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Studies on the Role of Microorganisms in Alimentation of *Galleria mellonella* Larvae

Badania na rolą drobnoustrojów w odżywianiu gąsienic mola woskowego

Исследование роли микроорганизмов в кормлении гусениц пчелиной моли

The data on the role of intestine flora in wax-moth alimentation are very scarce. The problem of microorganism participation in wax digesting could be solved by means of sterile cultures of insects (8, 9) and by the isolation of wax decomposing microorganisms from the alimentary tracts of the larvae (1, 2, 8). The results of these studies, however, were different. Rybicki (8) reports a very weak growth and development of insects in sterile conditions, while Waterhouse (9) did not observe any disturbances in insect development on an artificial diet with an addition of beeswax or compounds included in its composition.

Microorganisms from the alimentary tract of the larvae of *Galleria mellonella* growing on wax have not been identified. Wax decomposition was established on the basis of an increase of medium acidity or changes in the consistence and quantity of wax added to the medium (1, 2, 8). The study should throw some light on the problem of interrelationships between the insect and bacteria.

MATERIALS AND METHODS

Sterile cultures of insects were used in the studies. They were obtained by placing imagines (from 4 to 6) in Petri dishes with pieces of folded sterile waxed paper. Petri dishes were kept at room temperature or at 30°C. Every 24 hours the dishes were checked for the presence of eggs. The eggs were sterilized with a 10 per cent solution of formalin and then rinsed six times with sterile distilled water, then with a scalpel the eggs were transferred to sterile plastic bowls. Every bowl contained a strictly determined number of eggs. The bowls with eggs were then placed in jars containing sterile wax. The wax was sterilized in an autoclave under a pressure of 0.5 atm. for 45 min. Sterility was checked by pouring nutrient broth into every fifth jar of the series.

The jars were kept in a thermostat at 30° C and 75—80 per cent humidity. After four days of incubation, the larvae hatched, quickly left the bowls, and moved to the wax. The number of larvae hatched was determined on the basis

of empty egg shells counted with the aid of a magnifying glass, without opening the jars.

In sterile jars without wax, the sterility of larvae was checked, 24 hours after hatching, by grinding them onto the plates with nutrient agar.

The growth of the insects was observed until maturity. The number of pupae and imagines was recorded.

The sterility of the larvae, pupae, and adults was checked during the experiments by taking out from every jar first one larva, then one pupa, and then one imago, every week. Inoculations were performed, as described previously, both from the surface and from the alimentary tract. The experiments were carried out in three series. Every series included 5 jars.

Analogical experiments were conducted with sterile larvae on wax infected previously with a suspension of *Streptococcus faecalis* which is a bacterium which constantly accompanies these insects.

Table 1. Development of *Galleria mellonella* in sterile conditions

Medium	Percentage of	
	larvae transformed into pupae	pupae transformed into imagines
Beeswax	91.46	87.01
Beeswax + vitamins	73.11	86.76
Beeswax + vitamins + casein	67.01	92.30
Beeswax + casein	75.00	91.30
Beeswax + *Streptococcus faccalis*	77.33	93.10
Control:		
Non-sterile beeswax + sterile eggs	73.61	88.67
Sterile beeswax + non-sterile eggs	61.32	81.54
Non-sterile beeswax + non-sterile eggs	90.19	96.19

Composition of vitamins in μg/g of beeswax (9): thiamine 25, riboflavine 25, nicotinic acid 100, pyridoxine 25, folic acid 5, mesoinositol 50, biotin 1, B_{12} 1.

Comparisons were made between the growth of sterile cultures on wax alone, on wax with an addition of B group vitamins, of vitamins and casein, of casein and the culture whose only microorganism was *Streptococcus faecalis*.

Examinations were also made in order to find out if in the microflora of *Galleria mellonella* there are wax or palmitic acid-decomposing bacteria. Bacterial strains isolated from the wax-moth were inoculated on a mineral medium (3, 4) and solidified with wax agar or palmitic acid agar. Control was provided by the same medium without wax or palmitic acid. From among the strains able to grow on this medium we selected 5 strains belonging to various systematic groups, and checked their growth on a liquid mineral medium with an addition of glucose and wax or palmitic acid, or of wax and palmitic acid only. Media with an addition of glucose and without any addition constituted controls.

Emulsion of wax or palmitic acid was obtained by soniphication of mineral medium with wax or palmitic acid sterilized in an autoclave.

The cultures were grown on a shaker at 37°C. Bacterial growth was checked every 24 hours by inoculating plates with nutrient agar.

Chemically pure palmitic acid and yellow wax produced by the Gur Company were employed. Since the composition of wax was not determined, its fatty acid content was assessed by gas chromatography.

