Making Milk Protein: The Single Most Important Ingredient is EVERYTHING

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Summary

The manner in which lactating dairy cows react to additional protein in the diet is certainly at least partly dependent on the amino acid (**AA**) proportions in that protein. The commonly held model follows the "uneven barrel staves" analogy of one clearly most limiting AA in the metabolizable protein (**MP**). In fact, this model is clearly an oversimplification. Microbial protein production in the rumen responds to AA balance in the rumen degraded protein and does not always follow the simple single limiting AA model. Within the cow, the biological drive to produce milk protein is separable from the requirement for the most limiting AA needed to sustain that drive. This protein secretion drive is dependent on energy, endocrine, and even non-limiting AA facets of feeding. The presence of nearly co-limiting AA, variance among diets, and variation among cows, makes interpretation of experimental data based only on the single limiting AA model a bad idea. Since even well designed experiments do not, and maybe cannot, include all necessary treatment combinations, other possible reasons for milk protein yield responses should be considered unless they are eliminated by the experimental design.

Introduction

This talk is a product of the American Dairy Science Associations 27th Discover Conference (https://www.adsa.org/Meetings/DiscoverConference.aspx) which was the second conference in that series that focused on AA in dairy cattle feeding. There were many worthwhile topics covered in that conference by expert speakers, but many of the talks may have been heard through this filter: that dairy cow milk protein yield responses are explained by the ability to meet the required delivery of the most limiting essential AA in the MP flow. This model is shown in Figure 1. This model has been applied successfully in monogastric meat animals and in experimental rodent models. By providing the most limiting AA (methionine in Figure 1, typical of chickens fed corn-soy diets) the dietary protein can be lowered. Thus, the methionine stave is lengthened while the others are shortened. This allows optimal rate of gain and protein production efficiency by feeding less crude protein (**CP**), but with a better biological value. After dealing with the most limiting AA, the concept can next be extended to a second limiting AA (lysine in Figure 1), etc. An important implication of this model is that the animal will not respond to addition of any secondary limiting AA unless the first limiting AA is dealt with first. Also if 2 AA are exactly equally co-limiting, the animal can only respond to both but neither fed alone. I think this model is useful and important, but I think assuming it explains all responses is a bad idea if it excludes knowing other possible explanations or even other experimental scenarios to test. I was

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asked to summarize the Discover conference for a group in Australia, and that talk led to this invitation. While I did work in AA and proteins early in my career, and somewhat thereafter, in this paper, I draw heavily on the talks presented at this Discover conference by Jeff Firkins, Mark Hannigan and Alex Hristov. They are not co-authors of this paper, and any opinions expressed or mistakes are on me, but much of what is written here was based on my interpretation of what they presented.

Protein and Energy Feeding

Any separation between protein and energy in ruminant feeding is a tenuous separation at best. Energy characteristics of the diet drive microbial protein yield in a tightly coupled process. Part of this linkage is shown in Figure 2.Without deriving energy from fermentable carbohydrates, microbes cannot reproduce. They must reproduce in order to provide a daily output of microbial protein to the small intestine, an important part of the MP for the cow. But Figure 2 should not be interpreted only as energy driving MP yield. If microbial production is limited by some deficiency of the quantity, or quality, of the rumen degradable protein (**RDP**), then carbohydrate fermentation can suffer as the rumen microbial population fails to maintain itself. This reduction in carbohydrate disappearance reduces volatile fatty acid absorption for the host cow and can limit carbohydrate disappearance in the rumen. The latter may enhance fill and limit intake. So the 2 processes are completely interdependent in the rumen. As we shall see, they are interdependent in the host animal metabolism as well.

Energy characteristics of the diet effect response to dietary protein in other ways as well. Changing dietary energy source can increase or decrease intake, which then changes intake of protein at any fixed protein concentration in the diet. Experiments with cows are almost always with ad libitum intake, so this effect can never be ignored. As intake increases, rate of passage increases, and this increases the proportion of dietary protein that is rumen undegraded if the degradation rate stays the same. Energy intake and nature of dietary energy also affects milk protein yield and drive to produce milk protein, which will be partly discussed later. The flip side of this is that increasing dietary protein concentration can influence energy nutrition of the cow. Increased dietary protein concentration may increase intake which affects energy balance and also further increases AA intake.

Microbial Protein

Microbial protein makes up about half of the MP available to the lactating cow. It is relatively constant in its AA make up. Based on the proportions of AA in this protein, it looks like rumen bacteria is a good match for milk protein, at least in methionine and lysine (Figure 3). It is similarly a pretty good match in histidine, which has about the same concentration as methionine in both milk and rumen bacteria. However, the reader must be aware that this is only true if the efficiency of converting methionine to milk protein is exactly the same as for lysine and other AA. Not shown in Figure 3 is the histidine content of milk and bacteria. These concentrations in milk are similar to methionine, although His in bacteria may be slightly lower (Volden and Harstad, 1998). Histidine in feeds ranges from 1.7 to 3.2% of CP.

Because the AA pattern of microbes differs from that in the feed, rumen undegraded protein (**RUP**), and is fairly constant and of good quality, maximizing microbial growth is important. Figure 4 shows that microbial population growth rate can respond to differences in AA. In this in vitro experiment, microbial growth was clearly stimulated by adding all

20 AA compared to only using ammonia. This shows that the AA content of the RDP is important. What is more interesting is that removing leucine and valine from this mix of AA reduces microbial growth. That would suggest that one or both of these is limiting for this population if we want to apply the barrel to a mixed population of microbes. However, if we now remove the third branched chain AA, isoleucine, we restore most of the lost growth! And even more interesting is the same thing happens with the 2 aromatic AA, tyrosine and phenylalanine. Removing tyrosine reduced growth and then additionally removing phenylalanine restored it. These are examples of an imbalance or antagonism, and is likely due to competition among microbial strains, but it definitely does not fit the concept of a single limiting AA. Also, note that this relates to the ideal composition of the AA content of the RDP, not the RUP. Granted these are extreme changes, the RDP will never be totally devoid of any AA as is done in these in vitro experiments. Still, it shows the potential influence of AA in the RDP, and these AA are not methionine, lysine, and histidine, which are commonly the ones that are thought to be limiting in RUP.

