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DEVELOPMENT OF A NEGATIVE STAIN FOR
A DIRECT MICROSCOPIC COUNT FOR
RUMINAL MICROORGANISMS

Thesis for the Degree of M. S.
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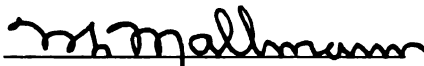
Development of a Negative Stain for a Direct
Microscopic Count for Ruminal Microorganisms.

presented by

Karl Kereluk

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DEVELOPMENT OF A NEGATIVE STAIN FOR A DIRECT MICROSCOPIC
COUNT FOR RUMINAL MICROORGANISMS

(An Abstract)

A number of methods for the counting of ruminal microorganisms has been developed (Kohler, 1940; Van der Wath, 1941, 1948; Bortree, 1948; Gall, 1949). Gall has developed the better method in as much as her total counts are higher than those by any of the other methods. The writer has observed that stains made by her method have given some difficulty in use. It was the purpose of this research to find another stain which would be satisfactory.

A survey of the majority of the negative stains, which could be applicable to this method, was made. Those stains which were unsatisfactory are: congo red, indulin, methyl blue, aniline blue, and rose bengal.

The following stain preparation was found to be satisfactory:

Nigrosine, water soluble, certified by the Commission on Standardization of Biological Stains	10.0 gm
Distilled water	100.0 ml
Formalin (40%)	0.5 ml

One part of the aqueous nigrosine stain is added to three parts of 95 percent ethyl alcohol and filtered through a Seitz filter twice.

A control smear should be made and examined microscopically. The smear should have an even distribution of the gray-black color without any perforations in the curtain of stain.

Gall's method was followed with three exceptions:

1. A two millimeter loop was used in the place of a three millimeter loop.
2. A circle with an area of four square centimeters was used in place of a square (Moir, 1951).
3. The above described stain was used in the place of Gall's.

Counts on feces from cattle were run in duplicate using the above described stain in Gall's method and the chamber counting method. The modified stain, a direct microscopic count method, showed a higher number of organisms than did the chamber counting method.

M. Gallmann

**DEVELOPMENT OF A NEGATIVE STAIN FOR A DIRECT MICROSCOPIC
COUNT FOR RUMINAL MICROORGANISMS**

by
Karl Kereluk

A THESIS

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- Introduction -

Of all the symbiotic relationships between mammals and micro-organisms, the most important to mans' economy is found in the ruminants (Hastings, 1944). The ruminating animals such as cattle, sheep and goats consume and digest large quantities of pasture grass, hay or other cellulose-containing roughage feeds. It has been shown that micro-organisms are chiefly responsible for the decomposition of cellulose in the rumen and that investigators have failed to detect the presence of the necessary cellulose-digesting enzymes in the digestive secretions of the host animal (McAnally, Phillipson, 1944).

There is a distinct need for a satisfactory method of determining microscopically the number of bacteria in rumen contents. Methods employing a positive staining of the bacteria have been unsatisfactory due to difficulty in distinguishing between debris and microorganisms and inability to see the minute micrococci apparently common to rumen contents.

Although the counting chamber method is perhaps better than the stained preparation it is difficult to use and the results may not be too accurate.

A third method consists of a stained preparation using negative staining.

Each of the latter two methods have been recommended

for use. Each has its limitations. In this study an attempt was made, first, to improve the negative staining technique of Gall; and second, to check the improved negative staining techniques derived against the chamber method.

- Historical Review -

There seems little doubt that cellulose-digesting micro-organisms are extremely important in the digestion of plant materials in ruminants (Tappener, 1884, Baker, 1935). However, attempts to grow them and to study them under controlled conditions have been uniformly unsuccessful. Some of the rumen protozoa have been shown to digest cellulose (Hungate, 1942). The removal of these protozoa does not impair the rate of cellulose digestion and it is concluded that other organisms also exercise this function (Becker, Schulz, Emmerson, 1929).

Henneberg (1922) applied direct microscopy to the detection of the micro-organisms concerned in the digestion of structural cellulose. He showed the preponderance of micro-organisms that underwent a blue color reaction with iodine. He observed the cellulolytic bacteria within the eroded cavities of plant material. He emphasized the relationship of the micro-organisms and cellulose on this basis.

Baker and Harris (1947-48) confirmed the work of Henneberg. They observed and distinguished between a free iodophile population consisting of micro-organisms suspended in the ruminal or caecal liquids and a fixed iodophile population attached to and acting upon structural cellulosic mater-

ial of the ingesta.

The morphological characteristics of the iodophiles were studied in detail by Baker (1942) who distinguished the following forms:

1. Oscillospira guillemondi, a colorless spore-forming oscillarian.
2. A giant Spirillum, divided by transverse septa into spherical or ovoidal compartments.
3. Large sarcina Packets.
4. An unidentified navicular organism (bacterium forming rosette-shaped oscillations of 5 to 30 units.
5. Coccoid chains of 2 to 8 units.

Elsdon (1948) states that two criteria must be fulfilled before an organism can be said to be a functional member of the rumen population. First, the organism must perform a chemical reaction known to occur in the rumen; second, the organism must be present in the rumen in sufficient numbers to perform this reaction. He also pointed out that the isolation in pure culture and study of organisms responsible for the chemical changes in the rumen are most important, but that analysis of rumen population will not be achieved by the "haphazard application of standard bacteriological procedures."

Gall and Huhtanen (1951) state that not all bacteria present in the rumen are true rumen organisms. Contamination of the rumen is constantly occurring from outside sources. Since there are about one hundred billion bacteria

per gram of rumen content, only the organisms present in one million per gram, or not outnumbered by more than one to 100,000 by other bacteria, are considered significant. As the rumen is an anaerobic organ, it would seem that an organism must be able to carry an anaerobic metabolism in order to survive.

The following is their criterion for judging a true rumen bacterium: "1. The bacterium must be anaerobic. 2. The bacterium must be demonstrated to be present in at least one million per gram of rumen contents. 3. The bacterium must be isolated repeatedly under these conditions. At least ten isolations of the same pure "type" culture, as shown by selected screen tests, are considered minimum. 4. The bacterium must be isolated in the animals in at least two geographical localities. 5. The bacterium must produce end products which can be metabolized by other rumen bacteria to end products found in the rumen. All five of these conditions should be met before a bacterium is accepted as a true rumen bacterium."

Since micro-organisms play such an important role in the breakdown of cellulose in the rumen, a total count of the bacteria present would help to shed some light on the complex problem.

Kohler (1940) was among the first to attack the problem of finding a method of counting micro-organisms present in the rumen. His technique involved centrifuging a suitable

dilution of rumen sample at 800 to 1,000 rpm, concentrating the sample by a bacterial filter, and staining the bacteria with carbol fuchsin. Then 0.01 ml of the material is spread on a cover slip over an area $30 \times 2700 \text{ } \mu^2$ and examined microscopically. It was difficult to distinguish cocci from debris in the sample. Rods and cocci were counted separately. In the case of chains of organisms, each cell was counted separately. The direct count for the rods averaged 2,373,900,000 per ml; and for both rods and cocci, the average count was 12,980,100,000 per ml.

Van der Wath (1941) reported a method of counting the infusoria from the rumen of sheep. Material for the infusorial count was always withdrawn at 9 A. M. After shaking the sample vigorously, one ml was drawn into a wide mouth pipette and added to seven or eight ml of corrosive-sublimate-alcohol fixative. After washing with alcohol-iodine and 70 percent alcohol, the sample was stained with borax-carmin. The stained material was suspended in 3 ml of oil of cloves in which it was preserved. After diluting to one to ten or one to 100 in oil of cloves, a drop of known volume from a capillary pipette was placed on a glass slide and covered with a cover slip. The total number of infusoria per drop was counted from which the number per ml of ingesta was then calculated.

