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ORIGINAL RESEARCH ARTICLE

The lactic acid bacteria involved in the production of bee pollen and bee bread

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Summary

Recently a large flora of lactic acid bacteria (LAB) was identified in the honey stomach of the honey bee *Apis mellifera*. In this study, the presence of this flora in bee pollen and bee bread was investigated. Pollen was collected from the legs of honey bees, and both two week old and two month old bee bread were also obtained for the study. Bacterial isolates cultivated from these bee products were identified using 16S rRNA gene analyzes. The majority of the honey stomach LAB flora was recovered in a viable state from both the pollen and the two week old bee bread, but not from the two month old bee bread. It is demonstrated for the first time that bee bread is probably fermented by the honey stomach LAB flora that has been added to the pollen via regurgitated nectar from the honey stomach. This discovery helps to explain how honey bees standardize the production of bee bread and how it is stored. The presence of the honey stomach LAB and its antimicrobial substances in bee bread also suggests a possible role in the defence against honey bee diseases since the bee bread is consumed by both the larvae and the adult bees.

Bacterias del ácido láctico implicadas en la producción polen de abeja y pan de abeja

Resumen

Recientemente una gran flora de bacterias del ácido láctico (BAL) fue identificada en el estómago de la abeja de la miel *Apis mellifera*. En este estudio se ha investigado la presencia de esta flora en el polen de las abejas y en el pan de abejas. El polen se obtuvo de las patas de las abejas, y el pan de abejas fue también recolectado a las dos semanas y a los dos meses de edad para el estudio. Cepas de bacterias cultivadas a partir de estos productos de la abeja se identificaron mediante análisis del gen ribosomal 16S. La mayoría de la flora estomacal BAL fue recuperada en un estado viable, tanto en el polen como en el pan de abejas a las dos semanas de edad, pero no en el pan de abejas a los dos meses de edad. Por primera vez se demuestra que el pan de abejas es probablemente fermentado por la flora BAL del estómago de la miel que se añade al polen a partir de néctar regurgitado del estómago. Este descubrimiento ayuda a explicar cómo las abejas regulan la producción de pan de abejas y la forma en que se almacena. La presencia en el estómago de BAL y de sustancias antimicrobianas en el pan de abejas también sugiere una posible función en la defensa contra las enfermedades de la abeja de la miel, ya que el pan de abejas es consumido por adultos y larvas de abejas.

Keywords: LAB, bee pollen, bee bread, honey stomach flora, fermentation, *Lactobacillus*, *Bifidobacterium*.

Introduction

Lactic acid bacteria (LAB) are a group of phenotypically related bacteria commonly found in healthy organisms as symbionts in their normal flora (Gasbarrini *et al.*, 2008). We recently described an endogenous LAB flora composed of a probable twelve different LAB species from the genera *Lactobacillus* and *Bifidobacterium* (Olofsson and Vásquez, 2008; Vásquez *et al.*, 2009). This flora was shown to be present in the honey stomach of the honey bee *Apis mellifera* from both Sweden and the USA (Vásquez *et al.*, 2008) and varied

numerically with different types of nectar collected by the bees (Olofsson and Vásquez, 2008). We concluded that this LAB flora has probably evolved in mutual dependence with the honey bee, the LAB obtaining a niche in which nutrients were available, the honey bee in turn being protected by the LAB from harmful microorganisms.

Honey bees use pollen as a source of vitamins, proteins, fatty acids, lipids, sterols, minerals and carbohydrates (Loper *et al.*, 1980). They collect pollen and store it in the colony as bee bread which is then consumed by adult bees and fed to the larvae. Bee bread is produced by a lactic acid fermentation, probably performed by microorganisms such as bacteria or yeasts or both (Chevtchik, 1950; Foote, 1957; Haydak, 1958; Pain and Maugenet, 1966; Egorova, 1971; Gilliam, 1979a; Gilliam, 1979b; Gilliam *et al.*, 1989) but the precise mechanisms have never been fully understood.

Initially bee bread is composed of bee pollen, which contains pollen, nectar and secretions from the bee's salivary glands. Bee pollen is collected by foraging bees and is transported on their hind legs in a specialized pollen basket back to the hive (Michener, 1974). It is then packed into cells of the brood comb by house bees who eventually seal it with a drop of honey. After two weeks, it is chemically changed by what is believed to be a natural fermentation caused by the intervention of different microorganisms (Chevtchik, 1950; Pain and Maugenet, 1966). After this two week fermentation period (Pain and Maugenet, 1966), bee bread will last for many months. As in other fermented products, when the process is completed, a high content of lactic acid and other metabolites preserve bee bread from spoilage by microorganisms. The chemical composition of bee bread differs from that of bee pollen; for example, bee bread has a higher acidity due to lactic acid and contains large amounts of vitamin K (Haydak, 1942).