Efficiency and Co-Limiting Amino Acids

The efficiency with which an AA is converted to the same AA in milk protein cannot be a biological constant. Amino acids are subject to an obligatory waste.This is dependent on absorption, but if we consider only MP which by definition is absorbed, there is almost certainly some minimal catabolism of AA post-absorptively which cannot be avoided. It is likely that as the absolute requirement is approached, this catabolism may increase. Even if this is not true in an individual cow, it will almost certainly look this way in a group of cows. What is of most consequence is that once we exceed the requirement, then we induce inefficiency (Figure 5). Therefore, anytime the efficiency of use of an AA is calculated from a study, we must consider where we are in the supplementation range (Figure 6). If a second limiting AA is closely co-limiting, adding the first will result in reduced efficiency of this first limiting AA.

Figure 7 is a summary I did quite a while ago when the main supplements for AA where methionine is alone or supplemented with lysine. Therefore, studies were done as either methionine supplemented or both, but not lysine without methionine. Note there is a small response in protein concentration to supplemental methionine alone, and this small response was significant; therefore, it must have been pretty consistent across studies. The response to both methionine and lysine is clearly larger. Does this mean methionine is consistently first limiting and lysine is very close? This would mean that even small amounts of added methionine cross over to lysine becoming first limiting. This is the only logical interpretation under the barrel model, but is it correct?

Although methionine and lysine have received the most attention, it is clear that histidine is also important. Figure 8 shows a response fo histidine alone, with not additional statistical response to additional methionine or lysine on top. There may be some indication of a response with methionine added. According to the barrel model, this means histidine is first limiting, and maybe methionine is second limiting and lysine is not important. However, this study did not test the effects of either methionine or lysine alone. By the barrel model, we assume the cows would not have responded, but we do not know this for sure from the data.

Figure 9 shows the response to histidine, methionine and lysine alone and separately. Is there a small response to each of the AA that

then sums up? If each is exactly co-limiting, there should be no response to any of them alone. Maybe some cows are first limiting in methionine, others in histidine and still others in lysine. If they are very closely co-limiting in the diet, that kind of cow-to-cow variation would not be surprising. The strategy in this and many other more recent AA studies is to add AA as a replacement for dietary protein. This study, which is a very well designed study with already many treatments and controls, does not answer the question of what would have happened if these AA were added to the high MP diet. The barrel model would suggest we would not get an increase as long as the high MP was high enough, but we do not know that from these data.

Figure 10 diagrams a problem with studies where a high protein diet is compared to a low protein diet with one or more added AA. These kind of studies should really be run as a factorial if they want to prove that the AA addition substitutes for the added protein. Just comparing a high protein diet to a low protein diet plus AA and seeing no difference proves very little. At least a low protein diet should be included (as in the study in Figure 9). But even then, if we want to say the added AA allowed us to lower protein with no loss in production, a fourth diet high in protein with the same AA should be tested. In other words, we want to know if there is an interaction (non-parallel lines) that show the added protein removes the AA response (and vice versa) or if there is simply a response to both protein and AA. An example of 2 studies which actually applied this type of factorial is shown in Figure 11. Neither study follows the barrel hypothesis showing that adding AA lowers the protein required for maximum production, actually both show a positive response to added methionine on the higher protein diets. Granted the response to methionine on the low protein diets was negative, which is certainly not the typical response, but the point is many studies never measure the interaction and the evidence for the interaction (which would occur under the barrel model) is simply lacking.

If Not the Barrel, What Else?

Molecular biologists like to give nicknames to molecules that regulate cell behavior. One of these is a protein called mTOR. When it is phosphorylated, it is activated. When it is activated, it increases milk protein synthesis. Figure 12 shows that when mammary cells are incubated in vitro with either insulin or essential AA, then mTOR is activated. The effect seems additive. Let us compare that to what we know added protein and insulin do to a live cow as shown in Figure 13. Here we see that adding both insulin and infusing casein into the cow each increase milk protein synthesis independently. Note that if the basal diet was limiting milk protein yield by starving the mammary gland of AA, it is difficult to explain the insulin response. What is most interesting in these data is there is synergy (shown by the significant interaction) so that together insulin and casein really increase milk protein synthesis more than can be explained by adding their effects. Presumable, insulin preps the mammary gland to make more milk protein (likely at least partly due to mTOR), and at the same time, the added casein amplifies this mTOR effect while providing any needed added AA to actually make the protein. What is perhaps most interesting is that while a mixture of essential AA quadruples mTOR, the effect is reduced by omitting AA like leucine, arginine, and isoleucine (Figure 14). All this suggests that poor patterns of AA may not just limit milk protein production by depriving it of needed AA in the protein assembly process, but that the balance of AA, including one not 'limiting', can be part of the stimulatory messaging that promotes more milk protein secretion.

The mTOR story is intriguing. The complete biology of this almost certainly includes other mechanisms that can explain responses "outside the barrel". It is important to keep our minds open about what causes responses of increased milk protein yield in cows. Many data sets that may "fit" the barrel model do not really firmly prove it and should be understood accordingly. A summary of the interacting factors discussed in this paper are shown in Figure 15.

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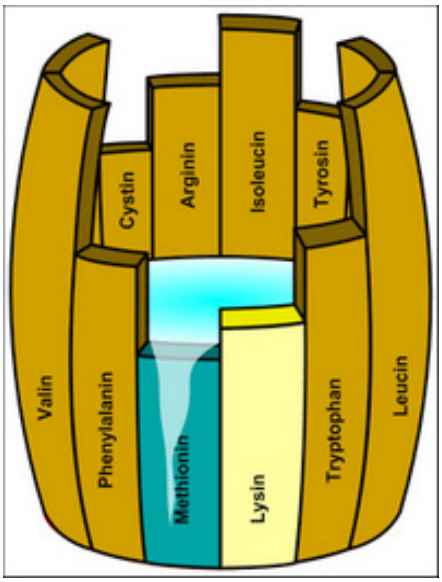

Figure 1. Classic description of amino acid balance. Amino acid levels (represented by stave length) is expressed as fraction of the requirement, not in absolute concentration in the diet.

