Phenotypic and Genotypic Characterization of Some Lactic Acid Bacteria Isolated from Bee Pollen: A Preliminary Study

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In the present work, five hundred and sixty-seven isolates of lactic acid bacteria were recovered from raw bee pollen grains. All isolates were screened for their antagonistic activity against both Gram-positive and Gram-negative pathogenic bacteria. Neutralized supernatants of 54 lactic acid bacteria (LAB) cultures from 216 active isolates inhibited the growth of indicator bacteria. They were phenotypically characterized, based on the fermentation of 39 carbohydrates. Using the simple matching coefficient and unweighted pair group algorithm with arithmetic averages (UPGMA), seven clusters with other two members were defined at the 79% similarity level. The following species were characterized: *Lactobacillus plantarum*, *Lactobacillus fermentum*, *Lactococcus lactis*, *Pediococcus acidilactici*, *Pediococcus pentosaceus*, and unidentified lactobacilli. Phenotypic characteristics of major and minor clusters were also identified. Partial sequencing of the 16S rRNA gene of representative isolates from each cluster was performed, and ten strains were assigned to seven species: *Lactobacillus plantarum*, *Lactobacillus fermentum*, *Lactococcus lactis*, *Lactobacillus ingluviei*, *Pediococcus pentosaceus*, *Lactobacillus acidipiscis* and *Weissella cibaria*. The molecular method used failed to determine the exact taxonomic status of BH0900 and AH3133.

Key words: lactic acid bacteria, numerical clustering, pollen, functional foods, UPGMA, 16S rRNA gene sequencing

Beehive products (honey, pollen, propolis and royal jelly) are natural functional foods that have gained increased attention in society [1]. Pollen, the male gametophyte of flowering plants, is a high-energy material collected by honeybees and other insects and stored as a food reserve. Pollen has been used traditionally by humans as a supplementary food and in alternative medical treatments. It has been used medically in prostatitis, bleeding stomach ulcers and some infectious diseases [2].

Because of its complex content, bee-pollen has a very important nutritional value in the human diet [3]. Since the middle of the last century, the bee-pollen microflora has been investigated [4–10]. However, little is known about the occurrence of lactic acid bacteria (LAB), and the roles they play, in pollens. Only a few reports are available in the literature considering this field. Occurrence of lactobacilli in pollens was reviewed by Gilliam [7]. In another study, Vásquez and Olofsson [11] identified lactobacilli isolated from pollen grains. These isolates were identified based on PCR and 16S rRNA gene sequencing. Other LAB representative genera (*Lactococcus*, *Lactobacillus*, *Pediococcus*, *Enterococcus* and *Leuconostoc*) were recovered and identified based on phenotypic traits [4]. In the latter investigation, in vitro studies indicated that several strains inhibit Gram-positive and Gram-negative pathogenic bacteria. Additionally, several members of LAB are known to produce antibacterial substances such as organic acids and bacteriocins. Antagonism towards undesirable microorganisms is an important criterion for LAB being used as bio-preservations or biocontrol agents. It seems that pollens are a suitable ecological niche for various microorganisms and an important source for the isolation of new strains belonging to the LAB group with

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Table 1. Indicator bacteria used and their growth conditions

Indicator species	Strain no.	Origin	Growth medium	Incubation conditions
Gram-positive bacteria				
Bacillus subtilis	20302	CLAM[a]	BHI[c]	37°C
Enterococcus faecium	H421	CLAM	MRS[d]	30°C
Listeria innocua	3030	CLAM	BHI	37°C
Staphylococcus aureus	25923	ATCC[b]	BHI	37°C
Gram-negative bacteria				
Escherichia coli	25922	ATCC	BHI	37°C
Salmonella typhimurium	1717	CLAM	BHI	37°C
Pseudomonas aeruginosa	27853	ATCC	BHI	37°C
Shigella sp.	96415	CLAM	BHI	37°C

[a] Collection of Laboratory for Applied Microbiology
[b] American Type Culture Collection
[c] Brain heart infusion broth
[d] De Man, Rogosa and Sharpe broth

antagonistic activity against harmful bacteria. Species or subspecies identification of such strains is recommended. In fact, physiological and biochemical criteria used for LAB identification are often ambiguous because most of the bacteria have very similar nutritional requirements and grow under similar environmental conditions. Therefore, clear identification to the species level by simple phenotypic tests may sometimes be difficult [12]. Indeed, additional molecular-based characterization approaches such as 16S rRNA gene sequencing, 16S-ARDRA, PFGE and other methods are used for assignment of a given LAB strain to its taxonomic status.

The present work describes isolation and phenotype-based numerical clustering of LAB isolated from pollen grains collected in some Algerian areas; 16S rRNA gene sequencing-based characterization of selected strains was investigated. Also, their antagonistic activity against Gram-positive and Gram-negative bacteria was evaluated.

