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Identification of a Lactic Acid Bacterial flora within the honey intestinal tract of *Apis mellifera* from different regions of Bulgaria

ABSTRACT

In the present study, we analyzed LAB microflora in the intestinal tract of bees *Apis melifera* on the territory of Bulgaria. Sixteen isolates were collected from the honey stomach of the honeybee *Apis mellifera*. Poor growth was recorded when strains were incubated anaerobically in the presence of D-glucose as sole carbon source. All of isolated strain showed fructophilic characteristics fermenting fructose faster than glucose. The isolates were Gram-stained and tested for catalase reaction. The 16S rRNA genes from extracted DNA of bacterial colonies were amplified with polymerase chain reaction using universal primers 27F and 1492R and were sequenced. The 10 isolated strains yielded five distinct 16S sequences of *Lactobacillus plantarum, L. pentosus, L. iwatensis, L. kunkeei, Weissella Confusa*. DNA of sequenced strains were amplified with specific primers in order to confirm the genus of the samples LBMA-1 (CTCAAAACTAAACAAAGTTC) and R16-1 (CTTGTACACACCGCCCGTCA). Carbohydrate fermentation reactions were recorded using API 50CH. For detection of enzymes activities of strains was used API ZYM. These strains can be good candidates for potential application as probiotics in honeybees and also as natural food preservatives, which, in turn, may be useful in the food industry.

Key words: fructofilic lactic acid bacteria, *Apis melifera,, Lactobacillus kunkeei, Weissella confusa*

Introduction

Fructophilic lactic acid bacteria (FLAB) are a special group of lactic acid bacteria (LAB) which prefer fructose over glucose as growth substrate (Endo et al., 2009). They are found in fructose-rich niches, e.g. flowers and fruits. Moreover, the organisms can be found in fermented foods made from specific fruits, including wine, fermented cocoa beans and fermented durian-based condiments (Endo et al., 2012; Leisner et al., 2005; Papalexandratou et al., 2011). Fructobacillus spp. and Lactobacillus kunkeei are representatives of these microorganisms, and a few novel species have recently been classified as members of this interesting group (Endo et al., 2010; Endo et al., 2011).

Quite recently FLAB were found in the gastrointestinal tracts of several flower- or fructose-related insects, including bees, tropical fruit flies and giant ants (He et al., 2011; Koch & Schmid-Hempel, 2011; Thaochan et al., 2010), whose diets are fructose-rich. Of these insects, honeybees are economically and agriculturally important for honey production and especially for crop pollination, which links to human food production. However, despite the importance of these insects in nature and in our lives, populations of honeybees are reported to have decreased considerably during the last decade and to be still decreasing worldwide, mainly by reason of Colony Collapse Disorder (CCD) (Bromenshenk et al., 2010). To understand and to prevent the disorder, microbial interactions, both symbiotic and pathogenic, have recently been studied (McFrederick et al., 2012; Moran et al., 2012), and findings have indicated that honeybees carry specific microbiota dissimilar to other

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animals, including humans. FLAB, especially *L. kunkeei*, have been found to be one of the dominating bacterial species in several bees kept or captured in different regions (McFrederick et al., 2012; Vásquez et al., 2012). LAB have been successfully applied as probiotics to contribute to health in humans and various companion and farm animals (Berge & Wierup, 2012; Salminen & Isolauri, 2008). As LAB are important components in their gastrointestinal tract, with a reported impact on the intestinal barrier mechanism (Miyauchi et al., 2012), it is not surprising that LAB, especially FLAB, may be involved with bee health. In the present study, FLAB were isolated from honeybees. The isolates were biochemically characterized for future application.

Materials and Methods

Sample collection

Thirty bees were collected from each point of region of Bulgaria. The bees were dissected and their stomach and intestines were crushed and cultivated in broth.

