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Bulletin of Entomological Research (2015) **105**, 470–476 doi:10.1017/S0007485315000292
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Microbiota associated with pollen, bee bread, larvae and adults of solitary bee *Osmia cornuta* (Hymenoptera: Megachilidae)

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Abstract

Using cultivation-dependant method, we isolated 184 strains from fresh and old bee bread, pollen, larvae and adults of solitary bee *Osmia cornuta*. The 16S rDNA sequencing of 79 selected isolates gave the final species-specific identification of strains. Phylogenetic analysis indicated that microbiota isolated from five different sources were represented with 29 species within three different phyla, Firmicutes with 25 species, Actinobacteria with only one species and Proteobacteria with three species of Enterobacteriaceae. Bacterial biodiversity presented with Shannon–Wiener index (H') was highest in the alimentary tract of adults and old bee bread ($H' = 2.43$ and $H' = 2.53$, respectively) and in the same time no dominance of any species was scored. On the contrary, results obtained for Simpson index (D) showed that in pollen samples the dominant species was *Pantoea agglomerans* ($D = 0.42$) while in fresh bee bread that was *Staphylococcus* sp. ($D = 0.27$). We assume that microbial diversity detected in the tested samples of solitary bee *O. cornuta* probably come from environment.

Keywords: microbiota, *Osmia cornuta*

(Accepted 10 March 2015; First published online 21 April 2015)

Introduction

Like in other animals, extensive populations of microorganisms are common symbionts primarily of the digestive system of insects, but also of all other parts of their body. Their coexistence with the host have different impact on host health and has been extensively investigated in the past, especially questions related to their specific role and origin in the insect body (Mattila *et al.*, 2012). Microbiota, especially gut microorganisms of insects provide numerous benefits for the host such as protection against various parasites and pathogens (Koch & Schmid-Hempel, 2011), digestion of pollen grains (Roulston & Cane, 2000), protection from the toxic compounds encountered in the environment (Mullin *et al.*, 2010), communication and activity in immunomodulation. Bee represent economical as well as ecological important group of insect concerning their involvement in pollination in different ecosystems (Klein *et al.*, 2007). In the recent years, still unexplained, decline of the honey bee and other pollinators across the world was observed (Potts *et al.*, 2010). This situation prompted researches to investigate microbiome of insects that are important to humans, such as honey bee (*Apis mellifera* L.) or bumble bees (*Bombus* spp.) (Martinson *et al.*, 2011; Engel *et al.*, 2012) to determine their possible role in bee decline. It seems that geographical location specificities and environmental differences do not influence on the microbiome of *A. mellifera* gut. Similarly, *Bombus terrestris* have distinct but not so diverse gut bacterial communities (Koch *et al.*, 2012). Additionally, in social bees vertical transmission of bacterial symbionts could be detected (Martinson *et al.*, 2012).

In relation to foregoing, an investigation of microorganisms related with solitary bee started in the last few years. The solitary bee *Osmia cornuta* (Latr.) is known as excellent

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orchards pollinator across Europe where it is used for about 30 years already (Krunić *et al.*, 2001; Bosch & Kemp 2002). The biology of this species is relatively known, as well as approaches for augmentation under natural conditions (Krunić & Stanisavljević, 2006*a*, *b*). Except Europe, it was also introduced in North America (Torchio *et al.*, 1987). It is species native to Europe, except for northern part, and active in nature during early spring from the end of February to the end of April. Their solitary life cycle starts with emerging of males and females, followed by mating. Females are nesting in various hollows such as dried stalks of the common reeds (in the past used for covering houses). Females collect pollen and nectar from flowers and store them in a nest cell, where lay an egg. The larvae are hatching for 2–3 days and immediately start feeding on the mixture of pollen and nectar mass (fresh bee bread). Solitary bees unlike honey bee have completely different life style in which during the larvae development there is no mouth-to-mouth contact with adults and probability of bacteria transmission in this way does not exist. The accompanying fauna of this solitary bee was extensively studied (Krunić *et al.*, 2005), but there are no data about diversity of microorganisms related to this species. *O. cornuta* with several related species have a very important role as alternative pollinators. Even more, it was shown that there is a synergistic effect in pollination when honey bee and non-*Apis* bee were used (Brittain *et al.*, 2013). Honey bee hive is potential source of pathogens of other bee species, while on the other hand, solitary bees may act as a source of honey bee pathogens (Ravoet *et al.*, 2014).

