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CONTENTS

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JOURNAL OF AGRICULTURAL RESEARCH

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RELATION OF COMMERCIAL HONEY TO THE SPREAD OF AMERICAN FOULBROOD[1]

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INTRODUCTION

The relation and importance of commercial honey to the spread of American foulbrood of bees has occupied the attention of the beekeeping industry more or less prominently for many years. The theory has been promulgated that honey which has not come from disease-free apiaries is dangerous because of the possibility of its disseminating American foulbrood. A few States and at least one foreign country require that honey intended for interstate shipment be accompanied by a certificate from the bee inspector of the State in which the honey originated to the effect that such honey was produced in apiaries free from American foulbrood.

It is a well-established fact that honey taken directly from the combs of the brood chambers of colonies affected by American foulbrood is capable of producing the disease if fed to healthy colonies. Since commercial beekeeping practice bans the extracting of honey from the brood nest, it is difficult to understand how heavily infected honey, in large quantities, could get on the market. Whether honey from supers that have been on colonies affected with American foulbrood is of serious importance in transmitting the disease is still open to question. White (*30, p. 35*)[3] says: "The likelihood that the disease will be transmitted by combs from diseased colonies, which contain honey but no brood, probably is frequently overestimated." On the other hand, Millen (*23*) found that combs built from foundation and completely filled above an excluder with honey from colonies that had been destroyed by American foulbrood produced disease in all of 10 colonies made from package bees to which one comb each of the honey had been given. Corkins (*8*) expressed the belief, as a result of preliminary studies, that "Extracted honey produced above an excluder in a colony in the early stages of American foulbrood is insignificant in the spread of this disease through commercial honey." The conflicting nature of these observations emphasizes the need for further research before the certification of honey is required as a means of alleviating the foulbrood situation.

In both animal and plant disease bacteriology it is known that pathogenic microorganisms may vary considerably, even within indi-

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[3] Reference is made by number (italics) to Literature Cited, p. 284.

vidual species, in virulence and in ability to produce disease. Furthermore, as stated by Zinsser (*31, p. 188–189*)—

> Whether or not infection occurs depends also upon the *number of bacteria* which gain entrance to the animal tissues. A small number of bacteria, even though of proper species and of sufficient virulence, may easily be overcome by the first onslaught of the defensive forces of the body. Bacteria, therefore, must be in sufficient number to overcome local defenses and to gain a definite foothold and carry on their life processes, before they can give rise to an infection. The more virulent the germ, other conditions being equal, the smaller the number necessary for the production of disease. The introduction of a single individual of the anthrax species, it is claimed, is often sufficient to cause fatal infection; while forms less well adapted to the parasitic mode of life will gain a foothold in the animal body only after the introduction of large numbers.

In the case of American foulbrood the quantity of infectious material that honey must carry in order to produce disease in a colony has never been determined. White (*30, p. 20, footnote 1*) states, in connection with inoculating healthy colonies experimentally with *Bacillus larvae:*

> It was found that less than one scale is sufficient disease material to produce a considerable amount of disease in the colony. In some experiments one scale, therefore, might supply all the spores needed although the use of a somewhat greater quantity of material is advisable in most instances.

While infected honey no doubt does become mixed with disease-free honey, it is probable in many cases that, because of the practice of using large settling and storage tanks, infected honey would be so diluted with spore-free honey as to make the spore content insufficient to produce infection even if fed to healthy bee larvae. Therefore, one object of these investigations was to determine the minimum number of spores of *Bacillus larvae* in honey necessary to produce American foulbrood in healthy colonies of bees as correlated with the infectivity or spore content of the average commercial honey.

In order to obtain information relative to this subject, experiments were conducted in the apiary over a period of five years. In these experiments honey or sugar sirup with a known content of spores of *Bacillus larvae* was fed to healthy colonies and the minimum number of spores that would produce infection was determined. At the same time laboratory studies were carried on with cultures of spores of *B. larvae*, concerning certain growth phases of the organism, particularly the minimum number of spores that would produce vegetative growth on artificial culture media. Methods for demonstrating the presence or absence of spores of *B. larvae* in samples of commercial honeys were also investigated, and these honeys were studied in relation to their infectiousness as correlated with the spore-feeding experiments. These three phases of the investigation will be discussed in the order mentioned.

MINIMUM NUMBER OF SPORES OF BACILLUS LARVAE NECESSARY TO PRODUCE DISEASE IN HEALTHY COLONIES OF BEES

METHODS OF PROCEDURE

Location of Experiments

These investigations were started during the summer of 1926 in a small experimental apiary located about half a mile from the bee culture laboratory of the Bureau of Entomology at Somerset, Md. The location at Somerset was undesirable, however, because of its

close proximity to the apiary connected with the laboratory and to other privately owned colonies of bees, necessitating extreme precautions to prevent spread of the disease. In 1927 the experimental work was transferred to the Intermountain States bee culture field laboratory at Laramie, Wyo.[4] In Wyoming an ideal isolated location was found about 14 miles east of Laramie in the Medicine Bow National Forest, the nearest colonies of bees being at least 14 miles away and probably farther. Since this location is more than 8,000 feet above sea level, there is only a slight nectar flow from wild flowers, which assures the immediate use of any inoculated sirup fed to colonies of bees. In fact, after the middle of the summer it was found necessary in most cases to feed the experimental colonies with uninoculated sugar sirup in order to prevent starvation.

In 1927 and 1928 the colonies used for experimentation were located in two yards between a quarter and a half mile apart. The arrangement of the colonies in the two yards was such as to prevent drifting as much as possible. In 1929 and 1930, in order to limit still further the danger of transmission of disease because of drifting or robbing, 20 colonies were stationed in pairs, so arranged as to minimize the danger from drifting, in 10 isolated locations at least a quarter of a mile apart.

Make-up of Colonies

Five-frame nucleus hives were used for the spore-feeding experiments. The colonies were prepared either with two or three frames of brood, honey, and adhering bees taken from healthy colonies, together with a young laying queen, or, as in 1927, 1928, and 1929, by placing a 2-pound package of bees containing a laying queen on foundation or on combs containing honey from healthy colonies and feeding them sugar sirup. During a good honey flow these small colonies were allowed to build up in the apiary connected with the laboratory until they consisted of three or four frames of brood before they were moved to the isolated locations. The bees making up the colonies used for the feeding experiments from 1927 to 1930 at Laramie, Wyo., were all from the same general strain.

Material Used for Inoculation

Spores of *Bacillus larvae* were obtained from American foulbrood scales in combs taken from diseased colonies located in the States of Maryland, Iowa, and Wyoming. The strain used at Somerset, Md., was obtained from a sample sent to that laboratory for diagnosis. Two different strains were used at Laramie during 1927, 1928, and 1929, one obtained from a diseased colony in the experimental apiary belonging to the University of Wyoming and one obtained from a beekeeper at Lander, Wyo. In 1930 three other strains were used in the feeding experiments, one from Iowa and two from apiaries in Wyoming.

Preparation of Spore Suspensions

In preparing the spores for feeding to the healthy colonies, scales were removed from the combs by means of sterile forceps (the necessary precautions being taken against contamination) and placed in

[4] This laboratory is maintained cooperatively by the University of Wyoming and the U. S. Department of Agriculture.

a flask containing 50 c c of sterile water and glass beads. After the scales had softened in the water, the flask was shaken for one-half hour to insure complete maceration of the scales. The suspension was then filtered through two thin layers of sterile absorbent cotton into another sterile flask in order to remove any lumps or débris.

In preparing the stock suspensions of spores, at first 75 to 100 scales were taken by counting. Later it was found that the average American foulbrood scale weighs 0.0223 g. Therefore, the 100 scales for the stock suspensions were obtained by weight, the scales being weighed in a sterile covered glass dish before they were deposited in the flask of sterile water.

After the suspension had been filtered and tested for contamination and was ready for use, the number of spores per cubic centimeter was determined by the following method: By means of a blood-diluting pipette giving a dilution of 1 to 20, the spore suspension was diluted with a weak solution of carbol fuchsin and a drop placed in the counting chamber of a Helber bacteria-counting cell 0.02 mm deep and ruled in squares of 0.0025 mm^2 each.[5] With the use of two 15× eyepieces in a binocular microscope and a 1.8-mm oil-immersion objective, the spores in 25 squares of the Helber chamber were counted. Then by means of the formula

$$\frac{\text{Total spores counted} \times \text{dilution} \times 20{,}000 \times 1{,}000}{\text{Number of squares counted}}$$

the approximate number of spores per cubic centimeter in the suspension was determined.

Later this method was checked by the method of Breed and Brew (*2*) for counting bacteria in milk. With the aid of a binocular microscope having two 15× eyepieces and a 1.8 mm oil-immersion objective, the area of a circle etched on an ocular micrometer disk was determined by means of a stage micrometer. One one-hundredth cubic centimeter of a 1 to 100 dilution of the stock suspension of spores was placed on a glass slide on which 1 cm^2 had been ruled with a diamond pencil. This was mixed with a small loopful of carbol fuchsin stain and the whole spread over the 1 cm^2 of surface[5] and allowed to dry uniformly. The number of spores per cubic centimeter of the stock suspension was determined according to the formula

$$\frac{\text{Area 1 cm}^2}{\text{Area of circular field}} \times \frac{\text{total number of spores counted} \times \text{dilution} \times 100.}{\text{number of circular fields counted}}$$

These two methods were found to check fairly closely within the limits of the precision of the methods used in counting. Furthermore, by both methods it was found that in the majority of cases 100 scales in 50 c c of water give approximately 5,000,000,000 spores per cubic centimeter for each suspension made up in this way. Therefore, this number was used as a standard for making all dilutions.