A Petroff-Hauser bacterial counting chamber for making ruminal counts is described by Van der Wath (1948). After

thoroughly shaking the fixed ruminal sample, a final dilution of one to 400 is made. Three-tenths ml of 1 percent Nile blue sulphate is added to the final dilution as the stain. A Thomas-Zeiss pipette is filled with the final dilution and the counting chamber filled. The chamber is allowed to stand for three or five minutes in order to allow the bacteria to settle. Ten blocks of nine small squares are counted (giving the total number of bacteria in 90 squares) and the total bacteria computed by a known formula. Van der Wath states that these counts are, of course, not necessarily true total counts, since an unknown percentage of organisms penetrates into or becomes absorbed by food particles. The total count as given is, therefore, always considered as less than the true count.

Bortree et al. (1948) used a chamber counting method similar to Van der Wath's, modified by using another stain and by the number of small squares counted. At present in this laboratory, the counting method of Bortree et al. (1948) is being used for a ruminal bacteria count. The method will be described under procedures.

Gall et al. (1949) were the first to describe a counting method employing a negative stain. The principle used in the counting method is the same as that of the direct milk count; however, certain modifications were made to adapt the technique to the special purpose of counting ruminal bacteria.

- Methods of Procedure -

The counting method of Bortree et al. (1948) is as follows:

Preparation of the stain: Ten ml of ethyl alcohol is saturated with crystal violet (gentian violet). One ml of this solution is added to 49 ml of distilled water. The stain is heated to 60 C., filtered and used.

Usual procedure and dilutions: Five ml of rumen ingesta is added to 10 ml of 10 percent formalin and shaken. Three ml of this sample is pipetted into 22 ml of sterile distilled water and again shaken. One ml is pipetted into eight ml of sterile distilled water and one ml of stain is added. This is mixed well and heated over a bunsen burner until it bumps slightly. The counting chamber is filled by using a Thoma-Zeiss blood pipette. The counting chamber is of the Petroff-Hauser type. One-hundred small squares on the top and the bottom of the ruled area are counted and the average of the number of bacteria per 100 squares is calculated. By using the following formula the total number of bacteria in one ml can be determined:

$$\begin{array}{l} \text{No. of bacteria} \\ \text{per ml of sample} \end{array} = \frac{\text{Ave. no. of bacteria per 100 squares} \times \text{dilution} \times 20 \times 20 \times 50 \times 1,000}{\text{No. of small squares counted}}$$

20 x 20 = size of small squares or 400

50 = depth of material with cover slip on the chamber

1,000 = conversion factor to change mm to ml

No. of small squares = 100 (after average has been found)

Recently a slight modification has been made in dilutions

and in the counting of the organisms in the chamber. A fecal specimen¹ is diluted one to ten and used as the sample. Ten ml of the sample is pipetted into a test tube containing five ml of 40 percent formalin. The dilution is now one to 15. One ml of the above dilution is pipetted into seven ml of sterile distilled water. This gives a dilution of one to 120. One-half ml of the one to 120 dilution is pipetted into a test tube containing four ml of sterile distilled water and one-half ml of crystal violet stain. This final dilution is one to 1,200. A portion of the final dilution is pipetted into the counting chamber and the organisms are counted. Two hundred small squares are then counted, and by using a modification of the first formula the following calculation is made:

$$\begin{array}{l} \text{No. of bacteria} \\ \text{per ml of sample} \end{array} = \frac{\text{No. of bacteria per 200 squares} \times \text{dilution (1,200)} \times 20 \text{ million}}{200 \text{ squares}}$$

The disadvantages of the Petroff-Hauser chamber counting method are:

1. Movement of the bacteria in the chamber. This takes time as the chamber on the microscope must remain stationary until the bacterial movement has stopped. This waiting takes from two to five minutes.
2. There is a lack of clear differentiation between micro-organisms and plant debris. The direct staining method

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For the work on this problem, fecal material was used in the place of ruminal material because of the accessibility of the samples.

- stains both the bacteria and plant debris making it hard to distinguish between the two.
3. The filled chambers cannot stand for a long period of time as evaporation will take place in the chamber.
 4. It is very cumbersome to fill the counting chamber. Time, practice, and patience are needed to do a satisfactory job.

Call et al. (1949) described a counting method for ruminal bacteria using a counting method similar to a direct milk count. Certain modifications were made to adapt the technique to the special purpose of counting ruminal bacteria. In brief, the method is as follows: "A 0.01 ml portion of the properly diluted sample is mixed with a 3 mm loopful of saturated methyl alcohol solution of water-soluble nigrosine and spread evenly over a 2 x 2 cm area of a very clean slide. This slide is dried quickly on a very hot electric plate and 10 to 20 fields counted." In following her inadequate description of the counting technique, these difficulties were noted:

1. When using a clean slide (following her cleaning methods), the sample would be repelled by the loop containing the stain and run over the 2 x 2 cm square, marked off by a wax pencil, before contact could be made. This may be due to static charges. A rapid contact of stain and sample gives a similar result. This phenomenon occurred frequently enough to warrant mention.

2. The marking of a 2 x 2 cm square on the glass slide would always leave many particles of pencil wax within the area which was to be used for counting.
3. There is no mention of a control smear made on the stain alone. As there is no specification made on the filtering, the filtering was done with ordinary laboratory filter paper. If bacteria were present in the stain preparation they would, therefore, be counted on the slide. To rule out doubt that the organisms seen on the slide are organisms from the sample, a control smear should be made and the stain preparation containing bacteria should be discarded.
4. When the 0.01 ml sample and the 3 mm loopful of stain are successfully mixed, the slide is rocked side to side to effect an even color. Slight shrinkage immediately takes place on the edge of the smear. The slide is placed on the very hot electric plate to dry. The smear dries unevenly or concentrates toward the center. On microscopic examination of the smear, cracking is seen in the concentrated area (about one-fourth of the total smear). The cracking and the concentration interfere with the counting of ruminal organisms.

Gall stated the following about the stain: "A large supply should preferably be made, so that it can be used over a long period, since minor adjustment in the amount of dye desired to give the proper black background must be made with each lot, to suit the individual." As mentioned,

her stain is a saturated methyl alcohol solution of a water soluble nigrosine. The only adjustment which could be made would be the dilution. This lightens the background and reduces contrast. Due to the unpredictable behavior of the stain, no counts could be made.

Cumley (1935) maintains that the dye is derived from varying degrees of sulfonation; and the manufacturers make little claim as to the possibility of duplication of any batch that has proven satisfactory for any special purpose. This may explain the unpredictable behavior of the stain.

- Experimental Studies -

It was the purpose of this research to find a negative stain which would overcome the difficulties encountered in Gall's method.

A direct staining method was ruled out, as a direct stain only stains the cytoplasmic membrane and protoplasm while the cell wall and slime layer are not stained. This decreases the visibility of the organism. A direct staining method would increase the time necessary to find the smear with the oil immersion lens, inasmuch as a high dilution of the sample is needed to secure 10 to 30 organisms per field and it is difficult to focus on a smear when there are so few organisms present.

A negative stain will increase the visibility of microorganisms. Definition of small micrococci is important. Baker (1931, 1939) noted the action of small cocci in the

disintegration of the cell wall substance. The cocci were present in clearly defined zones of erosion. The size of the cocci was 0.25 to 0.9 mu and 0.1 to 0.2 mu.

Knaysi (1945) made a comparative study of the cell width of Bacillus cereus and Bacillus mycoides. Measurements of the living cell in the medium in which they were growing agreed with measurements on similar cells stained by a method showing the cell wall. In stained smears in which the cell wall was not visible, the cell appeared much smaller and represented the shrunken mass of protoplasm. The cell wall and slime layer are not stained with a negative stain but the stain outlines the cell wall and slime layer. The organisms measure larger than their true size owing to the fact that the colloidal dye film retracts on drying.

