This is to certify that the

thesis entitled

METABOLIC EFFECTS OF MALIC
ACID IN RUMINANTS

presented by

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has been accepted towards fulfillment
of the requirements for

Masters degree in Dairy Science

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Date ~~April~~ 11, 1979

METABOLIC EFFECTS OF MALIC ACID IN RUMINANTS

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A THESIS

Submitted to
Michigan State University
in partial fulfillment of the requirements
for the degree of

MASTER OF SCIENCE

Department of Dairy

1979

ABSTRACT

METABOLIC EFFECTS OF MALIC
ACID IN RUMINANTS

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In the *in vitro* study gas production, volatile fatty acid production and ph changes were used to estimate the effect of malic acid on the rumen fermentation rate. Malic acid increased gas production and volatile fatty acid production.

In the milk production trial 32 lactating Holstein cows were randomly allotted to 4 treatment levels of malic acid (0, 70, 105, and 140 grams/day) fed during a 100 day treatment period. The group receiving highest malate had significantly higher milk persistency than controls (95 vs. 88%). Early lactation cows receiving malic acid were significantly higher in total rumen volatile fatty acids.

In the nitrogen balance study 6 steers (420 kilograms) were randomly assigned to a 3 x 3 Latin Square receiving 0, 100, or 200 milligrams/kilogram body weight of malic acid per day. Rumen propionate was significantly higher in animals receiving malic acid than in controls.

ACKNOWLEDGEMENTS

The author expresses appreciation to his major professor, Dr. Robert M. Cook, for continued encouragement, assistance and guidance during graduate study.

Gratitude is extended to the Department of Dairy Science for financial support in the form of a Graduate Research Assistantship.

Thanks is rendered to Dow Chemical Company for financial backing enabling this research to be conducted.

The author is grateful to Dr. Melvin T. Yokoyama, Dr. C. A. Reddy and Dr. John T. Huber for aid and advice while serving on the guidance committee.

Appreciation is conveyed to Laurie Allison, Sheri Schlotz, Denise Schlotz, Karen Wernette and Kathy Wood for analysis of samples and compilation of data.

Last and foremost, recognition is given to my wife, Elizabeth Jean, for her patience and understanding throughout this graduate school program.

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INTRODUCTION

Research in animal agriculture consists of conducting experiments to determine the origin of various biological and physical processes so that the system may be more fully understood. The goal is to use this new knowledge to increase the productivity and efficiency of animal agriculture.

The ruminant, by virtue of the microbial population inhabiting its rumen, is unique in its ability to digest feeds that are metabolically less available to other animals. The bovine is able to produce milk and meat which are two high quality foods. Thus, the bovine has the capacity to convert feeds of low nutritional value to high quality food for humans.

Digestion of feeds in the rumen is an important process in ruminant nutrition. If microbial fermentation is at the optimum level, maximum intake and utilization of feeds can be achieved. Certain microbial growth factors have been identified which have been shown to be able to increase feed efficiency and nitrogen retention in ruminants. This is accomplished by increasing the growth rate of rumen bacteria enabling more complete digestion of feeds to occur in the forestomach of the ruminant. The end result is a more efficient system whereby the animal receives more available nutrients than was possible with a less desirable rumen fermentation.

In animal agriculture today the cost benefit ratio of microbial growth factors must be considered. Industry must know if the increase in feed efficiency provided by supplying microbial growth factors is substantial enough to justify research, manufacturing, and marketing costs of the microbial growth factors.

There has been considerable effort put forth recently in studying feed additives for beef and dairy cattle. Malic acid has been shown to increase feed efficiency and nitrogen retention in beef and dairy cattle presumably by increasing the rumen fermentation efficiency. However, little research has been conducted with dairy cattle concerning the effect malic acid might have on milk production, milk composition, feed intake, body weight changes, and feed efficiency.

The objective of this thesis was to develop an _in vitro_ technique to assess the effects of microbial growth factors such as malic acid on the rumen fermentation rate and to determine if supplemental malic acid increases the utilization of nutrients by ruminants and enhances milk yields in lactating dairy cows.

LITERATURE REVIEW

In Vitro Technique for Studying the Rumen Fermentation Rate

Most of the methods used for measuring *in vitro* rumen microbial activity have been a measure of fiber disappearance during a specified time interval. The measurement of microbial activity by fiber disappearance in forty eight hours may not show differences between treatments even though there were differences at some time prior to forty eight hours. Thus, a system was needed that could quickly test the effects of chemicals on the rumen fermentation rate.

Production of gas as measured manometrically has been used by Hungate (16), McBee (25), Perez (27), Quin (29), and Reid (30). Like volatile fatty acid production, gas production data needs to be considered cautiously because of its lack of specificity. Gas can be produced from a variety of substrates by a mixed culture of rumen microorganisms. Furthermore, CO_2 can be released from carbonate buffered medium by the acid produced and care must be taken to account for it. Nevertheless, this parameter has been used successfully and will be important in future studies of rumen fermentation.

The *in vitro* method of using gas production rates to measure microbial net growth was developed by ElShazly and Hungate in 1965 (11). They found that if substrate was in excess and the optimum dilution was used, fermentation progressed at the maximal rate and was proportional to total microbial cells. This technique was used with some modifications for rapidly determining the effects of different microbial growth factors on the rumen fermentation rate.

Malic Acid

Malic acid is an important natural organic acid. It is widely dispersed among the vegetables of the world and is the most abundant of the acids found in fruit. For example, the fruit of lychee has malic acid present in it totaling eighty percent of the nonvolatile acids (6). Malic acid is found in strawberries (28), grapes (17), peaches (20), and peas (36). In the wine industry malic acid concentrations are monitored to give an idea of the stability of the wine in question (32). Since wine is a product of certain yeasts transforming sugars to alcohol, the breakdown of malic acid by lactic bacteria reduces acidity producing a more stable wine. Malic acid is the predominant organic acid in many plants (12). It is in grasses (4, 8), silages (31), and legume forages (31) in varying amounts.

In the Animal Kingdom malic acid plays a key role in carbohydrate metabolism. It serves as the precursor of pyruvate and oxalacetate (19).

Malic acid is used in the food industry as an anti-spattering agent for margarine, as a metal chelating agent to inactivate heavy metals, and in the extraction of pectin from fruit waste (23). Malic acid has applications in the pharmaceutical industry as a component of compounds used to treat hepatic disfunction (23).

Examples of chemical uses of malic acid are as an essential ingredient of insect repellents, hydrogen peroxide stabilizers, and as an algicide. In cosmetics malic acid is used in teeth cleaning tablets, toothpastes and mouthwashes (23).

Malic acid contains an asymmetric carbon. Thus, it exists in both dextrorotary and levorotatory forms. The form used in this research was obtained from Dow Chemical Company and is a racemic mixture of D and L isomers. The acid found in nature is the levorotatory configuration (35).

Malic acid is a key intermediate in the metabolism of bacteria (2, 10, 14, 15, 22, 24, 33). This is important to ruminants because of the symbiotic relationship between the ruminant and its microbial population. Since malic acid is a key intermediate in microbial metabolism, the concept arises as to whether it is a limiting growth factor for these microbes.

Malic acid has been shown to stimulate the growth of rumen bacteria on lactate media (21). This occurs because malic acid is a source of oxalacetate which is limiting. The oxalacetate deficiency arises because of the need for glucose synthesis. A limitation of this important metabolic intermediate could limit microbial growth. Thus, malic acid increases oxalacetate which is used in propionate and glucose formation and other biosynthetic reactions to increase the rumen microbial fermentation efficiency.

Studies on certain species of yeasts indicate that the L form of malic acid is utilized to a greater extent than the DL mixture (5). However, research conducted with rats show both the L and DL forms are metabolized equally with no apparent difference in utilization of either form (9).

In ruminants malic acid increases propionate production when added to the rumen (34). This is associated with more efficient utilization of energy sources from the rumen. This is very important to ruminants especially if the ratio of forage to concentrate in the ration is high. The reasoning being that propionate is converted to glucose in the liver in ruminants, increased propionate production will increase glucose production in the ruminant. This makes more energy available for body processes including milk production.

Malic acid functions in the tricarboxylic acid cycle to supply a source of oxalacetate (19). Oxalacetate is necessary for the production of carbohydrate from all precursors except glycerol and L-glycerophosphate (19). The precursors are propionate and lactate produced from fermentation of forages and concentrates (34).

Feeding Trials

Experiments have shown that the L isomer of malic acid is twice as effective as the DL mixture in improving nitrogen retention in sheep (34). This is expected since it is the L form that occurs in nature.

The supplementation of malic acid to steers on high forage rations containing urea increased protein digestibility, and nitrogen retention (34).

The addition of malic acid to sheep on high forage rations increases nitrogen retention (34). However, adding malic acid to steers on a high concentrate diet was not successful in increasing digestibility of nitrogen or dry matter or retention of nitrogen (34).

A study was recently completed at Utah State University (1). It was found that feeding 107 grams malic acid per head daily gave increases in milk production.

Two other studies have fed malic acid to lactating dairy cattle (34). In the first study cows receiving 70 grams malic acid per head per day produced 4.5 pounds more milk per day than controls. The solids corrected milk was also significantly increased and the treatment group gained more weight. Feeding 28 grams malic acid per head daily gave no significant increase in milk production or weight gain. In the second study cows receiving 70 grams were more efficient in converting energy to weight gain and milk production. This treatment group gained more weight and produced more milk than controls or cows receiving 35 grams malic acid daily.

In conclusion previous studies of the effects of feeding malic acid to lactating dairy cattle have been with cows in average production and only a limited number of animals were used. Also, previous studies have not considered metabolic indices which may indicate the mechanism of action of the compound. This study was undertaken to develop and utilize an *in vitro* technique for measuring the effects of malic acid on the rumen fermentation rate and then use applied research to determine the effects of malic acid on lactating dairy cattle. A nitrogen balance experiment was also performed to help elucidate the mechanism of action through which malic acid increases productivity in ruminants.

Table 1. Physical properties of malic acid.

Formula	HOCHCOOH / CH_2COOH
Molecular weight	134.07
Melting point	Racemic DL-form, 131-132°C
Physical shape	White crystals
Forms	Natural L-levorotatory Synthetic DL-racemic mixture
Solubility	
Ethanol	39.16 grams/100 ml
Ether	1.41 grams/100 ml
Chloroform	0.04 grams/100 ml
Heat of combustion	-320.1 kcal/mole
Heat of solution	-4.0 kcal/mole
Viscosity, 50% aqueous solution	6.5
Odor	Odorless
Specific gravity, D_4^{20}	1.601

MATERIALS AND METHODS

I. *In Vitro Experiments*

Variations occurred in the amount and kind of substrates utilized and in the composition of the culture media (Table 2).

The source of the rumen fluid was a mature, nonlactating Holstein cow equipped with a permanent rumen fistula. The cow was on an all corn silage ration with access to a trace mineralized block throughout the experimental sampling period.

Rumen fluid samples were collected by removing whole rumen contents via the fistula and squeezing it through cheesecloth into a dewar. The dewar had been warmed with hot water to prevent changes in rumen fluid temperature. The lid was placed on the flask and rumen fluid was immediately transferred to the laboratory.

In the laboratory one hundred milliliter aliquots of rumen fluid were transferred to one-pint flasks containing substrates. The rumen fluid was stirred gently to insure uniform aliquots. The flasks were preincubated at $39^{o}C$. The flasks were gased with CO_2 to exclude oxygen and then closed with a rubber stopper provided with a tube having a three-way stopcock. The stopcock connected the flask to a ten milliliter glass syringe. The stopcock allowed the syringe to be emptied without exposing the incubation to the atmosphere. Gas measurements started after a thirty minute equilibration period. The time interval between rumen sampling and incubation was approximately fifteen minutes.

Table 2. Composition of the *in vitro* media.

Component	Experiment I	II	III	IV
Buffer, ml*	200	200	200	200
Rumen fluid, ml	100	100	100	100
Sodium bicarbonate, mg	--	3000	1000	500
Cellulose, g	--	5	5	--
Amylose, g	--	5	--	--
Concentrates, g	--	--	5	5

Component	Experiment V	VI	VII
Buffer, ml*	200	200	200
Rumen fluid, ml	100	100	100
Sodium bicarbonate, mg	1000	3000	3000
Cellulose, g	--	--	5
Amylose, g	--	7.5	2.5
Concentrates, g	5	--	--

* Hungate buffer: 1 part A & 1 part B & 4 parts double distilled water.

A = 0.3% KH_2PO_4, 0.6% NaCl, 0.3% $(NH_4)_2SO_4$, 0.06% $MgSO_4$, 0.06% $CaCl_2$

B = 0.3% K_2HPO_4

Gas production was measured using a ten milliliter water-lubricated glass syringe. The volume of gas forced into the syringe was read every five minutes. The incubation time was two hours (not including the thirty minutes of preincubation). The ph of each incubation mixture was measured before and after each incubation period (initial ph was 7.0 for each flask).

One sample was taken prior to incubation and treated as the others to serve as the zero time control. When the incubation was complete, samples from each flask were collected, placed in ice, and later centrifuged at 10,000 x gravity for fifteen minutes. The supernatant was recovered for volatile fatty acid determination.

II. Effects of Malic Acid on Milk Production

Thirty-two lactating Holstein cows (16 in mid and 16 in early lactation) were randomly allotted to four treatment groups of eight cows each. The statistical design was a randomized complete block design. Treatment groups were balanced for milk production, age, days after calving and breeding groups. Treatments consisted of four levels of malic acid (0, 70, 105, and 140 grams/day) fed during a one hundred day treatment period.

Feeding Regime:

The cows were fed once daily in a stanchion-type barn. Water was free choice. Corn silage and alfalfa hay were mixed together in relative proportions of sixty kilograms corn silage to five kilograms alfalfa hay. This mixture was identified as mix-three and was fed to the cows ad libitum. Concentrate was fed at the rate of one kilogram concentrate for each two and one half kilograms milk. The concentrate was placed into the feed bunk on top of mix-three so that the concentrate was totally consumed. Weighbacks of feed occurred each morning at 6 am. Cows were fed between 8 am to 11 am each morning.

Cows were fed quantities of mix-three to enable a ten percent weighback to occur each day. Feed records were checked every two-three days to insure that a ten percent weighback was occurring. The amount of concentrate fed was adjusted every three days based on changes in milk production.

Three different concentrates were fed: D200, D208 and D209. The composition of each concentrate is shown in Table 3.

The early lactation cows were adapted to nonprotein-nitrogen during the first four weeks after they calved. D208 concentrate was used for this and as the four weeks elapsed, D208 (contains NPN) replaced D200 as the concentrate source. The malic acid was in D209 and was fed as follows: 0, 4, 6, or 8 pounds D209 was fed so that each cow received 0, 70, 105, or 140 grams malic acid per day. Thus, the amount of D209 a cow received during the one hundred day treatment period stayed the same and it was D208 that fluctuated depending on the animal's previous three days milk production. D208 and D209 were of similar composition except for the malic acid content of D209.

