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Microorganisms associated with pollen, honey, and brood provisions in the nest of a stingless bee, *Melipona fasciata* *

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Summary — Spore-forming bacteria belonging to the genus *Bacillus* were isolated from brood cell provisions, honey from honey storage pots, and pollen from pollen storage pots obtained from a nest of a stingless bee, *Melipona fasciata. Bacillus megaterium, B circulans,* and *B alvei* were identified. Few other microorganisms were found. These bacteria produced a variety of enzymes including esterases, lipases, proteases, aminopeptidases, phosphatases, and glycosidases that could convert food into more digestible products for storage. *Bacillus* species could also secrete chemicals such as antibiotics and fatty acids to inhibit competing microorganisms which could cause spoilage of stored food, particularly in tropical environments.

***Melipona fasciata* / *Bacillus* / microbiology / honey / pollen**

INTRODUCTION

Both solitary and social bee species have physical, physiological, behavioral, and chemical adaptations which control spoilage of food stores rich in protein, lipid, and carbohydrate content (Roubik, 1989). These adaptations, in addition to potential mutualistic relationships with microbes and other organisms, may be particularly important in perennial bee species that rely on stored food in tropical environments.

Meliponinae are eusocial, stingless bees native to the tropical forest. Colonies of *Melipona fasciata panamica* Cockerell in Panamá contain 200–1000 adult bees and brood populations 5 times greater than the number of adults (Roubik, 1983). Nests are located in tree cavities and consist of an inner grouping of comb cells for brood and large, ellipsoidal pots for separate storage of honey and pollen. The bees use secreted wax mixed with plant resins (cerumen) to construct the storage pots, and the resin may serve as a biocidal agent (Michener, 1974; Roubik, 1983). The nest entrance is a hole which is large enough for only 1 bee. Plant resins, other exudates, secreted wax, and mud used for nest construction, as well as the nest site, protect the nest from water and predators.

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Nursing workers of stingless bees provision brood cells with larval food (pollen and honey mixed with glandular secretions) prior to oviposition by the queen. Each larva is mass-provisioned with the total amount of food required for adult development. Since the cell is sealed after oviposition, the larva feeds in a closed environment.

We have examined the stored food of various temperate-zone and tropical solitary and social species of Apoidea for microorganisms in an effort to not only determine microbial species which can survive in these acidic niches but also to obtain some indication as to whether bees use microorganisms to pre-digest, convert, ferment, and/or preserve stored food. Pollen stored by honey bees, *Apis mellifera,* is called bee bread and differs biochemically and microbiologically from floral and corbicular pollen of the same plant species (Gilliam, 1979a, b; Loper *et al,* 1980; Standifer *et al,* 1980; Gilliam *et al,* 1989). Yeasts (Gilliam, 1979a), spore-forming bacteria belonging to the genus *Bacillus* (Gilliam, 1979b), and molds (Gilliam *et al,* 1989) may play a role in the conversion of pollen to bee bread.

Less diverse microbiological associations have been found with other bees. Machado (1971) reported an association between the pollen stores of *M quadrifasciata* and a *Bacillus* similar to *B pumilus.* The *Bacillus* appeared to predigest the pollen, and elimination of the bacterium with an antibiotic led to colony death. The *Bacillus* appeared in large numbers in the glandular secretion deposited on the pollen and honey layers in the cells and in pollen; it was found in smaller numbers in honey. At least 1 species of *Bacillus* was found in the larval food of 13 stingless bee species. Gilliam *et al* (1984, 1985) examined larval provisions of *Anthophora* sp, *Centris pallida,* and an obligate necrophage of the genus *Trigona* and found that all contained 1–5 species of the genus *Bacillus* but no other microorganisms. The same species of *Bacillus (B pumilus, B megaterium, B subtilis, B circulans, and B licheniformis)* were found in the entirely glandular cell provisions made by the *Trigona* in tropical wet forest and in pollen collected and stored as bee bread by *A mellifera* in the Arizona desert. Larval provisions of solitary, soil-nesting bees contained only *B circulans* in the case of *Anthophora,* but provisions of *Centris* yielded this species as well as *B coagulans, B firmus,* and *B megaterium.*

The *Bacillus* species that Gilliam *et al* (1984, 1985) isolated from provisions of bees grew in acidic conditions and at high osmotic pressure and produced numerous enzymes involved in protein, lipid, and carbohydrate metabolism. *Bacillus* species are widely recognized for their ability to secrete a number of extracellular enzymes in large quantities at the same time. They are also producers of antibiotics and fatty acids. Thus, they could potentially participate both in the metabolic conversion of food for later use by the bees and in the control of competing spoilage microbes.