[5] Mm^2 and cm^2 are the abbreviations for square millimeter and square centimeter, respectively, recently adopted by the Style Manual for United States Government printing.

After a considerable number of counts had been taken in making up several stock suspensions of spores, counting was eliminated and the spore content of the stock suspensions was standardized according to the method described by Gates (*11*, p. 114), as follows: "The opacity of a bacterial suspension is measured by the length of a column of the suspension required to cause the disappearance of a wire loop." An instrument known as a suspensiometer was used for this purpose. The use of this method saved considerable time and labor without appreciably affecting the precision of the counts. One liter of a 50 per cent solution of sugar in water was used as the standard quantity of inoculated sirup fed to each experimental colony. A series of dilutions of the original stock suspension containing 5,000,000,000 spores was made by adding different quantities of the spore suspension to 1 liter of sugar sirup. In this way the approximate total number of spores in each liter of sugar sirup to be fed to colonies of bees was known.

Method of Inoculating Colonies

In 1926 at Somerset, Md., the sugar sirup containing the various dilutions of spores was fed to the colonies by means of galvanized-iron troughs that were hung inside the hives after two combs had been removed. In these troughs sterile excelsior was placed for the bees to walk on in order to prevent them from drowning. This method was found unsatisfactory, however. At Laramie, Wyo., the sugar sirup containing the spores was first placed in Boardman feeders, but owing to the danger of robbing at the entrance of the hives, the method finally used was to invert the jars in holes bored in the hive covers. In this way any leakage into the hives was cleaned up by the bees without danger of causing robbing. To prevent the jars from being broken or knocked over, box covers were placed over them and fastened to the hive covers. Each colony was usually inoculated only once with an individual dilution of spores. Duplicate colonies were inoculated with each dilution of spores. Uninoculated check colonies were placed among those that were inoculated.

PRIMARY OBSERVATIONS

Observations of the condition of the brood were made at least once a week, and sometimes oftener, after the colony was given the liter of inoculated sirup. In 1926 at Somerset, Md., as soon as diseased larvae appeared in a colony, the colony was killed and at once removed from the apiary. Because of the isolated location near Laramie, Wyo., the colonies were left until the end of the brood-rearing season, when final observations were made.

The results of the spore-feeding experiments are shown in Table 1.

TABLE 1.—*Results of spore-feeding experiments* [a]

[Duplicate colonies of bees (A and B) were used in the first 4 years, and triplicate colonies (A, B, and C) in 1930]

Total number of spores fed	Extent of foulbrood in—																		
	1926		1927				1928, repeat		1928				1929				1930, final		
			During season		Final				During season		Final		During season		Final				
	A	B	A	B	A	B	A	B	A	B	A	B	A	B	A	B	A	B	C
5,000,000,000	+	?+	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----
2,500,000,000	+	+	-----	-----	-----	-----	-----	-----	+	+	+	+	-----	-----	-----	-----	-----	-----	-----
1,000,000,000	+	+	-----	-----	-----	-----	-----	-----	+	+	−*	+	-----	-----	-----	-----	-----	-----	-----
750,000,000	+	+	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----
500,000,000	+	+	+	-----	−*	-----	-----	-----	+	+	−*	+	+	+	-----	-----	-----	-----	-----
350,000,000	+	0	+	-----	+	-----	-----	-----	+	-----	+	-----	-----	-----	-----	-----	-----	-----	-----
200,000,000	+	?+	+	+	−*	−*	+	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----
175,000,000	-----	-----	+	?+	+	−*	-----	+	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----
150,000,000	-----	-----	?+	+	−*	−*	−	−	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----
125,000,000	-----	-----	0	?+	0	−*	0	−	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----
100,000,000	0	0	0	?+	0	−*	0	−	+	+	−*	+	-----	-----	-----	-----	-----	-----	-----
75,000,000	-----	-----	+	?+	−*	−*	-----	+	0	+	0	+	+	0	+	0	-----	-----	-----
50,000,000	0	0	0	0	0	0	-----	-----	0	+	0	−*	0	+	0	−*	0	0	0
25,000,000	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	0	0	0	0	0	0	0
10,000,000	0	0	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	0	0	0	0	-----	-----	-----
5,000,000	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	0	0	0	0	-----	-----	-----
2,500,000	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	0	0	0	0	-----	-----	-----
1,500,000	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	0	0	0	0	-----	-----	-----
500,000	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	0	0	0	0	-----	-----	-----
100,000	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	0	0	0	0	-----	-----	-----
Controls	1+,12−0		1+,2−0		1+,2−0		1−0		1+,3−0		1+,3−0		8−0		8−0		2−0		

[a] +, Positive American foulbrood; ?+, probable American foulbrood, very slight and unconfirmed and disappearing by end of brood-rearing season; 0, no disease found during season; −*, disease cleaned out by end of brood rearing; −, no recurrence in second season.

In 1926 a total of 200,000,000 spores fed to a colony was the smallest number that produced disease; in 1927, on the other hand, 75,000,000 was the smallest number. However, in the latter year the spores were obtained from another locality in which environmental conditions were quite different. In an effort to obtain check results, the feeding experiments were repeated in 1928. Through an error in making up the spore dilutions, which was not discovered until too late for rectification, no colony received less than 50,000,000 spores. This season one colony of the pair receiving an inoculation of 50,000,000 spores became infected. The feeding experiments were repeated again in 1929, with dilutions of spores from 75,000,000 down to 100,000—considerably less than the minimum number in 1928. Again only one colony of the pair receiving a total of 50,000,000 became infected. As a result of two years' experiments this was found to be the apparent minimum number of spores of *Bacillus larvae* capable of producing infection when fed in 1 liter of sugar sirup. In 1930 spores from three different localities were fed in duplicate to six healthy colonies in dilutions of 50,000,000 and 25,000,000 without producing disease.

It is therefore apparent that a certain minimum number or mass of spores is required to start the initial action capable of producing American foulbrood in healthy larvae. Under the conditions of these experiments this minimum number was approximately 50,000,000 spores of inoculum per liter of sirup.

SECONDARY OBSERVATIONS

During the first three years of the experiments, or previous to 1929, at which time the experimental colonies were isolated in pairs, certain of the uninoculated control colonies developed disease, 1 out of 13 in

1926, 1 out of 3 in 1927, and 1 out of 4 in 1928. It was assumed that the disease was probably not spread by robbing, since no active robbing was observed at any time. In practically every case where a control colony became infected, it was so located in relation to the inoculated colonies that drifting of young nurse bees during play flights could account for the spread of the disease, in one or two cases quite definitely so. In 1929 all eight uninoculated colonies, although they were not located with the inoculated colonies but were within robbing range of all, remained free from disease. The prevention of drifting apparently eliminated the casual spread of disease.

Occasionally a colony of bees affected with American foulbrood will try to clean out the diseased remains, often removing parts of the scales and sometimes actually tearing a comb down to the midrib in order to do this. White (*30, p. 34–35*) states:

> There is considerable evidence to support the belief that occasionally in cases of light infection the disease may disappear unaided by treatment. * * * It should be emphasized that such a course for the disease, if it occurs at all, is unusual. Although American foulbrood spreads more or less rapidly within an infected colony, the fact remains that it frequently does not.

Lineburg (*16*) in 1925 reported that in two colonies which were diseased in the spring the disease apparently disappeared later in the season. Three colonies were divided and used for making increase in June and July, but all remained free from disease, at least until the end of that season. Further observations were not reported. Corkins (*8*) in 1928 reported five colonies which were given combs containing scales of American foulbrood at the beginning of the honey flow of 1927 and developed no disease up to July 10, 1928. Two other colonies were observed to have cleaned out the disease and remained healthy for an entire season. However, during the several years of his experimental work on American foulbrood, the writer never observed a colony in which the disease was permanently cleaned out until 1927. In that year, of 16 colonies inoculated with various dilutions of spores, 4 colonies, 2 of which received more than the probable minimum dose causing infection, showed no disease during the season. The disease completely disappeared by the end of brood rearing in 10 of the 12 other colonies that had showed either positive or probable disease some time during the summer. In 1928 package bees were placed on the combs of seven of these colonies that had apparently cleaned out the disease during the previous summer and on two that had been inoculated with presumably a sufficient number of spores but which had remained healthy. Three of the seven developed disease again the second season, while four remained healthy during the entire season. Neither of the two inoculated colonies that had remained free from disease in 1927 developed it in 1928. Of the 11 colonies inoculated in 1928 that developed disease, 4 cleaned up the disease by the end of the brood-rearing season and 2 inoculated colonies showed no disease. In 1929, 1 of the 2 colonies developing disease cleaned up by the end of the brood-rearing season, making a total of 15 cases in which the disease was cleaned up by the end of brood rearing. Two of the colonies inoculated with the minimum infectious dose or more showed no disease during that summer.

