaragiri and Erdman, 1997). Supplementing rumen-protected methyl donors may help fulfill the daily methyl group requirement, and possibly improve the overall production and health of dairy cows during the transition period (Zom et al., 2011; Osorio et al., 2013; Osorio et al., 2014).

The availability of MET and CHOL is important for various biological functions. For instance, MET together with Lys are the most-limiting amino acids (AA) for milk synthesis (NRC, 2001). Being the only essential sulfur-containing AA, MET acts as the precursor for other sulfur-containing AA such as cysteine (Cys), homocysteine and taurine (Brosnan and Brosnan, 2006). It has been estimated in lactating goats that as much as 28% of absorbed MET could be used for CHOL synthesis (Emmanuel and Kennelly, 1984). Hence, it is thought that rumen-protected CHOL supplementation could spare MET to help cows achieve better overall performance (Hartwell et al., 2000; Pinotti, 2012). Current recommendations for duodenal supply of Lys and MET to maximize milk protein content and yield in established lactation are 7.2 and 2.4% of MP, respectively (NRC, 2001). In terms of production performance, a Lys:Met ratio close to 2.8:1 of MP during the periparturient period by supplementing rumen-protected MET was beneficial (Osorio et al., 2013).

As a lipotropic agent, MET is directly involved in very low density lipoprotein (VLDL) synthesis via the generation of S-adenosylmethionine (SAM), the most important methyl donor (Martinov et al., 2010). In turn, SAM can be used to methylate phosphatidylethanolamine (PE) to generate PC, which is essential for VLDL synthesis (Auboiron et al., 1995). In the context of VLDL synthesis and liver lipid metabolism, CHOL-containing nutrients (mainly in the form of PC) are indispensable for the synthesis and release of chylomicrons and VLDL (Pinotti et al., 2002). Thus, supplementation of rumen-protected MET and/or CHOL (Zom et al., 2011) may increase hepatic triacylglycerol (TAG) export and consequently decrease lipodosis.

The immune system benefits greatly from proper nutrition, which in turn prepares the cow for periods of stress, reducing adverse effects and enhancing recovery. These concepts become of central importance when applied to the transition cow, as a

successful transition to lactation sets the stage for a profitable lactation, with optimal production, reproduction, and health, avoiding premature culling. Metabolic disorders are common during this time and can easily erase the entire profit potential for dairy cow farms (Drackley, 1999). The immune dysfunction during transition (Kehrli et al., 1989; Waller, 2000) and the state of oxidative stress (Abuelo et al., 2015) can lead to a cow that might be hyposensitive and hyporesponsive to antigens, hence, more susceptible to infectious diseases such as mastitis (Mallard et al., 1998).

## RUMEN-PROTECTED METHYL DONORS AND PRODUCTION PERFORMANCE

To date, the reported effects of rumen-protected MET and/or CHOL supplementation on dairy cow production performance have been inconsistent. Although previous studies from our group and others have observed beneficial effects from MET (Chen et al., 2011; Osorio et al., 2013) or CHOL (Pinotti et al., 2003; Zom et al., 2011) supplementation, other studies did not detect significant improvements on peripartal production performance with MET (Socha et al., 2005; Ordway et al., 2009; Preynat et al., 2009) or CHOL (Guretzky et al., 2006; Leiva et al., 2015) supplementation. Similarly, studies evaluating whether CHOL alone or in combination with MET provide equal or different benefits in terms of production performance also yielded different results. For instance, MET and CHOL supplementation both led to greater DMI in previous studies (Ardalan et al., 2010; Sun et al., 2016). In contrast, only a MET effect was observed in the most recent transition cow study from our group (Zhou et al., 2016c). Production performance results from peripartal MET and CHOL supplementation studies are summarized in Table 1.

Production performance benefits in response to MET supplementation during the periparturient period are likely associated with an enriched AA and sulfur-containing antioxidant pool. Inadequate MET availability could limit the utilization of other circulating AA according to von Liebig's hypothesis which is commonly described with the analogy of the water barrel with broken staves (Mitchell and Block, 1946). The fact that circulating MET concentration decreased markedly through parturition and was not restored to prepartum levels until 28 d postpartum (Zhou et al., 2016b) suggest increasing MET availability during this period could potentially benefit production performance. Additionally, enhancing MET availability (Graulet et al., 2005) is likely to increase its entry into the 1-carbon metabolism cycle in liver which consequently increases the production of downstream compounds such as Cys. Glutathione (GSH) is another downstream compound arising in the MET cycle that can supply AA such as Cys to the mammary gland for milk synthesis (Pocius et al., 1981).

