Characterization of fructophilic lactic microbiota of *Apis mellifera* from the Caucasus Mountains

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Abstract Microbial symbionts of honeybee colony are considered as promising tools to support the honeybee population welfare. The majority of existing honeybee microbiota studies is focused on genetic description of the honeybee-associated microbiome fingerprints. The lack of a deeper knowledge on the bacterial community colonizing the honeybee niche, which may be helpful in encouraging industrial applications of this microbiota, led us to undertake this study. The biodiversity of the cultivable fructophilic lactic acid bacteria (FLAB) isolated from adult honeybee intestine and beebread samples was studied. Phenotypic properties of probiotic interest, such as the adhesive potential using in vitro models and adhesion determinants, were also investigated. Antibiotic resistance profiles as reliable markers to evaluate the impact of long-term and current exposure of honeybees to antibiotics were phenotypically determined on the isolated lactic acid bacteria (LAB). The mannose-specific adhesion and high cell surface hydrophobicity found in the studied FLAB isolates sheds light on the effective adaptation of microbiota to specific ecologic niche. It is the first report of phenotypically detected antibiotic resistance profiles of honeybee endogenous bacteria and the first account of minimum inhibitory concentration (MIC) values for four antibiotics used in beekeeping practice.

Keywords Fructophilic lactic acid bacteria · Honeybees · Microbial adhesion · Caucasus

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Introduction

The honeybee, *Apis mellifera*, together with other pollinator insects, provides critical services to ecosystem thanks to the pollination of flowering plants. The decline of honeybee colonies, the consequent decrease of their productivity, and the impaired pollination efficacy became a major concern for modern agriculture, which foresees world food shortages in the future if no remedy against all or even part of honeybee pathogens is found (Klein et al. 2007; Becher et al. 2013; Tirado et al. 2013). This pollinator crisis has stimulated scientific interest and new series of trials to understand the etiology of the diseases and the countermeasures against the honeybee pathologies, which are decimating their populations. A combination of multiple stress components is responsible for the increased mortality of honeybee colonies. The protection of honeybee populations requires a strategy targeting simultaneously several causes that might exert synergistically detrimental effects (Becher et al. 2013).

Research on honeybee gut symbiotic microbial composition, which is important for the protection of honeybees from different stressors (Wu et al. 2013), resulted in the elaboration of a microbial resource management (MRM) approach, analogous to what

is successfully applied in different biological systems (Crotti et al. 2012). Bacteria, along with fungi residing in the gastrointestinal tract (GIT) of insects, represent a part of this particular and dynamically changing ecosystem. Recent findings showed that honeybee microbiome is different from what can be observed in other living organisms (Endo and Salminen 2013). This peculiar and unique set of intestinal honeybee bacteria is the result of a long-term co-evolution based on symbiotic interactions between the colonizers and their host (Evans and Armstrong 2006; Engel et al. 2012).

Functions of honeybee gut microbiota remain largely unexplored. Recent studies indicate the possible involvement of honeybee symbionts, particularly lactic acid bacteria (LAB), in the beebread production and its protective role from deterioration during storage. Antimicrobial activities expressed against causative agents of American foulbrood (AFB) and European foulbrood (EFB), *Paenibacillus larvae* and *Melissococcus plutonius*, respectively (Forsgren et al. 2010; Vásquez et al. 2012), demonstrate that MRM may be a promising tool for the control of bacterial diseases in the beekeeping. LAB were identified as active immunomodulators, inducing the generation of various antibacterial peptides in the host honeybees (Evans and Lopez 2004; Yoshiyama et al. 2013). Comparative analysis of the microbiome contents of the honeybee gut suggests that different microbial species display peculiar functional characteristics, such as the formation of biofilms and uptake of nutrients, which are expressed in response to interactions with the host (Engel et al. 2012).

