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**Omasal, Reticular Sampling Techniques, and Dual-flow Continuous Culture System for
Assessing Ruminant Digestion, Nutrient, and Microbial Protein Flow out of the Rumen and
Canola Meal as a Protein Supplement for Lactating Dairy Cows**

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Background:

This paper is divided in three components: 1) Comparing the omasal and the reticular sampling techniques; 2) Elaborating on the dual-flow continuous culture system; and 3) Summarizing recent studies on canola meal as the main protein supplement for lactating dairy cows. The goal is to illustrate some peculiarities among these three different methodologies and to point out recent findings on canola meal research.

Introduction:

Ruminant animals, also known as pre-gastric fermenters, have their stomach divided in four compartments: rumen, reticulum, omasum, and abomasum. The rumen and reticulum harbor a large symbiotic microbial population that intensively ferments dietary feed components, that is then passed on to the omasum. The omasum is a major site of water absorption, and much less fermentation occurs because omasal feed retention time is substantially shorter compared to the rumen-reticulo compartment. After passing through the omasum, the modified feed components

and the ruminal microbial population are delivered to the abomasum, the true stomach in which acidic and animal enzymatic digestion starts.

In order to quantify the nutrients that are available for absorption in the small intestines it is crucial to understand what happens to nutrients prior to reaching that point. The challenge with ruminant animals, as opposed to non-ruminants, is that the rumen-reticulo microbial population greatly changes the chemical and physical characteristics of feed; therefore, the nutrients available for absorption are critically different from dietary nutrients. Over the years, several techniques have attempted to quantify the fermentation process that occurs in the rumen. In this article I am going to focus on two of these techniques; omasal and reticular sampling techniques.

Omasal sampling technique:

In order to assess ruminal digestion and ruminal microbial activity, ruminant nutrition studies have been extensively used abomasal and duodenal digesta collected from cannulated animals. According to Huhtanen et al. (1997), three major problems may be associated with these techniques: 1) abomasal secretions may interfere with ruminal digestion measurements, 2) abomasal and duodenal surgical procedures are more elaborate and require longer animal recovery times than ruminal cannulation, and 3) cannulas at the abomasum and duodenum demand extensive maintenance.

Attempts at collecting omasal digesta are not new (Ash, 1962; Engelhardt and Hauffe, 1975; Rupp et al., 1994). However as pointed out by Huhtanen et al. (1997), these procedures required either omasal cannulation or collection of digesta passing the omaso-abomasal orifice via a sleeve that was secured to the orifice and exteriorized through an abomasal cannula, which made these procedures even more involved than abomasal or duodenal cannulation, and thus have had limited success.

Huhtanen et al. (1997) tested the feasibility of inserting a sampling device into the omasum for up to 3 weeks via the ruminal cannula for the purpose of sampling digesta leaving the reticulo-rumen. The authors concluded that omasal sampling could be useful for partitioning digestion between the rumen and the lower gastrointestinal tract. Moreover, they observed that the composition of omasal digesta samples was biased when a single marker was used; however, using a double-marker method resulted in fairly small coefficients of variation for measurements of ruminal digestion variables.

Ahvenjärvi et al. (2000) further presented another two advantages of omasal sampling over post-ruminal sampling: 1) substantially less endogenous Nitrogen is secreted into the rumen than into the duodenum, and 2) since rumen microbes are not digested in the abomasum, digesta Nitrogen flow can be separated into particle- and liquid-associated bacteria, protozoa and soluble and insoluble dietary Nitrogen fractions. Ahvenjärvi et al. (2000) were the first to publish data comparing omasal versus duodenal sampling. In their study they used four lactating cows cannulated in the rumen and duodenum fed grass silage based diets. Moreover, to assess digesta flow, the authors used a triple-marker method based on Co-EDTA, Yb-acetate and indigestible neutral detergent fiber (iNDF). They observed that organic matter (OM) flow was significantly lower into the omasum than the duodenum, indicating an endogenous organic matter secretion into the abomasum. Furthermore, they reported that neutral detergent fiber (NDF), acid detergent fiber (ADF), and minerals flows were significantly higher into the omasum, indicating fiber digestion and mineral absorption in the omasum. The authors concluded that omasal sampling could be a promising alternative to duodenal sampling as a means of investigating ruminal digestion in cattle. However, they pointed out that accuracy and precision of omasal flows are more dependent on marker techniques than duodenal flows that are typically based on a single marker method.

