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Original article (Orijinal araştırma)

Isolation, identification and characterization of probiotic properties of bacterium from the honey stomachs of Yigilca honeybees in Turkey

Yığılca (Türkiye) bal arılarının bal midelerinden bakteri izolasyonu, tanımlanması ve probiyotik özelliklerinin karakterizasyonu

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Summary

Honeybees are considered as a key species in nature for their vital role in the maintenance of almost all life on earth. However, the massive death of honeybee stocks worldwide, largely due to colony collapse disorder, is causing international concern. In order to avoid these losses, new approaches must be sought. In previous studies, the probiotic properties of the bacteria found in the bodies of honeybees are thought to have an active role in providing resistance against pathogens. Consequently, in this study, it is aimed to isolate probiotic lactic acid bacteria from honey stomachs of the healthy honeybee, to examine the effect of these bacteria against pathogenic bacteria and to use these bacteria to boost the immune system of bees. For this purpose, between 2015 and 2016, probiotic bacteria were screened from honey bees that provided by DAGEM (Düzce University, Beekeeping Research, Development and Application Center, Yığılca, DÜZCE). The inhibitory activity of the obtained bacteria against the bee pathogen *Melissococcus plutonius* (Trüper and de 'Clari, 1998) (Enterococcaceae) was determined by *in vitro* agar well diffusion. The bacterium with the desired characteristics were identified by biochemical, physiological and 16s rDNA analysis as *Lactobacillus kunkeei* (Edwards, 1998) (Lactobacillaceae) and its probiotic nature was investigated. With the evaluation of these findings, future preparations of the isolate are expected to support the bee immune system and, as a result, to produce resistant honeybees without resorting to treatment with antibiotics.

Keywords: Bacterium, honeybee, isolation, *Lactobacillus kunkeei*, probiotics

Özet

Balarıları, dünyadaki hemen hemen bütün canlıların idamesi için hayati değer taşıyan anahtar türler olarak nitelendirilmektedir. Ancak, tüm dünyada bal arısı stoklarının büyük oranda kitlesel ölümlerine yol açan koloni çöküşü, uluslararası endişeye sebep olmaktadır. Bu kayıpları önlemek için yeni yaklaşımların ortaya çıkarılması gerekmektedir. Yapılan çalışmalarda, arıların vücutlarında bulunan probiyotik özellik taşıyan bakterilerin, arıların patojenlere karşı direnç sağlamasında aktif bir rol üstlenecekleri düşünülmektedir. Bu bağlamda, bu çalışmada sağlıklı arıların bal midesinden probiyotik özellikli laktik asit bakterilerinin izole edilmesi, bu bakterilerin arılarda hastalık etmeni patojenlere karşı etkisinin incelenmesi ve bu bakterilerin arıların bağışıklık sistemini güçlendirmek maksadıyla kullanılması amaçlanmıştır. Bu amaç doğrultusunda, 2015-2016 yılları arasında, DAGEM (Düzce Üniversitesi, Arıcılık Araştırma, Geliştirme ve Uygulama Merkezi, Yığılca, DÜZCE)' den temin edilen arıların bal midesinden probiyotik özellikli bakteri taraması yapılmıştır. Elde edilen bakterilerin arı patojeni *Melissococcus plutonius* (Trüper and de' Clari, 1998) (Enterococcaceae)' a karşı inhibisyon aktivitesi *in vitro* agar kuyu difüzyon metodu ile belirlenmiştir. Ardından istenen özelliklere sahip bakteri biyokimyasal, fizyolojik ve 16s rDNA analizi ile moleküler olarak *Lactobacillus kunkeei* (Edwards, 1998) (Lactobacillaceae) olarak tanımlanmış ve probiyotik doğası incelenmiştir. Sonuçlar değerlendirildiğinde, elde edilen izolatın gelecekte preparatlarının hazırlanarak, arıların bağışıklık sistemlerini destekleyeceği ve sonuçta, antibiyotikler ile kimyasal tedavi yöntemlerine başvurulmadan dirençli arıların üretileceği düşünülmektedir.

Anahtar sözcükler: Bakteri, balarısı, izolasyon, *Lactobacillus kunkeei*, probiyotikler

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Introduction

Honeybees produce honey, beeswax, royal jelly, propolis and bee venom. Although they provide these exceptional products to human beings, even more importantly, bees, together with wasps and hornets, carry out pollination in cultivated crops requiring cross pollination and thus ensure the superior quantity and quality of the plants (Crane & Walker, 1984; Free, 1993; Özbek, 2002).