EXPERIMENTAL

Isolation of LAB from pollen

Previous studies [4] indicate that pollen samples taken from pollen traps are highly contaminated, especially by enterococci. Furthermore, dilution-based LAB isolation is not a sufficient manner for the recovery of these bacteria from pollen grains, and full negative results are unavoidable with this method. Hence, a simple technique is used for the efficient isolation of LAB. In the field during flowering seasons, honey bee foragers are kinched (catched) by a sterile stainless steel or plastic forceps. The pollen pellets (60 samples from 10 clean regions; six subsamples from each sampling site; in Bordj Bou Arreridj and Sétif) are then collected by a humidified sterile cotton swab by gently touching the pollen pellets attached to the posterior legs of the bee. At this time, we can discuss the facts regarding bee-collected pollen from a microbiological point of view. The swabs were transported to the laboratory at low temperature (5°C) and analyzed or further maintained with refrigeration until use. Because LAB isolation is enrichment-based method, the quantity of pollen recovered is not important. Each swab was then introduced into a capped glass tube containing 15 mL Elliker broth pH 6.5 [13], and incubated anaerobically at 30°C for at least two days. Afterwards, serial dilutions were prepared from each tube in peptone water (0.1% peptone and 0.1% of Tween 80), and from the appropriate dilution, aliquots (100 µL) were spread and cultured on the following media: M17 agar (Fluka) incubated for 72 hr at 30°C for lactococci and streptococci, LBS agar [14] after anaerobic incubation (BBL GasPack System) at 30°C for 72 hr for lactobacilli, Glucose Yeast Extract Agar [15] incubated at 30°C for 48 hr to isolate leuconostocs and pediococci, and D-MRS agar [16] incubated at 25°C for 72 hr to isolate carnobacteria. Enterococci were treated in a separate study. From each culture, 10–30 colonies were randomly picked up and further purified on MRS agar (pH 6.5). Pure isolates were maintained at –20°C in MRS broth containing glycerol (20%, v/v final concentration).

Screening for antagonistic activity

For detection of antagonistic activity, an agar well diffusion assay was used according to Schillinger and Lucke [17]. Bacterial species used as indicator

microorganisms were listed in Table 1. LAB isolates were subcultured twice (1% inoculums, 24 hr, 30°C) in 10 mL MRS broth (Fluka). The non-LAB were subcultured twice (1% inoculum, 24 hr, 37°C) in 10 mL BHI broth (Fluka) and kept frozen at –20°C in BHI broth supplemented with 20% glycerol. Cell-free supernatants from LAB cultures were obtained by centrifuging the cultures (8,000 g/10 min at 4°C), and then the pH of each supernatant was adjusted to 6.5 using 5N HCl followed by filtration through a cellulose acetate filter with a pore size of 0.2 µm. Before supernatant neutralization, an antimicrobial assay was performed for all isolates. All experiments were performed in duplicate, and the results were displayed as the mean value of the experiments. Isolates showing antagonistic activity against one or more indicator bacteria (with an inhibition zone diameter of more than 5 mm) were subjected for phenotypic characterization.

Phenotypic identification of bioactive isolates

Isolates exhibiting antagonistic activity were selected on the basis of Gram staining, morphology, tetrad formation and catalase activity. Catalase-negative and Gram-positive rods and cocci were selected and screened for the production of CO_2 from glucose (in MRS broth, containing Durham inverted tubes, without beef extract and citrate). Ability to grow at 10 and at 45°C was evaluated in MRS broth after incubation for 7 days and 48 hr, respectively. Growth in MRS containing 6.5 or 18% NaCl, as well as growth in MRS with pH 4.4 and 9.6, was studied. Acid production from carbohydrates (glycerol, erythritol, D-arabinose, L-arabinose, ribose, D-xylose, L-xylose, adonitol, galactose, D-glucose, D-fructose, D-mannose, L-sorbose, rhamnose, dulcitol, inositol, mannitol, sorbitol, amygdalin, arbutin, salicin, cellobiose, maltose, lactose, melibiose, saccharose, trehalose, inulin, melezitose, D-raffinose, starch, glycogen, xylitol, gentiobiose, D-lyxose, D-tagatose, D-arabitol, L-arabitol, and gluconate) was evaluated by miniaturized assay in 96-well flat-bottom microtiter plates [18]. Sterile carbohydrate solutions (Institut Pasteur, France) were added to the basal medium (MRS without glucose and meat extract and with 0.16 g/L bromocresol purple, pH 7.0) at a final concentration of 1%. Cells were harvested from overnight cultures by centrifugation (10,000 g, 5 min, 4°C), washed twice in sterile phosphate buffer (pH 7.0) and suspended in sterile saline (0.85% NaCl). This suspension was used to inoculate sterile microtiter plates (96 flat-bottom wells), which were incubated in anaerobiosis for 7 days at 30°C. Esculin hydrolysis was assessed by adding 2 g/L esculin (Sigma) and 5 g/L ferric ammonium citrate (Sigma) to the basal medium.

Fermentation of each of the 39 carbohydrates was interpreted as follows: positive (+), complete change to yellow; weakly positive (w), change to green; and negative (-), no change at all. Esculin hydrolysis (revealed by a change to a darker color) was interpreted as positive (+), while no change was negative (-). Strains were tested in duplicate to determine the test reproducibility.

Genotypic identification of selected LAB strains

Sample preparation prior to PCR amplification

As described by Rodas et al. [19], selected LAB isolates (10 strains displaying a remarkable assimilatory pattern) were grown in MRS agar at 30°C for 2 days. One single colony was picked up from plates and suspended in 20 µL of sterile distilled water. These suspensions were used for PCR reactions without further processing.