Ipharraguerre et al. (2007) further attempted to evaluate nutrient flow from omasal versus duodenal sampling. The authors used three ruminally and duodenally cannulated cows fed corn based diets. To assess digesta flow from the omasum, the authors used a triple-marker method based on Co-EDTA, Yb-chloride, and iNDF, whereas digesta flow at the duodenum was determined using Co-EDTA, Yb-chloride, and Cr_2O_3 individually as single markers. The authors observed great variation and biologically unrealistic data from duodenal flow. For example, NDF digested in the rumen ranged from 4.8 to 39.7% depending on marker used. In addition, starch digested in the rumen varied from -40.4 to 9.0% from samples collected in the duodenum. This may have happened due to several reasons, including: 1) small number of animals, 2) sampling errors, 3) duodenal cannula type, 4) errors associated with analytical marker determination, and 5) inaccurate marker methodology (that may have led to unrepresentative sample). The authors also observed issues with samples collected from the omasum. They reported a higher ruminal starch digestion [86% versus 48% from compiled data published by Reynolds et al. (1997)], higher NDF ruminal digestion, and higher microbial nonammonia-N (NAN). Reasons for these observations may be related to overestimation of small particles and fluid phase compared to large particles when omasal sampling was conducted. The work of Ipharraguerre et al. (2007) exemplifies the challenges of accurately quantifying digesta flow from either the omasum canal as well as from the duodenum. Adequate animal numbers, proper sample collection, precise and accurate analytical determinations, and sound marker methodology are essential components of microbial and nutrient flows determinations.

As discussed earlier, there are very limited direct comparisons between omasal versus abomasal/duodenal digesta. To address this, Huhtanen et al. (2010) used an extensive data set comprised of 32 studies [totaling 122 diets, obtained when feeding North American diets ($n = 36$) based on alfalfa silage, corn silage, and corn grain and North European diets ($n = 86$), comprising

grass silage supplemented with barley-based concentrates] to evaluate the accuracy and precision of the omasal sampling technique. This was done by investigating the relationships between ruminal and total digestion of NDF; among intake, apparent and true ruminal digestion of OM; and between omasal NAN flow and milk protein yield. The authors used a mixed model regression analysis with random study effect to evaluate these relationships. It is important to mention that in all studies included in this analysis digesta flow was quantified using a triple-marker method. The authors found that standard deviations of ruminal NDF and true OM digestibility were smaller than typically reported in duodenal sampling studies using only chromic oxide as a flow marker. They also reported that the relationship between total and ruminal NDF digestion was consistent with biologically expected results. Furthermore, according to the authors, the close relationship between omasal flow of NAN and milk protein yield provided further confidence in the accuracy and reliability of omasal flow measurements. One important explanation for the smaller prediction errors of the Huhtanen model, which indicates higher precision of the omasal sampling data when compared with published duodenal sampling data, is the marker technique used rather than sampling site per se.

In a companion publication, Broderick et al. (2010) used the same dataset to evaluate the precision and accuracy of the omasal sampling technique for quantifying ruminal-N metabolism and to assess the relationships between NAN flow at the omasal canal and milk protein yield. The authors used linear regressions to predict microbial-N flow to the omasum from intake of dry matter (DM), OM, or total digestible nutrients (TDN). These linear regressions indicated an average of 32 and 68% for rumen-undegraded protein (RUP) and rumen-degraded protein (RDP), respectively. Moreover, this analysis indicated that the NRC 2001 overestimates RUP flow on average by 22% and underestimates microbial-N flow on average by 26%. The authors reported that zero ruminal N-balance, which corresponds to omasal crude protein (CP) flow equal to CP