In this context, honeybees, as the most important pollinator insects worldwide (Corby-Harris et al., 2014), hold an exceptional position in Turkish agriculture. Bees are considered a key species in nature because of their vital role in the maintenance of almost all life on earth (Özbek, 2002). Apiculture is practiced widely throughout the world and has always had an important place in agriculture. Turkey, having a favorable climate and rich vegetation, is very suited to beekeeping.

Colony collapse disorder (CCD), however, has led to a large mass die-off of commercial honeybee stocks worldwide, causing international concern (Cox-Foster et al., 2007; Rangberg et al., 2015). Colony collapse disorder is characterized by the rapid death of adult bees. Although the reason for this mass die-off is not clearly understood, it is known to be caused by a number of pathogens (Cornman et al., 2012; Rangberg et al., 2015). Regrettably, in this regard, it must be emphasized that the density of the bee population in Turkey has been declining significantly over about 30 years (Özbek, 2002). Consequently, bee diseases have begun to receive increasing attention in recent years.

One of the biggest problems in beekeeping is attempting to treat honeybee diseases with chemical treatments. Limited success is achieved after chemical treatment, and there are problems, such as a danger to human health with chemical residues in the honey (Barganska et al., 2011). Problems are experienced in export markets for honey from treated bees, so attempts are made to sell this honey in the domestic market. As a result, the products cannot be sold easily and at their proper value.

At the same time, in order for the honeybees to be healthy, under the chemical treatment practices, antibiotics are frequently used against bacterial diseases (Mutinelli, 2003). Antibiotic use weakens the immune system of the bees and leads to antibiotic-resistant bacterial pathogens (Doğaroğlu & Samancı, 2006; Barganska et al., 2011). Unfortunately, the fight against these bacteria is self-defeating. In previous studies, positive effects have been shown by the resistance of the bacterial flora in the bodies of bees against disease (Gilliam, 1997).Thus, the idea arises that if the naturally occurring microbial flora in the bodies of the bees are supported, the bees may be more resistant to disease (Tajabadi et al., 2013) In particular, the bacteria with probiotic properties found in the honey stomachs or intestines of honeybees have been observed to provide resistance against other bee pathogens (Forsgren et al., 2010). Therefore, this study presents a new approach to combat bee diseases. Firstly, bacteria with potential probiotic properties were isolated from the honey stomach of healthy bees. Then the inhibitory activity of these bacteria against a bacterial pathogen of bees was evaluated.

A possible application of this study would be to use preparations of the probiotic bacterial isolate from the honey stomachs of healthy honeybees, identified as *Lactobacillus kunkeei* (Edwards, 1998) (Lactobacillaceae), in the beekeeping sector to support the bee immune system and accordingly, to produce resistant bees without resorting to antibiotic treatments.

Material and Methods

Sampling

The bees and all the standard bee products used in the study were provided by DAGEM (Düzce University, Beekeeping Research, Development and Application Center). The bee samples were brought to the laboratory in sterile 20-ml tubes containing 0.1% peptone-NaCl solution. After surface sterilization, the bee honey stomachs were removed in a sterile cabinet and the specimens were spread onto MRS agar and M17 agar, and incubated at 30-37ºC for 24-48 h. At the end of the incubation period, pure cultures were obtained by considering the colony morphologies and microscopic appearance. These pure cultures were stored at -20ºC in a 30%-glycerol MRS broth.

Identification of isolates

Identification of the bacterial strain was based on morphological, and biochemical characteristics, as described in Bergey's Manual of Systematic Bacteriology (Krieg & Holt, 1984). Gram staining, color and shape of the colonies were determined. For molecular identification of the strain, firstly, genomic DNA was extracted according to Sambrook et al. (1989). The PCR amplification of 16S rDNA genes using genomic DNA was performed using oligonucleotide primers (UNI16S-L: 5'-ATT CTA GAG TTT GAT CAT GGC TTC A-3' and UNI16S-R: 5'-ATG GTA CCG TGT GAC GGG CGG TGT TGT A-3') and then sequencing of the amplified DNA fragments was performed by Macrogene, Inc., Europe. Comparison of the 16S rDNA gene sequences with entries in the updated GenBank database was conducted using the BLAST program.