Amplification and sequencing of 16S rRNA gene

The protocol of Rodas et al. [19] was used for 16S rRNA gene amplification using primers pA (8-AGAGTTTGATCCTGGCTCAG-28) and pH (1542-AAGAGGTGATCCAGCCGCA-1522) [20]. DNA amplification was carried out in a 50 µL PCR mixture containing 200 µM dNTP, 1 µM of each primer, 20 mM Tris-HCl (pH 8.4), 50 mM KCl, 2 mM $MgCl_2$, 6 U of Taq DNA polymerase and 1 µL of the cell suspension. Each PCR cycle consisted of an initial denaturation time of 5 min at 94°C followed by 35 cycles of amplification comprising a denaturation step for 30 sec at 94°C, annealing at 56°C for 30 sec, and extension at 72°C for 1 min. Reactions were completed with 5 min elongation at 72°C followed by cooling to 10°C. PCR products were resolved by electrophoresis at a constant voltage (200 V) in 1.2% (w/v) agarose in 0.5 x TBE (45 mM Tris-HCl, 45 mM boric acid and 1 mM EDTA pH 8.0), gels were stained with ethidium bromide (0.5 µg/mL). Amplification products were purified using a PCR purification kit (Qiagen, Germany) and sequenced using the BigDye® Terminator v3.1 Cycle Sequencing Kit and an automated DNA sequencer (ABI Prism® 3100 Avant Genetic Analyzer, Applied Biosystems). The nucleotide sequences of the 16S rRNA gene of all the isolates were analyzed and determined by the BLAST program on the NCBI website (http://www.ncbi.nlm.nih.gov/). The alignments were analyzed to construct a phylogenetic tree and to compare similarities among the sequences by the neighbor-joining method [21] using MEGA software version 4.0. Bootstrap analysis was used to evaluate the tree topology of the data by performing 1,000 resamplings. The sequences were deposited in the GenBank database using the web-based data submission

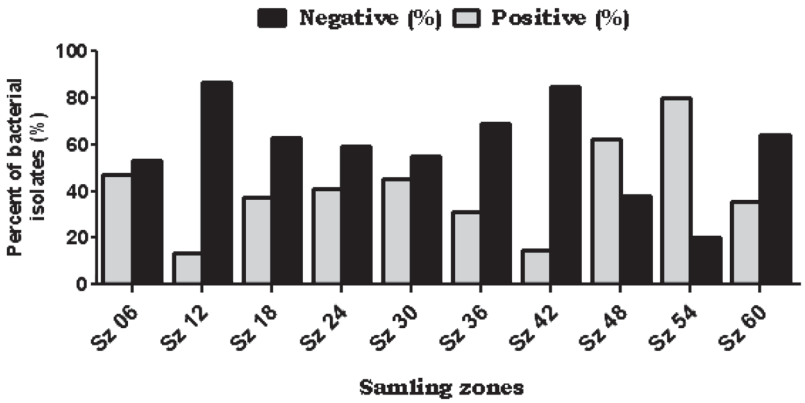

Fig. 1. Percent distribution of antagonism among 567 LAB strains isolated from pollen samples. Samples Sz06, Sz18, Sz30, Sz36 and Sz54 were from BBA, whereas samples Sz12, Sz24, Sz42, Sz48 and Sz60 were from Sétif. Statistical analysis indicates that the total percentage of potent strains (40.74 ± 6.31) is significantly different ($p=0.003 < 0.05$) from that of non-potent strains (59.26 ± 6.31). Percent distributions of potent strains were extremely different from each other ($p<0.0001$) except for sampling zones Sz36 and Sz60 ($p<0.01$). Also, there were no statistical differences between the percentage of potent strains of sampling zones Sz06 and Sz30 ($p>0.05$) or sampling zones Sz18 and Sz60 ($p>0.05$). Percent distributions of non-potent strains among sampling zones were significantly different from each other ($p<0.05$) except for sampling zones Sz06 and Sz30, Sz12 and Sz42 and Sz18 and Sz24 and Sz18 and Sz60, which do not differ statistically ($p>0.05$).

tool Sequin (http://www.ncbi.nlm.nih.gov/Sequin).

Statistical analysis

Hierarchical cluster analysis was carried out with Statistica 6 software (Statsoft Italia, Padua, Italy). The Euclidean distance, unweighted pair group method with arithmetic mean (UPGMA) and an index of similarity were used for the analysis of carbohydrate fermentation. A two-way ANOVA test was used for comparing antimicrobial activity of LAB.

RESULTS

Isolation and antagonism among LAB strains

From the ten sampling zones, distributed across two provinces, six samples were collected from each zone. However, a total of 567 isolates were recovered from pollen grains. Growth of yeasts and molds on isolation agar media was observed and confirmed by microscopic examination. Also, catalase-positive bacterial colonies were encountered. Presumptive LAB cells were carefully selected based on catalase reaction, cell shape, motility and Gram staining. All Gram-positive, catalase-negative cocci and rods were purified on suitable agar media to homogeneity. The 567 pure LAB strains were screened for their antagonistic activity by agar well diffusion assay against eight undesirable bacteria belonging to Gram-positive and Gram-negative ranks (*Staphylococcus aureus* ATCC 25923, *Bacillus subtilis* CLAM 20302, *Enterococcus faecium* CLAM H421, *Listeria innocua* CLAM 3030, *Shigella* sp. CLAM 96415, *Escherichia coli* ATCC 25922, *Salmonella typhimurium* CLAM 1717, and *Pseudomonas aeruginosa* ATCC 27853). After a random screening process, using neutralized supernatant cultures, 216 stains found to potentially inhibit at least one indicator bacteria, whereas 351 were not. Statistical analysis indicated that total percentage of potent strains (40.74 ± 6.31) was significantly different ($p=0.003 < 0.05$) from that of non-potent strains (59.26 ± 6.31). From all sampling zones, except zones Sz48 (62.06%) and Sz54 (80.00%), the percentage of antagonistic LAB strains was below 46 (Fig. 1).