The late lactation cows had a three week adaptation period to NPN starting approximately one hundred fifty five days into their lactation. All groups were fed the same basal ration of mix-three and concentrate during treatment.

Milk Data:

Individual milk weights were recorded twice daily. Milk composition (percent fat, protein and total solids) was determined weekly

Table 3. Composition of concentrates used in the production trial (kilograms).

D200

Ground Shelled Corn	392.4
Ground Oats	192.8
Soybean Meal	247.2
Deflourinated Phosphate	15.9
Trace Mineralized Salt	9.1
Vitamin A & D*	4.5
Sugar Cane Molasses	45.4

D208

Ground Shelled Corn	469.9
Ground Oats	235.9
Soybean Meal	104.3
Trace Mineralized Salt	9.1
Deflourinated Phosphate	13.6
Limestone	4.5
Urea	20.0
Vitamin A & D*	4.5
Sugar Cane Molasses	45.4

D209

Ground Shelled Corn	446.8
Ground Oats	224.1
Soybean Meal	104.3
Trace Mineralized Salt	9.1
Deflourinated Phosphate	13.6
Limestone	4.5
Malic Acid	34.9
Urea	20.0
Vitamin A & D*	4.5
Sugar Cane Molasses	45.4

* Vitamin A contained 4409 international units per kilogram.

Vitamin D contained 441 international units per kilogram.

for each cow. A composite milk sample was taken on Tuesday afternoons and Wednesday mornings. Milk was analyzed for fat by the Milkoscan 300 machine, protein by the Orange G Dye Binding method and for total solids by drying 25 grams in a forced air oven for 3 hours.

Herd Management:

All cows were weighed seven days after the beginning of treatment and biweekly thereafter.

The cows were housed in a stanchion-type barn which was completely enclosed. The milking parlor was approximately twenty meters from the cows and was attached to the barn where the cows were housed.

The cows were milked in a double-eight herringbone milking parlor twice daily at 4:00 am and 3:00 pm. After being milked, the cows were allowed to exercise in an outside lot for one hour before returning to their stanchions. Herd health and husbandry programs were conducted by the Michigan State University Dairy Research Barn management personnel.

Rumen Fluid and Blood Collection and Analysis:

Rumen fluid and coccygeal tail vein blood samples were collected biweekly for each animal during the treatment period. The animals were sampled two hours after feeding.

Rumen fluid samples were taken with a stomach tube, speculum and suction pump. Rumen fluid was analyzed for volatile fatty acids and ammonia. Ph was determined.

Volatile fatty acids (VFA) were determined with a Hewlett-Packard gas chromatograph, model 5730A, equipped with a model 7671A automatic sampler and an Integrator-Recorder, model 3880A. The column was packed with graphited carbon, Carbowax B. Nitrogen was the carrier gas. The acid standards contained 0.1N each of acetic, propionic, isobutyric, butyric, 2-methylbutyric, isovaleric and valeric acids in double distilled water. The carrier gas flow rate was forty milliliters per minute. The temperature program was initiated at $155^{\circ}C$ for four minutes and progressed to $190^{\circ}C$ at the rate of 4° per minute. Rumen fluid samples were first centrifuged at 10,000 x gravity for fifteen minutes and then the supernatant stored at $-4^{\circ}C$ until analyzed. Rumen ph was determined using a standard Beckman ph meter.

Rumen ammonia concentrations were determined by using the phenol-hypochlorite colorimetric procedure (26), plasma urea was determined by using the phenol-hypochlorite colorimetric procedure (18), and plasma glucose by the glucose oxidase and peroxidase method (37).

Feed Sampling and Analysis:

The silage was sampled three times per week. A composite sample was made every two weeks and analyzed for dry matter and crude protein (3). Concentrates were also analyzed for dry matter and crude protein (3).

The dry matter of silages and concentrates was determined by placing duplicate representative samples in an oven set at $90^{\circ}C$ for twenty four hours (3). Samples were allowed to cool in a dessicator and then weighed.

Crude protein content of silages and concentrates was determined by the Macro-Kjedahl procedure (3).

III. Nitrogen Balance Trial

Six Holstein steers (420 kilograms) fitted with rumen cannulae were used in an experiment designed to test the effects of feeding malic acid on ration digestibility and nitrogen utilization as well as rumen ammonia, plasma urea, plasma ammonia and volatile fatty acid concentrations. The experimental design was a 3 x 3 Latin Square. There were two animals per treatment. Malic acid was fed at the level of 0, 100 or 200 milligrams per kilogram body weight per day.

Feeding Procedure:

Steers were fed once daily at 8:00 am. The diets were composed of shelled corn and corn silage (ad libitum) on a 1:1 dry matter basis. Urea was supplemented to increase total dietary protein to 12% on a dry matter basis. A mineral supplement was also fed.

Each morning the steers were fed individually according to how much feed had been consumed the previous day. The malic acid was carefully mixed with the diet of each individual steer.

Management:

After a five-week adaptation to the corn-urea diet, malic acid was fed for three periods of twelve days each. Feed intakes were recorded throughout each twelve day period. Urine and feces were collected for the last seven days of each twelve day period. When the experiment was completed, each steer had received all malic acid treatments.

The steers were housed in metabolism stalls. Feces were collected each morning and afternoon.

Blood and rumen fluid samples were taken before feeding, and then every two hours for twelve hours on the last day of each treatment period.

Rumen Fluid and Blood Collection and Analysis:

Rumen fluid samples were taken with an aspirator and plastic tube through the rumen fistula. Rumen fluid was analyzed for ammonia and volatile fatty acids as previously described.

The coccygeal tail vein blood samples were analyzed for plasma urea and plasma ammonia (18, 26).

Feed Sampling and Analysis:

Daily feed samples were taken and stored at $-4^{\circ}C$. Feeds were composited for each period and analyzed for dry matter, nitrogen and acid detergent fiber (3).

Feces and Urine Sampling and Analysis:

Daily fecal samples were stored at $-4^{\circ}C$. Composite samples were made for each seven day period for each animal and then analyzed for dry matter, nitrogen, and acid detergent fiber (3).

Daily urine samples were stored frozen at $-4^{\circ}C$. A composite sample was made for each seven day period for each animal. Urine samples were analyzed for nitrogen (3).

RESULTS

I. *In Vitro* Experiments

Experiment I was a time study to determine the point after feeding when the rumen fermentation rate was maximal. Whole rumen contents were incubated for two hours. The highest rate of gas production occurred from samples obtained two hours after feeding. The first three hours after feeding samplings gave the highest gas production. By five hours after feeding, the gas production had diminished (Table 4). Thus the rumen microbial population was multiplying at its maximum capacity at two hours after feeding. This should be when microbial growth factors such as malic acid would be in short supply. It is important to the rumen microbial population to have all essential nutrients present at optimum levels in order to achieve maximum growth rates. This enables more complete digestion of feeds to occur in the rumen because the number of bacteria is increasing at the maximal rate and more feed is broken down and absorbed. This creates a more efficient rumen fermentation. Two hours after feeding was the period selected for sampling of rumen fluid for demonstrating the effects of malic acid on the rumen fermentation.

Experiment II compared the gas production rates when amylose or cellulose were substrates. As expected the amylose fermentation was much faster than the cellulose (Table 4). When solka floc was used as the only substrate source, the fermentation rate was too slow to detect differences between treatments. In the rumen the starch

Table 4. Gas production in the *in vitro* experiments.

Experiment	Treatment	Total gas produced (ml)	Time after feeding (hours)
I	---	3.0	0
	---	4.0	0
	---	27.8	1
	---	27.6	1
	---	31.2	2
	---	31.3	2
	---	23.8	3
	---	17.0	3
	---	11.2	4
	---	13.7	4
	---	22.0	5
	---	20.6	5
	---	21.2	6
	---	21.6	6
	---	22.0	7
	---	21.5	7
	---	20.1	8
	---	21.2	8
	---	18.1	9
	---	18.8	9
	---	20.5	10
	---	20.1	10
II	Control	0	0
	Control	2.4	0
	5 grams starch	159.0	0
	5 grams starch	151.3	0
	5 grams cellulose	2.8	0
	5 grams cellulose	4.0	0
	Control	52.7	2.5
	Control	65.4	2.5
	5 grams starch	205.5	2.5
	5 grams starch	192.1	2.5
	5 grams cellulose	74.6	2.5
	5 grams cellulose	62.7	2.5
	Control	32.7	5
	Control	39.7	5
	5 grams starch	172.3	5
	5 grams starch	167.7	5
	5 grams cellulose	35.0	5
	5 grams cellulose	31.5	5
	Control	25.2	7.5
	Control	24.7	7.5
	5 grams starch	154.0	7.5
	5 grams starch	154.0	7.5
	5 grams cellulose	26.9	7.5
	5 grams cellulose	25.4	7.5

Table 4. Continued

Experiment	Treatment	Total gas produced (ml)	Length of Incubation (hours)
III	Control	35.8	2
	Control	33.4	2
	5 grams cellulose	34.3	2
	5 grams cellulose	32.7	2
	5 grams concentrate	90.3	2
	5 grams concentrate	93.5	2
IV	Control	88.1	1
	Control	90.0	1
	100mg malic acid	94.0	1
	100mg malic acid	98.3	1
	300mg malic acid	94.6	1
	300mg malic acid	94.9	1
	500mg malic acid	97.2	1
	500mg malic acid	97.3	1
V	Control	115.5	1
	Control	118.5	1
	100mg malic acid	117.2	1
	100mg malic acid	123.9	1
	300mg malic acid	130.8	1
	300mg malic acid	128.1	1
	600mg malic acid	119.6	1
	600mg malic acid	119.8	1
VI	Control	281.6	2
	Control	277.6	2
	600mg malic acid	282.7	2
	600mg malic acid	285.6	2
	900mg malic acid	288.1	2
	900mg malic acid	289.4	2
	1200mg malic acid	291.2	2
	1200mg malic acid	290.2	2
VII	Control	184.8	2
	Control	185.6	2
	600mg malic acid	192.6	2
	600mg malic acid	190.5	2
	900mg malic acid	190.9	2
	900mg malic acid	192.4	2
	1200mg malic acid	202.1	2
	1200mg malic acid	205.7	2

fermenting bacteria multiply much faster than the cellulose fermentors. Fermentors of readily available carbohydrate rapidly digest soluble sugars and starches whereas the cellulose fermentors must first attach to the fibrous substrate. Also, the cellulolytic population does not fluctuate nearly as much as the amylolytic. This enables members of the latter to increase more rapidly as substrates become available for digestion.

Experiment III was a time study to determine at what time after feeding the carbohydrate fermentors were at their peak in terms of fermentation rate. Two and one half hours after feeding was when the rumen fluid sampled gave the maximum fermentation of amylose (Table 4).

It became clear that fluctuation in the activity of rumen fluid samples from day to day made it difficult to identify amounts of carbohydrate or cellulose desired for optimum fermentation in the *in vitro* experiments. Total microbial cell counts or some other method is needed to determine the population density of the rumen fluid before levels of substrate required per flask can be defined.

After the *in vitro* technique had been refined, the effects of malic acid on the rumen fermentation rate was tested. Experiments IV, V, VI, and VII determined the effects of malic acid on the rumen fermentation rate. The levels used ranged from one hundred milligrams to twelve hundred milligrams malic acid added per bottle of incubation media. These amounts of malic acid were arrived at by considering levels fed during the production trial. Malic acid was fed at 0-140 grams per head per day. Thus, 70 grams malic acid in a 70 kilogram rumen was 0.001 grams malic acid per milliliter of fluid. The total volume of the *in vitro* incubation media was 300 milliliters so 70 grams fed translated into 0.3 grams malic acid per flask. Malic acid increased the gas production and volatile fatty acid production over controls. In all *in vitro* experiments volatile fatty acid production was directly correlated to gas production.

II. Milk Production Trial

The effect of malic acid on energy utilization for both early and mid lactation cows is shown in Table 5. Treatments A, B, C, and D correspond to 0, 70, 105, and 140 grams malic acid fed per head per day respectively. Cows fed the high level malic acid were most efficient in terms of milk produced per megacalorie of feed ingested.

When malic acid was fed the efficiency was greater for the mid lactation cows than for the early lactation cows (Tables 6 & 7). However, differences between treatments were not significant.

The high level of malic acid significantly increased the persistency of lactation over controls (Table 8). Since malic acid enhances milk production the cost of manufacturing and marketing malic acid as a feed additive should be examined to determine if it is profitable to use malic acid as a commercial feed additive.

There were no significant differences between treatments for total dry matter intake (Table 9).

There also were no significant differences between treatments for roughage dry matter intakes although stage of lactation tended to influence roughage dry matter intake (Table 10). This was because the early lactation cows were producing more milk so were receiving more concentrate in the ration than mid lactation cows. Consequently, the early lactation cows consumed less roughage.

The control group of cows weighed the lightest (Table 11). After balancing the animals for milk production, breeding groups, age

Table 5. The effects of malic acid on energy utilization, early and mid lactation combined.

	TREATMENTS			
	A	B	C	D
FEED CONSUMED				
SILAGE (kg)	23.0	23.8	23.7	22.3
CONCENTRATE (kg)	11.0	10.4	11.1	10.9
TOTAL (kg)	34.0	34.2	34.8	33.2
ENERGY CONSUMED				
SILAGE (0.51 Mcal/kg)	11.7	12.1	12.0	11.3
CONCENTRATE (1.76 Mcal/kg)	19.4	18.3	19.6	19.2
TOTAL (Mcal)	31.1	30.4	31.6	30.5
ENERGY REQUIREMENTS				
BODY WEIGHT (kg)	570.5	609.2	637.8	620.4
NE FOR MAINT. (Mcal)	9.9	10.4	10.8	10.5
ENERGY AVAILABLE FOR MILK				
(ENERGY INTAKE - MAINT.) (Mcal)	21.2	20.0	20.8	20.0
EFFICIENCY				
MILK PRODUCED (kg)	26.3	24.6	26.0	26.0
kg MILK/Mcal	1.24	1.23	1.25	1.30

Table 6. The effects of malic acid on energy utilization in mid lactation cows.

	TREATMENTS			
	A	B	C	D
FEED CONSUMED				
SILAGE (kg)	25.9	25.3	23.5	24.4
CONCENTRATE (kg)	9.4	9.2	10.3	9.7
TOTAL (kg)	35.3	34.5	33.8	34.1
ENERGY CONSUMED				
SILAGE (0.51 Mcal/kg)	13.1	12.8	11.9	12.4
CONCENTRATE (1.76 Mcal/kg)	16.6	16.2	18.2	17.0
TOTAL (Mcal)	29.7	29.0	30.1	29.4
ENERGY REQUIREMENTS				
BODY WEIGHT (kg)	581.6	650.8	674.8	640.8
NE FOR MAINT. (Mcal)	10.0	10.9	11.2	10.8
ENERGY AVAILABLE FOR MILK				
(ENERGY INTAKE - MAINT.) (Mcal)	19.7	18.1	18.9	18.6
EFFICIENCY				
MILK PRODUCED (kg)	21.6	20.4	22.8	22.2
kg MILK/Mcal	1.10	1.13	1.21	1.19

Table 7. Effects of malic acid on energy utilization in early lactation cows.