Phylogenetic analysis

The nucleotide sequences of the 16S rRNA genes were edited with EditSeq. The 16S rRNA gene sequences of the isolate from *Apis mellifera* L., 1761 and of six closely related strains (DAT822, Genbank Accession AB777210.1; NRIC 0778, AB559822.1; YH-15, NR_026404.1; 100-1, JQ009336.1; ANRIC 0777, B559821.1) were used in the phylogenetic analysis. The phylogenetic analysis was performed via the neighbor-joining method and carried out with MEGA 5.0 software (Tamura et al., 2004). The reliability of the phylogram was tested by the bootstrap analysis of 1000 replicates using MEGA 5.0.

Determination of resistance to low pH

Active bacterial culture (1 mL) was centrifuged at 10000 g for 5 min at 4ºC. The cells were then precipitated and the supernatant was removed. The bacterial pellet was suspended in 1 mL-solutions of PBS buffers with pH values of1.0, 2.0 and 3.0 and incubated for 3 h at 37ºC. Spectroscopic measurements were taken at 0 h and 3 h after the incubation (Maragkoudakis et al., 2006; Turhan Eryılmaz, 2011).

Determination of pepsin resistance

Active bacterial culture (1 mL) was centrifuged at 10 000 g for 5 min at 4ºC. The cells were then precipitated and the supernatant was removed. The bacterial pellet was suspended in PBS buffers containing pepsin (3 mg/mL, Merck, Kenilworth, NJ, USA) atpH values of 1.0, 2.0 and 3.0 and incubatedat 37ºC for 3 h. Spectroscopic measurements were taken at 0 h and 3 h after the incubation (Maragkoudakis et al., 2006; Turhan Eryılmaz, 2011).

Determination of pancreatin resistance

Active bacterial culture (1 mL) was centrifuged at 10 000 g for 5 min at 4ºC. The cells were then precipitated and the supernatant was removed. The bacterial pellet was suspended in PBS (pH 8.0) buffer containing pancreatin (1 mg/mL, Merck) and incubated at 37ºC for 3 h. Spectroscopic measurements were taken at 0 h and 3 h after the incubation (Maragkoudakis et al., 2006; Turhan Eryılmaz, 2011).

Determination of hemolytic activity

Bacterial cultures (18 h) were grown as stab cultures in a Columbia-agar medium containing 5% human blood. After incubation at 35ºC for 48 h, in the areas surrounding the colonies a bright-green zone was formed of α-hemolytic colonies and a clear zone of β-hemolytic, while the unformed zones were considered as γ-hemolytic (Maragkoudakis et al., 2006; Turhan Eryılmaz, 2011).

Determination of antibiotic susceptibility

An antibiotic disk diffusion method was used. The cell density of the isolate cultured at 37ºC for 18 h was adjusted to 10^6 and added to the solid medium. Antibiotic discs were then placed on the solid medium. After incubation at 30ºC for 18 h, the resistance or susceptibility was determined by measuring the diameter of the resulting inhibition zones (Wilkins et al., 1972; Turhan Eryılmaz, 2011).

Determination of antimicrobial activity

A well diffusion method was used. A 48-h bacterial culture was centrifuged at 13 000 g for 15 min. The supernatant was filtered with a 0.45-µm membrane, and stored at +4ºC for later use. Soft agar containing indicator bacteria (10^7 cells/mL) was poured into Petri dishes, allowed to solidify and wells opened. The culture supernatant (100 µL) was added to the wells. After sufficient incubation for indicator bacteria to develop, any inhibition zones were measured. The bacterial strains as indicator bacteria used in this study were obtained from the American Type Culture Collection (ATCC) and were as follows: *Enterococcus faecalis* ATCC 29212, *Salmonella typhimurium* ATCC 14028, *Klebsiella pneumonia* ATCC 13883, *Escherichia coli* ATCC 35218, *Proteus vulgaris* ATCC 13315, *Listeria monocytogenes* ATCC 7644, *Yersinia pseudotuberculosis* ATCC 911, *Pseudomonas aeruginosa* ATCC 27853, *Staphylococcus epidermidis* ATCC 12228, *Staphylococcus aureus* ATCC 25923, *Enterobacter cloacae* ATCC 13047, *Bacillus subtilis* ATCC 6633.

