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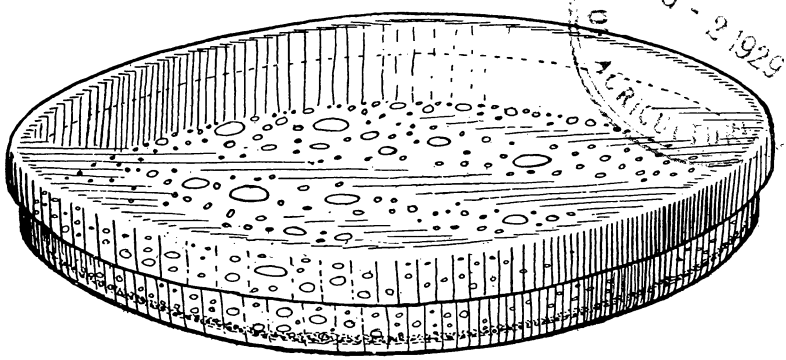

DOTS AND CIRCLES IN THIS SHALLOW GLASS DISH SHOW THE APPROXIMATE SIZE OF COLONIES OF BACTERIA AFTER GROWTH ON AGAR.

HOW BACTERIA IN MILK ARE COUNTED.

SUMMARIZED BY
F. H. HALL
FROM BULLETIN BY
JAMES D. BREW AND W. D. DOTTERRER.

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* Connected with Grape Culture Investigations. † On leave.

POPULAR EDITION *

OF

BULLETIN NO. 439.

HOW BACTERIA IN MILK ARE COUNTED.

F. H. HALL.

Until a few years ago no practicable method was known by which bacteria in milk could actually be counted. "Counts" have long been made, it is true, but these have been made by some cultural method in which the tiny plants themselves were not seen and counted, but "colonies" — little spots and circles that developed about the points where one or more bacteria were embedded in some jelly-like material suited to their growth.

Agar plate counts of bacteria.

Agar, a gelatinous substance derived from seaweed, has usually been the basis of this culture medium. Water, beef extract, peptone and often milk sugar are added to this, making a translucent jelly, fluid when warm, but solid when cold. A definite, small quantity of the milk sample is diluted with germ-free water to 10, 100, 1000, 10,000 or even more times its original volume and a measured amount of this dilution is placed in shallow, circular glass dishes. To this is added a much larger quantity of the warm and fluid agar; the mixture is then shaken to insure uniform distribution of the diluted milk and its contained bacteria. As the fluid agar hardens on cooling, the bacteria are caught and held wherever they happen to be. If the bacteria are single and separated in the diluted milk, then they lie well separated from each other in the hardened agar. If, as is more frequently the case, they are in groups of two to thousands of individuals, then each of these groups forms a single center of growth. These "agar plates" are placed where the temperature is right to induce the growth of the bacteria and then held for several days, usually either two days at a warm temperature, or five days at ordinary room temperatures. Bacteria multiply very rapidly, sometimes doubling in number every twenty minutes, so at the end of a few days, under these favorable conditions, each center of

* This is a brief review of Bulletin No. 439 of this Station on The Number of Bacteria in Milk, by James D. Brew and W. D. Dotterrer, with Preface by Robert S. Breed. Those interested in the detailed data of the investigations and the discussion of the technical relationships involved will be furnished, on application, with a copy of the complete bulletin. Names of those who so request will be placed on the Station mailing list to receive future bulletins, regular or popular edition as desired.

growth has become a mass of individual bacteria forming a " colony " large enough to be seen and counted. Unfortunately for the accuracy of this count, the tendency of the bacteria to grow in milk in masses which cannot be separated into their component individuals causes this count to be one of the number of centers of growth, rather than a count of the number of bacteria present. It has long been known that this was the case; but since there has been no available method of determining the amount of the error thus introduced, and since counts made from duplicate samples of milk agreed fairly well when made carefully, it has become the general custom to speak of these counts as counts of the " number of bacteria " in milk. So general has this custom become among American dairy bacteriologists, and so seldom is the fact mentioned that a single colony may represent *many bacteria* in the milk, not merely *one*, that " counts " of colonies and of numbers of bacteria have to most people meant the same thing.