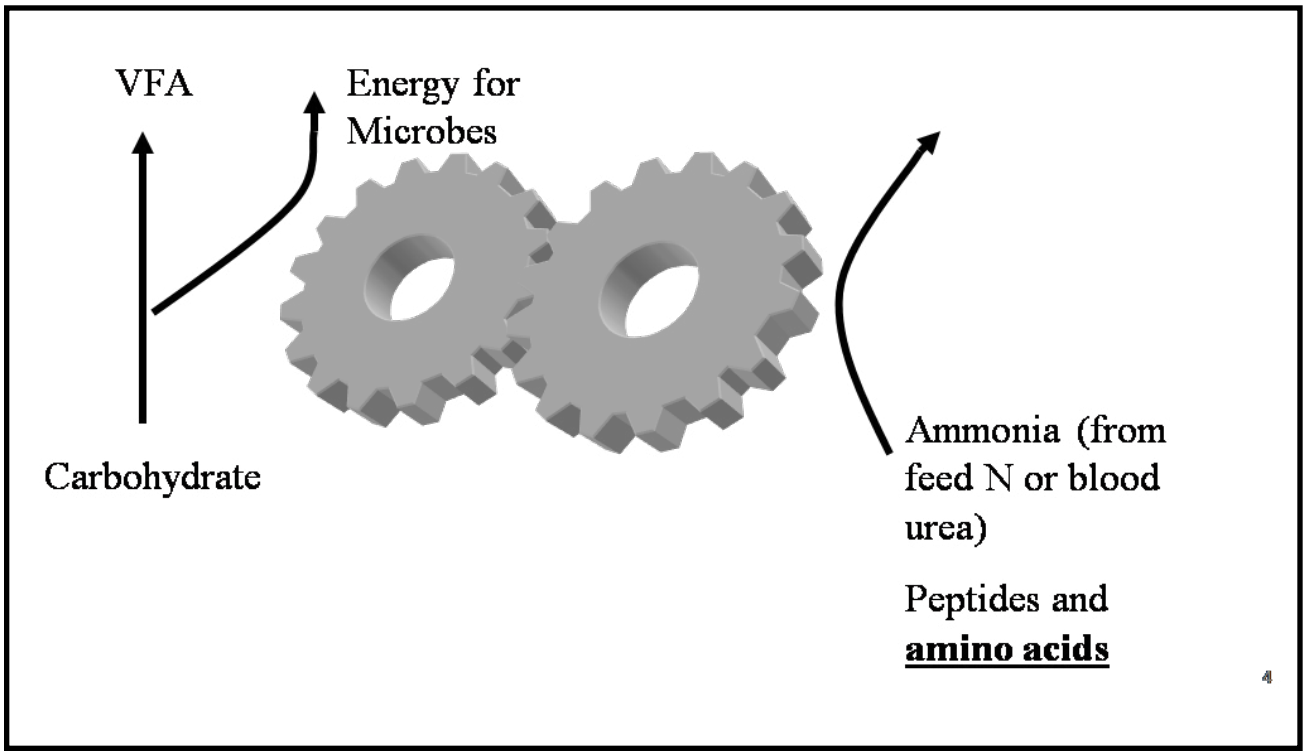

Figure 2. Microbial energy, mostly derived from fermentation of carbohydrate, drives microbial reproduction which is microbial protein yield. Microbial reproduction can also be limited by the amount or nature of the rumen degraded protein (**RDP**) in the diet. If the microbial population replacement is limited by the dietary RDP, energy fermentation, production of volatile fatty acids for the host cow, and degradation of dietary carbohydrate is reduced as well (**VFA** = volatile fatty acids).

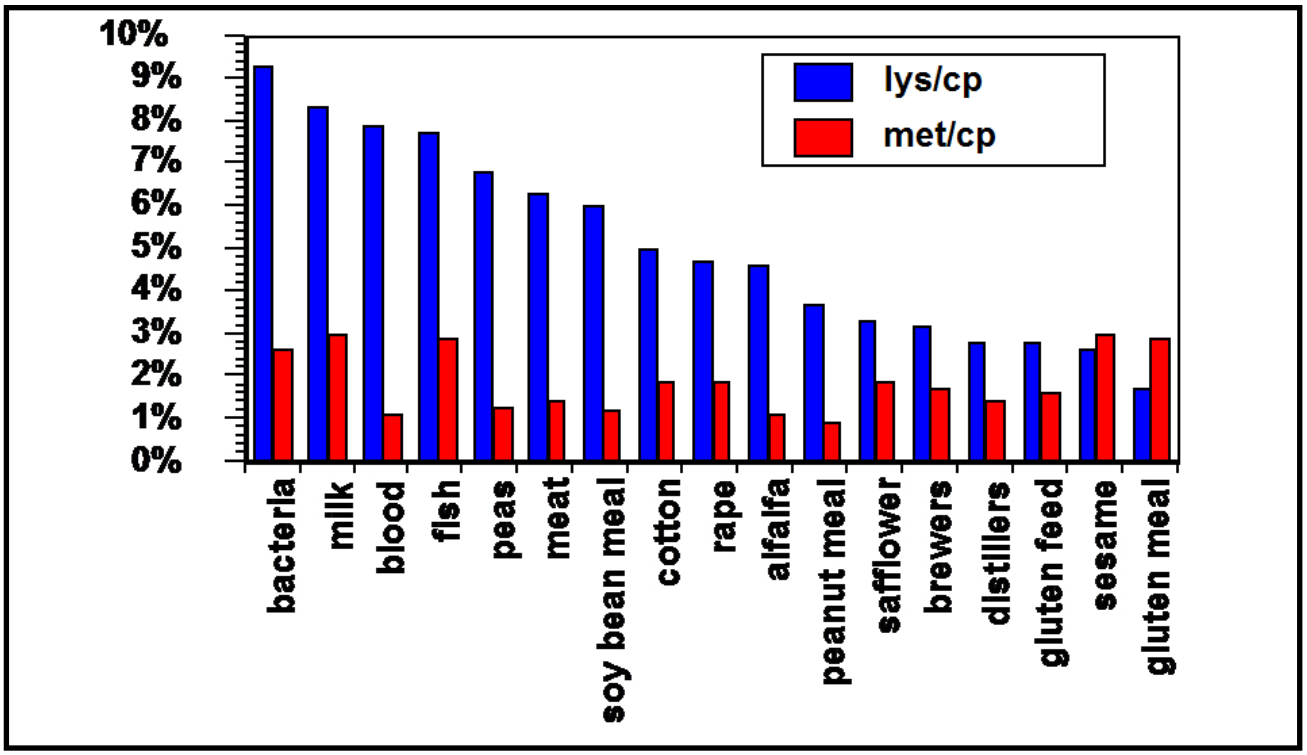

Figure 3. Content of methionine (**met**) and lysine (**lys**) in milk, rumen bacteria, and various feed sources. If lysine and methionine are used with identical efficiency to make milk protein, then rumen bacteria are a pretty good source for milk protein , but with methionine more limiting than lysine. There is no reason to assume the efficiency of utilization is the same as pathways of catabolism are quite separate.