Isolation of fructophilic LAB

One 100µl of the fortified culture in broth was transferred in to the new media – FYP and composed of (L_1) 10g D-fructose/D-glucose, 10g yeast extract, 5g polypeptone, 2g sodium acetate, 0.5g Tween 80, 0.2g $MgSO_4.7H_2O$, 0.01g $MnSO_4.4H_2O$, 0.01 g $FeSO_4.7H_2O$, 0.01g NaCl, 0.05g cycloheximide and 0.05g sodium azide (pH 6.8) and incubated at 30°C for 24 h. Colonies were selected based on shape, size streaked onto FYP agar to obtain pure cultures and Gram stained. Catalase activity was recorded using 3% (v/v) H_2O_2. Gram-positive and catalase negative isolates were inoculated into FYP broth and GYP broth (identical to FYP broth, but fructose replaced with 1%, w/v, glucose), respectively, and incubated anaerobically at 30°C of 24 h.

Grouping and identification of FLAB isolates

The genus affiliation of the strains was confirmed by PCR amplification with genus-specific primer pairs designed by (Dubernet et al., 2002) which included 21 mer primer LbLMA-1 (lactobacilli specific sequence) coupled with a 21 mer universal sequence R16-1 from the flanking terminal region of the 16S rRNA gene were then applied to determine the genera of the type strains. The PCR product was detected by 1.5% agarose gel electrophoresis.

Biochemical Characterization

Carbohydrate fermentation reactions were recorded using API 50CH (BIOMERIEUX), according to the manufacturer's instructions. Readings were taken daily for 7 days at 30°C. For detection of enzymes activities of strains was used API ZYM (BIOMERIEUX) according to the manufacturer's instructions. Growth characteristics on D-glucose and D-fructose and requirement of external electron acceptors for D-glucose dissimilation were determined in GYP broth. Oxygen utilization as an electron acceptor was determined in GYP broth under aerobic conditions on an orbital shaker (120rpm). Incubation was at 30°C for 7 days. Catalase activity was determined for aerobically cultured cells by the addition of 3% (v/v) H_2O_2 as described previously (Endo et al., 2009). Production of lactic acid, acetic acid, and ethanol from D-glucose was determined after 3days of growth at 30°C in FYP broth. Gas production from D-glucose was determined by using a Durham tube.

Results

In total, 14 strains of LAB were isolated from all intestinal tract of honeybees (Figure 1) All these strains grew very well in FYP broth but poorly in GYP broth, and all were thus regarded as FLAB and subjected to genotyping. All strains are catalase negative and Gram positive. The confirmation of the affiliation of the strains as member of the genus *Lactobacillus* was performed with lactobacilli genus-specific primer (LbLMA1) (Figure 2). Only 3 strains amplified an expected fragment of about 250bp. These results proved their belonging to the genus *Lactobacillus.* Carbohydrate fermentation reactions using API 50CH separate all strains in three groups (Figure 3). Detection of enzymes activities using API ZYM separate strains relatively three groups (Figure 4).

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Figure 1. *Intestinal tract of honeybee.*

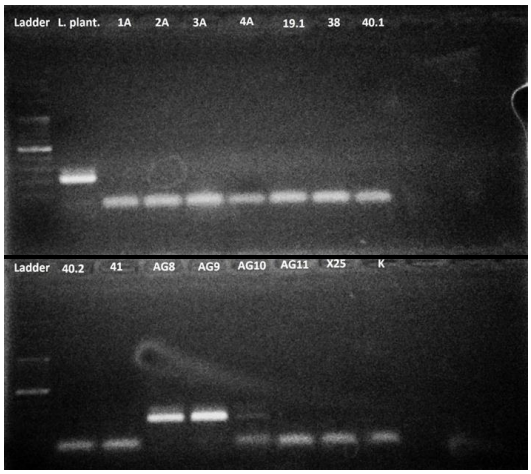

Figure 2. *Gel electrophoresis of PCR products amplified with lactobacili genus-specific primer (LbLMA1).*

