



Original article

Insight into probiotic properties of lactic acid bacterial endosymbionts of *Apis mellifera* L. derived from the Polish apiary

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ABSTRACT

Taking into account that fructophilic lactic acid bacteria (FLAB) can play an important role in the health of honey bees and can be used as probiotics, phenotypic properties of probiotic interest of *Lactobacillus kunkeei* (12 strains) and *Fructobacillus fructosus* bacteria (2 strains), isolated from *Apis mellifera* gastrointestinal tract, have been studied. We have evaluated survival of tested FLAB in honey bee gut, their susceptibility to antibiotics (ampicillin, erythromycin, tylosin), cell surface hydrophobicity, auto-aggregation ability, co-aggregation with model pathogenic bacteria, biofilm formation capacity, and effect of studied FLAB, added to sucrose syrup bee diet, on longevity of honey bees. The tested FLAB exhibited good gastrointestinal tract tolerance and high antibiotic susceptibility, which are important criteria in the screening of probiotic candidates. It was also found that all FLAB studied have high cell surface hydrophobicity and fulfil next selection criterion for their use as probiotics. Symbionts of *A. mellifera* showed also auto- and co-aggregation capacities regarded as valuable features for biofilm formation and inhibition of pathogens adhesion to the bee gut cells. Biofilm-development ability is a desired characteristic of probiotic lactic acid bacteria. As indicated by quantitative crystal violet-stained microplate assay and confocal laser scanning microscopy imaging, all studied *A. mellifera* gut isolates exhibit a biofilm positive phenotype. Moreover, it was also documented, on honey bees kept in cages, that supplementation of *A. mellifera* sucrose diet with FLAB decreases mortality and improves significantly longevity of honey bees. Presented research showed that *A. mellifera* FLAB symbionts are good candidates for application as probiotics.

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1. Introduction

On the health and survival of honey bees significant impact has the composition of the intestinal microflora (Crotti et al., 2013; Gilliam, 1997; Kwong et al., 2017; Kwong and Moran, 2016;

Raymann and Moran, 2018). In the maintaining of the proper gut microbial ecosystem particular role play lactic acid bacteria (LAB), which are natural inhabitants of the honey bee gastrointestinal tract (Endo and Salminen, 2013; Forsgren et al., 2010; Pachla et al., 2018; Pattabhiramaiah et al., 2012; Vásquez et al., 2012). Honey bee LAB symbionts have been shown to be responsible for nutrient assimilation by their hosts, triggering immune response of bees, elimination of pathogens, and sustaining microflora homeostasis in the honey bee gut (Asama et al., 2015; Evans and Lopez, 2004; Raymann and Moran, 2018; Vásquez and Olofsson, 2009). It is known that lactic acid bacteria produce antimicrobial substances which eliminate pathogenic microorganisms (Asama et al., 2015; Crotti et al., 2013; Evans and Lopez, 2004; Forsgren et al., 2010;

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Jack et al., 1995; Pachla et al., 2018; Servin, 2004; Vásquez et al., 2012; Wu et al., 2014). They also ferment lactose as well as other sugars and produce lactic and acetic acids as the end-products which acidify the gastrointestinal tract and inhibit the growth of some harmful bacteria (Carina Audisio et al., 2011; Forsgren et al., 2010; Pachla et al., 2019, 2018). Honey bee are subjected to infection by different parasites, i.e.: bacteria (*Paenibacillus larvae*, *Melissococcus plutonius*), fungi (*Nosema ceranae*, *Nosema apis*), mites (*Varroa destructor*), and many different viruses (Chen and Siede, 2007; Dussaubat et al., 2012; Forsgren et al., 2010; Killer et al., 2014; Ptaszyńska et al., 2018; Strauss et al., 2015). The colonization of honey bee alimentary tract by LAB is important feature for their hosts in disease prevention (Raymann and Moran, 2018; Vásquez and Olofsson, 2009). To shape a proper honey bee gut community and improve bee health, supplementation of a pollen substitute diet of the honey bee colonies with honey bee-specific lactic acid bacteria, which colonize a gastrointestinal tract and exhibit beneficial effect on honey bee colonies, is suggested (Kazmierczak-Baryczko and Bożena, 2006; Pătruică et al., 2013; Szymaś et al., 2012). It was documented that lactic acid bacteria, used as a sugar syrup supplements, may not only destroy the pathogens by production of lactic and acetic acids and modulation of the immune response but also by synthesis of hydrogen peroxide and bacteriocins (Butler et al., 2013; Olofsson et al., 2016; Pachla et al., 2018). All these data suggest, that honey bee LAB symbionts, exhibiting therapeutic and antibacterial activity, can be considered as a natural, alternative tool to antibiotics in combating the infection diseases. It is especially important in the era of antibiotics overuse and the rapid spread of antibiotic-resistant infections.

The objective of this paper was to evaluate the potential probiotic properties of lactic acid bacteria isolated from the intestinal tract of *Apis mellifera* derived from Polish apiary. Parameters such as: survival in honey bee gut, cell surface hydrophobicity, auto- and co-aggregation capacities, ability to form biofilm, and the impact of studied LAB, added to sugar syrup, on health and longevity of honey bees were studied.

2. Materials and methods

2.1. Bacterial strains and culture conditions

In this paper, 12 *L. kunkeei* strains and 2 *F. fructosus* ones, previously isolated from a healthy honey bee hives, were aerobically cultured in FYP broth/agar medium or MRS agar medium with 0.5% CaCO₃ at 30 °C as described by Pachla et al., (2018). *L. acidophilus* ATCC4356 (ATCC, Manassas, VA, USA), used as control strain for antibiotic susceptibility analysis of LAB, was cultured in MRS agar/broth medium (Pachla et al., 2018). For co-aggregation assays, the commercially available pathogens such as: *Escherichia coli* ATCC 25922, *Klebsiella pneumoniae* ATCC 700603, *Paenibacillus larvae* LMG 9820 were cultured as described earlier (Pachla et al., 2018).