In this paper, we report the results of microbiological examinations of pollen, honey, and brood provisions from *M fasciata* which were conducted to determine additional associations of *Bacillus* species with bees.

MATERIALS AND METHODS

In July, a nest of *M fasciata panamica* was removed from a tree trunk which had been transported to Panamá City from 50 km NE in Colón Province along the El Llano-Cartí Road. It was freeze-dried for shipment by air to Tucson where it was immediately stored at – 20 °C until samples were taken for microbiological analyses. Brood cells and storage pots from which

material was to be collected were first swabbed with sterile distilled water to remove any visible debris and then surface-sterilized twice with 70% ethanol. Cappings were removed with sterile surgical instruments taking care to avoid contamination of the pot and cell contents with any capping material. Because the honey was too viscous to be removed with a hypodermic needle and syringe, it was mixed with a sterile spatula before direct plating with an inoculating loop. Pollen from individual storage pots was removed with a sterile spatula, and the content of each pot was placed in a separate sterile bottle and mixed. Approximately half of the content of each bottle was individually homogenized in 1 ml of sterile distilled water in a sterile glass tissue grinder and then plated. After removal of the egg or larva, brood provisions from individual comb cells were removed with a sterile spatula, homogenized individually in 1 ml of sterile distilled water, and plated.

Honey from 8 honey storage pots; pollen from 11 pollen storage pots; and brood provisions from 17 comb cells, from each of which a single egg or small larva had been removed, were plated and analyzed for bacteria, yeasts, and molds by procedures previously described (Gilliam *et al,* 1985) except that blood agar was omitted from the isolation media. Thus, nutrient agar, TYG (tryptic soy agar with glucose and yeast extract), Czapek solution agar, YM-1 agar, and thioglycollate medium incubated at 25 °C and 37 °C were the isolation media. Thioglycollate medium was used to test for the presence of microaerophilic and anaerobic organisms, and any growth in these tubes was transferred to nutrient agar and TYG plates. When growth on these media was poor, plates of brain heart infusion agar were also inoculated. Plates were incubated aerobically, under 5% Co_2, and anaerobically to determine whether the organisms were facultative, microaerophilic, or anaerobic. Isolates were maintained, tested, identified, and then assessed for production of 19 enzymes with the API ZYM system (Analylab Products) as previously described (Gilliam *et al,* 1985).

RESULTS

Table I shows the results of microbiological isolations from honey, pollen, and brood provisions. Microbes were found in 71% (n = 17) of brood provision, 50% (n = 8) of honey, and 27% (n = 11) of pollen samples. *Bacillus* species were the only microorganisms found other than a mold from honey from 1 storage pot and Gram-variable, pleomorphic, rod-shaped, anaerobic bacteria from 1 pollen sample and from 2 samples of brood provisions. The latter organisms formed clear colonies on agar media, but growth was poor on all media except thioglycollate.

Bacillus species were isolated from 59% of brood provision, 50% of honey, and 18% of pollen samples. The most common species isolated from all 3 sample types was *B megaterium. Bacillus circulans* and *B alvei* were the only other *Bacillus* species present and were isolated from a few honey and brood provision samples but not from pollen. Overall, 45 isolates of *B megaterium,* 6 of *B circulans,* and 3 of *B alvei* were obtained. Since our intent was to determine the number of samples of each type that contained various microorganisms, no attempt was made to quantify the number of microorganisms per unit volume or weight. However, it should be noted that in order to facilitate plating of the samples, pollen and brood provisions were homogenized in water, but honey was not. This procedure should not affect determination of the presence or absence of microorganisms in material from a cell or storage pot because of the number of plates and tubes of media inoculated. However, it may have allowed isolation of more organisms per sample from honey than from either pollen or brood provisions.