The 216 LAB strains showing inhibition zones against at least one indicator bacterium (without pH neutralization of spent cultures), according to the first antimicrobial screening assay, were further subjected to determination of the antibacterial activity of neutralized cell-free supernatants obtained by centrifugation. Neutralization of culture supernatant pH (6.5) eliminates the effect of acidity on the target bacteria. Furthermore, incubation of LAB under anaerobic conditions minimizes hydrogen peroxide production. Following the second screening assay using eight indicator bacteria (four Gram negative

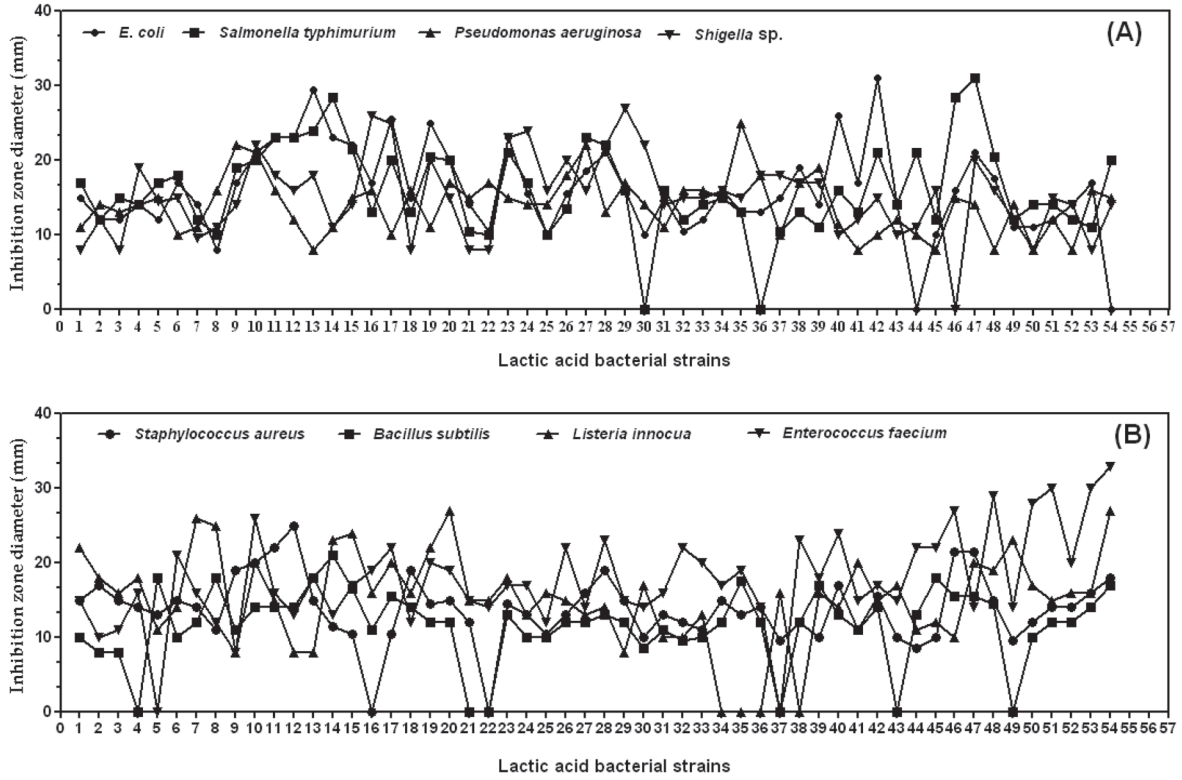

Fig. 2. Antibacterial activity (inhibition zone diameter, mm) of 54 pollen residing lactic acid bacterial strains against Gram-negative and Gram positive indicator bacteria (A) *Escherichia coli* ATCC 25922, *Salmonella typhimurium* CLAM 1717, *Pseudomonas aeruginosa* ATCC 27853 and *Shigella* sp. CLAM 96415. Significant statistical differences were observed ($p=0.0071$, $p<0.05$) among antimicrobial potential of LAB strains, whereas indicator pathogenic bacteria themselves could not affect the variation in antimicrobial activity ($p=0.1033$, $p>0.05$) as determined by two-way ANOVA test. (B) *Staphylococcus aureus* ATCC 25923, *Bacillus subtilis* CLAM 20302, *Enterococcus faecium* CLAM H421, and *Listeria innocua* CLAM 3030. Two-way ANOVA analysis indicated that there were no significant statistical differences among the antimicrobial actions of LAB strains ($p>0.05$), but is resulted from the indicator pathogenic bacteria themselves and extremely significant differences were obtained ($p<0.0001$).

and four Gram positive), 54 potent LAB strains were obtained (Fig. 2). Figure 2A. presents the variation in the antagonistic activity against Gram-negative human and animal pathogenic bacteria. The activity was measured from the zone of inhibition (mm) around the well. This quantified activity varied from 10 to 27 mm for the majority of LAB strains studied (Fig. 2). Furthermore, only two strains (strains 43 and 54) could not inhibit *E. coli* ATCC 25922, two other strains (strains 30 and 36) were inactive against *Salmonella typhimurium* CLAM 1717, and strain 46 was inactive against *Shigella* sp. CLAM 96415 (Fig. 2A). In addition, only five strains were able to produce a zone of inhibition in between 29 and 35 mm against indicator strains, for example, strains 12 and 41 against *E. coli* ATCC 25922 and strains 14, 46 and 47 against *Salmonella typhimurium* CLAM 1717 (Fig. 2A). However, it was observed that *Pseudomonas aeruginosa* ATCC 27853 resisted the inhibitory potential as compared with the other indicator bacteria (Fig. 2A).