2.2. Survival of lactic acid bacteria in honey bee gut - test in vivo

The analysis of survival of two LAB strains, i.e.: *L. kunkeei* CH1 and *F. fructosus* V5 in the gastrointestinal tract of honey bee was performed in two experimental replicates as follows. The one-day-old honey bees, were obtained from the apiary (Puławy, Poland) as described by Ptaszyńska et al., (2016) and kept in laboratory in the wooden cages, 40 bees per each cage, at 30 °C and at humidity of 60%. Bacteria for experiment were grown overnight in FYP broth at 30 °C (Pachla et al., 2018), collected by centrifugation at 5,000 rpm for 15 min, suspended in 0.85% (w/v) sterile saline

solution to OD₆₀₀ ~ 1 (~2x10⁸ bacterial cells ml⁻¹), and such fresh bacterial suspensions were concentrated by centrifugation and added separately to 35% sucrose syrup in the number ~ 1x10⁸ cells per ml of sucrose syrup and used as a feeding for bees for the first 24 h of experiment. For the next 66 h, the bees were fed only sucrose syrup. The control honey bees were fed only 35% sucrose syrup throughout the duration of the experiment. 10 honey bees were removed from the control cages at the beginning of the experiment (0 h) and 24, 48, 72 h after the beginning of the experiment as well as from cages in which bees were fed sucrose syrup supplemented with LAB at 0 h (soon after administration of sucrose syrup with LAB to honey bees) and after 18, 24, 42, 48, 66, 72, and 90 h from the beginning of the experiment. To determine the survival of LAB in the insect's digestive system, the honey bees were anaesthetized by chilling at 4 °C, their guts were removed by pulling the last segment of the abdomen, and the midgut was separated from the rest of gut with a sterile scalpel as described earlier (Pachla et al., 2018). Ten midguts, from both honey bee groups at each time point, were homogenized and serially diluted to determine viable cell count (colony forming units - CFU) of lactic acid bacteria in the bee gut. Serial LAB dilutions were plated on MRS agar medium supplemented with 0.5% CaCO₃ (w/v) and plates were incubated at 30 °C in 5% CO₂ atmosphere as described earlier (Pachla et al. 2018). The survival of LAB in the honey bee gastrointestinal tract was determined as viable cell number per one midgut at 18, 24, 42, 48, 66, 72, and 90 h of the experiment. The results were analyzed by GraphPad Prism software and presented as CFU per honey bee gut.

2.3. Survival of lactic acid bacteria in honey bee guts – test in vitro

For isolation of bee guts, the one-day old honey bees were anaesthetized by chilling at 4 °C and the guts were isolated as described above. For each sample ten midguts were used. The midguts were homogenized, using a sterile tissue grinder pestle, and next, mixed thoroughly (using vortex) with: (I) 10 µl of 30% (w/v) sucrose syrup and 10 µl of 0.6% NaCl containing 10⁷ of fresh lactic acid bacterial cells (*L. kunkeei* CH1 or *F. fructosus* V5) prepared as described above and (II) 10 µl of 30% sucrose syrup and 10 µl of 0.6% NaCl. The samples were incubated anaerobically at 30 °C for 24 h. To determine viable cell counts of lactic acid bacteria in the honey bee guts, the serial 10-fold dilutions of homogenized gut material at 0 h (directly after adding bacteria) and 6, 12, and 24 h after adding bacteria were done, plated on MRS agar medium with 0.5% CaCO₃ (w/v), incubated at 30 °C in 5% CO₂ atmosphere as described earlier (Pachla et al., 2018) for 24–48 h, and the survival rate of lactic acid bacteria was calculated as:

$$\% \text{ survived cells} = \frac{\text{Viable LAB cell counts in honey bee guts at 0, 6, 12, 24 h}}{\text{Viable cell counts at 0 h (10}^7 \text{ CFU)}}$$

These studies were performed with two replicates. The results of experiment were analyzed by GraphPad Prism software and presented as % survived bacterial cells.

2.4. Determination of antibiotic resistance

Antibiotic susceptibility testing of lactic acid bacteria was conducted for three antibacterial agents: ampicillin as inhibitors of cell wall synthesis as well as erythromycin and tylosin as inhibitors of protein synthesis (EFSA, 2012; Reybroeck et al., 2012). The minimum inhibitory concentrations (MIC) and minimum bactericidal concentrations (MBC) were determined by the microdilution broth method, with some modifications. First, the antibiotics were dissolved in dimethyl sulfoxide (DMSO), filter-sterilized, and diluted to the concentration 64 µg/ml in FYP medium (Pachla et al.,

2018). Subsequently, using the same FYP medium, serial two-fold dilutions of antibiotics, ranging from 64 to 0.0625 ml, were made. The sterile 96-well polystyrene microtiter plates (Nunc, Denmark) were prepared by adding 200 μ l of appropriate dilution of the tested antibiotic per well. Bacterial inocula, from FYP broth cultures incubated at 30 °C for 20 h, were prepared in sterile 0.85% NaCl to a turbidity equivalent of 0.5 McFarland standard, and 2 μ l of bacterium inoculum was added to the wells (final bacterium density 1.5×10^6 CFU/ml). DMSO control (DMSO at a final concentration of 10% and inoculum), a positive control (inoculum without antimicrobial agent), and negative control (the tested antibiotics without inoculum) were included in each microplate. The plates were incubated at 30 °C in 5% CO₂ for 72 h. The MIC values were determined visually as the lowest concentration of antibiotic, at which bacterial growth was inhibited in comparison to an antibiotic-free control wells. Minimal bactericidal concentration (MBC) was determined by subculturing, onto FYP agar medium, 5 μ l of the bacterial culture from each well that showed complete growth inhibition, from the last positive one as well as from the positive control. The plates were incubated at 30 °C in 5% CO₂ for 72 h and the MBC value was recorded as the lowest concentration of antibiotic that resulted in a greater than $\geq 99.9\%$ reduction in the number of live bacteria (3 logarithms) compared to positive control. *L. acidophilus* ATCC4356, sensitive to studied antibiotics, was used as a reference strain. Each experiment was repeated in triplicate.

2.5. Measurement of cell surface hydrophobicity

The LAB cell surface hydrophobicity was determined according to the MATH (microbial adhesion to hydrocarbon) method with some slight modifications using the aliphatic petroleum hydrocarbon n-hexadecane (Sigma, purity $\geq 99\%$) as the solvent (Vinderola and Reinheimer, 2003). The bacteria were grown in FYP broth for 24 h at 30 °C (Pachla et al., 2018), harvested by centrifugation (5,000 rpm, 15 min, 5 °C), washed twice with PBS buffer (pH 7.4), and suspended in the same PBS solution. The initial absorbance of the bacterial suspension at 560 nm (A_0) was adjusted to 1.0 optical unit using spectrophotometer (Biorad, Germany). Three milliliters of cell suspension was dispensed into clean and dry round-bottom test tube and 600 μ l of hexadecane was added. The mixture of bacteria and hydrocarbon was vortexed for 2 min, and next, the tube was set aside to rest for 1 h at 30 °C to allow phase separation. The lower aqueous phase was carefully removed with a sterile Pasteur pipette (paying attention not to take the hydrocarbon layer into the pipet) and its absorbance at 560 nm was recorded (A_t) in quartz measurement cell. Cell surface hydrophobicity in percentage terms (H%) was calculated using the following formula:

$$H\% = (1 - A_t/A_0) \times 100$$

The results of two independent experiments, each with two replicates, were reported as mean values \pm standard deviations (SD).