	TREATMENTS			
	A	B	C	D
FEED CONSUMED				
SILAGE (kg)	20.2	22.4	23.8	20.2
CONCENTRATE (kg)	12.6	11.6	12.0	12.1
TOTAL (kg)	32.8	34.0	35.8	32.3
ENERGY CONSUMED				
SILAGE (0.51 Mcal/kg)	10.2	11.4	12.1	10.2
CONCENTRATE (1.76 Mcal/kg)	22.2	20.5	21.1	21.4
TOTAL (Mcal)	32.4	31.9	33.2	31.6
ENERGY REQUIREMENTS				
BODY WEIGHT (kg)	559.3	567.7	600.8	600.0
NE FOR MAINT. (Mcal)	9.6	9.7	10.3	10.3
ENERGY AVAILABLE FOR MILK				
(ENERGY INTAKE - MAINT.) (Mcal)	22.8	22.2	22.9	21.3
EFFICIENCY				
MILK PRODUCED (kg)	31.1	28.7	29.3	29.9
kg MILK/Mcal	1.36	1.29	1.28	1.40

Table 8. The effects of malic acid on persistency of lactation.

TREATMENT	WEEK 1	2	3	4	5	6	7	8	9	10	11	12	13	14	AVG.
A	98.0	94.5	92.1	95.0	94.7	92.2	92.1	91.5	85.2	77.6	84.6	81.4	80.4	78.2	88.2
B	100.3	92.6	94.7	96.9	95.0	92.9	88.9	89.1	88.0	84.3	87.1	85.1	79.2	77.2	89.6
C	102.2	95.3	95.5	97.0	96.7	96.0	93.1	89.1	88.0	86.6	85.0	75.5	77.1	73.8	89.3
D	104.8	103.5	100.3	102.3	99.5	98.8	97.2	98.5	93.0	89.6	90.1	85.6	84.1	81.8	95.0*

Standard error = 2.3

* Significant at p .05.

Table 9. The effects of malic acid on dry matter intake (kg) for all cows (cow # on left side of column).

A		B		C		D	
1430	18.4	1345	19.1	1350	20.3	1442	21.1
1359	18.5	1435	21.2	1448	20.0	1328	17.7
1397	18.9	1456	16.3	1352	17.1	1364	16.1
1407	19.2	1376	17.9	1449	20.5	1410	18.2
1415	17.4	1282	17.3	1400	17.8	1269	18.2
1458	18.8	1321	17.4	1263	20.1	1369	16.8
1387	17.3	1417	21.6	1418	19.2	1302	18.7
1385	21.7	1377	16.6	1419	17.2	1390	17.9
AVG.	18.8		18.5		19.0		18.1

Standard error = 0.5

Table 10. The effects of malic acid on roughage dry matter intake (kg) for all cows (cow # is on the left side of column).

A		B		C		D	
1430	7.5	1345	7.6	1350	9.9	1442	9.1
1359	7.6	1435	9.6	1448	8.6	1328	6.7
1397	6.3	1456	8.0	1352	6.4	1364	7.2
1407	9.5	1376	8.5	1449	10.9	1410	8.2
1415	9.7	1282	9.8	1400	9.3	1269	9.8
1458	11.2	1321	10.1	1263	10.9	1369	9.8
1387	9.3	1417	12.8	1418	10.0	1302	9.5
1385	11.3	1377	7.9	1419	7.7	1390	8.4
AVG.	9.1		9.3		9.2		8.6

Standard error = 1.4

Table 11. The effects of malic acid on average body weights (kg) for treatment period for all cows (cow # is on the left side of the column).

A		B		C		D	
1430	488.7	1345	576.4	1350	671.6	1442	558.9
1359	631.9	1435	606.1	1448	566.6	1328	584.2
1397	539.7	1456	546.9	1352	597.8	1364	679.8
1407	577.0	1376	541.1	1449	567.4	1410	577.1
1415	589.8	1282	635.6	1400	585.7	1269	636.1
1458	592.4	1321	707.7	1263	772.1	1369	583.3
1387	571.5	1417	672.8	1418	698.7	1302	724.7
1385	572.9	1377	586.9	1419	642.6	1390	619.2
AVG.	570.5		609.2		637.8		620.4

and days after calving, it was impossible to also evenly distribute the cows among the treatment groups according to weight. Since weight was a less important factor than those mentioned, it was given less attention.

After treatments started early lactation cows lost and then regained weight. Late lactation cows were constantly gaining weight during treatment. Cows generally loose weight during the first third of the lactation due to the drain of energy from body tissue reserves caused by high milk production. Cows gain this weight back during the last two-thirds of the lactation.

All groups receiving malic acid had significantly lower total dry matter intake as a percent of body weight than the controls (Table 12). No significant linear dose response was observed although the trend existed. Since the heavier cows received malic acid and produced more milk but consumed less feed as a percent of body weight, malic acid increased the feed efficiency of the cows. Roughage dry matter intake (percent of body weight) was not significantly different between treatments (Table 13).

The percent fat in milk was not significantly different between treatments (Table 14). Since percent fat in the milk is a major factor in determining the price the farmer receives for milk, it is important to know malic acid does not decrease the fat test. There were no significant differences between treatments for percent protein or total solids in milk (Table 15 & 16).

Plasma glucose concentrations were not different between treatments (Table 17). Malic acid could have been converted to propionate in the rumen, the propionate converted to glucose by the rumen microbes, and the glucose used up by the rumen microbes in various biosynthetic

Table 12. The effect of malic acid on total dry matter intake (kg/100kg) body weight (cow # is on the left side of column).

A		B		C		D	
1430	3.76	1345	3.31	1350	3.02	1442	3.77
1359	2.93	1435	3.50	1448	3.53	1328	3.02
1397	3.49	1456	2.98	1352	2.85	1364	2.37
1407	3.33	1376	3.30	1449	3.61	1410	3.14
1415	2.94	1282	2.71	1400	3.04	1269	2.85
1458	3.17	1321	2.45	1263	2.60	1369	2.87
1387	3.02	1417	3.20	1418	2.74	1302	2.58
1385	3.78	1377	2.88	1419	2.67	1390	2.89
AVG.	3.30		3.04*		3.01*		2.94*

Standard error = 0.129

* Significant at p .05.

Table 13. The effects of malic acid on roughage dry matter intake (kg/100kg) body weight for treatment period for all cows (cow # is on the left side of column).

A		B		C		D	
1430	1.527	1345	1.312	1350	1.473	1442	1.625
1359	1.197	1435	1.588	1448	1.525	1328	1.150
1397	1.165	1456	1.468	1352	1.066	1364	1.057
1407	1.651	1376	1.575	1449	1.917	1410	1.418
1415	1.642	1282	1.548	1400	1.590	1269	1.537
1458	1.930	1321	1.430	1263	1.409	1369	1.685
1387	1.623	1417	1.900	1418	1.425	1302	1.313
1385	1.977	1377	1.353	1419	1.202	1390	1.363
AVG.	1.589		1.522		1.451		1.394

Standard error = 0.072

Table 14. The effects of malic acid on % fat in milk (cow # is on the left side of column).

A		B		C		D	
1430	3.08	1345	3.34	1350	3.58	1442	3.37
1359	3.07	1435	3.31	1448	3.26	1328	2.81
1397	2.65	1456	3.66	1352	2.77	1364	3.66
1407	3.38	1376	3.61	1449	3.88	1410	3.62
1415	3.39	1282	3.12	1400	3.58	1269	3.61
1458	4.10	1321	3.82	1263	3.82	1369	3.10
1387	3.89	1417	3.81	1418	3.55	1302	3.69
1385	3.38	1377	3.45	1419	3.48	1390	3.76
AVG.	3.37		3.52		3.49		3.45

Standard error = 0.10

Table 15. The effects of malic acid on % protein in milk (cow # is on the left side of column).

A		B		C		D	
1430	3.03	1345	2.86	1350	3.14	1442	3.17
1359	3.06	1435	3.22	1448	3.44	1328	3.13
1397	3.03	1456	3.35	1352	2.98	1364	3.19
1407	3.10	1376	3.23	1449	3.25	1410	3.07
1415	3.41	1282	3.26	1400	3.66	1269	3.32
1458	4.12	1321	3.93	1263	3.94	1369	3.79
1387	3.71	1417	3.68	1418	3.39	1302	3.79
1385	3.69	1377	3.38	1419	3.71	1390	3.79
AVG.	3.39		3.36		3.44		3.41

Standard error = 0.07

Table 16. The effects of malic acid on % total solids in milk (cow # is on the left side of column).

A		B		C		D	
1430	11.61	1345	11.93	1350	12.65	1442	12.02
1359	11.77	1435	12.08	1448	12.09	1328	12.02
1397	10.71	1456	12.73	1352	11.12	1364	12.66
1407	12.17	1376	12.56	1449	12.93	1410	12.31
1415	12.15	1282	11.54	1400	12.60	1269	12.46
1458	13.43	1321	13.08	1263	13.01	1369	12.07
1387	12.96	1417	12.51	1418	11.68	1302	12.67
1385	12.57	1377	12.25	1419	12.65	1390	13.23
AVG.	12.17		12.34		12.34		12.43

Standard error = 0.18

Table 17. The effects of malic acid on plasma glucose concentration in milligrams/100 ml of plasma (cow # is on the left side of column).

A		B		C		D	
1430	41.8	1345	48.1	1350	45.7	1442	43.6
1359	50.7	1435	45.5	1448	46.1	1328	44.4
1397	45.9	1456	43.0	1352	43.1	1364	49.8
1407	48.2	1376	44.8	1449	44.2	1410	47.2
1415	44.3	1282	53.1	1400	45.6	1269	52.1
1458	52.3	1321	49.9	1263	50.3	1369	46.8
1387	48.6	1417	51.6	1418	55.0	1302	48.1
1385	51.7	1337	49.6	1419	51.0	1390	43.1
AVG.	47.9		48.2		47.6		46.9

Standard error = 1.12

reactions.

Plasma urea and rumen ammonia levels were not different between treatments (Tables 18 & 19). In the rumen malic acid is a potential source of oxalacetate and alphaketoglutarate needed for trapping free ammonia (6). This nitrogen is then incorporated into rumen microbial protein. When low quality protein is fed to dairy cattle, isobutyrate and other precursors directly involved in the synthesis of essential amino acids are limiting microbial growth in the rumen (13). It has been demonstrated that certain branched chain fatty acids stimulate production in ruminants and malic acid may function at the trans-amination reaction step to help trap free ammonia and direct it into microbial protein synthesis (6).

However, if malic acid had increased the utilization of free rumen ammonia, plasma urea and rumen ammonia levels should have been lowered by the malic acid. Less ammonia would have been converted to urea by the liver and plasma urea concentrations would have been lowered. If malic acid enhanced ammonia uptake by the rumen microbes for microbial protein synthesis, rumen ammonia levels should have been lowered by malic acid. Further investigation will be conducted in the form of a nitrogen balance trial to determine the role of malic acid in the rumen.

Rumen ph was not different between treatments (Table 20).

Total volatile fatty acid concentrations in the rumen were not significantly different between treatments (Table 21). Total rumen acetate levels were not significantly different (Table 22).

It was found that volatile fatty acid concentration was increased significantly in the early lactation cows fed malic acid but not in the mid lactation cows. Thus, all groups receiving malic

Table 18. The effects of malic acid on plasma urea concentrations in milligrams/100 ml of plasma (cow # is on left side of column).

A		B		C		D	
1430	14.66	1345	13.31	1350	11.83	1442	16.45
1359	13.15	1435	14.80	1448	16.48	1328	13.22
1397	13.57	1456	13.77	1352	13.76	1364	11.59
1407	13.52	1376	14.34	1449	14.04	1410	13.15
1415	16.64	1282	15.92	1400	12.76	1269	12.49
1458	17.49	1321	12.98	1263	14.83	1369	13.84
1387	15.28	1417	12.39	1418	17.03	1302	16.18
1385	15.35	1377	15.88	1419	17.23	1390	15.95
AVG.	14.96		14.17		14.74		14.11

Standard error = 0.58

Table 19. The effects of malic acid on rumen ammonia concentration in milligrams/100 ml of rumen fluid (cow # is on the left side of column).

A		B		C		D	
1430	10.50	1345	15.27	1350	13.53	1442	23.78
1359	13.97	1435	17.46	1448	16.55	1328	13.37
1397	19.88	1456	22.59	1352	17.33	1364	14.90
1407	13.91	1376	19.06	1449	21.90	1410	20.97
1415	22.62	1282	18.22	1400	13.96	1269	15.68
1458	23.16	1321	14.13	1263	18.56	1369	23.55
1387	16.62	1417	19.54	1418	15.14	1302	16.74
1385	25.28	1377	25.41	1419	23.97	1390	13.09
AVG.	18.24		18.96		17.62		17.76

Standard error = 1.50

Table 20. The effects of malic acid on rumen ph (cow # is on the left side of column).

A		B		C		D	
1430	7.1	1345	7.0	1350	7.1	1442	7.1
1359	7.3	1435	6.9	1448	7.0	1328	6.4
1397	7.0	1456	7.1	1352	6.6	1364	7.1
1407	7.3	1376	6.8	1449	7.1	1410	7.1
1415	6.9	1282	7.0	1400	6.9	1269	7.2
1458	7.0	1321	7.0	1263	7.1	1369	6.4
1387	7.2	1417	7.1	1418	7.3	1302	7.0
1385	6.9	1377	6.8	1419	6.8	1390	7.2
AVG.	7.1		7.0		7.0		6.9

Standard error = 0.07

Table 21. The average concentration of total VFA content in mmoles/100 ml rumen fluid (cow # is on the left side of column).

A		B		C		D	
1430	7.848	1345	9.056	1350	8.743	1442	8.318
1359	6.612	1435	10.556	1448	9.461	1328	10.556
1397	8.504	1456	8.699	1352	11.233	1364	7.604
1407	6.796	1376	9.639	1449	7.622	1410	9.127
1415	9.638	1282	9.723	1400	9.857	1269	7.533
1458	9.552	1321	8.458	1263	9.111	1369	12.069
1387	8.674	1417	8.996	1418	7.079	1302	9.907
1385	10.520	1377	11.095	1419	11.153	1390	8.355
AVG.	8.518		9.528		9.282		9.184

Standard error = 0.41

Table 22. The effects of malic acid on the average concentration of total acetate content in mmoles/100 ml rumen fluid (cow # is on left side of column).

