

# THE EFFECT OF POLLEN ON THE SPORULATION OF *BACILLUS LARVAE* (WHITE)<sup>1</sup>

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The study of *Bacillus larvae* (White) and the disease of honeybees engendered by it has been retarded by the failure of the microorganism to form more than a few endospores when grown on artificial media. The production of endospores in this species is important because spores alone are capable of transmitting American foulbrood (Tarr, 1937) and because spores of this organism have been shown to be the source of a promising antibiotic effective against the tubercle bacillus and members of the *Brucella* group in artificial culture (Holst, 1945).

The low percentage of sporulation on laboratory media is unexpected inasmuch as large numbers of spores are formed in infected bee larvae. In fact, it is difficult to find the vegetative stage of *B. larvae* in honeybee larvae dead of American foulbrood. It thus appears that factors present in the bee larvae but not in usual laboratory media induce spore formation. Such factors may be physical, environmental, nutritional, or combinations of these and others. The addition of crushed bee larvae to nutrient agar has been shown to increase spore formation by *B. larvae* (White, 1907; Tarr, 1937). This indicates that the active sporulation-inducing agent may be a nutritional factor.

Such a nutritional factor could either be produced by the larvae or be present in their food. The foulbrood microorganisms are believed to gain entry to the bee larvae orally and then to develop extensively in the alimentary canal with a subsequent high production of spores (Sturtevant, 1924). Therefore, it was considered more likely that a nutritive factor for sporulation of the microorganism would be present in the food of the larvae. Since pollen is one of the principal foods of bee larvae, it was decided to study the effect on spore formation of the addition of pollen or chemical fractions of pollen to basal media on which good growth, but poor sporulation, is obtained.

## EXPERIMENTAL PROCEDURES AND RESULTS

*Organism used.* This investigation was conducted with three strains of *Bacillus larvae* isolated from pieces of honeycomb infected with the American foulbrood microorganism. The strains showed no variation in morphology and are designated as strains only by reason of their being isolated from widely separated localities. Strain A was obtained from central Pennsylvania, strain

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B from Ohio, and strain G from Florida. One of the strains, A, was confirmed as being typical *B. larvae* by Dr. H. Katznelson of Ottawa, Canada. The cultures were carried on the stock culture medium and tested periodically for purity by the use of diagnostic broth (Holst and Sturtevant, 1940). As a further check for purity, the organisms were tested for their inability to grow on nutrient agar, which is characteristic of this species.

*Composition of the media.* The culture media consisted of aqueous extracts of pollen and carrots added to a basal substrate. The pollen extract was made by grinding the desired amount of pollen in a mortar and pestle with a small amount of distilled water. The mixture was finally diluted to a total volume of 500 ml with distilled water and allowed to stand 20 minutes with occasional stirring. It was then filtered through a Buchner funnel using a no. 2 Whatman filter paper. The filtered extract was incorporated in media to yield a volume of 1 liter. Thus, to prepare a medium containing 1 per cent pollen, an extract from 10 g of pollen was prepared.

The carrot extract was prepared by macerating 100 g of cleaned carrots with 225 ml of distilled water, allowing the mixture to stand 30 minutes, and filtering through a Buchner funnel using a Whatman no. 2 paper. This procedure yields about 200 ml of filtered carrot extract. The use of carrot extract was discontinued after it was found that it had no effect on sporulation. It was used in early portions of the work because of its reported action (Holst and Sturtevant, 1940).

The basal medium had the following composition: Difco neopeptone, 10 g; Difco yeast extract, 10 g;  $K_2HPO_4$ , 0.5 g; Difco agar, 15 g; and distilled water, 250 ml. The pH was adjusted to 6.8 to 7.0 with N NaOH. For use, the media were prepared by adding 500 ml of the pollen extract, and 200 ml of the carrot extract or 200 ml distilled water, to 250 ml of basal substrate immediately prior to sterilization. The media were sterilized in the autoclave at 120 C for 15 minutes.

The pollen had been gathered by honeybees during different seasons of the year from areas in central Pennsylvania and later stored in glass jars for varying periods of time before being used in these experiments.

