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Properties of different lactic acid bacteria isolated from *Apis mellifera* L. bee-gut

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Summary

Eight strains belonging to *Lactobacillus* spp. and five to *Enterococcus* spp. were isolated from the gut of worker *Apis mellifera* L. bees. Studies based on 16S rRNA sequencing revealed that AJ5, IG9, A15 and CRL1647 strains had a 99% identity with *Lactobacillus johnsonii*, while SM21 showed a 99% similarity with *Enterococcus faecium*. *L. johnsonii* CRL1647, AJ5 and IG9 were high lactic acid producers (values were between 177 and 275 mM), and *in vitro* they inhibited different human food-borne pathogens and *Paenibacillus larvae*, the American foulbrood agent. This bacterium was the most sensitive to the lactic acid effect being inhibited by 44 mM of this metabolite. *L. johnsonii* CRL1647, AJ5 and IG9 also presented important surface properties. These cells showed between 77% and 93% of auto-aggregation. The preliminary study of the chemical nature of the aggregating factors revealed that the molecules involved in the surface of each *L. johnsonii* strain were quite complex; and something of a peptidic nature was mainly involved. *E. faecium* SM21 produced bacteriocin-like compounds with anti-*Listeria* effects. Furthermore, a band close to 6.0–7.5 kDA was detected by SDS-PAGE studies, and the entA, B and P structural genes were amplified by PCR reactions. For the first time, bee-gut associated *L. johnsonii* and *E. faecium* strains have been isolated, identified, cultivated and some of their functional properties reported.

Introduction

The microbiota associated to the honeybee *Apis mellifera mellifera* L. is complex and far from being fully understood, or even known. It has been

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described as being mainly composed of yeasts, Gram-positive bacteria (such as *Lactobacillus rigidus apis*, *L. constellatus*, *Bacillus* spp., *Streptococcus* and *Clostridium*), and Gram-negative or Gram variable bacteria (*Achromobacter*, *Citrobacter*, *Enterobacter*, *Erwinia*, *Escherichia coli*, *Flavobacterium*, *Klebsiella*, *Proteus* and *Pseudomonas*) (Rousseau et al., 1969; Gilliam and Morton, 1978; Gilliam and Prest, 1987; Rada et al., 1997; Mohr and Tebbe, 2006). Gilliam (1997) reported that these bacteria were likely to be endemic in the alimentary tract of adult bees and were neither dependent on seasonal nor food factors. Glinski and Jarosz (1995), also suggests that this bees' normal microbiota comes from pollen consumption or through contact with older bees. The presence of intestinal enterobacteria in hibernating honeybees was also extensively discussed by Lyapunov et al. (2008).

Interestingly, only five studies have been carried out regarding the presence of lactic acid bacteria associated with honeybee microbiota, and their isolation and identification have yielded inconclusive results (Rada et al. 1997; Gilliam, 1997; Máchová et al., 1997; Kačániova et al. 2004, Olofsson and Vásquez 2008). In some cases, bacteria considered as probable members of *Lactobacillus* group, produced catalase and reduced nitrate (Gilliam, 1997). These properties are negative for this genus (Hammes et al. 1992). Kačániova et al. (2004) could not detect *Lactobacillus* spp. at all. Thus, only these scientific articles report the presence of culturable bee-gut *Lactobacillus*, *Bifidobacterium* or *Enterococcus* strains associated with the intestinal tract of the honeybee.

Lactic acid bacteria are important inhabitants of the intestinal tract of man and other mammalian and vertebrate animals. *Lactobacillus* and *Enterococcus* are members of this family and are also present in food and fermentation processes. These microorganisms disclose interesting properties not only for the food industry but also for health (Naidu et al., 1999). The antimicrobial potential of these bacteria includes, among others, the synthesis of compounds such as lactic acid, short-chain-volatile-fatty acids, and bacteriocin-like molecules (Jack et al. 1995; Wilson et al. 2005). Antagonistic studies are generally directed towards food-spoilage and/or pathogenic microorganisms related to the host or product from which the lactic acid bacteria were isolated. Different microorganisms affect the honeybee and the most serious bacterial agent may be *Paenibacillus larvae*, a sporulated Gram-positive bacillus that causes the American foulbrood disease in larvae (Williams, 2000). An indiscriminate antibiotic use against this bee pathogen has brought about not only chemoresistant strains, but also honey contamination (Evans, 2003).

The aim of this work was to isolate, characterize, cultivate, preserve and pheno and genotypically identify lactic acid bacteria associated to bee gut in order to improve the understanding of this microbial environment. Different properties were also analyzed such as their surface characteristics and *in vitro* antimicrobial spectra against the bacterial bee pathogen, and in a broader sense, against human foodborne pathogen bacteria.

Materials and methods

Honeybee intestinal tract sampling

Intestine samples of summer adult worker bees from different hives of El Galpón (Salta, Argentina) were used to obtain homogenates in physiological solution (0.85% w/w NaCl). The whole intestinal tracts (esophagus to rectum) were analyzed and aseptically handled to avoid contamination with the external surface of the bee body.

Culture media and growth conditions for lactic acid bacteria isolation

Known aliquots of the different homogenates were sown on the *Streptococcus* selective medium (MSS agar) devised in our laboratory (tryptone 1.4%; glucose 0.5%; sodium citrate 0.1%; NaCl 0.4%; sodium azide 0.022%; meat peptone 0.5%; sodium sulphite 0.022%; L-cysteine 0.022%; pH 6.5; Audisio et al., 2005), on MRS agar (Britania, Argentina) and on brain-heart infusion agar (BHI, Britania, Argentina). The plates were incubated at 37 °C for 24–48 h except for the MRS plates which were incubated at 37 °C for 48–72 h under microaerophilic conditions. In all cases, the incubation period was extended until visible colonies appeared. White, small, round colonies from the MSS and BHI agar were recovered and placed into BHI broth and incubated at 37 °C. Colonies with similar characteristics from MRS agar were transferred to MRS broth.

