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MiniReview

Identification and roles of non-pathogenic microflora associated with honey bees

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Abstract

Microorganisms associated with honey bees, *Apis mellifera*, and their food include bacteria (Gram-variable pleomorphic bacteria, *Bacillus* spp., and Enterobacteriaceae), molds (primarily aspergilli and penicillia), and yeasts (mainly *Torulopsis* spp.). Eggs, prepupae, pupae, and worker bees emerging from cells as adults are usually free of internal microbes. Microorganisms acquired by larvae through ingestion of contaminated food are usually eliminated through the single defecation that occurs at the end of the feeding period prior to pupation. Emerging adult bees acquire intestinal microflora by food exchange with other bees in the colony and through consumption of pollen. Biochemical contributions of microorganisms to honey bees; the role of microorganisms in the conversion, enhancement, and preservation of pollen stored as bee bread in comb cells; and the production of antimycotic substances by molds and *Bacillus* spp. from honey bee colonies that are resistant to the fungal disease, chalkbrood, are discussed. An association of *Bacillus* spp. with bees including honey bees, stingless bees, and solitary bees from tropical and temperate zones appears to have evolved in which female bees inoculate food sources with these bacteria whose chemical products contribute to the elaboration and/or protection from spoilage of food that is stored in the nest. This association is ancient based on results from stingless bees preserved in amber for 25–40 million years. It is concluded that bees, their products, and their associated microorganisms are potential sources of bioactive products including antimicrobial compounds.

Keywords: Honey bee; *Bacillus* spp.; Gram-variable pleomorphic bacteria; Mold; Yeast; Pollen

1. Introduction

Honey bees, *Apis mellifera*, are social insects which live in perennial colonies consisting of the egg-laying queen, drones whose only known function is to mate with the queen, and workers that perform various duties during their life. Workers are the most numerous individuals within the colony. Under normal conditions, the younger and intermediate-aged adult workers confine their activities such as cell cleaning, care and feeding of brood, and food storage to the interior of the hive; adults 20 or more days old are foragers. The diet of workers varies with age. The hypopharyngeal glands of young adult worker bees are activated by consumption of pollen to produce brood food (worker jelly and royal jelly). Pollen consumption begins when the adult emerges, reaches a maximum at 5 days, but cintinues until 15–18 days.

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Nectar or honey is the carbohydrate energy source for adult bees and is necessary for wax secretion by workers generally 12–18 days old and for flight by foragers. In contrast, royal jelly is seemingly the primary component of the diet of the queen bee throughout her entire life.

Insect pathology had its beginnings early in the development of beekeeping and the establishment of sericulture when certain abnormalities in honey bees and silkworms were noted by those who reared them for their useful products. Concentrated research efforts have been devoted to diseases of honey bees, but these have not been matched with regard to non-pathogenic microorganisms and their possible roles in the bee colony. This paper summarizes results on the non-pathogenic microflora associated with honey bees and on the contributions of microorganisms to the levels of biochemicals in bees, preservation of food stored in the nest, and resistance to disease. Information gained from these studies may lead to development of technologies that utilize microbes to increase bee productivity.

2. Isolation and identification of microorganisms

Microflora associated with honey bees and their food was determined by examination and subsequent isolation and identification of bacteria, yeasts, and molds from brood, adult worker bees, virgin and mated queen bees, nectar, and pollen. In addition, microbiological studies of food stored in the nests of over 25 species of social and solitary tropical and temperate-zone non-*Apis* bees were conducted. Many selective and specially formulated media were employed. Almost all microbial species associated with bees grew well under aerobic conditions or under 5–10% CO_2. Obligate anaerobes were rare.

Isolates were tested and identified using appropriate tests and taxonomic keys. These included the conventional methods of Gordon et al. [1] for bacteria belonging to the genus *Bacillus*, API 20E kits (Analytab Products) for Enterobacteriaceae, the methods of Wickerham [2] and Lodder [3] for yeasts, and those of Raper and Fennell [4] and Raper and Thom [5] as well as other specialized keys for molds. Databases of some available systems and procedures for identification of species of bacteria and yeasts were inadequate for microorganisms associated with bees and often gave erroneous results. Frequently, more variability existed within various microbial species associated with bees than has been reported for the same species from other sources.