It is possible that, in the high altitude of Laramie, and in similar places where the air is very dry, the scales of American foulbrood

become dried without adhering so tenaciously to the cell walls as they do in more humid climates at lower altitudes. These observations indicate the necessity of further work on the resistance of bees to the disease and variation in virulence of different strains of the organism.

INOCULATION OF INDIVIDUAL BEE LARVAE WITH DEFINITE NUMBERS OF SPORES OF BACILLUS LARVAE

In the light of the results of the foregoing experiments, in which colonies were inoculated with presumably a quantity of spores sufficient to produce infection but in which no disease developed, the question arises as to what became of the spores in the sugar sirup, some of which presumably were fed to healthy larvae. In those colonies developing disease that received a minimum number of spores, how many spores did each larva developing the disease receive? In order to obtain information on these points, a preliminary series of experiments was planned in which individual larvae were inoculated with known numbers of spores.

Toumanoff (*29*) reports that he was unable to cause infection by giving individual larvae a drop of a rich emulsion of a culture of *Bacillus larvae* in salt solution. He found that many of the larvae so treated were removed from the cells by the bees, and those remaining failed to develop disease. He further found that larvae given only uninoculated salt solution were also removed in the same way. Therefore, in the present experiments sugar sirup was used instead of salt solution. In a comb from a healthy colony containing numerous coiled larvae, a drop of an uninoculated 50 per cent solution of sugar in water was placed in each cell containing a larva, as near the mouth parts of the larva as possible. The rim of each cell so treated was marked with a paint consisting of 1 part of liquid white shellac, 1 part of a paint pigment, and 4 parts of ethyl alcohol. The sugar sirup was slightly colored with water-soluble eosin in order to aid in determining the effect. Frequent observations showed that practically all larvae that were fed this colored sugar sirup developed normally and were sealed over, the pigment markings still being present on the edges of the cappings. In most of the cells a residue of colored sirup could be observed for several hours after the larvae had fed.

A series of 5-frame nuclei was prepared, each containing one or two combs having a large number of unsealed larvae. A set of dilutions of spores was made from a stock suspension with a sterilized 50 per cent sugar sirup in such a way that each 0.01 c c of the dilution would contain an approximate known number of spores, as indicated in Table 2. Sterilized 2 c c Luer tuberculin hypodermic syringes graduated in 0.01 c c, the needles of which had been blunted, were used in inoculating the cells containing coiled larvae. Fifty or more coiled larvae at least 4 days old were each given 0.01 c c of a dilution of spores, each dilution being given to larvae in one comb in a separate colony, and the cells so inoculated were distinctively marked. A few larvae that had just been sealed also were inoculated by puncturing the capping with the inoculating needle and depositing the 0.01 c c in the cell. Observations were taken at the end of 24 hours and at frequent intervals thereafter until the end of the brood-rearing season.

TABLE 2.—*Results of inoculation of individual healthy bee larvae with known numbers of spores of bacillus larvae, 1930*

Spores fed each larva in 0.01 cubic centimeter of dilution	First inoculation, July 14		Second inoculation, Aug. 1			Third inoculation, Aug. 19							Fourth inoculation, Aug. 21							
	Colony No.	Larvae developing disease Aug. 1	Colony No.	Larvae developing disease		Colony No.	Length of time isolated from nurse bees	Effect on larvae of being isolated from nurse bees	Larvae developing disease				Colony No.	Length of time isolated from nurse bees	Effect on larvae of being isolated from nurse bees	Larvae developing disease			
				Aug. 19	Oct. 7				Aug. 26	Sept. 9	Sept. 19	Oct. 7				Aug. 26	Sept. 9	Sept. 19	Oct. 7
Number		*Number*		*Number*	*Number*		*Hours*		*Number*	*Number*	*Number*	*Number*		*Minutes*		*Number*	*Number*	*Number*	*Number*
50,000,000	----	----	----	----	----	12	1	Larvae removed	0	0	0	0	12	5	Larvae removed	0	0	0	0
25,000,000	----	----	----	----	----	13	1	----do	0	0	0	0	13	10	----do	0	0	0	0
10,000,000	----	----	----	----	----	20	¾	Larvae not removed	0	[1] 13	----	----	20	15	Larvae not removed	0	[a] 9	----	----
7,500,000	----	----	----	----	----	15	1	Three-fourths of larvae removed.	0	0	0	0	15	20	----do	0	0	0	0
5,000,000	----	----	14	0	0	4	¾	One-half of larvae removed.	0	0	0	0	4	30	One-half of larvae removed.	0	0	0	0
1,000,000	----	----	34	0	0	16	1	Larvae not removed	0	0	0	0	----	----	----	----	----	----	----
500,000	----	----	16	0	0	22	1	----do	0	0	0	0	----	----	----	----	----	----	----
250,000	----	----	18	0	0	----	----	----	----	----	----	----	----	----	----	----	----	----	----
100,000	----	----	22	0	0	----	----	----	----	----	----	----	----	----	----	----	----	----	----
75,000	----	----	26	0	0	----	----	----	----	----	----	----	----	----	----	----	----	----	----
50,000	----	----	28	0	0	----	----	----	----	----	----	----	----	----	----	----	----	----	----
25,000	----	----	4	0	0	----	----	----	----	----	----	----	----	----	----	----	----	----	----
10,000	----	----	15	0	0	----	----	----	----	----	----	----	----	----	----	----	----	----	----
7,500	----	----	20	0	0	----	----	----	----	----	----	----	----	----	----	----	----	----	----
5,000	12	0	12	0	0	----	----	----	----	----	----	----	----	----	----	----	----	----	----
1,000	13	0	13	0	0	----	----	----	----	----	----	----	----	----	----	----	----	----	----
750	14	0	----	----	----	----	----	----	----	----	----	----	----	----	----	----	----	----	----
500	15	0	----	----	----	----	----	----	----	----	----	----	----	----	----	----	----	----	----
250	18	0	----	----	----	----	----	----	----	----	----	----	----	----	----	----	----	----	----
100	22	0	----	----	----	----	----	----	----	----	----	----	----	----	----	----	----	----	----
75	26	0	----	----	----	----	----	----	----	----	----	----	----	----	----	----	----	----	----
50	28	0	----	----	----	----	----	----	----	----	----	----	----	----	----	----	----	----	----
25	4	0	----	----	----	----	----	----	----	----	----	----	----	----	----	----	----	----	----
10	15	0	----	----	----	----	----	----	----	----	----	----	----	----	----	----	----	----	----
5	20	0	----	----	----	----	----	----	----	----	----	----	----	----	----	----	----	----	----
1	34	0	----	----	----	----	----	----	----	----	----	----	----	----	----	----	----	----	----
0	32	0	----	----	----	----	----	----	----	----	----	----	----	----	----	----	----	----	----

[a] Number of cells showing American foul-brood remains.

In the first series of inoculations the number of spores fed each larva ranged from approximately 5,000 down to 1. None of the larvae inoculated developed disease. (Table 2.) Later a second series of inoculations was made. The same colonies were used because of the limited number available, but the larvae inoculated were in a different comb in each colony and a different color was used to mark the cells. In these inoculations the number of spores fed ranged from 5,000,000 down to 1,000 per larva. No disease developed from this set of inoculations.

It was thought possible that the nurse bees might be removing most, if not all, of the inoculated sugar sirup before the larvae had had time to ingest a sufficient number of spores to bring about infection. Therefore, in a third series of experiments each inoculated comb was placed in a screen-wire queen-nucleus introducing cage, and this cage was put back in the colony for periods ranging from one-half to one hour before the unprotected comb was replaced in the colony, thus theoretically giving the larvae time to ingest some of the sugar sirup before the nurse bees had access to the inoculated cells. In these tests the larvae were kept from the bees so long that many of them, becoming hungry, were starting to crawl from the cells. The number of spores fed ranged from 50,000,000 down to 500,000 per larva. Twenty-four hours after the larvae were fed it was found that all receiving 50,000,000 and 25,000,000 spores had been removed from the cells, while those receiving a smaller number of spores were either partly removed or remained in the cells, according to the strength of the dilution and the length of time that the larvae were kept away from the nurse bees. (Table 2.)

Two days later another set of larvae was inoculated with the same dilutions as were previously used for these colonies but on the other side of the same combs. In this series the combs were kept away from the bees for periods ranging from 5 minutes for the heaviest dilution to 30 minutes for the weakest. Again all the larvae receiving the 50,000,000 and 25,000,000 spores were removed, while those receiving the 5,000,000, which were kept from the bees for half an hour, were partly removed, and those receiving 7,500,000 or 10,000,000 were not removed. Apparently there are two factors concerned in the removal of the larvae—the length of time they are kept away from the bees and the amount of foreign matter in the sirup, as indicated by the spore content, that is given to the larvae.