Lactic acid bacteria phenotypic characterization

Morphological observations were carried out using Gram staining. Carbohydrate fermentation patterns (APICH50, Biomérieux) and phenotypical identification according to basic biochemical tests were carried out for *Lactobacillus* (Hammes et al., 1992). *Enterococcus* strains were also characterized

according to typical biochemical tests for this genus (Devriese et al., 1992).

Antimicrobial spectrum analyses

Antimicrobial substance synthesis by the isolated lactic acid bacteria was analyzed in the cell-free supernatants (CFS), obtained from cultures of the lactobacilli (strains AJ5, A15, IG9 and CRL1647) and enterococci (strains SM21 and CA12) in MRS and BHI broths, respectively. After 24 h of incubation at 37 °C and under the appropiate atmosphere for each strain, the CFS were recovered by centrifuging (10,000*g*, 10 °C, 15 min), filter-sterilized (0.22 μm) and kept at 4 °C until used. *Listeria monocytogenes* 99/287 was provided by Instituto "Dr. Carlos Malbrán" (Buenos Aires, Argentina). *Bacillus cereus* C1 was supplied by Dr. Morea (ISPA, Bari, Italy). *Escherichia coli* 0157:H7 was supplied by Dr. Raya (CERELA, Argentina). *Staphylococcus aureus* 29213 from ATCC (American Type Culture Collection). Six different *P. larvae* strains (I, II, lll, IV, Azul and C) were provided by Dr. Terzolo and Engineer Borracci (INTA-Balcarce, Argentina). *P larvae* 7 and 35 were from Unidad de Bacteriología of CIDEFI (La Plata, Buenos Aires – Argentina). *P. larvae* strains were activated in MPYGP (Dingman and Stahly, 1983) agar at 37 °C for 72 h in a microaerophilic atmosphere. The other indicator strains were grown in BHI broth at 37 °C for 24 h. All strains were kept at −20 °C in MRS or in BHI broth plus glycerol (20%).

The different CFS were split off in two fractions: one was kept unmodified and the other was adjusted to pH 6.0 with NaOH 2.0 N; both aliquots were filter-sterilized (0.22 μm pore size cellulose acetate filter) and kept at 4 °C until used. Different lawns of the human pathogen strains were obtained on BHI agar (1.5%) for the agar diffusion assay. *P. larvae* cell suspensions were prepared by recovering the colonies grown on MPYGP agar, with a sterile cotton swab soaked in buffered-peptone water. They were resuspended in 2 mL of MPYGP broth (ca. 1×10^7 CFU/mL) and 300 μL were inoculated in 10 mL of molten MPYGP agar. Twenty five microliters of each CFS were placed in wells (5 mm diameter) cut in agar plates previously sown with the indicator strains. The plates were incubated at 30 °C for 12–24 h and examined for inhibition halos. For the microplaque technique, 100 μL of each CFS fractions were placed into 96 wells microplaque and 10 μL of the different indicator strain cell suspensions (final concentration varies from 10^6 to 10^8 CFU/mL) were added. Then, the microtitulation plaques were incubated at 37 °C for 2, 4 and 24 h under the optimal conditions for the tested bacteria. Lack of turbidity in the wells sown with the pathogen indicated its inhibition by the sample under analysis. Peptone–water (0.1%) dilutions were made and viable indicator cell number was determined by plate counting. In the particular case of *P. larvae*, aliquots of 5 μl of those suspensions that presented slight turbidity were sown on MPYGP agar and the plates were incubated in order to determine *P. larvae* residual viability. All assays were carried out in triplicate.

Phylogenetic analyses of selected bacteria

Lactobacilli CRL1647, A15, AJ5 and IG9 and the *Enterococcus faecium* SM21 were selected for genotypical characterization and further analyses. Their total genomic DNA were extracted from overnight MRS or BHI cultures by alkaline lysis (Pospiech and Neumann 1995). Two oligonucleotides, based on the report of Daffonchio et al. (1998), were used to determine 16S rDNA of the isolate: S-D-BACT-1494-A-S-20 (GTCGTAACAAGGTAGCCGTA) and L-D-BACT-0035-A-A-15 (CAAGGCATCCACCGT). PCR amplification was performed in a 25 μL reaction mix containing 2.5 μL 10X STR reaction buffer (Promega), 20 ng total DNA, 0.5 μM of each primer and 1 U of Taq DNA polymerase (Promega). Amplification consisted of an initial denaturation step at 94 °C for 5 min, followed by 35 cycles at 94 °C for 1 min, 50 °C for 2 min and 72 °C for 2 min. A final extension step consisting of 7 min at 72 °C was included (Sabaté et al., 2009). The PCR amplification fragments were resolved by agarose 0.8% gel electrophoresis at 75 V for 1 h 30 min. The gel was stained with ethidium bromide, and the bands were visualized under UV illumination at 254 nm. The 16S rDNA amplified with PCR was purified and the DNA sequencing carried out by Macrogen Services. Sequences were compared and aligned with sequences from the GenBank database by using the BLAST program of the National Center for Biotechnology Information (NCBI; http://www.ncbi.nlm.nih.gov) network server. Phylogenetic trees, based in the 16S-rRNA gen sequences (1,339 bp) of the isolate and culturable lactic acid bacteria were constructed using the Tree Builder program of the Ribosomal Database Project II (http://rdp.cme.msu.edu) (Cole et al. 2009). The neighbour-joining tree was bootstrapped 1000 times. *Lactobacillus acidophilus* (acc. Number 780913) was used as an outgroup.

Physicochemical characterization of the antagonistic compounds

The concentration of all organic acids was determined by HPLC. Prior to the assay, CFS from

MRS (lactobacilli) and BHI (enterococci) cultures were deproteinized. In brief, 1 mL of sample was added to 2 mL of a solution in 1.8% $Ba(OH)_2$, adding 2 mL of a solution of 2% $ZnSO_4$. The mixture was vortex shaken, allowed to settle for 10 min and centrifuged at 3500*g* for 5 min. Each supernatant was filter sterilized (0.45 μm) before the HPLC analyses. The sample amount injected was 20 μL. The column temperature was 55 °C and the flow rate of the H_2SO_4 10 mM mobile phase was 0.6 mL/min. Detection was carried out by determining the refraction index using a 2142 LKB Differential Refractometer. The chromatography column (Rezec Organic Acid, Phenomenex) had a diameter of 7.8 mm and a length of 300 mm. Peak profiles, integration, and quantification were obtained with a CR601 Shimadzu chromatopac integrator (Shimadzu Corporation, Analytical Instrument Division, Kyoto, Japan). All tests were carried out in duplicate.