3. Microbiology of brood and adults of worker honey bees and of adult queen bees

Eggs, prepupae, pupae, and worker honey bees emerging as adults from their comb cells are usually free of internal microorganisms [6–8]. Naturally occurring antimicrobial systems abound in bees and their food [9,10] and undoubtedly play a role in maintaining this generally microbe-free environment. However, some larvae acquire microorganisms that are associated with adult bees, pollen, and combs through ingestion of contaminated food [11]. These contaminants are usually eliminated through the single defecation that occurs at the end of the larval feeding period prior to pupation.

Newly emerged adult worker bees are inoculated with microorganisms when they begin to feed. Microbial inoculation and colonization of the gut occur within 4 days after emergence [7] as a result of pollen consumption and through food exchange with older bees in the colony [6,7,12]. Since 82% ($n = 142$) of the nectar samples that we have examined from various species of native and cultivated crop plants from the Sonoran Desert of Arizona contained no microbes, nectar does not appear to have a direct role in inoculation of bees [12,13]. However, the antimicrobial systems of nectar preclude the establishment of invading microorganisms [10].

The intestinal microflora of mature worker bees may vary somewhat with the age of the bee, the season, and geographical location, although some species of microorganisms are found consistently [14–16]. Gram-variable pleomorphic bacteria of uncertain taxonomic status, *Bacillus* spp., Enterobacteriaceae, molds, and yeasts dominate [16]. The body surface of both nurse and foraging adult worker honey bees is relatively free of microorganisms ([17], Gilliam, unpublished). This is probably due in large part to grooming behavior. Generally, less than 20% of bees examined had surface microorganisms. Fungi including molds and yeasts were more

frequently isolated than were bacteria. More foraging bees than nurse bees yielded positive results since foragers have a greater chance of body contamination through activities outside the hive that would also decrease opportunities for in-hive grooming.

3.1. Gram-variable pleomorphic bacteria

Gram-variable pleomorphic bacteria are the most common intestinal microorganisms in adult worker and queen honey bees [16–18]. We have also isolated them from honey bee larvae and their feces [11,17]; floral pollen and corbicular pollen (from pollen baskets on the legs of returning foraging worker honey bees) (Gilliam, unpublished); bee bread (pollen stored in comb cells of the hive) and a few samples of honey from honey bee colonies [17]; frass from larvae of the greater wax moth, *Galleria mellonella*, a destructive pest of honey bee colonies which consumes bee products including larvae and stored pollen [19]; pollen and larval provisions (pollen and honey mixed with glandular secretions) from the nest of *Melipona fasciata*, a stingless bee [20]; and larval provisions of other species of stingless bees from Panama (Gilliam, Roubik, Buchmann and Lorenz, unpublished). These bacteria exhibit extreme variability in reaction to the Gram stain and have both rod and coccoid forms. On the basis of microscopic morphology, they may be mistaken for other forms such as Gram-negative rods, Gram-positive cocci, or yeasts when Gram stains are not performed periodically on growing cultures throughout extended incubation. Some strains and/or species have been referred to as *Bacterium* or *Achromobacter eurydice* [21] and assigned to genera such as *Lactobacillus* [22], *Corynebacterium* [23], and *Bifidobacterium* [24]. Not only is their taxonomic position uncertain, but it is unclear whether all researchers are working with the same bacterial species. Because of difficulties in isolation and maintenance of most of these organisms, it is likely that the most readily isolated and most easily maintained strains have received the greatest attention.