Determination of inhibition the bee pathogen, *Melissococcus plutonius*

The inhibitory activity of the isolate obtained in this study against *Melissococcus plutonius* (Trüper and de 'Clari, 1998) (Enterococcaceae), the cause of European foulbrood, was determined using the diffusion method of Padilla et al. (1996) in vitro. The bacterium was cultivated for 72 h in MRS broth at 30ºC. The culture was then centrifuged (10000g,15 min) and filtered with a 0.45-µm membrane. A10^6-CFU/mL suspension of *M. plutonius ATCC 35311* was spread to the surface of Columbia agar supplemented with blood. The wells were cut into the inoculated agar plates. Bacterial supernatants (100 µL) were placed in these wells. The appearance of the inhibition zone around the wells was determined after 16-18-h incubation.

Results

This study isolated bacteria with probiotic features from in the honey stomach of the Yigilca honeybee, which appears as a unique ecotype, in Düzce, Turkey (Kekeçoğlu, 2007, 2009). Priority was given to bacterium in the honey stomach that grew well on MRS agar. The isolate obtained (designated HD1) from the honey stomach formed a smooth, creamy, circular Gram-positive colony (Figure 1). Biochemical tests indicated it was indole (-), amylases (-) and catalase (-). The molecular diagnostics with PCR amplification (Figure 2) revealed it has 99% 16S rRNA sequence similarity with the six strains of *Lactobacillus kunkeei*, tested and a phylogenetic tree was constructed using these reference bacteria (Figure 3).

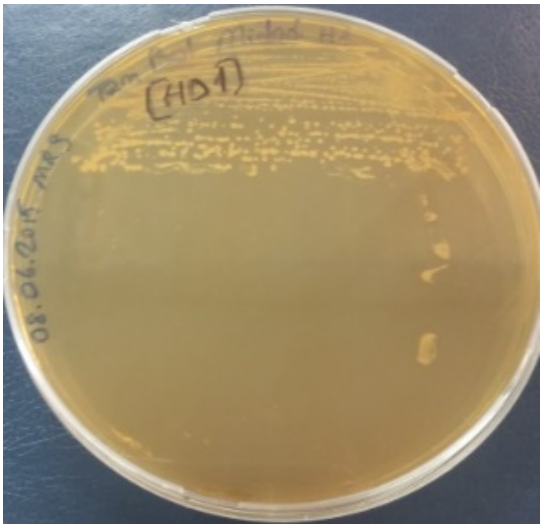

Figure 1. The isolate HD1 colony formation on MRS.

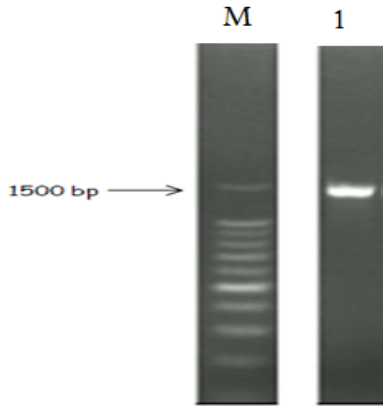

Figure 2. (1) PCR profiles of 16S rDNA fragments amplified from *Lactobacillus kunkeei* HD1 isolated from honeybee stomach, (M) marker.

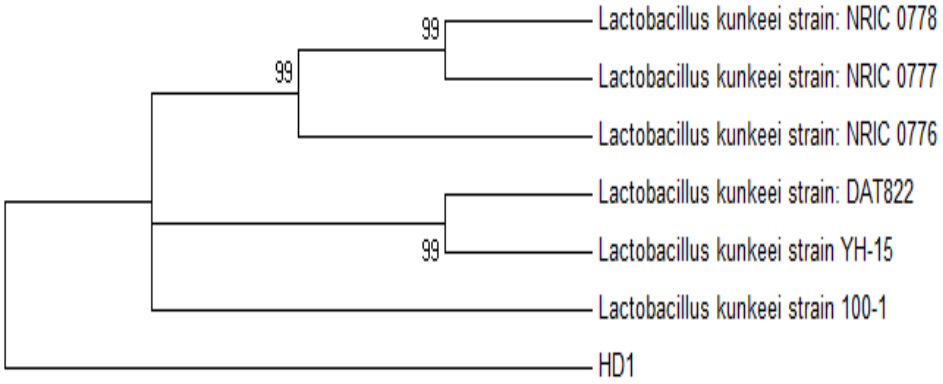

Figure 3. Neighbor-joining tree of isolate HD1.