Bacteriocin-like substances production

Bacteriocin-like substances production was determined by the well-diffusion assay with *L. monocytogenes* 99/287 as the indicator strain. Trypsin, proteinase K, pronase E, α-chymotrypsin and pepsin (final concentration of 1 mg/L in phosphate buffer pH 7.00, 0.05 M), were assayed to test the proteinaceous nature of the inhibitors. Catalase, α-amylase and lipase effects (final concentration of 1 mg/L in phosphate buffer pH 7.00, 0.05 M), were also analyzed. Heat resistance was studied after CFSs were heated to 70 °C for 30 min, 100 °C for 15 min and 121 °C for 15 min (in an autoclave) while another aliquot without thermal treatment was used as control. Then, they were cooled and tested for antimicrobial activity.

To determine bacteriocin molecular size, the peptides present in the CFS of *E. faecium* SM21 were separated by Tricine-SDS-PAGE as described by Schäger (2006). Polyacrylamide concentration on the separating gel was 16% (w/v). Electrophoresis was conducted at a constant voltage of 50 V for 1 h, 75 V for 2 h and 150 V for 1 h. A low rainbow molecular weight marker with sizes ranging from 3.5 to 38 kDa (Amersham Pharmacia, Germany) was used. The gel was fixed with an isopropanol (20% v/v) – acetic acid (10% v/v) solution, exhaustively washed with distilled water, and overlaid on BHI agar inoculated with *L. monocytogenes* 99/287.

The presence of enterocin-encoding genes was studied by PCR amplification with primers for the following well-known enterococcal bacteriocins: enterocin A; enterocin B; enterocin P; enterocin 31 and enterocin L50A/B (du Toit et al., 2000). Total genomic DNA from strains was used. PCR products were analyzed by electrophoresis (85 V for 1 h 5 min) on 2% (w/v) agarose gels in 1 × TAE buffer. Gels were stained GelRed (Invitrogen) and observed under UV light. The sizes of the amplified fragments were determined using 100-bp DNA Ladder (Invitrogen) as a molecular weight marker. As a positive control, the entA, B and P bacteriocin-gene sequenced of *E. faecium* CRL1385 were used (accession numbers to the GeneBank database GQ369790, GQ369791 and FJ57726, respectively).

Lactobacillus cell surface properties

When AJ5, IG9 and CRL1647 cells were grown in an MRS (Britania, Argentina) medium, discernible cell grouping appeared to the naked-eye, which adhered to the tube walls or sedimented to the bottom of the tube, due to this, their aggregation properties were studied. These properties were analyzed according to Handley et al. (1987) with some modifications. Lactobacilli cells were washed twice with PBS (NaCl 0.8; KH_2PO_4, 0.034; K_2HPO_4, 0.121, pH 7.4), resuspended in the same buffer and adjusted to $OD_{600}=0.60\pm0.05$. Cell suspensions (2 mL) were centrifuged at 2000*g* for 15 min and optic density (OD) at 600 nm was measured, without agitation, at room temperature every hour for 4 h. The percentage of autoaggregation was expressed as: $A\%=(A_0-A_t)/A_0\times100$, where A_0 represents the absorbance (A_{600}) at 0 h and A_t represents the absorbance (A_{600}) taken at 1, 2 and 3 hours.

Preliminary physic-chemical studies of molecules involve in the lactobacilli surface interactions

In order to approach the chemical nature of the molecules involved in the surface adsorption phenomena, *L. johnsonii* cells were put in contact with solutions of either 1 mg/mL of trypsin and pepsin at 37 °C for 1 h, lipase and sodium m-periodate at 25 °C for 1 h, and heated to 100 °C for 15 min. All enzymes were from Sigma and were prepared in their optimal buffer. Following this, the aggregation assay was performed.

Statistical analyses

All analyses were carried out according to the Tukey test and the results considered significant at the $P<0.05$ level. Assays were carried out in duplicate.

Results

Screening and phenotypic characterization of lactic acid bacteria associated to bee-gut

Different types of colonies developed on the surface of BHI agar plate after 1 day of incubation at 37 °C, and after 48 or 72 h on the surface of MRS agar. Thirteen microorganisms were purely isolated and showed biochemical characteristic of lactic acid bacteria: small, round, opaque and white colonies, non-sporulated, cocci (5) or rods (8) Gram-positive, non-motile cells, catalase negative and nitrate negative. Classic biochemical tests revealed that the eight bacilli were homofermentative and belonged to *Lactobacillus* genus. The strains CRL1647 and IG9 were characterized as *acidophilus* and *crispatus*, respectively; but the species of the other strains could not be determined and they were designated as *Lactobacillus* spp. The cocci were grouped into the *Enterococcus* genus. Basic biochemical tests revealed that, at least, SM21, CA12 and SM7 can be considered as *E. faecium* (Table 1).

Antimicrobial spectrum

The agar-diffusion assay was used to ''quick screen'' the antimicrobial spectra of the 13 lactic bacteria strains isolated from the bee gut. It was observed that only *Lactobacillus* AJ5, IG9, A15 and CRL1647 were able to inhibit *S. aureus* ATCC29213, *B. cereus* C1, *E. coli* O157:H7, *L. monocytogenes* and *P. larvae*; but strain A15 failed to inhibit two of the eight *P. larvae* strains tested. While, *E. faecium* SM21 only exhibited anti-*L. monocytogenes* activity. The lactobacilli antagonistic effects disappeared in all cases after adjusting CFS pH to 6.0, but the coccus kept its antilisteria activity under this new condition (see Table 2).