RESULTS

The function of microorganisms in wax-moth nutrition was examined in sterile cultures. The results of the experiments are presented in Table 1. The percentage of larvae undergoing complete transformation does not show any significant differences in all the modifications of the experiments.

It was noticed, however, that in the first period of life the larvae grew more slowly in sterile conditions. Control larvae obtained the length of 1 cm in the first week and they were easy to count. Sterile larvae, on the other hand, obtained that body length only after 12—14 days. The delay in the first period of growth did not affect further development.

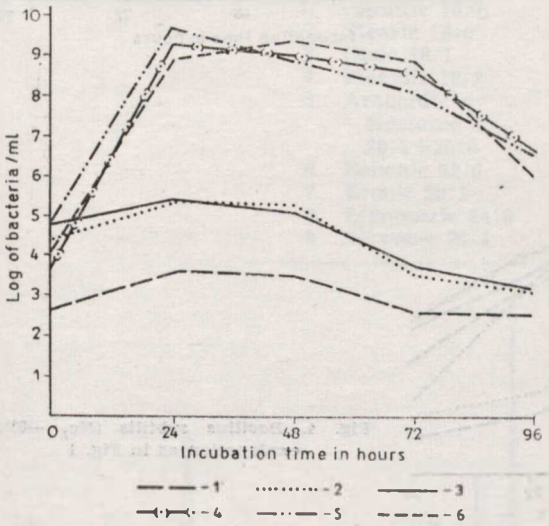

Fig. 1. *Streptococcus faecalis* (No. 9); 1 — mineral medium, 2 — mineral medium + palmitic acid, 3 — mineral medium + beeswax, 4 — mineral medium + glucose, 5 — mineral medium + glucose + beeswax, 6 — mineral medium + glucose + palmitic acid