In this study, we investigated diversity of microbiota within fresh and old bee bread, freshly provisioned pollen, larvae and gut of adults of solitary bee *O. cornuta* by cultivation-dependant method to determine characteristic strains for each sample. We aim to determine possible origin and transfer route of microorganisms related to this solitary bee.

Material and methods

Sampling

The nests with brood and adult bees were obtained from managed population near Belgrade (near the Grocka town: Latitude 44°41′31.5″N Longitude 20°41′31.3″E). Sampling of the freshly collected pollen, fresh bee bread, larvae, adults and old bee bread was performed during one life cycle of *O. cornuta*. During spring, pollen was collected from the abdominal scopa of active females, before entering nest; active females were captured when returning from bee pasture, and young larvae (at second and third instars which feeding on bee bread) were extracted from nest cells. While in the autumn, only old bee bread was sampled from the nests. Samples from the field were placed in separate sterile tubes each containing 5 ml sterile physiological solution (0.85% w/v NaCl). Each tube was shaken vigorously and immediately transported to the laboratory in Belgrade, situated 30 km from the sampling field. Intestine samples (from the honey crop to rectum) of adult bees, and whole larvae were used to obtain homogenates in physiological solution (0.85% w/v NaCl). All samples were aseptically handled to avoid contamination with the external surface of the bee body. Dissected gut were aseptically placed into ethanol (70% w/v) for 5 min and subsequently rinsed in sterile water (for 5 min). Whole larvae were surface sterilized in the same way. Five samples were analyzed from each type of sample.

Microbiological analysis

First approach for isolation of bacteria was plating of bacterial dilution onto appropriate growth media as follows: 1 ml of mixture prepared from each sample in 0.85% (w/v) sterile physiological solution was transferred to 9 ml of the same solution and mixed for 1 min. Obtained dilutions were plated on different media: M17 (Merck GmbH, Darmstadt, Germany) supplemented with 0.5% (w/v) glucose (GM17), MRS (Merck GmbH), MSS (Audisio *et al.*, 2011) and LA (Luria-Bertain) agar plates. All agar plates were incubated at 30 °C, 37 °C and 45 °C for 72 h under aerobic and anaerobic conditions, respectively using the Anaerocult A (Merck GmbH) in anaerobic jars. From each sample, all colonies that grown on agar plates after incubation period was selected and streaked on new agar plates.

The second approach was the enrichment of lactic acid bacteria (LAB) that were eventually present in a small number in the samples. For this purpose, 300 µl of the previously prepared dilution were transferred into 5 ml of 10% skimmed milk. Tubes were incubated at 30 °C and 45 °C under anaerobic conditions for 24 h. After the incubation, loopful of grown culture was streaked in parallel on MRS, GM17, MSS and LA agar plates and incubated for additional 48 h at 30 °C and 45 °C under anaerobic conditions. From each plate, all colonies were picked and streaked on new plates in order to obtain pure colonies. Purity of the isolates was checked by streaking again and sub-culturing on fresh agar plates of the isolation media, followed by microscopic examination (Olympus U-RFL-T, BX51, GmbH, Hamburg, Germany). The pure cultures were stored at −80 °C in appropriate media with 15% (w/v) glycerol. Overall 184 isolates were obtained and after catalase test, Gram staining and microscopy, 79 were chosen for further analyses.