Presently, beside various pests and pathogens, honeybees are facing multiple new stressors, such as climate change, habitat loss, environmental pollutants, and contaminants from beekeeping practice (Potts et al. 2010; Smodis Skerl et al. 2010; Pilatic 2012). These new factors can also unbalance, to some extent, the endogenous microbial communities residing in bee GIT. Also, the antibiotic treatments can reduce the diversity of endogenous microbes, enriching the pool of antibiotic resistance genes, hence reinforcing the pathogens and impairing the community of microbes beneficial to the host bee (Tian et al. 2012). Despite the efficacy of antibiotics against AFB and EFB or nosemosis, their usage in beekeeping is limited in the EU by the established maximum residue level (MRL) in the bee products, resulting in a zero tolerance for the presence of traces of antimicrobial agents in honey (Reybroeck et al. 2012; Levy and Marshall 2013). Nevertheless, some antibiotics are still widely used outside Europe, e.g., oxytetracycline and tylosin in the USA (Murray et al. 2009) and in other parts of the world (Kaufmann and Kaenzig 2004; Ortelli et al. 2004; Vidal et al. 2009). In this regard, the application of probiotics may be a reliable problem-solving, soft, and biocompatible approach helping the honeybee colonies to preserve their unique microbiota, diminishing, thus, the necessity of drastic use of antibiotics. The candidate probiotic bacteria should possess highly specific properties to strengthen or maintain the health of the host, including: modulation of immune responses, inhibition of epithelial invasion, production of antimicrobial substances, and adhesive properties (Rolfe 2000; Mercenier et al. 2008; Duary et al. 2011; Salminen and van Loveren 2012).

Fructophilic lactic acid bacteria (FLAB) is a specific group of LAB which has been characterized and described only recently. They prefer fructose as growth substrate and inhabit only fructose-rich niches (Endo and Salminen 2013). Because honeybees are high-fructose-consuming insects, probiotic candidates were searched within FLAB. For the isolation of FLAB strains, foreguts separated from adult honeybee GIT and stored beebread samples were chosen. The foregut is an important organ which is used by bees for food production, collection, transport, and disposal of nectar in the hive. Furthermore, the honeybee nurses feed the larvae with foregut (crop) contents and this is the mechanism by which larval food is inoculated with LAB (Vásquez et al. 2012).

The geographic localization and biological diversity, including microbial diversity, are making the Caucasus range a key Euro-Asiatic ecologic niche. Its diversity of elevations, climates, and humidities presents to the dwelling insects huge adaptive challenges. Hence, the study of honeybee microbiota from Georgian bees may provide results that are also valid for the honeybee populations from other parts of the world.

The aim of the present study was to identify the cultivable FLAB isolated from the Georgian honeybees and to evaluate their diversity, according to the genotypic profiles, the adhesive properties, and the antibiotic resistance.

Materials and methods

Isolation of bacterial strains and growth conditions

Fifty-seven samples obtained from adult worker bee intestines and 25 samples of beebread were collected from 16 apiaries located mainly in the Caucasus Mountains and Kolkheti valley. For sampling, bee colonies without any visible signs of bacterial diseases and nosemosis were chosen. The bees were dissected, and their foregut was separated and homogenized in physiological solution. The beebread samples were homogenized in sterile physiological solution. For isolation of the honeybee FLAB, homogenized samples were spread on Lactic Bacteria Differential Agar medium (LBD, HiMedia, M1087) containing fructose as the unique source of carbohydrates. Plates were incubated in anaerobic conditions at 37 °C during a period of 48 h. Different bacterial colonies were collected and purified on de Man, Rogosa, and Sharpe (MRS) agar plates (Oxoid) supplemented with 0.1 % L-cysteine and 2.0 % fructose (Vásquez et al. 2012). Selected bacterial isolates were propagated in MRS broth supplemented with the same components and then maintained as frozen

stocks at −80 °C in the presence of 150 ml/l glycerol as the cryoprotective agent.