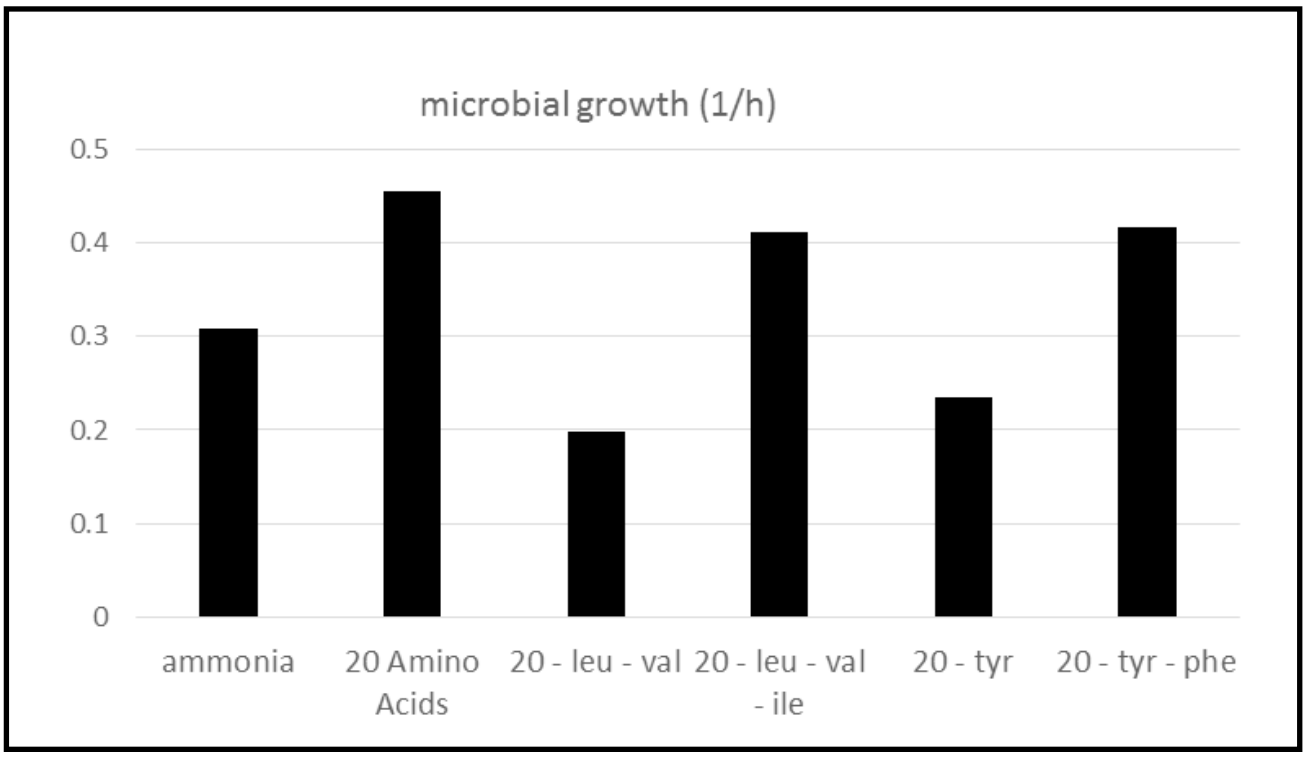

Figure 4. Response of a mixed rumen microbial population to sequential subtraction of amino acids from the media. (Kajikawa et al., 2005)