Genotypic characterization

Total DNA from 79 selected bacteria were isolated by modified methods previously described (Hopwood *et al.*, 1985). For polymerase chain reaction (PCR) of 16S rRNA gene, a set of specific primers was used: UNI16SF (5′-GAGA GTTTGATCCTGGC-3′) and UNI16SR (5′-AGGAGGTG ATCCAGCCG-3′) (Jovcic *et al.*, 2009). KapaTaq DNA Polymerase (Kapa Biosystems, Inc., Boston, MA, USA) was used to amplify DNA fragment by PCR using a GeneAmp PCR system 2700 thermal cycler (Applied Biosystems, Foster City, CA, USA). PCR products were purified with QiAquick PCR purification kit (QIAGEN) according to the protocol of the manufacture and sequenced by the Macrogen Sequencing Service (Macrogen, Nederland). Obtained sequences were analyzed in the NCBI database using BLAST similarity search. Hits with identities above 93–97% were treated as likely same species but different subspecies/strains and with more than 97% declared as the same species, subspecies and strain. The phylogeny was inferred using the Maximum-Likelihood method based on the Kimura 2-parameter model (Kimura, 1980), with 1000 bootstrap replications. Initial tree(s) for the heuristic search were obtained by applying the Neighbor-Joining method to a matrix of pair wise distances estimated using the Maximum Composite Likelihood (MCL) approach. The analysis involved 96 nucleotide sequences (79 isolates + 17 reference strains) and there were 834 positions in the final dataset. Phylogenetic analysis was done using Mega 6 software (Tamura *et al.*, 2013). The bacterial

16S rRNA gene sequences were deposited in EMBL GenBank under accession numbers HG799933 through HG800012.

Statistical analysis

To determine species richness (*S*) classical ecology index was used for all type of sources used in this study, Shannon–Wiener index (*H′*) for presentation of general biodiversity and Simpson's index (D) for evaluating dominance of the species in each sample, as follows:

$$S = \Sigma^N; H' = \Sigma^N p_i \log_2(p_i); D = 1 - \Sigma^N (p_i)^2$$

where N is the number of species and p_i is the number of isolates belonging to one species in the analyzed sample.

Results

After incubation at different conditions on MRS, GM17 and LA medium 184 microbial strains were isolated from all samples. Incubation of samples in the MSS medium did not yield in growth of any bacteria. The most numerous bacteria (70 isolates) were nonsporogenous, Gram-positive and catalase-positive facultative anaerobic cocci (Table 1). Another large group of bacteria (24 isolates) were sporogenous, Gram-positive and catalase-positive aerobic rods. Based on preliminary classification 79 bacterial isolates were selected for further examinations. Except bacteria, in all analyzed samples 27 yeast isolates were obtained; but in this study we did not perform their further identification. Final identification allowed us to determined 29 species. Gram-negative bacteria were represented with only three species within family Enterobacteriaceae of Gammaproteobacteria (fig. 1). On the other hand, Gram-positive bacteria were represented with 24 species from the class Bacilli and only one species from class Clostridia (*Clostridium baratii*), all from phylum Firmicutes. Also, gram-positive was *Curtobacterium flaccumfaciens*, only representative of Actinobacteria. The genus *Bacillus*, with six identified species, had representative strains in all samples. *Enterococcus* species (three determined in total) were present in all samples, except pollen, while representatives of family Leuconostocaceae (overall four species) were not isolated only in larvae sample. Streptococci (three species identified) were isolated only from alimentary tract of adults while *Lactobacillus kunkeei* was detected only in fresh bee bread. Staphylococci were represented in alimentary tract, old bee bread and pollen samples with six species. Sole representatives of *Paenibacillus polymyxa*, *C. baratii* and *Serratia marcescens* were isolated from old bee bread samples. Only pollen samples harbored isolates of *Pantoea agglomerans* and *C. flaccumfaciens*. Phylogenetic analysis indicated that bacterial microbiota isolated from fresh bee bread, larvae, adults, old bee bread and pollen belongs to three different phyla (fig. 1), Firmicutes, Actinobacteria and Proteobacteria. Expressed as a percentage of all isolated microbial strains in this study the most abundant were Firmicutes (almost 75%), with representatives of the genus *Staphylococcus* (38.04%), LAB (23.37%) and *Bacillus* sp. (13.04%), while the species from *Enterobacteriaceae* family were present in lower percentage (8.15%).