2.6. Auto-aggregation assay

The auto-aggregation assay was performed according to Honey Chandran et al., (2018) with some small modifications. Briefly, the bacteria were grown in FYP broth at 30 °C (Pachla et al., 2018), harvested at the stationary phase, collected by centrifugation (3,500 rpm, 15 min, 5 °C), washed twice with PBS solution (pH 7.4), suspended in the same PBS buffer, and adjusted to the OD₅₆₀ = 1.0 (A_0) by measuring the absorbance in spectrophotometer (Biorad, Germany). Bacterial suspension in PBS buffer (4 ml) was vortexed for 10 s, incubated at room temperature for 24 h

without disturbing, and absorbance at 560 nm was measured at 0 h, after 1, 2, 4, 6, and 24 h from the beginning of the experiment. The auto-aggregation (Auto-A%) was expressed as the percentage decrease in absorbance after 1, 2, 4, 6, and 24 h relative to that of original suspension as follow:

$$\text{Auto-A\%} = (1 - A_t/A_0) \times 100$$

where, A_0 is the initial optical density of bacterial suspension and A_t represents the absorbance of bacterial suspension after 1, 2, 4, 6, and 24 h from the initial optical density (Nikolic et al., 2010). The data of two independent experiments, each with two replicates, are presented as means \pm standard deviation (SD).

2.7. Co-aggregation assay

In the co-aggregation assay (Co-A%), suspensions of lactobacilli and commercially available potentially pathogenic strains: *Escherichia coli* ATCC 25922, *Klebsiella pneumoniae* ATCC 700603, *Paenibacillus larvae* LMG 9820 were prepared as described earlier (Pachla et al., 2018). Equal volumes (2 ml) of suspensions of tested LAB strain and pathogenic strain, of the same optical density (OD₅₆₀ = 1.0), were mixed together in pairs, vortexed for 10 s, and then proceeded as in the case of auto-aggregation. Each strain studied was tested in duplicate. The co-aggregation percentage was calculated using the equation:

$$\text{Co-A\%} = (1 - A_t/A_0) \times 100$$

where, A_0 is the initial optical density of mixture of two bacterial suspensions and A_t represents the absorbance of mixture of two bacterial suspensions after 1, 2, 4, 6, and 24 h from the initial optical density (Nikolic et al., 2010). The co-aggregation assay was performed two times and data are presented as means \pm standard deviation (SD).

2.8. Quantitative determination of biofilm formation

Biofilm formation by studied lactic acid bacterial strains was investigated by the crystal violet-stained microplate assay according to Olofsson et al., (2016) with slight modifications. Briefly, bacteria were grown in FYP broth for 24 h at 30 °C. The absorbance of each bacterial culture was adjusted to 0.6 optical unit at 560 nm, diluted 100-fold in the same medium containing 1% or 5% fructose (w/v) and aliquot of 100 μ l of each individual culture, in both media was added into six wells in 96-well polystyrene MicroWell plate (Nunc Sigma-Aldrich). The microplate was covered with a sterile microporous sealing film (AeraSeal catalog no. BS-25) to allow gas exchange and prevent evaporation, and plate was incubated under static conditions at 30 °C for 72 h. Next, the culture supernatants were withdrawn from each well, microplate was air dry, and then, the wells were washed three times with 150 μ l of sterile PBS solution (pH 7.4) to remove unbound cells. The bacteria attached to the wall of each well were stained with 100 μ l of 0.4% crystal violet (CV) water solution for 30 min. Next, the CV was aspirated and the wells were rinsed three times with sterile water to remove unbound crystal violet. The plate was air-dried and the attachment of bacteria to well walls was quantified by adding 150 μ l of 95% ethanol to each dish well. The optical density (OD) was measured at 600 nm with a BioRad Microtiter Plate reader. The experiment was repeated two times for each strain tested.

2.9. Analysis of biofilms by confocal laser scanning microscope (CLSM)

24-h bacterial cultures in FYP liquid medium were equilibrated to OD₆₀₀ = 0.6, diluted 100-fold in the same medium with 5% fructose, and 100 μ l of each bacterial culture was added to six wells in 96-well polystyrene MicroWell plate (Nunc Sigma-Aldrich) and

biofilms were grown according to method described by Janczarek et al., (2015) with some modification. Microplates were incubated under static aerobic conditions at 30 °C for 48 h. Subsequently, the culture supernatants were discarded from the wells, each well was washed gently three times with 200 µl of 0.85% NaCl to remove planktonic cells, and biofilms were subsequently air dried for 30 min and stained in darkness for 30 min with 5 µM Syto-9 and 30 µM propidium iodide (PI) in 0.85% NaCl (LIVE/DEAD BacLight stain kit (Invitrogen) (Janczarek et al., 2015)). Next, the dyes were removed from the wells, the wells were washed three times with 0.85% NaCl, and biofilm structures were analyzed using the Confocal Laser Scanning Microscope LSM 5 PASCAL (Carl Zeiss, Germany). The images of biofilms were recorded using the fluorescence microscope Axiovert 200 M with the scanning head LSM 5 PASCAL (Carl Zeiss, Germany). The biofilm thickness was recorded using AIM 4.2 software (Carl Zeiss) in a multifaceted laser scan mode. The surface area occupied by the biofilm was measured at a magnification of 200x. To establish the ratios of live/dead cells in the biofilm, formed by each individual strain growing in FYP medium with 5% sucrose, two independent sets of images, separately for green - Syto-9 (live cells) and red - propidium iodide (dead cells) fluorescences, were collected from six wells for each strain. The Live/Dead bacteria ratio was based on 3 images from each biofilm. The images were recorded using the AxioVision 4.8 (Carl Zeiss) software and the ratio of live/dead cells was determined by multichannel fluorescence technique using AxioCam HR3 camera and 470 nm and 546 nm filters for the green and red channels, respectively. The ratio of live to dead cells and the percentage of the area covered by biofilm were calculated using ImageJ 1.43e software (Wayne Rasband, NIH, USA) (Beyenal et al., 2004; Ploux et al., 2007). The experiments were performed in duplicate and repeated twice.