*Determination of percentage of sporulation.* Lots of the media were slanted and used within 2 weeks of the time of preparation. The percentage of sporulation was determined by suspending the entire growth from a slant with a measured volume of water. Aliquots of this suspension were taken and slides were prepared by a modified direct microscopic technique. The slide was stained by the method of Huntoon, and the numbers of spores and vegetative cells were determined by microscopic count at a magnification of 950 diameters. Thirty to 50 fields were counted on each slide.

The Huntoon spore stain is described by Katznelson (1948) as follows: Make stain by mixing: 4 grams of acid fuchsin in 50 ml of 2 per cent aqueous acetic acid; and 2 grams of methylene blue in 50 ml of 2 per cent aqueous acetic acid. Cover the film with stain for 1 to 3 minutes. Wash in water (film is now a

bright red). Decolorize in a dilute solution of sodium carbonate until film turns blue, usually 4 to 9 seconds. (The dilute solution is made by adding 7 or 8 drops of a saturated  $\text{Na}_2\text{CO}_3$  solution to 225 ml of water.) Rinse immediately in water and examine. The spores are a purplish-red, the vegetative cells a light blue.

*Effect of pollen on sporulation.* A 2 per cent pollen medium was prepared, inoculated with the 3 strains of the organism, and incubated at 37 C. Spore counts were made at several intervals after inoculation, as shown in table 1. A marked and regular increased sporulation was noted with all 3 strains. The percentage of spores in the absence of pollen ranged from 0.4 to 16.8; in the presence of 2 per cent pollen the percentage of cells present as spores ranged from 17.2 to 83.9.

TABLE 1  
*Effect of pollen on sporulation of Bacillus larvae*

MEDIUM.....	I			II		
STRAIN.....	A	B	G	A	B	G
Incubation time	Percentage of sporulation					
days						
3	2.7	3.5	0.4	26.3	23.3	17.2
5	6.1	7.1	2.3	41.1	39.1	40.9
8	10.3	16.8	4.2	51.4	50.3	56.1
11	5.3	8.2	2.8	57.9	59.9	62.5
14	9.3	8.1	5.6	66.5	68.8	83.9

I. Basal carrot medium.

II. Basal carrot-pollen (2%) medium.

Various amounts of pollen were added to the basal carrot medium in order to determine the most favorable pollen concentration for sporulation. These media were inoculated with the 3 strains and incubated at 37 C. Figure 1 shows the average percentage of sporulation under these conditions for the 3 strains. A medium containing 2 per cent pollen is superior to the other concentrations employed. A marked inhibitory effect of 4 per cent pollen on sporulation is shown in these data.

Preliminary observations indicate that pollen has a marked effect on the growth of *B. larvae*. The entire growth on media each containing 0, 1, 2, 3, or 4 per cent pollen was suspended in 5 ml solution, and the amount of growth was determined by measuring the turbidity of the suspensions with a photometer. After a 5-day growth period the suspension of bacterial cells grown on media containing each of the foregoing concentrations of pollen gave the following readings in percentage of light transmitted: 50, 43, 47, 71, and 95 per cent, respectively. The marked inhibitory effect on the growth of *B. larvae* at pollen concentrations of about 3 per cent is apparent.

*Fractionation of pollen.* The sporeforming factor in pollen has been shown to be soluble in water. To test further the solubility behavior of the factor an aqueous extract of pollen was subjected to continuous extraction with ether for a period of 22 hours in a Soxhlet type extractor. The residual aqueous portion was freed of ether by heating to 56 C for 1 hour. The ether portion was evaporated to dryness and the residue dissolved in warm water. The resulting solution was a pale yellow color with a pH of 6.5. The two portions were then in-