intake, was obtained at dietary CP and RDP concentrations of 147 and 106 g/kg of DM, corresponding to ruminal ammonia-N and milk urea-N concentrations of 7.1 and 8.3 mg/100 mL, respectively. They also found that efficiency of microbial-N synthesis increased with dry matter intake (DMI), and milk protein yield was positively correlated to the efficiency of microbial-N synthesis and measured RUP concentration. The results presented in this study clearly indicated that the omasal sampling technique can generate valuable estimates of RDP, RUP, and ruminal microbial protein supply in cattle.

The omasal sampling technique allows in vivo measurement of ruminal protein degradation and escape, microbial protein and NAN flow out of the rumen, ruminal digestion of NDF, starch, and OM, as well as fatty acid flow out of the rumen. Additionally, this technique is a viable alternative to duodenal sampling, which is more expensive, more invasive, and harmful to the animals. Because this technique can be used in a broad range of animals (I have personally conducted omasal sampling not only on dairy cows but also goats, sheep, and beef cattle), is less invasive, and allows direct in vivo determination of microbial growth, digestion, and flow of nutrients; expanded training of researchers and adoption of this technique will improve our understanding of ruminant nutritional physiology and contribute to currently used models such as the NRC.

At the end of this article you will find figures that illustrate the omasal sampling technique, note that some of the presented devices can also be used for ruminal, reticular, and duodenal sampling as well. These pictures were taken at the U.S. Dairy Forage Research Center and at the University of Wisconsin-Madison.

Reticular sampling technique:

Performing omasal sampling as suggested by Huhtanen et al. (1997) and Ahvenjärvi et al. (2000) requires insertion of a tube (omasal tube, Figures 1 and 2) via rumen cannula that reaches

the reticulo-omasal orifice (Figures 3, 4, and 5) and a vacuum pump (Figure 6 and 7) attached to this omasal tube that generates enough vacuum to draw omasal digesta from the omasum into a collecting flask. There are several variations of the omasal tube (with and without weight that keeps the tube in place, with different shapes, forms, and materials), there are also several variations of vacuum pump (notably with or without alternating vacuum and pressure that prevents clogging of the omasal tube). It is also worthwhile to mention that correct positioning of the omasal tube requires some experience and practice, especially when dealing with large animals (such as dairy cows) and agitated animals (such as younger animals and some beef-cattle breeds). Because of those requirements, attention has been given to reticular sampling as an alternative to omasal and duodenal sampling. An advantage of reticular sampling compared with omasal sampling is that the former does not require a vacuum pump for sample collection.

Hristov (2007) argued that the reticulum, as the organ propelling digesta through the reticulo-omasal orifice, may regulate the flow of nutrients to the lower digestive tract (Sissons et al., 1984; Sutherland, 1988). Hristov (2007) also argued that the composition of reticular digesta may be fairly consistent (Dardillat and Baumont, 1992), and that particles found in the reticulum are likely to leave the reticulo-rumen (McBride et al., 1984) as opposed to return to the rumen. Therefore, Hristov (2007) first proposed that the reticulum digesta could represent digesta leaving the reticulo-rumen compartment and thus be used to estimate ruminal digestion, and microbial protein flow out of the rumen.

Hristov (2007) evaluated reticular, duodenal, and ruminal digesta using four cows in a 4×4 Latin square design experiment, in which the ruminal effects of four exogenous enzyme preparations were studied. Similarly to Ahvenjärvi et al. (2000), Hristov (2007) used a triple-marker method to estimate digesta flow and ^{15}N enriched $(\text{NH}_4)_2\text{SO}_4$ as a microbial marker. The author found that ruminal outflow of DM and OM was greater (by 17 and 28%) and that of NDF

was lower (by 14%) when estimated from duodenal sampling compared with reticular sampling. The author also reported no differences in the estimated flow of starch, NAN, and microbial-N between the reticular and duodenal sampling. Moreover, microbial-N flow estimated based on ruminal sampling was similar to those based on duodenal and reticular sampling. However, the ruminal technique grossly overestimated flow of DM, OM, and NDF. Hristov (2007) concluded that microbial protein outflow from the rumen may be estimated based on sampling of ruminal or reticular digesta, as opposed to duodenal sampling. Furthermore, the reticular sampling technique could also provide reliable estimates for ruminal digestibility of OM, N, and fiber fractions.