2.10. The survival of honey bees fed sucrose syrup supplemented with lactic acid bacteria - the cage experiment

A collection of 1-day-old honey bees, *Apis mellifera carnica*, was obtained from apiary (Puławy, Poland) and kept in wooden cages (40 bees/cage) as described earlier (Ptaszyńska et al., 2016). The honey bees were fed for seven days with a fresh, daily prepared 35% sucrose syrup (w/v) supplemented with: (T1) (treatment 1) - *L. kunkeei* CH1 in the number of $\sim 2 \times 10^8$ cells per ml of sucrose syrup, (T2) - *F. fructosus* V5 in the number of $\sim 2 \times 10^8$ cells per ml of sucrose syrup, (T3) - 12 studied *L. kunkeei* strains, each in the number of $\sim 1.7 \times 10^7$ cells per ml of sucrose syrup, (T4) - *F. fructosus* VIII1 and *F. fructosus* V5 strains - each in the number of $\sim 1 \times 10^8$ cells per ml of sucrose syrup, (T5) - 12 studied *L. kunkeei* strains and two *F. fructosus* strains - each in the number of $\sim 1.4 \times 10^7$ per ml of sucrose syrup. Control honey bees (T6) were fed 35% sucrose syrup without lactic acid bacteria. Bacterial cultures, for use as a bee food supplements, were prepared daily by culturing bacteria in FYP broth at 30 °C as described by Pachla et al., (2018). In total, for each of six honey bee treatments 120 honey bees were used. The mortality of bees, fed sucrose syrup supplemented with lactic acid bacteria and sucrose syrup without lactic acid bacteria, was scored every second day during 24 days of experiment. This experiment was repeated two times. The obtained results were statistically analyzed by SAS software (2002–2003) using the ANOVA (a group and variant effects were the experimental factors) and the Tukey's honestly significant difference (HSD) test (SAS Institute 2002–2003).

We want to emphasize that cage experiments on the survival of honey bees fed sucrose syrup supplemented with LAB was carried out and was supervised by assoc. prof. Aneta Ptaszyńska. The experiment was performed in the Institute of Biological Sciences of Maria Curie-Skłodowska University as part of the Innovation

Incubator project entitled: "The probiotic preparation based on bacterial strains of *Lactobacillus* and *Fructobacillus* genera isolated from the digestive tract of honeybees". The results obtained in the framework of this experiment are subject to all legal regulations.

3. Results

Two selected strains, i.e.: *L. kunkeei* CH1 and *F. fructosus* V5, able to survive in 35% sugar syrup and resistant to pH 3.5–11 (Pachla et al., 2018), in studies in vivo with honey bees fed for 24 h 35% sugar syrup containing lactic acid bacteria and next, for 66 h only sugar syrup, tolerated well the changes in pH throughout the honey bee intestinal tract and survived well in honey bee guts. The number of viable lactic acid bacteria in the midgut remained constant for the first 48 h of the experiment. In the subsequent 42 h, the number of live *L. kunkeei* CH1 and *F. fructosus* V5 bacteria decreased (Table 1). At the beginning of this experiment (0 h) the LAB were not found in the midgut of honey bees. The lactic acid bacteria were also not found in the midgut of honey bees fed only sucrose syrup (control group) for the entire duration of experiment (0, 24, 48, 72 h) (data not presented).

Temporary persistence of *L. kunkeei* CH1 and *F. fructosus* V5 bacteria, in the honey bee midgut content, was also found in our *in vitro* studies (Fig. 1). Bacteria examined survived well in the midgut content, without significant reduction of their number for the first 12 h after adding of lactobacilli. In the subsequent 12 h, the survival rate of *L. kunkeei* CH1 and *F. fructosus* V5 strains decreased dramatically to $\sim 17\%$ and $\sim 10\%$, respectively. It must be emphasized that the midgut contents of honey bees from control group (fed sugar syrup without LAB) were free from lactic acid bacteria throughout the duration of experiment (0–24 h) (data not presented).

The another question posed in this research concerned the resistance of the probiotic candidates to three antibiotics used in beekeeping practice, i.e. ampicillin, erythromycin, and tylosin (Reybroeck et al., 2012).

In this study, 14 fructophilic lactic acid bacteria, originating from *A. mellifera* guts, were tested for susceptibility to ampicillin, erythromycin, and tylosin by using serial two-fold dilution method to estimate the Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC) of antibiotics (Tejchman et al., 2017). Additionally, the mode of drug action, i.e. bacteriostatic (MBC/MIC > 4) or bactericidal one (MBC/MIC ≤ 4) was determined (Mogana et al., 2020). Overall, 12 *L. kunkeei* and 2 *F. fructosus* strains showed the high susceptibility to all three antibiotics used with MIC of 0.25–1, 0.25–05, 0.5–4 µg/ml for ampicillin, erythromycin, and tylosin, respectively (Table 2). All three drugs used exhibited bactericidal activity against most studied honey bee endosymbionts (MBC/MIC = 1–4) and bacteriostatic effect of ampicillin and erythromycin against *L. kunkeei* CH2 and *L. kunkeei* CH1 strains, respectively (MBC/MIC = 16) (Table 2).

The lactic acid bacteria were further evaluated for cell surface hydrophobicity associated with bacterial adhesion capacity to biotic and abiotic surfaces (Doyle, 2000; Goulter et al., 2009; Janashia et al., 2016). Bacterial cell surface hydrophobicity was determined according to simple MATH method based on bacterial adherence to the n-hexadecane (alkane hydrocarbon) (Rosenberg, 1984; Vinderola and Reinheimer, 2003). The cell hydrophobicity of the 13 strains ranged from 84% to 99%. Only one strain (*L. kunkeei* VI) showed lower hydrophobic cell surface, i.e. 77% (Fig. 2).

Other preliminary criterion used for selection of lactic acid bacteria with probiotic potential was their ability to form multicellular aggregates composed of the same strain cells (auto-aggregation,

Table 1
Survival of LAB in *A. mellifera* midgut (CFU/midgut) – test *in vivo*¹.

