An approach to the characterization of the honey bee hive bacterial flora

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SUMMARY

In this study we describe an approach to the characterization of the cultivable heterotrophic bacterial flora of honey bee hives. We assessed the bacterial abundance in brood combs and on hive floors at different times of the year, and identified several isolated bacteria. The number of bacteria recovered from brood combs was notably lower than the number obtained from hive floors. In addition, we observed an increase in bacteria recovered from brood comb samples from winter to summer, probably reflecting the activity of the colony during these seasons. In floor samples bacterial counts were not different among the different sampling times. Most of the identified isolates belonged to the genera Bacillus and Corynebacterium. Spores of Paenibacillus larvae subspecies larvae were only found in samples taken during summer of 2002, in accordance with an increase of American foulbrood incidence in Uruguay in the last year. The presence of several micro-organisms such as those identified as belonging to the genus Bacillus, potentially related to those that have biological control capabilities, and the differences between brood combs and floor-recovered bacteria encourage further research on the microbiology of honey bee hives and new strategies of pathogens control.

Keywords: bacteria, honey bees, hives, Bacillus, brood comb, bee hive floor

INTRODUCTION

Apicultural economic development strongly relies on the health status of honey bee colonies. Several bacterial diseases that affect honey bees have been described, including American foulbrood (AFB) and European foulbrood, caused by Paenibacillus larvae subspecies larvae (Heyndrickx et al., 1996) and Melissococcus plutonius (Allen & Ball, 1993), respectively. The presence of P. l. larvae in Uruguay has been reported already in larvae, honey and adult bees (Piccini & Zunino, 2001), but so far, studies related to the bacterial flora present in honey bee hives are lacking. It has been reported that the microbes most frequently found in honeycombs and adult bees belong to the Bacillus genus (Snowdon & Cliver, 1996). It is also well known that several soil bacteria, in particular Bacillus spp., produce antimicrobial substances (Kim et al., 1997). Thus, information about the interactions that occur between different bacterial species inside the hive and the dynamics of the bacterial community could be important in order to develop new approaches for disease control, avoiding the use of commercial antimicrobial substances such as antibiotics. In order to gain insight into the microbial ecology of honey bees, we determined the number of total viable aerobic and microaerophilic bacteria from two different parts of the hive at different seasons, and we also identified a number of frequently found bacterial isolates. In the present study we chose the brood combs and the floor of the hives as the sampling sites. The work was done in this way in order to assess differences between the place where the population resides and great activity takes place (brood combs), and the place where the colony wastes accumulate (floor).

MATERIALS AND METHODS

Samples

Samples were obtained from an experimental apiary, containing 12 beehives, that belongs to the National Institute for Agricultural Research (INIA), close to the city of Colonia, Uruguay. Samples were collected from the brood combs and from the floor of the hive during November (spring) of 2000 (S1), July (winter) of 2001 (S2) and January (summer) of 2002 (S3). Pieces of brood combs (0.9 g average weight ± 0.36 s.d.) were collected from each hive by random sampling and stored at –20 °C until processed. In 24 brood combs samples out of 36 (66.6%) there was presence of honey or pollen, and in eight samples out of 36 (22.2%) there was presence of larvae. To obtain floor samples, floor material (0.12 g average weight ± 0.045 s.d.) was scraped with an alcohol-disinfected spatula and the collected samples were stored at –20 °C until processed.

Bacterial enumeration

Comb samples were divided into small fragments using sterile scalpel blades and weighed. Then, 5 ml of sterile distilled water (SDW) were added and the mix was vigorously vortexed for 3 min. One 100 µl aliquots of the resultant suspension were spread in five replicates onto nutrient agar (NA) plates (Difco). When bacterial growth was confluent and plate counting was not possible, we performed serial decimal dilutions of the original sample in SDW and aliquots of each dilution were spread onto NA plates. Plates were incubated at 37 °C for 48 h and colony-forming units (cfu) were counted. This procedure was performed at least three times for each sample. Since we wanted to assess exclusively bacterial growth, we added 30 µg/ml of nystatin (Uru-farma) to the medium in order to avoid the growth of yeasts. The inhibitory concentration of nystatin for yeasts in this kind of sample was previously determined using different concentrations of the product for the same kind of samples (data not shown).