Among lactic acid bacteria, the genus *Lactobacillus* is one of the most important and includes about 174 species (Rezvani et al., 2016). Showing a wide distribution in different habitats, *Lactobacillus* species are located in the gastrointestinal tract of bees and many other animals (Mitsuoka, 1996; Schrezenmeir & de Vrese, 2001; Tannock, 2004; Fujisawa &Mitsuoka, 1996).

The genus *Lactobacillus* is reported to be the most dominant species found in the honey stomach of bee specieslike *A. mellifera*, *Apis dorsata* Fab., 1793 and bumblebees (Olofsson & Vasquez, 2008; Vásquez et al., 2009; Tajabadi et al., 2011, 2013). In particular, *Lactobacillus* species as probiotics strengthen the immune system of bees against pathogens and have been found to help honeybees survive and to provide significant advantages for the health of honeybees (Evans & Lopez, 2004; Forsgren et al., 2010; Tajabadi et al., 2013).

In an earlier study, *Lactobacillus apinorum*, *Lactobacillus mellifer*, *Lactobacillus mellis*, *Lactobacillus melliventris*, *Lactobacillus kimbladii*, *Lactobacillus helsingborgensis* and *Lactobacillus kullabergensis* were isolated from the honey stomach of the honeybee, *A. mellifera* (Olofsson et al., 2014).

Lactobacillus kunkeei can be found in fructose-rich media like honey, bee bread, wine and flowers (Vásquez et al., 2012; Endo, 2012; Asenjo et al., 2016). In recent studies especially, *Lactobacillus kunkeei* is reported to have been isolated from the intestine of honeybees during the summer (Corby-Harris et al., 2014; McFrederick et al., 2014, Asenjo et al., 2016).

In the present study, for the first time in Turkey, *Lactobacillus kunkeei* was isolated from the honey stomach of the Yigilca honeybee. The possibility of using this isolate (HD1) in Turkey to render individual honeybees strong against diseases is promising.

HD1 was found to be substantially resistant to low pH values and highly resistant after being mixed with pepsin at pH 2 and 3. Moreover, HD1 was also found to be quite resistant against pancreatin (Figure 4). In order for bacteria with probiotic properties to reach the intestinal microbiota, they must be resistant to the acidic medium of the stomach they are required to pass through (Millette et al., 2008). HD1 was determined to be highly durable. Problematically, HD1 grows well during the first passages and tests, but slows down after 5-6 passages. CFU counts were made, but clear data were not obtained because HD1is too sensitive to growth. Observations were made only to confirm absorbance data (Figure 4). In particular, if the bacterial density is reduced, colonies do not form. If the bacterial density is increased, the bacteria survive and form colonies.