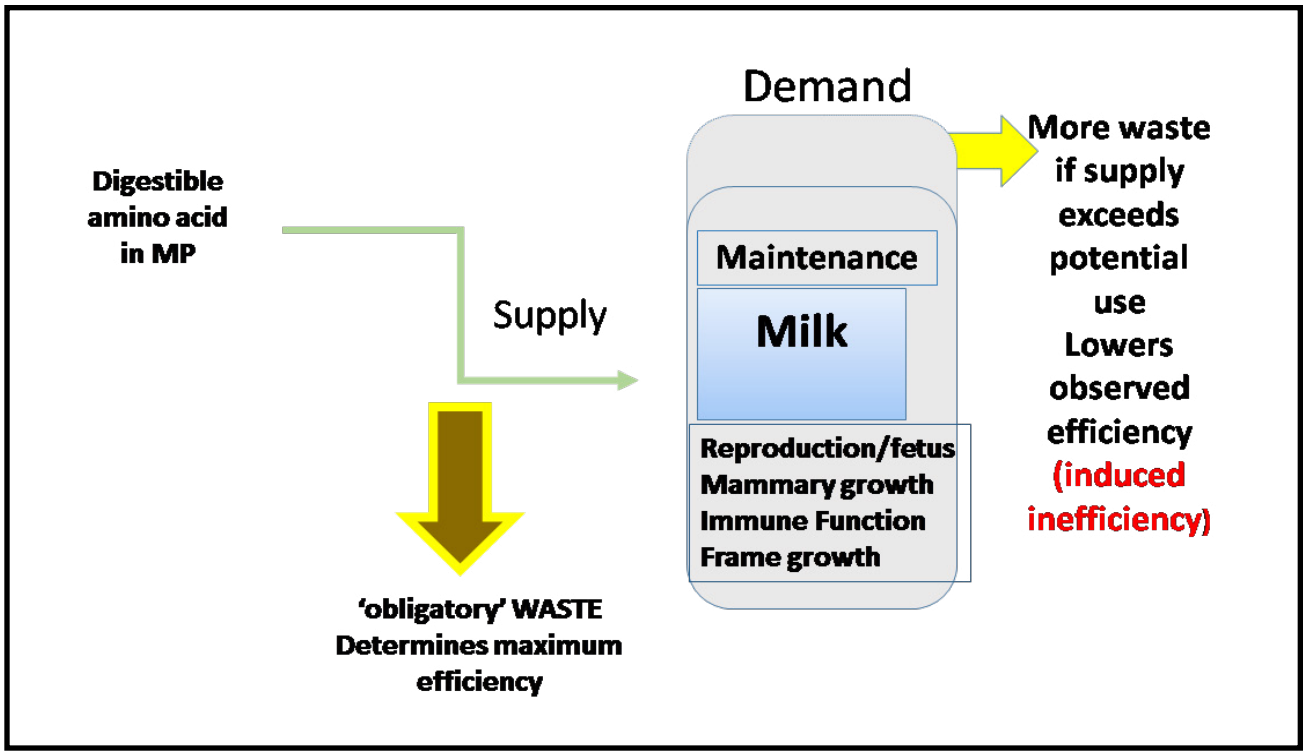

Figure 5. Amino acids are subject to obligatory and induced inefficiency. If amino acid supply is elevated above the potential milk secretion rate, inneficiancy is induced. Likewise if milk yield potential is reduced by some other cause, amino acid inefficiency of conversion to milk protein will also be induced (**MP** = metabolizable protein).

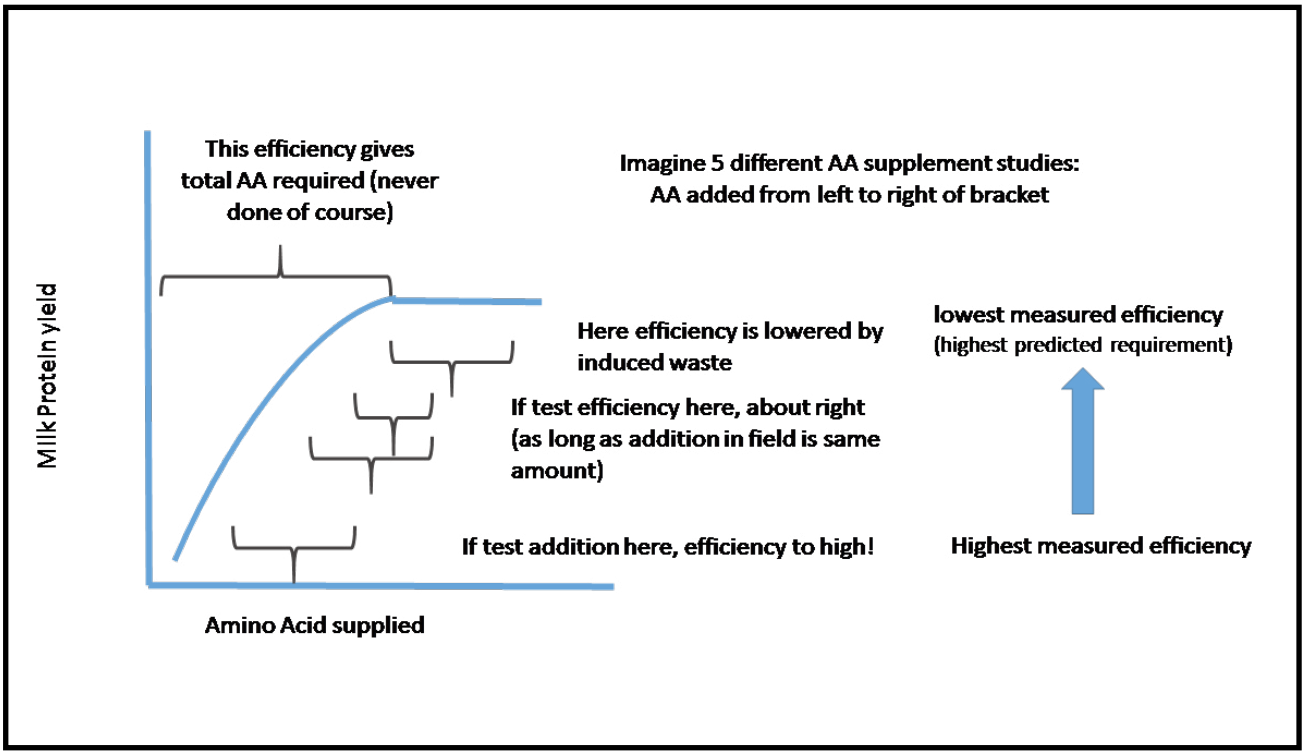

Figure 6. The efficiency with which an amino acid or total protein is used is dependent on the range over which it is fed and the overall maximal potential for milk yield (**AA** = amino acids).

	n	DMI	MYIE	PYIE	PPER	FYIE	FPER
		kg/d		g/d	%	g/d	%
Control	34	22.4	34.5	1033	2.99	1201	3.57
Methionine	60	22.3	34.3	1045	3.04	1214	3.61
SEM		.7	1.6	49	.04	60	.15
*P*value		.65	.28	.04	<.0001	.20	.03

	n	DMI	MYIE	PYIE	PPER	FYIE	FPER
		kg/d		g/d	%	g/d	%
Control	31	23.1	31.8	969	3.08	1116	3.56
Met + Lys	71	23.1	32.3	1011	3.16	1118	3.52
SEM		.8	1.5	41	.03	37	.08
*P*value		.99	.07	<.0001	<.0001	.87	.18

Figure 7. Response to only supplemental methionine or methionine plus lysine, for intake, milk yield (**MYIE**), protein yield (**PYIE**) and concentration (**PPER**), and fat yield (**FYIE**) and concentration (**FPER**). For a more recent review of different methionine supplement methods, see Zanton et al., 2014.

	basal	His	His Met	His Lys	His Met Lys
Milk (kg/day)	22.9*	23.6	23.7	24.2	23.7
Protein (g/day)	695*	721	728	717	729

*P < 0.05

Figure 8. Response to histidine (**His**), or histidine with added methionine (**Met**) and lysine (**Lys**). Note this makes a good case for His as the first limiting amino acid, with maybe methionine being second. But nowhere do we measure the effect of methionine and/or lysine alone (Vanhatlo et al., 1999).