A number of negative stains was tested for their suitability in a direct counting method as prescribed by Gall et al. (1949). A control smear of the stain and a smear with a 0.01 ml sample of rumen liquor (diluted one to 100) and the stain were made on the same slide. As no comparative counts were to be made, the area covered by the smear was not controlled.

It was important to have a clean slide for the preparation of the smears. The following method has been used for cleaning the slides: The slides were stored in a chromic acid cleaning solution for a few days, then rinsed in distilled water, dried, and stored in a clean dust-proof box. Prior to using the slide, they were flamed and allowed to cool.

Benian's Congo red method was the first staining technique to be tried.

Stain preparation: Congo red (80 percent dye content) - 2 gm
Distilled water - 100 ml

- Staining schedule:
1. Place a drop of the above stain on the slide.
 2. Mix the stain and the 0.01 ml diluted rumen sample and spread out into a rather thick film.
 3. After the film has dried, wash with 1 percent hydrochloric acid.
 4. Dry.
 5. Examine.

Results: The cells are unstained against a blue background. The blue background was heavily textured. (A texture similar to the crinkly finish caused by baking certain types of black enamel.) The textured background interfered with the visibility of the organisms. The texturing was found to be due to the formalin present in the original fecal sample. The formalin was added as a preservative. For further studies, fecal samples without formalin were used. However, this staining method was very unsatisfactory because large sheets of the smear float off when the 1 percent hydrochloric acid was added. The parts of the smear which remain on the slide show a very even distribution of the blue color. The stain gave a good contrast and the control smears show little or no interference from other organisms or artifacts. Various strengths (0.25%, 0.5%, 2%, 5%) of the hydrochloric acid were used to minimize flaking. This was unsuccessful.

A one-third percent hydrochloric acid solution made with 95 percent alcohol was tried and gave the same results.

Two and one-half, five, and ten percent aqueous solutions of anilin blue, indulin, rose bengal, and methyl blue were next tried. Each preparation was heated and filtered before using. Smears were made in the same manner as that used with the Congo red stain. All the stains showed poor contrasting backgrounds.

Two staining procedures were used. The first method was discarded after a series of 240 samples were tested because of a desire to find a method that would eliminate the objectionable features that were found.

In the first method the stain preparation, designated as nigrosine stain #1, was as follows:

Nigrosine, water soluble (American nigrosine certified by Commission on Standardization of Biological Stains as ordinarily satisfactory).....	10.0 gm
Distilled water.....	100.0 ml
Formalin (40 percent).....	0.5 ml
Ethyl alcohol (95 percent).....	5.0 ml

The nigrosine is added to 100 ml of distilled water and stirred until the nigrosine has completely dissolved. Next the formalin and ethyl alcohol are added. The stain is filtered twice through a Seitz's filter. The stain should produce an even dark gray color on the slide. Procedure: The sample (rumen or fecal) is diluted in distilled water so that there

are about five to twenty organisms per microscopic field (a dilution of one to 15,000). Ten ml of a one to ten dilution of the sample is placed in a test tube containing five ml of formalin, producing a one to 15 dilution. The sample is shaken vigorously for 30 seconds. One-tenth ml of the one to 15 dilution is pipetted into a dilution blank containing 99 ml of distilled water, producing a final dilution of one to 15,000. The diluted sample (one to 15,000) is vigorously shaken for 30 seconds.

A 0.01 ml sample is pipetted from the dilution blank and placed on a clean slide. Care should be taken in pipetting the diluted sample onto the slide. The tip of the pipette is brought into contact with the clean slide at the center of the inscribed circle and the 0.01 ml sample is released. The tip of the pipette is lifted from the slide and again gently touched to the slide, near the original sample, to dispell the last small drop which will cling to the pipette.

A loop made of nichrome wire, with an inside diameter of 2 mm is dipped into the prepared stain and is placed in contact with the 0.01 ml sample. By means of the nichrome loop the stain and sample are completely mixed and spread to form a circle which has an area of four square cm.

Moir (1951) used the nigrosine slide technique described by Gall et al. but modified the 2 x 2 cm square to a circle of the same area. He noted that less shrinking diffi-

culties were experienced with the circle than with the square and the resultant slides were more uniform in density. A circle with a radius of 1.13 cm gives an area of four square cm. The slide is placed over a guide and the smear is made.

After the stain and the sample are mixed and spread, the slide is rocked back and forth and side to side to achieve an even color. The slide is rapidly dried on a hot plate at 700 F. (A Sunbeam hot plate, model 14 B, was used.) The drying takes five seconds at the most. The slide is quickly removed, as a longer time on the hot plate will crack the slide. It has been found that 3 percent of the glass slides will break from the intense heat of the hot plate. The breakage is usually due to imperfections in the glass slide and not from the short exposure to the heat. If the break occurs through the smear, a duplicate slide should be made. After the slide has cooled, it is ready to be examined microscopically and the bacteria counted.

The microscope is calibrated by following the directions in the 9th edition of Standard Methods for the Examination of Dairy Products.

The bacteria will appear white against the dark gray of the negative stain (Fig. 1). Care should be exercised in the counting of the bacteria. Spherical aberration of the oil immersion lens will obscure the view of the periphery of the field. Therefore, by means of the fine adjustment screw the bacteria, which would be present in the peripheral area, can

be brought into focus.

Artifacts present in the smear will cause undue cracking, formation of a dark ring of stain around the artifact, and irregular white forms. Some artifacts will be stained also. Dirty slides will cause undue confusion as small grease spots will appear as bacteria.

"Strip counting" should be employed. The smear will be light gray in the periphery which will afford little or no contrast for the bacteria. The center of the field will be black containing large cracks and the bacteria will be obscured by the heaviness of the stain. The larger area between the extremes, the light and the dark, is the preferred area for the counting of the bacteria. Conditions here are optimum for contrast and for ease of vision. The "strip counting" should, therefore, be done in this area. Ten or 20 fields are counted.

Williams and Moir (1951) stated: "The relationship between the numbers of organisms counted in a nigrosine smear and the numbers actually present in the sample, however, is not nearly so satisfactory. It is exceedingly difficult to distinguish with certainty between artifacts and bacteria when their size is less than about 0.5 mu. The counts present in this study arbitrarily exclude all bacteria less than about 0.5 mu, even though the presence of more minute organisms can be demonstrated in stained preparations. As

a result, the number of organisms counted are slightly underestimated."

Baker (1931, 1939) showed the importance of small micrococci in the rumen and noted their size as 0.25 to 0.90 mu, and 0.1 to 0.2 mu. It is therefore indicated that organisms under 0.5 mu should be counted, otherwise a complete picture of the total free ruminal organisms could not be made. Thus in the strip counting, all organisms seen are counted.

In the previously described technique there is a valid objection to the fact that the center area of the stained preparation is too heavy and that shrinkage occurs around the edges. This forced strip counting, which may not be a representative count of the total smear.

Steps were taken to eliminate these objections by further modifications of the stain mixture.

To keep the smear in place during the quick drying on an electric hot plate, several experiments were tried. Varying concentrations of agar and gelatin (0.5, 1 and 1.5 percent) were added to the 99 ml dilution bottles in an attempt to increase viscosity. The 0.01 ml sample was pipetted to a clean glass slide. The usual procedure of mixing and drying was followed, but neither the agar or gelatin prevented the smear from concentrating toward the center. The higher concentrations of agar gave a textured background to the nigrosine stain and interfered with the counting of the bacteria.

A cationic surface active agent was added to the 99 ml dilution blank; but it, also, was unsuccessful in stopping the concentration.

Different dilutions of ethyl alcohol were tested. A stain was then found which would give a completely countable smear. The dilution of the aqueous nigrosine stain with ethyl alcohol has overcome the difficulty but has sacrificed some contrast. The following is the formula of the stain:²

Nigrosine, water soluble (certified by the Commission on Standardization of Biological Stains).....	10.0 gm
Distilled water.....	100.0 ml
Formalin (40 percent).....	0.5 ml

One part of the aqueous nigrosine stain is added to three parts of 95 percent alcohol and filtered twice through a Seitz filter.