It is very important to take into consideration that this was a study with four animals and one single diet. Therefore, the findings presented by Hristov (2007) require further evaluations with more animals and with diets comprised of different ingredients and different nutritional composition.

Krizsan et al. (2010) conducted a study to evaluate how reticular and omasal sampling compare in the assessment of nutrients and microbial protein flows. The authors used six cows in an experimental design with 3×2 factorial arrangement of treatments, in which basal diets consisted of grass silage differing in NDF content (41 – 64% of the diet) supplemented with two levels of concentrate (5 or 9kg/d as fed). In this study the triple-marker method was used to estimate digesta flow, similarly to Ahvenjärvi et al. (2000), and ^{15}N enriched $(\text{NH}_4)_2\text{SO}_4$ was used as a microbial marker. The authors did not observe sampling site \times diet interactions. Also, ^{15}N enrichment of bacterial samples did not differ between sampling sites. Furthermore, reticular and omasal sampling generated similar estimates of marker concentrations in reconstituted digesta, estimates of ruminal flow, and ruminal digestibility values for DM, OM, starch, and N. However, Krizsan et al. (2010) reported that composition of the large particle phase differed between the two sampling sites, NDF concentration was 2.2% higher in the omasal digesta

compared with the reticular digesta. Consequently, ruminal NDF digestibility was 2.7% higher when estimated by reticular sampling compared with omasal sampling. The authors concluded that nutrient and microbial protein outflow from the rumen could be measured using reticular sampling, which could be a promising alternative to omasal sampling as it interfered less with the animals and did not require elaborate sampling equipment.

It is somewhat surprising that Krizsan et al. (2010) observed higher NDF concentration in omasal samples compared to reticular samples. It is also surprising that the authors' concluded that reticular sampling interfered less with the animals since omasal and reticular sampling were performed in the same schedule and no measurements were taken to assess interference level.

The dual-flow continuous culture system:

The dual-flow continuous culture system is an *in vitro* technique developed by Hoover *et al.* (1976) that simulates rumen digestion, in which different feeds and artificial saliva are mixed with fresh rumen fluid. In the modern version of this system (Soder *et al.*, 2013), temperature, anaerobiosis, and flow rates are tightly controlled. This technique has been widely used to evaluate the effect of complete diets and individual feed ingredients on ruminal digestion, fermentation, microbial protein synthesis, and nutrient flow (Hristov *et al.*, 2012). The advantages of this technique are: 1) ability to test a large number of treatments 2) ability to test high concentrations of one specific ingredient, 3) reduced experimental time, 4) reduced amount of total feed used, 5) reduced experimental animal use, and 6) lower cost when compared to *in vivo* experiments.

Typically the *in vitro* experiments can be conducted with four to ten fermenters in experiments with seven days of adaptation and stabilization and three days of sample collection. Fermenters are typically inoculated with mixed ruminal fluid and receive N₂ gas continually (to maintain anaerobiosis) at a rate of 40 mL/min and temperature is maintained at 39 °C.

Each fermenter is typically fed 60-80 g (on dry matter basis) in one or several (i.e. 12 portions) per day. Fermenters volumes can range from 1000 to 1250 mL. Solid and liquid passage rates can be adjusted daily and rate of passage can mimic different physiological states (i.e. 5 and 10% for solids and liquids, respectively). This can be adjusted by regulating buffer input and filtrate removal rates. During the sampling period (typically the last three days), fermenters' effluents are maintained at 2 °C using a water bath to stop microbial and enzymatic activities.