Counts on new basis.

In 1911, however, a modification of earlier and little used microscopic methods of counting bacteria in milk was announced, which has since been perfected, and which possesses many advantages over the plate method for the counting of bacteria in unpasteurized milks, so that it is being rapidly introduced in milk inspection work. Instead of counting " colonies " after their development on a culture medium, the actual bacteria are counted under a high power microscope in the original milk distributed in a thin layer or " smear " on a glass slide and dried. By the microscopic method the results can be secured within a half hour of the time of sampling the milk, and the microscopic slides may be preserved as a record of the condition of the milk. The milk smear is cleared of fat. The bacteria are stained so that they show plainly and are killed in preparing the slide; therefore, no growth or colony development takes place.

By this method, also, the form, shape and size of the bacteria can be studied, giving much information regarding their character; and the actual numbers of the germs can be counted, whether they be alone, in pairs, in chains, or in groups. Occasionally groups are found so large and compact that the numbers must be estimated, not counted, but such groups are uncommon.

New method gives high counts.

Unless some uncontrolled error creeps into one or the other counts, the counts of individual bacteria by the microscopic method greatly exceed the counts of colonies obtained by the agar plate method. Consequently, consumers and others who take an intelligent interest in the question of milk quality must be warned that as the new method comes into more common use, they may expect larger numbers of bacteria to be reported as found in milk. The actual numbers of bacteria in the milk will not be larger, but the microscopic method will give figures that represent more

closely than does the plate method the number of germs really present.

Errors in count.

Because of the physical impossibility of counting the hundreds of thousands or even millions of bacteria which may be present in a single drop of milk, all counts are made from a very small amount of material. The final figures given, obtained as they are by multiplication of the count made, by a figure of the proper size to give results per cubic centimeter of milk, are in every case to be regarded as " estimates " rather than as " exact " counts. As estimates they are subject not only to the usual errors of estimates, but they are also subject to certain other errors which can be made plainer by a comparison with counts of more familiar objects, such as seeds.

Counting seeds compared with counting bacteria.

Suppose a farmer had several quarts of accidentally mixed seeds in which there were beet, clover, timothy, lettuce and tomato seeds, each quart unlike every other in number and proportion of the different kinds of seeds; and he wished exact information regarding each quart. After thoroly mixing them, he sows one one-thousandth of the seeds in each quart over circles containing one square rod each of clean soil, distributing them as evenly as he can. Now he can wait a few weeks to make his counts until the seeds have grown into seedling plants. He then selects a few areas each containing one square foot and counts all of the plants which have germinated from the seeds. Or, he can make his counts at once by distributing the seeds in a like fashion on white cloth, marking off areas of one square foot each upon the cloth and counting the seeds as they lie sharply contrasted against the background.

Certain errors may occur in the first count very similar to those which occur in making agar plate counts of bacteria. To make the comparison exact, we must assume that he is unable to distinguish the separate plants produced from each beet seed ball, so that all groups of beet plants are counted as if they were single plants. It may be assumed also that part of the tomato seed falls on ground that is so wet that it does not grow, altho it would have done so under proper conditions; or that some of the clover seeds fall on acid places in the soil and do not germinate. Some of the seed may also have been dead when they were planted. Assuming for the moment that he wishes a count of viable seeds only, then the latter may be disregarded. The other errors will all tend to make the count too low. But, however careful he may have been in preparing the soil for his seed count, seeds of weeds or hold-over seeds of these same kinds are liable to be present, to germinate and be counted; so his figures may be much too high. He cannot make any count of the seeds which will include the dead seeds as well as the living ones.