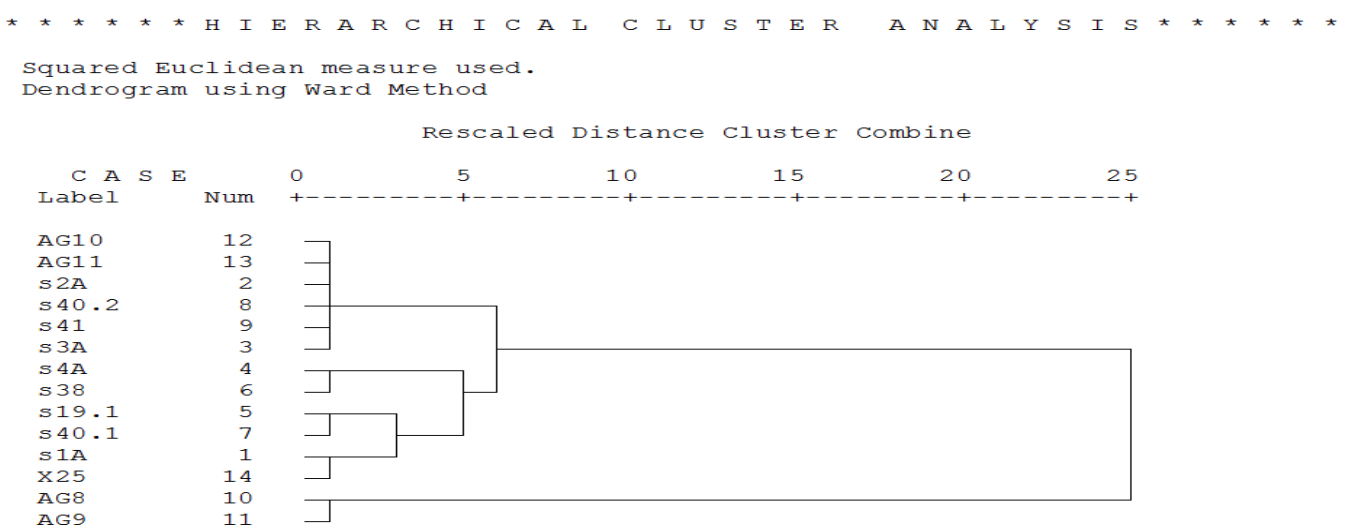

Figure 4. *API ZYM-dendogram.*

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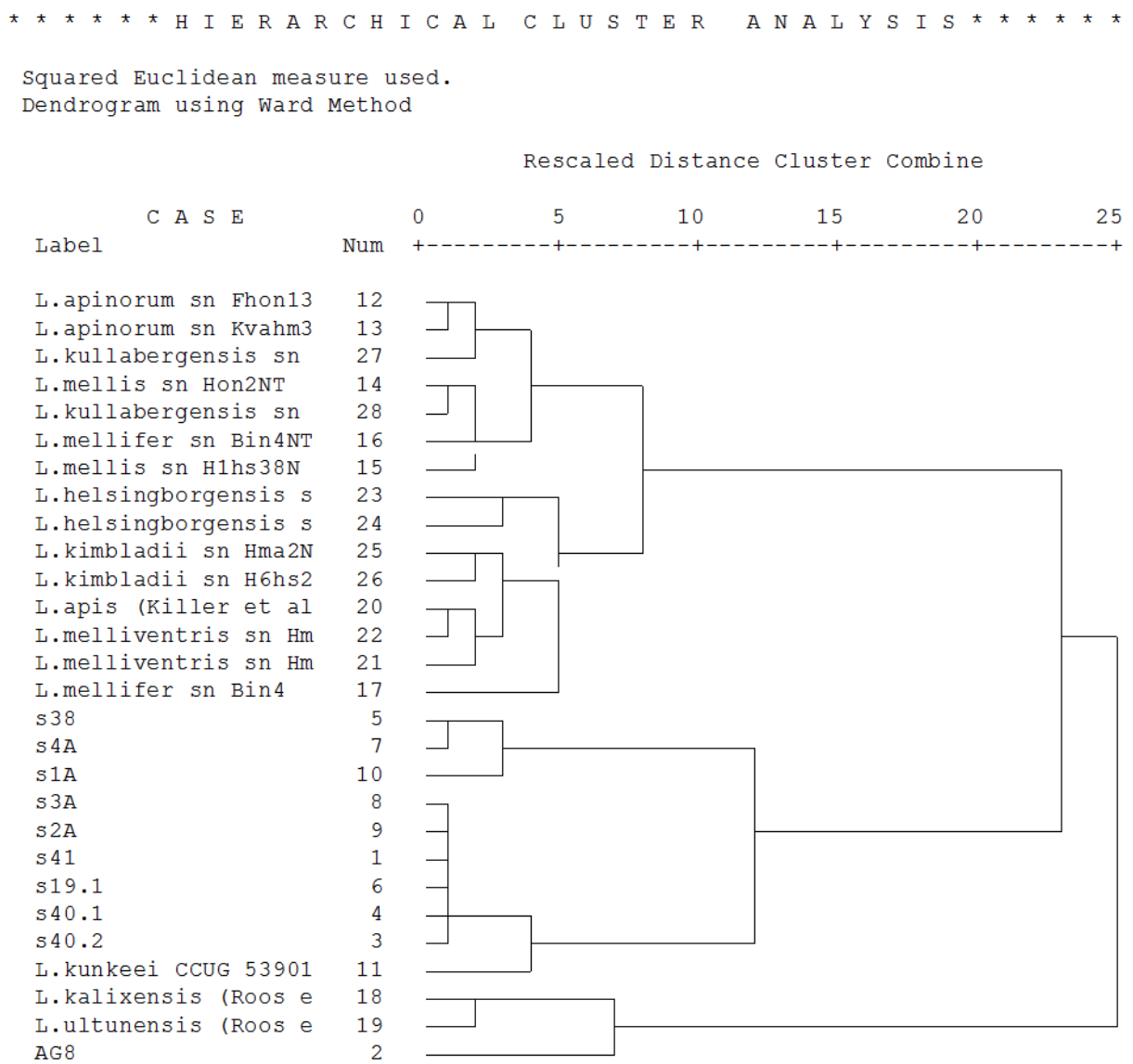

Figure 3. *API 50CH-dendogram.*

Discussion

FLAB are a newly identified and characterized LAB group, and their ecological role and potential amenable to applications have not as yet been well characterized. In the present study, we isolated FLAB strains from honeybees. This would suggest that honeybees and beehives are rich and effective sources for FLAB. All of the 14 FLAB isolates carbohydrate fermentation showed that strains are close to *Lactobaciluus kunkeei*. This result agrees with several previous finding whereby *L. kunkeei* has been found to be one of the most predominant LAB in honeybees (McFrederick et al., 2012; Neveling et al., 2012). *L. Kunkeei* is the FLAB species most frequently observed in nature and has been seen in flowers and fruit fermentation (Endo et al., 2009; Endo et al., 2012). To our knowledge, FLAB species have never hitherto been found in the intestinal tracts of vertebrates. This is consistent with the biochemical

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characteristics of FLAB; they prefer aerobic rather than anaerobic conditions for growth and cannot grow on glucose under anaerobic conditions. However, FLAB species also grow well under anaerobic conditions if fructose is supplied. In addition, FLAB have been found in several fructose-related insects, including bees (Koch & Schmid-Hempel, 2011; Neveling et al., 2012), suggesting that consumption of fructose as a major carbohydrate induces colonization of FLAB in the intestinal tract. There are few vertebrates consuming mainly flowers, fruits or high-fructose diets.

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