DNA extraction and RAPD-PCR

Total bacterial DNA was extracted from overnight MRS cultures using a Chelex-based method described in the MicroSeq protocol of the manufacturer's instruction (Life Technologies, Monza, Italy). The genomic DNA was used as a template in random amplified polymorphic DNA polymerase chain reaction (RAPD-PCR) fingerprinting using the M13 minisatellite core sequence (5′-GAGGGTGGCGGTTCT-3′) (Huey and Hall 1989). Amplification reactions were performed following the protocol described by Giraffa and Neviani 2000. The presence of PCR products was confirmed by 1 % agarose gel electrophoresis, and 1 Kb Plus DNA Ladder (Invitrogen, Milan, Italy) was used as a DNA molecular weight marker. The gels were stained with GelRed™ (Biotium, Hayward, CA, USA), according to the manufacturer's instructions. Gel images from RAPD-PCR analysis were photographed after overnight electrophoresis (1.5 V cm^{-1}) using the Kodak Electrophoresis Documentation and Analysis System 290 (EDAS 290, Celbio, Milan, Italy), equipped with the EDAS 290 imaging cabinet. The images were saved as TIFF files and processed with the pattern analysis software package BioNumerics™ (version 7.1; Applied Maths, Sint-Martens-Latem, Belgium).

Calculation of similarity of the PCR fingerprinting profiles was based on the Pearson product-moment correlation coefficient. A dendrogram was deduced from the matrix of similarities by the unweighted pair group method using arithmetic average (UPGMA) clustering algorithm (Vauterin and Vauterin 1992). The reproducibility of the RAPD-PCR fingerprinting patterns was evaluated by repeated running of DNA samples from duplicate amplifications of a control (Rossetti and Giraffa 2005).

Species identification by DNA sequencing

Representative isolates for each RAPD cluster were identified at the species level by sequencing a 500-bp fragment of the 16S rRNA. The 500-bp 16S rRNA fragment was amplified from the 5′ end of the gene using the forward primer 5′-GCYTAACACATGCAAGTCGA-3′ (46 *Escherichia coli* numbering) and reverse primer 5′-GTATTACCGCGGCTGCTGG-3′ (536 *Escherichia coli* numbering) (Carminati et al. 2014). For a few isolates, the whole 16S rRNA gene was sequenced by using the MicroSEQ Full Gene 16S rDNA PCR Kit (Life Technologies), according to the manufacturer's protocol. The DNA sequencing was performed using an ABI PRISM 310 automated DNA sequencer (Life Technologies), as previously described (Suárez et al. 2009). The sequence data were processed, and the alignments were performed using the pattern analysis software package BioNumerics™ (version 7.1; Applied Maths). The obtained sequences were compared with closely related sequences from NCBI GenBank using the BLASTN program (http://blast.ncbi.nlm.nih.gov/Blast.cgi) and aligned using the CLUSTALW software (http://www.ebi.ac.uk/Tools/msa/clustalw2/).

Study of adhesive properties

Yeast agglutination

Bacterial cells were washed and dissolved in 0.1 M of phosphate buffer saline (PBS, pH 7.2). Fifty microliters of a four-fold dilution of the initial bacterial suspensions in PBS was transferred to microtiter plates (Iwaki brand, SciTech Div. Asahi Techno Glass, Japan). To each well, 50 μl of buffer or buffer with methyl-a-D-mannopyranoside (final concentration 100 mM, Sigma Aldrich), as well as 100 μl of 1 % (v/w) of *Saccharomyces cerevisiae* cells, grown in yeast extract peptone dextrose (YPD) broth at 30 °C under ambient aeration and suspended in PBS, were added. The same suspension of *S. cerevisiae* alone was dispensed in the microtiter plates and used as a control for autoagglutination. Microtiter plates were shaken for 30 min at 28 °C. The induced visible yeast cell agglutination was assessed by visible light microscopy at 40-fold magnification. Agglutination with or without the addition of methyl-a-D-mannopyranoside was compared to evaluate the role of mannose in the adhesion mechanism (Zago et al. 2011). Tests were carried out in triplicate.