The results of the last two series of inoculations showed that in the colonies in which the larvae were not removed, or were not entirely removed, several larvae in the colony receiving 10,000,000 spores per larva developed disease, while those in the colonies receiving a smaller number remained healthy. (Table 2.) This work should be repeated with a different colony for each set of inoculations, although apparently the disease did not spread in the colonies used. Only one colony of the entire number developed disease. Although a certain degree of success was obtained, these results seem to bear out Toumanoff's (*29*) conclusion that the artificial infection of individual larvae is not brought about so easily as one had been in the habit of believing. Apparently, also, a considerable number of spores are necessary to establish an infection under these conditions.

MINIMUM NUMBER OF SPORES OF BACILLUS LARVAE PRODUCING VEGETATIVE GROWTH ON ARTIFICIAL CULTURE MEDIA

Bacteria are known to pass through a definite cycle of growth, particularly when cells from an old culture are transferred to fresh culture media. The growth stages have been described by Buchanan (*3; 13, Ch. V*), Henrici (*12*), and Winslow (*13, Ch. VI*) somewhat as follows: The initial stationary phase during which no growth takes place; the logarithmic phase when the organisms begin to divide, slowly at first but gradually accelerating; and so on through the complete cycle of growth. Henrici (*12, p. 21, 24*) has observed that—

> Various factors, as temperature; the size, the age, and previous history of the inoculum; and the composition and nutrient value of the medium, influence the growth curves of bacteria. * * * Of the various factors which influence the rate of growth and form of the growth curve, the initial number of cells introduced into a unit volume of medium seems to be one of the most important.

Robertson (*25*), in studies of cultures of certain protozoa, has shown that growth seems to be stimulated by the presence of other cells of the same type. This characteristic has been described at various times as mass action or communal activity.

Early in 1929, in conjunction with the spore-feeding experiments in the apiary, an investigation was started to determine whether there is a similar manifestation of mass action in the vegetative growth of spores of *Bacillus larvae* on artificial culture media. In a preliminary paper on this subject the writer (*27, p. 456*) made the following observations: Starting with a seeding of 5,000,000,000 spores of *B. larvae* on a suitable slanted solid culture medium, it was found at the end of 48 hours' incubation at 37° C. that growth had occurred in the original and in a diluted seeding containing 60,000,000 spores, but not in one containing 50,000,000 spores. Growth occurred in a diluted seeding containing only 5,000,000 spores after six days' incubation, and in one containing 700,000 spores after 10 days' incubation. (Table 4, Group 1.) These observations indicated that a certain initial mass of spores is necessary to start vegetative growth. Furthermore, although the growth results were rather irregular owing to the comparatively small number of cultures made, they seemed to show that, within certain limits, the smaller the seeding the longer the incubation period necessary to obtain germination of the spores and vegetative growth. From this preliminary work it was assumed that the lower limits of dilution of the stock suspension that would give growth on longer incubation had not been reached.

Ahrens (*1*) has observed, in cultural studies of scales treated with formalin solution for different lengths of time, that growth may occur in cultures from such scales after varying periods of incubation up to 30 days, depending on the length of treatment and the percentage of formalin in the solution. Burnside (*7*) states, in connection with studies of disinfection of American foulbrood combs by fumigation with formaldehyde gas, that "it is probable that if scales had been washed and the incubation period increased, growth of *Bacillus larvae* would have been obtained in some instances when negative results were recorded."

Therefore, a single trial series of cultures was run (No. 7, Table 4), the total incubation period being 30 days. Results from this set of cultures showed that in some cases growth was obtained after 30

days' incubation where no growth was observed after 10 days' incubation. Work on this phase of the problem was continued during the summer and fall of 1930. Several sets of cultures were made in which *Bacillus larvae* from eight different localities were used in a series of seedings with a decreasing number of spores for each lot of the organism and all incubated for 30 days. (Table 4, Group 2.)

METHODS OF PROCEDURE

Culture Media

A culture medium was used similar to that employed by the writer in the preliminary experiments (*27*) and also in earlier cultural work with *Bacillus larvae* (*26*)—that is, a combination of the medium made of yeast-extract and egg-yolk suspension and the carrot-extract medium of Lochhead (*18*). The yeast-carrot extract medium was prepared as follows:

(A)	Dried yeast	grams	10
	Peptone	do	10
	Buffer (sodium glycerophosphate)	do	2.5
	Water (distilled)	cubic centimeters	500

This solution was heated in flowing steam for one-half hour and, after a tablespoonful of siliceous earth had been added to assist in the filtration and clarification, it was filtered through filter paper on a perforated porcelain funnel with suction.

(B) Two hundred grams of cleaned carrots was macerated in a meat grinder, added to 500 c c of distilled water, and allowed to stand for at least 30 minutes, preferably longer. The macerated carrot was removed by filtration through fine muslin, as much liquid as possible being squeezed from the mass. The filtrate was then clarified by the addition of siliceous earth and filtration in the same manner as the yeast-extract medium.

(C) The final base medium was prepared by mixing 500 c c of A with 200 c c of B and adding 700 c c of a 3 per cent solution of washed agar.

The reaction of the medium was so adjusted that when 2 c c of sterile egg-yolk suspension, prepared as described in a previous paper (*26*), was added to 10 c c of the yeast-carrot extract base medium by means of the apparatus shown in Figure 1, and described previously (*26*), the pH value was 6.8. The medium was then sterilized in the autoclave at 15 pounds' pressure (sea level) for 15 minutes. After it had cooled to 45° C., 20 drops, or about 2 c c, of the sterile egg-yolk suspension was added to each tube of medium, mixed by shaking, and the medium was then allowed to solidify in a slanting position.

The Lochhead yeast-extract medium was tried without the addition of egg-yolk suspension, but although it gave good growth with the heavier seedings of spores, the combination medium was found to give more uniform germination and heavier vegetative growth with the more dilute seedings. The addition of the carrot extract, while possibly adding somewhat to the growth-producing qualities of the medium, served in these experiments as an indicator for vegetative growth because of the ability of *Bacillus larvae* to produce nitrite in the carrot-extract medium without the addition of potassium nitrate (*18*).

Preparation of Dilutions of Spores

The stock suspensions of spores of *Bacillus larvae* were made up as described earlier in this paper. A series of primary dilutions, each one-tenth of the preceding dilution, was then made up in sterile 125

c c flasks by adding 4 c c of a dilution to 36 c c of sterile water. The series of dilutions containing gradually decreasing numbers of spores per cubic centimeter to be used in inoculating the culture medium were then prepared as indicated in Table 4. Sterile burettes were used in adding the proper proportions of spore suspension or spore-suspension dilutions to the proper quantities of sterile water in sterile test tubes, in order to make up the desired series of dilutions containing approximately known numbers of spores.

Inoculation of Culture Medium

Swann has observed that in old cultures of anthrax a considerable percentage of spores are dead and therefore never germinate. Because of the possibility that some of the spores in the stock suspensions of *Bacillus larvae* might not be viable, an effort was made to determine the approximate proportions of viable and dead spores in the stock suspensions. Since the determination of viable spores of *B. larvae* by means of plate cultures is difficult because of the opaqueness of the special culture medium that is required, an attempt was made to determine the percentage of viable spores by the differential staining method of Burke (*4*) as modified by Koser and Mills (*15*). The procedure is as follows: A small quantity of the spore suspension is spread in a thin film on a slide and allowed to dry without heating. The slide, after immersion in a solution of carbol fuchsin at room temperature for two minutes, is washed in water and decolorized with absolute acetone for a few seconds, washed again, and immersed in Loeffler's alkaline methylene blue for two minutes, washed, dried, and examined. Very few solid-staining forms were observed in any of the suspensions examined, possibly one or two spores in several fields. It was therefore assumed that the number of nonviable spores could be considered as negligible and probably within the limits of the precision of the measurements as indicated by this procedure.

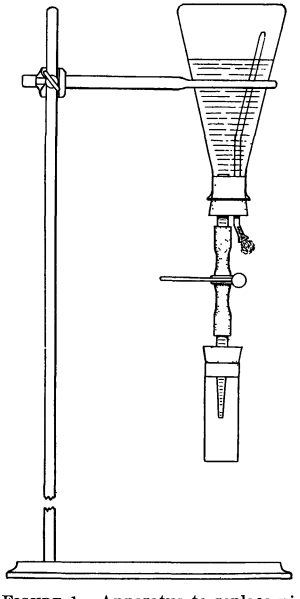

Figure 1.—Apparatus to replace pipetting of egg-yolk suspension

One cubic centimeter of each dilution was added to duplicate tubes of the slanted solid medium by means of sterile 1 c c pipettes, each cubic centimeter of inoculum containing an approximately known number of spores of *Bacillus larvae*. After inoculation the cultures were incubated at 37° C. In order to prevent the liquid in the tubes from drying out on long incubation, from time to time, as the water of condensation evaporated, 2 or 3 c c of sterile broth similar in composition to that of the base medium, without the egg, was added to each tube by means of the apparatus shown in Figure 1. A total of 556 cultures was made during this series of experiments.

Method of Making Observations

The culture tubes were incubated for 30 days at 37° C. Each tube was examined usually every 24 hours during this period. The presence or absence of vegetative growth was noted at each observation, and in cases of slight or doubtful growth the vegetative growth was checked both by microscopic examination of a stained smear and by testing for nitrite production in the culture medium by the sulphanilic acid and alpha-naphthylamine acetate test. After a large number of such observations had been made, it was found that vegetative germination of spores of *Bacillus larvae*, almost too slight to be seen, would give a definite pink color on the addition of the reagents.