Worker bees ($n = 43$) from free-flying colonies averaged 266 352–496 728 colony-forming units (cfu) of Gram-variable pleomorphic bacteria per intestine [16]. There was no seasonal effect on numbers of Gram-variable pleomorphic bacteria in adult worker honey bees, but a sharp and significant decrease occurred in bees at the age of 6 days which corresponds to the end of the period of maximum pollen consumption. This indicated a possible pollen connection with these organisms. However, less than 26% ($n = 496$) of floral and corbicular pollen and bee bread samples that were examined contained Gram-variable pleomorphic bacteria (Gilliam, unpublished). In contrast, 99% ($n = 100$) of worker bees (Gilliam, unpublished) and 88% ($n = 110$) of queen bees had these intestinal bacteria [18]. Thus, it appears likely that the bacteria are endemic in the alimentary tract of adult bees and are spread from the mouths of adults to larvae and food sources as has been reported for *Ac. eurydice* [22].

When fed to bees, both streptomycin [16] which is active against Gram-negative bacteria and penicillin G (Gilliam, Lorenz and Richardson, unpublished) which affects Gram-positive bacteria decreased the numbers of intestinal Gram-variable pleomorphic bacteria, further evidence that this group is comprised of more than one species.

We have been conducting morphological studies and biochemical tests on representative isolates of these pleomorphic bacteria ([25], Gilliam and Lorenz, unpublished). For isolation and maintenance, over 30 agar-based and liquid media with various concentrations and sources of nitrogen, carbohydrates, and supplements as well as pH, incubation temperatures, and oxygen requirements were tested. TYG agar (40 g Difco tryptic soy agar, 3 g yeast extract, 10 g glucose, and 1 ℓ distilled water) and broth were the best of those tested; however, they were not ideal.

Gram-variable pleomorphic bacteria were isolated on various enriched media incubated aerobically, under 10% CO_2, or anaerobically. Growth appeared as white, cream, tan, or clear pinpoint colonies which had a distinct pleasant ester aroma similar to that produced by many yeasts. The white colonies even had the typical appearance of yeast colonies.

Representative strains were subjected to numerous microbiological tests (Gilliam and Lorenz, unpublished) in an effort to determine differences and species. Media had to be modified, devised, and tested for each biochemical test required for taxonomic purposes. Results revealed that all of the strains may be facultative anaerobes. Most strains produced

catalase and reduced nitrates to nitrites. All strains produced acid from D-fructose; most produced acid from D-glucose, L-arabinose, and D-xylose. From these and other results, it was concluded that the Gram-variable pleomorphic bacteria associated with honey bees are comprised of perhaps as many as six species. Two of the isolates appeared to be members of the genus *Lactobacillus*, and another has been tentatively identified as *Bifidobacterium* sp., but a more accurate generic allocation requires 16S RNA sequence analysis.

These microorganisms appeared to contribute useful metabolites such as intestinal enzymes to honey bees [16]. However, better systems must be devised to produce sufficient in vitro growth and thus adequate amounts of biochemicals for testing to determine fully the contributions of these organisms to bees.

3.2. Enterobacteriaceae

Gram-negative bacterial rods belonging to the Enterobacteriaceae commonly found in honey bees included *Enterobacter cloacae, E. aerogenes*, and *Klebsiella pneumoniae* [15,16,26]. In Arizona, intestines of worker honey bees from free-flying colonies contained the greatest number of species and the highest mean counts (31 524 cfu; $n = 21$) during August through early November [16,26]. Enterobacteriaceae were most often found in the guts of worker bees at least 14 days old (Gilliam, unpublished). In December, 75% ($n = 40$) of foraging bees had intestinal Enterobacteriaceae [15]. Gram-negative bacterial rods were rarely associated with brood [11] but were found in intestines of queen bees [18].

3.3. Bacillus spp.

Spore-forming bacterial rods belonging to the genus *Bacillus* are common associates of bees. In a study of intestinal *Bacillus* spp. in 388 adult worker honey bees, 110 isolates belonging to 13 species were identified [27]. *Bacillus megaterium, B. subtilis, B. pumilus, B. licheniformis, B. circulans*, and *Paenibacillus alvei* were the predominant species. Bees ($n = 43$) from free-flying colonies contained an average of 190 cfu of *Bacillus* spp. per intestine in the spring (March–April) and 9638 cfu in the fall (September) [16].