As we previously suggested, this honey stomach LAB flora may be of importance for the health of the honey bees, their larvae and the entire colony. We speculated that the LAB flora could be involved in the fermentation of bee bread. To our knowledge, no previous work identifies any specific species within the genera of either *Lactobacillus* or *Bifidobacterium* that could be involved in the fermentation process of bee bread.

The honey bee is one of our most important pollinators that has recently come into focus because of the condition "Colony Collapse Disorder" or CCD (Cox-Foster *et al.*, 2007). Our aim was to concentrate on the beneficial bacteria of bees which we feel is as important as searching for the pathogens that may cause CCD. We believe that the newly found LAB flora is of critical importance for honey bee health, so wanted to establish whether honey stomach LAB are also involved in the production of bee bread from pollen and what role they may play in colony health.

Materials and methods

Bee pollen and bee bread

Bee collected pollen and bee bread were obtained from an apiary in Helsingborg in southern Sweden in June 2008 from colonies maintained using standard beekeeping practices.

Bee pollen pellets were picked off by hand from three individual bee's legs by the beekeeper as soon as foragers returned to the hive. Approximately two week old bee bread (a swarm of bees that was two weeks old was used) was collected from combs using sterile tweezers and put in a 10.0 ml tube. The samples were immediately transported to the laboratory for bacterial analysis. In addition, bee bread that was two months old was collected in Gainsville, Florida, USA, stored in airtight tubes, and transported to Sweden for comparison with the fresh bee bread. To identify and acquire the complete lactic acid bacterial flora within the pollen and bee bread, a 16S rRNA gene analysis was performed on all the bacteria using a pure-culture technique (Olofsson and Vásquez, 2008).

Procedure for isolates

Bee pollen weighing 20.0 µg and bee bread weighing 300.0 µg were placed in separate sterile 1.5 ml tubes, each containing 0.9 ml sterile physiological saline (0.9% w/v NaCl, 0.1% w/v Tween 80 and 0.1% w/v peptone). Each tube was shaken vigorously and a dilution series was produced using physiological saline. Pure cultures were obtained on two types of media, one containing tomato juice agar (Oxoid; Basingstoke, UK), the other Rogosa agar (Merck; Darmstadt, Germany). The isolates were cultivated anaerobically at 35ºC for 3-4 days. Ten to thirty bacterial colonies were picked randomly from each of the media involved, which contained 30-300 colonies each, and were subcultured to obtain pure isolates.

PCR of isolates

One of the colonies from the purified isolates was placed in 0.2 ml Thermo-Strips (Abgene; Surrey, UK) together with glass beads (0.106 mm, Sigma-Aldrich; St Louis, MO, USA) and 0.1 ml sterile water. The cells were disintegrated by being shaken for 45 min in an MS1 Minishaker (IKA Works; Wilmington, DE, USA). After centrifugation at 20,200 g for 5 min in a Galaxy mini-centrifuge (VWR; West Chester, Pennsylvania, USA), 1 µl of the supernatant was used in the PCR reaction that followed.

Amplification was conducted using primers designed to anneal the conserved regions of bacterial 16S rRNA genes. The forward primer ENV1 (5´-AGA GTT TGA TII TGG CTC AG- 3´) corresponded to positions 8-27 in *Escherichia coli* 16S rRNA, the reverse primer ENV2 (5´-CGG ITA CCT TGT TAC GAC TT- 3´) corresponded to positions 1511-1492 (Brosius *et al.*, 1978). The PCR reaction contained 5 µl 10 x PCR buffer (100 mM Tris-HCl, 15 mM $MgCl_2$, 500 mM KCl, pH 8.3), 200 µmol/l of each deoxyribonucleotide triphosphate, 2.5 U of Taq

Table 1. Bacteria in bee pollen. *Previously described phylotypes belonging to the genus *Lactobacillus*[1], the genus *Bifidobacterium*[2] and the family *Pasteurelaceae*[3]. **Phylotypes isolated from bee pollen; the number of identical sequences found are shown in brackets. ***The length of the compared sequence (the similarity to the closest related previously described phylotype is shown as a percentage within parentheses).