Results of enzymes produced by *Bacillus* species from stored food of *M fasciata* are presented in table II. *Bacillus* species from pollen produced 12 of the 19 enzymes for which tests were conducted, those from brood provisions produced 14, and those from honey produced 15. All

Table I. Microorganisms in stored food from a nest of *Melipona fasciata*.

Sample	*Microorganism*	*Number of isolations*
Brood provision–1	none	
Brood provision–2	none	
Brood provision–3	*B alvei*	1
Brood provision–4	*B circulans*	4
	B megaterium	4
Brood provision–5	*B megaterium*	3
Brood provision–6	*B megaterium*	2
Brood provision–7	*B megaterium*	5
Brood provision–8	*B alvei*	1
	B megaterium	1
Brood provision–9	none	
Brood provision–10	none	
Brood provision–11	none	
Brood provision–12	unidentified Gram-variable anaerobic bacterium	1
Brood provision–13	*B megaterium*	7
Brood provision–14	unidentified Gram-variable anaerobic bacterium	1
Brood provision–15	*B megaterium*	1
Brood provision–16	*B megaterium*	3
Brood provision–17	*B megaterium*	1
Honey–1	*B megaterium*	5
	B circulans	2
Honey–2	*B megaterium*	3
	unidentified gray mold	2
Honey–3	none	
Honey–4	*B alvei*	1
Honey–5	none	
Honey–6	*B megaterium*	2
Honey–7	none	
Honey–8	none	
Pollen–1	none	
Pollen–2	none	
Pollen–3	*B megaterium*	4
Pollen–4	none	
Pollen–5	unidentified Gram-variable anaerobic bacterium	2
Pollen–6	none	
Pollen–7	none	
Pollen–8	none	
Pollen–9	*B megaterium*	4
Pollen–10	none	
Pollen–11	none	

Table II. Enzymes produced by *Bacillus* species * isolated from stored food of *Melipona fasciata*. * *B alvei, B circulans,* and *B megaterium* were isolated from brood provisions and honey; *B megaterium* was isolated from pollen. ** V = variable.

Enzyme	*Brood provisions*	*Honey*	*Pollen*
Alkaline phosphatase	V**	+	V
Butyrate esterase	V	+	V
Caprylate esterase-lipase	+	+	+
Myristate lipase	V	V	V
Leucine aminopeptidase	V	+	+
Valine aminopeptidase	V	V	V
Cystine aminopeptidase	–	V	–
Trypsin	–	V	–
Chymotrypsin	V	+	+
Acid phosphatase	V	+	+
Phosphoamidase	V	+	+
α-Galactosidase	V	V	V
β-Galactosidase	V	V	V
β-Glucuronidase	–	–	–
α-Glucosidase	V	V	+
β-Glucosidase	V	V	–
N-Acetyl-β-glucosaminidase	–	–	–
α-Mannosidase	–	–	–
α-Fucosidase	V	–	–

strains tested from the 3 sources produced caprylate esterase-lipase; none produced β-glucuronidase, N-acetyl-β-glucosaminidase, or α-mannosidase. Other commonly produced enzymes were alkaline phosphatase, butyrate esterase, leucine aminopeptidase, chymotrypsin, acid phosphatase, phosphoamidase, and α-glucosidase. *Bacillus megaterium* strains were the most active producers of enzymes, followed by *B alvei,* and then *B circulans.* Enzymes produced in the highest concentrations (≥ 30 nanomoles) were leucine aminopeptidase and chymotrypsin by at least some strains of species isolated from all 3 sources.

Results from taxonomic tests also yielded biochemical data. All isolates produced catalase and fermented glucose with acid production, 88% fermented trehalose with acid production, 88% hydrolyzed casein, 76% liquefied gelatin, 72% digested litmus milk with most of them producing lipases that catabolized the fats in milk, 76% hydrolyzed tyrosine, 60% reduced nitrates to nitrites, 76% produced amylase, and 80% grew in 5% NaCl and at pH 5.7. Thus, proteolytic, glycolytic, and lipolytic enzymes were produced, and most isolates grew at low pH and in high osmotic pressure which may reflect the environment from which they were isolated.