A		B		C		D	
1430	4.663	1345	5.545	1350	5.409	1442	5.405
1359	4.282	1435	6.014	1448	6.216	1328	5.605
1397	4.819	1456	5.519	1352	6.263	1364	4.619
1407	4.125	1376	5.666	1449	4.912	1410	5.765
1415	6.114	1282	6.261	1400	5.908	1269	4.628
1458	6.205	1321	5.571	1263	6.049	1369	7.410
1387	5.884	1417	5.927	1418	4.571	1302	6.342
1385	6.831	1377	6.828	1419	6.895	1390	5.198
AVG.	5.365		5.916		5.778		5.622

acid in early lactation had significantly increased production of volatile fatty acids (Table 23). This increase in total volatile fatty acids was due to significant increases in acetate, isobutyrate, butyrate, 2-methyl butyrate and isovalerate (Tables 24, 26, 27, 28, and 29). Propionate and valerate were also higher but treatment effects were not significant (Tables 25 & 30).

The mode of action of malic acid is not clear. It increases volatile acid production in early lactation cows. Malic acid could trap free ruminal ammonia and increase microbial protein synthesis or it may supply a source of oxalacetate for gluconeogenesis by the rumen microbes. The nitrogen balance trial was next undertaken to determine more definitely the effect of malic acid on the rumen fermentation.

III. Nitrogen Balance Trial

Malic acid has been shown to increase nitrogen retention in growing steers and heifers and enhance milk yields in lactating cows. It has been proposed that malic acid promotes more efficient utilization of ammonia by rumen microbes. However, a more efficient uptake of ammonia by the rumen microbes was not reflected in the data taken from the lactating cows. Thus, the nitrogen balance trial was designed to examine the effects of malic acid on the plasma urea, plasma ammonia, rumen ammonia and volatile fatty acid production as well as nitrogen metabolism and digestibility.

The fistulated steers (420 kilograms) consumed approximately eighteen kilograms feed per head per day (Table 31). Their urinary excretion rate was about eight kilograms per head per day (Table 31). Their fecal excretion rate was approximately ten kilograms per head per day (Table 31).

Table 23. The effects of malic acid on the average concentration of total VFA content in mmoles/100 ml of rumen fluid for early lactation (cow # is on left side of column).

A		B		C		D	
1430	7.848	1345	9.056	1350	8.743	1442	8.318
1359	6.612	1435	10.556	1448	9.461	1328	10.556
1397	8.504	1456	8.699	1352	11.233	1364	7.604
1407	6.796	1376	9.639	1449	7.622	1410	9.127
AVG.	7.440		9.488*		9.265*		8.901**

Standard error = 0.48

* Significant at p .05

**Significant at p .10

Table 24. The effects of malic acid on the average concentration of acetate in mmoles/100 ml rumen fluid for early lactation (cow # is on left side of column).

A		B		C		D	
1430	4.663	1345	5.545	1350	5.409	1442	5.405
1359	4.282	1435	6.014	1448	6.216	1328	5.605
1397	4.819	1456	5.519	1352	6.263	1364	4.619
1407	4.125	1376	5.666	1449	4.912	1410	5.765
AVG.	4.472		5.686*		5.700*		5.348*

Standard error = 0.18

* p .05

Table 25. The effects of malic acid on the average concentration of propionate in mmoles/100 ml rumen fluid for early lactation (cow # is on left side of column).

A		B		C		D	
1430	1.979	1345	1.963	1350	1.840	1442	1.597
1359	1.306	1435	3.052	1448	1.759	1328	3.092
1397	2.400	1456	1.709	1352	3.115	1364	1.653
1407	1.486	1376	2.377	1449	1.438	1410	1.752
AVG.	1.793		2.275		2.038		2.024

Standard error = 0.28

Table 26. The effects of malic acid on the average concentration of isobutyrate in mmoles/100 ml rumen fluid for early lactation (cow # is on the left side of column).

A		B		C		D	
1430	.057	1345	.089	1350	.082	1442	.072
1359	.055	1435	.065	1448	.083	1328	.067
1397	.055	1456	.077	1352	.071	1364	.072
1407	.062	1376	.066	1449	.071	1410	.082
AVG.	.057		.074*		.077*		.073*

Standard error = 0.004

* p .05

Table 27. The effects of malic acid on the average concentration of butyrate in mmoles/100 ml rumen fluid for early lactation (cow # is on the left side of column).

A		B		C		D	
1430	.975	1345	1.184	1350	1.152	1442	1.005
1359	.811	1435	1.160	1448	1.156	1328	1.282
1397	.981	1456	1.142	1352	1.409	1364	.985
1407	.903	1376	1.211	1449	.979	1410	1.309
AVG.	.918		1.174*		1.174*		1.145*

Standard error = 0.053

* p .05

Table 28. The effects of malic acid on the average concentration of 2-methyl butyrate in mmoles/100 ml rumen fluid for early lactation (cow # is on the left side of column).

A		B		C		D	
1430	.059	1345	.118	1350	.083	1442	.087
1359	.051	1435	.082	1448	.084	1328	.077
1397	.065	1456	.084	1352	.111	1364	.109
1407	.083	1376	.079	1449	.079	1410	.069
AVG.	.065		.091*		.088*		.086**

Standard error = 0.008

* p .05

**p .10

Table 29. The effects of malic acid on the average concentration of isovalerate in mmoles/100 ml rumen fluid for early lactation (cow # is on the left side of column).

A		B		C		D	
1430	.042	1345	.069	1350	.064	1442	.054
1359	.045	1435	.044	1448	.060	1328	.049
1397	.037	1456	.060	1352	.058	1364	.062
1407	.042	1376	.056	1449	.054	1410	.062
AVG.	.042		.057*		.059*		.057*

Standard error = 0.004

* p .05

Table 30. The effects of malic acid on the average concentration of valerate in mmoles/100 ml rumen fluid for early lactation (cow # is on the left side of column).

A		B		C		D	
1430	.072	1345	.102	1350	.112	1442	.099
1359	.071	1435	.139	1448	.104	1328	.185
1397	.139	1456	.105	1352	.207	1364	.105
1407	.096	1376	.183	1449	.094	1410	.107
AVG.	.095		.132		.129		.124

Standard error = 0.018

Table 31. The effect of malic acid on feed intake, feces and urine excretion.

TREATMENT	L	H	H	C	C	L
PERIOD II						
FEED (kg)	17.0	18.6	14.8	24.6	19.8	17.6
FECES (kg)	9.6	7.3	7.1	14.8	11.6	10.6
URINE (kg)	5.4	8.5	6.4	9.2	6.2	7.1
TREATMENT	C	L	L	H	H	C
PERIOD III						
FEED (kg)	14.2	21.0	18.7	19.8	23.0	18.6
FECES (kg)	8.2	9.8	8.9	14.3	13.6	11.2
URINE (kg)	6.4	12.2	6.3	10.8	6.1	6.9
TREATMENT	H	C	C	L	L	H
PERIOD IV						
FEED (kg)	13.6	20.1	12.1	12.4	18.0	18.6
FECES (kg)	6.1	10.0	4.7	5.8	10.2	8.7
URINE (kg)	6.9	16.2	7.1	8.6	6.7	5.3

Percent dry matter digestibility of the ration increased with the level of malic acid fed but the treatment differences were not significant (Table 33).

The digestibility of acid detergent fiber in the ration increased as the level of malic acid fed increased but the treatment differences were not significant (Table 34).

Animals receiving malic acid showed trends of increased nitrogen retention as a percent of total nitrogen (fed and absorbed) but treatment differences were not significant (Tables 35 and 36).

Percent protein digestibility of the ration was not different between treatments (Table 37).

Rumen ammonia, plasma urea and plasma ammonia concentrations were not significantly different between treatments (Tables 38, 39 and 40).

Rumen propionate was significantly higher in animals receiving malic acid than controls (Tables 41, 42 and 43).

These results demonstrate that malic acid is affecting the rumen fermentation not by aiding in the incorporation of free ammonia but by stimulating the production of the volatile fatty acid propionate. This would increase gluconeogenesis in the rumen and liver and thus, increase the efficiency of the rumen fermentation.

Table 32. The effect of malic acid on daily dry matter intake (kg).

ANIMAL	TREATMENT		
	CONTROL	LOW	HIGH
435	6.0	7.8	5.6
436	8.3	8.9	8.6
437	5.0	8.0	6.8
438	11.4	5.1	8.4
439	9.7	7.4	9.1
440	7.9	8.1	7.7
AVERAGE	8.0	7.6	7.7

Standard error = 1.6

Table 33. The effects of malic acid on percent dry matter digestibility.

ANIMAL	TREATMENT		
	CONTROL	LOW	HIGH
435	71.4	74.3	77.2
436	75.0	78.7	83.7
437	79.4	77.3	79.4
438	74.2	77.0	66.6
439	74.6	74.7	74.6
440	71.8	72.1	78.1
AVERAGE	74.4	75.7	76.6

Standard error = 1.33

Table 34. The effects of malic acid on percent acid detergent fiber digestibility.

ANIMAL	TREATMENT		
	CONTROL	LOW	HIGH
435	56.7	60.3	62.7
436	61.8	63.6	69.8
437	74.9	67.8	63.0
438	57.4	68.0	43.8
439	52.3	58.1	55.3
440	45.7	46.6	65.1
AVERAGE	58.1	60.7	60.0

Standard error = 2.6

Table 35. The effects of malic acid on nitrogen retained as a percent of nitrogen fed (total nitrogen retained).

ANIMAL	TREATMENT		
	CONTROL	LOW	HIGH
435	29.4	38.7	27.1
436	24.9	49.9	45.0
437	17.7	44.6	11.6
438	34.4	21.9	36.6
439	49.3	37.4	46.2
440	42.0	33.0	42.6
AVERAGE	33.0	37.5	34.9

Standard error = 3.94

Table 36. The effect of malic acid on nitrogen retained as a percent of nitrogen absorbed (% digestible nitrogen retained).

ANIMAL	TREATMENT		
	CONTROL	LOW	HIGH
435	42.8	57.8	37.2
436	36.1	64.7	58.8
437	24.1	59.6	16.1
438	52.6	31.1	61.9
439	67.3	51.2	65.8
440	59.5	49.5	56.9
AVERAGE	47.1	52.3	50.7

Standard error = 5.05

Table 37. The effect of malic acid on percent protein digestibility.

ANIMAL	TREATMENT		
	CONTROL	LOW	HIGH
435	68.6	66.9	72.9
436	69.1	77.1	69.7
437	73.6	74.8	72.2
438	65.4	70.4	59.1
439	73.2	73.0	70.2
440	70.6	68.0	74.8
AVERAGE	70.1	71.7	69.8

Standard error = 1.53

Table 38. The effect of malic acid on rumen ammonia (mg %).

ANIMAL	TREATMENT		
	CONTROL	LOW	HIGH
435	15.19	19.77	13.94
436	18.43	17.99	14.12
437	17.33	19.47	20.07
438	17.35	13.83	19.02
439	8.74	11.32	15.44
440	15.58	13.00	11.78
AVERAGE	15.44	15.90	15.73

Table 39. The effect of malic acid on plasma urea (mg %).

ANIMAL	TREATMENT		
	CONTROL	LOW	HIGH
435	7.30	8.95	6.81
436	10.95	10.18	5.88
437	7.27	10.60	11.08
438	6.77	7.11	5.59
439	7.91	9.37	8.97
440	6.77	4.76	8.66
AVERAGE	7.83	8.50	7.83

Table 40. The effects of malic acid on plasma NH_3 (ug/100 ml).

ANIMAL	TREATMENT		
	CONTROL	LOW	HIGH
435	116.7	88.1	123.6
436	125.8	88.3	74.0
437	138.8	101.1	70.2
438	83.5	134.7	110.7
439	62.7	115.7	98.4
440	97.4	91.1	138.1
AVERAGE	104.2	103.2	102.5

Table 41. The effect of malic acid on rumen volatile fatty acid production (millimoles/100 ml).

	CONTROL						
	435	436	437	438	439	440	AVERAGE
ACETATE	6.780	7.110	6.654	5.322	3.682	6.231	5.963
PROPIONATE	1.727	1.877	1.844	1.643	1.320	1.361	1.629
ISOBUTYRATE	0.092	0.159	0.187	0.082	0.053	0.108	0.114
BUTYRATE	1.309	0.992	1.049	1.331	0.828	0.894	1.067
2-METHYL BUTYRATE	0.090	0.229	0.209	0.095	0.068	0.136	0.138
ISOVALERATE	0.084	0.124	0.154	0.056	0.034	0.094	0.091
VALERATE	0.171	0.268	0.302	0.255	0.079	0.202	0.213
TOTAL	10.253	10.759	10.399	8.784	6.064	9.026	9.215

Table 42. The effect of malic acid on rumen volatile fatty acid production (millimoles/100 ml).

	LOW						
	435	436	437	438	439	440	AVERAGE
ACETATE	6.803	4.587	5.868	6.199	7.000	5.490	5.991
PROPIONATE	2.022	1.301	1.883	1.894	2.416	1.897	1.902*
ISOBUTYRATE	0.131	0.155	0.156	0.133	0.192	0.080	0.141
BUTYRATE	1.520	0.973	1.123	0.948	1.117	1.197	1.146
2-METHYL BUTYRATE	0.149	0.141	0.131	0.139	0.183	0.172	0.152
ISOVALERATE	0.099	0.121	0.135	0.092	0.151	0.066	0.111
VALERATE	0.187	0.208	0.242	0.238	0.323	0.202	0.233
TOTAL	10.911	7.486	9.538	9.643	11.382	9.104	9.676

* p .05

Table 43. The effect of malic acid on rumen volatile fatty acid production (millimoles/100 ml).