If, however, he should count the seeds at once, as collected on the square foot areas of white cloth, he can see how many true seeds there are in each seed ball of the beet, and can count each of the other seeds separately. If he does not have a magnifying glass, or his eyes are poor he may overlook some of the small, light-colored timothy seeds, causing underestimates of the number present. On the contrary, all of the seeds present, including dead as well as viable seeds, will be seen and counted. In some cases this may be the count desired; but in others the desired count may be one of viable seeds only. In the latter case supplementary germination tests would show whether the error caused by the presence of dead seeds was significant.

The comparison between the difficulties met with in making satisfactory counts of these mixed seeds in the two ways just described are so nearly identical with those met with in making counts of bacteria by the agar plate and the microscopic methods that it will require very little explanation to make these difficulties clear. Who can doubt, however, were the two methods mentioned above the only ones by which a farmer could compare and count his seeds, that he would use the one in which he could see and count the actual seeds rather than the one by which he had to wait a considerable time and estimate the number of seeds from the plants produced? And this would be especially true if the error introduced by the compound nature of the beet seed balls was a large one. By the time the seeds have germinated and the plants appeared it might be altogether too late to use the knowledge in selecting seeds for the season's planting. Moreover, if the counts were to be used as standards for judging the quality of the seed from the commercial or the control standpoint, many disputes over the counts could be prevented and fraudulent practices stopped by the preservation of the samples of seeds; while the germinated seeds could not be preserved.

Application to counting of bacteria.

During the past few years some work done by the Bacteriological Department of the Station has given a good opportunity to compare the "counts" made by the two methods of counting bacteria, and to analyze them in such a way as to determine which of the errors discussed above are really important in making routine counts of bacteria in milk. The samples, 643 in number, were taken in the morning from cans brought to the City of Geneva for its milk supply, and were about equally distributed between night milk, usually about 16 hours old, and morning milk, not more than four hours old. The samples were brought directly to the laboratory, and plates for the agar counts and slides for the microscopic count were prepared as quickly as possible under conditions which made the resulting comparisons fair ones. The plates

were held for five days at room temperature and counted; then placed for two days at a higher temperature and counted again. Usually the latter count was the higher, owing to the development of new colonies from bacteria which do not grow at the lower temperature; and the higher count was used. Only " colonies " could be counted on the plates; and there was no way of telling whether these had developed from single bacteria or from many. Under the microscope the bacteria could be plainly seen, and two kinds of counts were made; one, of the whole number of individual bacteria, and the other, of the groups of bacteria. In the " group " count each individual separated from all others was counted as a " group," as well as the real groups of two or more bacteria.

If no irregularities occurred in the counts due to the presence of dead bacteria, or of organisms that failed to grow under these conditions, or to extra colonies on the plates developed from contaminations, or like things, then the counts of " colonies " on the plates should be greater than the number of " groups " and less than the number of individual bacteria. This proved to be the case in more than half of all of the counts (54 per ct., or 345 out of the 643 samples). If, however, all of the counts in which the number of individual bacteria was less than 30,000 per cubic centimeter are excluded (200 in number), nearly three-fourths of the remaining counts (71 per ct. of the 443 samples) show this normal relationship. These figures indicate then that the chief reason why agar plate counts tend to be smaller than the microscopic counts is because the bacteria exist in the milk as groups more frequently than as single individuals; and that irregularities in counts due to other causes are less important than those due to the clumping of bacteria.

Because of the importance of this effect, a further study was made of the average size of the " groups " of bacteria found in the milk, this being easily determined from an examination of the microscopic preparations. Thus, 3600 groups selected at random from milk of all grades were found to contain an average of 11.8 individuals; but these groups varied greatly in average size according to the type of bacterium. Thus " groups " of bacteria of the ordinary lactic acid types were found to be of small size, containing an average of only 3.1 individuals; while those of the streptococci (organisms which occur commonly in milk, frequently being derived from cows with garget) showed " groups " containing an average of 33.7 individuals. The average size of the " groups " in 580 of the 643 samples was 7.9; but there seemed to be a tendency for the size of the " groups " to vary with the grade of the milk. The better grades of milk containing very few bacteria showed " groups " containing an average of 2.2 individuals. The " groups " increased in size from this to an average of 12.9 individuals, and then again decreased to an average of 5.6 individuals as the bacteria developed

in number and there came to be more than 10,000,000 of these organisms for every cubic centimeter (about one-third teaspoonful).