Lochhead (*17, p. 14*) states:

> It was found, however, that ordinary nitrate-reducing species, such as *B. cereus* or *Es. coli*, which are able to form nitrites readily in nitrate media, were unable to produce nitrites in recognizable amount in the peptone-carrot media, though capable of doing so upon the addition of nitrates. *Bacillus larvæ* under the same condition readily forms nitrites without the addition of nitrate to the medium.

Despite this statement, a series of miscellaneous organisms was tested in standard nitrate broth, in carrot-extract broth, and on carrot-extract agar. Several organisms that commonly reduce nitrates and a few that do not were used. (Table 3.) Observations were made at short intervals during the first 24 hours. Most of these organisms gave positive nitrite tests within a few hours after inoculation in all the media used, but in the carrot-extract medium the nitrate had apparently disappeared in most cases after 24 hours' incubation, and in all cases after 48 hours. The same organisms on standard nitrate medium still gave positive tests after 48 hours' incubation. A positive nitrite test was obtained in cultures of *Bacillus larvae* that were incubated for 5 days and in one culture that was incubated for 4 days and then allowed to stand at room temperature for 16 days more before testing. Therefore, it appears probable—at least the results in Table 3 indicate—that in the case of many contaminating organisms having the power to reduce nitrite that might get into the culture tubes inoculated with spores of *B. larvae* the nitrite, if produced by the contaminating organism, would have disappeared after 48 hours' incubation, leaving contamination to be determined by gross appearence of the culture and microscopic examination. Nevertheless, in order to be sure that contaminating growth of any kind was not giving erroneous results with the nitrite test when this was used alone, any suspicious-looking growth in the culture tubes was examined under the microscope before it was tested with the reagents for nitrite production. Even though a positive nitrite test might be observed in some cases, the contaminations were recorded only as such.

Observations and Results

In no instance was positive growth obtained in cultures inoculated with less than 50,000 spores, even after 30 days' incubation, and growth with 50,000 spores was obtained from only two of the eight lots of spores used, namely, Nos. 19 and 23. (Table 4.) In the other six strains the minimum number of spores that produced positive growth ranged from 5,000,000 to 70,000.

TABLE 3.—*Nitrate reduction by various miscellaneous organisms in standard nitrate broth and carrot-extract media during different periods of time*[a]

Organism	Standard nitrate broth					Carrot-extract broth							Carrot-extract agar					
	3 hours	6 hours	8 hours	24 hours	48 hours	3 hours	6 hours	8 hours	10 hours	14 hours	16 hours	24 hours	3 hours	6 hours	24 hours	48 hours	5 days	20 days
Escherichia communior	3+	4+	4+	4+	4+	2+	−	−	−	−	−	−	+	+	−	−		
Escherichia coli	−	3+	4+	4+	4+	−	+	−	−	−	−	−	+	+	−	−		
Eberthella typhi	−	3+	4+	4+	4+	−	−	−	−	−	−	−	+	±	−	−		
Alcaligines fecalis	+	3+	4+	±	−	Tr.	2+	2+	+	+	−	−	2+	+	+	−		
Aerobacter aerogenes	3+	4+	4+	4+	4+	+	−	−	−	−	−	−	Tr.	Tr.	±	−		
Pseudomonas aeruginosa	−	3+	4+	−	−	+	2+	2+	+	±	−	−	+	+	+	−		
Serratia marcescens	−	3+	4+	4+	4+	−	+	−	−	−	−	−	Tr.	Tr.	+	−		
Staphylococcus albus	−	−	−	−	−	−	−	−	−	−	−	−	±	−	−	−		
Bacillus subtilis				−	−							−			−	−		
Bacillus mesentericus				−	−							−			−	−		
Bacillus larvae				2+	4+							2+			2+	4+	4+	4+

[a] +, 2+, 3+, and 4+ indicate relative degrees of reaction; Tr. indicates trace; − indicates no reaction; ± indicates that the reaction was doubtful.

TABLE 4.—*Vegetative growth in cultures of Bacillus larvae with varying dilutions of a stock suspension*

Method of making dilutions			Spores per cubic centimeter inoculated ($\times 10^3$)	Strains of bacillus larvae [a]													
				Group 1 (preliminary experiments)						Trial series	Group 2						
Primary dilution taken	Quantity of primary dilution taken	Water added		No. 1	No. 4	No. 5	No. 6A	No. 6A2	No. 6D	No. 7	No. 12	No. 13	No. 14	No. 15	No. 19	No. 20	No. 21	No. 23
	Cubic centimeters	*Cubic centimeters*																
	Original		*Number*															
	(A)	0	5,000,000	+2 +2	+2 +2	+2	+2	+6	+10	+5	+2 +2	+2 =2	+2 +2	+2 +2	+2 +2	+2 +2	+2 +1	+2 +2
	8	2	4,000,000			+2												
A	6	4	3,000,000			+2												
	4	6	2,000,000			+2												
	2	8	1,000,000			+2												
	1 (B)	9	500,000	+2 +2	+2 +2	+2	+2	+6	+10	+5	+2 +2	+2 +2	+2 +2	+2 +2	+2 +2	+2 +2	+2 +2	+2 +2
	8	2	400,000		+2	+2	+2											
	6	4	300,000		+2	+2	+2											
	4	6	200,000		+2	+2	+2											
B	2	8	100,000		±2?	+2	+2											
(1A+9H_2O)	1.8	8.2	90,000			+2	+2											
	1.6	8.4	80,000			+2	+6											
	1.4	8.6	70,000			−2	+6											
	1.2	8.8	60,000			−2	+2											
	1.0 (C)	9.0	50,000	±2±2	±2?−2	−2	−6	+6	+10	+5+30	+3 +3	+3 +3	+3 +3	+2 +2	+2 +2	+4 +4	+5 +5	+4 +4
	8	2	40,000			−2	−6	−6	+10	+10+10	+3 +3	+3 +5	+4 +4	+2 +2	+2 +2	+4 +4	+5 +5	+4 +4
	6	4	30,000			−2	+6	−6	+10	+10+15	+3 +3	+5 +5	+4 +4	+2 +2	+2 +2	+4 +6	+5 +5	+4 +4
	4	6	20,000			−2	−6	+6	+10	+10+30	+3 +3	+5 +5	+4 +4	+2 +2	+3 +3	+4 +6	+7 +7	+4 +4
C	2	8	10,000			−2	−6	+6	+10	−30−30	+7 +8	+5 +7	+6 ×	+3 +3	+3 +3	+4 +6	+7+14	+4 +4
(1B+9H_2O)	1.8	8.2	9,000			−2	+6	+6	−10	−10+30				+4 +4	+6 +6	+6 +6	+9+27	+4 +4
	1.6	8.4	8,000			−2	−6	+6	+10	−30−30				+4 +4	+6+16	+6+11	−27−30	+4 +4
	1.4	8.6	7,000			−2	−6	+6	−10	+10−10				+4 +4	−30−30	+6 +6	−30−30	+4 ×
	1.2	8.8	6,000			−2	−6	−6	+10	−30−30				+5 +6	+6−30	+6 +6	+7−30	+4 +4
	1.0 (D)	9.0	5,000	−2−2	−2 −2	−2	−6	+6	+10	−10+30	+7−30	+14−30	+12−30	+6 ×	−30−30	+11+14	−30−30	+4 +4
	8	2	4,000						+10	−10−30	+7+12	× ×	+6+10	−30 ×	+8−30	−30×	+17−30	+4 +4
	6	4	3,000						+10	−10+30	+6+14	+ 7×	+10+10	−30−30	+8×	+6+12	+27+27	+6 ×
	4	6	2,000						−10	−10+30	+6−30	+17−30	+20−30	−30−30	+8−30	+27−30	−30−30	+6 +6
D	2	8	1,000						−10	−10−30	+10+14	+21×	−30−30	−30−30	−30−30	−30−30	−30−30	+6 ×
(1C+9H_2O)	1.8	8.2	900						−10	−10+30	+6×	−30×	+10+16	−30−30	−30−30	+25−30	−30−30	−30−30
	1.6	8.4	800						−10	−10+30	+14+22	+21−30	−30×	−30−30	−30−30	+20−30	+27−30	−30−30
	1.4	8.6	700						+10	−10−30	−30−30	+30×	−30×	−30−30	+8+10	+30−30	+27−30	+8×
	1.2	8.8	600						−10	−10+30	+27−27	−30−30	−30×	+13−30	−30−30	−30−30	−30−30	+6−30
	1.0 (E)	9.0	500	−2−2	−2 −2	−2	−6	−6	−10	−10−30	−30−30	−30−30	−30×	−30−30	−30−30	−30−30	+27+27	−30−30