For Gram-positive indicator bacteria, the majority of inhibition diameters were between 10 and 20 mm for *Staphylococcus aureus* ATCC 25923 and *Bacillus subtilis* CLAM 20302 and between 10 and 30 mm for *Enterococcus faecium* CLAM H421 and *Listeria innocua* CLAM 3030 (Fig. 2B). However, 14 strains were inactive against indicator bacteria. Strains 5, 21, 22, 37, 43 and 49 were inactive against *Bacillus subtilis*; strains 16 and 22 were inactive against *Staphylococcus aureus*; strains 34, 35, 36 and 38 were inactive against *Listeria innocua*; and strains 5 and 37 were inactive against *Enterococcus faecium*. In addition, it was noted that *Listeria innocua* and *Enterococcus faecium* were more susceptible to the action of LAB strains than *Bacillus subtilis* and *Staphylococcus aureus*. For the latter bacterium, the

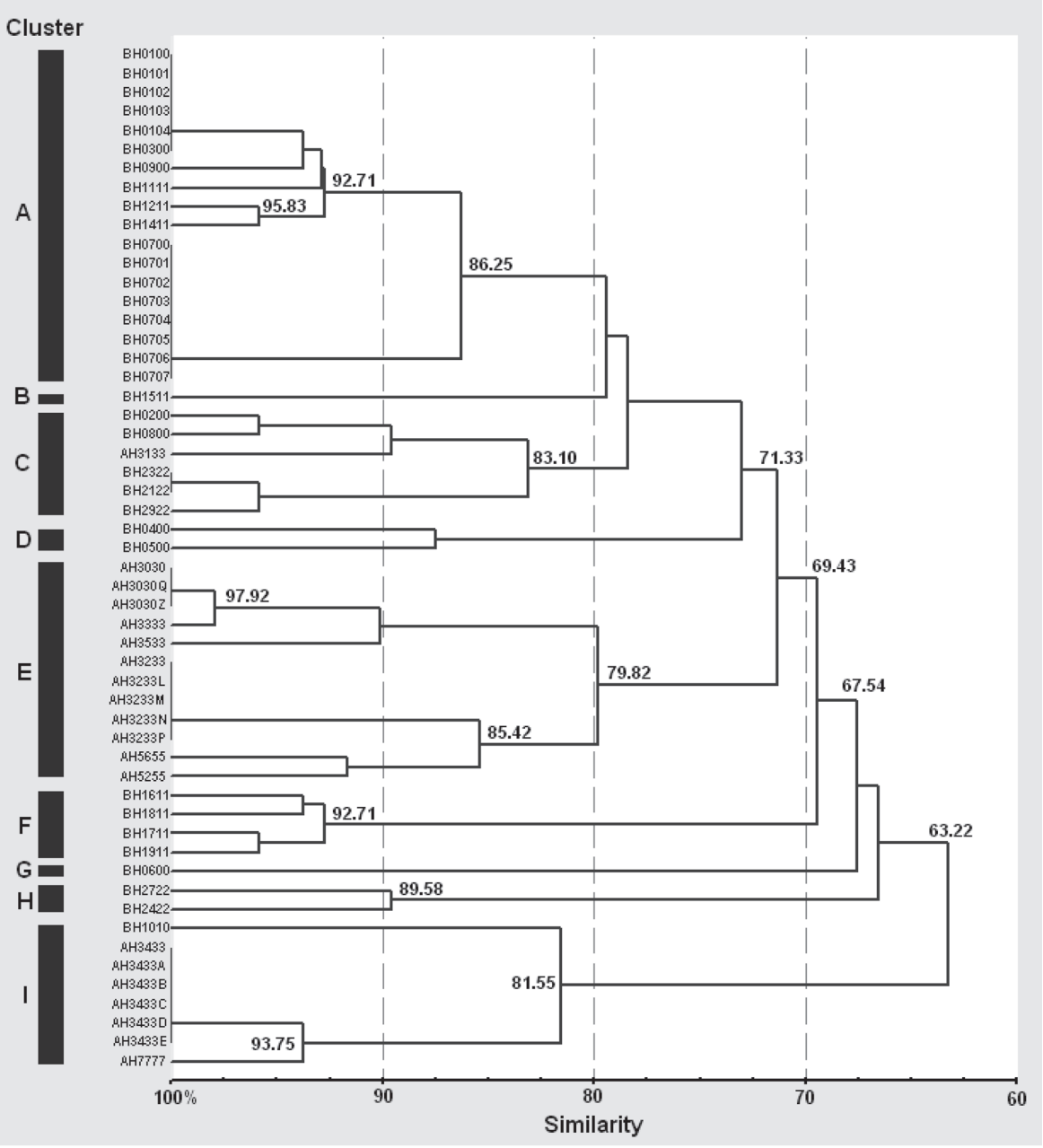

Fig. 3. Dendrogram of combined phenotype profiles of 54 potent antagonistic lactic acid bacteria isolates as determined by carbohydrate fermentation and physiologic traits. Cluster analysis was carried out based on the simple matching coefficient and unweighted pair group algorithm with arithmetic averages (UPGMA). Codes of strains and clusters are indicated on the left hand side of the figure.

inhibition diameters were between 10 and 33 mm (Fig. 2B).