Previously described phylotypes*	Phylotypes from bee pollen (and numbers)**	Sequence lengths (and % similarity)***
Bma5[1]	Bip2Rk9 [1]	1000 (99.6)
Hma8[1]	Bip2Rk5 [3]	1000 (99.6)
Biut2[1]	0	
Hma2[1]	BipRk13 [2]	210 (100.0)
Hma11[1]	Bip2Rk1 [6]	1000 (100.0)
Hon2[1]	Bip2Rk12 [2]	1050 (99.8)
Bin4[1]	0	
Fhon2[1]	Bip1tok5 [2]	1000 (100.0)
Bma6[2]	0	
Bin7[2]	Bip1Rk3 [8]	1150 (100.0)
Hma3[2]	0	
Bin2[2]	Bip2Rk11 [1]	700 (100.0)
Lv2[3]	Bip1tok1 [2]	1000 (100.0)
Trm1[3]	Bip1tok2 [2]	1000 (99.4)

DNA polymerase (Roche Diagnostics; Mannheim, Germany), 10 pmol of each primer and 1-10 µl template in a total volume of 50 µl. Amplification was performed using a Mastercycler (Eppendorf; Hamburg, Germany) as follows: 30 cycles at 95°C for 15 s, 48°C for 30 s and 72°C for 90 s followed by an elongation step at 72°C for 10 min.

Sequencing and bacterial identification

PCR products originating from isolates were sequenced by a sequencing company using universal primer ENV1. For identification, these 16S rDNA sequences were searched against GenBank (National Centre for Biotechnology Information; Rockville Pike, Bethesda, MD, USA) using the Advanced BLAST similarity search option, accessible from the homepage of the National Centre for Biotechnology Information (http://www.ncbi.nlm.nih.gov/) and the software RDP (Ribosomal Database Project II), accessible from the homepage (http://rdp.cme.msu.edu/). Furthermore, the obtained 16S rDNA sequences were searched against the previously described sequences for the honey stomach LAB flora (Olofsson and Vásquez, 2008; Vásquez *et al.*, 2009) using the software programs BioEdit (version 6.0.7) (Hall, 1997) for editing and Clustal X (version 1.81) (Thompson *et al.*, 1997) for alignment and sequence comparison. The partial sequences were approximately 1000 base pairs (range 210-1060 bp)

Results

Bee pollen

Sequences from thirty two isolates were identified, and all of them were most closely related to either the LAB or the bacteria belonging to the *Pasteurelaceae* family previously found by us (Olofsson and Vásquez, 2008; Vásquez *et al.*, 2009). Of the twelve different members of the LAB flora phylotypes, eight (67%) were represented in the bee pollen. Six of the eight *Lactobacillus* phylotypes (Bma5, Hma8, Hma2, Hma11, Hon2, and Fhon2) and two of the four *Bifidobacterium* phylotypes (Bin7 and Bin2) were present (Table 1). Furthermore two of the four different phylotypes belonging to the *Pasteurelaceae* family (Trm1 and Lv2) were represented in the bee pollen (Table 1).

Bee bread

From the two week old Swedish bee bread, sequences from thirty one isolates were identified, and all of them were most closely related to either the LAB or the bacteria belonging to the *Pasteurelaceae* family previously found by us (Olofsson and Vásquez, 2008; Vásquez *et al.*, 2009). Of the twelve different members of the LAB flora phylotypes nine (75%) were represented in the bee bread. Six of the eight *Lactobacillus* phylotypes (Bma5, Hma8, Hma11, Hon2, Bin4 and

Table 2. Bacteria in bee bread. *Previously described phylotypes belonging to the genus *Lactobacillus*[1], the genus *Bifidobacterium*[2] and the family *Pasteurelaceae*[3]. **Phylotypes isolated from bee bread; the number of identical sequences found are shown in brackets. ***The length of the compared sequence (the similarity to the closest related previously described phylotype is shown as a percentage within parentheses).