Agglutination with concanavalin A

Bacterial strains were propagated in MRS broth (supplemented with 0.1 % L-cysteine and 2.0 % fructose) at 37 °C for 18 h. The cells were harvested by centrifugation (2000 × g, 10 min, 20 °C) and washed three times with sterile 0.01 M PBS. Pellets were suspended in PBS to reach about 6×10^{10} cells/ml. Concanavalin A (ConA, Sigma Aldrich) was dissolved in PBS at a concentration of 0.1563 mg/ml. Twenty-five microliters of cell suspensions were dispensed in the microtiter plates (Iwaki brand, Sci Tech Div. Asahi Techno Glass, Japan) and mixed by pipetting up and down with an equal volume of ConA solution; sterile PBS was used as a negative control. The plates were left at room temperature for a period of 1 h. Agglutination of bacterial cells was observed under visible-light microscopy at 40-fold magnification and, after visual evaluation, was expressed as no agglutination (−), weak agglutination (+), and strong agglutination (++).

To evaluate the role of different carbohydrates in the bacterial agglutination with ConA, tests were carried out with the addition of D-glucose, D-mannose, and D-galactose (Sigma) using sterile water as a negative control. The carbohydrate solutions in PBS buffer (1 % w/v) were mixed separately with equal volumes of ConA solution (0.3120 mg/ml concentration) and left at room temperature for 10 min. Next, they were mixed with equal

volumes of bacterial cell suspensions and left at room temperature for a period of 1 h. The reaction was observed and assessed as described above (Kim et al. 2006). The experiments were carried out in triplicate.

Cell surface hydrophobicity

Cell surface hydrophobicity was determined according to the method of microbial adhesion to hydrocarbons (MATH) as described by Vinderola and Reinheimer (2003), using hexadecane as the solvent. The isolates were grown in MRS broth for 16–18 h at 37 °C. Bacterial cells were harvested by centrifugation (9500 rpm, 5 min, 5 °C) from overnight MRS cultures, washed twice, and suspended in 60 mM of potassium phosphate buffer (pH 6.5). The initial absorbance (A_0) at 560 nm of the suspension was adjusted to 0.90–1.10 optical units. Three milliliters of cell suspension was dispensed in clean and dry round-bottom test tubes, followed by the addition of 600 μl of hexadecane. The tubes were vortexed for 2 min. The tubes were left for 1 h at 37 °C to allow phase separation. The lower aqueous phase was carefully removed with a sterile Pasteur pipette and absorbance (A_1) at 560 nm was recorded. Cell surface hydrophobicity in terms of percent (H %) was calculated using the following formula: H % = $(1 − A_1/A_0) \times 100$.

Determination of antibiotic resistance

Antibiotic susceptibility and the minimum inhibitory concentration (MIC) of different antibiotics were determined by the microdilution test, with some modifications. Growth medium was optimized by mixing Iso-Sensitest broth (70 % *v/v*, Oxoid) and MRS broth (30 % *v/v*, Merck) supplemented with L-cysteine (0.1 %) and fructose (2 %) pH 6.7. Inoculation of MIC microtiter test plates containing different antibiotics (test ranges: 2–1024 μg ml^{-1} for oxytetracycline and lincomycin; 0.0625–32 μg ml^{-1} for rifampicin and kanamycin) and preparation of inocula to reach a final inoculum density of 10^4 bacteria/ml were performed according to Rossetti et al. (2009). After anaerobic incubation of the inoculated plates at 37 °C for 48 h, the MIC of a given antibiotic was evaluated visually as the lowest concentration at which no growth was observed. All tested antibiotics were purchased from Sigma.