Phylogenetic identification and analyses of selected isolates

Five strains were selected due to their antimicrobial spectra and their species-specific identifications were derived using a 16S-rRNA sequence analysis. Comparison of the near complete 16S rRNA gene sequence of the bee-gut associated CRL1647, AJ5, IG9 and A15

Table 1. Characterization of the most representative lactic acid bacteria associated with summer adult worker bee gut samples.

Isolates	Characters	Tentative identification
IG9, CRL1647, AJ5, A15 C22, SM16, SM18, CA22	G+, long rods, non motile, non sporulated, nitrate (−), Catalase (−), arginine (+), esculin (+), growth at 37 °C (+), grwoth at 45 °C (+), glucose (+) without gas production	*Lactobacillus* spp.
IG9, CRL1647 AJ5, A15	Gluconate (−), esculin (+), melezitose (−), sacarose (+), Glycerol (−), fructose (+), mannose (+), cellobiose (+), Rhamnose (−)[a], sorbitol (+), manitol (+), D−arabinose (+) Salicin (w+), trehalose (+), xylitol (−), L-fucose (+), Gentibiose (+), maltose (+), arbutine (+), tagatose (+), Raffinose (+), amygdaline (+), melibiose (−)[a], lactose (−)[a]	*L. acidophilus* CRL1647 *L. crispatus* IG9 *Lactobacillus* spp. A15 *Lactobacillus* spp. AJ5
CA12, SM21, SM7, CA10, CA11	G(+), cocci in short chains, immobile, non sporulated, nitrate (−) Catalase (−), growth at 15, 37 and 45°C (+), growth at pH 9.6 (+); Growth at 6.5% NaCl (+), Voges Proskauer (−), arginine (+), Esculin (+), glucose (+)	*Enterococcus* spp.
CA12, SM21, SM7	Cellobiose (+), melibiose (+), melezitose (−), sacarose (−), Starch (+), glycerol (+), L-arabinose (+), ribose (+), fructose (+), Galactose (+), manitol (+), D-mannose (+), rhamnose (+), Sorbitol (+), lactose (+), salicin (+), trehalose (+), xylitol (−), D-arabinose (+), gentibiose (+), maltose (+), arbutine(+), Amygdaline (+), gluconate (+)	*Enterococcus faecium*

+, positive; w+, weak positive; −, negative.
[a](+) for AJ5 and A15 strains.

Table 2. Property profile of the selected lactic acid bacteria isolated from the bee-gut of *Apis mellifera* L.

	CRL1647	AJ5	IG9	A15	SM21
Phylogenetic identification	*L. johnsonii*	*L. johnsonii*	*L. johnsonii*	*L. johnsonii*	*E. faecium*
Genbank accession code	EU428007	EU428008	EU780913	EU428009	EU428012
Lactic acid synthesis (mM)	177±7	275±8	185±5	106±8	34±5
Bacteriocin production	-	-	-	-	+
Surface property	+	+	+	-	-
Inhibitory spectrum:					
P. larvae	8/8	8/8	8/8	6/8	0/8
L. monocytogenes	3/3	3/3	3/3	3/3	3/3
S. aureus ATCC29213	+	+	+	+	-
B. cereus C1	+	+	+	+	-
E. coli O157:H7	+	+	+	+	-

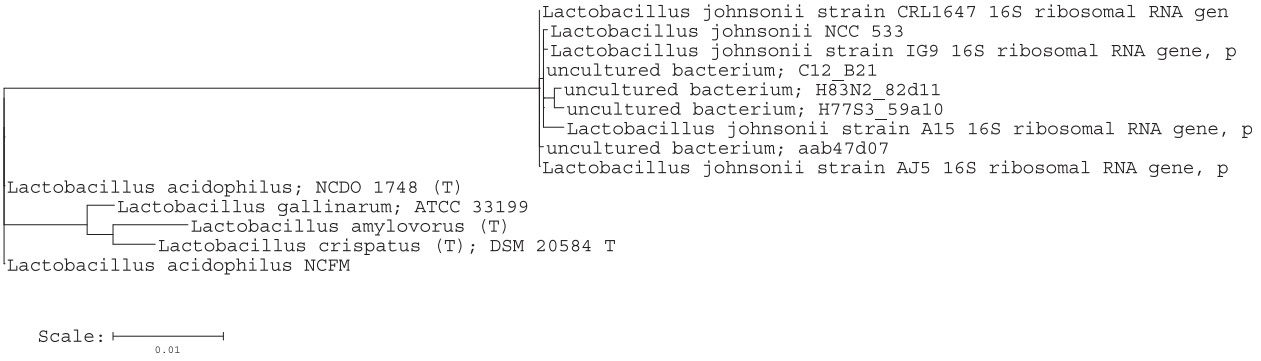

Figure 1. Phylogenetic tree, based in the 16S-rRNA gen sequences (1339 bp) of the four *L. johnsonii* strains culturable lactic acid bacteria from bee-gut, was constructed using the Tree Builder program of the Ribosomal Database Project II (http://rdp.cme.msu.edu) (Cole et al., 2009).

strains with the corresponding *Lactobacillus* sequences from the GenBank database showed all strains lay in the evolutionary clade of *Lactobacillus*. The four strains had a 99% identity with known *Lactobacillus johnsonii*, and their sequences were introduced into the GenBank database (Table 2). A phylogenetic tree of these lactobacilli was built with the neighbour-joining algorithms, using different sequences from the *L. acidophilus* group with, at least, 95% identity as shown in Figure 1. *L. johnsonii* NCC533, a type strain with its complete genome known, was also included in this analysis. It can be seen that the bee-gut associated strains are closely related to *L. johnsonii*.

A similar analysis was done with SM21 strain confirmed as an *E. faecium* with a 99% identity with *E. faecium* strain DSM20477 (=ATCC19434), a type strain, and other known and reported sequences (see Table 2 and Figure 2).