Apart from providing MET, GSH (as a potent intracellular antioxidant) may contribute to better overall performance by alleviating oxidative stress and subsequent inflammation. Previous work has demonstrated a positive effect of MET supplementation on intrahepatic GSH concentration during the peripartal period (Osorio et al., 2014; Zhou et al., 2016a). Such effect may be directly associated with MET supplementation considering that it can be incorporated upstream in the *de novo* synthesis pathway for GSH (Halsted, 2013). Both in vitro (Hartman et al., 2002) and in

vivo (Tabachnick and Tarver, 1955) studies using radioactive-labelled MET demonstrated hepatic incorporation of [<sup>35</sup>S] into GSH. Therefore, the higher hepatic GSH concentration observed in MET-supplemented cows helps alleviate oxidative stress and contributes to greater DMI through an overall alleviation of the inflammatory status (Zhou et al., 2016a).

Although CHOL does not contain sulfur, MET can be generated in tissues like the liver from CHOL when homocysteine accepts a methyl group from CHOL through betaine (Wong and Thompson, 1972; Li and Vance, 2008). Hence, if comparable MET can be generated in response to CHOL supplementation, similar production performance benefits would be expected. Despite the fact that milk yield, DMI, and milk composition benefits were not observed in CHOL-supplemented cows in a recent transition cow study from our group (Zhou et al., 2016c), lactation performance benefits were detected in previous studies, indicating that CHOL may exert its lactation benefits through various means. For instance, the increase in hepatic mRNA expression of carnitine transporter suggested an increase in fatty acid uptake capacity and intracellular transport in CHOL cows, which was associated with reduced liver TAG accumulation (Goselink et al., 2013). Additionally, the CHOL supplementation from precalving through early lactation led to increased glycogen in liver tissue, implying a benefit to liver metabolism (Piepenbrink and Overton, 2003). Furthermore, CHOL can be used to generate PC, which is essential for VLDL synthesis and help reduce liver lipodosis by promoting TAG export (Pinotti et al., 2002).

#### RUMEN-PROTECTED METHYL DONORS AND METABOLISM

During the transition period cows will normally experience an increase in adipose tissue lipolysis due to changes in hormones such as insulin (decrease) and growth hormone (increase), and consequently blood non-esterified fatty acid (NEFA) concentrations increase. Once NEFA reach the liver these can be oxidized to provide energy, partially oxidized to produce ketone bodies, or esterified to triglyceride (TAG). A major organelle within hepatocytes where NEFA oxidation takes place is the mitochondria, and carnitine is essential for transport of NEFA from cytosol into mitochondria for subsequent  $\beta$ -oxidation (Drackley, 1999). Methionine is essential for carnitine synthesis (Carlson et al., 2007), thus, the greater hepatic concentration of carnitine (82.1 vs 37.5 nmol/g of tissue) that was detected in MET-supplemented cows indicates a greater bioavailability of MET to methylate Lys (Osorio et al., 2014).

Previous studies reported no significant effect of CHOL on blood glucose or BHBA concentrations (Guretzky et al., 2006; Zahra et al., 2006; Zom et al., 2011). In a recent study from our group (Zhou et al., 2016c), the tendency for lower BHBA in response to CHOL supplementation agreed with the greater glucose concentration. Although speculative, the pattern of BHBA and glucose detected in CHOL-supplemented cows was associated with numerically lower negative energy balance as a result of lower milk production. The exact mechanisms for the lower milk yield in these cows that maintained greater blood glucose is not known.

**Table 1.** Summary of production performance results from transition cow studies supplementing rumen-protected MET or CHOL.