E $(1D+9H_2O)$	8	2	400	....	....	....	....	....	−10−30	−30−30	−30−30	−30−30	−30−30	−30−30	−30−30	−30×	−30−30
	6	4	300	....	....	....	....	....	−10+30	−30−30	−30−30	−30−30	−30−30	−30−30	−30−30	+27−30	−30−30
	4	6	200	....	....	....	....	....	−10−30	−30−30	−30−30	−30−30	−30−30	−30−30	+11−30	−30−30	−30−30
	2	8	100	....	....	....	....	....	−10−30	−30−30	+30−30	−30−30	−30−30	−30−39	−30−30	−30−30	−30−30
	1.8	8.2	90	....	....	....	....	....	−10−30	−30−30	−30−30	−30−30	−30−30	−30−30	−30−30	−30−30	+8−30
	1.6	8.4	80	....	....	....	....	....	−10−30	−30−30	+30−30	−30−30	−30−30	−30−30	−30−30	−30−30	+8−30
	1.4	8.6	70	....	....	....	....	....	−10−30	−30−30	−30−30	−30−30	−30−30	× −30	−30−30	+27+27	+6−30
	1.2	8.8	60	....	....	....	....	....	−10−30	−30−30	−30−30	−30−30	−30−30	−30−30	−30−30	−30−30	+8−30
	1.0 (F)	9.0	50	−2−2	−2 −2	−2 −6	−6	−10	−10−30	−30−30	−30−30	−30−30	−30−30	+13−30	−30−30	−30−30	+11−30
F $(1E+9H_2O)$	8	2	40	....	....	....	....	....	−10−30	−30−30	−30−30	×−30	−30−30	−30−30	−30−30	....	....
	6	4	30	....	....	....	....	....	−10−30	−30−30	−30−30	−30−30	−30−30	−30−30	−30−30	....	....
	4	6	20	....	....	....	....	....	−10−30	−30−30	−30−30	−30−30	−30−30	−30−30	−30−30	....	....
	2	8	10	....	....	....	....	....	−10−30	−30−30	−30−30	−30−30	−30−30	−30−30	−30−30	....	....
	1 (G)	9	5	−2−2	−2 −2	−2 −6	−6	−10	−10−30	−30−30	−30−30	−30−30	−30−30	−30−30	−30−30	−30−30	−30−30
G $(1F+9H_2O)$	8	2	4	....	....	....	....	....	−10−30	−30−30	−30−30	−30−30	−30−30	−30−30	−30−30	....	....
	6	4	3	....	....	....	....	....	−10−30	−30−30	−30−30	−30−30	−30−30	−30−30	−30−30	....	....
	4	6	2	....	....	....	....	....	−10−30	−30−30	−30−30	−30−30	−30−30	−30−30	−30−30	....	....
	2	8	1	....	....	....	....	....	−10−30	−30−30	−30−30	−30−30	−30−30	−30−30	−30−30	....	....
	1 (H)	9	0.5	−2−2	−2 −2	−2 −6	−6	−10	−10−30	−30−30	−30−30	−30−30	−30−30	−30−30	−30−30	....	....

[a] Numbers in these columns refer to number of days of incubation before observations were made; + indicates vegetative growth; − indicates no vegetative growth; × indicates contamination; ± indicates that it was impossible to tell whether or not there was growth; ±? indicates that the positive was more doubtful than the negative. Where two numbers are given for a dilution, two cultures of this dilution was carried.

139707—32——2

The length of the incubation period in relation to the decreasing number of spores used varied greatly with the different lots of spores, even with the duplicate inoculations of each lot. Table 5 gives the results of positive cultures obtained in relation to the period of incubation and the dilution of the spores. The coefficient of correlation (*14, p. 179*) for the positive cultures only, in relation to length of incubation and dilution of spores, was found to be 0.3558 ± 0.0440. While this does not show a strong correlation, it indicates that with the smaller numbers of spores there is a tendency for growth to take place with longer periods of incubation. However, when the cases of positive growth were correlated with the dilution and incubation time on the basis of the percentage of positive cultures to negative cultures for each observation period of incubation time, an insignificant negative correlation was obtained. Apparently there is a variable uncontrollable factor present, more obvious when spores are used from different lots of the organism, which makes it impossible to correlate the other factors closely. However, the data summarized in Table 6 indicate that, of the 120 cultures made with seedings of between 5,000,000,000 and 9,000,000 spores per seeding, 98.33 per cent showed growth at the end of 10 days' incubation, while 100 per cent (120 cultures) showed growth after 30 days' incubation. This is 56.87 per cent of the 211 total cultures showing growth after 30 days.

TABLE 5.—*Summary of the positive vegetative cultures of Bacillus larvae in relation to length of incubation period and with varying dilutions of the stock suspension*

Number of spores per cubic centimeter inoculated	Number of positive cultures after days of incubation																												Number of cultures		
	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	Positive	Negative	Total
5,000,000,000	16																													16	0	16
500,000,000	16																													16	0	16
50,000,000	4	6	4	2																										16	0	16
40,000,000	4	3	6	3																										16	0	16
30,000,000	2	4	5	4	1																									16	0	16
20,000,000		6	5	2	1	2																								16	0	16
10,000,000		2	2	1	4	3	1						1																	14	0	14
9,000,000			4		4			1																		1				10	0	10
8,000,000			4		2					1					1															8	2	10
7,000,000			3		2																									5	4	9
6,000,000			2	1	4	1																								8	2	10
5,000,000			2		1	1				1	1		2																	8	7	15
4,000,000			2		1	1	1		1		1					1														8	4	12
3,000,000					3	1	1		2		1		1													2				11	2	13
2,000,000					3		1									1			1							1				7	9	16
1,000,000					1				1				1							1										4	10	14
900,000					1				1						1									1						4	10	14
800,000													1						1	1	1					1				5	10	15
700,000							2		1																	1			2	6	7	13
600,000					1							1														1				3	12	15
500,000																										2				2	13	15
400,000																														0	15	15
300,000																										1				1	15	16
200,000													1																	1	15	16
100,000																													1	1	15	16
90,000							1																							1	15	16
80,000							1																						1	2	14	16
70,000					1																					2				3	12	15
60,000							1																							1	15	16
50,000										1		1																		2	14	16
0–40,000																														0	123	123
Total positive cultures	42	21	39	13	30	9	9	1	6	3	3	2	7	0	2	2	0	0	2	2	1	0	0	1	0	12	0	0	4	211		
Total negative cultures	514	493	454	441	411	402	393	392	386	383	380	378	371	371	369	367	367	367	365	363	362	362	362	361	361	349	349	349	345		345	
Total cultures																																556

TABLE 6.—*Summary of positive vegetative cultures of Bacillus larvae grouped in relation to size of seedings and length of incubation period*

Number of spores per seeding	10 days' incubation				11 to 30 days' incubation				Final observations after 30 days' incubation								
	Number of positive cultures	Number of negative cultures	Percentage positive	Percentage negative	Number of positive cultures	Number of negative cultures	Percentage positive	Percentage negative	Total number of positive cultures	Total number of negative cultures	Total number of all cultures	Percentage positive	Percentage negative	Percentage positive of total of all positive cultures	Percentage negative of total of all negative cultures	Percentage positive of total of all cultures	Percentage negative of total of all cultures
5,000,000,000–9,000,000	118	2	98.33	1.66	2	0	1.67	0	120	0	120	100	0	56.87	0	21.58	0
8,000,000–500,000	48	123	28.07	71.93	31	92	18.12	53.80	79	92	171	46.20	53.80	37.44	26.66	14.21	16.55
400,000–50,000	4	138	2.82	97.18	8	130	5.63	91.55	12	130	142	8.45	91.55	5.69	37.68	2.16	23.38
40,000–0	0	123	0	100	0	123	0	100	0	123	123	0	100	0	35.65	0	22.12
Total	170	386	30.58	69.42	41	345	7.37	62.05	211	345	556					37.95	62.05

Of the 171 cultures made with seedings between 8,000,000 and 500,000 spores per seeding, 48, or 28.07 per cent, showed growth at the end of 10 days' incubation, while 79, or 46.20 per cent, showed growth after 30 days' incubation. The latter number is 37.44 per cent of the 211 total cultures showing growth after 30 days' incubation.

Of the 142 cultures made with seedings between 400,000 and 50,000 spores per seeding, only 4, or 2.82 per cent, showed growth at the end of 10 days' incubation, while 12, or 8.45 per cent, showed growth after 30 days' incubation. The latter figure is 5.69 per cent of the 211 cultures showing growth after 30 days' incubation.

Of the 123 cultures made with seedings of 40,000 or fewer spores per seeding, no growth was obtained after 30 days' incubation.

Of the 556 cultures made with all seedings, 30.58 per cent showed growth at the end of 10 days' incubation and 69.42 per cent showed no growth. The 170 positive cultures after 10 days' incubation is 80.57 per cent (not shown in Table 6) of the 211 total positive cultures obtained. In the interval between the 10 and 30 day incubation periods, 19.43 per cent (not shown in Table 6) of the 211 total positive cultures, or another 7.37 per cent of all cultures made, showed growth, making a total of only 37.95 per cent of all cultures which showed growth at the end of 30 days' incubation, with 62.05 per cent still showing no growth.