To estimate species diversity in five types of different analyzed samples, the general index of species biodiversity (*H′*) and index for evaluating dominance (*D*) of the species were calculated. The basis for calculation was 79 strains identified by 16S rDNA sequencing in combination with classical microbiological identification of the strains isolated. Obtained results are presented in fig. 2. The greatest number of species was obtained in the alimentary tract of adults (OCAT) as well as in old bee bread (OCOB), which also showed the highest species diversity. From OCAT we isolated 18 species and from OCOB 14 species (fig. 2). In both samples, no dominance of any species was scored. The species diversity was the lowest in the larvae (OCL) as only tree bacterial species were isolated, and two of them were present only in this sample, *Bacillus cereus* and *Enterobacter aerogenes*. On the other hand, yeasts were the most abundant in this sample. Similar situation was observed in the last two samples, fresh bee bread (OCFB) and in pollen (OCP). In these samples diversity is higher than in larvae and the presence of yeasts was relatively high. Results obtained for Simpson index (*D*) showed that in pollen (OCP) samples the dominant species was *P. agglomerans* ($D = 0.42$) while in fresh bee bread (OCFB) that was *Staphylococcus* sp. ($D = 0.27$).

Table 1. Preliminary classification of microbial strains isolated from the nests of *O. cornuta* according to morphological characteristics analyzed.

Morphological characteristics	Number of strains
Rod-shaped bacteria	
Aerobes, sporogenous, Gram-positive and catalase-positive	24
Aerobes, nonsporogenous, Gram-positive and catalase-positive	2
Anaerobes, sporogenous, Gram-positive and catalase-negative	2
Facultative anaerobes, sporogenous, Gram-positive and catalase-positive	1
Facultative anaerobes, nonsporogenous, Gram-positive and catalase-negative	16
Facultative anaerobes, nonsporogenous, Gram-negative and catalase-positive	15
Coccoid bacteria	
Facultative anaerobes, nonsporogenous, Gram-positive and catalase-positive	70
Facultative anaerobes, nonsporogenous, Gram-positive and catalase-negative	21
Ovoid cocci, facultative anaerobes, nonsporogenous, Gram-positive and catalase-negative	6
Fungi	
Yeasts, aerobes, anaerobe or facultative anaerobes, Gram-positive and catalase-positive	27
Total	184

Discussion

European orchard bee *O. cornuta* is very important for pollination of almond, apricot, pear, apple and several other rosaceous fruit plants (Krunić & Stanisavljević, 2006*b*). Given that pollinators in general, and honey bees in special, are in decline, knowledge of diversity of microbiota related to the alternative pollinators could be of great importance since the health status of bees (as for any other animal) depending on presence and activity of commensal microorganisms. Moreover, Koch & Schmid-Hempel (2011) stated that specific microbiota could be important for bee health. Due to its importance, we conducted this comprehensive study of bacterial diversity within