Bacillus spp. were the predominant microorganisms in feces of worker larvae [11]. From feces of 20 larvae, 44 isolates of *Bacillus* spp. were obtained. Seventeen of these were *B. megaterium*, and 19 were *B. subtilis*. *Bacillus cereus* and *B. megaterium* were the most common *Bacillus* spp. in the intestines of queen bees [18].

3.4. Molds

The most frequently found molds in the alimentary canal of worker honey bees belonged to the genera *Penicillium* and *Aspergillus*. Commonly identified species included *P. frequentans*, *P. cyclopium*, *A. flavus*, and *A. niger* [17,28–30]. Other molds that were often associated with intestines of workers bees were *Cladosporium cladosporioides* and *Alternaria tenuissima*. Not all bees contained molds, and the numbers of those that did varied from colony to colony. Reasons for these differences appear to be related to season, and as discussed in Section 5, to disease resistance.

In December, 100% ($n = 40$) of the foraging workers contained intestinal molds [28]; in March through May, only 20% ($n = 45$) of adult worker bees had intestinal molds [30]; and in another study, no molds were detected in bees ($n = 22$) in March–April, but means of 190 cfu per bee intestine ($n = 21$) were obtained in September [16]. Thus, molds were more prevalent in worker bees in the fall and winter months.

However, feces from 50% of worker larvae contained molds in May, but only 25% had molds in September ($n = 20$) [11]. Most of the molds from larval feces were penicillia. Molds occurred less frequently than Gram-variable pleomorphic bacteria or *Bacillus* spp., a situation similar to that in intestines of adult worker bees [16]. Only 5% ($n = 110$) of queen bees contained molds [31]. Most species associated with larval feces and queens were also found in worker bees.

3.5. Yeasts

Intestinal yeasts were most frequently encountered in worker bees from colonies that were caged, diseased, fed deficient diets, fed antibiotics, or exposed to pesticides [16,30,32,33]. Thus, they appeared to be indicators of stress conditions in honey bees in Arizona. They were also more prevalent in bees in the

spring. In one study, no intestinal yeasts were isolated from bees ($n = 21$) from free-flying colonies in the fall, but mean counts of 2454 cfu ($n = 22$) per bee were obtained in the spring [16]. However, intestinal yeasts were isolated from bees from caged colonies in both seasons ($\bar{x} = 44$ cfu per bee, $n = 135$ in the fall; $\bar{x} = 3528$ cfu, $n = 165$ in the spring). When caged bees were fed streptomycin, the numbers of intestinal yeasts increased ($\bar{x} = 712$ cfu, $n = 135$ in the fall; $\bar{x} = 16\,924$, $n = 143$ in the spring). Species most frequently isolated were *Torulopsis magnoliae, T. glabrata, Candida parapsilosis*, and *Hansenula anomola* [30,33]. Yeasts were rare in healthy brood [8,11], and only one of 110 queen bees contained a yeast [31].

3.6. Microflora of honey bees from feral colonies

Honey bees from feral colonies located in rock caves, overhangs, or holes in central Arizona contained the same kinds of intestinal microorganisms as honey bees from managed colonies housed in wooden hives in southern Arizona [34]. This finding demonstrated the constancy of the microflora in honey bees from both feral and managed colonies and from areas separated by approximately 289 km.

4. Microbiology of food sources of honey bees

The antimicrobial systems that have evolved to protect the food of honey bees from growth or establishment of most invading microorganisms were reviewed by Burgett [10]. Acidity, high osmotic pressure, and hydrogen peroxide are the major systems in nectar and honey. Glucose oxidase from the hypopharyngeal glands of worker bees and from bacteria of the genus *Gluconobacter* is responsible for gluconic acid, the major acid in honey, and for the liberation and accumulation of hydrogen peroxide. These components as well as others would also contribute to the antimicrobial properties of brood food produced by the hypopharyngeal glands. Thus, these food sources are less interesting than pollen from the standpoint of microbial diversity.

