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Studies on the Alimentary Tract of the Merino Sheep in South Africa. XII.—A Technique for the counting of Ruminal Bacteria.

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IN the course of researches on the digestive processes in the rumen of sheep it was realised that a reliable method of estimating the bacterial population in the ruminal mass would be invaluable. In attempting to evolve such a technique several difficulties had to be overcome. Firstly, the method to be employed, secondly the most effective fixative and stain, and thirdly, the best time to make the counts so as to obtain most uniform results. With regard to the lastnamed point, it was found best to withdraw ruminal ingesta approximately 12 hours after completion of feeding.

It was realised at the outset that the plating and Wright's methods could not be employed. In plating rumen contents the medium usually becomes so overgrown by various bacteria and fungi, that it becomes impossible to distinguish colonies. Selective media were also tried but without success. According to Leishmann (1910) the results with Wright's method are not consistent and errors of 50-100 per cent. may occur. The enumeration of bacteria (for the purposes of standardising bacterial emulsions for vaccines) in a haemocytometer chamber, was suggested by Mallory and Homer Wright (1908). They employed a well 0·02 mm. deep, an optically plane coverslip, a $^1/_{16}$ inch dry lens and no staining fluid. In 1908 Leith Murray likewise used an ordinary Thoma-Zeiss haemocytometer with a well 0·1 mm. deep, an ordinary coverslip, a $^1/_{12}$ oil immersion lens and a staining solution of weak Giemsa.

TECHNIQUE.

For the purposes of this work, the author selected a Petroff-Hauser bacterial counting chamber, which has a well 0·02 mm. deep. This chamber has a reinforced precision coverslip, optically plane and 0·18 mm. thick. The apparatus can be used with dark and bright field illumination and is suitable for all achromatic oil immersion lenses as well as dry lenses. Further advantages are (Glynn, Powell *et al*, 1913) that almost all the bacteria settle to the bottom of the chamber in five to ten minutes, when accurate counts can be made. Bacteria adhering to the under surface of the coverslip, and any still floating in the chamber, are easily enumerated. The optical definition of the bacteria is good owing to the small quantity of fluid.

After trying out several stains and methods of killing the bacteria suspended in the ruminal fluid, a five per cent. carbolic solution or 30 per cent. hydrogen peroxide was found to be most suitable, although not entirely effective, probably due to the great variety of organisms present.

Fresh rumen ingesta has a definite decolorising action on stains, and unless the concentration is increased several times beyond that required for ordinary bacterial emulsions, the stain is reduced to such an extent that it loses its staining power almost completely. A further point that had to be considered was the fact that staining is most effective when a stain is so used that its hydrogen-ion concentration is changed as little as possible, so that the stain is allowed to act at the pH where it stains best. Thus a stain which would be effective at a pH of approximately 6·8 to 7·2 had to be sought. For this purpose nile blue sulphate in a one per cent. concentration was found to be most suitable. If a stronger concentration is used the organisms are inclined to clump. After considerable experimentation the following routine method was adopted in preparing material for counting:—

> Ruminal ingesta is withdrawn into a sterilised glass tube and chilled for fifteen to thirty minutes in ice cold water, so as to stop fermentation and break the froth. After this the tube is shaken for a few seconds to mix the material and one cubic centimeter of it is drawn into a wide-mouthed 2 c.c. pipette. This is run into a small 3 c.c. glass tube with stopper. To this 0·25 c.c. of a 5 per cent. carbolic acid solution, or 30 per cent. hydrogen peroxide is added. The mixture is stirred with a thin glass rod and then allowed to stand for one hour. After this 0·3 c.c. of nile blue sulphate is added and the mixture well stirred. After staining for an hour 1 c.c. of the stained material is pipetted off and mixed with 257 c.c. of sterilised distilled water, the pipette being washed out well in this water. In this way a dilution of 1 in 400 is obtained which was found to be the most suitable dilution for ordinary counts. When bacterial populations were low the dilution was reduced to 1 in 200. Unstained bacteria can be counted in a similar way, but it was found that by staining, eyestrain was much reduced and accuracy increased.
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> After the final dilution is made the suspension is allowed to stand for two minutes so as to allow the coarser material to settle. With a Thoma-Zeiss pipette a drop of the emulsion is allowed to pass under the coverslip by capillary attraction. The counting chamber is then placed in position on a level microscopic stage and allowed to stand for three to five minutes in order to allow the bacteria to settle. Ten blocks of nine small squares are then counted, giving the total number of bacteria in 90 squares. The volume of each square being 0·0008 cubic mm., the total volume is 0·072 cubic mm. If N = total number of organisms in 0·072 cubic mm., then with a dilution of 1 in 400 there would be 400N organisms in 0·072 cubic mm. Thus in one cubic mm. there would be $\frac{400N}{\cdot 072}$ organisms. If the number of bacteria in 90 small squares is known, the total number per cubic mm. could thus be calculated by multiplying with $\frac{400}{\cdot 072}$ for a dilution of 1 in 400 or $\frac{200}{\cdot 072}$ for a dilution of 1 in 200 and so forth.

These counts are of course not necessarily true total counts, since an unknown percentage of organisms penetrate into or become adsorbed to food particles. The total count as given must, therefore, be considered always

as being less than the true count would be. In examining food particles and ruminal fluid microscopically after fixation and staining for bacteria, it is seen that only a very small proportion of the organisms is not counted, due to intimate relationship with food particles.

Throughout the investigations Zeiss Huygens oculars 7 × and a Zeiss Achromate 40 × dry lens were used with excellent results.

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