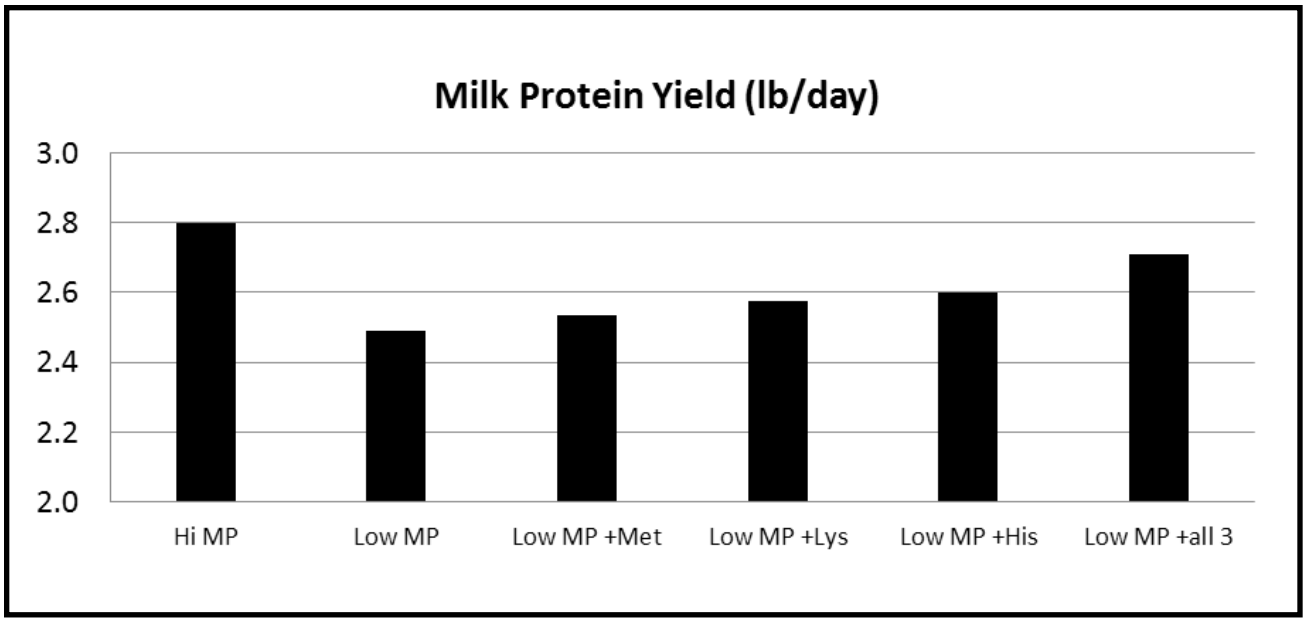

Figure 9. Response to histidine (**His**), methionine (**Met**), and lysine (**Lys**) separately and combined. This study tests each amino acid separately and only gets a significant response to the three combined. This study also has a low and high metabolizable protein diet to serve as negative and positive control. Note there is no test of added amino acids to the high MP diet (Giallongo et al., 2016) (**MP** = metabolizable protein).

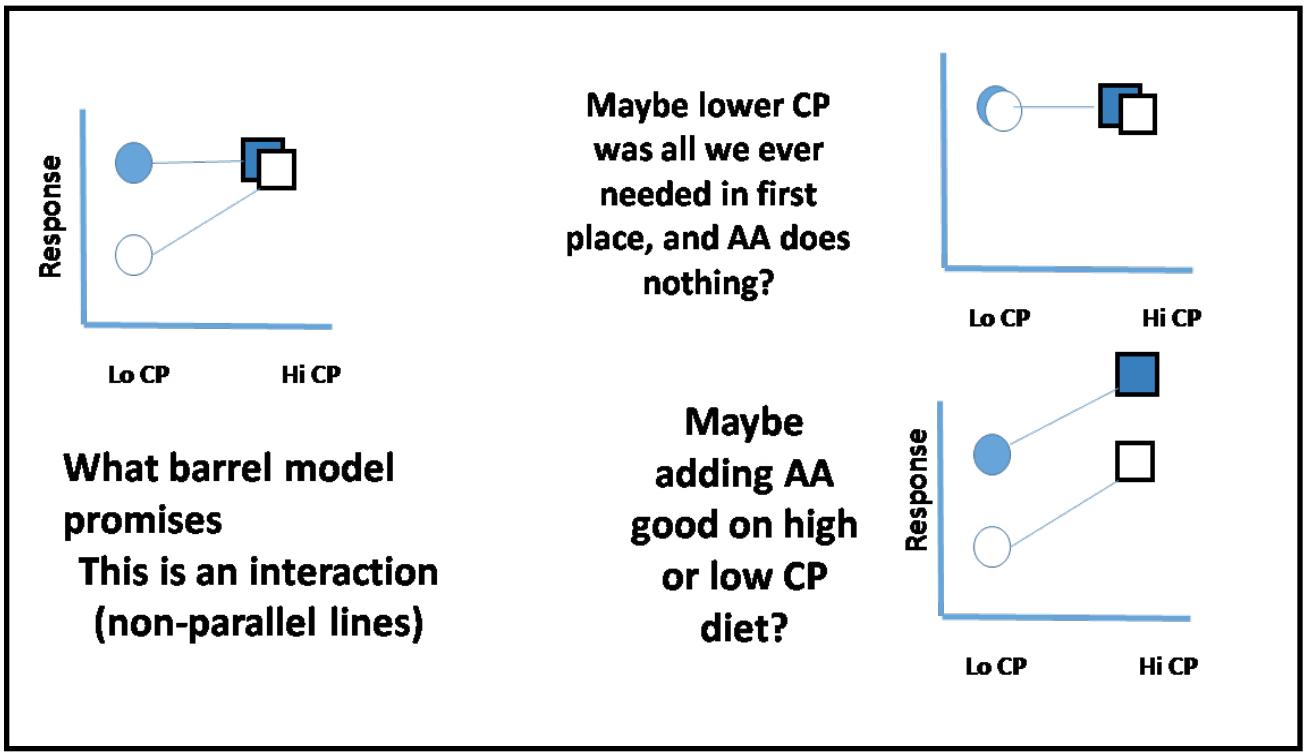

Figure 10. Open squares mean no amino acid (**AA**) added, closed means amino acid added. Studies often include the closed circle (low protein plus amino acid) and the open square (high protein with no amino acids). To really prove the barrel model can be used to safely lower dietary protein, the graph should look like the upper left response. The upper right response says any diet is ok, and low protein without amino acid is probably the cheapest diet to feed. The lower right figure says animals respond to both more protein and added amino acids.