	HIGH						
	435	436	437	438	439	440	AVERAGE
ACETATE	7.671	5.733	6.272	4.700	5.477	7.159	6.169
PROPIONATE	4.175	2.660	1.923	1.738	1.459	3.296	2.542*
ISOBUTYRATE	0.125	0.080	0.111	0.195	0.136	0.148	0.132
BUTYRATE	1.358	1.526	1.203	0.659	0.857	1.278	1.147
2-METHYL BUTYRATE	0.162	0.139	0.137	0.075	0.115	0.200	0.138
ISOVALERATE	0.132	0.072	0.139	0.087	0.087	0.144	0.110
VALERATE	0.414	0.224	0.152	0.099	0.151	0.377	0.236
TOTAL	14.037	10.434	9.937	7.553	8.282	12.602	10.474

* p .05

DISCUSSION

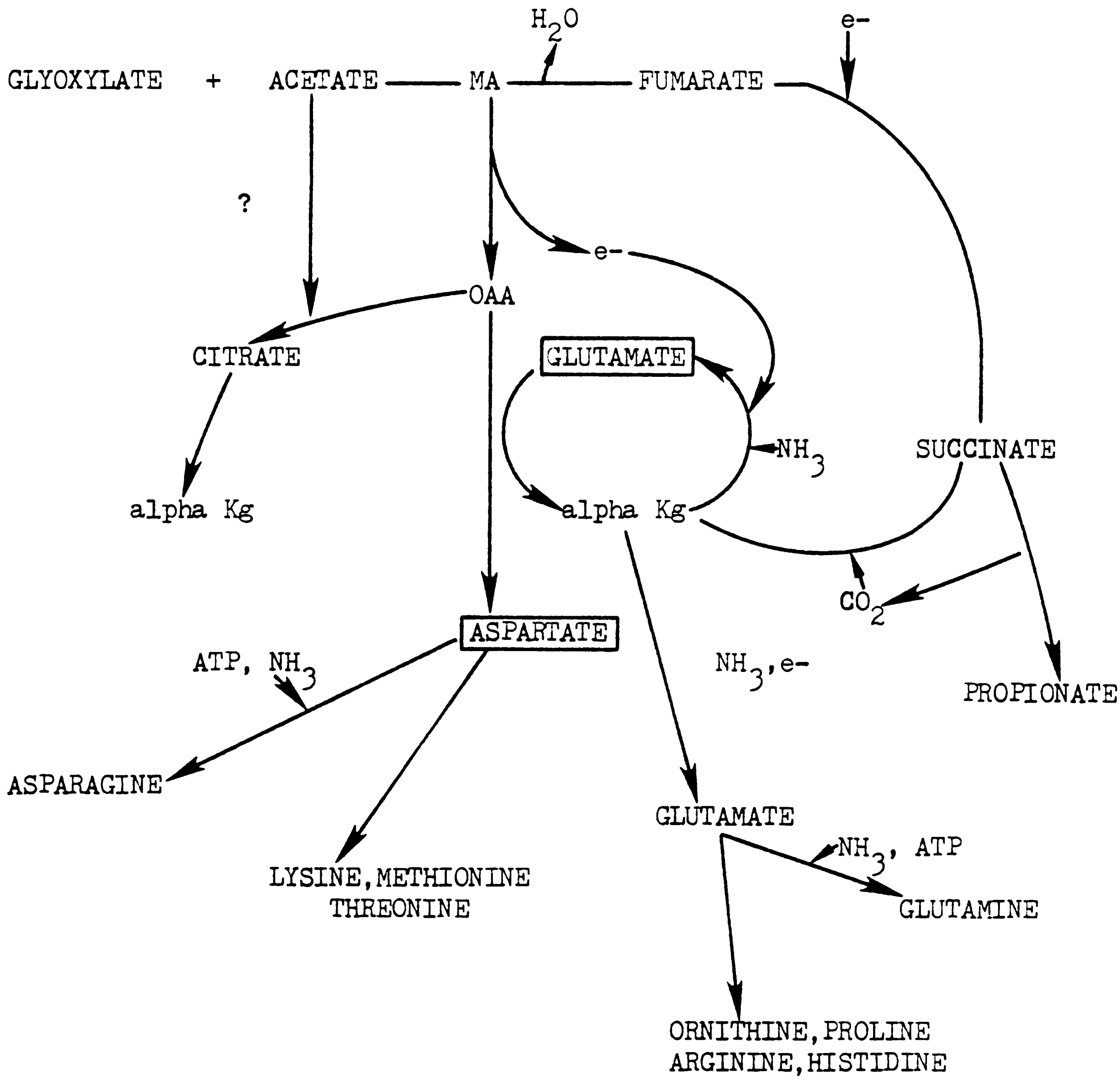
In the rumen alpha-ketoglutarate is considered to be the universal compound for trapping free ammonia for formation of amino groups for microbial protein synthesis. However, there has always been a question as to what is the source of alpha-ketoglutarate in the rumen. It is not from citrate since anaerobic bacteria do not oxidize compounds to carbon dioxide and water and Krebs cycle enzymes are not present in the rumen to any great extent.

However, succinate is a major metabolic intermediate in the rumen and malic acid can provide a source of succinate. Allison has shown that succinate can be directly carboxylated to form alpha-ketoglutarate (2). Also, Wolin demonstrated that certain rumen bacteria, when grown on lactate media, have increased growth when supplied malic acid (22). This is due to an oxalacetate deficiency. Oxalacetate is drawn off for formation of various cellular constituents namely carbohydrate. Malic acid is thus providing a source of oxalacetate.

The aspartate transamination, like the glutamate transamination, may be important but its significance is not known at this time. Glutamate and aspartate carbon are utilized for the synthesis of several essential amino acids.

Thus, malic acid, for the reasons previously mentioned, is a key intermediate in rumen fermentation and deserves further study both *in vitro* and *in vivo*.

REACTIONS IN THE RUMEN



SUMMARY AND CONCLUSIONS

In vitro rumen fermentation experiments, one milk production study, and one nitrogen balance trial were conducted to investigate the effects of malic acid on rumen fermentation and milk production.

1. The *in vitro* experiments showed that malic acid increases the fermentation rate by increasing gas and volatile fatty acid production.

2. Malic acid significantly increased the persistency of lactation over controls (95 vs. 88%). The early lactation cows receiving malic acid had increased total volatile fatty acid production due to significant increases in acetate, isobutyrate, butyrate, 2-methyl butyrate and isovalerate. Propionate was also higher but treatment effects were not significant.

3. The nitrogen balance trial was undertaken to determine the mode of action of malic acid in ruminants. Malic acid treatments significantly increased rumen propionate over controls.

In conclusion further research is needed to determine if malic acid could be incorporated into rations for growing beef cattle. This study indicates malic acid increases propionate production which has been shown to increase growth rates of growing beef cattle. Malic acid increased lactation persistency seven percent. Manufacturing and marketing costs for malic acid would have to be lower than the income from the additional milk produced to make malic acid profitable for lactating dairy cattle.

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APPENDIX

Table 44. The effects of malic acid on the analysis of variance for persistency of lactation for all cows.

AOV - Persistency

Observation	d.f.	s.s.	m.s.
Trt	3	233.92	77.97
S of L	1	1530.42	1530.42
(Trt)x(S of L)	3	91.47	30.49
Block	3	653.61	217.87
Error(8-1)(4-1)	21	891.28	42.44
Total	31		

Treatment

Block	C	1	2	3
1	2	2	2	2
3	2	2	2	2
4	2	2	2	2

$$SS_{TL} = 379.3^2 + 325^2 + 397.8^2 + 320.4^2 +$$

$$\frac{359.4^2 + 400.9^2 + 332.2^2 + 380.3^2}{4} - 261{,}961.31 - 233.92 - 1530.42$$

$$SS_{TL} = 263{,}817.12 - 263{,}725.65 = 91.47$$

Table 45. The effects of malic acid on the persistency (blocked according to milk production) (cow # is on the left side of column) and sum of squares of persistency is for all cows.

1		2		3		4	
1397	85.5	1359	95.8	1430	100.0	1407	98.0
1345	87.6	1435	98.5	1456	94.6	1376	117.1
1352	89.4	1350	92.0	1448	103.5	1449	95.4
1328	88.1	1442	104.7	1410	106.3	1364	101.8
1385	85.9	1415	73.6	1387	78.1	1458	87.4
1377	69.2	1417	91.1	1282	74.3	1321	85.8
1419	81.4	1263	77.8	1418	82.0	1400	91.0
1302	82.6	1390	88.1	1269	94.4	1369	94.3
X	669.7		721.6		733.2		770.8

$$SS_B = \frac{669.7^2 + 721.6^2 + 733.2^2 + 770.8^2}{8}$$

$$SS_B = 262{,}614.92 - 261{,}961.31 = 653.61$$

$$SS_L = \frac{1558.3^2 + 1337.0^2}{16} - 261{,}961.31 =$$

$$SS_L = 263{,}491.73 - 261{,}961.31 = 1530.42$$

Table 46. The effects of malic acid on test of the significance of persistency of lactation for all cows.

H: $t_i = 0$ (trt)

$$f = \frac{MS_t}{MS_e} \quad \text{vs } f\ ,\ t-1,\ (t-1)\ (r-1)$$

$$f = \frac{77.97}{42.44} = 1.84 \qquad f \quad .25,\ 3,\ 21 = 1.48$$

$$f \quad .10,\ 3,\ 21 = 2.36$$

Standard error of treatment means

$$yi \pm \quad \frac{MSE}{r} = \frac{42.44}{8} = 2.3$$

	Treatment		
A	B	C	D
88.2±2.3	89.6±2.3	89.3±2.3	95.0±2.3

Did blocks decrease experimental error used to test treatment effects?

$$f = \frac{MS_D}{MS_E} = \frac{217.87}{42.44} = 5.13 \qquad \text{vs. } f\ ,\ r-1,\ (t-1)\ (r-1)$$

$$f \quad .025,\ 3,\ 21 = 3.82$$

$$f \quad .005,\ 3,\ 21 = 5.73$$

What about treatment block interaction? It must be not significant in order to use MS_E to test treatments.

$$SS_{NA} = \frac{32 \quad 207.2^2}{(233.92)(653.61)} = 8.98$$

$$f = \frac{8.98}{891.28 - 8.98/20} \qquad \text{vs}$$

$$f \quad .25,\ 1,\ 20 = 1.40$$

$$f = 8.98/44.1 = 0.20$$

∴ treatments and blocks don't interact

Test of interaction of treatments and stage of lactation

$$f = \frac{MS_{TXL}}{MS_E} = \frac{30.49}{42.44} = 0.72 \quad \text{vs} \quad f \quad .50,\ 3,\ 21 = .815$$

$\therefore$ no interaction exists between treatments and stage of lactation

Table 47. The effects of malic acid on the orthogonal test of persistency of lactation for all cows used to determine which treatments are different.

A vs all others

-3, 1, 1, 1

$(+3\ (705.6) = (716.8 + 714.4 + 760))^2 = 5535.4$

$\frac{5535.4}{96} = 57.7 \qquad \frac{57.7}{42.4} = 1.36$

B vs C & D

0, -2, 1, 1

$(+2\ (716.8) - (714.4 + 760))^2 = 1664.6$

$\frac{1664.6}{48} = 34.7 \qquad \frac{34.7}{42.4} = 0.82$

C vs D

0, 0. 1. -1

$(714.4 - 760)^2 = 2079.4$

$\frac{2079.4}{16} = \frac{130.0}{42.4} = 3.1 \qquad P \quad .10$

D vs A, B, C

-3, 1, 1, 1

$(3\ (760) - (2136.8))^2 = 20{,}506.2$

$\frac{20{,}506.2}{96} = 213.6 \qquad \frac{213.6}{42.4} = 5.0 \qquad P \quad .05$

C.V. = f 1, n-t

32-

1, 21

A vs D

-1, 0, 0, 1

$(705.6 - 760)^2 = 2959.4$

$\frac{2959.4}{16} = 185.0 \qquad \frac{185}{42.4} = 4.36 \qquad P \quad .05$

Linear Dose Response

0, -1, 0, 1

$(-716.8 + 760)^2 = 1866.2 \qquad f \quad .10, 1, 21 = 2.96$

$\frac{1866.2}{16} = \frac{116.6}{42.4} = 2.75 \qquad P \quad .12$

Table 48. The effects of malic acid on the analysis of variance for total dry matter intake (all cows).

Observation	d.f.	s.s.	m.s.
Trt	3	13.3	4.4
S of L	1	6.2	6.2
(Trt) x (S of L)	3	10.9	3.6
Block	3	75.3	25.1
Error	21	260.9	12.4
Total	31		

$$SS_y = 54{,}094.6 - 53{,}728.0 = 366.6$$

$$SS_T = \frac{(331.04^2 + 325.6^2 + 335.7^2 + 318.8^2)}{8} = 53{,}728 = 13.3$$

$$SS_B = 53{,}803.3 - 53{,}728 = 75.3$$

$$SS_L = \frac{662.61^2 + 648.61^2}{16} - 53{,}728 = 6.2$$

$$SS_{TxS} = 53{,}758.4 - 53{,}728 - 13.3 - 6.2 = 10.9$$

H: $t_i = 0$ (For DMI)

$$f = \frac{MST}{MSE} \text{ vs } f\ \ ,\ t-1,\ (t-1)\ (r-1)$$

$$f = \frac{4.4}{12.4} = 0.35 \qquad f_1,\ 50,\ 3,\ 21 = 0.8$$

No differences

Std. error for Total DMI

$$\frac{MSE}{r} = 1.24$$

Table 49. The effects of malic acid on the analysis of variance for roughage dry matter intake (all cows).

Observation	d.f.	s.s.	m.s.
Trt	3	11.7	3.9
S of L	1	108.1	108.1
(Trt) x (S of L)	3	23.5	7.8
Block	3	80.2	26.7
Error	21	1725.4	82.2
Total	31		

$SS_y = 14,667.8 - 12,718.9 = 1948.9$

$SS_T = 12,730.6 - 12,718.9 = 11.7$

$SS_B = 12,799.1 - 12,718.9 = 80.2$

$SS_L = \frac{(290^2 + 348.04^2)}{16} - 12,718.9 = 108.1$

$SS_{TxS} = \frac{(21,103.8 + 30,345.1)}{4} - 12,718.9 - 11.7 - 108.1 = 23.5$

H: t_i = (For R DMI)

$f = \frac{MST}{MSE}$ vs f , t-1, (t-1) (r-1)

$f = \frac{3.9}{82.2} = 0.05$ f 50, 3, 21 = 0.8

No differences

H: L = 0=0

$f = \frac{108.1}{82.2} = 1.3$ f .25, 1, 21 = 1.40

Stage of lactation tends to influence roughage DMI

Std. error for R. DMI = 3.20

Table 50. The effects of malic acid on analysis of variance for total dry matter intake/100 kg body weight for all cows.

Observation	d.f.	s.s.	m.s.
Trt	3	0.613	0.204
S of L	1	0.949	0.949
(Trt) x (S of L)	3	0.155	0.052
Block	3	0.037	0.012
Error	21	2.795	0.133
Total	31		

$$SS_y = 307.461 - (302.912) = 4.549$$

$$SS_T = \frac{26.457^2 + 24.367^2 + 24.106^2 + 23.524^2}{8} - 302.912 = 0.613$$

$$SS_B = \frac{24.617^2 + 24.886^2 + 24.780^2 + 24.171^2}{8} - 302.912 = 0.037$$

$$SS_L = \frac{51.982^2 + 46.472^2}{16} - 302.912 = 0.949$$

$$SS_{TxS} = \frac{13.524^2 + 13.103^2 + 13.035^2 + 12.320^2 + 12.933^2 + 11.264^2 + 11.071^2 + 11.204^2}{4} - 302.912 - 0.613 -$$

$$0.949 = 0.155$$

H: $t_i = 0$ (For total DMI/100 kg BW)

$$f = \frac{MS_T}{MS_E} \text{ vs. } f\ ,\ t-1,\ (t-1)\ (r-1)$$

$$f = \frac{0.204}{0.133} = 1.53 \qquad f\ \ .25,\ 3,\ 21 = 1.48$$

$$f\ \ .10,\ 3,\ 21 = 2.36$$

Std. error (for total DMI/100 kg bw)

$$= \frac{MS_E}{r} = .129$$

Table 51. The orthogonal tests of total dry matter intake/100 kg body weight used to determine which treatments are different.