Clustering structure and analysis

Fifty-four bacteriocin-like producing isolates of LAB obtained from different raw pollen samples were characterized according to the method of Axelsson [22], Bergey's Manual [23] and the prokaryotes [24]. Preliminary, the diversity was studied based on a phenotypic approach. Microplates containing 39 different carbon sources and 10 other physiological traits were used to determine the phenotypic profiles of the 54 isolates of

LAB. The reproducibility of the fermentation tests was 100%. The similarity coefficient cluster analysis resulted in five major clusters (A, C, E, F and I, containing three or more strains) defined at the 79.0% similarity level (Fig. 3). The following four other minor clusters were defined at the 79% similarity level (B, D, G and H, two 2-member cluster and two 1-member clusters).

Major clusters (A, C, E, F and I)

Cluster A), *Lactobacillus plantarum*, *Lactobacillus paraplantarum* and *Lactobacillus* sp., comprised 18 isolates from Bordj Bou Arreridj (BBA) (33.33% of the total isolates). The cluster contained eight other *Lactobacillus plantarum* strains, one strain of *Lactobacillus paraplantarum* and eight *Lactobacillus* sp. strains. All strains were homofermentative, and 55.5% of the isolates in this cluster fermented L-arabinose. They did not ferment xylose, adonitol, β-methyl-xyloside, starch, arabitol and rhamnose. Alpha-methyl-D-mannoside was variously fermented. The same pattern was noted for gluconate, lactose and sorbitol. Members of this cluster grew at 10 and 45°C in the presence of 6.5% NaCl and at pH 4.4.

Cluster C), *Lactobacillus plantarum*, *Lactococcus lactis* subsp. *lactis*, and *Lactobacillus* sp., contained three *Lactobacillus* sp. members, one strain of *Lactococcus lactis* subsp. *lactis* and two *Lactobacillus plantarum* strains. All were homofermentative and grew well at 10 and 45°C, in the presence of 6.5% NaCl and under alkaline conditions (pH, 9.6). They all fermented ribose, hexoses, cellobiose, lactose, saccharose, trehalose and B-gentiobiose, but they did not ferment erythritol, D-arabinose, xylose, adonitol, β-methyl-xyloside, dulcitol, α-methyl-D-glucoside, starch, xylitol, L-arabitol, D-lyxose, D-tagatose, D-fucose and L-fucose. They variously fermented L-arabinose, rhamnose, inositol, mannitol, sorbitol, α-methyl-D-mannoside, melibiose, melezitose, D-raffinose, D-arabitol, gluconate and amygdalin.

Cluster E), *Lactococcus lactis*, *Pediococcus pentsaceus* and *Pediococcus acidilactici*, contained five strains of *Lactococcus lactis* subsp. *lactis* (AH3030, AH3031, AH3032, AH3333 and AH3533), six strains of *Pediococcus pentosaceus* (AH3233, AH3233 L, AH3233 M, AH3233 N, AH3233 and AH5655), and one strain of *Pediococcus acidilactici* (AH5255). All strains of this group were homofermenters and grew well at 45°C. The strains AH3030, AH3031, AH3032 and AH3333 did not grow at 10°C or in the presence of 6.5% sodium chloride. They did not ferment D-arabinose, β-methyl-xyloside, rhamnose, dulcitol, inositol, sorbitol, α-methyl-D-mannoside, α-methyl-D-glucoside, melibiose and melezitose, but they did ferment ribose, galactose, D-glucose, D-fructose, N-acetyl-glucosamine, amygdalin, arbutin, esculin, salicin and cellobiose. Furthermore, L-arabinose, D-xylose, D-mannose, mannitol, lactose, B-gentiobiose, maltose and saccharose were variously fermented. In addition, only the strain, *Lactococcus lactis* AH3533, was able to ferment starch and gluconate.

Cluster F), this cluster contained four strains of *Lactobacillus* sp., BH1611, BH1711, BH181 and BH1911. They grew at pH 4.4, at 10 and 45°C and in the presence of 6.5% sodium chloride, but they did not grow at pH 9.6. They fermented ribose, xylose, hexoses, α-methyl-D-mannoside, N-acetyl-glucosamine, amygdalin, arbutin, salicin, cellobiose, maltose, lactose, melibiose, saccharose, trehalose and D-raffinose. They did not ferment melezitose, gluconate, inositol, mannitol, rhamnose, D-arabinose and β-methyl-xyloside.