In order to detect the presence of P. l. larvae, the remaining suspension was heated at 80 °C for 15 min and aliquots of 100 µl were inoculated onto J agar (Hornitzky & Nicholls, 1993) supplemented with 9 µg/ml of naldixic acid to avoid the growth of Paenibacillus alvei (Sigma) (J/Nal) (Alippi, 1995). Plates were
incubated under microaerophilic conditions (5 to 10% CO2) at 37 °C for 72 h to detect *P. l. larvae* growth. The colonies were also examined for detection of AFB clinical symptoms. Floor samples were weighed, suspended in 1 ml of SDW and vortexed for 3 min. Serial dilutions in SDW of the resultant suspension were done and aliquots of each dilution were spread on NA in five replicas. Plates were incubated at 37 °C until growth was observed and cfu were counted. The remaining suspension was subjected to the same heating treatment and culture conditions already described for comb samples. After plate counting, the number of cfu per gram of sample (cfu/g) was calculated.

**Isolate identification**

Different isolates were observed to be pure prior to identification and were selected according to the following phenotypic traits: colony morphology, cellular morphology and Gram staining and catalase reaction. Those phenotypes that were more frequently isolated were identified using the BBL-CRYSTAL GP kit for Gram-positive bacteria (Becton Dickinson) and BBL-Enterotube II kit for Enterobacteriaceae (Becton Dickinson). When identification was not possible using the kits we performed additional standard biochemical or bacteriological tests, including oxidase activity, glucose oxidation-fermentation, urea hydrolysis, motility at 36 °C (Gerhardt et al., 1994), and growth on Rogosa selective medium (de Waard et al., 2002). *P. l. larvae* was identified by colony shape and margins, microscopic examination of Gram-stained smears and the next biochemical test: catalase production, Voges-Proskauer reaction, gelatine liquefaction (12%), indol production, starch hydrolysis, manitol usage and growth on nutritive broth (Alippi, 1992).

**Statistical analysis**

Mann-Whitney one-tailed non-parametric analysis was used to compare cfu/g from brood combs and hive floors among different seasons. Observed differences were considered significant at *P* ≤ 0.05.

**RESULTS**

**Bacterial enumeration**

When the numbers of cfu/g were analysed, we found that bacterial numbers in brood combs samples were notably lower than those observed in floor samples, showing ranges of approximately 10^1 to 10^5 in brood combs and from 10^3 to 10^7 in floor samples (fig. 1).

A significant increase in cfu/g of brood comb samples was observed from winter (S2) to summer (S3) (*P* = 0.0001) (fig. 1A). Although we did not find a significant variation between brood comb bacterial counts from spring (S1) to winter (S2) (*P* = 0.068), a decrease in cfu/g could be observed in nine out of 12
There are several studies about bacteria present in different organs of honey bees and bee larvae (Snowdon & Cliver, 1996; Jayaprakash et al., 2003). In this study, the cultivatable heterotrophic bacterial flora sampled from two different places in the beehive, brood comb and hive floor, was assessed. We observed that the number of bacteria recovered from the material located on hive floors was markedly higher than the number of bacteria present in brood comb samples. As it has been described, fatty acids present in pollen, especially linoleic, linolenic, myristic and lauric acids, have inhibitory properties against several micro-organisms including P. l. larvae and B. cereus (Manning, 2000). In addition, Gilliam (1979) found that Bacillus subtilis, which produces antibiotics that are active against Gram-positive bacteria, was the most common bacterial species trapped in almond pollen. Therefore, our findings could be explained by the presence of antimicrobial substances in pollen and honey (Tichy & Novak, 2000), which were in high amounts (almost in 70% of the samples) in brood combs. A further possibility that cannot be ruled out is that hygienic behaviour of adult bees may also be also responsible for the lower number of bacteria detected in brood combs (Spivak & Gilliam, 1998). We also found a decrease in the number of total bacteria obtained in floor samples taken during winter that could be related to the decline of honey bee populations in this season. Besides, bacterial numbers increased significantly from winter to summer in brood comb samples. This could be related to bee population recovery during the warmer seasons.

It is important to consider that in a preliminary assay we found that the use of other culture media (Luria-Bertani and Brain-Heart Infusion agar) did not significantly change the recovery of bacteria (data not shown). We also verified that bacterial growth was not affected by the chosen concentration of nystatin used to avoid the presence of yeasts.

Although storage at –20 °C could result in loss of some of the less robust organisms, we did not notice variation in the number of total recovered bacteria when control samples were frozen and thawed twice (data not shown).

**Isolate identification**

Forty-four different isolates were selected according to their different phenotypic traits and were identified using the commercial kit or complemented with standard tests. Most of the identified isolates (14 out of 44) belonged to the Bacillus genus (table 1), but we also detected bacteria belonging to the genera Brevibacillus, Corynebacterium, Lactobacillus, Paenibacillus, Proteus and Leifsonia (Evtushenko et al., 2000).

