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Responses of Rumen Microflora to Whey Products Added to High-Grain Low-Roughage Rations

Virgil L. Metzger

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RESPONSES OF RUMEN MICROFLORA TO WHEY PRODUCTS ADDED
TO HIGH-GRAIN LOW-ROUGHAGE RATIONS

BY

VIRGIL L. METZGER

A thesis submitted
in partial fulfillment of the requirements for the
degree Master of Science, Major in
Dairy Science, South Dakota
State University

1971
RESPONSES OF RUMEN MICROFLORA TO WHEY PRODUCTS ADDED
TO HIGH-GRAIN LOW-ROUGHAGE RATIONS

This thesis is approved as a creditable and independent
investigation by a candidate for the degree, Master of Science,
and is acceptable as meeting the thesis requirements for this
degree. Acceptance of this thesis does not imply that the conclusions
reached by the candidate are necessarily the conclusions of the major
department.

Thesis Adviser                      Date

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VLM
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INTRODUCTION

Many factors present in livestock feeds affect the numbers and types of protozoa and bacteria in the rumen. A small particle size of feed and a fast rate of passage of the feed through the ruminant decreases protozoal numbers. Full feeding of a concentrate ration and an acid pH in the rumen also tend to reduce protozoa numbers. Bacteria numbers tend to increase as the protozoa numbers decrease.

Knowledge of rumen microflora population changes on different rations is lacking. It is known that restricted-roughage rations will generally cause lower protozoal numbers. Since whey is an available source of lactose and energy, the bacteria and protozoa numbers could be affected. In ruminants, the proper growth of the rumen microbial population is a good indication of efficient feed utilization. Good growth of the rumen microbial population when adding whey to the ration will indicate its efficient feed utilization. Adding whey products to restricted-roughage rations of cows caused milk fat production to return towards normal (5, 26, 27); however, it was not known which component in whey was responsible for correcting milk fat depression.

This study was undertaken to determine the population changes of the rumen microflora when cows were fed whey or whey products on a high-concentrate low-roughage ration. The objectives of the study were as follows:

1. To determine the total numbers of protozoa per milliliter of rumen fluid.

2. To classify the protozoa into the five main genera.
3. To determine total numbers of bacteria by the direct microscopic count and roll tube methods.

4. To determine the types of bacteria as either starch fermenters, lactose fermenters, or proteolytic bacteria.
REVIEW OF LITERATURE

The relationship between protozoa and bacteria is one of the least understood areas in rumen microbiology, particularly in the area of quantitative relationships (29). Eadie (18) and Bryant and Small (12) stated that the numbers of bacteria in nonfaunated animals are greater than in normal faunated ones. The difference can be due to competition for food or to consumption of the bacteria by the protozoa. The fact that protozoa ingest and presumably digest rumen bacteria indicates that part of the effect is due to this ingestion, but the extent to which the protozoa utilize nitrogenous constituents of the feed and whether these are used in preference to the bacteria has not been established (29). The importance of the source of feed nitrogen for the activity and numbers of the protozoa has been reported (37), with possible interrelationships between the protozoa and the bacteria.

According to Becker and Talbott (4) the existence of a wealth of protozoan life in the rumen and reticulum of cattle was first mentioned by Grube and Delafond in 1843. Since that time numerous workers have studied the rumen microbes. Researchers at Iowa State (4) classified the first two divisions of the rumen protozoa into three classes: Sarcodina, Mastigophora, and Infusoria. The class Sarcodina consists of nonciliated ameobae, and the two other classes include all ruminal ciliates. Ciliates, belonging to the class Infusoria, are very predominant in rumen fluid. These include the genera- Isotricha, Dasytricha, Diplodinium, Entodinium, and Ophryoscolex. A survey of the rumen ciliate population in a series of adult sheep by Abou Akkada and
El-Shazly (1) showed that a mixture of *Entodinium*, *Isotricha*, *Ophryoscolex*, and *Polyplastron* species was found in the rumen contents of Egyptian sheep. No *Epidinium* and a negligible number of *Dasytricha* were also observed.

Protozoa populations in the rumen

Warner (44) studied some factors influencing the rumen microbial population. In sheep fed once daily, the concentration of protozoa in the rumen changed with the time after feeding. These changes in concentration were reflected in changes in the proportion of dividing cells. One animal at different times or different animals on the same ration of any dietary regime had very different rumen microbial populations. These differences were particularly marked in the case of some organisms. Feeding different quantities of the same ration had little effects on the concentration of rumen protozoa, provided the ration was above minimal level. Starvation for a few days or prolonged undernutrition had a marked effect also. Some organisms were drastically reduced in numbers or died out completely. When the qualitative nature of the diet was changed, about ten days were needed to complete the major adjustment in the rumen protozoal population.

Christiansen et al. (15) conducted a series of trials studying the influence of level of feed intake, rate of passage of ingesta, physical form of ration and feeding diethylstilbestrol upon rumen protozoal populations in lambs. Full feeding a pelleted high-concentrate ration resulted in very small rumen protozoal numbers. As the particle size
of the ration was reduced, the number of rumen protozoa decreased. Diethylstilbestrol not only prevented the disappearance of protozoa from lambs fed high-concentrate pelleted ration, but it also increased protozoal numbers in all rations studied.