Results

Identification of bacterial strains

A total of 86 strains were studied. They were composed of 25 strains isolated from pollen samples and 61 strains from gut samples. Each strain corresponded to a distinct colony morphotype. RAPD-PCR fingerprinting allowed to genotype LAB isolates, which were grouped into 30 clusters (with an intracluster similarity coefficient >79 %, which corresponded to the reproducibility of the applied RAPD-PCR method). Fifteen clusters out of 30 were represented by single strains. Thirty strains representing each RAPD cluster were selected and identified by 500-bp 16S rRNA sequencing: *Lactobacillus kunkeei* 1 (KX180927), 6 (KX180928), 14p (KU158309), 18 (KX180929), 21 (KX180943), 22 (KX197199), 23a (KX180930), 23p (KX226337), 28 (KX180931), 38 (KX226339), 48b (KX180932), 55 (KX180933), 59 (KX180934), and 63b (KX226342); *L. casei* 45 (KX180935); *Fructobacillus fructosus* 32 (KX180939) and 49a (KU158312); *F. tropaeoli* 21p (KX180940) and 46 (KU158311); *F. pseudoficulneus* 57 (KU158313); *Enterococcus durans* 42s (KX180941); *E. faecalis* 31 (KX226338), 40 (KX226340), 43 (KX180942); *Bifidobacterium. asteroides* 26p (KU158310); and five unidentified strains belonging to *Lactobacillus* spp. 60 (KX180936), 61 (KX226341), 62s (KX180937), 63s (KX180938), and 64s (KX226343).

The 86 strains studied belonged to the genera *Lactobacillus* (72 strains), *Fructobacillus* (eight strains), and *Enterococcus* (five strains). Only one strain was identified as *B. asteroides*. Among the 72 *Lactobacillus* strains, *L. kunkeei* was found to be the most frequently isolated species (66 strains, which were grouped into 16 different RAPD clusters), followed by *L. casei* (one strain), while five strains remained unidentified. A complete 16S rRNA gene sequencing of two out of the five unidentified strains allowed to identify one of them as *L. kunkeei*, while the sequence of the other strains perfectly matched with *Lactobacillus* sp. M4, a strain isolated from honeybee gut by a Korean group (GenBank accession number KF543103.1) (unpublished data). The *Fructobacillus* strains belonged to the species *F. fructosus* (three strains), *F. tropaeoli* (three strains), and *F. pseudoficulneus* (two strains). The *Enterococcus* strains belonged to the species *E. faecalis* (four strains) and *E. durans* (one strain).

Twenty-four strains representing all identified species and covering the most distinguishable RAPD clusters were selected for further characterization.

Adhesive properties

Yeast agglutination

Among the 24 strains tested, only one strain of *F. fructosus* (strain 49a) was able to agglutinate *S. cerevisiae* cells (Fig. 1 left). This ability was lost after the addition of methyl-D-mannopyranoside (Fig. 1 right).
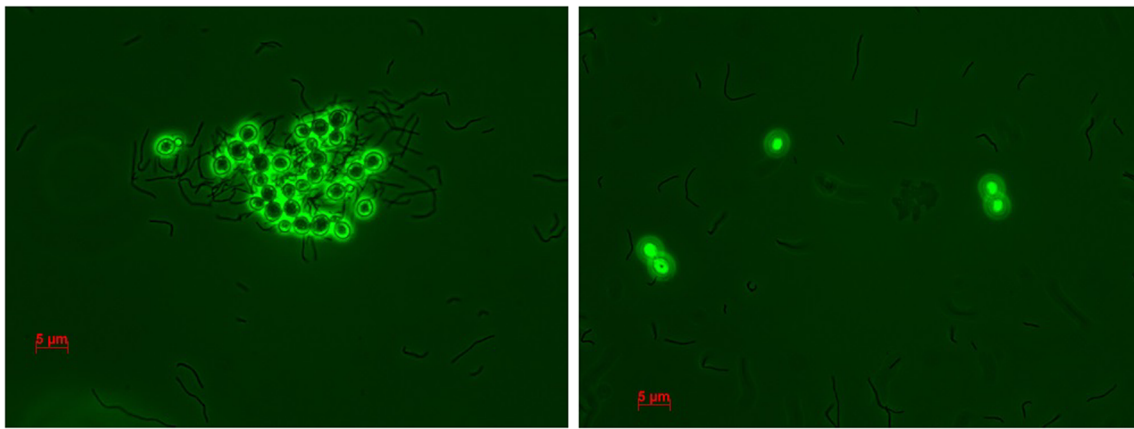
Fig. 1 Agglutination of *Saccharomyces cerevisiae* with *Fructobacillus fructosus* 49a as visualized by fluorescence microscopy (staining with SYBR Green): *left* without and *right* with methyl-D-mannopyranoside (magnification ×100)