The smear is made in the same way as with nigrosine stain #1 with two exceptions: 1. A nichrome loop calibrated so it will deliver 0.01 ml of the sample is used in the place of a pipette. 2. The final dilution of the fecal or rumen sample is one to 3,000.

"Strip counting" is not employed with this stain (nigrosine #2) for it does not concentrate forming an uncountable area in the center of the smear. There is a slight shrinkage only on the very edge of the smear caused by the initial

2

This stain will be referred to as "nigrosine stain #2".

drying. Ten or twenty fields on the smear are counted.

Fecal samples from cattle were run in duplicate using the chamber counting method (Bortree, 1948) and the modified Gall's slide counting method. The nigrosine stain #2 was used in the place of Gall's stain.

- Results -

Two hundred and forty samples were counted using nigrosine stain #1. Sixty-eight percent of these samples were higher by the direct microscopic method than by the chamber method.

Two hundred and thirty-seven samples were counted using nigrosine stain #2. The direct microscopic method gave 89.5 percent higher counts than did the chamber counting method.

Figures 2 to 8 show the fluctuation in counts for each cow, of the 237 samples tested, over a four months period.

Five two-hour rumen samples were taken from one cow; the first sample was taken at 8 A. M., the other samples at 10 and 12 A. M., 2 and 4 P. M. The counts by the direct microscopic method showed a higher number of bacteria present in the samples than did the chamber count (Fig. 9).

- Discussion -

The first nigrosine stain preparation developed (nigrosine #1) gives a smear with a large uncountable area. When the smear is dried, this stain shrinks and concentrates toward

the center of the smear. The center portion cracks and the stain is so heavy the bacteria are obscured. "Strip counting" must be employed.

To overcome these problems, the nigrosine stain was diluted with ethyl alcohol. The increased amount of alcohol allowed the smear to dry more quickly thus eliminating the concentrated center area. It was found that one part of aqueous nigrosine stain to three parts 95 percent ethyl alcohol gave the least shrinkage without a great loss of contrast. When lesser amounts of ethyl alcohol are added to the stain preparation, cracks will appear in the smear. When greater amounts are added to the stain, the contrast is less, making it difficult to count the microorganisms.

The direct microscopic counting method using the nigrosine stain gave consistently higher counts than did the chamber counting method. When nigrosine stain #1 was used, only 32 percent of 240 samples were higher by the chamber counting method. With the elimination of the objectionable features in the nigrosine stain #1, the counts were still higher. Only 10.5 percent of 237 samples were higher by the chamber counting method. Not only does the direct microscopic method give higher counts, but it is easier and faster to use.

In the chamber counting method, there are three separate steps when making the dilutions of the fecal sample. A Thoma-Zeiss pipette is used to transfer the fecal sample to

the counting chamber. The chamber must rest on the stage of the microscope until movement of the bacteria has stopped. In the direct microscopic counting method, the dilutions take only two steps, and the use of a nichrome loop facilitates a quick transfer of the sample to the slide.

When examining the microscopic field of the counting chamber, it is hard to differentiate between plant debris and bacteria for this method stains them both. The negative stain makes possible a rapid differentiation of plant debris and bacteria. The bacteria are not stained and appear as white micro-organisms against the black of the stain. The plant debris is slightly stained and, therefore, becomes part of the background.

- Conclusions -

1. A direct microscopic counting method using a negative stain is presented.
2. The formula for the negative stain is presented.
3. The direct microscopic count using a nigrosine stain has several advantages over a chamber counting method:
 - (a) There is no movement of the bacteria.
 - (b) Smears can be stored for future checking and reference.
 - (c) The method is faster and simpler.
 - (d) A rapid differentiation between plant debris and bacteria is possible.
 - (e) The counts are usually higher.

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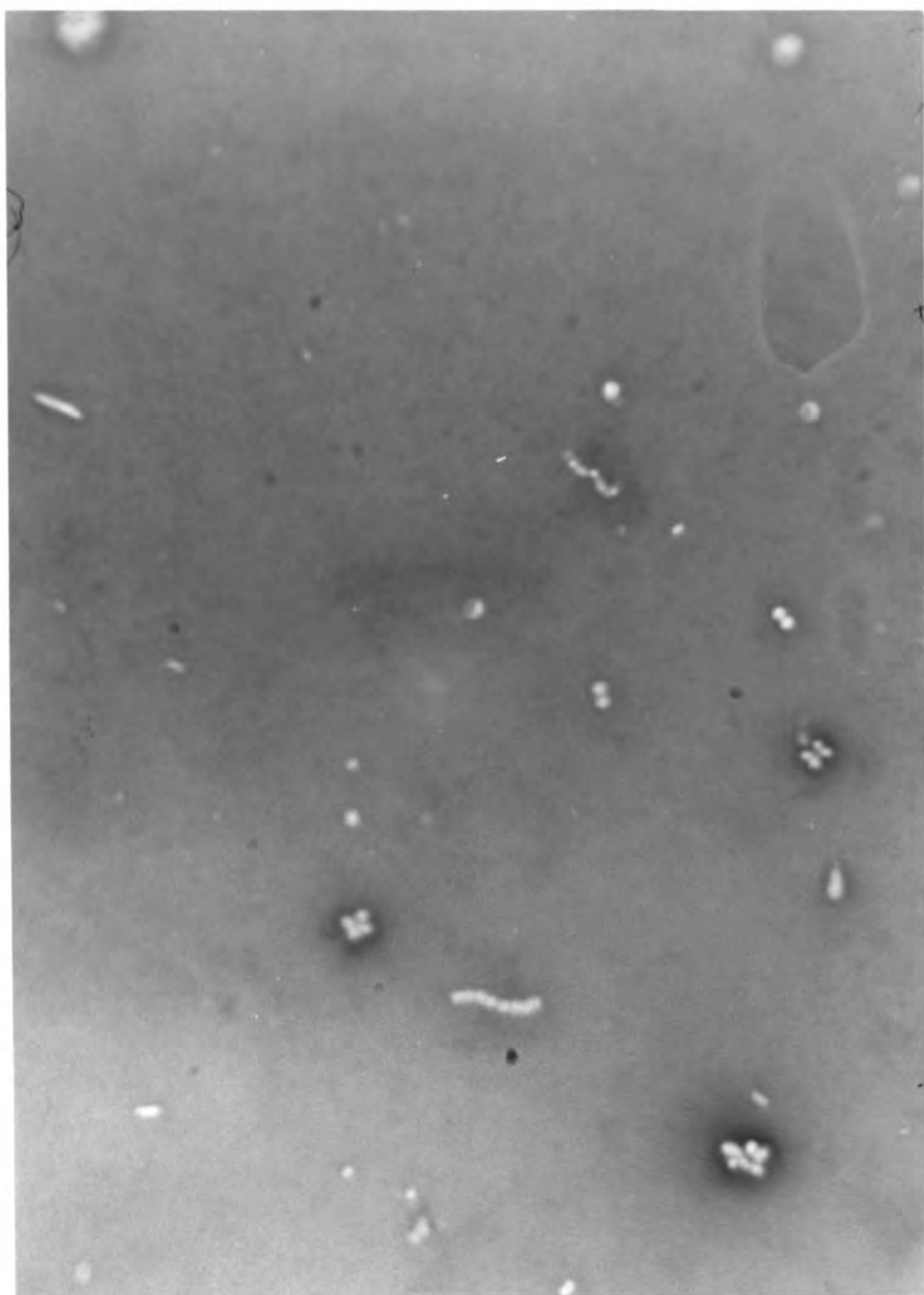