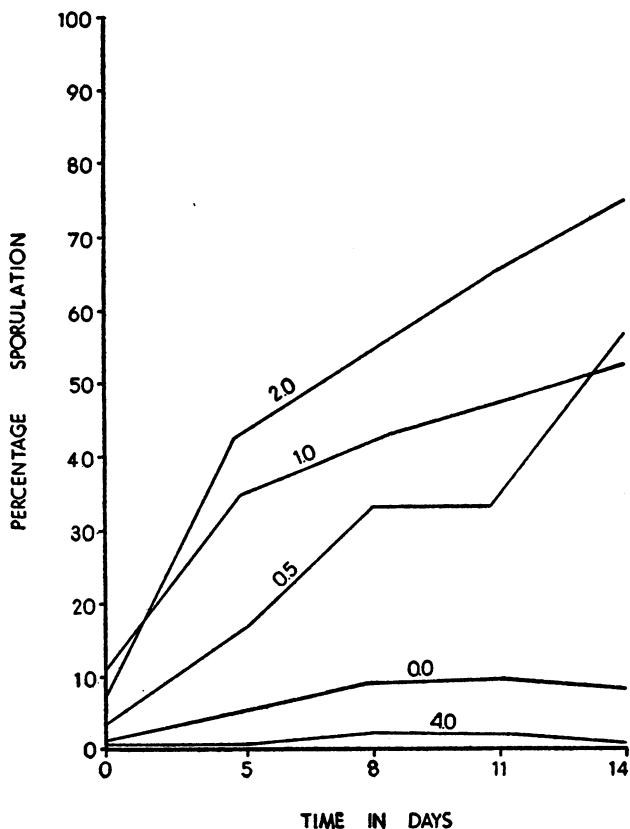


Figure 1. The effect of pollen concentration on endospore production. Figures on curves indicate percentage of pollen.

corporated into media at concentrations equivalent to 2 per cent pollen. Table 2 shows the percentage of sporulation of cells of strain A on these media. Similar results were obtained with other strains. The medium prepared from the ether extract is seen to favor spore production; that prepared from the residual water extract is not favorable for sporulation. No cause is apparent for the abnormally low spore production on the latter medium.

The high solubility of the active fraction in ether is indicated by the following: The ether fraction and the residual water extract were mixed, shaken, and allowed to stand for 1 hour. The solutions were separated and each was tested

for the presence of the sporulation-inducing factor. The aqueous portion showed only 0.3 per cent sporulation in 4 days, but the ether portion showed 10.3 per cent in the same period of time as compared with 8.6 per cent found with the original aqueous pollen extract. The active sporulation-inducing factor of pollen, therefore, is highly soluble in ether and may easily be separated from an aqueous extract of pollen by simple ether partition.

## DISCUSSION

The present investigation has shown that some factor present in aqueous and ether extracts of ground pollen is capable of very significantly increasing the percentage of spores produced by *Bacillus larvae* on an artificial medium. The fact that several lots of pollen from central Pennsylvania were successfully employed in the experiments indicates that the active factor is not limited to a

TABLE 2  
*Solubility of active fraction of pollen*

MEDIUM.....	I	II	III
Incubation time	Percentage of sporulation		
<i>days</i>			
4	5.1	0.1	17.9
8	17.3	0.1	32.4
12	26.4	0.1	30.7

- I. Basal pollen medium. Control not ether-extracted.  
 II. Basal pollen medium. Portion of pollen preferably soluble in water.  
 III. Basal pollen medium. Portion of pollen preferably soluble in ether.

particular flora. The factor likewise seems rather stable, since its activity can be demonstrated after heating at 120 C for 15 minutes.

The inhibitory action of large concentrations of pollen on vegetative growth confirms an earlier report by Lochhead (1926). The failure of earlier workers to observe the stimulatory effect of pollen on spore formation by *B. larvae* may be due to the fact that they might have used such large concentrations of pollen that a very slight growth resulted. It is worthy of note that although heavy inocula are needed to initiate growth on the basal pollen-carrot medium, substantially higher vegetative growth is obtained on such medium than on the reference medium of Holst and Sturtevant (1940).

This sporulation-promoting power in pollen may indicate that some nutrilitic not present in previously used media is required for sporulation in addition to those nutrients needed for the vegetative growth of this organism. The question can also be raised as to whether a specific factor is essential for sporulation in other bacilli. In particular, *Bacillus popilliae* (Dutky) like *B. larvae* has to date failed to sporulate on artificial media although it readily forms spores in its host, the Japanese beetle (Dutky, 1947). Further work on the isolation of the sporulation-promoting factor of pollen is in progress.

## SUMMARY

Sporulation of *Bacillus larvae* on a medium of peptone, yeast extract, dipotassium phosphate, carrot extract, and pollen extract is reported. The increase in sporulation due to pollen is 10 to 50 per cent, and is greatest with a concentration of 2 per cent pollen.

The fraction of the pollen responsible for sporulation is soluble in ether in preference to water.

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