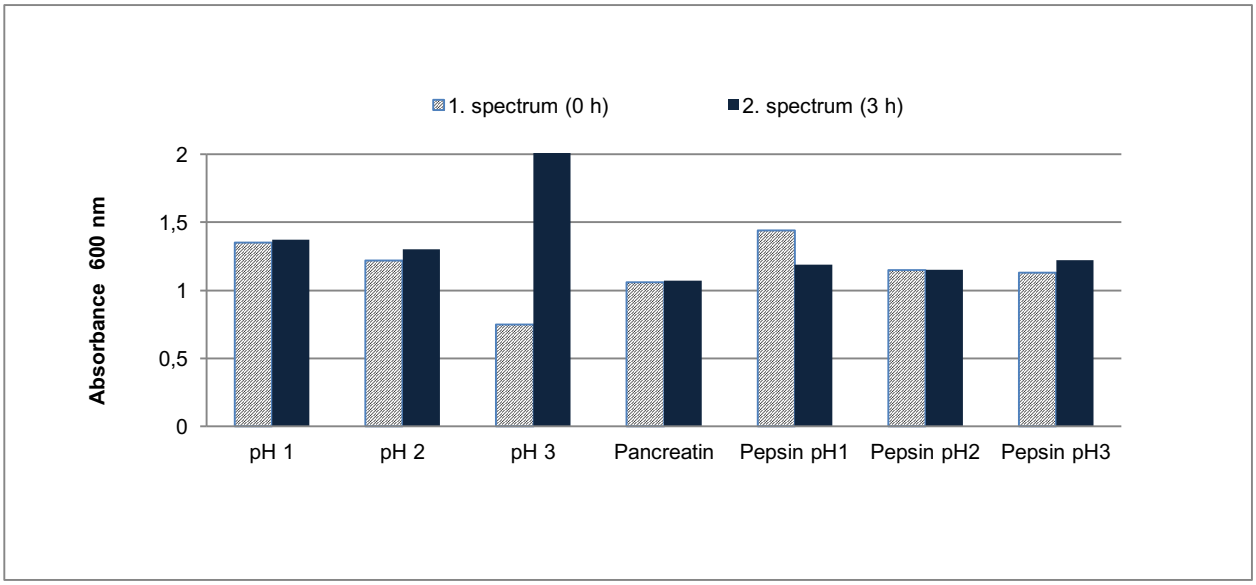

Figure 4. Tolerance to different conditions of *Lactobacillus kunkeei* HD1 isolated from honeybee stomach.

HD1 was found to exhibit γ-hemolytic activity and sensitive to multiple antibiotics (Table 1). Non-hemolytic activity and antibiotic resistance are prerequisites for the selection of probiotic strains (Hawaz, 2014). HD1 was found to be resistant to streptomycin (10 µg) and tobramycin (10 µg), and sensitive to azitromycin (15 µg), cefdinir (5 µg), ciprofloxacin (5 µg), gentamicin (10 µg), erythromycin (10 µg) and imipenem (10 µg).

Table 1. Antibiotic susceptibility of isolate HD1

Antibiotics and Inhibition zone diameter (mm)							
AZM	CD	CIP	CN	E	IPM	S	TOB
30	30	12	17	16	41	-	-

AZM: azitromycin (15 µg); CD: cefdınır (5 µg); CIP: ciprofloxacin (5 µg); CN: gentamicin (10 µg); E: erythromycin (10 µg); IPM: imipenem (10 µg); S:, streptomycin (10 µg); TOB: tobramycin (10 µg); -: no inhibition.

HD1 was determined to inhibit most of the indicator bacteria (Table 2). For two indicator strains, the inhibition was determined to be bacteriostatic because after 16-18 colonies formed in the initially large inhibition zones and the zones became smaller (Table 2).

Table 2. Inhibition activity of isolate HD1

Indicator Bacteria / Inhibition Zone (mm)											
(1)	(2)	(3)	(4)	(5)	(6)	(7)	(8)	(9)	(10)	(11)	(12)
-	-	BS	12	-	18	13	15	-	BS	-	-

1) *Enterococcus faecalis*; 2) *Salmonella typhimurium*; 3) *Klebsiella pneumonia*; 4) *Escherichia coli*; 5) *Proteus vulgaris*; 6) *Listeria monocytogenes*; 7) *Yersinia pseudotuberculosis*; 8) *Pseudomonas aeruginosa*; 9) *Staphylococcus epidermidis*; 10) *Staphylococcus aureus*; 11) *Enterobacter cloacae*; 12) *Bacillus subtilis*; BS: bacteriostatic activity; -: no inhibition.

In vitro, HD1 was found to be highly inhibitory (17 mm zone) to *Melissococcus plutonius,* the bacterial pathogen that causes European foulbrood in honeybees, a globally important honeybee brood disease (Haynes et al., 2013).

Discussion

It is suggested that preparations of the eco-friendly *Lactobacillus kunkeei* HD1*,* thanks to its probiotic properties, could be used in the beekeeping sector to support the bee immune system and produce resistant bees without resorting to treatment with antibiotics. It is hoped that future studies on the inhibitory activity of HD1 against *M. plutonius* may prove to be encouraging. Just as probiotics from fermented food products are thought to have broad application in pharmaceutical preparations (Salminen et al., 1998), this bacteria with probiotic properties might also have the potential to be used in a variety of applications.

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References

Asenjo, F., A. Olmos, P. Henríquez-Piskulich, V. Polanco, P. Aldea, J. A. Ugalde & A. N. Trombert, 2016. Genome sequencing and analysis of the first complete genome of *Lactobacillus kunkeei* strain MP2, an *Apis mellifera* gut isolate. PeerJ, 4: e1950, DOI:10.7717/peerj.1950.

Barganska, Z., M. Slebioda & J. Namiesnik, 2011. Determination of antibiotic residues in honey. Trends in Analytical Chemistry, 30 (7): 1035–1041.