Cluster I), contained strains of *Pediococcus pentosaceus* and *Lactobacillus plantarum*. Members of this cluster (seven strains of *Pediococcus pentosaceus* and one strain *Lactobacillus plantarum* BH1010) were homofermenters. All strains were able to grew at 10 and 45°C, at pH 4.4 and in the presence of 6.5% NaCl. However, D-arabinose, L-arabinose, β-methyl-xyloside, galactose, D-glucose, D-fructose, α-methyl-D-glucoside, N-acetyl glucosamine, amygdalin, arbutin, salicin, saccharose, xylitol and D-lyxose were fermented by members of this group. Adonitol was fermented by all strains except *Pediococcus pentosaceus* AH7777 and *Lactobacillus plantarum* BH1010. In addition, none of the strains fermented ribose, xylose, D-mannose, L-sorbose, rhamnose, dulcitol, sorbitol, α-methyl-D-mannoside, trehalose, D-raffinose, starch, gentiobiose, gluconate and D-arabitol. Melezitose, inositol, mannitol, maltose and lactose were fermented only by *Lactobacillus plantarum* BH1010.

Minor clusters and stragglers (B, D, G and H)

Cluster B contained one strain of *Lactobacillus fermentum*; cluster D contained two strains, *Lactobacillus fermentum* and *Lactobacillus* sp. One strain identified as *Lactobacillus plantarum* was assigned to cluster G. However, two other strains, *L. fermentum*, were grouped in cluster H. All strains were heterofermenters and grow at 45°C and in the presence of 6.5% of sodium chloride with the exception of strain BH2422. Members of cluster H were able to grow at pH 9.6 compared with strains of the other clusters. None of the strains fermented erythritol, adonitol, β-methyl-xyloside, D-lyxose,

Table 2. Phenotypic profiles of the 54 potent LAB isolates as determined by carbohydrate fermentation

	Cluster A 18 isolates	Cluster B (BH1511)	Cluster C 6 isolates	Cluster D (BH0400 BH0500)	Cluster E 12 isolates	Cluster F 4 isolates	Cluster G (BH0600)	Cluster H (BH2722 BH2422)	Cluster I 8 isolates
D-Arabinose	5.5	0	0	0	0	0	0	50	100
L-Arabinose	55.5	100	33.33	100	41.66	0	0	100	100
Ribose	44.5	100	100	50	100	100	100	100	0
Adonitol	0	0	0	0	0	0	0	0	75
β-M-xyloside	0	0	0	0	0	0	0	0	100
D-Mannose	100	0	100	50	58.33	100	0	0	0
Rhamnose	0	100	50	0	0	0	0	50	0
Inositol	0	0	33.33	50	0	0	0	0	12.5
Mannitol	100	100	83.33	50	41.66	0	0	50	12.5
Sorbitol	50	100	83.33	0	0	100	0	50	0
Alpha-M-D-mannoside	38.88	0	50	50	0	100	0	50	0
β-M-xyloside	0	0	16.2	50	0	50	0	0	100
Cellobiose	100	0	100	50	100	100	0	50	25
Maltose	100	100	100	50	91.66	100	100	100	12.5
Lactose	10	100	100	100	41.66	100	100	100	12.5
Melibiose	100	100	66.7	50	0	100	100	100	87.5
Saccharose	100	100	100	100	41.66	100	100	100	100
Trehalose	100	0	100	50	91.66	100	0	0	0
Melezitose	100	100	83.33	50	0	0	0	50	12.5
D-Raffinose	94.5	100	50	0	0	100	100	100	0
Starch	0	0	0	0	8.33	75	0	50	0
Gentiobiose	100	100	100	50	91.66	0	0	100	0

Numbers indicate the percent positive reaction of each test for the isolates of each cluster.

D-tagatose, D-fucose, L-arabitol and 2-keto-gluconate. However, strain BH2422 (cluster H) was also unable to ferment D-arabinose. Table 2 highlights the different carbohydrates variously fermented by members of the clusters obtained at the similarity level of 79% (A-I).

Genotypic characterization of selected LAB strains

Sequences of the 16S rRNA gene (approximately 1,500 bp) of ten LAB isolates, BH0900, BH1511, AH3133, BH0500, AH5655, AH3030, BH1711, BH0600, BH2422, and AH3433A, were determined. The 16S rRNA nucleotide sequences of the isolates were aligned with homologous regions from various LAB, and a phylogenetic tree was constructed by the neighbor-joining method (Fig. 4). The BLAST analysis of 16S rRNA gene sequences of the selected strains showed alignments of these sequences with reported 16S rRNA genes in the gene bank. The nucleotide sequences were deposited in GenBank, and accession numbers for strains BH0900, BH1511, AH3133, BH0500, AH5655, AH3030, BH1711, BH0600, BH2422 and AH3433A were obtained (KF178303, KF178304, KF178305, KF178307, KF178308, KF178306, KF178310, KF178311, KF178312 and KF178309, respectively). However, the 10 isolates were assigned to seven species, *Lactobacillus plantarum*, *L. fermentum*, *Lactococcus lactis*, *L. ingluviei*, *Pediococcus pentosaceus*, *L. acidipiscis* and *Weissella cibaria*.