The initial growth phases as described by Buchanan (*3; 13, Ch. V*) are clearly more marked with spores than with simple vegetative organisms, since there is a varying length of time necessary for spores to germinate and start growing after implantation in a suitable medium. In the light of observations on other spore-forming organisms, it is probable that this factor, which seems to cause variations in the germination time of *Bacillus larvae* even within a lot from a single source, is what has been termed "dormancy." Burke (*5, p. 283*), working with *Clostridium botulinum*, found:

> The individual (unheated) spores in a given culture of *Cl. botulinum* vary greatly in the time required for germination under optimum growth conditions. The majority germinate relatively quickly, but a few lie dormant for a longer time. One hundred and forty-four days is the maximum period of dormancy recorded here * * *.

Burke states:

> The primary factors which cause the spore to lie dormant for long periods of time under optimum growth conditions are believed to be inherent in the spore itself. It is thought that relative permeability of the spore wall is one of the factors. Environmental conditions may secondarily modify the period of dormancy.

Burke, Sprague, and Barnes (*6, p. 560*) observed the same phenomenon with such non spore-bearing bacteria as *Bacillus coli* (=*Escherichia coli*). They found that spores of *B. subtilis* remained dormant 39 days and those of *B. megatherium* 90 days, although a large majority developed in 4 or 5 days. They believe:

> Dormancy must be considered a factor in infection. It reduces the chances of infection by reducing the number of organisms that would otherwise start to grow at one time. Since the cells begin to multiply at different times, the body has an opportunity to initiate defensive reactions before all the cells develop. If dormant for a sufficient period, the organisms will be excluded from the body before development takes place.

Swann (*28*) has observed that there is a variation in the germination time of anthrax spores, depending on the age and condition of the spores.

Morrison and Rettger (*24, p. 339*) recently stated—

> Because of the marked variability of germination, depending upon the stimuli supplied in the environment, the deduction is made that bacterial spores in the process of germination are vitally active bodies having requirements for metabolic function which are the same as or more exacting and specific than those of the vegetative cells.
>
> Experimental evidence is presented to show that the dormancy of aerobic bacterial spores is largely, if not entirely, determined by conditions in the environment of the spores, and that these factors must be taken into consideration, perhaps specifically for each species, before so-called "inherent" or "normal" dormancy of bacterial spores can be established.

This phase of the work with *Bacillus larvae* is being repeated with the organism obtained from a single source in an effort to determine the importance of this variable factor of dormancy.

SPORES OF BACILLUS LARVAE IN COMMERCIAL HONEY

A few instances have been reported in the bee journals, such as that by Merrill (*22*), in which American foulbrood has developed as a result of bees having access to cans of infected honey that have been carelessly thrown out. Without doubt in some cases honey has been allowed to get on the market from infected colonies through negligence of the beekeepers and without being diluted by mixing or blending with honey from disease-free apiaries. On the other hand, Fracker (*10, p. 379–380*) has shown, by a study of disease-inspection statistics for Wisconsin:

> 1. In Wisconsin the introduction of this disease into the State and into many individual localities is definitely known to have been in specific importations of bees and equipment.
> 2. Cases of infection in which the source appears to be infected honey in the channels of trade are comparatively rare.
> 3. Even near such a large center as Milwaukee the infection percentage is greatest in localities of active movement, such as greenhouse areas, and is relatively low within the city itself.
> 4. Towns and cities of from 3,000 to 40,000 which have been natural markets for infected honey from near-by counties, have remained for years free from disease either until the present or until infected bees and equipment were introduced.
> 5. No new centers of infection are known to have been started since the policy of limiting movement of bees and equipment was begun in 1919.
> 6. These observations appear to be confirmed by conditions in the South, in spite of the fact that the period of active flight of the bee tends to continue through the peak of honey distribution.

Furthermore, F. L. Thomas, State entomologist of Texas, in an unpublished manuscript states:

> The largest of the estimates with reference to the quantity of honey that is brought into Texas in a year is 19 carloads. Most of this honey is produced in California, Colorado, New Mexico, Utah, and Wyoming. * * *
>
> If 19 carloads of foulbrood-infected honey are distributed annually in this State, it seems reasonable to suppose that our inspectors would have a hard time to keep this disease within bounds. In fact, I would expect to find that the inspectors would be gradually losing ground in their attempts to eradicate this menace. A large share of the honey which is imported is sold in west and northwest Texas where practically no bees are kept. The amount which is distributed in the beekeeping territory of the State is evidently less dangerous than is commonly supposed. The following facts, I think, will prove this statement.
>
> During the period September 1, 1920, to August 31, 1926, the inspection work has been carried into 100 counties. Fifty-six counties were found to be free

from contagious or infectious diseases of bees, but in the other 44 counties American foulbrood has been present.

An average of 668 beekeepers have been visited each year and 38,661 colonies examined with the result that an average of 430 colonies, or 1.11 per cent, have been found to be diseased.

American foulbrood is found now in only 23 counties, 21 of the 44 counties having been cleaned up. In 12 of the counties where disease occurs, only 30 colonies were found to be infected out of 7,642 examined—less than 0.4 of 1 per cent. Six counties had one diseased colony each.

About 40 per cent of the beekeepers and 60 per cent of the colonies are reinspected from year to year; the remainder, being free of disease and considered out of danger, are dropped and "new territory" is taken over and examined for presence of foulbrood. By "new territory" is meant beekeepers and their colonies visited and inspected for the first time. An average of 228 diseased colonies are discovered each year in "new territory." This is 1.6 per cent of the total number of colonies examined in this territory.

The reinspection which has been made in the counties where disease has been present shows that there have been both gains and losses. But a net gain has resulted which has averaged 21 beekeepers and 368 colonies freed from American foulbrood and quarantine annually.

From these facts it is easily seen that definite and really rapid progress in eradicating the disease is being made. Rarely do our inspectors find new outbreaks of disease that can not be traced to careless beekeeping methods, bees robbing infected and weakened colonies, or to the use of old and infected equipment.

It is not my intention to imply that honey is not a carrier of American foulbrood. The above evidence simply indicates that the honey which has been imported into Texas has not been as dangerous a source of disease to bees as is sometimes thought.

Practically no work has been reported on the microbiology of honey other than that in connection with the spoilage of honey through fermentation by yeasts (*19*, *21*), and no work appears to have been done on the *Bacillus larvae* spore content of commercial honey. In 1925 the writer undertook to devise a method for demonstrating, at least qualitatively, the presence or absence of spores of *B. larvae* in honey and their significance in relation to the results of the spore-feeding experiments. Difficulties were encountered in obtaining cultures of *B. larvae* from honey. It was impossible to obtain vegetative growth of this organism, even when a considerable number of spores had previously been added to honey, because of the difficulty of eliminating contaminating organisms that developed rapidly in the honey, completely overgrowing any possible vegetative growth of *B. larvae* before it could get well started. Therefore, methods of concentrating the spores from the honey and of identifying them by means of microscopic examination were attempted. Because spores of *B. larvae* have a characteristic appearance in stained smears (*20*, *p. 9*), it was assumed that this method might give at least tentative evidence.

METHODS OF PROCEDURE

The first method attempted was the filtration of honey diluted with water through a membrane of ether-alcohol collodion or through filter paper impregnated with an acetic acid solution of collodion (*9*). Apparatus was devised in which both suction and pressure were tried in this filtering process. Stained smears were made of the sediment retained on the surface of the filter. In several cases spores of *Bacillus larvae* were observed in stained smears of the sediment filtered out of honey known to have a large spore content. However, with honey containing fewer spores it was found impossible to concentrate them on a small enough area of filter in sufficient

numbers to recover and identify them under the microscope. Even with a comparatively large filtering surface, the process was so slow that the diluted honey would frequently start to ferment before it had all passed through the filter. A filter of smaller area would become clogged, preventing the passage of a sufficient quantity of honey.

Several unsuccessful attempts were made to recover spores of *Bacillus larvae* from honey by centrifuging samples diluted with an equal quantity of water. After considerable experimentation with honey of known spore content, it was found that it was necessary to dilute the honey to a much greater extent—1 part to at least 9 of water—in order to throw the spores down with the sediment. Apparently the specific gravity of these spores is so low that on centrifuging they remain in suspension in only slightly diluted honey.

The procedure finally used for demonstrating the presence of spores of *Bacillus larvae* in honey is as follows: Five c c of warmed honey is thoroughly mixed with 45 c c of distilled water in a 50 c c cone-shaped centrifuge tube made of heat-resistant glass. Duplicate quantities of each sample of honey are made up for examination. The diluted honey is then centrifuged at 2,000 revolutions per minute for one-half hour. Because of the difficulty of obtaining a satisfactory stained smear from the sediment thrown down in the presence of the sugars of the honey solution, all but 2 c c of the solution in each centrifuge tube is drawn off by means of a 50 c c pipette. Another 45 c c of distilled water is added, the sediment is thoroughly shaken up in the water, and the tubes are centrifuged again for 20 minutes. After all but 2 c c or less of the wash water has been removed, 0.01 c c of the sediment is removed by means of a capillary pipette and smeared on a cover glass over a surface of 1 cm^2, a small loopful of carbol fuchsin being mixed with the material before it is allowed to dry. After drying by gentle heat, the cover glass is mounted on a slide by means of a drop of distilled water and the smear is examined with an oil-immersion objective. Spores of *B. larvae* are identified by their size and shape in conjunction with their distinctive habit of breaking loose from the stained mass of the smear and of showing a delicate Brownian movement in the thin film of water between the two pieces of glass. In a few samples only one or two spores were seen in numerous fields examined or the spores did not have the typical appearance of spores of *B. larvae*. In such cases another test, in which twice as much honey was used, was made from the sample.