Purser and Moir (38) found that adding unextracted linseed to a basal diet caused a significant decrease in protozoal concentration while adding urea and gluten caused an increase in protozoal concentration. Greater protozoal concentrations were associated in each case with high levels of ammonia in the rumen. Protozoal numbers were higher when a diet containing 60% roughage and 40% concentrate was supplemented with condensed corn fermentation extractives (32). The energy level of the diet had a marked influence on protozoal numbers with the greater populations being found on high energy diets.

*Diplodinium* and *Entodinium* spp. were greater with the high energy diets. Clarke (16) found that fluctuations in total numbers of *Isotricha* appeared to be related to the diet and dietary regime of the host. When the host was fed chaffed red clover hay for two hours daily a marked rise in *Isotricha* numbers occurred during feeding. When the host was fed fresh red clover the *Isotricha* numbers decreased.

Patton et al. (36) studied the effect of feeding methionine hydroxy analog on the concentration of protozoa in the rumen of sheep. Protozoal concentration was significantly higher in the grain plus methionine hydroxy analog fed sheep (P<0.01) over the grain alone group, but significantly lower than the grain plus hay fed sheep. Eadie (18) studied the development of rumen microbial populations in lambs and
calves under various conditions of management. Certain large bacteria were developed in isolated lambs without direct contact between animals. Diet was the governing factor in the establishment of rumen ciliates. Feeding a fat depressing diet of pelleted forage showed rumen protozoa were almost completely absent during the secretion of low-fat milk (14). It was suggested that defaunation was a primary factor responsible for the change in composition of the milk with depressed fat tests.

**Carbohydrate metabolism**

The rumen protozoa which have been examined are largely carbohydrate fermenters (24). The holotrichs differ biochemically from the ophryoscolecids. The holotrichs utilize mostly soluble sugars. Glucose and fructose and some of their compounds are the only sugars readily fermented by both the *Dasytricha* and *Isotricha*. However, galactose, maltose and cellulose are fermented by *Dasytricha* only. The ophryoscolecids seem to ferment only insoluble polysaccharides, which are swallowed and digested. The outer membranes of the ophryoscolecids are tougher, and possibly therefore less permeable than those of the holotrichs. This may explain why the ophryoscolecids can use only those carbohydrates which occur in solid, ingestible form. The enzymes found in any species correspond generally with the sugars on which the organism feeds. The holotrichs have a rich source of invertase. *Entodinium caudatum* possesses only amylase and maltase and thus ferments only granular starch. Other ophryoscolecids with a wider range of foods possess in addition xylanase and cellulase (25).

The rumen protozoa can be assigned to three different groups
according to Gutierrez (20); one consisting of the genus Diplodinium and related types which ingest quantities of fibrous plant material; a second is the genus Entodinium which actively digest starch but seldom are found to contain the cell walls of plant; and a third group, the holotrichs, which seldom contain plant materials. This latter group includes the species Isotricha prostoma, I. intestinalis and the smaller Dasytricha ruminantium. These holotrichs were separated to determine their individual characteristics and to estimate their significance to the host. The fermentation products of the protozoa were determined and carbon dioxide, hydrogen, lactic, acetic and butyric acids were found to be produced by both Isotricha and Dasytricha. All three species of holotrichs deposited reserve food when fed sucrose, fructose, glucose, inulin and raffinose. Cellobiose and salicin were used only by Dasytricha but to a lesser extent than glucose. Holotrichs were found to produce 240 g of fermentation acids per day in 100 kg of rumen contents. Increased Isotricha numbers in cultures suggested than under culture conditions it divided once every 48 hours.

Using a suitable buffer at 39 C, Sugden and Oxford (41) found the life of all three species of holotrich ciliates of the sheep's rumen could be extended for one or more days by the addition of glucose, fructose, galactose, sucrose, cellobiose, raffinose, inulin, bacterial levan, salicin or melibiose. Mannose, glucosamine, or galactosamine were definitely toxic in that they shortened protozoan life. This toxic effect was also observed in the presence of glucose. Williams et al. (46) found that rumen ciliate Ophryoscolex caudatus fermented starch with the production of acetic, butyric, and lactic acids plus CO₂ and
H₂O. Cellulose was not significantly metabolized although pectin was rapidly attacked when using the Warburg apparatus.

Suspensions of *Dasytricha ruminantium* and of mixed *Isotricha intestinalis* and *I. prostoma* were prepared from the rumen liquor of sheep and used by Howard (24). The *D. ruminantium* suspensions, but not the isotrichs, were able to ferment galactose, maltose, and cellobiose. Some naturally occurring β-glucosides were more readily fermented by *D. ruminantium* than by the isotrichs. Products of galactose fermentation by *D. ruminantium* were the same as those formed from glucose by the same species. Howard (24) in another study used cell-free extracts, nearly free from bacteria, to prepare suspensions of the two genera of rumen holotrich protozoa, *Dasytricha* and *Isotricha*. He investigated the ability of these extracts to split a number of di-, tri-, and polysaccharides. Hydrolytic activity of the extracts corresponded to the ability of the living protozoa to ferment the substrates. *Dasytricha ruminantium* extracts contained appreciable cellobiase and β-glucosidase activity, and moderate maltase activity. Extracts of mixed *Isotricha intestinalis* and *I. prostoma* contained hardly any maltase, a trace of cellobiase and a small amount of β-glucosidase. Neither genus gave extracts able to hydrolyse lactose, melibiose, trehalose, melezitose or xylobiose. Considerable invertase activity was found in the protozoal extracts, especially those from *Isotricha*. Sucrose, raffinose, inulin and a bacterial levan were all hydrolysed, but not melezitose.