Honey bees obtain proteins, amino acids, lipids, carbohydrates, vitamins, and minerals from pollen. Foraging worker bees collect floral pollen that they transport to the colony in corbiculae on the legs. This pollen is then packed into cells of the brood comb by other, generally younger, bees; a small cover of honey is deposited on the pollen to prevent spoilage when the pollen is not being consumed rapidly. This store of pollen, which has undergone chemical changes, is called bee bread. Bee bread is consumed by adult bees and is fed to larvae.

Our studies of floral and corbicular pollen and of bee bread stored over time in comb cells of the hive, all from the same plant species, demonstrated that pollen from a flower changes microbiologically and biochemically as soon as a honey bee collects it [35–39]. Bees moisten pollen with regurgitated nectar or honey to facilitate packing into the corbiculae, add glandular secretions, and inoculate it with microbes.

Fungi (molds and yeasts) and *Bacillus* spp. were the predominant microbes in pollen and bee bread. Of the total microbial isolates ($n = 391$) from pollen and bee bread, 55% of the pollen and 85% of the bee bread isolates were fungi (Gilliam, unpublished). It appeared that honey bees engaged in 'microbial farming' by inoculating pollen with specific microorganisms as they collected and packed it for transport to the colony. Examples of the microbes that were introduced by the bees were the yeast *T. magnoliae*, bacteria belonging to the genus *Bacillus*, and the molds *Aureobasidium pullulans, P. corylophilum, P. crustosum*, and *Rhizopus nigricans*. Most of the organisms isolated from corbicular pollen and bee bread were also associated with honey bee colonies, particularly in the guts of adult worker honey bees [11,17,19,35–37].

A microbial succession occurred due to addition by bees of microbes which replaced many of those associated with floral pollen. Bacteria including Gram-positive cocci, coryneforms, and Gram-negative rods comprised 49% of the microflora of floral pollen, decreased to 28% in corbicular pollen, and were rare (4%) in bee bread (Gilliam, unpublished). The only *Bacillus* species that was associated with floral pollen was *B. subtilis* which increased in corbicular pollen and in bee bread stored in comb cells for 1 week [36]. In addition to *B. subtilis*, corbicular pollen yielded *B. circulans*, *B. licheniformis*, *B. megaterium*, *B. pumilus*, and atypical *B. subtilis* strains. *Bacillus* spp. comprised only 2% of the microbes in floral pollen, increased to 20% in corbicular

pollen, and then remained at 11% in bee bread (Gilliam, unpublished).

The majority of molds were penicillia, Mucorales, and aspergilli [37]. Floral pollen had the largest number of mold isolates but the fewest species. Floral pollen, corbicular pollen, and bee bread stored over time in comb cells differed in the predominant molds present (*Mucor* sp. in floral pollen, penicillia in corbicular pollen and in bee bread stored for 1 week, aspergilli and penicillia in bee bread stored for 3 weeks, and aspergilli in bee bread stored for 6 weeks).

Floral pollen also had the largest number of different yeast species which decreased in corbicular pollen and bee bread [35]. The predominant species were *Cryptococcus albidus, Kloeckera apiculata*, and *Candida guilliermondii* var. *guilliermondii* in floral pollen; *C.g.* var. *guilliermondii* and *T. magnoliae* in corbicular pollen; and *T. magnoliae* in bee bread.

5. Roles of microorganisms in the honey bee colony

Efforts continue to assess the roles of non-pathogenic microorganisms in the honey bee colony. The areas of interest include biochemical contributions of intestinal microorganisms to honey bees, conversion and preservation of pollen stored in comb cells as bee bread, and disease resistance.

5.1. Biochemical contributions of microorganisms to honey bees

These studies have involved two approaches. First, various antibiotics were fed to bees to eliminate specific microorganisms or groups of microorganisms from adult worker bee guts. The guts were then analyzed over the life of the bees for microbial content and various biochemicals including enzymes, amino acids, aminosugars, carbohydrates, lipids, riboflavin, and fecal purines. A non-pollen protein source was fed to bees as a control for assessing pollen contributions. In the second approach, intestinal microorganisms were analyzed for secondary metabolites including enzymes and antimicrobial substances.