Study	DMI	Milk	Protein	Fat	Dosage	Product	Duration	Cows
<b>Methionine studies</b>								
Overton et al., 1996	-	+	-	-	17 g	RPM (Degussa)	-7 to 126	24
Piepenbrink et al., 2003	-	+	-	-	2.34% or 2.7% MP pre; 2.36 or 2.63% MP post	Alimet	-21 to 84	48
Socha et al., 2005	-	-	-	-	10.5 g	SM	-14 to 105	48
Ordway et al., 2009	-	-	-	-	Met:Lys = 3.0:1	MS and SM	-21 to 140	60
Preynat et al., 2009	-	-	-	-	15.3 g	Mepron-85	-21 to 112	60
Ardalan et al., 2010	+	-	/	/	14.4 g	SM	-28 to 70	40
Osorio et al., 2013	+	+	+	+	0.19% MS or 0.07 % SM DM	MS and SM	-21 to 30	56
Zhou et al., 2016	+	+	+	+	0.08 % DM	SM	-21 to 30	81
Sun et al., 2016	+	+	+	+	15 g	Mepron-85	-21 to 21	48
<b>Choline studies</b>								
Hartwell et al., 2000	-	-	-	-	0,6,12 g	Capshure	-21 to 120	48
Pinotti et al., 2003	-	+	-	+	20 g	Overcholine	-14 to 30	26
Piepenbrink et al., 2003	-	+	-	+	11, 15, 19 g	ReaShure	-21 to 63	48
Guretzky et al., 2006	-	-	-	-	15 g	ReaShure	-21 to 21	42
Lima et al., 2007	+	+	+	+	15 g	ReaShure	-25 to 80	369
Elek et al., 2008	-	+	+	+	25/50 g pre/post	Norcol-25	-21 to 60	32
Ardalan et al., 2010	+	+	/	/	14 g	Col 24	-28 to 70	40
Zom et al., 2011	+	-	+	-	15 g	ReaShure	-21 to 42	38
Leiva et al., 2015	-	-	+	-	9.4/18.8 g pre/post	CholiPearl	-21 to 45	23
Zhou et al., 2016	-	-	-	-	15	ReaShure	-21 to 30	81
Sun et al., 2016	+	+	+	+	15	ReaShure	-21 to 21	48

## IMMUNONUTRITIONAL ROLE OF METHYL DONORS

In addition to being considered one of the most-limiting AA for milk production, MET and several of its metabolites display an immunonutritional role, i.e. they help support and boost certain activities of the immune system in humans (Grimble, 2006; Li et al., 2007). Since these properties have been tested on immune-suppressed human subjects with positive outcomes (Van Brummelen and du Toit, 2007), we hypothesize that enhancing MET supply would have a positive effect on immune function in the transition period, where cows seem to be in an immuno-compromised state. A study with mid-lactation cows reported that supplementation with 30 g/d of rumen-protected MET compared with 0 or 15 g/d led to greater T lymphocyte proliferation in vitro in response to various mitogens (Soder and Holden, 1999). Since human lymphocytes seem to have an absolute requirement for MET to proliferate (Hall et al., 1986), these results were not unexpected.

In one of our previous studies (Osorio et al., 2013) where transition cows were fed either Smartamine (0.07 %DM) or Metasmart (0.19 %DM) from -21 to +30 days relative to parturition, we observed increased phagocytosis (pathogen killing ability) in neutrophils, the cells that make up the first line of defense in the animal immunity. In a follow-up study feeding Smartamine (0.08 %DM) between -21 and 30 days relative to parturition (Zhou et al., 2016a) we detected both greater in vitro blood neutrophil phagocytosis and oxidative burst (another pathogen-killing mechanism) from day 1 post-calving through day 28 postpartum. Furthermore, supplementation with rumen-protected MET optimized the response to lipopolysaccharide (LPS) (a component of bacteria cell walls), by controlling the inflammatory ability of the immune cells (Vailati-Riboni et al., unpublished). This is very relevant during the transition period, as cows might mount an excessive inflammatory response to pathogens, creating more damage than benefit (Jahan et al., 2015). One possible mechanism could be related to the ability of MET to influence the oxidative status of periparturient cows as it is a precursor for glutathione and taurine (Atmaca, 2004). Through its chloramine metabolites, taurine has a well-known immuno-modulatory capacity (Schuller-Levis and Park, 2004).