Heald and Oxford (22) found suspensions of bacteria-free holotrich ciliates from sheep's rumen contents produced acids and gas at a rapid
rate under anaerobic conditions from the following soluble carbohydrates: glucose, fructose, sucrose, raffinose, inulin, rye-grass levan, and to a much lesser degree cellobiose. This indicated that the rumen ciliate protozoa do possess a vigorous carbohydrate metabolism in the absence of bacteria.

Protein metabolism

Gutierrez and Hungate (21) noticed an interrelationship between certain bacteria and the rumen ciliate Dasytricha ruminantium. It was concluded that the holotrich rumen ciliates derive at least some of their nitrogenous requirements from the ingestion of associated bacteria. Moir and Somers (35) found that a daily ration given as a single feed gave the lowest nitrogen retention. The same ration also caused the greatest fall in bacterial counts and the lowest protozoal counts. The highest ruminal ammonia nitrogen levels were also associated with this treatment.

Klopfenstein et al. (31) studying the effects of defaunation found that the presence of protozoa increased rumen ammonia concentration, but plasma urea levels did not always show corresponding increases. It was suggested that lysine may have been the limiting amino acid when the lambs were defaunated, but that no one amino acid was consistently limiting after protozoa were established. Plasma amino acid concentrations were lower in faunated than in defaunated lambs. Faunation resulted in greater dry matter digestion. Greater protozoal concentrations were associated with high levels of ammonia in the rumen (38). These workers added unextracted linseed to a basal ration and found a
significant decrease in both rumen ammonia levels and protozoal concentrations. When urea supplement supplying 8 g of nitrogen was fed with the basal ration there were significantly greater protozoal concentrations than when one-half or three-fourths of the supplement was infused into the rumen ten hours after feeding or when the urea supplement was infused continuously. They also noticed a significantly greater protozoal concentration when urea was added isonitrogenously to a basal ration, but addition of gluten decreased protozoal level.

Quinn et al. (39) noted both groups of ciliates, the oligotrichs and holotrichs, utilized amino acids as their principal nitrogen sources when these are supplied in micromolar concentrations. However, at millimolar concentrations, amino acids are toxic, possibly from excessive ammonia formation arising from ciliate deaminase activity. The nitrogenous needs of holotrich ciliate protozoa of sheep seem to be best met by whole grass juice. An alcoholic precipitate from boiled and cleared grass juice was a better nitrogenous supplement than cleared rumen liquor. The ash from this alcoholic precipitate could extend protozoan life in the absence of a nitrogenous supplement (41). They presumed this effect could be due to trace metal which may be Ti, Mo, Cr, Co, or V.

According to Williams et al. (46) the protein sources; cottonseed, soybean, and linseed oil meals and the amino acids; DL-alanine, DL-valine, and DL-leucine were utilized by the protozoa, whereas ammonia was an end product of nitrogenous metabolism.
Bacteria populations in the rumen

The rumen contents of cows fed mostly roughage in the form of alfalfa hay and of other cows fed mostly grain were diluted serially in trypticase-soy-semi-solid medium (3). With most of the samples, growth occurred in the $10^{-10}$ dilution of rumen material. From the high roughage samples the organisms found in greatest numbers were Gram positive, pleomorphic, anaerobic rods that resembled Lactobacillus bifidus. They fermented glucose almost entirely to acetic and lactic acids in a molar ratio of 2:1. From high grain samples the organisms found in largest numbers were Gram positive cocci that occurred mainly in pairs and tetrads with characteristics similar to those of the genus Pediococcus. They converted glucose primarily to lactic and acetic acids in a molar ratio of 10:1. None of the rumen isolates digested cellulose, but they fermented starch and a variety of simple sugars.

According to Maki and Foster (34) total direct microscopic counts from samples containing no roughage were two to three times as high as those from animals that were fed roughage. Isolates from the $10^{-8}$ or $10^{-9}$ dilutions were characterized by the acids they produced from glucose. The predominant organisms from the rations without roughage converted glucose mainly to acetic, lactic, or succinic acids. Studying different methods for isolation, Gall et al. (19) found rumen contents of both sheep and cattle contained about $10^{10}$ bacteria per g of fresh material.

The crude wet rumen contents of hay-fed sheep has been shown by Heald et al. (23) to contain a population of $10^8$ viable and facultatively
anaerobic bacteria per g. These bacteria were mostly Gram-positive cocci capable of fermenting a wide range of soluble carbohydrates including starch and inulin but not mannitol. The mannitol-fermenting population was of the order of $10^6$ organisms per g, consisting mostly of Gram-negative rods, including coliform bacteria.

No correlation between rations and differences in the microbiota could be detected by Hungate (28) when the cows were fed a constant ration. Direct microscopic and culture counts were made on the rumen contents of cows on a ration of timothy hay plus various amounts of cottonseed meal, rolled barley, and salt. *Streptococcus bovis* was identified by its rapid growth in the feed medium and showed counts ranging between $2 \times 10^5$ and $1.4 \times 10^8$ per g. Numerous cellulose-digesting bacteria were also identified.

Bryant and Small (12) conducted a study of microorganisms in the rumens of three calves inoculated with whole rumen contents from a mature cow, and of two calves not inoculated and isolated from other ruminants immediately after birth. Inoculation of normally raised calves had little effect on the time of establishment of predominant bacteria typical of mature cattle, but had considerable effect on the age at which ciliate protozoa became established. It was concluded that the lack of normal populations did not have any drastic effect on the animals feed consumption, growth, or health through an age of 17 weeks.