Lactic acid synthesis and antagonistic effect analyses

The lactic acid production by *L. johnsonii* CRL1647, IG9, AJ5, A15 and *E. faecium* SM21 was determined by HPLC. Interestingly, even though the pH values of the lactobacilli cultures were similar with an average value of 3.8±0.5; the lactic acid concentration profile was characteristic of each strain. *L. johnsonii* AJ5 synthesized the largest amount of this metabolite followed by IG9, CRL1647 and A15 (Table 2). While, *E. faecium* SM21, also a homofermentative lactic acid bacteria, acidified BHI broth at pH 4.5 and produced the lowest concentration of lactic acid. BHI broth was selected to culture *Enterococcus* spp. because it was determined in the laboratory that MRS broth was not as efficient for cocci growth.

E. coli O157:H7, *S. aureus* ATCC29213, *B. cereus* C1 and the three *L. monocytogenes* assayed showed the same behaviour against lactic acid; all of them were inhibited by 177 mM of this acid (Table 2). But, 88±5 mM had no impact on cell viability, independently of the detection method, i.e. well-diffusion agar or direct contact in microplaques. In particular, Figure 3A demonstrates the effect of 177 mM lactic acid synthesized by *L. johnsonii* CRL1647 on *L. monocytogenes* 99/287 non-proliferative cells viability.

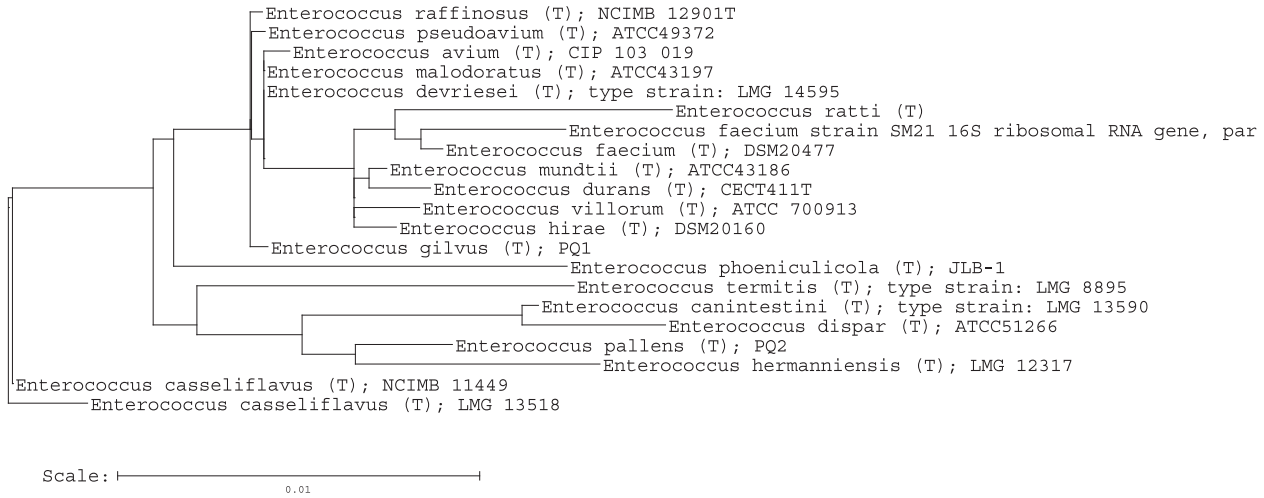

Figure 2. Phylogenetic tree, based in the 16S-rRNA gen sequences (1339 bp) of the *E. faecium* SM21 culturable lactic acid bacterium from bee-gut, was constructed using the Tree Builder program of the Ribosomal Database Project II (http://rdp.cme.msu.edu) (Cole et al., 2009).

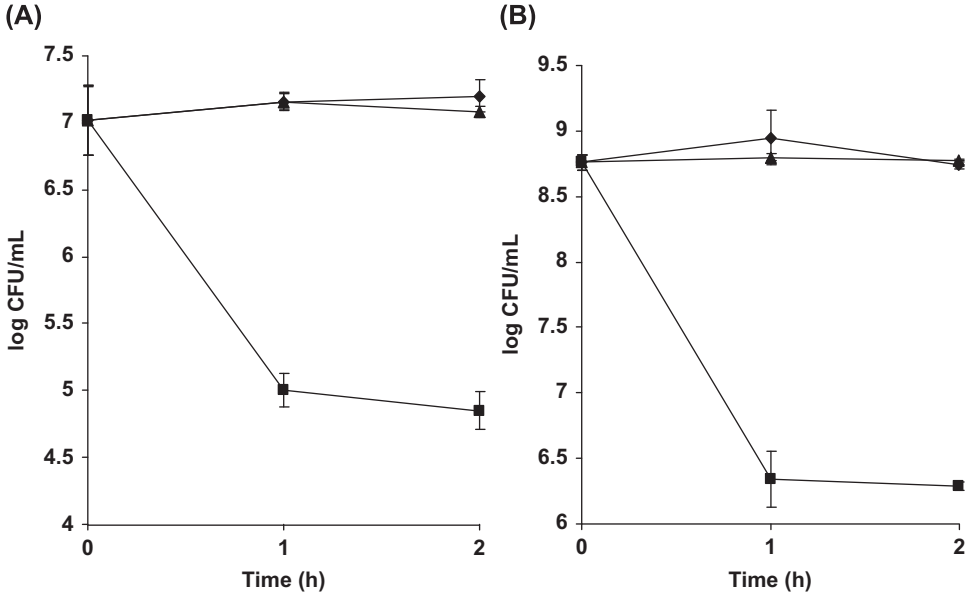

Figure 3. *Listeria monocytogenes* 99/287 inhibition by effect of: (A) lactic acid produced by *L. johnsonii* CRL1647 (♦ control; ▲ 177 mM lactic acid at pH 6.0; ■ 177 mM lactic acid at pH 3.9) and (B) bacteriocin-like compounds produced by *E. faecium* SM21 (▲ control; ■ crude CFS at pH 6.0; ♦ CFS at pH 6.0 and trypsin treated).

The vegetative cells of *P. larvae* were the most sensitive to lactic acid action; no colonies were detected on MPYGP agar after a 2 h contact period with 44 mM of lactic acid. These results were confirmed by the microplaque technique when different aliquots of cultures, from wells of the microplaque with slight turbidity, showed a viable plate count number three orders less in the log of CFU/mL than the control (Figure 4).