Previously described phylotypes*	Phylotypes from bee bread (and numbers) **	Sequence lengths (and % similarity) ***
Bma5[1]	Bib2Rk5 [7]	1060 (100.0)
Hma8[1]	Bib2tok4 [3]	1000 (99.7)
Biut2[1]	0	
Hma2[1]	0	
Hma11[1]	Bib2tok1 [1]	1000 (100.0)
Hon2[1]	Bib3rk1 [3]	1000 (99.8)
Bin4[1]	Bib3tok5 [1]	1000 (100.0)
Fhon2[1]	Bib3tok4 [6]	1050 (100.0)
Bma6[2]	Bib3Rk8 [1]	1060 (100.0)
Bin7[2]	Bib3Rk9 [4]	1060 (100.0)
Hma3[2]	Bib3Rk7 [2]	950 (99.7)
Bin2[2]	0	
Lv2[3]	Bib3tok2 [1]	1060 (98.2)

Fhon2) and three of the four *Bifidobacterium* phylotypes (Bin7, Hma3 and Bma6) were present (Table 1). Furthermore one of the four different phylotypes belonging to the *Pasteurelaceae* family (Lv2) was represented in the bee bread (Table 2). No LAB were isolated nor any bacteria belonging to the *Pasteurelaceae* family were found from the two month old American bee bread.

The total flora

Altogether, eleven of the twelve previously described honey stomach LAB phylotypes were recovered from either the bee pollen or the two week old bee bread samples including 63 isolates. Only phylotype Biut2 was not found in the bee pollen or the bee bread. Two of the four previously described phylotypes belonging to the *Pasteurelaceae* family were represented but phylotype Bib3tok2 was only related to the previously described phylotype Lv2 by 98.2%. In summary both the bee pollen and the bee bread samples were dominated by the recently described honey stomach LAB.

Discussion

The majority of the honey stomach LAB flora that we recently isolated (Olofsson and Vásquez, 2008) was detected and viable in both the bee pollen and the two week old bee bread in this study. The bee pollen contained eight, and the bee bread contained nine of the twelve honey stomach LAB phylotypes, together eleven of the twelve. While the numbers of the different phylotypes found was almost the same in both products, the individual members varied (Table 1 and 2). This could be partly explained by the fact that the bee bread did not contain the same kinds of pollen as in the bee pollen loads. It is therefore probable that more extensive sampling of both the bee pollen and bee bread would show all of the honey stomach LAB flora members in both products. We previously found that different nectars have different effects on the LAB growth, which explains their varying numbers and appearance. In addition, we found that phylotype Bin4 may have originated from the honey stomach even if it was not verified. In this study, Bin4 was detected in the bee bread flora, which suggests that it probably originates from the honey stomach.

When honey bees make bee bread, pollen, nectar and salivary gland secretions are all mixed, and most probably the honey stomach LAB are integrated via the nectar that is regurgitated from the honey stomach. In the hive, the pollen is packed in cells and later sealed

with a drop of honey. The temperature in the hive is kept around 35°C all year round and the water content of pollen and bee bread is around 24% (Herbert and Shimanuki, 1978). The environment in the bee bread is at least microaerophilic and the bacteria are provided with sugars from both the nectar and the honey, which also contains numerous other nutrients. This is the perfect environment for the honey stomach LAB, since they are very aero tolerant (unpublished data). In summary, the honey stomach LAB seems to be well adapted for growth in bee bread.

No viable honey stomach LAB were detected in the two month old bee bread. This could be explained by the fermentation process of the bee bread, started by the honey stomach LAB, in which a great amount of lactic acid and antimicrobial substances are produced. This makes the immediate environment very acidic and hostile which generally prevents all bacterial growth. The pH of bee pollen ranges between 4.1 and 5.9 but in the fermented bee bread that contains lactic acid, the pH ranges between 3.8 and 4.3 (Herbert and Shimanuki, 1978).

Two of the four previously described phylotypes belonging to the *Pasteurelaceae* family were represented in the bee pollen, but only one of the phylotypes was detected in the bee bread. They have been found as isolates in our previous work, in honey stomachs, the hindgut, larvae and even in honey and by other researchers (Jeyaprakash *et al*., 2003; Babendreier *et al*., 2007) as clones sampling the hindgut. In addition, these bacteria are often detected when sampling the honey stomach (unpublished data). These novel bacteria must be described and analyzed, as it seems likely that they are honey bee specific and share the same niche as the LAB. However, it is too early to speculate if they are involved in the production of bee bread.

In this study, no other microorganisms were isolated except the honey stomach LAB and the *Pasteurelaceae* bacteria because only media and growth conditions well suited for these bacteria were used.