Thorley et al. (42) determined the numbers and kinds of predominating bacteria and most probable numbers (MPN) of cellulolytic bacteria in cattle fed ground and pelleted roughage. Counts were significantly
higher when animals were fed ground grass \((15.7 \times 10^9 \text{ per g})\) as compared to long grass \((10.5 \times 10^9 \text{ per g})\). The MPN of cellulolytic bacteria were similar for the two animals when long grass was fed, but these counts were depressed on one animal when it was fed ground grass. The results indicate changes in the rumen fermentation occur when high levels of ground forage are fed, due partially to changes in the distribution of the microbial species. The effects of diet, time after feeding, and position sampled on numbers of viable bacteria in the bovine rumen were noted in a study by Bryant and Robinson (10). The numbers of bacteria estimated by colony counts obtained from different positions in the rumen were determined at 1.0, 2.5, 5.5, and 10 hours after feeding four heifers at 12-hour intervals at the maintenance level of intake. Numbers were lowest at one hour and increased significantly between 1, 2.5, and 5.5 hours. Only when pellets were fed were the numbers at one hour not significantly lower \((P<0.01)\) at a given time than in the dorsal rumen. Numbers in the reticulum were essentially the same as those in the ventral rumen when hay or hay and grain was fed, but were higher \((P<0.01)\) when pellets were fed and lower \((P<0.05)\) when silage was fed. The results suggested that grinding of hay affects the numbers of bacteria per unit of weight of digesta passing on to the omasum.

**Enumeration of rumen bacteria**

Studies have shown evidence that the rumen contains a great variety of bacterial species, many of which are present in large numbers in one
animal held under one set of conditions (2, 3, 7, 28, 47). Most of the more significant groups have been isolated and it is doubtful that future studies will disclose as many new groups as have been found. Heald et al. (23) found that a basal nutrient agar, consisting of buffered and very highly clarified rumen liquor, supplemented only with vitamin-free acid hydrolysate of casein, tryptophan, a fermentable carbohydrate and agar would grow rumen bacteria very well.

The inorganic salts-rumen fluid-sugar-cellulose medium, according to King and Smith (30), was superior to the highly organic medium with respect to: (a) total counts, (b) cellulolytic counts, (c) diversity of cellulolytic morphotypes recovered, and (d) ability to support growth of cellulolytic isolates from the other medium. Bryant and Burkey (11) studied methods for cultural counts and the isolation and determination of some characteristics of some of the more numerous groups of bacteria in the rumen of cows. Samples of the rumen contents were diluted in an anaerobic dilution solution and cultured in an anaerobic culture medium. The morphology, Gram reaction, motility, relations to oxygen, and ability to produce hydrogen sulfide, to liquefy gelatin, to hydrolyze starch and cellulose, and to produce acid from glucose, xylose, and cellobiose were determined for each of 896 strains of bacteria isolated.

Doetsch et al. (17) noted rumen fluid contained unknown substances essential for optimal growth of some bacteria. An improved rumen fluid agar medium was developed that permitted the growth of about double the numbers of bacteria from ruminal contents grown in medium used previously
(8). The main modifications made were the use of rumen fluid clarified by centrifugation, reducing the concentration of the sugar energy sources (glucose by cellobiose) and inclusion of soluble starch as an additional energy source and changing the reducing agent from cysteine to a combination of cysteine and sodium sulfide. A simple medium and dilution count technique was evolved for determining the viable count of rumen bacteria (47). Observations were made of the homogeneity of rumen contents, of variations in count with time after sampling and between cows, and of variation from day to day, hour to hour, before and after feeding, and on different diets.

Caldwell and Bryant (13) noted that colony counts were similar between a rumen fluid-agar medium (RFM) and medium 10 without rumen fluid. The medium was identical to the RFM except for the replacement of rumen fluid with $1.5 \times 10^{-6}$M hemin, 0.2% trypicase, 0.05% yeast extract, and a $6.6 \times 10^{-2}$M volatile fatty acid mixture similar to that in rumen fluid. Single deletion of trypicase, yeast extract, or the volatile fatty acid mixture from the medium 10 significantly reduced colony counts. The results showed that the medium 10 is suitable for enumeration and isolation of many predominant rumen bacteria. The effect of enzymatic hydrolysate of casein, $\text{NH}_4$, a mixture of volatile fatty acids, hemin and ruminal fluid on growth of 89 freshly isolated strains of predominant culturable ruminal bacteria was studied by Bryant and Robinson (9). They used a basal medium containing glucose, cellobiose, or maltose as energy source, minerals, cysteine, and $S^-$ as reducing agents, and $\text{H}_2\text{CO}_3$-$\text{HCO}_3^{-}$ buffer. The results indicated that
most strains of ruminal bacteria can be grown in defined media, and suggested the relative lack of importance of organic nitrogen compounds, such as amino acids, in the nutrition of these bacteria.