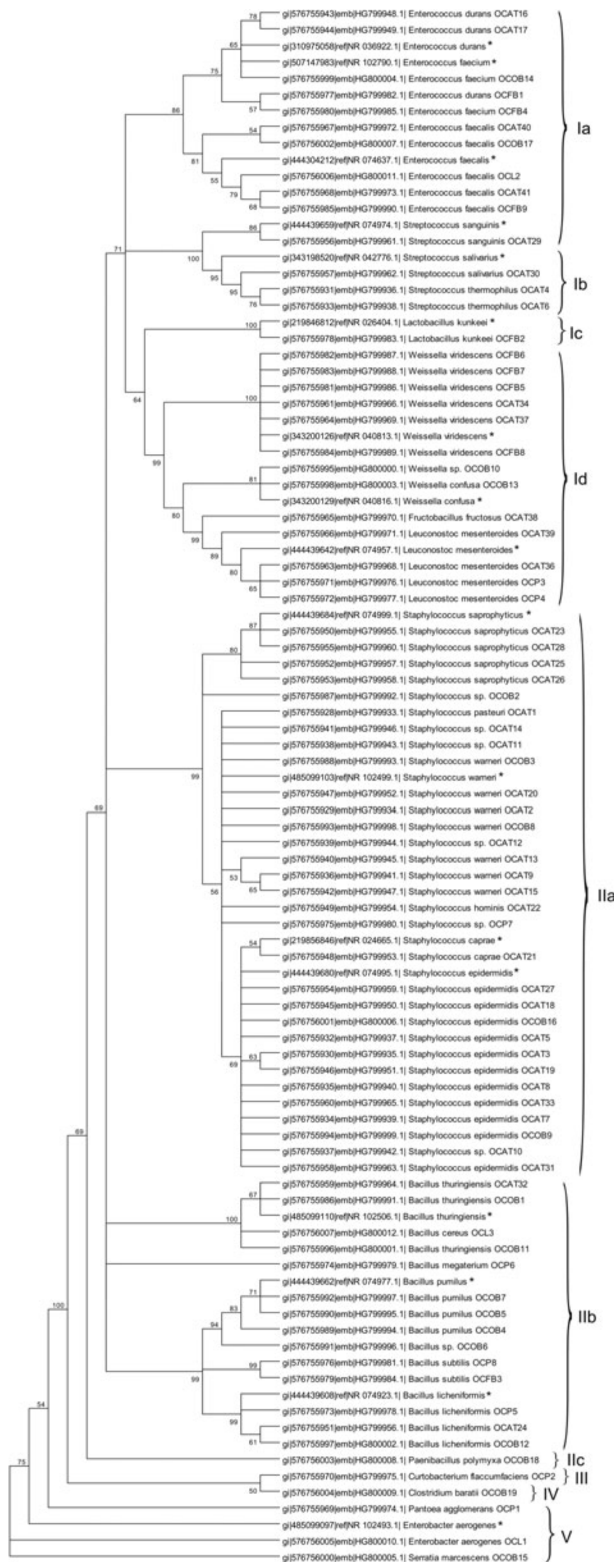
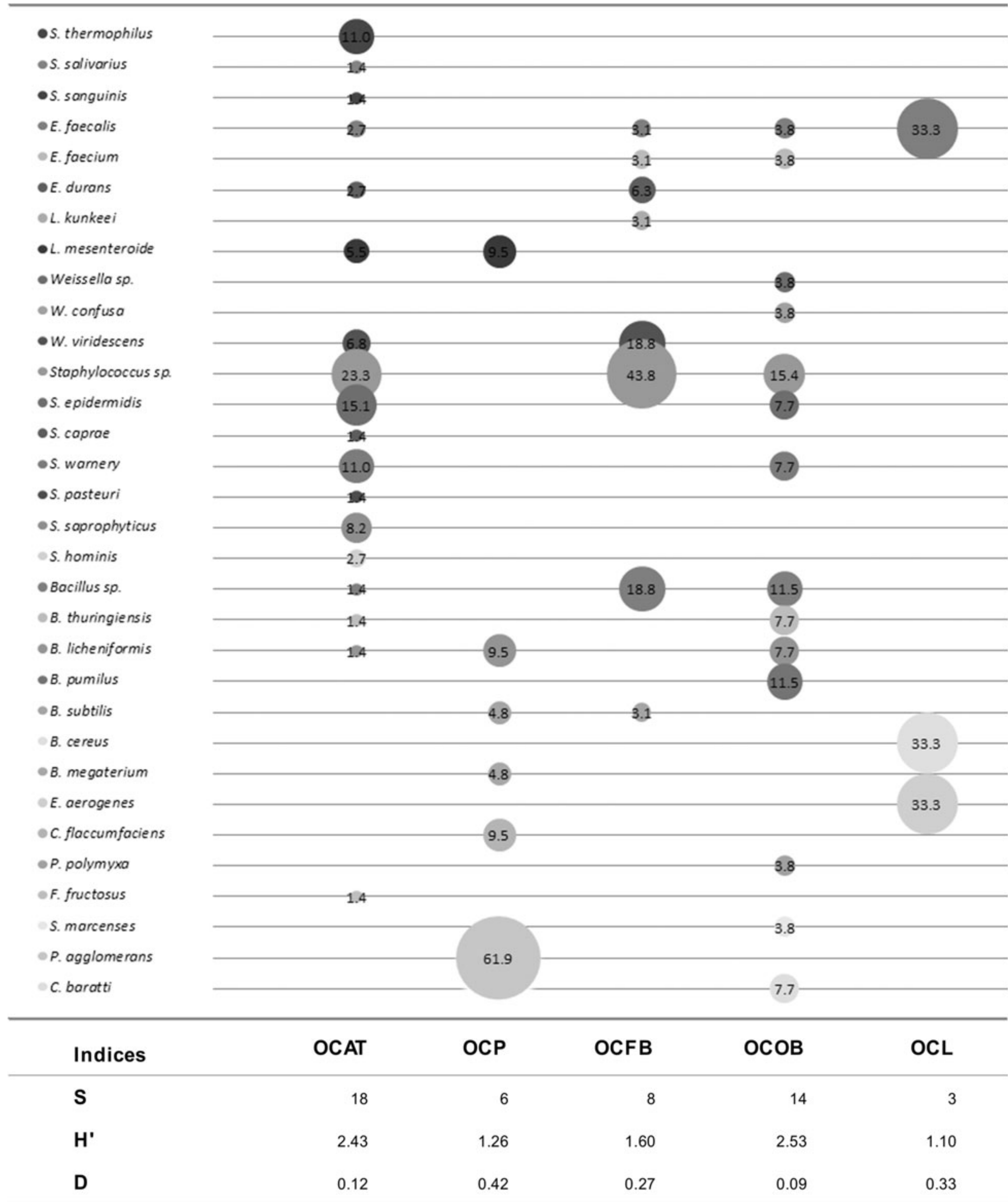