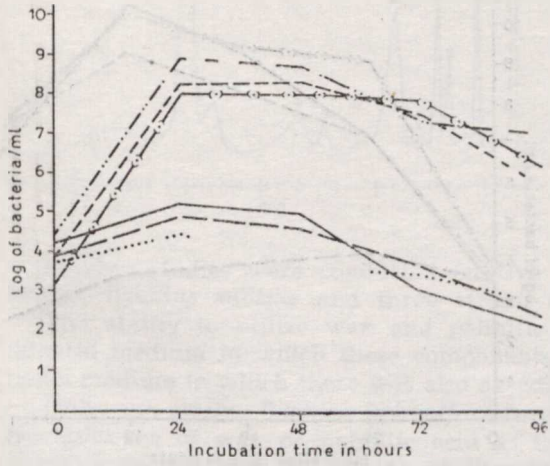

Fig. 2. *Streptococcus faecalis* (No. —238); explanation as in Fig. 1

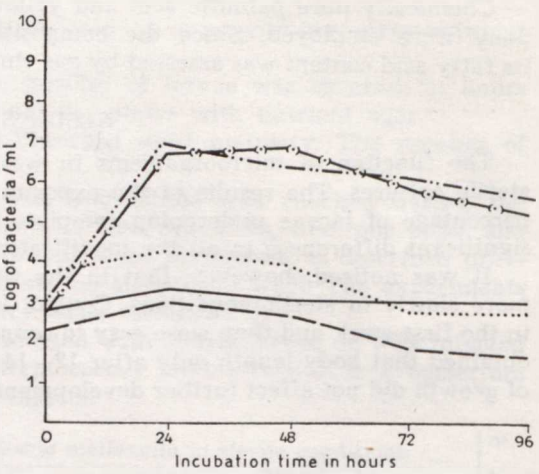

Fig. 3. *Streptococcus faecalis* (No. —205); explanation as in Fig. 1

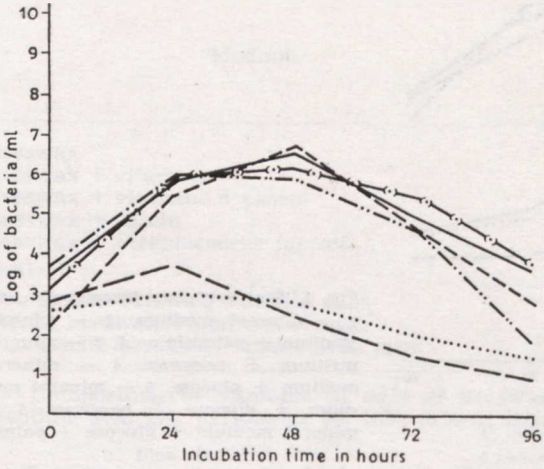

Fig. 4. *Bacillus subtilis* (No. —53); explanation as in Fig. 1

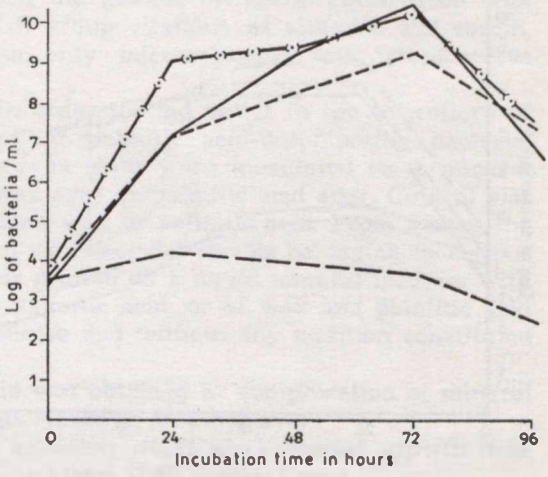

Fig. 5. *Bacillus licheniformis* (No. —4); explanation as in Fig. 1

Full development of insects from the moment of hatching till the appearance of imagines lasted from 43 to 54 days, with the larval period 26—35 days, and the pupal period, 9—10 days. We also observed egg-laying by adults and hatching of the second generation larvae.

Initial experiments on the ability to decompose wax and palmitic acid of bacterial strains occurring in the alimentary tract of larvae were carried out on a mineral medium solidified with agar enriched by these compounds. The ability to grow on this medium was shown by 31 strains belonging to bacilli and catalase-negative cocci.

Table 2. Fatty acid contents in beeswax (in %%)

No.	Name of acid	Content in %
1.	Palmitic 16:0	14.4
2.	Stearic 18:0	2.2
3.	Oleic 18:1	3.4
4.	Eleaidic 18:2	0.2
5.	Arachidic + eicosenic 20:1+20:0	4.2
6.	Behenic 22:0	0.9
7.	Erucic 22:1	2.8
8.	Lignoceric 24:0	1.4
9	Nervonic 24:1	8.0

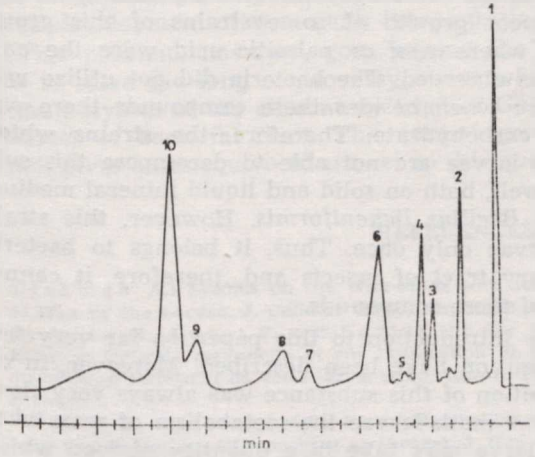

Fig. 6. Chromatogram of fatty acids in beeswax from the GUR Co.; 1 — solvent (13% BF_3 in methanol), 2 — palmitic acid, 3 — stearic acid, 4 — oleic acid, 5 — oleidic acid, 6 — arachidic + eicosenic acids, 7 — behenic acid, 8 — erucic acid, 9 — lignoceric acid, 10 — nervonic acid

Further studies were conducted on five selected strains *Bacillus licheniformis*, *Bacillus subtilis* and three strains of *Streptococcus faecalis*.

The ability to utilize wax and palmitic acid was examined on a liquid mineral medium in which these components were the sole source of carbon, and a medium in which there was also an addition of glucose.

Only one strain, *Bacillus licheniformis*, showed the ability to multiply in the presence of wax or palmitic acid as the only sources of carbon, while *Bacillus subtilis* and the strains of *Streptococcus faecalis* did not grow on

this medium. Growth on the medium which, besides wax or palmitic acid, contained glucose proved utilization of sugar only (Figs. 1, 2, 3, 4, 5).

The yellow beeswax of the GUR Co., used in the experiments, was assessed for the contents of fatty acids. Results obtained are shown in Fig. 6 and Table 2.

DISCUSSION OF RESULTS

The question of the participation of microflora in the process of digesting wax has givn rise to many controversies. Rybicki did not succeed in obtaining a regular growth of the wax-moth in axenic conditions while the bacterial strains isolated from the alimentary tract changed, though very slowly, the consistency of beeswax. Waterhouse (9), on the other hand, obtained a normally growing bacteria-free culture of *Galleria mellonella* on his artificial medium and showed that under such conditions the larvae digested wax compounds as well as in the presence of microflora. Those wax compounds which were not metabolized in sterile conditions were not utilized with the participation of bacteria, either.

In the present paper an attempt was made to solve the problem of the participation of microflora in the nutrition of wax-moth larvae both by observing the growth of sterile cultures and by looking for wax-decomposing microorganisms in intestinal flora.