Typically multiple daily samples are collected and analyzed for DM, OM, NDF, ADF, EE, Ash, and bacterial populations. Individual minerals, amino acids, and fatty acids can also be analyzed. Other analyses may include pH (if not controlled), total and individual VFA, NH₃-N, bacterial purine determinations, and ¹⁵N enrichment.

Using canola meal as the main protein supplement for lactating dairy cows:

Historically, soybean meal has been the most common protein source in dairy cattle production. However, in recent years, increasing production of canola has given rise to greater availability of canola meal as a protein supplement for livestock feeding (Harker et al., 2012). Greater access to CM has made it a viable alternative to soybean meal for lactating dairy cows (Hickling, 2008).

Brito and Broderick (2007) observed a numeric increase in milk and protein yields when CM replaced equal supplemental protein from solvent-extracted SBM in 16.5% CP diets fed to dairy cows.

Faciola and Broderick (2013) found that replacing soybean meal with canola meal with different forage sources (alfalfa silage or corn silage) consistently increased yields of milk and true protein, and reduced MUN. In this study, it was observed that canola meal was as a more effective CP supplement than soybean meal when fed in alfalfa silage-based to lactating dairy

cows, and that relative response to canola meal declined as corn silage replaces alfalfa silage in the ration, possibly due to greater contribution of microbial protein as a proportion of MP when corn silage was fed.

Recent studies (Broderick et al. 2015; Faciola and Broderick 2014; Paula et al. 2015b) have shown that diet formulation using canola meal instead of soybean meal at the same level of dietary crude protein can increase milk yield and milk protein content and it can also increase nitrogen utilization, potentially lowering environmental nitrogen excretion. Causes for that may be canola meal's lower protein degradation rates and favorable amino acid profile.

Recent meta-analyses of results published in peer-reviewed journals showed greater DMI and yield of milk and milk components when CM substituted for several commonly fed proteins (Martineau et al., 2013). These meta-analyses reported that replacing SBM with CM significantly increased milk protein yield (Martineau et al., 2013) and increased intake and yield of milk and milk components (Huhtanen et al., 2011). Martineau et al., 2013, reviewing 49 different diets showed that canola meal improved milk yield by 0.64 kg per day and increased protein yield when compared to other protein ingredients fed at the same level.

Concentration of RUP in different canola meals may vary from 38-50% of CP (Broderick et al., 2014); however, preliminary studies indicated that this difference appears to not affect milk production and efficiency of N utilization in the rumen (Paula et al. 2015a, Paula et al. 2015b).

Amino acid supplementation (Lys and Met) does not appear to improve production when canola meal was fed to lactating dairy cows (Broderick et al. 2015, Broderick and Faciola 2014). However, this has not been tested extensively.

Overall conclusions:

Rumen outflow of different nutrients and microbial protein can be estimated using samples collected by reticular, omasal, and duodenal techniques.

Major limitations for reticular sampling technique are: 1) inadequate ruminal outflow sample representation, and 2) lack of more robust literature data.

Major limitations for omasal sampling technique are: 1) elaborate equipment needed for sample collection, and 2) difficulties during sample collection such as lack of personnel experience, animal size, and animal behavior.

Major limitations for duodenal sampling technique are 1) abomasal secretions contaminations, 2) more elaborate surgical procedures and longer animal recovery times, 3) delicate maintenance of duodenally-cannulated animals, and 4) since rumen microbes are digested in the abomasum, digesta Nitrogen flow cannot be separated into particle- and liquid-associated bacteria, protozoa and soluble and insoluble dietary Nitrogen fractions.

However, in every case, it is likely that adequate animal numbers, proper sample collection, precise and accurate analytical determinations, and sound marker methodology are more important than sampling site per se when determining ruminal nutrient digestion and ruminal microbial protein flow.