Agglutination with concanavalin A

All 24 strains were unable to self-agglutinate, while 17 strains (ten *L. kunkeei*, two *F. fructosus*, three *F. tropaeoli*, and two *Enterococcus*) showed agglutination properties in the presence of ConA (Fig. 2 left; strain *L. kunkeei* 21p shown as an example; Table 1). Strains of *L. casei*, *Lactobacillus* spp., *F. pseudoficulneus*, and *B. asteroides* did not agglutinate with ConA. The agglutination was always inhibited in the presence of D-mannose (Fig. 2 right), while it always took place in the presence of D-glucose and D-galactose (Table 1).

Hydrophobicity

Tests on the microbial adhesion to hydrocarbons (MATH test) demonstrated genus-dependent differences in cell surface hydrophobicity among the tested strains, with the highest values for *L. kunkeei*, *F. tropaeoli*, and *F. pseudoficulneus* (about 90 %), followed by *F. fructosus* and *Lactobacillus* spp. (about 80 %). *Lactobacillus casei* and *E. faecalis* had intermediate values (about 50 %), while *E. durans* showed the lowest hydrophobicity (6 %).

Antibiotic resistance

Due to the lack of approved standards applicable for antibiotic susceptibilities in honeybee gut bacteria, the isolated FLAB were tested with the most frequently exploited antibiotics used in Georgia for beekeeping manipulations (Table 2). Overall, the strains of *L. kunkeei* showed the highest resistance to oxytetracycline and kanamycin, with MICs of about 256–512 and 128–256 μg/ml, respectively. The strains of *Fructobacillus* species were less resistant, with MIC values between 64 and 128 μg/ml for both oxytetracycline and kanamycin. *Lactobacillus casei*, *Lactobacillus* spp., *B. asteroides*, *E. durans*, and *E. faecalis* appeared to be the most sensitive (Table 2). Conversely, all the strains tested resulted, with few exceptions, highly sensitive to rifampicin and lincomycin.
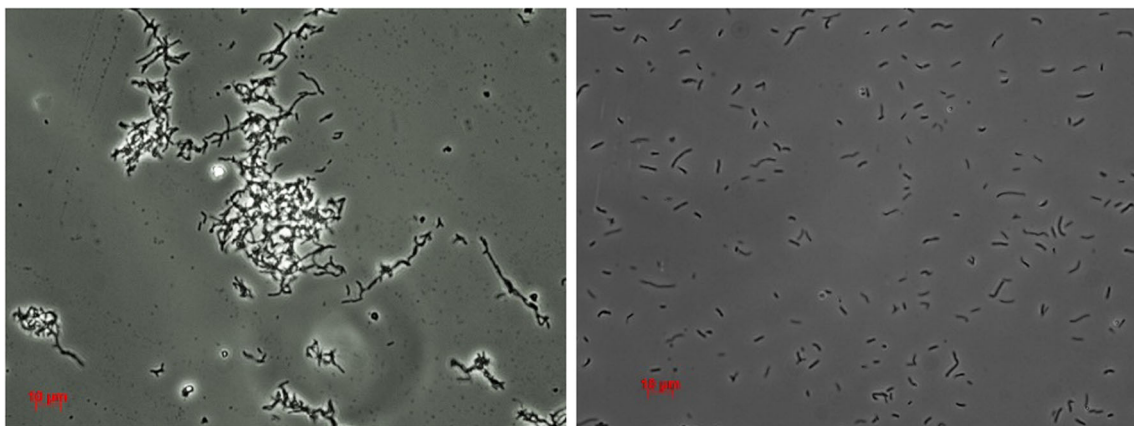
Fig. 2 Agglutination of *Lactobacillus kunkeei* 21p with concanavalin A (ConA) as visualized by bright-light microscopy: *left* without and *right* with D-mannose (magnification ×40)