After cultures of heated combs and floor samples on J/Nal, we isolated P. l. larvae from seven out of 12 colonies from S3 (fig. 1). P. l. larvae was not recovered from any sample corresponding to S1 or S2. Among the P. l. larvae positive samples, five were from brood combs and three were from floor samples. No AFB symptoms were seen in any of the 12 colonies at any time.

**DISCUSSION**

There are several studies about bacteria present in different parts of honey bees and bee larvae (Snowdon & Cliver, 1996; Jayaprakash et al., 2003). In this study, the cultivatable heterotrophic bacterial flora sampled from two different places in the beehive, brood comb and hive floor, was assessed. We observed that the number of bacteria recovered from the material located on hive floors was markedly higher than the number of bacteria present in brood comb samples. As it has been described, fatty acids present in pollen, especially linoleic, linolenic, myristic and lauric acids, have inhibitory properties against several micro-organisms including P. l. larvae and B. cereus (Manning, 2001). In addition, Gilliam (1979) found that Bacillus subtilis, which produces antibiotics that are active against Gram-positive bacteria, was the most common bacterial species trapped in almond pollen. Therefore, our findings could be explained by the presence of antimicrobial substances in pollen and honey (Tichy & Novak, 2000), which were in high amounts (almost in 70% of the samples) in brood combs. A further possibility that cannot be ruled out is that hygienic behaviour of adult bees may also be also responsible for the lower number of bacteria detected in brood combs (Spivak & Gilliam, 1998). We also found a decrease in the number of total bacteria obtained in floor samples taken during winter that could be related to the decline of honey bee populations in this season. Besides, bacterial numbers increased significantly from winter to summer in brood comb samples. This could be related to bee population recovery during the warmer seasons.

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**Identification of isolates using commercial kits** was not completely effective, probably because these kinds of kits are designed for clinical strains. For this reason, some isolates could be identified only to genus level.

The prevalence of Bacillus species among the identified isolates is in agreement with previous reports, where it is described that bacteria isolated from different organs of the queen honey bee are mostly B. megaterium, B. cereus, B. subtilis and B. brevis (Gilliam, 1978). Furthermore, we also found P. alvei, a common inhabitant of the beehive (Djordjevic et al., 2000), Proteus, a genus previously
associated with honey bees (Snowdon & Cliver, 1996) that includes an insect pathogen species (P. nyxofaciens, Rozalsky et al., 1996), and members of the Enterobacteriaceae family belonging to the Enteric Group 60. Enteric Groups are defined as groups containing biochemically similar strains whose classification needs further study. Particularly, Enteric Group 60 was first thought to be inactive strains of Morganella. This hypothesis was ruled out and there is no clue to its correct taxonomic position (Farmer et al., 1985).

The environment that surrounds honey bee hives could provide important sources of bacteria, especially those commonly found in soil. In the case of honey, the primary sources of microbial contamination include pollen, dust, air, soil and the digestive tract of honey bees (Snowdon & Cliver, 1996). This may explain the presence of Corynebacterium pseudotuberculosis, the causative organism of caseous lymphadenitis in sheep and goats (Connor et al., 2000) that was isolated in this study. Its presence in the bee hive samples could be explained by the fact that the apiary is located in a farming area. Thus, the source of this organism was probably the surrounding vegetation or water resources, being carried inside the hive by foraging honey bees.

The genus Lactobacillus comprises the largest group of rod-shaped organisms within the lactic acid bacteria. These bacteria are widespread in nature and are frequently found in association with the intestinal tracts and mucous membranes of a wide range of animal species (Lawson et al., 1994). Lactobacillus and related bacilli have been previously been isolated in A. m. scutellata and A. m. scutellata and even from other honey bee subspecies (Jayaprakash et al., 2003). Lactobacillus spp. are being intensively studied for probiotic usage. The frequent presence of native Lactobacillus strains associated with honey bees confirmed in this study could encourage research related to the use of probiotics or related products for apiculture.

Along with bacteria from environmental sources, bacterial pathogens can also be found inside the hive. In Uruguay, P. larvae has been detected in both larvae and adult bee samples (Piccini & Zunino, 2001) and even in the honey (Antúnez et al., 2004). In this study, P. larvae spores were detected in S3 samples, corresponding to the summer of 2002. This finding correlates with an observed increase of AFB incidence in Uruguay during the last year, particularly in the region where the sampled hives were located.

Knowledge about the microbial community of the bee hive can be important for the development of biological control strategies based on the use of native bacterial strains. The high incidence of Bacillus species observed among our isolates is an interesting finding that could be taken in account in order to reach this objective. A preliminary survey using different Bacillus spp. strains from this study showed that some of them have inhibitory activity against P. alvei (data not shown). Moreover, it could be important not only to study in more detail the relationship among the different bacterial species, but also the response of the bacterial community to different environmental factors.

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