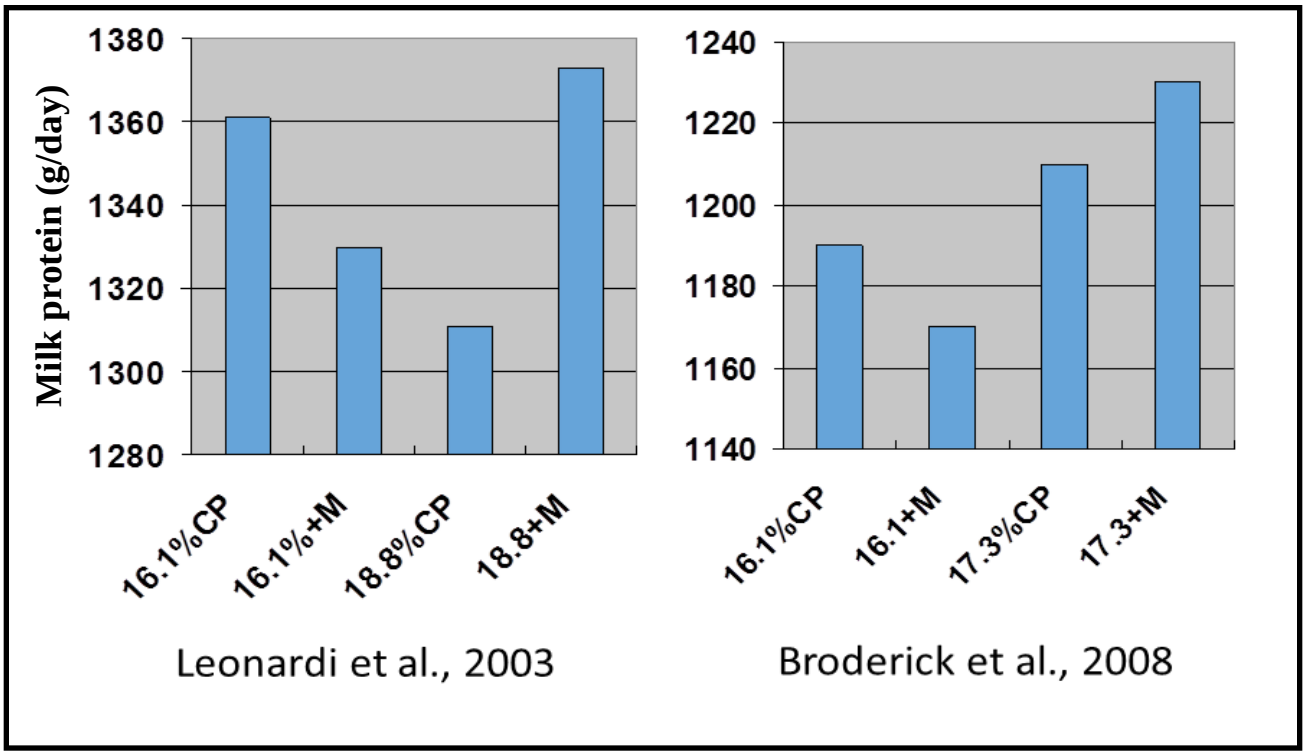

Figure 11. Studies that did factorial treatments of protein and amino acids. These studies actually only showed a response to methionine (**M**) on the high protein diets.