Despite the interconnection between MET and CHOL (via the one-carbon metabolism), there is a paucity of data on CHOL and the bovine immune response. Immune cells lack the ability to convert CHOL into MET through the betaine pathway, which in bovine is present only in liver and kidney (Lambert et al., 2002). In a recent study (Zhou et al., 2016a), compared with feeding MET feeding 60 g/d of Reashure (rumen protected CHOL) from -21 to 30 days in milk had no effect on immune cell killing capacity of neutrophils and monocytes (another cell type of the animal immune response). However, there are data generated using other animal models. For example, supplementation of choline in the diet improved immune indices in both fish (Wu et al., 2013) and suckling rats (Lewis et al., 2016). Authors did not speculate on the mode of action, but most probably CHOL efficacy is mediated by betaine (a choline derivate). Data from broilers revealed that dietary betaine supplementation improved intestinal health, and induced a boost in the intestinal immune response to a coccidiosis challenge (Klasing et al., 2002).

## METHIONINE AND LIVER FUNCTION: BALANCE BETWEEN INFLAMMATION AND OXIDATIVE STRESS

The periparturient inflammatory response is characterized by an increase in the hepatic production of positive acute-phase proteins (posAPP), such as haptoglobin and serum amyloid A (SAA), and a concomitant decrease in the production of negative APP (negAPP) such as albumin (Bertoni et al., 2008). At the level of liver, the well-established triggers of these responses are the pro-inflammatory cytokines IL-6, IL-1 $\beta$ , and TNF- $\alpha$  (Kindt et al., 2007). In contrast, oxidative stress is driven by the imbalance between the production of reactive oxygen metabolites (ROM) and the neutralizing capacity of antioxidant mechanisms in tissues and in blood. Pro-inflammatory cytokines have also been identified as a cause of oxidative stress, hence, linking the two conditions (Sordillo and Mavangira, 2014).

Both inflammation and oxidative stress reduce liver function in periparturient dairy cows (Bionaz et al., 2007; Trevisi et al., 2012). Using changes in plasma concentrations of albumin, cholesterol, and bilirubin, Bertoni and Trevisi (2013) developed the liver functionality index (LFI), which characterizes the extent of the inflammatory response and helps predict consequences on health and well-being of the cow. For instance, a low LFI value is indicative of a pronounced inflammatory response, suggestive of a more difficult transition from gestation to lactation, whereas a high LFI is suggestive of a smooth transition. In our experiments, supplementation of MET consistently increased blood albumin (Osorio et al., 2014; Zhou et al., 2016a) well above the concentration range in cows with adequate postpartum liver function (Bionaz et al., 2007). Furthermore, supplementation with rumen-protected MET decreased the concentrations of inflammation-related biomarkers such as ceruloplasmin, haptoglobin, IL-1 $\beta$ , and SAA (Osorio et al., 2014; Zhou et al., 2016a). MET supplementation increased liver glutathione and antioxidant capacity (Osorio et al., 2014; Zhou et al., 2016a). Using the cow data from the Zhou et al. experiment, compared with 35% of the cows without MET supplementation ending-up in the Low LFI group, only 10% of the MET-supplemented cows ended-up in the Low LFI group, hence, supporting the existence of a positive effect of MET supplementation on liver function.

## THE LINK BETWEEN INTAKE AND HEALTH

The transient inflammatory-like status around parturition appears to be a “normal” aspect of the adaptations to lactation (Bradford et al., 2015), with its positive or negative impact depending on its degree. Cows that approach parturition with a greater (but still subclinical) level of circulating cytokines have greater inflammation and oxidative stress, and lower liver function often through 30 days in milk together with lower milk yield and lower postpartum DMI (Bertoni et al., 2008; Trevisi et al., 2015). In addition to their fundamental function in immunity, cytokines (ILs), interferons (IFNs) and TNF- $\alpha$  also elicit pathophysiological effects. This leads to what is commonly known as “sickness behavior”, whose primary manifestation is satiety. Similar to how cows react during an inflammatory state around parturition, the reduction in DMI around calving is an example of this behavior. In mice, these cytokines have been shown to reduce meal

size and duration, as well as decrease meal frequency and prolong inter-meal intervals (Plata-Salaman, 1995). Furthermore, cytokines directly affect the hypothalamus; IL-1 $\beta$  and IFN act directly and specifically on the glucose-sensitive neurons in the brain "satiety" and "hunger" sites (Plata-Salaman, 1995). Thus, the increased DMI observed when feeding rumen-protected MET (Smartamine or Metasmart) can be partly explained by a reduction in inflammation, as it directly (at the hepatic level and by dampening the immune cell overresponse) and indirectly (reducing oxidative stress) decreases circulating pro-inflammatory cytokines.

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