Strain	18 h	24 h	42 h	48 h	66 h	72 h	90 h
CH1^a	5.03x10 ⁵ ± 1.00x10 ⁵	6.50x10 ⁵ ± 5.57x10 ⁴	5.67x10 ⁵ ± 3.51x10 ⁴	4.47x10 ⁵ ± 4.04x10 ⁴	5.27x10 ⁴ ± 7.02x10 ³	2.93x10 ⁴ ± 4.51x10 ³	1.73x10 ⁴ ± 3.06x10 ³
V5^b	3.45x10 ⁵ ± 5.89x10 ⁴	5.47x10 ⁵ ± 5.03x10 ⁴	3.10x10 ⁵ ± 5.57x10 ⁴	4.07x10 ⁵ ± 3.06x10 ⁴	5.43x10 ⁴ ± 6.51x10 ³	1.70x10 ⁴ ± 9.54x10 ³	1.23x10 ⁴ ± 3.21x10 ³

¹ Values are means ± SD;

^a *L. kunkei*;

^b *F. fructosus*

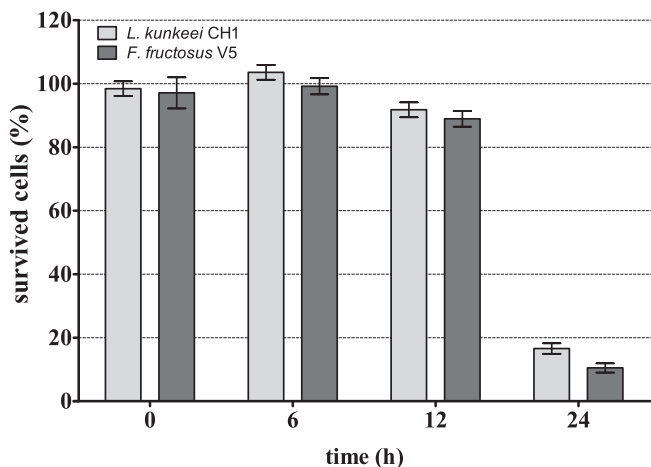


Fig. 1. Survival rate (%) of LAB in *A. mellifera* midguts – test *in vitro*. The values are means and error bars represent standard deviations.

self-aggregation) or the cells of genetically different strains (co-aggregation) (Honey Chandran and Keerthi, 2018; Nikolic et al., 2010). The tested *A. mellifera* gut isolates, i.e. six strains representing *L. kunkei* and two classified as *F. fructosus* showed ability to auto-aggregation. The LAB self-aggregation increased as a function of time and was the highest at 24 h of bacteria incubation at room temperature (18–20 °C) in PBS, i.e. ranged between 24.3 and 37.8% in the case of *L. kunkei* strains and between 42.2 and 46.5% in the case of *F. fructosus* bacteria (Table 3). The co-aggregation capacities of *A. mellifera* symbionts with potential pathogenic strains increased also along with the incubation time and achieved the highest values after 24 h of these bacteria mixture incubation in PBS at room temperature. The co-aggregation efficiency was dependent not only on lactic acid bacterial strain but was also dependent on potential pathogenic strain. It was especially high between: *F. fructosus* V5 × *E. coli* (79.7%), *F. fructosus* VIII1 × *E. coli* (77.9%), *F. fructosus* V5 × *P. larvae* (75.4%) after

24 h of bacteria incubation at room temperature in PBS, particularly low between all studied lactic acid bacteria and *K. pneumoniae*, and low between *L. kunkei* Z3 and *E.coli* after 24 h of experiment (Fig. 3).

In this paper the biofilm formation ability of lactic acid bacteria isolated from intestinal tract of healthy honey bees and structure of biofilms formed by them were determined in experiments *in vitro*. Bacteria were grown in polystyrene microtiter plates at 30 °C without shaking as described in materials and methods (2.8., 2.9.). The ability of LAB to biofilm formation was evaluated by quantitative technique using crystal violet to stain bacteria (Christensen et al., 1985) and qualitative method using for visualization of bacteria, analysis of their viability and investigation of the structure of biofilm formed by them, two fluorescent dyes, i.e. SYTO-9 to identify live bacterial cells with intact DNA and propidium iodide to stain dead cells with damaged membrane (Boulos et al., 1999).

The ability to form biofilm was assayed first by measuring the optical density of crystal violet stained bacteria adsorbed to hydrophobic surface of microtiter plates (Fig. 4). Mass of biofilm formed by LAB, measured by the absorbance at 600 nm, was similar in all studied strains grown in FYP medium with 1 or 5% of fructose but bacterial cell density was twice higher in biofilms formed by LAB grown in broth with 5% than in biofilms of bacteria grown in FYP medium with 1% fructose. Based on the biomass of biofilm formed by studied lactic acid bacteria (OD_{600 nm}) *L. kunkei* and *F. fructosus* honey bee gut symbionts were classified as strongly adherent to substratum, according to Christensen et al., (1985).

In the next experiment, biofilms formed by five *L. kunkei* and two *F. fructosus* honey bee endosymbionts were visualized by confocal laser scanning microscopy (CLSM) using LIVE/DEAD stains, specific for live or dead cells, respectively (Boulos et al., 1999). Observation of 48 h old biofilms by confocal microscopy revealed their early step formation, mainly with one-two layers of cell aggregates joined to polystyrene surface of microtiter plate, in some places biofilms were composed of three or more layers of bacterial cell aggregates (Supplementary Figure 1). The size of bio-

Table 2
Antibiotic susceptibility of LAB to ampicillin, erythromycin, and tylosin.

Antibiotic	Strain	Strain														
		III1 ^a	VI1 ^a	VI3 ^a	VI4 ^a	VI6 ^a	VI4 ^a	CH1 ^a	CH2 ^a	CH3 ^a	Z1 ^a	Z3 ^a	Z5 ^a	V5 ^b	VIII1 ^b	<i>L. acidophilus</i> ATCC 4356
Ampicillin	MIC (ml)	0,25	0,5	0,5	0,5	0,5	0,5	0,5	0,5	1	0,5	0,5	0,5	0,5	0,5	0,25
	MBC (ml)	0,5	0,5	0,5	0,5	0,5	0,5	1	8	1	1	1	0,5	0,5	0,5	0,5
	MBC/MIC	2	1	1	1	1	1	2	16	1	2	2	1	1	1	2
Erythromycin	MIC (ml)	0,5	0,25	0,25	0,5	0,25	0,25	0,5	0,25	0,5	0,5	0,25	0,25	0,5	0,25	
	MBC (ml)	2	1	0,5	1	0,5	0,5	8	1	2	2	2	0,5	0,5	0,5	
	MBC/MIC	4	4	2	2	2	2	16	4	4	4	2	2	1	2	
Tylosin	MIC (ml)	2	1	1	2	2	1	2	2	4	2	2	0,5	2	2	1
	MBC (ml)	4	2	2	4	2	2	4	2	8	4	4	2	4	4	2
	MBC/MIC	2	2	2	2	1	2	2	1	2	2	2	4	2	2	2

MIC - minimum inhibitory concentration; MBC - minimum bactericidal concentration

^a *L. kunkei*;

^b *F. fructosus*;