Fig. 1. Phylogeny of the analyzed taxa represented by the bootstrap consensus tree based on the 16S rRNA gene sequences. Branches corresponding to partitions reproduced in less than 50% bootstrap replicates are collapsed. Closely related type and reference strains are indicated in parentheses together with accession numbers from GeneBank. Cluster **I**, Lactobacillales comprise: **Ia** = family Enterococcaceae, **Ib** = family Streptococcaceae, **Ic** = family Lactobacillaceae and **Id** = family Leuconostocacea; Cluster **II**, Bacillales comprise: **IIa** = family Staphylococcaceae, **IIb** = family Bacillaceae and **IIc** = family Paenibacillaceae; Cluster **III** = *Clostridium baratii*; Cluster **IV** = *Curtobacterium flaccumfaciens*; Cluster **V** = family Enterobacteriaceae which served as the out-group. Proteobacteria – Cluster V, Actinobacteria – Cluster III and Firmicutes – Cluster I, II and IV.

fresh and old bee bread, pollen, larvae and adults of *O. cornuta*. To our knowledge, this is the first report on microbiota associated with *O. cornuta* bee. All bacteria isolated were classified in three different phyla, Firmicutes, Actinobacteria and Proteobacteria. For solitary bee with similar life style, *O. bicornis*, six different phyla were reported (Mohr & Tebbe, 2006; Keller *et al.*, 2013). In addition to phyla that we detected, Acidobacteria, Bacteriodetes and Chloroflexi were also reported in *O. bicornis*. However, in both species the same three phyla were the dominant one. Bearing in mind that these two species of bees are phylogenetically closely related, but without the complete overlapping host plant spectra, which has been shown in recent study (Haider *et al.*, 2014), it appears that both of the bees have very similar composition of the microbiota in their nest and in their alimentary tract.

In digestive tract of *O. cornuta*, we detected representatives of 16 species, all Firmicutes. These results are different from the one reported for the gut of *A. mellifera*, where except Gram-positive bacteria multitude of Proteobacteria were detected (Brochier *et al.*, 2002). Nevertheless, this finding corroborate the one obtained for *O. bicornis* (Keller *et al.*, 2013) where only few Enterobacteriaceae were observed in samples other than gut, indicating that they have only marginal importance in *O. bicornis* microbiota and could be explained with different life style of solitary bee in comparison with honey bee. Additionally, offspring of solitary bees develop in completely different microenvironment than those in the beehive. Their development and characteristics depend greatly of pollen of host plants used for bee bread formation. It is not surprising that species from genus *Bacillus* were identified in all samples analyzed and *Paenibacillus* in old bee bread. Strains from genus *Bacillus* have physiological and morphological characteristics, which allow their prevalence, since the production of different antimicrobial substances may enable their better adaptation to different environmental conditions. In addition, these antimicrobial compounds could have beneficial impact for the bee since it could kill pathogenic bacteria before they develop and infect the nests (Sabaté *et al.*, 2012). Study of bacteria diversity in the nests of *O. bicornis* (Keller *et al.*, 2013) also showed numerous isolates from these two genuses. Changes in the gut structure during insects development are connected with different physiological conditions, which could be selective criteria for gut microbiome development. Even more, metamorphosis in mosquitoes resulted in complete loss of gut bacteria with adults containing no bacteria in their guts (Moll *et al.*, 2001). However, changes in the gut development, specific adaptation of bacteria to live in the insect guts and transmission of bacteria between adults and offspring could have significant effect on the development of specific microbiota in different life stages. In addition, simple passage from environment to insects gut does not necessary mean that those bacteria ingested with food or in other way could