Bacteriocin-like compounds synthesis

E. faecium SM7, SM21 and CA12 produced compounds with strong anti-*L. monocytogenes* activity. The antagonistic effect had no significant difference when the filtered cell-free supernatants were adjusted to pH 6.0 with NaOH. Also lipase, catalase and α-amylase did not affect the anti-*Listeria* activity, but disappeared after a treatment

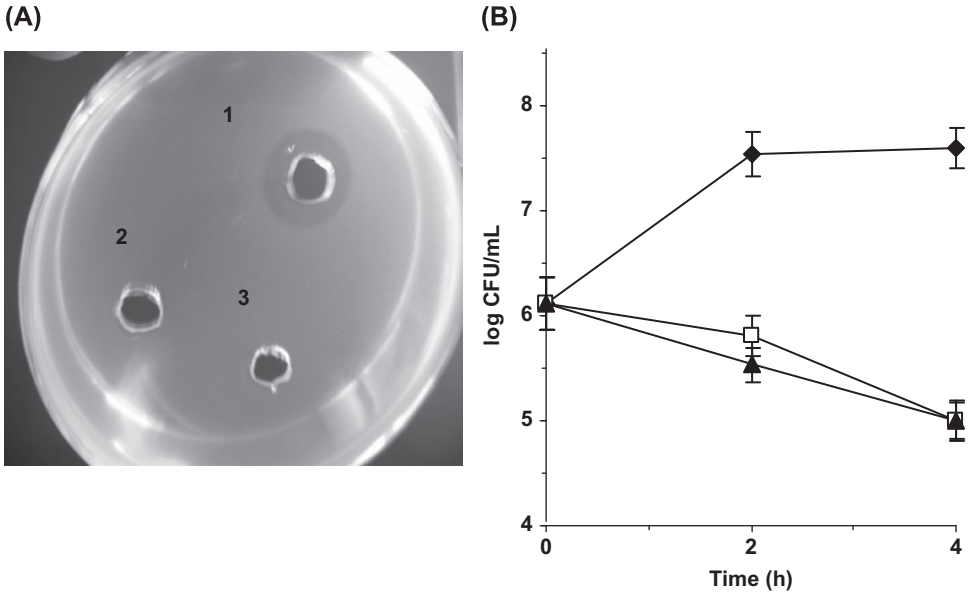

Figure 4. *Paenibacillus larvae* IV response in the presence of different CFS from *L. johnsonii* CRL1647. (A) By the well-difussion assay (**1**: pH 3.9 and 177 mM lactic acid; **2**: pH 5 and 177 mM lactic acid, and **3**: pH 5 and 44 mM lactic acid) and (B) by the microplaque technique ((♦) control of *P. larvae* IV cells, (▲) *P. larvae* IV cells in contact with 177 mM lactic acid; and (□) in contact with 44 mM lactic acid).

with pepsin, α-chymotrypsin, trypsin, pronase E and proteinase K, confirming their peptidic nature. The compounds were also highly thermostable keeping their anti-listeria effect after autoclave sterilization (121 °C for 15 min).

A bactericidal effect of *E. faecium* SM21 cell-free supernatant on *L. monocytogenes* 99/287 non-proliferative cells viability was observed (Figure 3B). Also, it was confirmed that the antagonistic effect was mainly due to bacteriocin-like compounds because when the CFS was treated with trypsin, its antilisteria effect disappeared (Figure 3B).

In particular, the analysis of *E. faecium* SM21 antimicrobial peptides by Tricine-SDS-PAGE, and further antimicrobial assays against *L. monocytogenes* 99/287, showed a broad band with a molecular weight of approximately 6.0–7.5 kDa (Figure 5A). The analyses of different structural enterocin genes in the DNA of *E. faecium* SM21 by PCR reactions was also done and revealed that the entA, B and P genes were amplified (Figure 4B). The size of the fragments of entA (126 bp) and entB (168 bp) were identical to those obtained for *E. faecium* CRL1385, the strain used as the positive control. The specific PCR fragment of 120 bp, corresponding to the entP gene, was also amplified. Moreover, no specific PCR fragments were detected with the set of primers for bacteriocins L50A/B and 31 (Figure 5B).

L. johnsonii surface properties

The presence of cells adhered to the tube wall were observed only in three of the fourteen lactobacilli isolated from bee-gut, as shown in Figure 6A for *L. johnsonii* CRL1647. A similar situation was observed for *L. johnsonii* AJ5 and IG9 cultures. After 3 h, without agitation, a reduction in the OD (600 nm) values were determined from 24-hour-old cells of IG9 and AJ5 (93.2% and 83.4%, respectively) resuspended in PBS. While, *L. johnsonii* CRL1647 showed 77.3% of autoaggregation. By scanning microscopy analyses, lactobacilli cells were aligned like bricks in a wall and some random coils were also observed (Figure 6B). Flocculation was not suspected as this phenomena is more common in yeast than in bacteria cells (Table 3).

The preliminary study of the chemical nature of the autoaggregating factors, revealed the molecules involved on the surface of each *L. johnsonii* strain were quite complex. All being sensitive to trypsin, sodium m-periodate and highly thermostable. But, only those of AJ5 were affected by pepsin. Also lipase action was more pronounced on IG9 cells autoaggregation (Table 4).