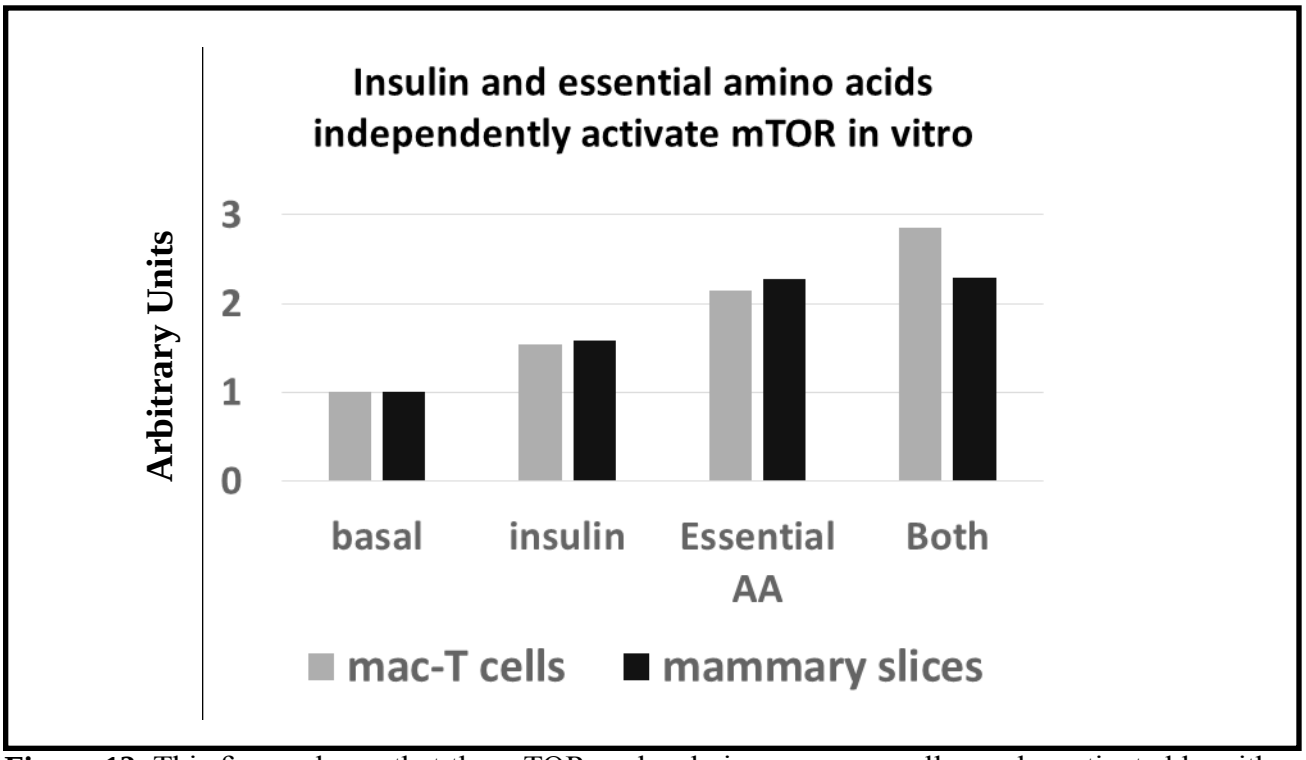

Figure 12. This figure shows that the mTOR molecule in mammary cells can be activated by either insulin or a mixture of essential amino acids (**AA**). Once activated (by phosphorylation), the mTOR protein should signal the mammary cells to make more protein (but protein synthesis not measured here) (Appuhamy et al., 2011).

	Control	Insulin	Casein	Both	P < 0.05
Insulin, ng/ml	1.5	6.3	1.6	7.3	insulin (I)
Milk, kg/day	26.3	27.0	28.6	30.5	casein (C)
Protein, kg/day	0.81	0.84	0.89	1.04	C, I, C*I

Figure 13. Insulin or abomasal casein increase milk protein secretion separately, but when combined, the effect is more than additive. This could be due to increased mTOR signaling by both and also increased supply of amino acids for protein building blocks (Griinari et al., 1997).

In vitro, direct effect of essential amino acid removal on mTOR

Note: Essential AA with biggest effects are not thought of as limiting

Treatment	mTOR
	Fold of +EAA
+EAA	1.00
−Lys	1.08
−Thr	0.88
−Phe	0.82
−Trp	0.73
−His	0.71
−Met	0.65
−Arg	0.51*
−Val	0.56
−Leu	0.53*
−Ile	0.43*
−EAA	0.24*
SEM	0.19
P value	0.01

Figure 14. Addition of a complete mixture of essential amino acids (**EAA**) quadruples mTOR activity in vitro in mammary cells, but omitting arginine, leucine, or isoleucine from this mixture reduces the mTOR stimulation. Note these are not usually thought to be limiting amino acids for cows, and probably aren't as building blocks for milk protein, but may help other signals that increase the potential for milk protein yield. (Appuhamy et al., 2012)

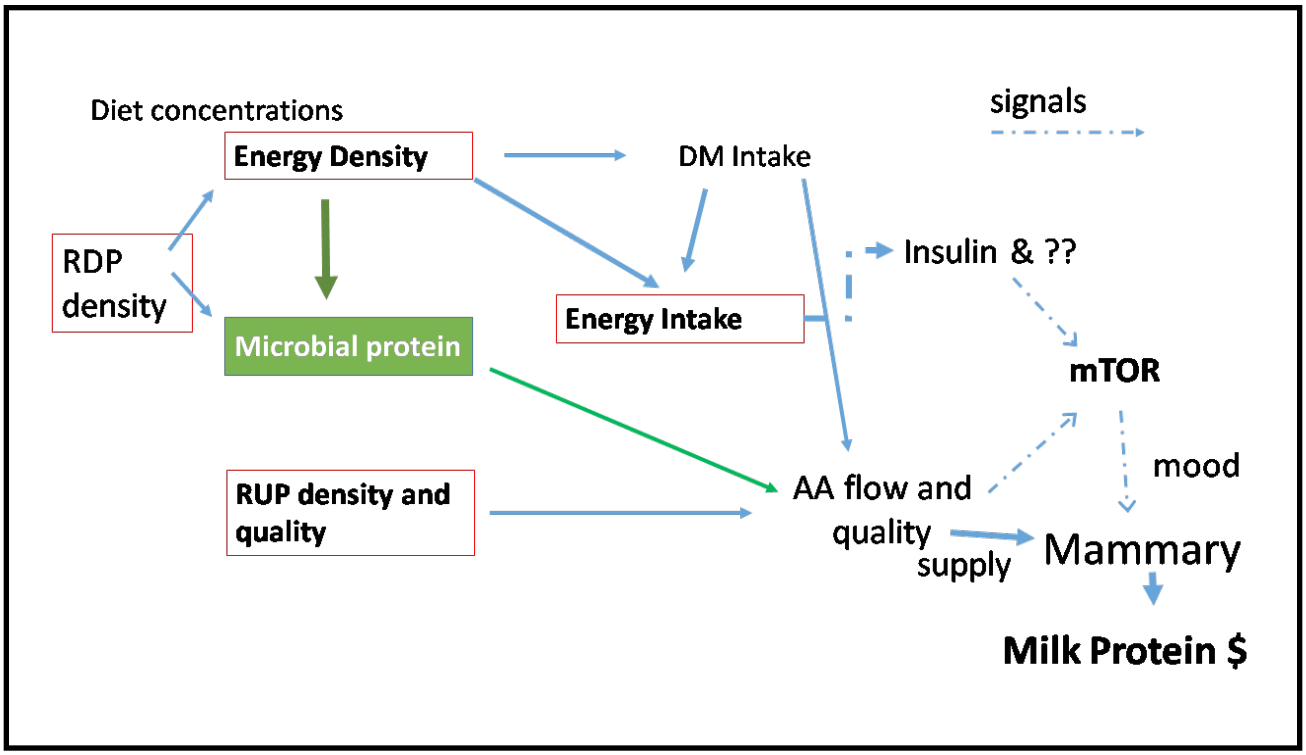

Figure 15. This figure shows how the interactions discussed in this paper tie together in a lactating cow. Supply of energy, amino acid supply and endocrine (insulin) and cellular control proteins (mTOR) play a part in getting the mammary gland in the "mood" to make milk protein while supplying the required energy and amino acid building blocks for protein synthesis (**RDP** = rumen degradable protein and **RUP** = rumen undegradable protein).