The dual-flow continuous culture system appears to be a useful tool to assess DM, OM, NDF, ADF, EE, AA, and FA digestions and flows, it can also quantify microbial growth, different microbial populations and ruminal fermentation parameters. This can be especially useful for screening forages, testing feed additives, and specific feed ingredients. However, just like with any other *in vitro* methodology, caution must be taken when extrapolating data obtained *in vitro* to *in vivo* situations.

Recent studies have shown that diet formulation using canola meal instead of soybean meal at the same level of dietary crude protein can increase milk yield and milk protein content and it can also increase nitrogen utilization, potentially lowering environmental nitrogen excretion.

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Figures:



Figure 1: Omasal tube.



Figure 2: Omasal tube close-up view.

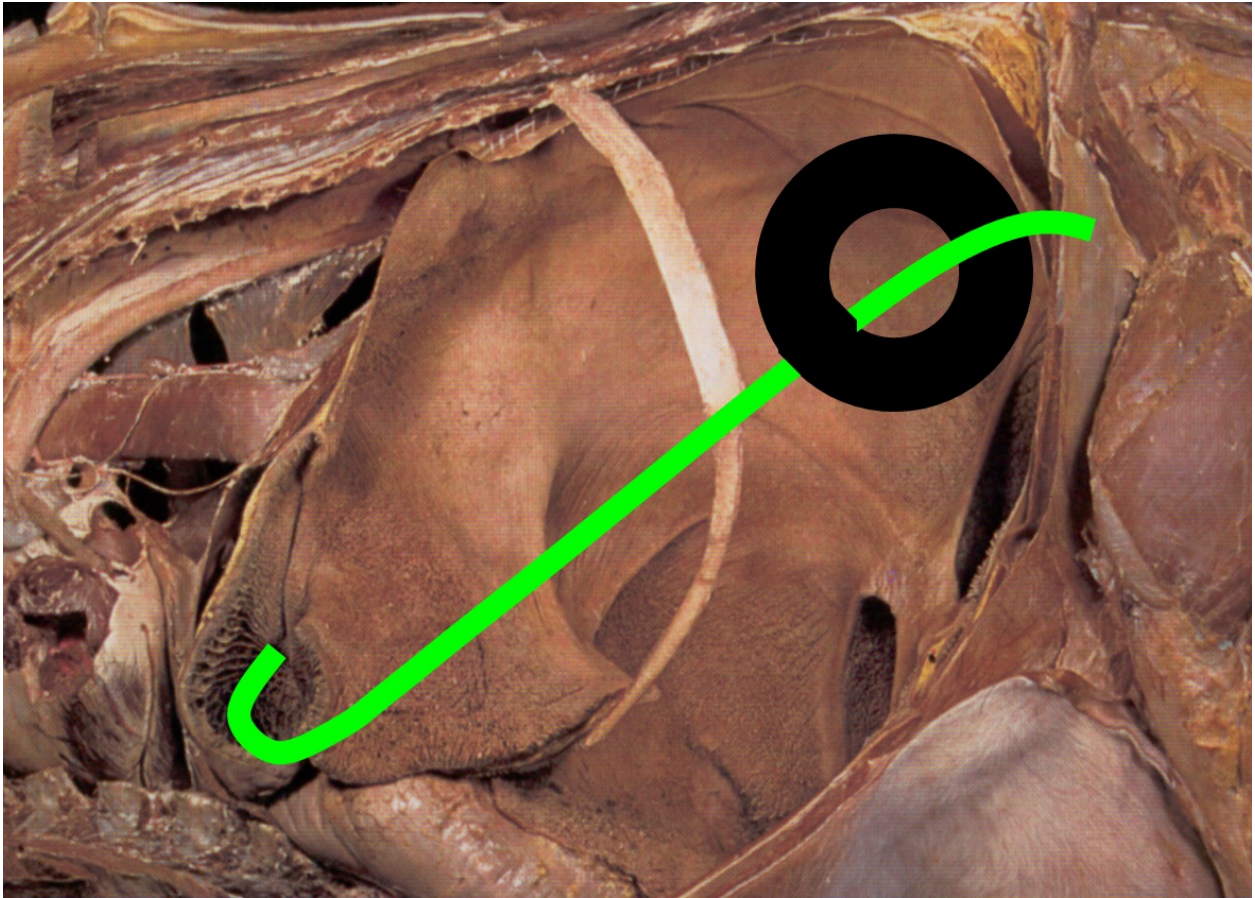


Figure 3: Omasal tube placement (view from left side of the animal).