Indices	OCAT	OCP	OCFB	OCOB	OCL
S	18	6	8	14	3
H'	2.43	1.26	1.60	2.53	1.10
D	0.12	0.42	0.27	0.09	0.33

Fig. 2. Relative abundance (%) of different bacteria species isolated from five different samples. Species richness (*S*), Shannon–Wiener (*H′*) indicating general biodiversity and Simpson's index (*D*) for evaluating dominance of the isolated strains are presented from each sample. OCAT – *O. cornuta* alimentary tract, OCP – *O. cornuta* pollen, OCFB – *O. cornuta* fresh bee bread, OCOB – *O. cornuta* old bee bread and OCL – *O. cornuta* larvae.

adapt to specific gut conditions. While in social bees, vertical transmission of bacteria was detected as evolutionary advantage to maintain permanent bacterial community in the gut (Martinson *et al.*, 2012) for solitary bee we could not gain the same conclusion, which was in accordance with their completely different life style. Consider that, we could see some consistency in appearance of some bacterial species in particular samples. That was especially right for bacteria obtained in samples of adults and old bee bread. Seven species, regardless of taxonomic affiliation, were present in both samples. Since those two samples have also greatest species richness, we can assume that they were good substrates for development and preservation of bacteria. Contrary to that, samples of pollen and fresh bee bread were 'too young' for developing of considerable number of bacteria, e.g. they stay below the radar. Similar can be stated for larvae, which were particularly difficult for sampling due to their small size.

Lactobacillus kunkeei was linked with *A. mellifera* in some studies (Mohr & Tebbe, 2006; Olofsson & Vásquez, 2008) while in some could not be detected (Cox-Foster *et al.*, 2007; Martinson *et al.*, 2011). The floral transmission of this strain and other acidophilic bacteria was suggested previously (McFrederick *et al.*, 2012) while differences in obtained results are explained with different seasonal sampling or methodology applied. However, we detected this species only in fresh bee bread, not in pollen and gut samples. In the *O. cornuta* alimentary tract, the presence of *Fructobacillus fructosus* was confirmed. Like *L. kunkeei*, *F. fructosus* is classified as 'obligatory' fructophilic lactic acid bacterium (Endo *et al.*, 2009) associated with flower. We suggest that they originate from flowers, although we did not detect any lactobacilli in the pollen sample, and probably participate in fermentation process in which bee bread was produced. Their abundance is changed during time in the similar manner as in food fermentation processes, for which the dynamic changes of microbiota are characteristic. Some kind of selective effect that is inevitable when using cultivation-dependent method could be the reason why we did not detect both species in both type of samples as well as changes in microbial community present at different samples at the time of collecting. Another of our result that supports the hypothesis that solitary bee microbiota originated from environmental bacteria is detection of bacteria from genus *Clostridium* and *Bacillus*. In study of microbial diversity in nests of *O. bicornis* species, bacteria from all three mentioned genuses were identified (Keller *et al.*, 2013).

According to our findings, we hypothesized that microbiota associated with solitary bee *O. cornuta* is introduced through transfer of strains that come from environment. Further investigation of selected strains for their antimicrobial and fermentation abilities will provide greater insight into the specific role of each species in particular sample analyzed.

Acknowledgments

The Ministry of Education, Science and Technological Development of the Republic of Serbia (Grant numbers 173026, 173019 and 43001) supported this work.

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