DISCUSSION

These results confirm the association of *Bacillus* species with stored food of bees. However, not all samples of honey and pollen from storage pots and of brood provisions from comb cells contained *Bacillus* species. This could be related to storage time of the materials in pots and cells or to the particular worker bees that participate in pollen-processing and brood-cell provisioning. Although the consistency of pollen from different storage pots ranged from moist to almost dry, no pattern of microbial growth appeared to be associated with this. If *M fasciata* does utilize *Bacillus* species to convert, ferment, enhance, and/or preserve food, this process may occur during collection of nectar and pollen, as storage pots are filled, before brood cells are sealed, and during the 5–10 d delay before eggs hatch. Since a higher percentage of provisioned cells (71%) than of pollen (27%) or honey (50%) stored in pots contained microbes, it would be of value to examine the microbial flora of the intestines of larvae and adult bees, the glandular secretion of adults, empty pots and cells, and the contents of pots and cells as they are being filled.

It is of interest to note that *B megaterium* and *B circulans,* species isolated in this study, were also found in larval provisions of the necrophage of the genus *Trigona* and of *C pallida* and in pollen collected and stored by *A mellifera*; only *B circulans* was found in larval provisions of *Anthophora* sp (Gilliam *et al,* 1984). To our knowledge, the isolation of *B alvei* from honey and brood provisions of *M fasciata* is a new record. *Bacillus alvei* is also associated with healthy *A mellifera* colonies (Gilliam and Morton, 1978; Gilliam, 1985) and is a secondary organism associated with European foulbrood disease of honey bees.

Of special importance is the relative absence of microorganisms other than *Bacillus* species in the stored food of *M fasciata.* This indicates that some mechanism exists to retard microbial growth which could cause spoilage, particularly in tropical environments. Our results demonstrate that the *Bacillus* species isolated are metabolically active and produce numerous enzymes. Thus, these bacteria could produce chemicals such as fatty acids and antibiotics that inhibit competing organisms (fungi and other bacteria) as well as enzymes that convert food into more digestible products for storage. High acidity and osmotic pressure of the stored food may also play a role in limiting spoilage organisms, particularly other bacteria.

Results of API ZYM tests showed that all *Bacillus* species isolated from pollen, honey, and brood provisions produced caprylate esterase-lipase which degrades lipids of intermediate chain length (8 carbons). All *Bacillus* species from honey also produced butyrate esterase which cleaves short-chain fatty acids at ester linkages, but this property was variable with strains from brood provisions and pollen. However, fewer isolates from all 3 sources produced myristate lipase which cleaves longer-chain fatty acids from lipids. Thus, the major lipolytic activity of the isolates was against hydrocarbons of intermediate chain length based on results with the substrates available for testing. High lipolytic activity was also observed in the conversion of litmus milk to a yellow transparent fluid by lipases of the *Bacillus* species which catabolized milk fats.

Extensive phosphatase activity, both acid and alkaline, was produced by most of the isolates. These enzymes are involved in fat and/or phosphate absorption.

Enzymes involved in protein catabolism were produced by the isolates. Leucine aminopeptidase was the most abundant

aminopeptidase followed by valine aminopeptidase. These enzymes liberate amino acids from proteins and polypeptides. Chymotrypsin was the most abundant protease, and proteolytic activity was also demonstrated by hydrolysis of casein and liquefaction of gelatin by most isolates. Almost all isolates also produced phosphoamidase which acts on phosphoamides.

α-Glucosidase was the most commonly produced glycosidase. It hydrolyzes carbohydrate substrates such as sucrose, maltose, trehalose, and melezitose. Fewer isolates produced galactosidases. α-Galactosidase hydrolyzes melibiose and raffinose, and β-galactosidase hydrolyzes lactose. In taxonomic tests, most isolates fermented glucose and trehalose and hydrolyzed starch.

Delage-Darchen and Darchen (1982) used the API ZYM system to test various glands and the midgut of a *Melipona* species, *M beecheii,* for 19 enzymes and found all but α-galactosidase and β-glucuronidase present in some samples. Later, these authors (Delage-Darchen and Darchen, 1984) compared results of enzymatic activity of glands and midguts from 4 species of stingless bees and from *A mellifera* and concluded that differences existed between species, but all lacked β-glucuronidase as did the *Bacillus* species that we isolated from food of *M fasciata.* Thus, determinations of microbes associated with various bee species and their food, enzyme production by the microorganisms, and enzymology of bee digestive systems are required to assess adaptations of particular bee species and their associated microbial flora to the environment.