Effect of grouping of bacteria on count.

In the making of the agar plates the samples of milk are diluted with germ-free water from 10 times to 10,000,000 times or more and these groups of bacteria are more or less broken apart; but they are never so broken apart that the resulting colonies each represent a single bacterium. In the making of the " smear " on the microscopic slide on the other hand, only a small portion of milk is taken, but this is not diluted, so the bacteria remain in groups as they were in the original sample. It has thus far proved impossible to control or measure the effect of the breaking of the groups in such a way that it is possible to correctly estimate from the plates what the total number of individual bacteria would be as seen under the microscope; nor to reverse the process and tell from a microscopic slide what the probable agar plate count would be in the case of individual samples of milk.

Effect of dead bacteria on count.

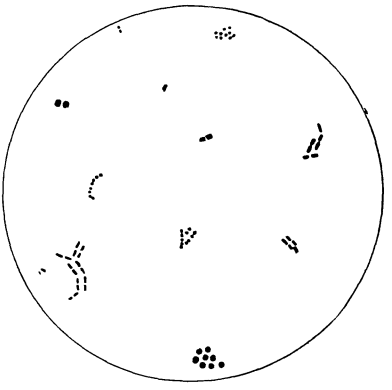
A few cases occurred (19 per ct., or 123 samples out of 643) in which the colony count did not even equal the " group " count, to say nothing of the individual count. Why is this? It must be remembered that to form colonies on the plates the bacteria must grow. To grow they must be alive, and even if alive must find conditions suitable for growth, just as in the case of the seeds. Bacteria are plants, and die as do other living things. The question, therefore, arises whether any of the discrepancies between the agar plate and the microscopic counts may be due to the presence of these dead bacteria. If such were present in the samples examined they must be included in the comparatively small group of samples where the plate count was smaller than expected. That the presence of dead bacteria was not the cause of all or even a majority of the 123 counts of this type, is indicated by two things; first, that bacteria almost invariably thrive and grow rapidly in fresh milk, and second, that the same effect is produced by the presence of living bacteria which do not find conditions suitable for growth on the agar even where conditions are favorable to their growth. Every gardener knows how difficult it is to get every seed to grow. Just in the same way, bacteriologists have found it impossible to so prepare a culture medium as to make it suitable for the growth of all bacteria. So well known and common is this effect that it is reasonable to assume that this played the larger part in causing the 123 samples to show the low plate counts.

Effect of contaminations in planting.

In discussing the counts of seeds it was pointed out that hold-over seeds might grow and thus increase the count. In the series of 643 counts there were 175 samples (27 per ct.) in which the plate counts were larger even than the individual counts as made under the microscope. In a very few instances the discrep-

ancy was very large. Yet a careful renewed search thru the microscopic preparations failed to show any such large numbers of bacteria as were indicated by the agar plates. The most natural explanation of these irregularities is that extra colonies developed on the plates from bacteria which got into them from outside sources, and this was taken to be the case until it was noticed that all but 32 of the 175 counts were made on milk samples containing less than 30,000 individual bacteria per cubic centimeter. The localization of the majority of these counts among the milk samples containing few bacteria indicates that the majority of the counts of this type were caused thru the mathematical impossibility of getting a fair average when only a small number of objects are counted, from which to estimate a very much larger number.