It is known that a balance between the endogenous microflora within humans is of crucial importance for our health. As demonstrated by various researchers, a disturbed balance in the human gut is implicated in disease (Moore and Moore, 1995; Guarner, 2005). The microflora of insects is much less complex than in humans, but Ryu *et al*. (2008) have shown that in the fruit fly *Drosophila,* a mutualistic relationship between the endogenous gut flora and their host was discovered. In addition, they demonstrated that the fly's normal flora was sufficient to suppress the growth of pathogenic bacteria. The same may be true for the honey bee; damage to their microbiota community in the honey stomach (the beneficial honey stomach LAB flora), may implicate a cascade of invading pathogens. The honey stomach LAB flora could therefore be the first line of defence bees have to protect themselves from ingested pathogens. Organic acids such as formic acid, which is produced by bifidobacteria (Van der Meulen *et al*., 2006) and both lactic and acetic acid, hydrogen peroxide, diacetyl, benzoate, and bacteriocins which are usually produced by LAB, are all antimicrobial substances. This means that the honey stomach LAB may be of considerable importance in the preservation of bee bread and in protecting honey bees and their larvae against pathogens. The chemical composition of pollen and nectar varies by plant species, which in turn may affect the growth of the LAB in the bee bread and therefore its quality.

There are no negative nutritional changes in bee bread compared to bee pollen (Herbert and Shimanuki, 1978) due to the lactic acid fermentation but bee pollen will deteriorate unless it is dried or frozen. The main differences that are known today seem to be that bee bread contains much more lactic acid but much less starch compared to bee pollen (Haydak and Palmer, 1942; Herbert and Shimanuki, 1978). Lactic acid bacteria such as lactobacilli and bifidobacteria are known to produce large amounts of lactic acid using starch (Zhang and Cheryan, 1991; Korakli *et al*., 2002). Notably, B vitamins are essential for honey bee larval nutrition (Serian-Back, 1961; Haydak and Dietz, 1965, 1972; Andersson and Dietz, 1976). Pollen is a rich source of Vitamin B but lactobacilli and bifidobacteria could produce at least Vitamin B7, B11 and B12 (Noda *et al*., 1994; Crittenden *et al*., 2003; Pompei *et al*., 2007; Santos *et al*., 2008) and B12 is not available from plant products.

In previous work, it has been suggested that other microorganisms apart from the honey stomach LAB could be involved in bee bread production, for example bacteria or yeast from the genera *Escherichia*, *Streptococcus* (Chevtchik, 1950), *Pseudomonas*, *Sacharomyces* (Pain and Maugenet, 1966), *Bacillus* and *Torulopsis* (Gilliam, 1979b; Gilliam, 1979a). It has also been mentioned that *Lactobacillus* bacteria could be involved (Chevtchik, 1950; Haydak, 1958; Egorova, 1971) but without pointing out any specific described *Lactobacillus* species. Pain and Maugenet (1966), tried to produce synthetic bee bread by inoculating a single species from the *Lactobacillus* genus onto sterilized pollen, which resulted in an unappetizing product. They therefore suggested that yeasts could play a key role in bee bread production. In the light of our results, a better result could probably be achieved using the complete honey stomach LAB flora which includes different species from both the genera *Lactobacillus* and *Bifidobacterium*.

The purpose of this work was to detect the honey stomach LAB flora in bee pollen and bee bread. These bacteria probably play a key role in the lactic acid fermentation of bee bread and are probably added to bee bread from the bee pollen that foragers bring in containing regurgitated nectar with the honey stomach LAB. This study confirms that lactic acid bacteria are involved in the production of bee bread and we elucidate for the first time which kind of LAB are involved: it is the honey stomach LAB belonging to the genera *Lactobacillus* and *Bifidobacterium.* This answers the question of how honey bees have standardized the fermentation process of bee bread.

The relationship and how or if other microorganisms are involved together with the role of the honey stomach LAB flora have to be further investigated.

We have suggested that the honey stomach LAB flora could be important for the health of the honey bees and the production and storage of their honey (Olofsson and Vásquez, 2008). The results from this study demonstrate that this flora is also important for the production of bee pollen and the production and storage of bee bread. Furthermore, since bee bread, containing antimicrobial substances produced by the LAB flora, is fed to both larvae and adult bees, the importance of these bacteria may be crucial for the defence against pathogens and even help to explain some of the causes of CCD. Conclusively, much more work needs to be done in a near future to understand the microbiota symbionts of honey bees and their role in bee health.

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