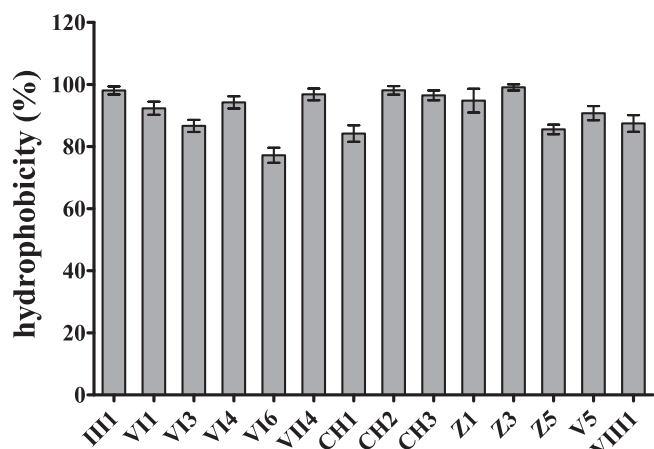


Fig. 2. Hydrophobicity (%) of *L. kunkeei* (III1, VI1, VI3, VI4, VI6, VII4, CH1, CH2, CH3, Z1, Z3, Z5) and *F. fructosus* (V5, VIII1) strains to n-hexadecane. The values are means and error bars represent standard deviations.

film was measured as the area colonized by bacterial aggregates and the height of aggregated bacteria (Table 4). Some differences between bacteria in surface covered with biofilm were noted; with minimum of surface coated with biofilm of 40.89% and maximum comprising 76.39% of plate substratum. The differences in the height of the biofilm formed by studied bacteria were also noted and the mean and the maximum biofilm heights are presented in Table 4.

Viability of lactic acid bacteria in biofilm formed in polystyrene microplates was assessed by CLSM using LIVE/DEAD staining of bacteria. Green fluorescence pointed out that bacteria were viable, red one was indicator of dead cells. The percentage of live cells in biofilms formed by *L. kunkeei* bacteria was very high (94.92–96.20%) compared to that in the biofilm created by *F. fructosus* strains (22.11 – 36.93%) (Table 4).

The last question posed in this paper concerned the effect of *A. mellifera* endosymbiotic LAB on the longevity and survival rate of healthy honey bees kept in cages, in laboratory conditions. Supplementing of the honey bee diet with lactic acid bacteria, naturally present in the bee gut, promoted the honey bee survival and significantly decreased the bee mortality compared to the mortality of control bees fed sugar syrup without LAB (Fig. 5). The honey bees fed 35% sucrose syrup without LAB exhibited a high mortality (80% ± 0.48%) after 24 days of experiment. The mortality percentage of honey bees, fed sugar syrup containing lactic acid bacteria, ranged from 51.7% to 56.7%. The especially low honey bee mortality was found when bees were fed sugar diet containing *L. kunkeei* CH1 strain (51.7%).

Table 3
Auto-aggregation (%) of LAB strains presented as a function of time¹.

Strain	1 h	2 h	4 h	6 h	24 h
VI1 ^a	1,1 ± 0,40	2,6 ± 0,62	4,9 ± 0,67	6,3 ± 0,76	24,3 ± 3,86
VI3 ^a	2,7 ± 0,72	6,7 ± 0,45	9,2 ± 0,80	10,0 ± 0,30	30,0 ± 1,45
VII4 ^a	1,9 ± 0,57	3,5 ± 0,82	6,2 ± 1,48	7,0 ± 1,05	31,1 ± 2,67
CH1 ^a	0,4 ± 0,53	2,6 ± 0,57	4,5 ± 0,70	6,0 ± 0,42	36,4 ± 1,57
CH3 ^a	1,8 ± 0,40	2,3 ± 0,30	5,1 ± 1,21	7,3 ± 0,83	33,1 ± 2,95
Z3 ^a	0,4 ± 0,51	2,9 ± 0,36	5,1 ± 0,40	5,6 ± 0,55	37,8 ± 2,11
V5 ^b	0,7 ± 1,21	9,1 ± 0,85	17,6 ± 0,66	21,2 ± 1,05	42,2 ± 1,31
VIII1 ^b	9,2 ± 1,17	22,0 ± 1,05	28,4 ± 1,13	31,1 ± 0,39	46,5 ± 1,73

¹ Values are means ± SD;

^a *L. kunkeei*;

^b *F. fructosus*

4. Discussion

One of the most important global problem in keeping of honey bees, the key pollinators of agricultural and horticultural crops, is spreading of pathogens in honey bee colonies (Crenna et al., 2017; Klein et al., 2007). The high density of honey bees within the colony, the trophallaxis among members of a community, and feeding of the honey bee larvae by the nurse bees favor horizontal transmission of the diseases among honey bee individuals (Chen et al., 2006; Forfert et al., 2015). For these reasons, the use of probiotics, as alternative to antibiotics, in preventing and combating diseases in honey bees, is of particular importance. Probiotics are defined as live, safe microorganisms, which positively affect a health and promote a longevity of their hosts (AFRC and FULLER, 1989; FAO/WHO, 2002). It was found that probiotic bacteria function better, when they are used as supplements in the diet of organisms from which they were originally isolated (Ptaszyńska et al., 2016; Saarela et al., 2000). In the present study, fructophilic lactic acid bacteria isolated from the intestinal tract of healthy Polish honey bees (Pachla et al., 2018) were assessed for the functional probiotic properties and beneficial effects on the health and the longevity of honey bees.

Important features of probiotic bacteria, used as a honey bee food supplement, is their ability to survive a passage through the bee intestinal tract (Gaggia et al., 2018; Kumar and Kumar, 2015; Zuo et al., 2016). Studied *L. kunkeei* bacteria, which are resistant to low pH and dominant members of midgut of summer honey bees, were expected to survive well in the bee intestinal tract after their reintroduction (Pachla et al., 2018). It is worth underlining, as some studies showed, that probiotic bacteria usually disappear from the intestinal tract within a couple of weeks after discontinuation of probiotic administration and that “permanent colonization of gut is seldom, if ever occurs” (Alander et al., 1999; Duncan, 2013).

It is known that the usage of antibiotics in beekeeping is prohibited in the EU Member States, with zero tolerance for the presence of antibiotics in honey (Reybroeck et al., 2012). However, some EU Member States (Belgium, France), as well as UK, Switzerland, USA, Canada, India, Argentina have established limits for antibiotics presence in honey and accepted the use of some antibiotics for treatment American and European foulbrood, and noseosis in honey bee colonies (Reybroeck et al., 2012).