We are continuing microbiological examinations of provisions of additional bee species to investigate further the association of bees and *Bacillus* species. Such a survey is necessary before evolutionary and ecological associations are made clear. It will also be useful for detailed studies to ascertain whether female bees of some species inoculate food sources with microbes that play a role in the metabolic conversion, enrichment, and/or preservation of food.

Résumé — Microorganismes associés au pollen, au miel et aux provisions pour le couvain, dans le nid de *Melipona fasciata.* Continuant à mettre en évidence l'association des abeilles et des bactéries sporulantes et Gram-positives appartenant au genre *Bacillus*, nous avons étudié du point de vue microbiologique la nourriture stockée dans le nid de *Melipona fasciata panamica.* Nous avons prélevé du miel dans 8 pots à miel, du pollen dans 11 pots à pollen et des provisions pour couvain dans 17 cellules et étalé ces produits sur des milieux de culture spécifiques afin de caractériser les bactéries, les levures et les champignons. Des espèces de *Bacillus* ont été isolées dans 59%, 50% et 18%, respectivement, des échantillons de provisions pour le couvain, de miel et de pollen. L'espèce isolée la plus commune dans les 3 types d'échantillons est *B megaterium. Bacillus circulans* et *B alvei* ont été isolés dans quelques échantillons de miel et de provisions pour le couvain, mais pas dans le pollen. Quelques autres microbes ont été trouvés (tableau I).

Des tests biochimiques de taxonomie et des tests avec le système API ZYM sur 19 enzymes ont montré que les espèces de *Bacillus* isolées produisaient des enzymes protéolytiques, lipolytiques et glycolytiques (tabelau II), qui pouvaient participer à la transformation métabolique de la nourriture en produits de stockage plus digestes. Puisque les espèces de *Bacillus* sont largement connues comme producteurs d'antibiotiques et d'acides gras, elles sont susceptibles de protéger également la

nourriture contre une éventuelle décomposition due à des microorganismes tels que les champignons ou d'autres bactéries.

***Melipona fasciata* / *Bacillus* / microbiologie / miel / pollen**

Zusammenfassung — Mikroorganismen in Pollen, Honig und den Brut-Nahrungsvorräten im Nest einer Stachellosen Biene, *Melipona fasciata.* Im Zuge der fortgesetzten Bemühungen, das Zusammenleben von Bienen und grampositiven, sporenbildenden Bakterien aus der Gattung *Bacillus* nachzuweisen, wurden mikrobiologische Untersuchungen der Futtervorräte im Nest einer Stachellosen Biene, *Mellipona fasciata panamica,* durchgeführt. Es wurden Honig aus 8 Honig-Vorratstöpfen, Pollen aus 11 Pollen-Vorratstöpfen und das Brutfutter aus 17 Wabenzellen auf mikrobiologische Medien ausgebracht, um Bakterien, Hefen und Pilze zu isolieren. *Bacillus*-Arten wurden aus 59% des Brutfutters, 50% des Honigs und 18% der Pollenproben isoliert. Die häufigste Art, vorhanden in allen drei Proben-Typen, war *B megaterium. Bacillus circulana* und *B alvei* wurden aus einigen wenigen Honig- und Brutfutter-Proben, aber nicht aus Pollen isoliert. Es wurden nur wenige andere Mikroben gefunden (Tabelle I).

Biochemische Tests für die Taxonomie und Tests auf 19 Enzyme unter Benutzung des API ZYM-Systems ergaben, daß die isolierten *Bacillus*-Arten proteolytische, lipolytische und glykolytische Enzyme erzeugten (Tabelle II), die zur metabolischen Umwandlung der Nahrung in leichter verdauliche Produkte für die Einlagerung beitragen könnten. Da *Bacillus*-Arten allgemein als Erzeuger von Antibiotika und Fettsäuren bekannt sind, könnten sie die Nahrung auch vor der möglichen Zersetzung durch Mikroorganismen, wie Pilze und andere Bakterien, schützen.

***Melipona fasciata* / *Bacillus* / Mikrobiologie / Honig / Pollen**

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