Table 1 Strain agglutination in the presence of concanavalin A (ConA) and influence of carbohydrates on the agglutination properties

Strains	Agglutination in the presence of ConA			
	Alone	+ D-mannose	+ D-glucose	+ D-galactose
L. kunkeei 1	+	−	+	+
L. kunkeei 6	+	−	+	+
L. kunkeei 18	+	−	+	+
L. kunkeei 21	+	−	+	+
L. kunkeei 22	−	−	−	−
L. kunkeei 23a	+	−	+	+
L. kunkeei 28	+	−	+	+
L. kunkeei 48b	+	−	+	+
L. kunkeei 55	+	−	+	+
L. kunkeei 59	+	−	+	+
L. casei 45	−	−	−	−
Lactobacillus spp. 60	−	−	−	−
Lactobacillus spp. 62s	−	−	−	−
Lactobacillus spp. 63s	−	−	−	−
F. fructosus 32	+	−	+	+
F. fructosus 49a	+	−	+	+
F. tropaeoli 21p	+	−	+	+
F. tropaeoli 46	+	−	+	+
F. tropaeoli 50	+	−	+	+
F. pseudoficulneus 54	−	−	−	−
F. pseudoficulneus 57	−	−	−	−
E. durans 42s	+	−	+	+
E. faecalis 43	+	−	+	+
B. asteroides 26p	−	−	−	−

Discussion

The 86 cultivable fructose-fermenting LAB isolated from Georgian honeybee and beebread samples were found to belong to four bacterial genera, with *Lactobacillus* as the most prevalent. Among lactobacilli, the species *L. kunkeei* was the most frequently isolated, as also demonstrated in other similar studies (Olofsson and Vásquez 2008; Vásquez and Olofsson 2009; Neveling et al. 2012). The genera *Fructobacillus* and *Enterococcus* were less represented, and only one strain of *Bifidobacterium* was identified.

The abundance and ratio of *Fructobacillus* and bifidobacteria were in agreement with the data of Vojvodic et al. (2013) on bacteria isolated from honeybee larval instars. *Fructobacillus* prefers fructose as growth substrate and inhabits only fructose-rich niches, such as the honeybee ecosystem (Endo et al. 2009; Endo and Salminen 2013). Even *Enterococcus* strains were usually associated with the intestinal tract of the honeybee (Carina Audisio et al. 2011). In regards to bifidobacteria, *B. asteroides* is the only species

frequently isolated from the hindgut of social insects (Bottacini et al. 2012).

The ability to agglutinate yeast cells, the co-aggregation of bacterial cells in the presence of ConA, and the cell wall hydrophobicity overall showed a high adhesion propensity of the species *L. kunkeei*, as well as of the genus *Fructobacillus*. In particular, *F. fructosus* displayed yeast cell agglutination ability in a mannose-specific manner, and *L. kunkeei*, *F. fructosus*, and *F. tropaeoli* showed a mannose-specific co-aggregation ability with ConA as well as the highest cell wall hydrophobicity. Highly hydrophobic cell wall surfaces serve as an efficient tool for LAB to anchor and survive in the host gut.

The abundance of mannose-specific adhesive strains in honeybee GIT could be one possible explanation for the rare manifestation of bacterial diseases in adult bees, which are frequently found in honeybee larvae. The absence of intestinal bacterial diseases in adult bees may be attributed to inefficient competition of exogenous bacterial pathogens for the colonization of GIT

Table 2 Minimum inhibitory concentration (MIC in μg/ml) of four antibiotics tested against honeybee lactic acid bacteria (LAB) strains