According to the updated guidance document of European Food Safety Authority (EFSA) (EFSA 2012), obligate heterofermentative *L. kunkeei* strains studied were defined as susceptible to ampicillin and erythromycin based on the cut-off values allowing to distinguish bacteria with antibiotic acquired resistance from antibiotic susceptible ones. The data from the serial two-fold dilution experiment showed that the growth of studied lactobacilli was inhibited at the minimum concentrations of ampicillin and erythromycin,

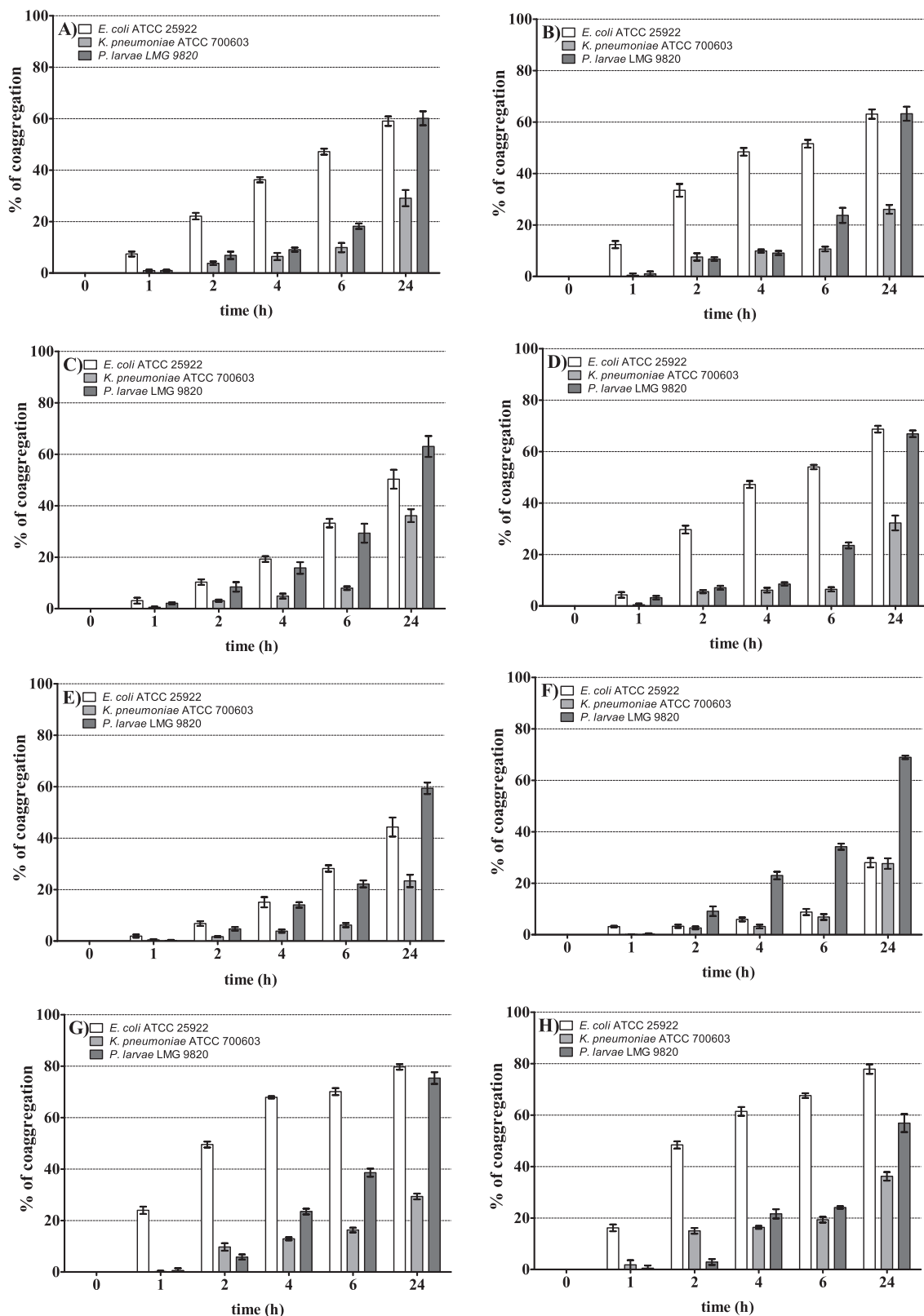


Fig. 3. Co-aggregation (%) of LAB strains: (A) *L. kunkeei* V11, (B) *L. kunkeei* VI3, (C) *L. kunkeei* VII4, (D) *L. kunkeei* CH1, (E) *L. kunkeei* CH3, (F) *L. kunkeei* Z3, (G) *F. fructosus* V5, (H) *F. fructosus* VIII1 with *E. coli*, *K. pneumoniae* and *P. larvae* presented as a function of time. The values are means and error bars represent standard deviations.

equal or lower than the cut-off values for these two drugs established by EFSA ($\leq 2 \mu\text{g/ml}$ for ampicillin and $\leq 1 \mu\text{g/ml}$ for erythromycin) (Table 2) (EFSA 2012). In EFSA guidance document (EFSA 2012) there is no information on tylosin cut-off values for

heterofermentative lactobacilli but MIC values of these bacteria allow to categorize them as tylosin susceptible. The high antibiotic susceptibility of all studied fructophilic lactic acid bacteria recommends these microbes for using as a honeybee food supplements

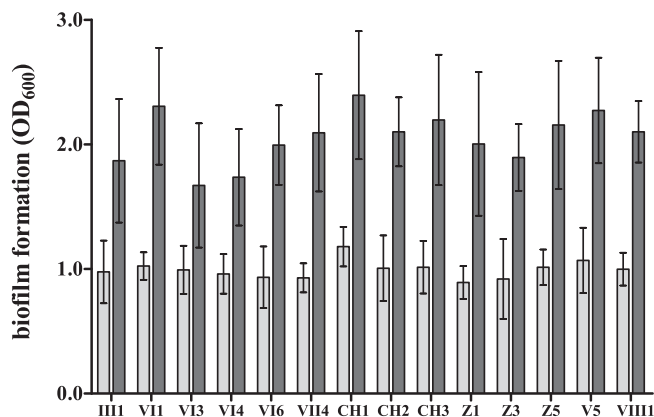


Fig. 4. Biofilm formation of *L. kunkeei* (III1, V11, V13, V14, V16, VII4, CH1, CH2, CH3, Z1, Z3, Z5) and *F. fructosus* (V5, VIII1) strains on FYP medium with 1% (light grey bars) or 5% (dark grey bars) fructose, quantified by crystal violet assay. The values are means and error bars represent standard deviations.

and points also to a detrimental role of these antibiotics for honey bee by the depletion of lactic acid bacteria number in the gut, which in turn leads to higher risk of bacterial and fungal diseases (Daisley et al. 2020; Forsgren et al. 2010; Honey Chandran and Keerthi 2018).