<u>A vs all others</u>
-3, 1, 1, 1

$$(3\ (26.457) - (24.367 + 24.106 + 23.524))^2 = 54.376$$

$$\frac{54.376}{12 \times 8} = 0.566 \qquad \frac{0.566}{0.133} = 4.26 \qquad P \quad .05$$

<u>B vs C & D</u>
0, -2, 1, 1

$$(2\ (24.367) - (24.106 + 23.524))^2 = 1.219$$

$$\frac{1.219}{6 \times 8} = 0.025 \qquad \frac{.025}{.133} = 0.188$$

<u>C vs D</u>
0, 1-1

$$(24.106 - 23.524)^2 = 0.159$$

$$\frac{.339}{2 \times 8} = 0.021 \qquad \frac{.021}{.133} = 0.159$$

C.V. = f , 1, 21

$$f_1\ .05,\ 1,\ 21 = 4.32$$
$$f_1\ .10,\ 1,\ 21 = 2.96$$
$$f_1\ .25,\ 1,\ 21 = 1.40$$

<u>D vs all others</u>
-3, 111

$$(3\ (23.524) - (26.457 + 24.367 + 24.106))^2 = 18.992$$

$$\frac{18.992}{96} = .198 \qquad \frac{.198}{.133} = 1.49 \qquad P \quad .25$$

A vs B

$(26.457 - 24.367)^2 = 4.368$

$\frac{4.368}{16} = 0.273 \qquad \frac{0.273}{.133} = 2.05 \qquad P \quad .25$

A vs C

$(26.457 - 24.106)^2 = 5.527$

$\frac{5.527}{16} = 0.345 \qquad \frac{0.345}{.133} = 2.59 \qquad P \quad .25$

Linear Dose Response

0, -1, 0, 1

$(24.367 - 23.524)^2 = 0.711$

$\frac{0.711}{16} = 0.044 \qquad \frac{.044}{0.133} = 0.331$

f .50, 1, 21 = .47
No linear response

A vs D

$(26.457 - 23.524)^2 = 8.602$

$\frac{8.602}{16} = 0.538 \qquad \frac{.538}{.133} = 4.04 \qquad P \quad .10$

B vs D

$(24.367 - 23.524)^2 = 0.711$

$\frac{0.711}{16} = 0.044 \qquad \frac{.044}{.133} = 0.33$

Table 52. The effects of malic acid on roughage dry matter intake kg/100 kg body weight for treatment period for all cows (cow # in on the left side of column), and the analysis of variance for roughage dry matter intake/100 kg body weight.

A		B		C		D	
1430	1.527	1345	1.312	1350	1.473	1442	1.625
1359	1.197	1435	1.588	1448	1.525	1328	1.150
1397	1.165	1456	1.468	1352	1.066	1364	1.057
1407	1.651	1376	1.575	1449	1.917	1410	1.418
1415	1.642	1282	1.548	1400	1.590	1269	1.537
1458	1.930	1321	1.430	1263	1.409	1369	1.685
1387	1.623	1417	1.900	1418	1.425	1302	1.313
1385	1.977	1377	1.353	1419	1.202	1390	1.363
AVG.	1.589		1.522		1.451		1.394
X^2	20.811		18.760		17.295		15.874

Tests for roughage DMI/100 kg B.W., all cows

Observation	d.f.	s.s.	m.s.
Trt	3	0.173	0.058
S of L	1	0.153	0.153
(Trt) x (S of L)	3	0.259	0.086
Block	3	0.356	0.119
Error	21	0.872	0.042
Total	31		

$$SS_y = 72.740 - \frac{(47.641^2)}{32} = 1.813$$

$$SS_T = \frac{12.712^2 + 12.174^2 + 11.607^2 + 11.148^2}{8} - 70.927 = 0.173$$

$$SS_B = \frac{10.538^2 + 12.197^2 + 12.071^2 + 12.835^2}{8} - 70.927 = 0.356$$

$$SS_L = \frac{22.714^2 + 24.927^2}{16} - 70.927 = 0.153$$

$$SS_{TxS} = \frac{5.54^2 + 5.943^2 + 5.981^2 + 5.25^2 + 7.172^2 + 6.231^2 + 5.626^2 + 5.898^2}{4} - 70.927 - 0.173 - 0.153 =$$

0.259

H: $t_i = 0$ (for roughage DMI/100 kg BW)

$$f = \frac{MS_T}{MS_E} \quad \text{vs.} \quad f\ , t-1, (t-1)(r-1)$$

$$f = \frac{.058}{.042} = 1.38 \qquad f\ .50, 3, 21 = 0.815$$

$$f\ .25, 3, 21 = 1.48$$

Standard error for roughage DMI/100 kg BW = 0.072

Table 53. The effects of malic acid on % fat in milk (cow # is on the left side of column) and the analysis of variance for % fat in milk for all cows.

A		B		C		D	
1430	3.08	1345	3.34	1350	3.58	1442	3.37
1359	3.07	1435	3.31	1448	3.26	1328	2.81
1397	2.65	1456	3.66	1352	2.77	1364	3.66
1407	3.38	1376	3.61	1449	3.88	1410	3.62
1415	3.39	1282	3.12	1400	3.58	1269	3.61
1458	4.10	1321	3.82	1263	3.82	1369	3.10
1387	3.89	1417	3.81	1418	3.55	1302	3.69
1385	3.38	1377	3.45	1419	3.48	1390	3.76
AVG.	3.37		3.52		3.49		3.45
X^2	92.217		99.285		98.293		96.149

Tests for % fat

Observation	d.f.	s.s.	m.s.
Trt	3	0.100	0.033
S of L	1	0.633	0.633
(trt) x (S of L)	3	0.381	0.127
Block	3	0.844	0.281
Error	21	1.725	0.082
Total	31		

$$SS_y = \frac{385.944 - (110.60^2)}{32} = 385.944 - 382.261 = 3.683$$

$$SS_T = \frac{26.94^2 + 28.12^2 + 27.92^2 + 27.62^2}{8} - 382.261 = 0.100$$

$$SS_B = \frac{25.57^2 + 28.11^2 + 27.79^2 + 29.13^2}{8} - 382.261 = 0.844$$

$$SS_L = \frac{53.05^2 + 57.55^2}{16} - 382.261 = 0.633$$

$$SS_{TxS} = \frac{12.18^2 + 13.92^2 + 13.49^2 + 13.46^2 + 14.76^2 + 14.20^2 + 14.43^2 + 14.16^2}{4} - 382.261 - 0.1 - 0.633 =$$

0.381

H: $t_i = 0$ (for % fat)

$$f = \frac{MS_T}{MS_E} \quad \text{vs.} \quad f\ ,\ t\text{-}1\ (t\text{-}1)(r\text{-}1)$$

$$f = \frac{0.033}{0.082} = 0.40 \qquad f\ \ .50,\ 3,\ 21 = 0.815$$

No difference between treatments

$$\text{Standard error} = \frac{MS_E}{r} = 0.10$$

Table 54. The effects of malic acid on % protein in milk (cow # is on the left side of column) and the analysis of variance for % protein in milk for all cows.

A		B		C		D	
1430	3.03	1345	2.86	1350	3.14	1442	3.17
1359	3.06	1435	3.22	1448	3.44	1328	3.13
1397	3.03	1456	3.35	1352	2.98	1364	3.19
1407	3.10	1376	3.23	1449	3.25	1410	3.07
1415	3.41	1282	3.26	1400	3.66	1269	3.32
1458	4.12	1321	3.93	1263	3.94	1369	3.79
1387	3.71	1417	3.68	1418	3.39	1302	3.79
1385	3.69	1377	3.38	1419	3.71	1390	3.79
AVG.	3.39		3.36		3.44		3.41
X^2	93.318		91.243		95.312		93.562

Test for % protein in milk

Observation	d.f.	s.s.	m.s.
Trt	3	0.023	0.008
S of L	1	2.163	2.163
(trt) x (S of L)	3	0.085	0.028
Block	3	0.248	0.083
Error	21	0.860	0.041
Total	31		

$$SS_y = 373.435 - \frac{(108.82^2)}{32} = 3.379$$

$$SS_T = \frac{27.15^2 + 26.91^2 + 27.51^2 + 27.25^2}{8} - 370.056 = 0.023$$

$$SS_B = \frac{26.57^2 + 27.41^2 + 26.57^2 + 28.27^2}{8} - 370.056 = 0.248$$

$$SS_L = \frac{50.25^2 + 58.57^2}{16} - 370.056 = 2.163$$

$$SS_{TxS} = \frac{12.22^2 + 12.66^2 + 12.81^2 + 12.56^2 + 14.93^2 + 14.25^2 + 14.70^2 + 14.69^2}{4} - 370.056 - .023 - 2.163 =$$

0.085

H: $t_i = 0$ (For % protein in milk)

$$f = \frac{MS_T}{MS_E} \quad \text{vs} \quad f\ ,\ t-1\ (t-1)\ (r-1)$$

$$f = \frac{0.008}{0.041} = 0.195 \qquad f\ \ .50,\ 3,\ 21 = 0.815$$

No differences between treatments

$$\text{Standard error} = \frac{MS_E}{r} = 0.07$$

Table 55. The effects of malic acid on % total solids in milk (cow # is on the left side of column) and the analysis of variance for % total solids in milk for all cows.

A		B		C		D	
1430	11.61	1345	11.93	1350	12.65	1442	12.02
1359	11.77	1435	12.08	1448	12.09	1328	12.02
1397	10.71	1456	12.73	1352	11.12	1364	12.66
1407	12.17	1376	12.56	1449	12.93	1410	12.31
1415	12.15	1282	11.54	1400	12.60	1269	12.46
1458	13.43	1321	13.08	1263	13.01	1369	12.07
1387	12.96	1417	12.51	1418	11.68	1302	12.67
1385	12.57	1377	12.25	1419	12.65	1390	13.23
AVG.	12.17		12.34		12.34		12.43
X^2	1190.09		1218.88		1221.49		1237.27

Test for total solids in milk

Observation	d.f.	s.s.	m.s.
Trt	3	0.279	0.093
S of L	1	1.758	1.758
(trt) x (S of L)	3	1.600	0.533
Block	3	2.218	0.739
Error	21	5.331	0.254
Total	31		

$$SS_y = 4867.73 - \frac{(394.22^2)}{32} = 11.186$$

$$SS_T = \frac{97.37^2 + 98.68^2 + 98.73^2 + 99.44^2}{8} - 4856.544 = 0.279$$

$$SS_B = \frac{95.92^2 + 99.42^2 + 97.38^2 + 101.50^2}{8} - 4856.544 = 2.218$$

$$SS_L = \frac{193.36^2 + 200.86^2}{16} - 4856.544 = 1.758$$

$$SS_{TxS} = \frac{46.26^2 + 49.3^2 + 48.79^2 + 49.01^2 + 51.11^2 + 49.38^2 + 49.94^2 + 50.43^2}{4} - 4856.544 - 0.279 - 1.758 =$$

1.600

H: $t_i = 0$ (for % total solids in milk)

$$f = \frac{MS_T}{MS_E} \quad \text{vs.} \quad f \quad t-1, (t-1)(r-1)$$

$$f = \frac{0.093}{0.254} = 0.366 \qquad f \quad .50, 3, 21 = 0.815$$

No differences between treatments but may have a treatment - S of L interaction P .25

Standard error = 0.18

Table 56. The effects of malic acid on plasma glucose concentration in milligrams/100 ml of plasma (cow # is on the left side of column) and the analysis of variance for plasma glucose concentration for all cows.

A		B		C		D	
1430	41.8	1345	48.1	1350	45.7	1442	43.6
1359	50.7	1435	45.5	1448	46.1	1328	44.4
1397	45.9	1456	43.0	1352	43.1	1364	49.8
1407	48.2	1376	44.8	1449	44.2	1410	47.2
1415	44.3	1282	53.1	1400	45.6	1269	52.1
1458	52.3	1321	49.9	1263	50.3	1369	46.8
1387	48.6	1417	51.6	1418	55.0	1302	48.1
1385	51.7	1377	49.6	1419	51.0	1390	43.1
AVG.	47.94		48.20		47.62		46.89
X^2	18480.41		18672.24		18260.40		17656.07

Test for plasma glucose

Observation	d.f.	s.s.	m.s.
Trt	3	7.737	2.579
S of L	1	116.285	116.285
(trt) x (S of L)	3	30.188	10.063
Block	3	9.262	3.087
Error	21	210.808	10.038
Total	31		

$$SS_y = 73{,}069.12 - \underset{72{,}694.84}{\frac{(1525.2)^2}{32}} = 374.28$$

$$SS_T = \frac{383.5^2 + 385.6^2 + 381.0^2 + 375.1^2}{8} - 72{,}694.84 = 7.737$$

$$SS_B = \frac{381.9^2 + 374.8^2 + 386.9^2 + 381.6^2}{8} - 72{,}694.84 = 9.262$$

$$SS_L = \frac{732.1^2 + 793.1^2}{16} - 72{,}694.84 = 116.285$$

$$SS_{TxS} = \frac{186.6^2 + 181.4^2 + 179.1^2 + 185.0^2 + 196.9^2 + 204.2^2 + 201.9^2 + 190.1^2}{4} - 72{,}694.84 - 7.737 - 116.285 = 30.188$$

H: $t_i = 0$ (for plasma glucose)

$$f = \frac{MS_T}{MS_E} \quad \text{vs.} \quad f\ ,\ t-1,\ (t-1)\ (r-1)$$

f = 2.579/10.038 = 0.26 f .50, 3, 21 = 0.82

Hi = interaction

$\frac{MS_{TxL}}{MS_E} = 1.0$ f .25, 3, 21 = 1.48 No differences between treatments

Standard error = 1.12

Table 57. The effects of malic acid on plasma urea concentration in milligrams/100 ml of plasma (cow # is on the left side of column) and the analysis of variance for plasma urea concentration for all cows.

A		B		C		D	
1430	14.66	1345	13.31	1350	11.83	1442	16.45
1359	13.15	1435	14.80	1448	16.48	1328	13.22
1397	13.57	1456	13.77	1352	13.76	1364	11.59
1407	13.52	1376	14.34	1449	14.04	1410	13.15
1415	16.64	1282	15.92	1400	12.76	1269	12.49
1458	17.49	1321	12.98	1263	14.83	1369	13.84
1387	15.28	1417	12.39	1418	17.03	1302	16.18
1385	15.35	1377	15.88	1419	17.23	1390	15.95
AVG.	14.96		14.17		14.74		14.11
X^2	1806.664		1619.058		1767.639		1616.362

Test for plasma ureas

Observation	d.f.	s.s.	m.s.
Trt	3	4.230	1.41
S of L	1	13.261	13.261
(trt) x (S of L)	3	5.173	1.724
Block	3	5.446	1.815
Error	21	57.093	2.719
Total	31		

$$SS_y = 6809.723 - \frac{463.88^2}{32} = 85.203$$

$$SS_T = \frac{119.66^2 + 113.39^2 + 117.96^2 + 112.87^2}{8} - 6724.52 = 4.230$$

$$SS_B = \frac{118.50^2 + 116.04^2 + 118.78^2 + 110.56^2}{8} - 6724.52 = 5.446$$

$$SS_L = \frac{221.64^2 + 242.24^2}{16} - 6724.52 = 13.261$$

$$SS_{TxS} = \frac{54.9^2 + 56.22^2 + 56.11^2 + 54.41^2 + 64.76^2 + 57.17^2 + 61.85^2 + 58.46^2}{4} - 6724.52 - 4.23 - 13.261 =$$

5.173

H: $t_i = 0$ (for plasma ureas)

$$f = \frac{MS_T}{MS_E} \quad \text{vs} \quad f \text{ , } t-1, (t-1)(r-1)$$

$$f = \frac{1.41}{2.719} = 0.52 \qquad f \quad .50, 3, 21 = 0.815$$

No difference between treatments

Standard error = 0.58

Table 58. The effects of malic acid on rumen ammonia concentration in milligrams/100 ml of rumen fluid (cow # is on the left side of column) and the analysis of variance for rumen ammonia concentration for all cows.