Wegner and Foster (45) isolated cultures from $10^{-8}$ dilutions of rumen fluid in roll tubes of a trypticase-phytone-glucose basal medium supplemented with rumen fluid. Almost one-fourth of the isolates failed to grow on subculture in the basal medium, unless air was enriched with rumen fluid or a mixture of fatty acids commonly found in the rumen. Proteolytic bacteria were isolated from the rumen of sheep receiving an adequate protein diet (2). He used selective anaerobic conditions to suppress the majority of bacteria which were not capable of utilizing casein. The most frequent organism was Bacillus licheniformis which existed in the vegetative form in the rumen, although present mostly in the form of spores in the hay fed to the animal.
EXPERIMENTAL PROCEDURE

Source of rumen samples

Five groups of three lactating Holstein cows each were fed one of five concentrate rations. The five rations included: (1) high-grain limited-roughage (control), (2) control + 14% dried whole whey\(^1\), (3) control + 5.9% dried whey molasses\(^2\), (4) control + 11.8% demineralized dried whey\(^3\), and (5) control + 9.8% lactose\(^4\). The composition of the rations is listed in Table 1. The whey fractions in the rations were balanced in the following manner: (a) the whey molasses diet contained the same amount of minerals as the 14% dried whole whey diet, (b) the demineralized whey diet contained the same amount of lactose as the dried whole whey diet, and (c) the lactose diet had the same amount of lactose as the dried whole whey diet.

Each trial consisted of a three-week standardization period followed by a six-week experimental period and a two-week post-experimental period. Rations during the standardization and post-experimental periods included alfalfa hay and corn silage ad libitum and the regular herd concentrate mix fed at the rate of 1 kg per 3 kg of milk produced. Experimental rations included 2.3 kg hay as the only forage and one of the five concentrate rations listed above fed ad libitum.

\(^1\) Dried whole whey, Foremost Foods Company, San Francisco, California.
\(^2\) Delactosed whey (some 65% of lactose removed), Valley Queen Cheese Factory, Incorporated, Milbank, South Dakota.
\(^3\) Nutritek 900, Foremost Foods Company, San Francisco, California.
\(^4\) Lactose, Foremost Foods Company, San Francisco, California.
Table 1. Composition of concentrate rations.

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</table>
Collection of rumen samples

Rumen fluid samples for protozoa and bacteria observations were taken every two weeks, starting the third week of the standardization period. The rumen samples were collected via stomach tube and syringe two to three hours after the morning feeding. After discarding the first 10 to 20 ml of rumen fluid drawn, approximately 30 ml of rumen fluid were collected in a 50 ml sample jar. All rumen fluid samples were then taken to the laboratory for further observations.

Preparation of rumen fluid samples for protozoa counting

A protozoa counting procedure of Luther (33) was used in this study. The sample of rumen fluid was thoroughly mixed and 10 ml was drawn by a wide bore pipette and placed in 10 ml of 20% formalin solution. After the formalinized sample was mixed thoroughly, 1 ml of the sample was transferred to a 10 ml volumetric flask and 3 drops of methyl green dye were added. The solution was then made to volume with 30% (v/v) glycerol solution.

The slides used for rumen protozoa counts were designed by Boyne et al. (6) and Luther (33). A 25.4 X 76.2 mm standard glass slide was fixed above a 38.1 X 76.2 mm glass slide. Strips of microscopic slides one millimeter in thickness were glued 20 mm apart on the larger slide for supports. The space between the slides permitted the flow of the counting solution containing protozoa to be held in place by surface tension. The slide was allowed to set 3 to 5 minutes for the protozoa to settle and then observed using a Bousch and Lomb microscope equipped with a 10X ocular and a 16 mm objective lens. A Whipple disc ocular,
composed of 10 squares wide and 10 squares long making a total area of one square millimeter, was inserted into the eye piece of the microscope. Thirty fields from each slide were counted. The total protozoa were counted and were classified under five predominant genera according to the scheme of Becker and Talboot (4): Isotricha, Dasytricha, Entodinium, Diplodinium, and Ophryoscolex.

Preparation of rumen samples for bacteria counts

Dilution blanks containing 99 ml of sterile phosphate (KH2PO4) buffered distilled water were used for preparing the samples to be cultured (43). In order to maintain conditions as anaerobic as possible during preparation of the dilutions, a stream of carbon dioxide was introduced into each dilution blank. Although high purity carbon dioxide was used, the precaution of scrubbing out any oxygen by passing it through heated copper shavings packed into a Pyrex glass column was used. The effluent end of the column containing the copper shavings was connected to a glass manifold. The glass manifold contained six outlets and attached to each outlet was a 76.2 mm 18 gauge hypodermic needle. The gas would then pass out through the needles into the dilution blanks. The purpose of the glass manifold was to facilitate the preparation of several dilution blanks and culture tubes at once.

Preparation of dilutions

An 11 ml sample of the rumen fluid was placed into one of the 99 ml dilution blanks which constituted the initial 1:10 dilution. From this initial dilution successive dilutions were prepared in order to
obtain thirty to three hundred colonies per tube (43).

The following dilutions were used for each group of organisms during each period of the experiment:

1. Total rumen bacteria: Dilutions of 1:1,000,000 and 1:10,000,000
2. Lactose fermenters: Dilutions of 1:100,000 and 1:1,000,000
3. Starch fermenters: Dilutions of 1:100,000 and 1:1,000,000
4. Proteolytic bacteria: Dilutions of 1:10,000 and 1:100,000
5. Direct microscopic count: The 1:1,000 dilution was used to make the direct microscope smear.