FIG. 1

COMPARATIVE FECAL COUNTS ON COW #1120

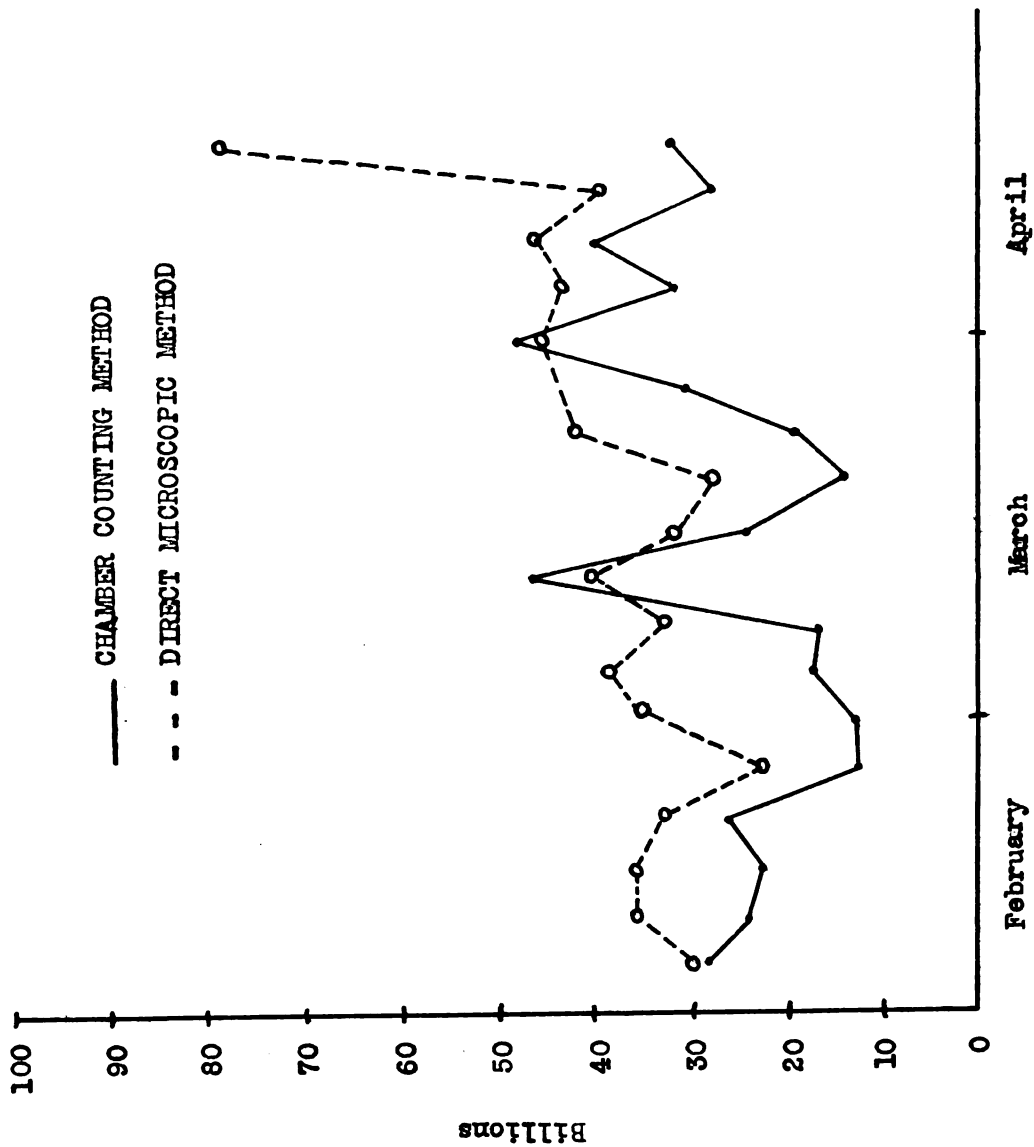


FIG. 2

COMPARATIVE FECAL COUNTS ON COW #1120

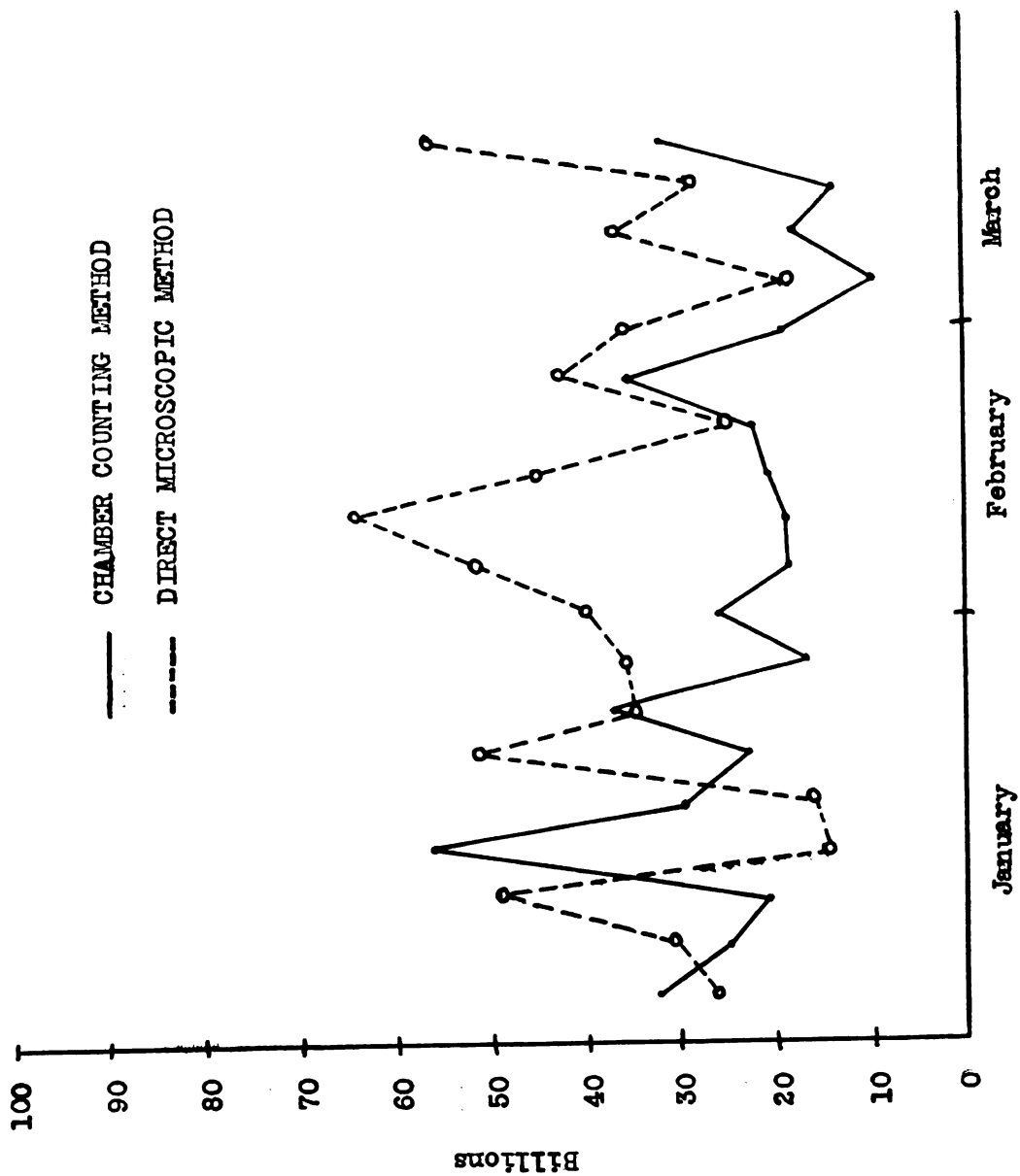


FIG. 3