A		B		C		D	
1430	10.50	1345	15.27	1350	13.53	1442	23.78
1359	13.97	1435	17.46	1448	16.55	1328	13.37
1397	19.88	1456	22.59	1352	17.33	1364	14.90
1407	13.91	1376	19.06	1449	21.90	1410	20.97
1415	22.62	1282	18.22	1400	13.96	1269	15.68
1458	23.16	1321	14.13	1263	18.56	1369	23.55
1387	16.62	1417	19.54	1418	15.14	1302	16.74
1385	25.28	1377	25.41	1419	23.97	1390	13.09
AVG.	18.242		18.96		17.618		17.76
X^2	2857.466		2970.721		2580.038		2658.037

Test for rumen ammonia

Observation	d.f.	s.s.	m.s.
Trt	3	8.802	2.934
S of L	1	29.453	29.453
(trt) x (S of L)	3	82.437	27.479
Block	3	29.045	9.682
Error	21	380.813	18.134
Total	31		

$$SS_y = 11{,}066.262 - \frac{(580.64^2)}{32} = 530.55$$

$$SS_T = \frac{145.94^2 + 151.68^2 + 140.94^2 + 142.08^2}{8} - 10{,}535.712 = 8.802$$

$$SS_B = \frac{157.25^2 + 142.55^2 + 136.27^2 + 144.57^2}{8} - 10{,}535.712 = 29.045$$

$$SS_L = \frac{274.97^2 + 305.67^2}{16} - 10{,}535.712 = 29.453$$

$$SS_{TxS} = \frac{58.26^2 + 74.38^2 + 69.31^2 + 73.02^2 + 87.68^2 + 77.30^2 + 71.63^2 + 69.06^2}{4} - 10{,}535.712 - 8.802 -$$

$$29.453 = 82.437$$

H: $t_i = 0$ (for rumen ammonia)

$$f = \frac{MS_T}{MS_E} \quad \text{vs} \quad f\ ,\ t-1,\ (t-1)\ (r-1)$$

$$f = \frac{2.934}{18.134} = 0.162 \qquad f\quad .50,\ 3,\ 21 = 0.815$$

No differences between treatments

f for S of L = 1.62 f .25, 3, 21 = 1.48
f for (trt) x (S of L) = 1.52 f .10, 3, 21 = 2.36

Standard error = 1.50

Table 59. The effects of malic acid on rumen pH (cow # is on the left side of column) and the analysis of variance for rumen pH for all cows.

A		B		C		D	
1430	7.1	1345	7.0	1350	7.1	1442	7.1
1359	7.3	1435	6.9	1448	7.0	1328	6.4
1397	7.0	1456	7.1	1352	6.6	1364	7.1
1407	7.3	1376	6.8	1449	7.1	1410	7.1
1415	6.9	1282	7.0	1400	6.9	1269	7.2
1458	7.0	1321	7.0	1263	7.1	1369	6.4
1387	7.2	1417	7.1	1418	7.3	1302	7.0
1385	6.9	1377	6.8	1419	6.8	1390	7.2
$\bar{x}$	7.1		7.0		7.0		6.9
x^2	402.05		387.91		390.93		385.83

Test for Rumen pH

Observation	d.f.	s.s.	m.s.
Trt	3	0.105	0.035
S of L	1	0.0025	0.0025
Block	3	0.4275	0.1425
S_{TxS}	3	0.0725	0.02417
Error	21	0.9113	0.04340
Total	31		

$$SS_y = 1566.72 - \frac{(223.8^2)}{32} = 1.5188$$

$$SS_T = \frac{56.7^2 + 55.7^2 + 55.9^2 + 55.5^2}{8} - 1565.20 = 0.105$$

$$SS_L = \frac{112^2 + 111.8^2}{16} - 1565.2 = 0.0025$$

$$SS_B = \frac{54.6^2 + 56.7^2 + 56.9^2 + 55.6^2}{8} - 1565.2 = 0.4275$$

$$SS_{TxS} = \frac{28.7^2 + 27.8^2 + 27.8^2 + 27.7^2 + 28.0^2 + 27.9^2 + 28.1^2 + 27.8^2}{4} - 0.105 - 0.0025 - 1565.2 =$$

0.0725

Test for Rumen pH

H: $t_i = 0$

$$f = \frac{MS_T}{MS_E} \quad \text{vs.} \quad f\ ,\ t-1,\ (t-1)\ (r-1)$$

$f\ \ .50,\ 3,\ 21 = .815$

$f\ \ .25,\ 3,\ 21 = 1.48$

$$f = \frac{0.035}{0.04340} = 0.81$$

No differences between treatments

Standard error = 0.07

Table 60. The effects of malic acid on the average concentration of total VFA content in mmoles/100 ml rumen fluid (cow # is on the left side of column) and the analysis of variance for total VFA concentration.

A		B		C		D	
1430	7.848	1345	9.056	1350	8.743	1442	8.318
1359	6.612	1435	10.556	1448	9.461	1328	10.556
1397	8.504	1456	8.699	1352	11.233	1364	7.604
1407	6.796	1376	9.639	1449	7.622	1410	9.127
1415	9.638	1282	9.723	1400	9.857	1269	7.533
1458	9.552	1321	8.458	1263	9.111	1369	12.069
1387	8.674	1417	8.996	1418	7.079	1302	9.907
1385	10.520	1377	11.095	1419	11.153	1390	8.355
AVG.	8.518		9.528		9.282		9.184
X^2	593.854		732.125		704.898		692.103

Test for total VFA

Observation	d.f.	s.s.	m.s.
Trt	3	4.470	1.490
S of L	1	4.022	4.022
(trt) x (S of L)	3	5.928	1.976
Block	3	14.264	4.755
Error	21	28.080	1.337
Total	31		

$$SS_y = 2722.980 - \frac{(292.094^2)}{32} = 56.764$$

$$SS_T = \frac{68.144^2 + 76.222^2 + 74.259^2 + 73.469^2}{8} - 2666.216 = 4.470$$

$$SS_B = \frac{82.024^2 + 70.329^2 + 68.144^2 + 71.597^2}{8} - 2666.216 = 14.264$$

$$SS_L = \frac{140.374^2 + 151.720^2}{16} - 2666.216 = 4.022$$

$$SS_{TxS} = \frac{29.76^2 + 37.95^2 + 37.059^2 + 35.605^2 + 38.384^2 + 38.272^2 + 37.200^2 + 37.864^2}{4} - 2666.216 - 4.022 -$$

$$4.470 = 5.928$$

H: $t_i = 0$ (for total VFA content, early and late)

$$f = \frac{MS_T}{MS_E} \quad \text{vs} \quad f\ ,\ t-1,\ (t-1)\ (r-1)$$

$$f = \frac{1.490}{1.337} = 1.11 \qquad \begin{array}{l} f \quad .50,\ 3,\ 21 = 0.815 \\ f \quad .25,\ 3,\ 21 = 1.48 \\ f \quad .10,\ 3,\ 21 = 2.96 \end{array}$$

H: early vs late

$$f = \frac{4.022}{1.337} = 3.008 \qquad P \quad .10$$

Standard error = 0.41

Orthogonal test for total VFA for all cows to determine which treatments are different in total VFA concentration in rumen fluid.

<u>A vs all others</u> P .11

-3, 111

$(3\,(68.144) - (76.222 + 74.259 + 73.469))^2 = 380.952$

$\frac{380.952}{96} = 3.968 \qquad \frac{3.968}{1.337} = 2.968$

Critical value = f , 1, 21 =

f_1 .25, 1, 21 = 1.40
f_1 .10, 1, 21 = 2.96
f_1 .05, 1, 21 = 4.32

<u>A vs B</u> P .10

$(68.144 - 76.222)^2 = 65.254$

$\frac{65.254}{16} = 4.078 \qquad \frac{4.078}{1.337} = 3.050$

<u>A vs C</u> P .25

$(68.144 - 74.259)^2 = 37.393$

$\frac{37.393}{16} = 2.337 \qquad \frac{2.337}{1.337} = 1.748$

<u>A vs D</u> P .25

$(68.144 - 73.469)^2 = 28.356$

$\frac{28.356}{16} = 1.77$

Table 61. The effects of malic acid on the average concentration of total acetate content in mmoles/100 ml rumen fluid (cow # is on the left side of column) and the analysis of variance for total acetate for all cows.

A		B		C		D	
1430	4.663	1345	5.545	1350	5.409	1442	5.405
1359	4.282	1435	6.014	1448	6.216	1328	5.605
1397	4.819	1456	5.519	1352	6.263	1364	4.619
1407	4.125	1376	5.666	1449	4.912	1410	5.765
1415	6.114	1282	6.261	1400	5.908	1269	4.628
1458	6.205	1321	5.571	1263	6.049	1369	7.410
1387	5.884	1417	5.927	1418	4.571	1302	6.342
1385	6.831	1377	6.828	1419	6.895	1390	5.198
AVG.	5.365		5.916		5.778		5.622
X^2	237.484		281.465		271.179		258.767

Test for acetate

Observation	d.f.	s.s.	m.s.
Trt	3	1.340	0.447
S of L	1	4.347	4.347
(trt) x (S of L)	3	3.103	1.034
Block	3	2.431	0.810
Error	21	8.807	0.419
Total	31		

$$SS_y = 1048.895 - \frac{(181.449)^2}{32} = 20.028$$

$$SS_T = \frac{(42.923^2 + 47.331^2 + 46.223^2 + 44.972)}{8} - 1028.867 = 1.340$$

$$SS_B = \frac{49.128^2 + 44.398^2 + 43.507^2 + 44.416^2}{8} - 1028.867 = 2.431$$

$$SS_L = \frac{84.827^2 + 96.622^2}{16} - 1028.867 = 4.347$$

$$SS_{TxS} = \begin{matrix} 17.889^2 + 22.744^2 + 22.80^2 + 21.394^2 + \\ 25.034^2 + 24.587^2 + 23.423^2 + 23.578^2 \end{matrix} - 1028.867 - 4.347 -$$

$$1.34 = 3.103$$

H: $t_i = 0$ (for acetate)

$$f = \frac{MS_T}{MS_E} \quad \text{vs} \quad f \quad t-1, (t-1)(r-1)$$

$$f = \frac{0.447}{0.419} = 1.067$$

f .50, 3, 21 = 0.815
f .25, 3, 21 = 1.48
f .10, 3, 21 = 2.96

Interaction

$$f = \frac{1.034}{0.419} = 2.468 \qquad P \quad .25$$

Orthogonal test for determining differences between treatments for total acetate in rumen fluid (all cows).

<u>A vs all others</u> P .25

$(3\ (42.923) - (47.331 + 46.223 + 44.972))^2 = 95.199$

$\frac{95.199}{96} = 0.992 \qquad \frac{0.992}{.419} = 2.368$

$C_1V_1 = f\ \ , 1, 21 =$

$f_1\ .50, 1, 21 = .471$
$f_1\ .25, 1, 21 = 1.40$
$f_1\ .10, 1, 21 = 2.96$

<u>A vs B</u> P .50

$(42.923 - 47.331)^2 = 19.430$

$\frac{1943}{16} = 1.214$

<u>A vs C</u> P .50

$(42.923 - 46.223)^2 = 10.890$

$\frac{10.89}{16} = 0.681$

<u>A vs D</u>

$(42.923 - 44.972)^2 = 4.198$

$\frac{4.198}{16} = 0.26$

Standard error = 0.23

Table 62. The effects of malic acid on the average concentration of total VFA content in mmoles/100 ml of rumen fluid for early lactation (cow # is on left side of column) and the analysis of variance for total VFA content.

A		B		C		D	
1430	7.848	1345	9.056	1350	8.743	1442	8.318
1359	6.612	1435	10.556	1448	9.461	1328	10.556
1397	8.504	1456	8.699	1352	11.233	1364	7.604
1407	6.796	1376	9.639	1449	7.622	1410	9.127
AVG.	7.44		9.488		9.265		8.901
X^2	223.813		362.023		350.226		321.741

Test for total VFA, early lactation

Observation	d.f.	s.s.	m.s.
Trt	3	10.182	3.394
Block	3	7.660	2.553
Error	9	8.407	0.934
Total	15		

$$SS_y = 1257.803 - \frac{(140.374)^2}{16} = 26.249$$

$$SS_T = \frac{29.76^2 + 37.95^2 + 37.059^2 + 35.605^2}{4} - 1231.554 = 10.182$$

$$SS_D = \frac{39.349^2 + 34.229^2 + 35.135^2 + 31.661^2}{4} - 1231.554 = 7.660$$

H: $t_i = 0$ (for VFA, early)

$$f = \frac{MS_T}{MS_E} \quad \text{vs} \quad f\ ,\ 3,\ 9$$

$f = \frac{3.394}{0.934} = 3.634$ P .10 f .10, 3, 9 = 2.81
f .05, 3, 9 = 3.86

Orthogonal test for total VFA for early lactation to determine which treatments are different in the average concentration of total VFA in rumen fluid.

A vs all others P .05

$$(3\ (29.76) - (37.95 + 37.059 + 35.605))^2 = 455.140$$

$$\frac{455.140}{48} = 9.482 \qquad \frac{9.482}{0.934} = 10.152$$

$f \quad .10,\ 1,\ 9 = 3.36$
$f \quad .05,\ 1,\ 9 = 5.12$
$f \quad .01,\ 1,\ 9 = 10.56$

A vs B

$$(29.76 - 37.95)^2 = 67.076$$

$$\frac{67.076}{8} = 8.38 \qquad \frac{8.38}{.934} = 8.98$$ P .05

A vs C

$$(29.76 - 37.059)^2 = 53.275$$ P .05

$$\frac{53.275}{8} = 6.659 \qquad \frac{6.659}{.934} = 7.130$$

A vs D

$$(29.76 - 35.605)^2 = 34.164$$

$$\frac{34.164}{8} = 4.2705 \qquad \frac{4.2705}{.934} = 4.57$$ P .10

Standard error = 0.48

Table 63. The effects of malic acid on the average concentration of acetate in mmoles/100 ml rumen fluid for early lactation (cow # is on left side of column) and analysis of variance for acetate.