Results revealed that intestinal biochemicals originate from the bees themselves, from pollen, and from microorganisms. For example, in addition to the concentrations of various enzymes produced by the bee, some trypsin, chymotrypsin, myristate lipase, acid phosphatase, and cystine aminopeptidase appeared to originate from pollen while levels of alkaline phosphatase, α-glucosidase, and β-glucosidase were increased by the presence of Enterobacteriaceae and Gram-variable pleomorphic bacteria in the bee [16]. Levels of total amino acids and most individual amino acids in bee guts were also increased by pollen and by microorganisms (Gilliam, McCaughey, Lorenz and Richardson, unpublished).

Results from the second approach demonstrated that *Bacillus* spp. from honey bees produce antimicrobial substances and numerous enzymes (Gilliam, unpublished). Also, some mold species from honey bees produced more antimicrobial substances in higher levels than the same species from other sources such as soil and food (Frisvad and Gilliam, unpublished; Gilliam, unpublished).

5.2. Microbial contributions to conversion and preservation of pollen

The conversion of pollen to bee bread and the accompanying biochemical changes have been postulated to result from microbial action, principally a lactic acid fermentation caused by bacteria and yeasts [40]. However, Pain and Maugenet [41] sterilized pollen with γ-irradiation, then seeded it with *Lactobacillus*, and determined that a pure lactic acid fermentation produced an unappetizing product of poor nutritive value for bees. They thought that yeasts played the most important role from a nutritional standpoint.

Examples of biochemical changes that we found during the conversion of pollen to bee bread were the addition of lipids to floral pollen by bees and/or microbes, a 115-fold increase in titratable acidity for free organic acids in corbicular pollen compared to floral pollen indicating active fermentation, and the superiority of the nutritive value and availability of amino acids in the protein of bee bread compared to corbicular pollen ([38,39], Standifer, McCaughey, Dixon, Gilliam and Loper, unpublished).

The fermentation and chemical changes of pollen stored in comb cells by honey bees may be processes similar to those that occur in green plant food materials that are ensiled and in foods of plant origin

that are fermented to prolong shelf-life and to improve palatability, digestibility, and nutritional value. Indeed, there are analogies in the microbiology and biochemistry of these processes and the production of bee bread. These include microbial succession; fermentation by fungi; the increased availability of amino acids; the enhancement of stability of silage by *Bacillus* spp., yeasts, and molds; and the production of organic acids to exert a preservative effect [35–37,39,42,43].

The microorganisms from pollen and bee bread were metabolically active and could produce compounds such as enzymes, vitamins, antimicrobial substances, organic acids, and lipids that contribute to the conversion of pollen and the stabilization of bee bread [35–37]. For example, the molds produced enzymes involved in protein, lipid, and carbohydrate metabolism [37]; 80% of the yeasts from bee bread fermented glucose and sucrose [35]; and *Bacillus* spp. from pollen and bee bread produced proteolytic enzymes and carbohydrases [36]. Also, aspergilli, penicillia, and Mucorales are utilized industrially for antibiotic, organic acid, enzyme, and lipid production; yeasts are sources of vitamins and enzymes; and *Bacillus* spp. are exploited for their ability to synthesize and secrete prodigious quantities of enzymes and are recognized for their production of antibiotics and fatty acids.