OBSERVATIONS

One hundred and ninety-one samples of honey were examined by this method. (Table 7.) Of these, 187 were regular commercial samples purchased in the open market and 2 were from the experimental apiary at Laramie. The other two were miscellaneous samples, one of which was obtained from a brood comb from a diseased colony and the other from a cappings melter which had been used with combs from an infected apiary.

TABLE 7.—*Results of the examination of samples of honey for the presence of spores of Bacillus larvae*

Source	Samples tested	Samples showing positive presence of spores resembling *Bacillus larvae*	Samples showing no evidence of spores [a]
Commercial samples from 30 States	187	15	172
Experimental	2		2
Miscellaneous	2	2	
Total	191	17	174

[a] 29 of these samples were doubtful on the first examination, but repeated examinations gave negative results in each case.

Of the 187 samples of commercial honey obtained from 30 different States or Territories, 15, or 8 per cent, showed the presence of a sufficient number of spores resembling spores of *Bacillus larvae* to be designated as positive. In 29 of the commercial samples, or 15.5 per cent, one or two doubtful spores were seen in each case, but on repeated examinations none of these samples could be considered positive. Two of the four miscellaneous samples from infected sources were also found to contain spores of *B. larvae*.

Five of the samples showing the presence of spores of *Bacillus larvae* were fed to healthy 5-frame colonies during the summer of 1930. These samples consisted of from a pint to a quart of honey. No evidence of American foulbrood appeared in any of the five colonies during the entire brood-rearing season.

In order to determine the approximate number of spores in the samples of honey in which the presence of *Bacillus larvae* was demonstrated, a series of dilutions of spores was prepared as described for the work with cultures. A stained smear was made of 0.01 c c of each dilution spread over a 1-cm^2 surface of cover glass mounted with water and examined with the oil-immersion objective. By this means a definitely recognizable number of spores could be found down to the dilution of 2,000,000 spores per cubic centimeter, with a few single spores seen in occasional fields down to the dilution of 500,000 spores per cubic centimeter. (Table 8.) Then 1 c c of each dilution was added to 5 c c of distilled water in 15 c c centrifuge tubes and centrifuged at 2,000 revolutions per minute for 20 minutes. A stained smear made from 0.01 c c of each sediment showed a definitely recognizable number of spores down to the 5,000-spore dilution, with one or two doubtful spores in several fields from the 500-spore dilution. The sample containing the 50,000-spore dilution, which would be comparable to the sugar sirup containing the minimum number of spores per cubic centimeter fed to colonies in the spore-feeding experiments that produced infection, showed a great many more spores in each field examined by this method than did the sample of commercial honey that showed the greatest number of spores. Therefore, until a better quantitative method is devised, it seems reasonable to believe, from the indications of the preliminary work on this problem, that, even though the presence of a few spores of *B. larvae* may be

demonstrated in 5 c c quantities from a comparatively small percentage of samples of commercial honey, the numbers are far below the minimum necessary to produce infection when such honey is used in healthy colonies of bees. Before definite conclusions can be drawn, it will be desirable to examine many more samples of commercial honey and to feed to healthy colonies samples of honey in which the presence of spores has been demonstrated.

TABLE 8.—*Microscopic examination of dilutions for spores of Bacillus larvae* [a]

Number of spores per cubic centimeter in each dilution	Direct examination of 0.01 cubic centimeter	Examination of sediment after centrifuging 1 cubic centimeter	Number of spores per cubic centimeter in each dilution	Direct examination of 0.01 cubic centimeter	Examination of sediment after centrifuging 1 cubic centimeter	Number of spores per cubic centimeter in each dilution	Direct examination of 0.01 cubic centimeter	Examination of sediment after centrifuging 1 cubic centimeter
5,000,000,000	+	+	10,000,000	+	+	100,000	−	+
4,000,000,000	+	+	9,000,000	+	+	90,000	-------	+
3,000,000,000	+	+	8,000,000	+	+	80,000	-------	+
2,000,000,000	+	+	7,000,000	+	+	70,000	-------	+
1,000,000,000	+	+	6,000,000	+	+	60,000	-------	+
500,000,000	+	+	5,000,000	+	+	50,000	−	+
400,000,000	+	+	4,000,000	+	+	40,000	-------	+
300,000,000	+	+	3,000,000	+	+	30,000	-------	+
200,000,000	+	+	2,000,000	+	+	20,000	-------	+
100,000,000	+	+	1,000,000	±?	+	10,000	-------	+
90,000,000	+	+	900,000	-------	+	5,000	−	+
80,000,000	+	+	800,000	-------	+	4,000	-------	-------
70,000,000	+	+	700,000	-------	+	3,000	-------	-------
60,000,000	+	+	600,000	-------	+	2,000	-------	-------
50,000,000	+	+	500,000	±?	+	1,000	-------	-------
40,000,000	+	+	400,000	−	+	500	−	±
30,000,000	+	+	300,000	−	+	50	−	−?
20,000,000	+	+	200,000	−	+	5	−	−

[a] + indicates that spores were found; − indicates that spores were not found, by microscopic examination; ± indicates that the result was doubtful; ±? indicates that the positive was more doubtful than the negative; −? indicates that the absence of spores was not definite.

SUMMARY AND CONCLUSIONS

As a result of five years' study it has been found that, in order to produce American foulbrood infection in a healthy colony of bees, the sugar sirup used for inoculation must contain a certain initial number of spores of *Bacillus larvae*. Seventy-three colonies were inoculated during this time with numbers of spores ranging from approximately 5,000,000,000 to 100,000 per colony; 30 of these colonies receiving 50,000,000 spores or less. Of these 30 colonies, 2 out of 11 receiving 50,000,000 spores showed infection, but no colony receiving less than that number of spores developed disease. Therefore, the minimum infectious dose of *B. larvae* for a colony of bees seems to be approximately 50,000,000 spores in 1 liter of sugar sirup.

Preliminary experiments in which individual bee larvae were given known numbers of spores of *Bacillus larvae* in 0.01 c c quantities of sugar sirup show that infection can be produced by this method, but with considerable difficulty. From 50 to 100 larvae were inoculated with each dilution of spores, ranging in number from approximately 50,000,000 spores to, theoretically, 1 spore per larva. The minimum infectious dose was found to be 10,000,000 spores per larva fed in 0.01 c c of sugar sirup. These results indicate that the

minimum dose of spores of *B. larvae* that will produce American foulbrood infection must be large.

The germination of spores of *Bacillus larvae* and vegetative growth on a suitable artificial culture medium resulting from the inoculation of 556 culture tubes with seedings varying from approximately 50,000,000,000 to 500 spores per culture also shows that a certain minimum initial number of spores in the inoculum is necessary in order to produce growth. This minimum number of spores producing vegetative growth on a medium consisting of yeast-carrot extract, egg-yolk suspension, and agar was found to be approximately 50,000 in 1 c c of suspension inoculated.

The production of nitrite in this medium by the vegetative growth of *Bacillus larvae* serves as a fairly delicate and reliable indicator of such growth.

There was a tendency for the seedings containing the smaller numbers of spores of *Bacillus larvae* to require a longer period of incubation than the larger seedings in order to produce vegetative growth. However, there was a considerable variation in the germination time of many of the seedings of spores, in one case a seeding of 9,000,000 spores requiring 27 days' incubation to produce growth and another of 70,000 spores requiring only 6 days. This variation, thought to be due to the variable character known as dormancy in bacterial spores, prevented more than a slight correlation.

In the group of cultures comprising seedings between 5,000,000,000 and 9,000,000 spores, only 1.67 per cent required more than 10 days' incubation to produce vegetative growth, 100 per cent having shown growth after 30 days. In the group of cultures comprising seedings between 8,000,000 and 500,000 spores, 71.93 per cent required more than 10 days' incubation, while 53.81 per cent showed no growth at the end of 30 days' incubation. In the group of cultures comprising seedings between 400,000 and 50,000 spores, 97.18 per cent required more than 10 days' incubation, while 91.55 per cent of the group showed no growth at the end of 30 days. Below 50,000 spores no growth was obtained. In other words, below a seeding of 9,000,000 spores an increasing number of the smaller spore seedings required a longer period of incubation. About 80 per cent of all the positive cultures were obtained during the first 10 days of incubation, although this was approximately only 30 per cent of all the cultures made; at the end of 30 days' incubation only about 38 per cent of all the cultures had shown any growth.

It was found possible to demonstrate the presence of spores of *Bacillus larvae* in 15 out of 187, or in 8 per cent, of the samples of commercial honey examined by means of the centrifuge and the microscope. The preliminary results indicate that, even though spores of *B. larvae* may be demonstrated in a certain percentage of samples of commercial honey, in most instances they are probably present in such small numbers as to be less than the minimum number, 50,000,000 per liter, found to be capable of producing disease, and therefore are ineffective in the spread of American foulbrood.

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