A		B		C		D	
1430	4.663	1345	5.545	1350	5.409	1442	5.405
1359	4.282	1435	6.014	1448	6.216	1328	5.605
1397	4.819	1456	5.519	1352	6.263	1364	4.619
1407	4.125	1376	5.666	1449	4.912	1410	5.765
AVG.	4.472		5.686		5.700		5.348
X^2	80.317		129.478		131.249		115.200

Total acetate for early lactation

Observation	d.f.	s.s.	m.s.
Trt	3	3.986	1.329
Block	3	1.382	0.461
Error	9	1.150	0.128
Total	15		

$$SS_y = 456.244 - \frac{(84.827)^2}{16} = 6.518$$

$$SS_T = \frac{17.889^2 + 22.744^2 + 22.8^2 + 21.394^2}{4} - 449.726 = 3.986$$

$$SS_D = \frac{22.232^2 + 21.110^2 + 22.163^2 + 19.322^2}{4} - 449.726 = 1.382$$

H: $t_i = 0$ (for acetate, early)

$$f = \frac{MS_T}{MS_E} = \frac{1.329}{0.128} = 10.383 \quad \text{vs} \quad f\ 3,\ 9$$

P .01 $\qquad f\ .01,\ 3,\ 9 = 6.99$

Orthogonal test for acetate for early lactation to determine which treatments are different in the average concentration of acetate in rumen fluid.

A vs BCD

$(3\ (17.889) - (22.744 + 22.8 + 21.394))^2 = 176.119$

$\frac{176.119}{48} = 3.669$ $\frac{3.669}{.128} = 28.66$ P .01

f .01, 1, 9 = 10.56

A vs B

$(17.889 - (22.744))^2 = 23.571$

$\frac{23.571}{8} = 2.946$ $\frac{2.946}{.128} = 23.02$ P .01

A vs C

$(17.889 - 22.8)^2 = 24.118$ P .01

A vs D

$(17.889 - 21.394)^2 = 12.285$

$\frac{12.285}{8} = 1.536$ $\frac{1.536}{.128} = 12.0$ P .01

Standard error = 0.18

Table 64. The effects of malic acid on the average concentration of propionate in mmoles/100 ml rumen fluid for early lactation (cow # is on the left side of column) and the analysis of variance for propionate.

A		B		C		D	
1430	1.979	1345	1.963	1350	1.840	1442	1.597
1359	1.306	1435	3.052	1448	1.759	1328	3.092
1397	2.400	1456	1.709	1352	3.115	1364	1.653
1407	1.486	1376	2.377	1449	1.438	1410	1.752
AVG.	1.793		2.275		2.038		2.024
X^2	13.590		21.739		18.251		17.913

Test for propionate, just early lactation

Observation	d.f.	s.s.	m.s.
Trt	3	0.466	0.155
Block	3	2.079	0.693
Error	9	2.859	0.318
Total	15		

$$SS_y = 71.493 - \frac{(32.518)^2}{16} = 5.404$$

$$SS_T = \frac{7.171^2 + 9.101^2 + 8.152^2 + 8.094^2}{4} - 66.089 = 0.466$$

$$SS_D = \frac{10.57^2 + 7.795^2 + 7.199^2 + 6.954^2}{4} - 66.089 = 2.079$$

H: $t_i = 0$ (for C_3 early)

$$f = \frac{MS_T}{MS_E} = \frac{.155}{.318} = 0.487$$

f , 3, 9

f .50, 3, 9 = 0.85

No differences between treatments

Standard error = 0.28

Table 65. The effects of malic acid on the average concentration of isobutyrate in mmoles/100 ml rumen fluid for early lactation (cow # is on the left side of column) and the analysis of variance for isobutyrate.

A		B		C		D	
1430	.057	1345	.089	1350	.082	1442	.072
1359	.055	1435	.065	1448	.083	1328	.067
1397	.055	1456	.077	1352	.071	1364	.072
1407	.062	1376	.066	1449	.071	1410	.082
AVG.	.057		.074		.077		.073
X^2	0.01314		0.02243		0.02370		0.02158

Test for isobutyrate, early lactation

Observation	d.f.	s.s.	m.s.
Trt	3	0.00095	0.00032
Block	3	0.00012	0.00004
Error	9	0.00054	0.00006
Total	15		

$$SS_y = 0.08085 - \frac{(1.126)^2}{16} = 0.00161$$

$$SS_T = \frac{.229^2 + .297^2 + .307^2 + .293^2}{4} - 0.07924 = 0.00095$$

$$SS_D = \frac{0.282^2 + .274^2 + .299^2 + .271^2}{4} - 0.07924 = 0.00012$$

H: $t_i = 0$ (for isobutyrate, early)

$$f = \frac{MS_T}{MS_E} = \frac{.00032}{.00006} = 5.33 \qquad P \quad .05$$

f 3, 9
f .05, 3, 9 = 3.86
f .01, 3, 9 = 6.99

Orthogonal test for isobutyrate for early lactation to determine which treatments are different for the average concentration of isobutyrate in rumen fluid.

A vs BCD

$(3\ (.299) - (.297 + .307 + .293))^2 = 0.0441$

$\frac{0.0441}{48} = 0.00092 \qquad \frac{0.00092}{0.00006} = 15.31$

P .01 vs f , 1, 9 =
f .01, 1, 9 = 10.56

A vs B

$(.229 - .297)^2 = 0.00462$

$\frac{.00462}{8} = 0.000578 \qquad \frac{0.000578}{.00006} = 9.63$

P .05 f .05, 1, 9 = 5.12

A vs C

$(.229 - .307)^2 = 0.00608$

$\frac{.00608}{8} = 0.00076 \qquad \frac{0.00076}{0.00006} = 12.7$ P .01

A vs D

$(.229 - .293)^2 = .00410$

$\frac{.00410}{8} = .00051 \qquad \frac{.00051}{.00006} = 8.5$ P .05

Standard error = 0.004

Table 66. The effects of malic acid on the average concentration of butyrate in mmoles/100 ml rumen fluid for early lactation (cow # is on the left side of column) and the analysis of variance for butyrate.

A		B		C		D	
1430	.975	1345	1.184	1350	1.152	1442	1.005
1359	.811	1435	1.160	1448	1.156	1328	1.282
1397	.981	1456	1.142	1352	1.409	1364	.985
1407	.903	1376	1.211	1449	.979	1410	1.309
AVG.	.918		1.174		1.174		1.145
X^2	3.38612		5.51814		5.60716		5.33726

Test for butyrate, early

Observation	d.f.	s.s.	m.s.
Trt	3	.18525	.06175
Block	3	.10456	.03485
Error	9	.10195	.01133
Total	15		

$$SS_y = 19.84868 - \frac{(17.644)^2}{16} = 0.39176$$

$$SS_T = \frac{3.67^2 + 4.128^2 + 4.582^2 + 4.078^2}{4} - 19.45692 = 0.18525$$

$$SS_D = \frac{4.856^2 + 4.128^2 + 4.582^2 + 4.078^2}{4} - 19.45692 = 0.10456$$

H: $t_i = 0$ (for butyrate, early only)

$$f = \frac{MS_T}{MS_E} = \frac{.06175}{.01133} = 5.45 \qquad P \quad .05$$

f .05, 3, 9 = 3.86
f .01, 3, 9 = 6.99

Orthogonal test for butyrate for early lactation to determine which treatments are different for the average concentration of butyrate in rumen fluid.

<u>A vs BCD</u>

$$(3\ (3.67)\ (4.697 + 4.696 + 4.581))^2 = 8.78530$$

$$\frac{8.78530}{48} = 0.18303 \qquad \frac{.18303}{.01133} = 16.154$$

P .01 $\qquad$ f .01, 1, 9 = 10.56
f .05, 1, 9 = 5.12

<u>A vs B</u>

$$(3.67 - 4.697)^2 = 1.05473$$

$$\frac{0.05473}{8} = 0.13184 \qquad \frac{.13184}{.01133} = 11.6 \qquad \text{P} \quad .01$$

<u>A vs C</u>

$$(3.67 - 4.696)^2 = 1.05268$$

$$\frac{1.05268}{8} = 0.13158 \qquad \frac{0.13158}{0.01133} = 11.61 \qquad \text{P} \quad .01$$

<u>A vs D</u>

$$(3.67 - 4.581)^2 = 0.82992$$

$$\frac{0.82992}{8} = 0.10374 \qquad \frac{0.10374}{0.01133} = 9.16 \qquad \text{P} \quad .05$$

$$\text{Standard error} = \frac{MS_E}{r} = 0.053$$

Table 67. The effects of malic acid on the average concentration of 2-methyl butyrate in mmoles/100 ml rumen fluid for early lactation (cow # is on the left side of column) and the analysis of variance for 2-methyl butyrate.

A		B		C		D	
1430	.059	1345	.118	1350	.083	1442	.087
1359	.051	1435	.082	1448	.084	1328	.077
1397	.065	1456	.084	1352	.111	1364	.109
1407	.083	1376	.079	1449	.079	1410	.069
AVG.	.065		.091		.088		.086
X^2	.01720		.03394		.03251		.03014

Test for 2-m B, early only

Observation	d.f.	s.s.	m.s.
Trt	3	.00179	.00060
Block	3	.00099	.00033
Error	9	.00211	.00023
Total	15		

$$SS_y = 0.11379 - \frac{(1.320)^2}{16} = 0.00489$$

$$SS_T = \frac{0.258^2 + 0.363^2 + 0.357^2 + .342^2}{4} - 0.1089 = 0.00179$$

$$SS_D = \frac{0.371^2 + 0.303^2 + 0.296^2 + 0.350^2}{4} - 0.1089 = 0.00099$$

H: $t_i = 0$ (for 2-m butyrate)

$$f = \frac{MS_T}{MS_E} = \frac{0.00060}{0.00023} = 2.61 \qquad f \quad .25$$

f .25, 3, 9 = 1.63
f .10, 3, 9 = 2.81
f .05, 3, 9 = 3.86

Orthogonal test for 2-m butyrate for early lactation to determine which treatments are different for the average concentration of 2-m butyrate in rumen fluid.

A vs all others

$(3\ (.258) - (.363 + .357 + .342))^2 = 0.08294$

$\frac{0.08294}{48} = 0.001728 \qquad \frac{0.001728}{0.00023} = 7.51$

P .05

f .10, 1, 9 = 3.36
f .05, 1, 9 = 5.12
f .01, 1, 9 = 10.56

A vs B

$(0.258 - .363)^2 = 0.011025$

$\frac{0.011025}{8} = 0.001378 \qquad \frac{0.001378}{0.00023} = 5.99$ P .05

A vs C

$(0.258 - 3.357)^2 = 0.00980$

$\frac{0.00980}{8} = 0.001225 \qquad \frac{0.001225}{0.00023} = 5.33$ P .05

A vs D

$(.258 - .342)^2 = 0.007056$

$\frac{0.007056}{8} = 0.000882 \qquad \frac{0.000882}{0.00023} = 3.83$ P .10

Standard error = 0.008

Table 68. The effects of malic acid on the average concentration of isovalerate in mmoles/100 ml rumen fluid for early lactation (cow # is on the left side of column) and the analysis of variance for isovalerate.

A		B		C		D	
1430	.042	1345	.069	1350	.064	1442	.054
1359	.045	1435	.044	1448	.060	1328	.049
1397	.037	1456	.060	1352	.058	1364	.062
1407	.042	1376	.056	1449	.054	1410	.062
AVG.	.042		.057		.059		.057
X^2	.006922		.013433		.013976		.013005

Test for isovalerate, just early lactation

Observation	d.f.	s.s.	m.s.
Trt	3	.0007955	.0002651
Block	3	.0000375	.0000125
Error	9	.0004928	.0000547
Total	15		

$$SS_y = 0.047336 - \frac{(0.858)^2}{16} = 0.0013258$$

$$SS_T = \frac{.166^2 + .229^2 + .236^2 + .227^2}{4} - 0.046010 = 0.0007955$$

$$SS_D = \frac{.213^2 + .207^2 + .224^2 + .214^2}{4} - 0.046010 = 0.0000375$$

H: $t_i = 0$ (for isovalerate)

$$f = \frac{MS_T}{MS_E} = \frac{.0002651}{.0000547} = 4.85$$

$f \quad .05, 3, 9 = 3.86 \qquad P \quad .05$

Orthogonal test for isovalerate for early lactation to determine which treatments are different for the average concentration of isovalerate in rumen fluid.

A vs all others

$(3\ (.166) - (.229 + .236 + .227))^2 = 0.037636$

$\frac{0.037636}{48} = 0.000784 \qquad \frac{0.000784}{0.0000547} = 14.3$

P .01

f .10, 1, 9 = 3.36
f .05, 1, 9 = 5.12
f .01, 1, 9 = 10.56

A vs B

$(.166 - .229)^2 = 0.003969$

$\frac{0.003969}{8} = 0.0004961 \qquad \frac{0.0004961}{0.0000547} = 9.1$ P .05

A vs C

$(.166 - .236)^2 = 0.0049$

$\frac{0.0049}{8} = 0.000612 \qquad \frac{0.000612}{0.0000547} = 11.2$ P .01

A vs D

$(.166 - .227)^2 = 0.003721$

$\frac{0.003721}{8} = 0.0004651 \qquad \frac{0.0004651}{0.0000547} = 8.5$ P .05

Standard error = 0.004

Table 69. The effects of malic acid on the average concentration of valerate in mmoles/100 ml rumen fluid for early lactation (cow # is on the left side of column) and the analysis of variance for valerate.

A		B		C		D	
1430	.072	1345	.102	1350	.112	1442	.099
1359	.071	1435	.139	1448	.104	1328	.185
1397	.139	1456	.105	1352	.207	1364	.105
1407	.096	1376	.183	1449	.094	1410	.107
AVG.	.095		.132		.129		.124
X^2	.038762		.074239		.075045		.066500

Test for valerate, early lactation only

Observation	d.f.	s.s.	m.s.
Trt	3	.0036075	.0012025
Block	3	.0088395	.0029465
Error	9	.011699	.0012998
Total	15		

$$SS_y = 0.254546 - \frac{(1.920)^2}{16} = 0.024146$$

$$SS_T = \frac{.378^2 + .529^2 + .517^2 + .496^2}{4} - 0.2304 = 0.0036075$$

$$SS_D = \frac{0.633^2 + 0.421^2 + .388^2 + .478^2}{4} - 0.2304 = 0.0088395$$

H: $t_i = 0$ (for valerate, early) P .50

$$f = \frac{MS_T}{MS_E} = \frac{.0012025}{.0012998} = 0.92 \qquad f\ .50,\ 3,\ 9 = 0.85$$

Standard error = 0.018

Table 70. The analysis of variance for propionate.

Source	Degree of Freedom	SS	MS	F	
Treatment	2	2.647	1.324	7.24	P .025
Period	2	3.158	1.579	8.63	
Animal	5	1.778	0.356	1.94	
Residual	8	1.467	0.183		
Total	17	9.05			