**Preparation of media**

The medium used for the total rumen bacteria count was described by Hungate (29). The rumen fluid-glucose-cellobiose agar (RGCA medium) was used as the basal medium for the lactose fermenters, starch fermenters, and proteolytic bacteria. Rumen fluid used in the medium was obtained by filtering rumen contents, obtained from a cow on an alfalfa hay-concentrate diet, through two layers of cheese cloth to remove the larger particles. The rumen fluid was stored in the refrigerator under carbon dioxide in rubber stoppered flasks after being autoclaved for 20 minutes at 15 pounds pressure. Before using the rumen fluid in the medium, it was centrifuged at 15,000 revolutions per minute for 20 minutes.

The RGCA medium as prepared by Bryant and Burkey (11) was used for the culturing of total count bacteria. The medium was boiled to dissolve the agar and placed in a 500 ml heavy-wall Pyrex glass Erlenmeyer flask. To the RGCA medium 120 ml of rumen fluid as described
previously was added and the solution again was boiled while passing carbon dioxide into the flask. The flask was then closed with a rubber stopper and wired in place to prevent the stoppers from blowing out during sterilization. The medium was sterilized in the autoclave for 20 minutes at 15 pounds pressure. After sterilization the medium was cooled in a water bath to a temperature of 45 to 50 C. The wire was cut, and as the stoppers were removed carbon dioxide was passed into the flasks to prevent the entrance of oxygen. Five milliliters of sterile 3% (w/v) cysteine hydrochloride and 20 ml of sterile 6% (w/v) sodium carbonate solution were added. The medium was then ready for use in the anaerobic culture tubes. Solutions of autoclaved cysteine hydrochloride and sodium carbonate were kept in dilution bottles in which carbon dioxide had replaced the air and then were stored under refrigeration.

Media for the other three groups of bacteria studied were prepared the same as the RGCA medium except that the glucose and cellobiose were omitted and the following ingredients added. (a) for the lactose fermenters, 0.5% (w/v) lactose; (b) for the starch fermenters, 0.2% (w/v) soluble starch; and (c) for the proteolytic bacteria, 1 ml of sterile skim milk was added per 100 ml of medium.

Preparation of culture tubes

The procedure of Bryant and Burkey (11) modified by Hungate (29) was used to prepare the culture tubes. Culture tubes 25 X 200 mm that had been previously sterilized without stoppers were used for preparing the roll tubes. The culture tubes were stoppered immediately with
sterile rubber stoppers upon their removal from the autoclave.

The method of preparing the culture tubes involved flushing sterile culture tubes containing nine ml of culture media with carbon dioxide as described previously. The tubes were stoppered and held in a 45 to 50C water bath until inoculated. The desired dilution of the inoculum was added with a sterile pipette and the tubes were flushed again with carbon dioxide. Then the rubber stoppers were inserted firmly into the culture tubes as the needles were withdrawn.

A roll tube was prepared in a manner similar to that used by Hungate (29). The tube was rolled by hand under the cold tap water so that an even film of agar covered the inside surface of the tube. An evenly spread film made for easier counting of the colonies. After solidification of the agar, the cultures were incubated at 39C for 72 to 96 hours. After incubation, colony counts were made on each tube. All dilutions were run in duplicate so that a more accurate count could be obtained.

**Statistical analysis**

Analysis of the rumen samples consisted of determining the total protozoa numbers and classifying them into one of the five genera. The different types of bacteria were counted and analysed to see if any difference was obtained between diets and time intervals. Least square analysis of variance computations were obtained using an IBM 360 computer. Additional tests computed on these results were run according to Steel & Torrie (40).
RESULTS AND DISCUSSION

Protozoa analysis of rumen samples

The sources of variation, degrees of freedom, mean square values, and levels of significance using the least square analysis of variance to analyze the protozoa and bacteria numbers are shown in Appendix Tables 1 and 2 respectively. Weeks zero and eight on the following figures designate the end of standardization and post-experimental periods respectively.

Total protozoa numbers are shown in Figure 1. Numbers ranged from a low $7.9 \times 10^4$ protozoa per ml in cows on the demineralized whey diet to a high of $2.5 \times 10^5$ protozoa per ml on the whey molasses diet during the standardization period. The highest numbers during the experimental period were $8.3 \times 10^5$ protozoa per ml on the whey molasses diet. The lowest total number of protozoa during the experimental period was $1.4 \times 10^4$ protozoa per ml of rumen fluid on the demineralized whey diet. The total protozoa count on the control, dried whole whey, and lactose diets were similar. There was no significant difference (P<0.05) between any of the diets on total protozoa numbers. The change of total numbers with time showed no significant difference (P<0.05).

Trends of the Isotricha numbers are shown in Figure 2. Isotricha numbers varied widely between the diets, however, no significant difference (P<0.05) was found between the diets. There was also no significant difference (P<0.05) in Isotricha between diets at the different time periods. Isotricha numbers ranged from a low of
Figure 1. Effect of rations on total rumen protozoa numbers. Weeks 0 and 8 indicate end of standardization and post-experimental periods respectively.
Figure 2. Effect of rations on rumen Isotricha numbers. Weeks 0 and 8 indicate end of standardization and post-experimental periods respectively.
2.2 \times 10^2 \text{ per ml of rumen fluid on the dried whole whey diet during the experimental period to a high of } 6.7 \times 10^4 \text{ per ml on the demineralized whey diet during the post-experimental period.}

Figure 3 shows the trends of the Dasytricha numbers on the different diets. No particular pattern was observed other than the fact that each diet had a lower number of Dasytricha at the end of the experimental period than during the standardization period. The cows on the demineralized whey diet contained no Dasytricha at the sixth week of the experimental period. Cows on the lactose diet had the highest number of Dasytricha at the sixth week of experimental period, having $1.2 \times 10^4 \text{ per ml of rumen fluid.}$ Numbers of Dasytricha returned toward normal during the post-experimental period.