5.3. Disease resistance

Chalkbrood is a fungal disease caused by the heterothallic ascomycete, *Ascosphaera apis*. It results in mummified larvae. Our efforts to develop control methods for chalkbrood are based on determination of the mechanisms that enable bee colonies to cope with and survive the disease, particularly those colonies that do not show clinical symptoms even when the pathogen is present in high concentration. Genetically determined hygienic behavior (uncapping of cells and removal of diseased and dead larvae) by nurse worker bees was found to be the primary mechanism of resistance or tolerance to chalkbrood [17,44]. Thus, bees can be selected and bred for resistance which is evidenced by elevated hygienic behavior; by decreased longevity of *A. apis* spores; and by reduced pathogen contamination of bees, brood, and stored food in the colony. A secondary mechanism of resistance is the addition during pollen collection and storage by bees of antagonistic molds and *Bacillus* spp. that inhibit the pathogen [17,45]. Bee colonies that are resistant or tolerant have more of these antagonists. Antimycotic substances active against *A. apis* were not produced by bees, larvae, bee bread, or honey [17]. However, bee bread and the guts of worker bees, the major sources of the pathogen [44,45], were the primary sources of the antagonistic microorganisms [17,45]. Thus, the antimycotic substances were produced by microorganisms that originated in worker bee intestines. These microorganisms were added to pollen by the bees.

Most of the antagonists were molds [17]. Species belonging to the Mucorales including *Mucor spinosus, Rhizopus arrhizus*, and *Rhizopus* sp. as well as aspergilli including *Asp. tamarii* produced the largest zones of inhibition against *A. apis* and are being tested as control agents by feeding them in pollen patties to bee colonies that have been selected and bred for susceptibility to chalkbrood and then inoculated with the chalkbrood pathogen ([18,45], Gilliam and Taber, unpublished). Twenty-seven strains of antagonistic endospore-forming bacteria were isolated and identified as belonging to six species (*Paenibacillus alvei, B. circulans, B. licheniformis, B. megaterium, B. pumilus*, and *B. subtilis*) [45]. They will be assessed for control potential.

5.4. Microorganisms associated with modern and ancient non-Apis bees

To extend knowledge on the association of microbes with bees, we isolated and identified microorganisms from the stored food of more than 25 species of eusocial stingless bees and solitary bees from tropical and temperate zones ([20,46–48], Gilliam, Rubik, Buchmann and Lorenz, unpublished). Results revealed *Bacillus* spp., exclusively or predominantly, or no microbes in the stored food of almost all bees examined. There were similarities in the species of *Bacillus* associated with food of different origins in the nests of diverse bee species from different geographical areas. This is particularly well demonstrated by comparison of results from a stingless bee, *Trigona hypogea* (= *necrophaga*), from Panama with those from honey bees from Arizona [36,48]. This *Trigona* is an obligate necrophage for which dead

animal tissue has replaced pollen as the sole protein source, and glandular provisions have replaced stored pollen. Worker bees of this species consume muscle and other tissue from vertebrate carcasses but do not carry unmodified pieces of animal flesh to the nest. Instead, the consumed flesh activates the hypopharyngeal glands to produce brood food by a mechanism analogous to that of nurse bees of *A. mellifera* which consume pollen to produce brood food via the hypopharyngeal glands. Five species of *Bacillus* (*B. circulans, B. licheniformis, B. megaterium, B. pumilus*, and *B. subtilis*) were the only microbes found in the glandular cell provisions produced by this *Trigona* in the tropical, wet forest. These are the same species found in almond pollen collected and stored as bee bread by honey bees in the Arizona desert.

Thus, we hypothesized that an association between *Bacillus* spp. and some bees may have evolved in which female bees inoculate food sources with these bacteria whose chemical products contribute to the pre-digestion, conversion, enhancement, and/or preservation of food that is stored in the nest [20,36,46–48]. Evidence supporting this view came from several studies. Analyses of the *Bacillus* spp. isolated from the stored food of various bee species showed that they produced a variety of extracellular enzymes including aminopeptidases, amylases, esterases, glycosidases, lipases, phosphatases, and proteases that were potential contributors [20,46–48]. Also, an association between the pollen stores of a stingless bee, *Melipona quadrifasciata*, and a *Bacillus* similar to *B. pumilus* was reported in which the *Bacillus* appeared to pre-digest the pollen, and elimination of the bacterium with an antibiotic led to destruction of comb cells by worker bees and eventual death of the colony [49]. The *Bacillus* was found in large numbers in pollen and in the glandular secretion that the bees deposited on honey and pollen layers in the cells. Furthermore, at least one species of *Bacillus* was present in the larval food of 13 species of stingless bees. *Bacillus* spp. were also isolated from the abdominal tissues of several additional species of tropical stingless bees [50] and were shown to be common associates of larvae, adults, and food (larval provisions and their components of pollen and nectar) of alfalfa leafcutting bees, *Megachile rotundata*, in Canada and may well be part of the resident microflora of the alimentary canal of this bee [51].