Recently, many researchers have shown that hydrophobic surface of bacterial cells play an essential role in bacteria aggregation, adhesion to the host cells, and is important cell property for the screening of probiotic bacteria (Doyle, 2000; Feng et al., 2017; Krasowska and Sigler, 2014; Nikolic et al., 2010; Rosenberg and Kjelleberg, 1986; Schiffer et al., 2019; Tuo et al., 2013). *A. mellifera* FLAB studied showed high cell surface hydrophobicity, that is, fulfil the next selection criterion for their use as probiotics. These bacteria exhibited also auto- and co-aggregation capacities regarded as valuable features for biofilm formation and inhibition of pathogens adhesion to the bee gut cells (Table 3, Fig. 3). The auto- aggregation and co-aggregation abilities of lactic acid bacteria, together with their antimicrobial activities and LAB adherence ability to the intestinal cells are desirable probiotic features in the removal of pathogens from the epithelium of honey bee gastrointestinal tract and all these features should be taken into account in the selection of potential probiotic lactic acid bacteria (FAO, 2001).

Interestingly, bacterial surface hydrophobicity together with aggregation are considered to be beneficial properties for biofilm formation. Biofilm, is defined as microbial community enclosed in a self-synthesized extracellular polymeric substances (EPS) composed of polysaccharides, proteins, and DNA and attached to biotic or abiotic surfaces (Costerton et al., 1995). Its development starts with the attachment of planktonic bacteria to a surface followed by cell division and formation of a complex three-dimensional biofilm structure composed of bacterial microcolonies embedded in EPS and separated by channels (Stanley and

Lazazzera, 2004). The biofilm formation capacity of LAB promotes the colonization of the gut of their hosts and prevents the adhesion of pathogens to intestinal tract by their competitive exclusion (Berríos et al., 2018; Faten et al., 2016; Gómez et al., 2016; Jalilsood et al., 2015; Tuo et al., 2013; Woo and Ahn, 2013; Wu et al., 2013).

The biofilm-forming ability of FLAB was documented by using quantitative test described by Christensen et al., (1985) and biofilm architecture was revealed by CLSM using LIVE/DEAD stains (Fig. 4; Table 4; Supplementary Figure 1). It is worth to pointing out that ability of lactic acid bacteria to form biofilm is considered to be important and desirable feature for selection of probiotic strains (Berríos et al., 2018; Saarela et al., 2000; Shokri et al., 2018) and therefore, studied honey bee gut endosymbionts were further studied for their health beneficial function and possibility to use them as *A. mellifera* feed supplements.

A positive impact of lactic acid bacteria on the longevity and mortality of honey bees, fed sucrose syrup supplemented with LAB, was supported in cage experiment carried out in laboratory conditions (Fig. 5). The positive effect of the gut-dwelling lactic acid bacteria on the survival of honey bees may be associated with improving gut microbial homeostasis, elimination of intestinal pathogens, stimulation of immune system, and with their participation in the host food digestion process (Evans and Lopez, 2004; Janashia et al., 2016; Olofsson and Vásquez, 2008; Servin, 2004; Vásquez et al., 2012).

Additionally, in order to be sure about the health beneficial function of examined honey bee gut endosymbionts and possibility to use them as *A. mellifera* feed supplements further studies will be undertaken in beekeeping practice.

5. Conclusion

The studies presented in this paper indicate that tested lactic acid bacteria, isolated from intestinal tract of honey bees, have a great probiotic potential for using them as *A. mellifera* food supplements but further investigations on *A. mellifera* colonies in apiaries are necessary to confirm the honey bee health-promoting properties of these bacteria. Indispensable are also studies supporting that lactic acid bacteria survive and retain functionality during manufacturing of probiotics under industrial conditions and during storage as probiotic products.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.sjbs.2020.12.040>.

Table 4
The parameters of biofilms formed by LAB¹.

Strain	V13 ^a	VII4 ^a	CH1 ^a	CH3 ^a	Z3 ^a	V5 ^b	VIII1 ^b
Ratio of live/dead cells	25.29 ± 1.78	22.63 ± 5.69	20.57 ± 1.99	21.06 ± 4.21	18.69 ± 2.87	0.59 ± 0.47	0.28 ± 0.22
Depth of biofilm – average thickness (µm)	13.30 ± 1.99	14.08 ± 1.21	16.62 ± 0.92	11.14 ± 1.54	15.64 ± 0.95	11.73 ± 1.19	11.93 ± 0.93
Depth of biofilm – maximum thickness (µm)	14.86 ± 0.78	17.40 ± 0.63	17.21 ± 0.19	12.71 ± 0.47	17.40 ± 0.37	13.69 ± 0.33	13.49 ± 0.96
Area covered by biofilm (%)	48.30 ± 4.09	47.58 ± 7.19	76.39 ± 4.53	60.66 ± 3.91	51.18 ± 2.55	40.89 ± 2.93	61.95 ± 4.80

¹ Values are means ± SD;

^a *L. kunkeei*;

^b *F. fructosus*

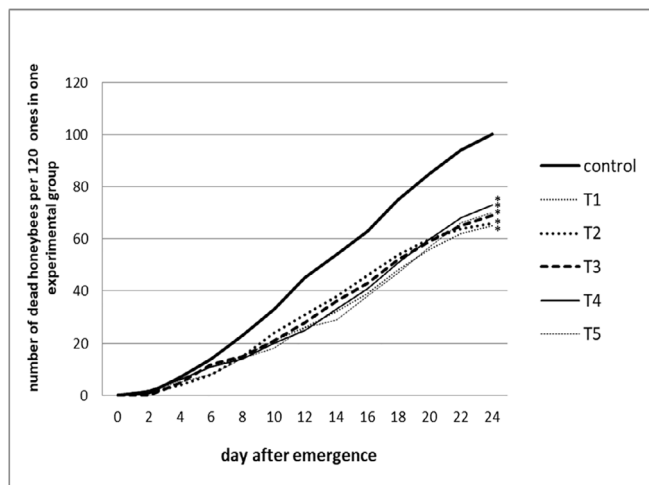


Fig. 5. Mortality of honey bees fed sucrose syrup supplemented with LAB – cage experiment. Honey bee groups treated with: (T1) - *L. kunkeei* CH1; (T2) - *F. fructosus* V5; (T3) – 12 studied *L. kunkeei* strains; (T4) - *F. fructosus* VIII1 and *F. fructosus* V5 strains; (T5) – 12 studied *L. kunkeei* strains and two *F. fructosus* strains. Control - sucrose syrup without lactic acid bacteria. Asterisks (*) indicates a statistically significant differences compared to control group ($p < 0.05$).

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