COMPARATIVE FECAL COUNTS ON COW #1121

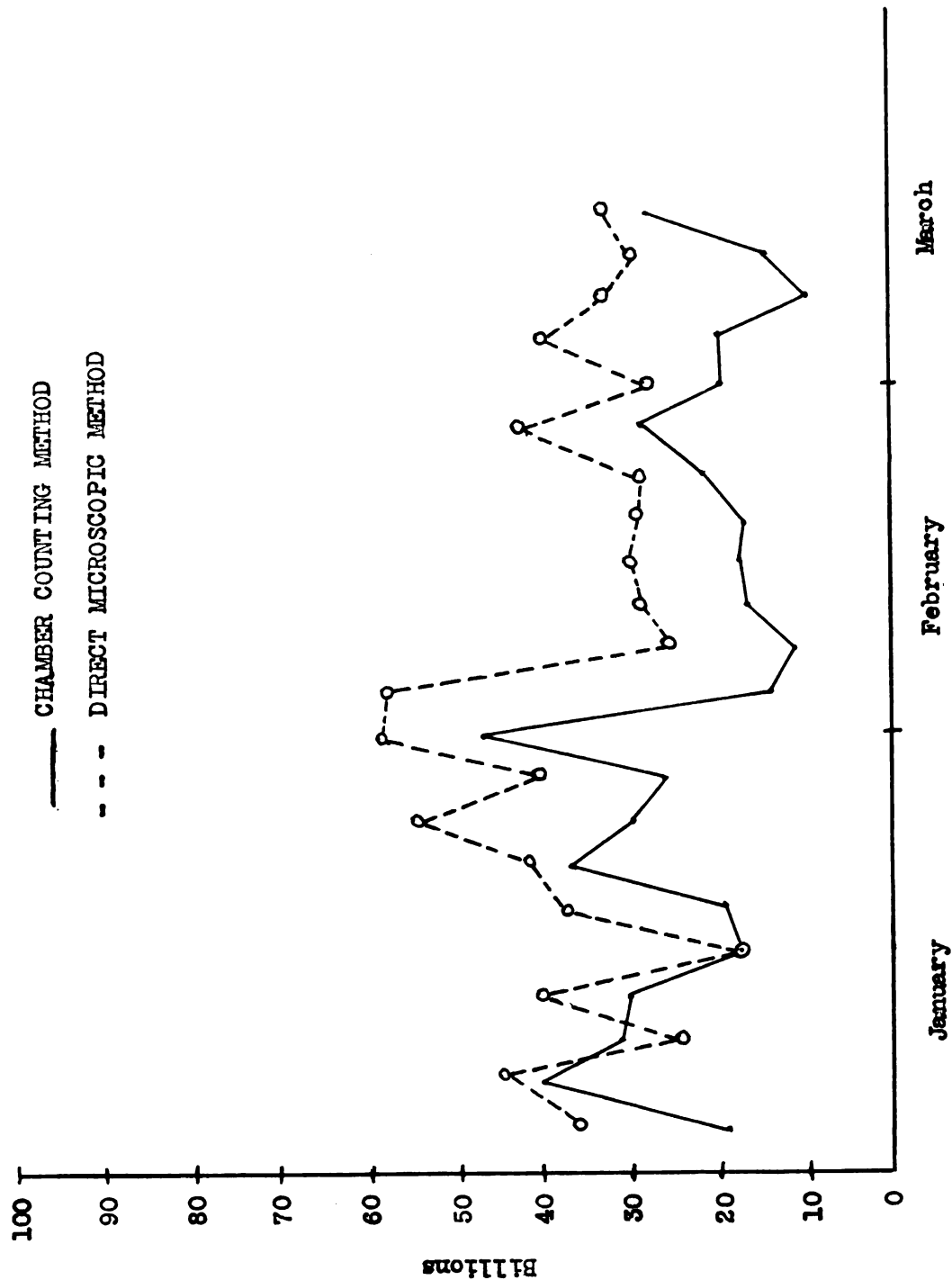


FIG. 4

COMPARATIVE FECAL COUNTS ON COW #2002

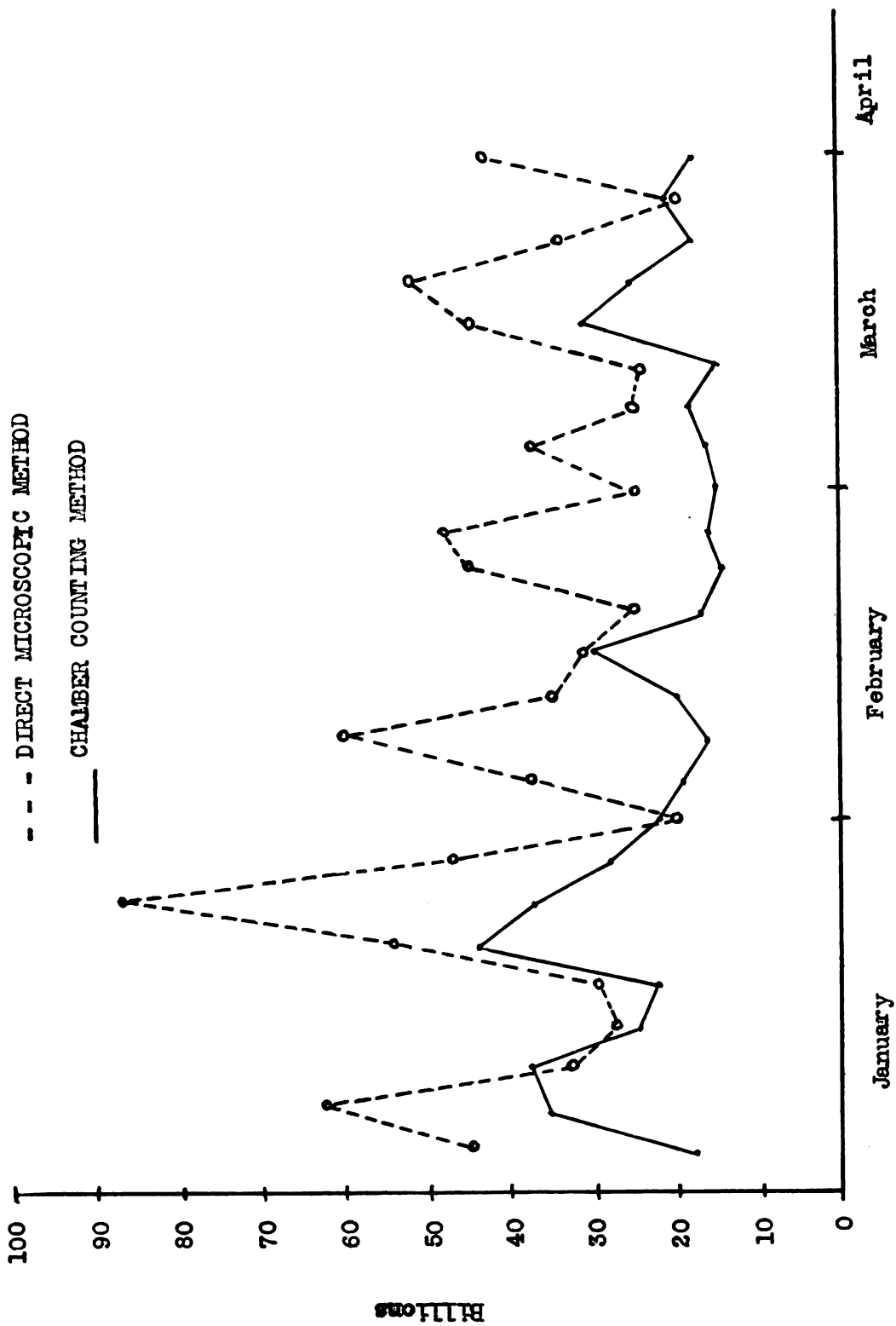


FIG. 5

COMPARATIVE FECAL COUNTS ON COW #556