Corby-Harris, V., P. Maes & K. E. Anderson, 2014. The bacterial communities associated with honey bee (*Apis mellifera*) foragers. PLoS ONE, 9 (4): e95056, DOI:10.1371/journal.pone.0095056.

Cornman, R. S., D. R. Tarpy, Y. Chen, L. Jeffreys, D. Lopez, J. S. Pettis, D. Van Engelsdorp & J. D. Evans, 2012. Pathogen webs in collapsing honey bee colonies. PLoS ONE 7(8): e43562, DOI:10.1371/journal.pone.0043562.

Cox-Foster, D. L., S. Conlan, Holmes, E. C. Palacios, G. Evans, J. D. Moran, N. A. Quan, P. L. Briese, T. Hornig, M. Geiser, D. M. V. Martinson, D. Van Engelsdorp, A. L. Kalkstein, A. Drysdale, J. Hui, J. Zhai, L. Cui, S. K. Hutchison, J. F. Simons, M. Egholm, J. S. Pettis & W. I. Lipkin, 2007. A metagenomic survey of microbes in honey bee colony collapse disorder. Science, 318: 283-287.

Crane, E. & P. Walker, 1984. Pollination Directory for World Crops, International Bee Research Association, London,183 pp.

Doğaroğlu, M. & T. Samancı, 2006. Balda Yörelere Göre Kalıntı Hile ve Orijin Tespit Projesi. Teknoloji ve Yenilik Destek Programları Başkanlığı (TEYDEB) Arıcılık Raporu, Ankara, Türkiye.

Endo, A., 2012. Fructophilic lactic acid bacteria inhabit fructose-rich niches in nature. Microbial Ecology in Health and Disease, 23: 10.3402/mehd.v23i0.18567, DOI:10.3402/mehd.v23i0.18563.

Evans, J. & D. Lopez, 2004. Bacterial probiotics induce an immune response in the honey bee (Hymenoptera: Apidae). Journal of Economic Entomology, 97: 752-756.

Forsgren, E., T. C. Olofsson, A. Vasquez & I. Fries, 2010. Novel lactic acid bacteria inhibiting *Paenibacillus* larvae in honey bee larvae. Apidologie, 41: 99–108.

Free, J. B., 1993. Insect Pollination of Crops, 2. Edition, Academic press, London, 684 pp.

Fujisawa, T. & T. Mitsuoka, 1996 Homofermentative *Lactobacillus* species predominantly isolated from canine feces. The Journal of Veterinary Medical Science, 58 (6): 591-593.

Gilliam, M., 1997. Identification and roles of non-pathogenic microflora associated with honey bees. FEMS Microbiology Letters, 155: 1-10.

Hawaz, E., 2014. Isolation and identification of probiotic lactic acid bacteria from curd and in vitro evaluation of its growth inhibition activities against pathogenic bacteria. African Journal of Microbiology Research, 8 (13): 1419-1425.

Haynes, E, T. Helgason, J. P. W. Young, R. Thwaites & G. E. Budge, 2013. A typing scheme for the honeybee pathogen *Melissococcus plutonius* allows detection of disease transmission events and a study of the distribution of variants. Environmental Microbiology Reports, 5: 525-529.

Krieg N. R. & J. G. Holt, 1984 Bergy's Manual of Systematic Bacteriology. Volume 1, Baltimore, The Williams and Wilkins Co., 964 pp.

Maragkoudakis, P. A., G. Zoumpopoulou, C. Miaris, G. Kalantzopoulos, B. Pot & E. Tsakalidou, 2006. Probiotic potential of *Lactobacillus* strains isolated from dairy products. International Dairy Journal, 16: 189-199.

McFrederick, Q. S., W. T. Wcislo, M. C. Hout & U. G. Mueller, 2014. Host species and developmental stage, but not host social structure, affects bacterial community structure in socially polymorphic bees. FEMS Microbiology Ecology, 88: 398-406.

Millette, M., F. M. Luquet, M. T. Ruiz & M. Lacroix, 2008. Characterization of probiotic properties of *Lactobacillus* strains. Dairy Science and Technology, 88 (6): 695-705.

Mutinelli, F., 2003. Practical application of antibacterial drugs for the control of honey bee diseases. Apiacta, 38: 149-155.