In making a microscopic count, it must be remembered that we do not examine the whole of the "smear" on the slide; since the field viewed under the microscope is exceedingly small and it would take altogether too much time to pass all of the thousands of fields in each smear under the microscope. Accordingly, we examine only one hundred of these fields, count the number of bacteria found, and multiply this number by the proper factor to give the approximate number per cubic centimeter. If the milk contains only a very few bacteria, we may not even find one in one hundred fields of the microscope, and so use too small a number in the computation. On the plates a much larger fraction of a cubic centimeter of milk is examined in those milks which contain few bacteria than in the case of the microscopic slides. In making comparisons of counts from low-count milk samples, the probability is that the microscopic count will be lower than it should be. This is quite probably what happened in the 143 cases in which the colony count on the agar was greater than the individual count on the slides. Many of these discrepancies were not large, and under these conditions where so few bacteria were present the microscope makes no mistake in placing it in the "good" or Grade A class even tho the number of bacteria found is not quite as large as the number found by the plating method. No difficulty of this sort arises in milks containing a sufficiently large number of bacteria to be found readily under the microscope. In the case of high-count milk, the



GROUPS OF BACTERIA IN POOR QUALITY MILK AS THEY APPEAR UNDER A MICROSCOPE.

advantage of examining a relatively large amount of milk is held by the microscopic and not by the plate method. It is possible that some bacteria are so small, or stain so lightly, that they are commonly overlooked under the microscope altho they would form colonies on the agar plates. There are few indications, however, of such instances, and it is believed that they are rare. The effect of such varieties if present would be to make the microscopic counts lower than they should be.

Comparison with counts of seeds.

Let us return for a moment to the hypothetical methods of counting seeds. The results of the studies on the counts of bacteria show that the commonly used agar plate counts are much too low because of an error similar to the one caused by counting the beet seed balls as a single seed. They also show that there is an error of smaller size due to the presence of living bacteria which do not grow; an error similar to the one caused by the failure of some of the seeds to grow. The indications are also that where the work of making bacteria counts is carefully done, errors due to contaminations, while they do occur, are much less important than would be the similar error caused by weed seeds present in soil.

None of these errors affect the microscopic counts, the errors in this case being largely those which come from the difficulty involved in making accurate counts of objects so tiny that they can not be seen except with high magnification under the microscope. The claim which is frequently urged against making counts of bacteria microscopically based on the fact that dead bacteria cannot be readily distinguished from living ones does not prove to be of any significance in the case of fresh unpasteurized milk; because of an apparent absence or practical absence of dead bacteria in such milk. There are circumstances, moreover, in which a total count of the bacteria is of greater importance than a count of the living bacteria; and in fresh milks the dead bacteria give just as much information concerning the past history of the milk as do the living ones. Under certain special circumstances where preservatives have fraudulently been added to the milk, it is especially desirable to be able to detect the dead bacteria; a thing which cannot be done by means of the agar plating method.

Conclusions.

In view of the manifest impossibility of so perfecting either the agar plate method or the microscopic method that absolutely accurate counts of bacteria can be made; or even of controlling the errors which have been discussed, it is fortunate that comparisons of real value can be made without counts more accurate than they now are when properly made. There is no necessity for us to be able to count the bacteria in milk so accurately as to tell the difference between a sample containing 5,657 bacteria and one containing 6,756 bacteria. In looking at a distant hillside, it is not possible to count the trees thereon. Never-

theless, it is ordinarily quite possible to form an accurate judgment as to the area covered by woods, by wooded pasture, and by meadows. Such a judgment may have as great a value for the purpose in hand as would one based upon an accurate count of the number of trees per acre of land. The fact that accurate counts of the number of bacteria in milk cannot be made does not prevent the use of either the agar plate method or the microscopic method as a means of distinguishing with a satisfactory degree of accuracy between milks containing few bacteria, a medium number, and a large number. The fact that this is all that is necessary for commercial and control work has been shown in the inspection work which has been done for the City of Geneva during the past three years, a description of which will be given in a forthcoming bulletin.

It is evident from all of this work that popular ideas of the number of bacteria in milk, based as they are upon the counts made by the plating method, must be revised. The results obtained by the microscopic method of counting bacteria in milk show these to be much too low.