Discussion

The scientific works where the presence of lactic acid bacteria associated to honeybee-gut is re-

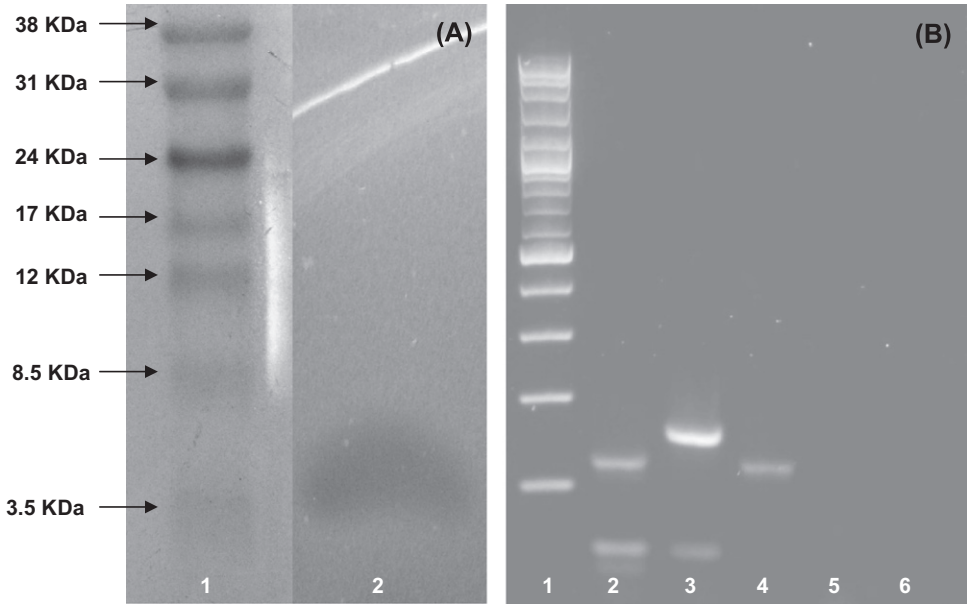

Figure 5. (A) Tricine-SDS-PAGE gel showing the zone of growth inhibition of *L. monocytogenes* 99/287 by the band of the CFS of *E. faecium* SM21. Lane 1: molecular mass marker; lane 2: CFS of *E. faecium* SM21; (B) Amplification of structural enterocin genes from DNA of *E. faecium* SM21. Lane 1: 100 bp Ladder (invitrogen), lane 2: ent A (126 bp), lane 3: ent B (162 bp), lane 4: ent P (120 bp), lane 5: ent bac 31 and lane 6: ent L50.

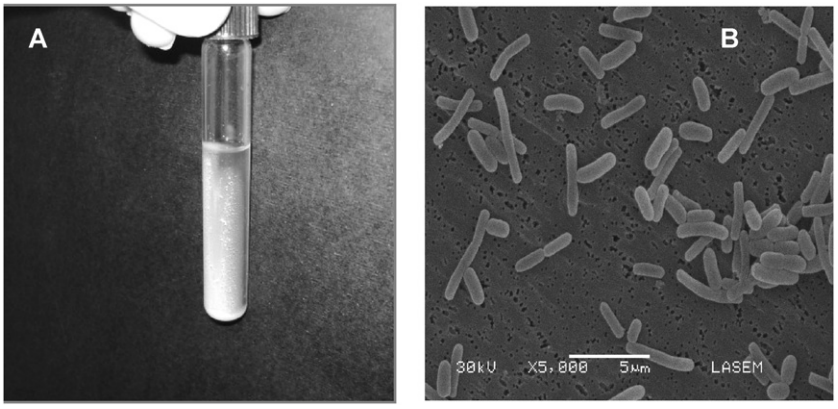

Figure 6. Auto-aggregating phenomena of *L. johnsonii* CRL1647 cells. (A) macroscopic observation, (B) scanning electron microscopy.

Table 3. Autoaggregation percentage of the different *L. johnsonii* cultures.

Strain	Time (h)			
	0	1	2	3
CRL1647	33.1±0.5	36.8±1.2	59.1±0.9	77.3±0.5
AJ5 4	36.6±0.6	49.6±0.8	71.4±0.4	83.4±0.7
IG9	31.6±1.1	81.7±1.1	92.1±0.8	93.2±1.1

ported are limited. They were carried out with different purposes. For example, Mohr and Tebbe (2006) revealed the presence of *Lactobacillus* in bee-gut only by a genetic analysis, but no isolations were carried out and the strains detected were uncultured. Mrázek et al. (2008) also analyzed the diversity of insect intestinal microbiota, without any strain isolation, and only reported the possible presence of *Bifidabacterium* sp. in honeyebees. Rada et al. (1997) were able to isolate *Lactobacillus* sp., *Enterococcus* sp. and some *Bifidobacterium* sp. when they were studying the microbiota in the honeybee digestive tract. However, they only characterized a *Bifidobacterium asteroides* by biochemical tests and analyzed the sensitivity of these bacteria to veterinary drugs.

In this work, *Lactobacillus* and *Enterococcus* strains were isolated from the gut of the summer adult worker *Apis mellifera* L. bees of the Argentinean Northwest region. The *Streptococcus*

Table 4. Autoaggregation percentage[a] of the different *L. johnsonii* strains after different treatments.

Treatment	Strains		
	CRL1647	IG9	AJ5
Control	77.3±0.5	93.2±0.7	83.4±1.1
Trypsin	30.8±1.2	59.4±0.3	45.3±0.5
Pepsin	75.1±0.5	90.3±1.1	65.3±0.8
Lipase	61.6±0.8	67.3±1.3	83.1±0.4
Sodium m-periodate	59.3±0.4	70.5±0.7	67.7±1.2
Boiling temperature for 15 min	75.6±1.1	92.4±0.6	82.8±0.7

[a]Each point corresponds to an average of at least four readings.

selective medium (MSS), modified by Audisio et al. (2005), was selective only for *Enterococcus* isolation. However *Lactobacillus* isolation was somewhat difficult. If MRS agar was prepared adjusting the pH close to 5.0 with acetic acid, as indicated by the manufacturer, scant colony development was observed and sometimes, there was no colony at all. On the other hand, if MRS agar was prepared without an acidic final pH, i.e. equal to 6.5, a higher number of typical lactic acid bacteria colonies were detected, including many others. Thus, to isolate lactobacilli from the bee-gut environment, the samples were seeded both on MRS at pH 6.5 and on MRS at pH 5.0, and small, round, opaque and white typical lactic acid bacteria colonies were picked up from both media.

The response of classic biochemical tests performed for *Enterococcus* (Devriese et al., 1992) allowed us to correctly identify some of the enterococci strains, confirmed by a phylogenetic analysis for the SM21 strain. The isolation of these microorganisms from bee-gut was an important step as there are only four scientific papers reporting their presence in the honeybee intestinal environment (Rousseau et al., 1969; Snowdon and Cliver, 1996; Rada et al., 1997; Kačániova et al., 2004) and with this information a clearer understanding of the bee-gut microbiota composition can be achieved.