Species	Strain number	Rifampicin	Lincomycin	Kanamycin	Oxytetracycline
L. kunkeei	1	0.250	0.125	256	512
L. kunkeei	6	0.0625	0.0625	16	32
L. kunkeei	18	0.250	0.125	128	512
L. kunkeei	21	0.0625	0.0625	64	256
L. kunkeei	22	0.125	0.125	128	256
L. kunkeei	23a	0.0625	0.0625	128	512
L. kunkeei	28	0.125	0.0625	128	256
L. kunkeei	48b	0.250	0.125	128	512
L. kunkeei	55	0.250	0.0625	128	512
L. kunkeei	59	0.0625	0.0625	256	512
F. tropaeoli	50	0.0625	0.0625	64	64
F. tropaeoli	21p	0.125	0.125	128	64
F. tropaeoli	46	0.125	0.125	128	64
F. pseudoficulneus	54	0.125	0.0625	64	128
F. pseudoficulneus	57	0.250	0.125	128	64
F. fructosus	49a	0.125	0.125	64	64
F. fructosus	32	0.250	0.125	128	64
L. casei	45	0.250	2	64	16
Lactobacillus spp.	62s	0.250	0.250	32	32
Lactobacillus spp.	63s	0.125	0.250	32	16
Lactobacillus spp.	60	2	32	32	16
E. durans	42s	0.0625	0.5	16	8
E. faecalis	43	4	32	128	64
B. asteroides	26p	1	0.0625	64	32

surfaces with honeybee endogenous bacteria. It was proposed that the immune status of honeybees might be modulated by the presence of the endogenous symbionts in their GIT (Vásquez et al. 2012). The resistance against pathogens is different in larvae because of the differences between their immune profiles with those of adult individuals. The agglutination ability and a mannose-specific adhesion could create the possibility of selection of specific bacterial strains shielding honeybees against their unicellular microsporidian parasites, such as *Nosema apis* and *Nosema ceranae*. This could also allow to hypothesize that GIT microbiota might play a role in the protection of honeybees against their intracellular fungal pathogens. The efficiency of conjoint probiotic therapy against the mucosal opportunistic fungal pathogen *Candida albicans* was recently reported by Köhler et al. (2012).

The absence or scarcity of consensus for antibiotic resistance criteria with respect to honeybee GIT bacterial species (EFSA Panel on Additives and Products or Substances used in Animal Feed 2012) and the lack of microbiological cut-off values make it difficult to assess and compare with specific standards the results obtained. The MIC values of the 24 endogenous honeybee

FLAB showed an overall resistance of these strains to high concentrations of oxytetracycline and kanamycin, and a sensitivity to low concentrations of rifampicin and lincomycin. The detection of oxytetracycline resistance phenotype in this study is coherent with the reported detection of genes encoding such resistance in the honeybee gut microbiota (Tian et al. 2012). Similarly to what is observed in human and animal-associated resistant bacteria, oxytetracycline resistance may have been acquired by horizontal gene transfer between and within commensal microflora because of frequent and extended use of this antibiotic in beekeeping practice (Murray et al. 2009; Tian et al. 2012). Co-resistance against kanamycin found in this study may be the effect of prolonged single-agent therapy with oxytetracycline and a co-mobilization of genetic determinants for both antibiotics (Tian et al. 2012).

High sensitivity of almost all the strains (except for the *E. faecalis* strain) to low concentrations of rifampicin and lincomycin could reveal a detrimental role of these antibiotics for honeybee gut microbiota, increasing the risks for depletion of resident FLAB strains and consequent exposure to fungal colonization. It should be pointed out that the use of rifampicin-based preparations as honeybee therapeutic agents

was introduced only recently (Gurgulova et al. 2003). However, as already demonstrated for oxytetracycline, the use of rifampicin-based treatments in beehives could lead to the emergence of new resistance genes in honeybee gut microbiota.

The results of this study can improve the understanding of many fundamental aspects of bacteria–insect interactions in honeybee GIT. More studies on the biochemical and microbiological properties of honeybee microbiota, especially the FLAB community, are still needed for a possible use of these microorganisms as probiotics, immune boosters, replacements, or supplements to antibiotic treatments.

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