Mechanisms for preservation and for protection of food stores and provisions from microorganisms that could cause spoilage are necessary for survival of bees. This is particularly important in perennial colonial stingless bees that rely on food stores in humid, tropical environments and in soil-dwelling bees whose pollen stores are susceptible to microbial attack [46,47]. One of these mechanisms might be the inhibition of potential spoilage microorganisms by secondary metabolites such as fatty acids and antimicrobial compounds that are produced by *Bacillus* spp. *Bacillus* spp. appear to be resistant to antimicrobial compounds associated with bees such as those found in glandular secretions and nectar [46].

Evidence that the bee-*Bacillus* relationship is ancient came from the report by Cano et al. [50] of the isolation, amplification, and sequencing of segments of *Bacillus* spp. DNA from abdominal tissue of four specimens of *Proplebeia dominicana*, an extinct species of stingless bee found in 25–40 million year old amber from the Dominican Republic. The DNA sequences were related to each other and to those of *B. circulans, B. firmus, B. pumilus*, and *B. subtilis*. These are species associated with extant bees, including stingless bees.

Further credence was given to the hypothesis by the subsequent report of the isolation of live *B. sphaericus* from abdominal tissue of *P. dominicana* entombed in Dominican amber [52]. Microscopy had demonstrated the presence of *Bacillus* spp. within the bees. It was noted that two other ancient *Bacillus* spp. were recovered and identified from Dominican amber and that more than 100 other bacterial isolates from ambers of various geological ages had been recovered but not evaluated. It would be interesting to know whether these additional isolates formed spores.

These reports generated excitement, publicity, and skepticism. Questions arose regarding possible contamination, and doubts were expressed about the claims that DNA and bacterial spores can survive for millions of years. However, as pointed out by Cano [53] and Postgate and Priest [54], the authors were aware of the contamination risk and thus were fastidious in their methods and controls to avoid contamination and to demonstrate that the *B. sphaericus* strain came from inside the bee. The more relevant question may concern identification

of the physical and chemical properties of amber that allow preservation of DNA and bacteria beyond theoretical limits. Cano [53] lists dehydration, high osmolality, oxygen-free environments, protection from UV radiation, and stable ambient temperatures as factors which make amber a suitable source of ancient DNA (and, one might presume, ancient bacterial spores). The last two factors result from the underground location of the Dominican amber mines. Rapid dehydration and anoxia would halt the in situ reproduction of *B. sphaericus*. In this case, the spores would be as old as the bee [54]. If, however, the spores are not as old as the bee, the most likely scenario is that the race of bacteria entombed within the bee was sustained by extraneous organic nutrient from the amber itself or from an outside source by entry through sub-microscopic fissures or faults in the amber structure [54].

6. Conclusions

Over 6000 microbial strains associated with bees and their food have been isolated and identified. More than 1000 are preserved by lyophilization for continuing studies of microbial contributions. Our aim has been to understand the relationships of bees and microbes in order to capitalize on this knowledge to increase bee productivity for pollination purposes. However, microorganisms associated with bees are novel sources of bioactive compounds that may have uses beyond the field of apiculture. For example, increasing problems of resistance of human pathogens to available antimicrobial compounds necessitate searches for new products. Augmentation of screening programs to include microbial taxa that are not the usual actinomycetes and molds from soils by inclusion of microbes from novel sources such as insects and analyses of components of insects themselves as well as their products might yield useful compounds. The honey bee colony is a promising subject for such studies.

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