Although there were differences between the phenotypical characterization of strains CRL 1647, IG9, AJ5, A15 and their genotypical profile, they were not surprising, as authors had already reported similar situations (Berger et al., 2007). Other articles have shown that there are six *Lactobacillus* strains of distinct homology groups (A1, A2, A3, A4, B1 and B2) that respond phenotypically to the characteristics of the *acidophilus* species, but a study with molecular biology tools revealed that the species may be *crispatus*, *gasseri*, *johnsonii*, *gallinarum*, *amylovorus* or *acidophilus* (Johnson et al., 1980; Pot et al. 1993). All the lactobacilli preselected in this work were homofermentative, i.e., they do not produce gas from glucose. These results agree with those reported by Olofsson and Vásquez (2008) who isolated and characterized many homofermentative lactobacilli from the honeybee stomach. They observed that lactobacilli and bifidobacteria were predominant within the lactic microbiota associated to the honeybee stomach, but failed to report the presence of *L. johnsonii* or *Streptococcus* or *Enterococcus* in this bee environment. However, in this work it was determined, by phylogenetic analyses, that the homofermentative lactobacilli associated to bee-gut were closely related to *L. johnsonii*. In particular, the IG9 strain showed a 97% (ca. 400 bp) identity with the 5′ end of *L. johnsonii* NCC533, a type strain with its complete genome known (Boekhorst et al., 2004).

A high *in vitro* susceptibility of *P. larvae* vegetative cells to a low pH, unreported so far for this bacterium, was observed in this work. The three *L. johnsonii* strains selected were able to inhibit the vegetative cells of the American foulbrood agent by lactic acid synthesis and 44 mM of this compound were necessary. *E. faecium* SM21, cultured in BHI broth, reached a pH close to 5.0 and produced 34 mM of lactic acid, but this concentration was not enough to affect *P. larvae* viability. Among the pre-selected lactobacilli and enterococci, the final pH values for the CFS used in the antagonistic tests were close to 4.0 or 4.5 in most cases, but organic acid concentration was variable and not all lactic acid bacteria selected, particularly enterococci, inhibited *E. coli* O157:H7, *S. aureus* ATCC29213, *B. cereus* C1 or *P. larvae*, by acidity. Although lactic acid production is an essential characteristic of the bacteria studied here, the amount of this metabolite that is synthesized depends on each particular strain. Susceptibility to the antagonistic activity of lactic acid also varies among the pathogenic microorganisms (Ogawa et al., 2001; Wilson et al., 2005). So, the present *in vitro* study revealed that certain *Lactobacillus* species, such as *L. johnsonii* exert

growth inhibition on the indicator microorganisms assayed. To the authors' knowledge, this would be the first report on *P. larvae* growth inhibition due to acidity generated by lactobacilli isolated from bee gut. *In vitro* inhibition of *P. larvae* by *Bacillus* sp. bacteria has been previously reported (Evans and Armstrong, 2005; Alippi and Reynaldi, 2006; Sabaté et al., 2009). However, *Bacillus* sp. is a genus normally related to the honeybee environment (Gilliam, 1987, 1997; Snowdon and Cliver, 1996).

The selected enterococci were remarkably active against *L. monocytogenes*. This inhibition was not due to lactic acidity but to a synthesis of bacteriocin-like compounds by four *E. faecium* of the nine isolated. These compounds were thermostable and showed both a proteinaceous nature and a bactericidal effect against *L. monocytogenes* 99/287; all these characteristics agree with those found for different enterocins produced by *E. faecium* isolated from other sources (Foulquié-Moreno et al., 2006). It has been informed that the structural gene of enterocin A is widely distributed among *E. faecium* strains, whereas that of enterocin B always occurs in the presence of enterocin A; besides, the high-frequency and variability of enterocin structural genes among enterococci of different origin by means of simple PCR reactions, without purification or sequentiation, was also reported (Poeta et al., 2007). This trend was confirmed in this paper and this would be the first case where these genes have been detected in enterococci associated with the bee digestive tract.

Bacterial aggregation/surface property at bacterial cell envelope level can be determined and quantified by the reduction of optic density values at different times and should not be confused/misunderstood with the flocculation phenomena (Handley et al., 1987; Kos et al., 2003). Flocculation is a phenomena more common/current for yeasts than bacteria. Furthermore, yeast flocculation is a non-sexual and reversible cell aggregation in which cells adhere to each other to form flocs (Zhao and Bai 2009). In this work, an important auto-aggregation was observed in three different *L. johnsonii* strains isolated from bee-gut. The assays carried out to determine the chemical nature of the factor involved in the lactobacilli auto-aggregation revealed in the cell envelope, different compounds for each strain; a lipopetide for IG9 and a glicopeptide for CRL1647 and AJ5. The involvement of proteinaceous bacterial surface compounds in lactobacilli adhesion has been analyzed and characterized for strains from human, fish and dairy product origins (Ventura et al., 2002; Ying et al., 2007). However, no reports exist on the study of this property in *Lactobacillus* with regards to insects, furthermore no analysis has been performed on honeybee-derived *Lactobacillus* until now. Further studies are needed to determine which molecules are present in the autoaggregation phenomena.

Even though this work analyzes human foodborne pathogens, it should be pointed out that the honeybee or its products are not *E. coli* O157:H7 or *L. monocytogenes* hosts or vectors. The origin of a strain was not considered to be an obstacle to ascertain its full antimicrobial potential; thus, the findings may open the way for different applications other than apiculture practices.

Conclusions

Lactobacillus and *Enterococcus* strains associated with adult worker bees were isolated and characterized to enhance the knowledge of microorganisms found in the intestinal environment of this insect. The presence of *L. johnsonii* has now been reported for the first time in the bee gut. Furthermore, this is the first report on *P. larvae* growth inhibition due to acidity generated by lactobacilli isolated from the bee gut ecosystem.

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