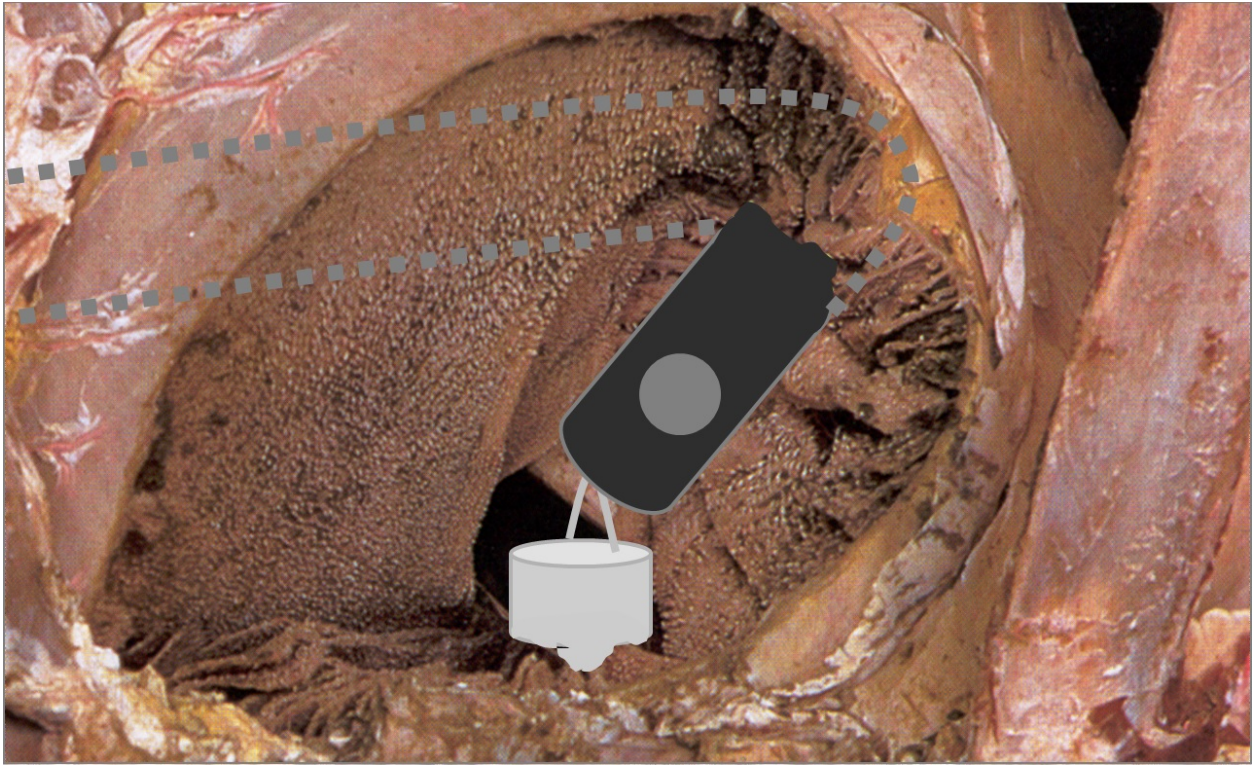


Figure 4: Omasal tube placement (view from right side of the animal).



Figure 5: Omasal tube placement and sample collection.