There was a significant difference ($P<0.05$) between diets in Dasytricha numbers. To determine which diets were different, Duncan's new multiple-range test was used (40). Dasytricha were significantly lower ($P<0.05$) on the control, dried whole whey, and demineralized whey diets than on the lactose diet. The demineralized whey and dried whole whey diets had significantly lower ($P<0.05$) Dasytricha than the whey molasses diet. No significant difference ($P<0.05$) was found between the time periods on Dasytricha numbers.

Entodinium numbers of the cows on the different diets are shown in Figure 4. The cows on the demineralized diet had lower Entodinium than the cows on the other diets, but changes from the different time intervals followed a similar pattern. The animals on the dried whole whey diet showed a considerable decline in Entodinium during the sixth week of the experimental period. All of the diets except the
Figure 3. Effect of rations on rumen Dasytricha numbers. Weeks 0 and 8 indicate end of standardization and post-experimental periods respectively.
Figure 4. Effect of rations on rumen Entodinium numbers. Weeks 0 and 8 indicate end of standardization and post-experimental periods respectively.
demineralized whey showed an increase in Entodinium the second week of the experimental period and then declined slowly to the end of the period. There was no significant difference (P<0.05) between the diets or time intervals on the Entodinium numbers.

Luther (32) observed Entodinium and Diplodinium numbers were greater with high-energy diets. Sizeable populations were maintained in the rumen when the diet was composed entirely of concentrates. All of the diets except the demineralized whey showed an increase in Entodinium numbers during the second week of the experimental period after being on the standardization ration. The demineralized whey diet showed a continual decrease in the Entodinium during the experimental period.

Figure 5 shows the numbers and trends of the Diplodinium. The Diplodinium tend to follow somewhat the same pattern as the Entodinium did. The control, whey molasses, and lactose diets showed an increase in Diplodinium during the second week of the experimental period, while the demineralized whey and dried whole whey diets decreased slightly. All diets showed approximately the same Diplodinium numbers during the standardization and post-experimental periods. The higher Diplodinium numbers during the experimental period follows the trends observed by Luther (32) in sheep on high-energy rations. No significant difference (P<0.05) between the diets and the different time periods was observed in Diplodinium numbers.

Ophryoscolex were practically extinct from the cows on the different diets. For this reason, they were omitted from any analysis. The Ophryoscolex are generally found in animals on largely all roughage
Figure 5. Effect of rations on rumen Diplodinium numbers. Weeks 0 and 8 indicate end of standardization and post-experimental periods respectively.
rations. Many times they are absent from certain groups of ruminants regardless of types of feed which the animals received.

**Bacteria analysis of rumen samples**

Direct microscopic counts are shown in Figure 6. The counts of $10^9$ are within the range observed by Hungate (28). There was no significant difference ($P<0.05$) between diets on direct microscopic counts, however, all of the diets had higher counts during the experimental period than at the standardization and post-experimental periods. Maki and Foster (34) observed total microscopic rumen counts were two to three times higher from cows fed concentrates than those from cows fed roughage.

A significant difference ($P<0.05$) existed between the time periods. To check which time periods showed the difference, Dunnett's test was applied (40). The control, dried whole whey, demineralized whey, and lactose diets had significantly higher ($P<0.05$) direct microscopic counts during the experimental period than during the standardization and post-experimental periods. The direct microscopic numbers from the whey molasses ration increased significantly ($P<0.05$) at the fourth week of the experimental period.

Total viable bacteria counts ranged from $9.2 \times 10^6$ to $2.0 \times 10^8$ as can be seen on Figure 7. All of the diets caused a drop in total count during the fourth week of the experimental period; however, the animals regained some of the loss in bacteria by the final week of the experimental period. The change from a high-energy diet during the experiment to a low-energy diet in the post-experimental period caused
Figure 6. Effect of rations on direct microscopic counts of bacteria in the rumen. Weeks 0 and 8 indicate end of standardization and post-experimental periods respectively.
Figure 7. Effect of rations on total viable rumen bacteria. Weeks 0 and 8 indicate end of standardization and post-experimental periods respectively.
a drop in total bacteria numbers (32). There was no significant difference (P<0.05) between diets or the different time intervals on total viable bacteria.

Bacteria classified as lactose fermenters are shown on Figure 8. The cows contained approximately $10^6$ to $10^7$ lactose fermenters per ml of rumen fluid. By using selective anaerobic conditions to suppress the majority of bacteria not capable of utilizing lactose, numbers of lactose fermenters were obtained. Cows on the demineralized whey diet had unstable growth of the lactose fermenters. The bacteria were highest during the standardization period, then subsided sharply during the second week of experimental period. The up and down trend of the lactose fermenters is prevalent in all of the diets throughout the experiment.