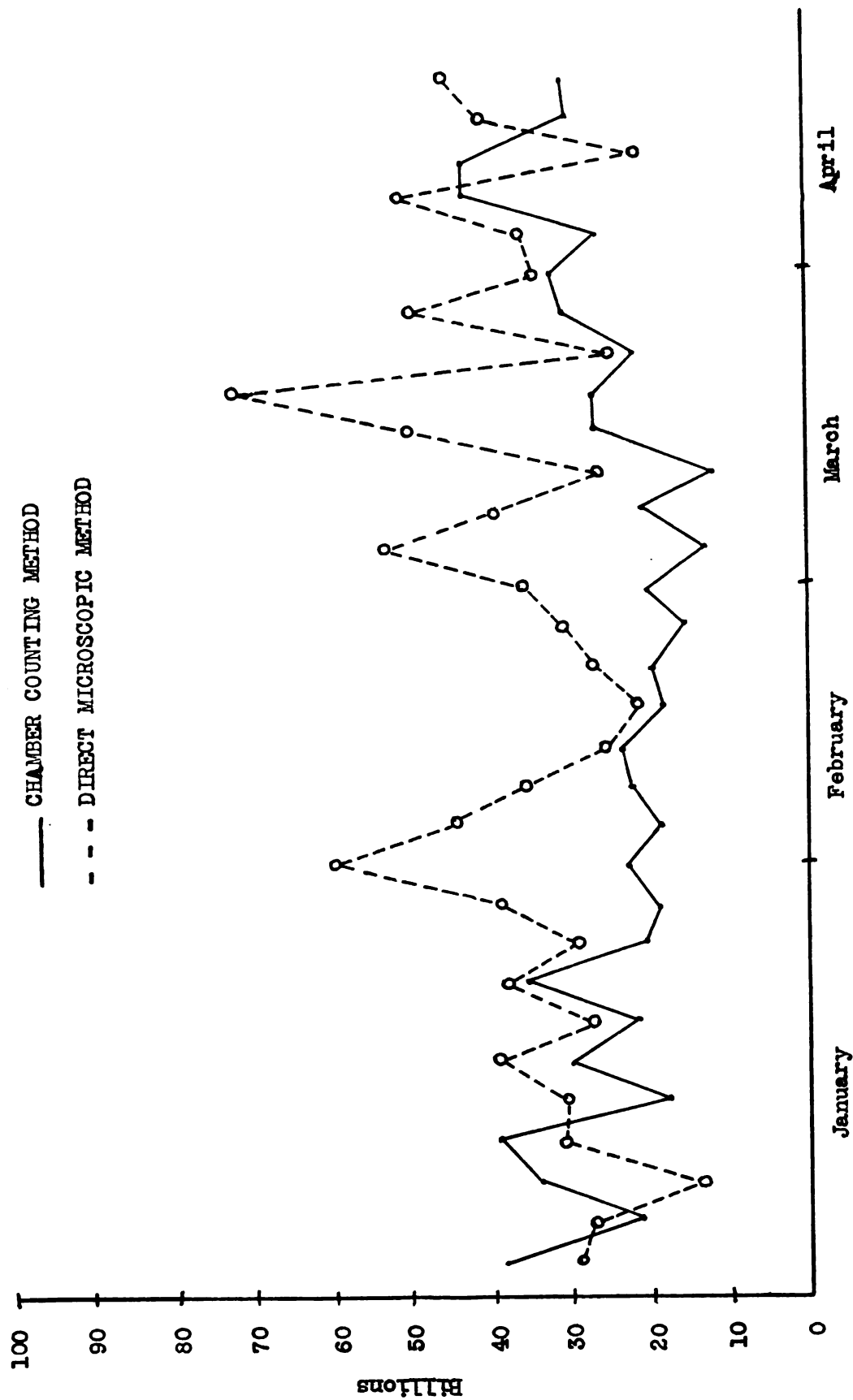


FIG. 6

COMPARATIVE FECAL COUNTS ON COW #558

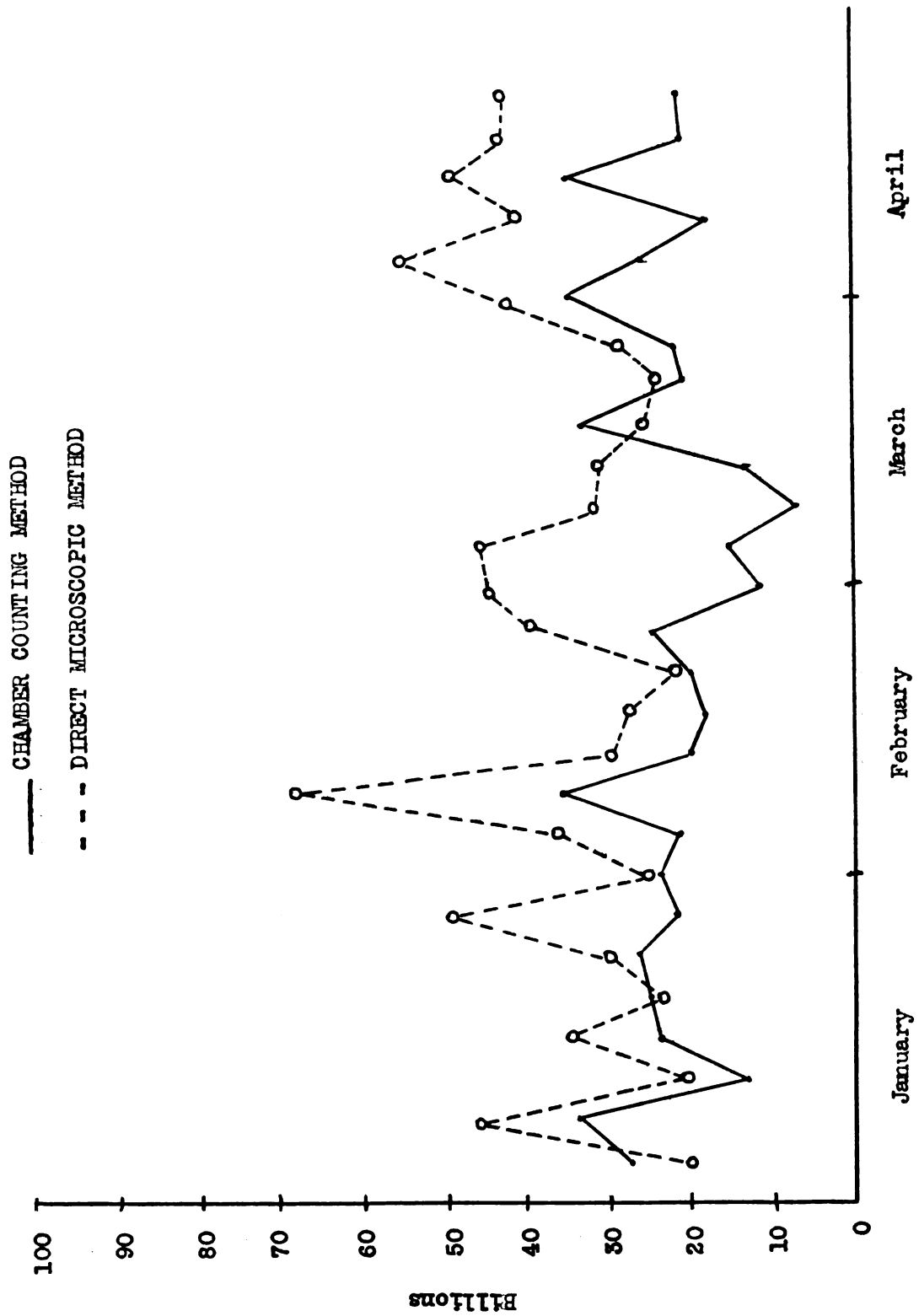


FIG. 7

COMPARATIVE FECAL COUNT ON COW #2003