If digesting of wax takes place with the participation of bacterial flora, then these microorganisms which have this ability should constantly accompany the larvae. In the initial experiments with agar mineral medium with wax or palmitic acid, we obtained poor growth of some strains of this group. However, on a liquid medium, where wax or palmitic acid were the only sources of carbon, no growth was observed. The bacteria did not utilize wax or palmitic acid even in the case when besides these compounds there was glucose, an easily metabolized carbohydrate. Therefore, the strains which constantly accompany wax-moth larvae are not able to decompose this substance. The only strain growing well, both on solid and liquid mineral medium with wax or palmitic acid, was *Bacillus licheniformis*. However, this strain was isolated from wax-moth larvae only once. Thus, it belongs to bacteria rarely occurring in the alimentary tract of insects and, therefore, it cannot play any role in the metabolism of these compounds.

As has been mentioned in the introduction to this paper, so far very few bacteria capable of wax decomposition have been described. Moreover, in all the cases described, the decomposition of this substance was always very slow. But in the alimentary tract of wax-moth larvae the metabolism of wax takes place very quickly. In a day a larva may take in a quantity of food which exceeds its body weight, of which a half is metabolized, the other half being excreted (5).

It seems more likely that bacterial flora utilizes indirect products of the transformation of lipid compounds. It is well known that streptococci, especially in an environment with low oxygen content, may utilize carbohydrates, multi-hydroxide alcohols and organic acids.

There have been several suggestions concerning the participation of bacteria in transformation of phosphate compounds in the organisms of both large and small wax-moths. It was reported (5) that both in the bodies and excreta

of *Galleria mellonella* and *Achroea grisella* there were large quantities of polyphosphates. Appearance of these compounds is connected with the intake of food containing no polyphosphates, which seems to support the opinion that they are formed during the process of digestion. Since the presence of these compounds is characteristic only of these two wax-eating insects, one may suppose that there is a connection between wax digesting and polyphosphate formation and that bacteria participate in this process (5, 6).

Cytological studies on the occurrence of enzymes and metabolites participating in the process of food transformation showed that they are localized almost exclusively in the medial intestine (7). However, no group of bacteria occurring in the alimentary tract of *Galleria mellonella* shows any specific localization in the intestine.

The growth of insects in sterile conditions shows indirectly that bacteria do not play any important role in the alimentation of the wax-moth.

The full cycle of insect development in axenic conditions is not disturbed. The insects pass through the same stages as controls. Lack of flora is not reflected in fertility, either.

The only disturbance observed in growth was a delay in the first period of life of the larvae, made up for in the further period of this stage. The phenomenon has not been explained. Observations on bacteria-free cultures concerned only one generation of insects.

Further studies on axenic cultures of moths carried out on several generations should make it possible to find out if the transformation of food takes place in the same way without microorganism participation as they do with microorganism participation. They should also reveal the reasons for the weak development of wax-moth larvae during the first week of life.

This problem, however, goes beyond the limits of this paper whose aim was to solve the long controversy about whether beeswax is digested owing to the enzymes of the alimentary tract of *Galleria mellonella* larvae, or to the enzymes of bacterial flora. The normal growth of sterile cultures of wax-moth gives an unequivocal answer to this question.

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STRESZCZENIE

W kulturach bezbakteryjnych owady rozwijają się normalnie. Pełny cykl rozwojowy przechodzą w takim samym czasie jak i te, których przewód pokarmowy zawiera mikroorganizmy. Jedynie w pierwszym okresie życia larwalnego zaznacza się powolny wzrost gąsienic. Motyle składają jaja, z których wylęga się drugie pokolenie aksenicznych larw.

Trawienie tak niezwykłego pokarmu, jakim jest woszczyna pszczela, zachodzi zatem dzięki enzymom przewodu pokarmowego gąsienic mola woskowego. Potwierdzeniem tego jest fakt, że wosk pszczeli ani jego główny składnik — kwas palmitynowy, nie są substratami rozkładanymi przez drobnoustroje stale towarzyszące owadom. Zdolność wykorzystywania tych związków ma *Bacillus licheniformis*, szczep przypadkowo izolowany z larw *Galleria mellonella*.

РЕЗЮМЕ

В стерильных культурах насекомые развиваются нормально. Время полного цикла развития у них такое же, как у насекомых, в пищеварительном тракте которых находились микроорганизмы. Только в первом периоде личиночной жизни наблюдался медленный рост гусениц. Моль откладывает яйца, из которых вылупливается второе поколение аксенических личинок.

Переваривание так необычного корма, каким является пчелиная вощина, происходит, следовательно, благодаря энзимам пищеварительного тракта гусениц. Подтверждением этого является факт, что ни пчелиный воск, ни его главная составляющая — пальмитиновая кислота — не являются субстратами, которые могли бы разложить микроорганизмы, сопутствующие насекомым. Эти соединения способны использовать *Bacillus licheniformis*, который является штаммом, случайно изолированным из личинок *Galleria mellonella*.