The five rations showed no significant difference (P<0.05) in lactose fermenters. Each diet tended to react in the same manner to these particular bacteria. Time intervals, however, showed a highly significant difference (P<0.01) in the lactose fermenters. Dunnett's test (40) was applied to these results to see which periods showed significance. Lactose fermenters decreased significantly (P<0.05) on the control and demineralized whey diets during the second and sixth weeks as compared to the standardization period. The rumens of all animals on the experiment contained significantly higher lactose fermenters during the fourth week and decreased significantly in numbers during the post-experimental period than on the standardization period. Cows on the control, demineralized whey and lactose diets decreased (P<0.05) during the sixth week as compared to the
Figure 8. Effect of rations on lactose fermenting organisms in the rumen. Weeks 0 and 8 indicate end of standardization and post-experimental periods respectively.
standardization period. Animals on the dried whole whey diet showed the greatest decline in lactose fermenters during the post-experimental period. The much lower energy diet during the post period as compared to the experimental could have caused the decline.

The starch fermenting bacteria shown on Figure 9 do not follow any particular pattern or trend in any two diets. All of the animals had starch fermenting bacteria in the range of $10^6$ to $10^7$ organisms per ml of rumen fluid. There was no significant difference ($P<0.05$) between diets on the starch fermenters. Time intervals showed no significant difference ($P<0.05$) in numbers either. The high levels of energy and starch in the high grain diets may have been the reason for not having much variation in the numbers of starch fermenting bacteria between rations.

Proteolytic bacteria were grown using casein as the source of protein in the medium. Figure 10 displays the pattern of the proteolytic count throughout the experiment. Each of the diets seemed to respond alike although not in the same degree. There was no significant difference ($P<0.05$) between rations and proteolytic bacteria. Time intervals, however, showed a highly significant difference ($P<0.01$) in the numbers of proteolytic bacteria. Each of the rations except the lactose diet showed a significant decrease in proteolytics during the post-experimental period as compared to the standardization and sixth week of the experimental period. Dunnett's test (40) also showed that cows on the control, whey molasses, demineralized whey and lactose diets increased significantly in proteolytics during the second
Figure 9. Effect of rations on starch fermenting organisms in the rumen. Weeks 0 and 8 indicate end of standardization and post-experimental periods respectively.
Figure 10. Effect of rations on proteolytic organisms in the rumen. Weeks 0 and 8 indicate end of standardization and post-experimental periods respectively.
week of the experimental period. The lower proteolytic bacteria counts at the post-experimental period indicates that the lower energy ration must have caused a decrease in the supply of protein, or the change in rations may have provided a different source of protein for the bacteria.
SUMMARY AND CONCLUSIONS

This study was undertaken to determine the responses of the rumen microflora in lactating cows on high-grain diets containing whey products.

Five groups of three lactating Holstein cows were fed one of five rations: (1) high-grain limited roughage (control), (2) control + 14% dried whole whey, (3) control + 5.9% dried whey molasses, (4) control + 11.8% demineralized dried whey, and (5) control + 9.8% lactose. Three trials were run, each containing one cow for each diet. The time span of the study was from April 1970 to October 1970. Each trial consisted of a three-week standardization period, a six-week experimental period, and a two-week post-experimental period. Rumen samples were obtained the third week of the standardization, and every two weeks after that.

Total protozoa were counted and classified into the five genera: Isotricha, Dasytricha, Diplodinium, Entodinium, and Ophryoscolex. All data were analyzed by the least square analysis method. Dasytricha numbers were significantly different (P<0.05) between the diets. Duncan's new multiple-range test (40) revealed Dasytricha numbers decreased (P<0.05) on the control, dried whole whey, and demineralized whey diets. There was no significant difference between diets on total protozoa or any of the other genera. Ophryoscolex numbers were so few that they were not analyzed. The analysis also showed no significant difference between time intervals of the three periods.

Total bacteria were measured by direct microscopic count and by the roll-tube method for total count, lactose fermenters, starch
fermenters, and proteolytic organisms. Analysis on the different
groups of bacteria revealed no difference (P<0.05) between diets;
however, differences in time intervals were found to be significant in
some of the groups of bacteria. Direct microscopic numbers increased
(P<0.05) on the control, dried whole whey, demineralized whey, and
lactose diets during the experimental period. Differences in time
periods occurred also in lactose fermenters and proteolytic organisms.
Each diet except the whey molasses showed an increase (P<0.01) in
lactose fermenters during the fourth week of the experimental period.
Proteolytic organisms decreased (P<0.01) on each diet except the
lactose ration during the post-experimental period.

Conclusions drawn from the study are:

(1) The total protozoa numbers were consistently lower on the
demineralized whey diet.

(2) Dasytricha numbers tend to vary more than the other genera of
protozoa.

(3) Minerals in the whey appear to facilitate the growth of protozoa.

(4) Proteolytic organisms tend to decrease after being on a high-
energy diet.

(5) The high-grain diets account for the majority of the fluctuations
in protozoa and bacteria numbers.
LITERATURE CITED


Appendix Table 1. The sources of variation, degrees of freedom, mean square values and levels of significance using the least squares analysis of variance to analyze protozoa numbers.

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<thead>
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<th>Source</th>
<th>Degrees of Freedom</th>
<th>Total No.</th>
<th>Isotricha</th>
<th>Dasytricha</th>
<th>Diplodinium</th>
<th>Entodinium</th>
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a highly significant at P<0.01.

b significant at P<0.05.
Appendix Table 2. The sources of variation, degrees of freedom, mean square values and levels of significance using the least squares analysis of variance to analyze bacteria numbers.

<table>
<thead>
<tr>
<th>Source</th>
<th>Degrees of Freedom</th>
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<sup>a</sup> highly significant at P<0.01.
<sup>b</sup> significant at P<0.05.