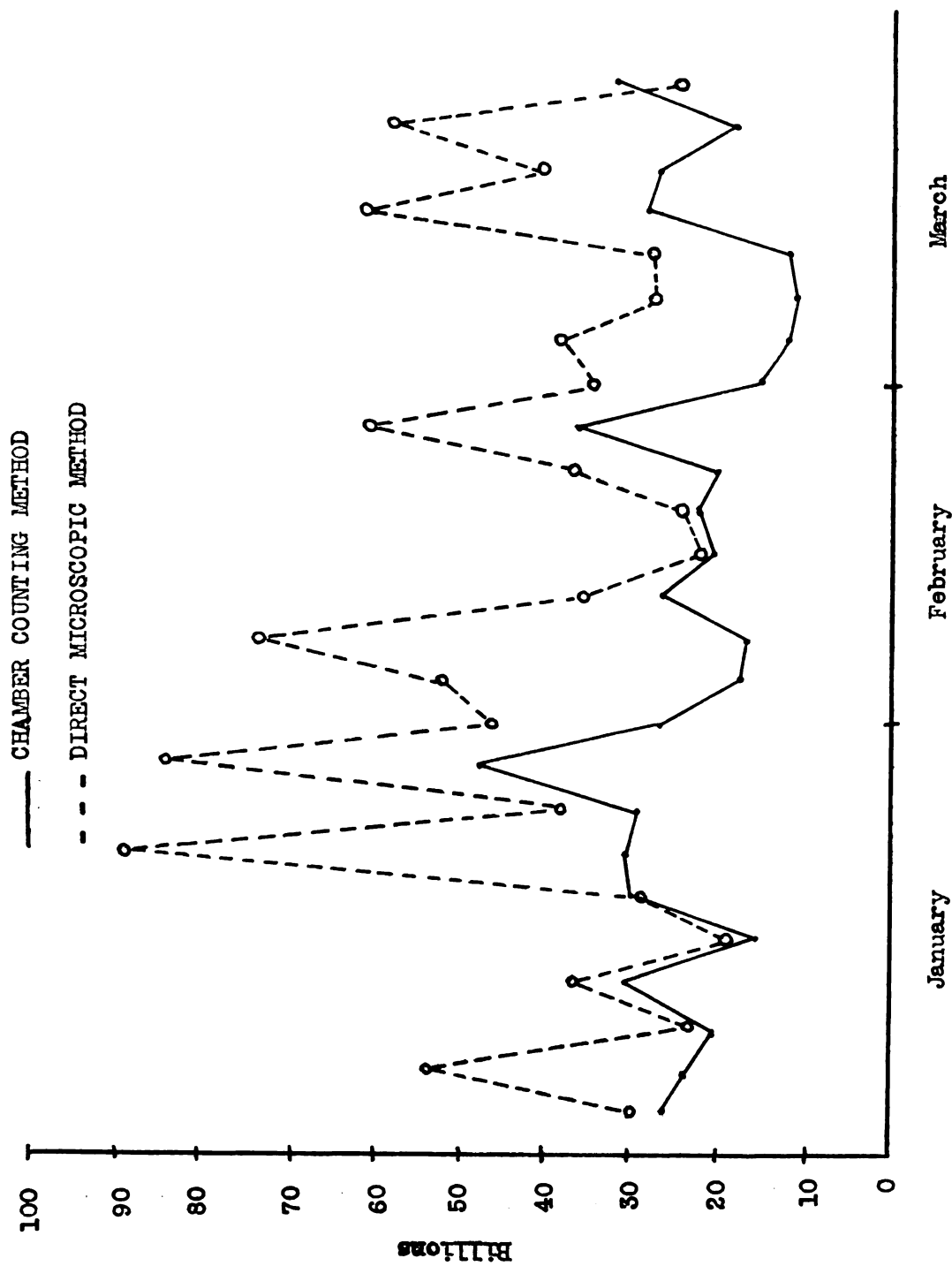


FIG. 8

BACTERIAL COUNTS OF THE RUMEN FOR COW #741

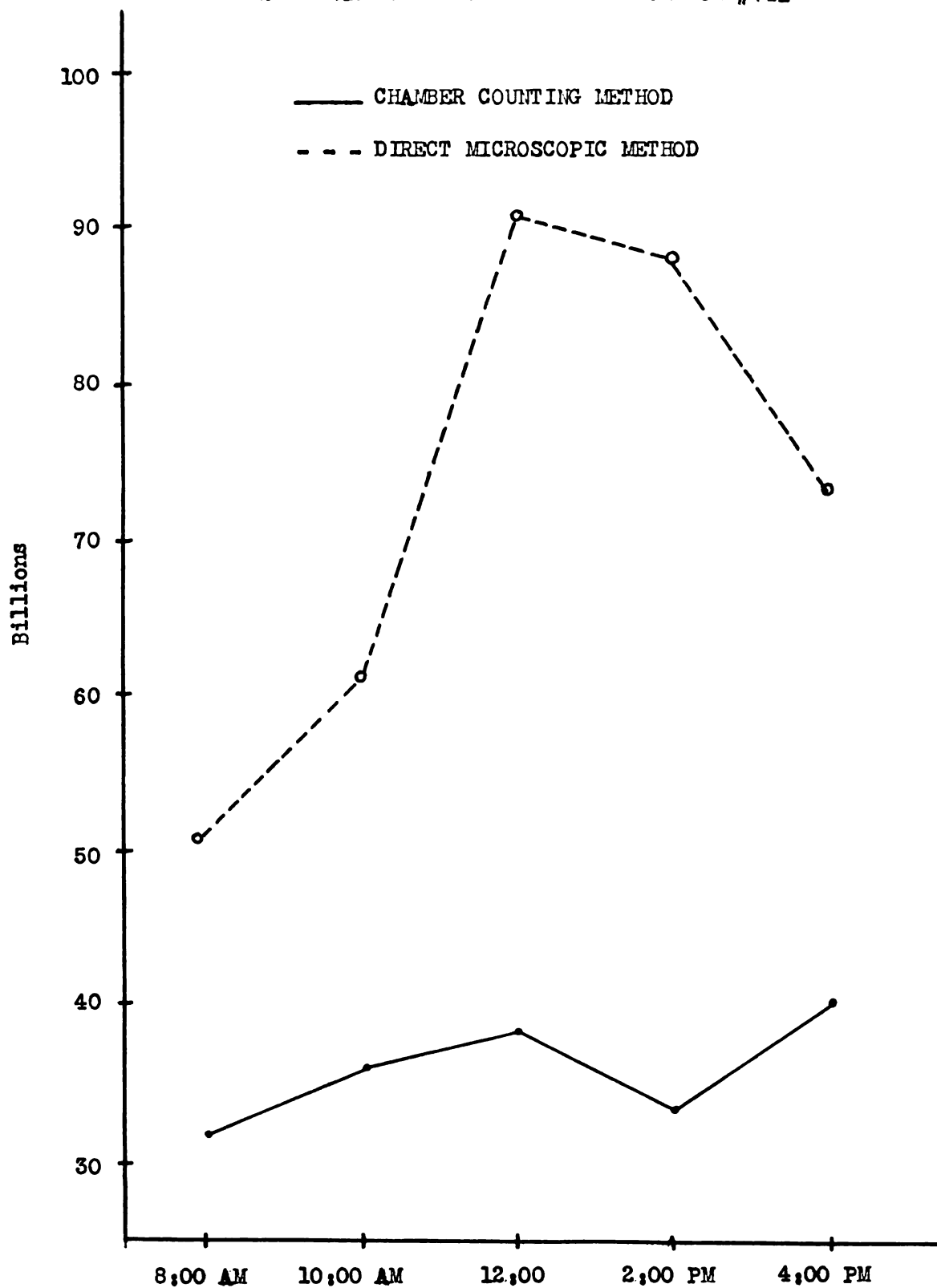


FIG. 9

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