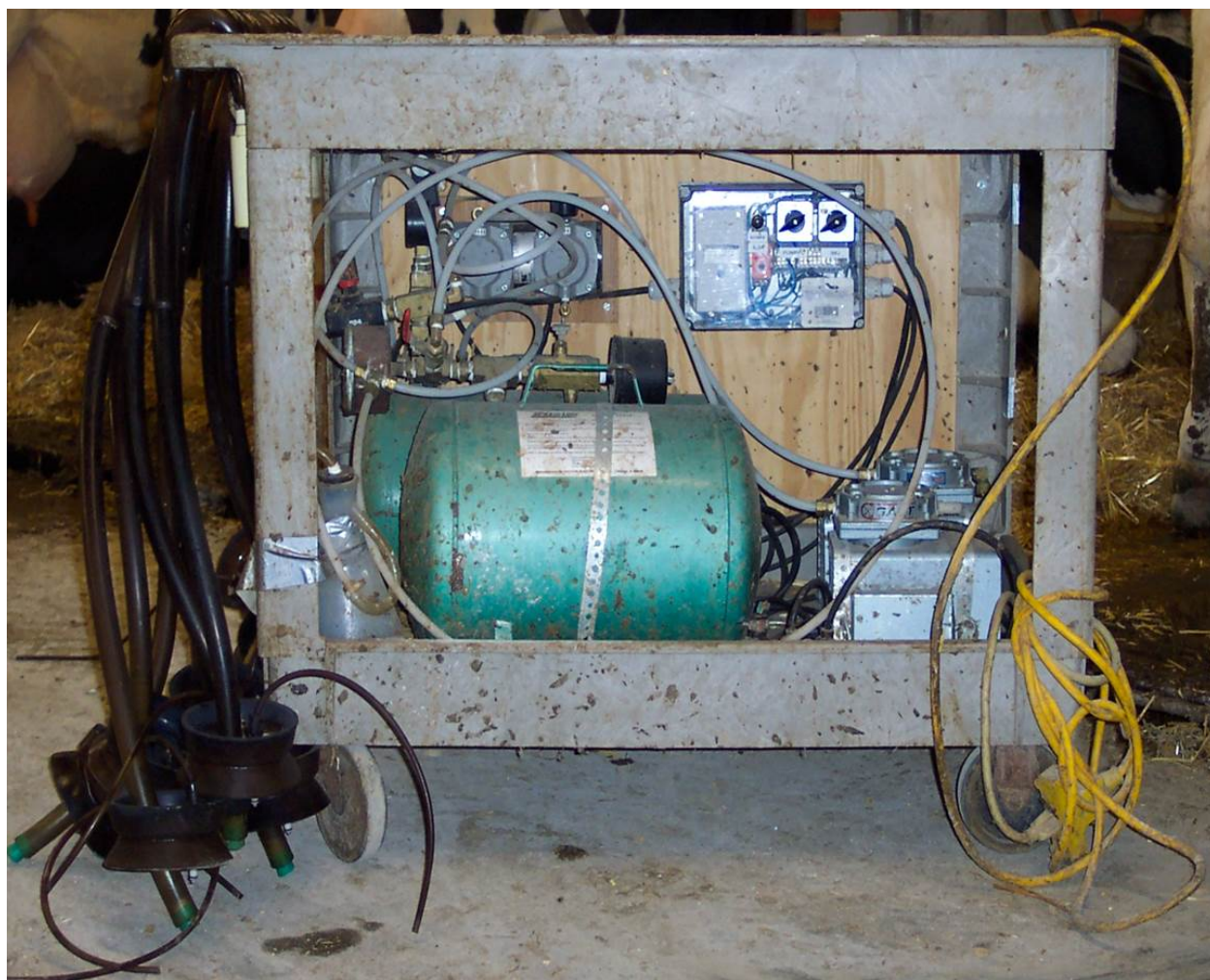


Figure 6: Dual vacuum pump (alternates pressure and vacuum).



Figure 7: Dual vacuum pump (alternates pressure and vacuum) cart view.



Figure 8: Triple-marker solution and syringe pump for infusion.



Figure 9: Ruminally cannulated cows and infusion lines.



Figure 10: Ruminally cannulated cows and infusion lines, close-up view.