Olofsson, T. & A. Vasquez, 2008 Detection and identification of a novel lactic acid bacterial flora within the honey stomach of the honeybee *Apis mellifera*. Current Microbiology, 57: 356-363.

Olofsson, T. C., M. Alsterfjord, B. Nilson, E. Butler & A. Vásquez, 2014. *Lactobacillus apinorum* sp. nov., *Lactobacillus mellifer* sp. nov., *Lactobacillus mellis* sp. nov., *Lactobacillus melliventris* sp. nov., *Lactobacillus kimbladii* sp. nov., *Lactobacillus helsingborgensis* sp. nov. and *Lactobacillus kulla*bergensis sp. nov., isolated from the honey stomach of the honeybee *Apis mellifera*. International Journal of Systematic and Evolutionary Microbiology, 64: 3109–311.

Özbek, H., 2002. Arılar ve doğa. Uludağ Arıcılık Dergisi, 3 (2): 22–25.

Padilla, C., P. Brevis, O. Lobos & E. Hubert, 1996. Bacteriocin activity of *Pseudomonas* sp. on enteropathogenic bacteria in an artificial aquatic system. Letters in Applied Microbiology, 23: 371–374.

Rangberg, A., G. Mathiesen, G. V. Amdam & D. B. Diep, 2015. The paratransgenic potential of *Lactobacillus kunkeei* in the honey bee *Apis mellifera.* Beneficial Microbes, 6 (4): 513-523.

Rezvani, M., Mendoza, M., Koci, M. D., Daron, C., J. Levy & H. M. Hassan, 2016. Draft genome sequence of *Lactobacillus crispatus* C25 isolated from chicken cecum. Genome Announcements, 4 (6): e01223-16.

Salminen, S., C. Boyley, M. C. Boutron-Ruault & J. H. Cummings, 1998. Functional food science and gastrointestinal physiology and function. British Journal of Nutrition, 80: 147–171.

Sambrook, J., E. F. Fritsch & T. Maniatis, 1989. Molecular Cloning: A Laboratory Manual. (Cold Spring Harbor Laboratory Press, 1659 pp.

Schrezenmeir, J. & M. de Vrese, 2001. Probiotics, prebiotics, and synbiotics-approaching a definition. The American Journal of Clinical Nutrition, 73: 361-364.

Tajabadi, N., M. Makhdzir, S. Nazamid, M. Shuhaimi, B. Rasoul & M. M. Y. Abdul, 2013. Identification of *Lactobacillus plantarum*, *Lactobacillus pentosus* and *Lactobacillus fermentum* from honey stomach of honeybee. Brazilian Journal of Microbiology, 44 (3): 717-722.

Tajabadi, N., M. Mardan, M. Y. Abdul Manap, M. Shuhaimi, A. Meimandipour & L. Nateghi, 2011. Detection and identification of *Lactobacillus* bacteria found in the honey stomach of the giant honeybee *Apis dorsata*. Apidologie, 42: 642-649.

Tamura, K., M. Nei & S. Kumar, 2004. Prospects for inferring very large phylogenies by using the neighbor-joining method. Proceedings of the National Academy of Sciences, 101 (30): 11030-11035.

Tannock, G., 2004. A special fondness for lactobacilli. Applied and Environmental Microbiology, 70: 3189-3194.

Turhan Eryılmaz, F., 2011. Vajinal Sekresyondan İzole Edilen Laktik Asit Bakterilerine Ait Bazı Suşların Potansiyel Probiyotik Özelliklerin Belirlenmesi. Ankara Üniversitesi, Doktora Tezi, Ankara, 95 s.

Wilkins, T. D., L. V. Holdeman, I. J. Abramson & W. E. C. Moore, 1972. Standardized single-disc method for antibiotic susceptibility testing of anaerobic bacteria. Antimicrobial Agents and Chemotherapy, 1 (6): 451-459.

Vásquez, A., E. Forsgren, I. Fries, R. J. Paxton, E. Flaberg, L. Szekely & T. C. Olofsson, 2012. Symbionts as major modulators of insect health: Lactic acid bacteria and honeybees. PLoS ONE 7 (3): e33188, DOI:10.1371/journal.pone.0033188.

Vásquez, A., T. Olofsson, D. Sammataro & K. Hartfelder, 2009. A scientific note on the lactic acid bacterial flora in honeybees in the USA-A comparison with bees from Sweden. Apidologie, 40: 417-417.