On the basis of phylogenetic data obtained, strain BH0900 showed similarity (99%) with *Lactobacillus plantarum* WCFS1 (075041.1) and *Lactobacillus pentosus* 124-2 (029133.1) as well as with *Lactobacillus paraplantarum* DSM 10667 (025447.1) and *Lactobacillus plantarum* NRRL B-14768 (042394.1). However, strain BH1511 shares 99% similarity with *Lactobacillus fermentum* IFO 3956 (075033.1) and 96% similarity with *Lactobacillus gastricus* Kx156A7 (029084.1). On the other hand, strains AH3133 and AH3030 share 99% similarity with *Lactococcus lactis* subsp. *lactis* NCDO 604 (040955.1), *Lactococcus lactis* subsp. hordniae NCDO 2181 (040956.1), *Lactococcus lactis* subsp. *cremoris* SK11 (074949.1), *Lactococcus lactis* subsp. *cremoris* NCDO 607 (040954.1) and 92% similarity with *Lactococcus plantarum* DSM 20686 (044358.1). Furthermore, 99% similarity was shared between isolate BH0500 and *Lactobacillus ingluviei* KR3 (028810.1). In addition, two strains, AH5655 and AH3433A, were closely related (99%) to *Pediococcus pentosaceus* DSM 20336 (042058.1) and showed more than 98% similarity to *Pediococcus stilesii* LMG 23082 (042401.1). One strain,

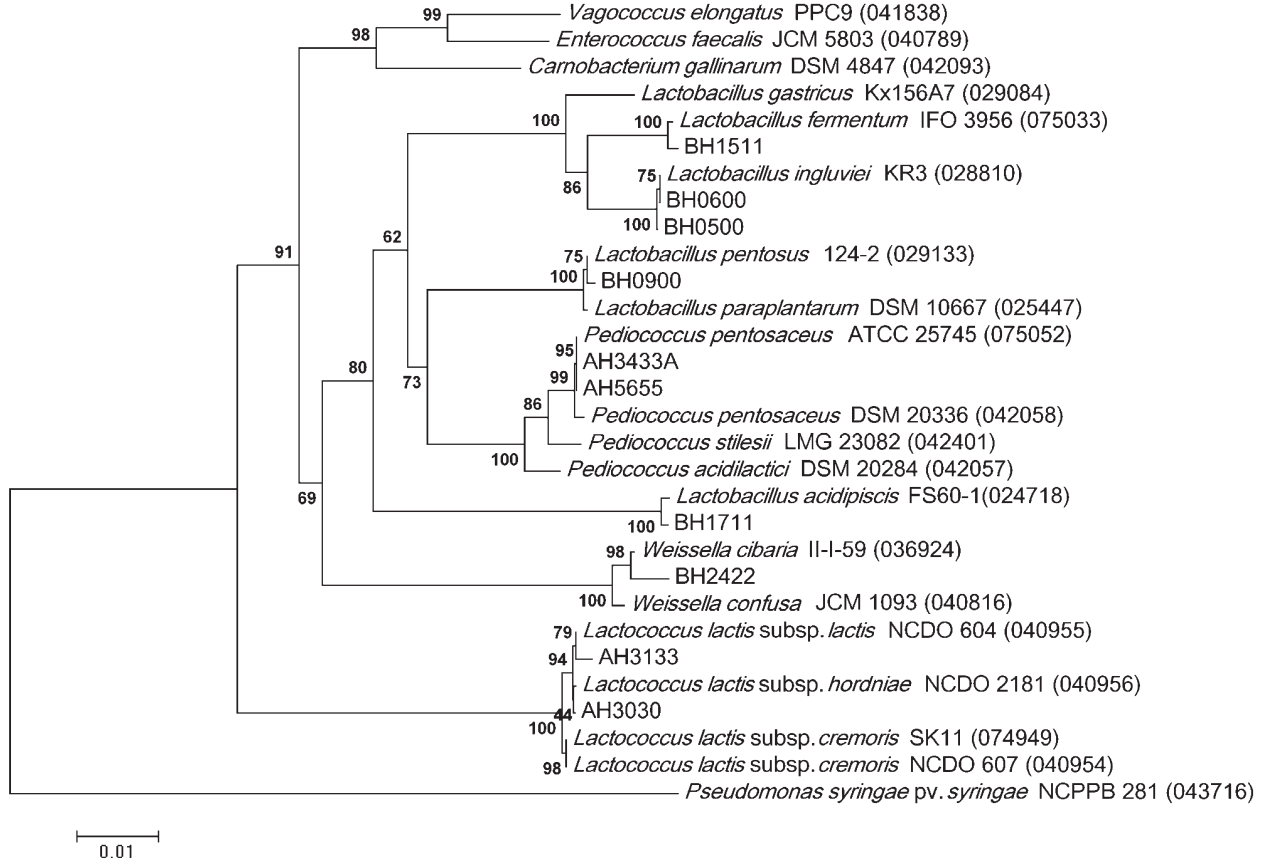

Fig. 4. Phylogenetic tree based on 16S rDNA sequence analysis, showing the phylogenetic placement of selected LAB strains isolated from pollen grains. The tree was constructed by the neighbor-joining method, and *Pseudomonas syringae* pv. *syringae* was used as the outgroup. Bootstrap values (expressed as percentages of 1000 replications) are shown at branch points.

BH1711, displayed 98% similarity with *Lactobacillus acidipiscis* FS60-1 (024718.1). Strain BH0600, which was phenotypically identified as *Lactobacillus plantarum*, was genotypically related (99%) to *Lactobacillus ingluviei* KR3 (028810.1). Finally, strain BH2422 was considered *Lactobacillus fermentum* based on phenotypic characterization; however, 16S rRNA gene sequence analysis revealed that this strain was related to *Weissella* and shared 99% sequence similarity with *Weissella cibaria* II-I-59 (036924.1).

DISCUSSION

Isolation of LAB and antagonistic activity

The effectiveness of LAB is a strain-dependant aspect. This trait may be obtained by genetic manipulation or, as is frequently the case, searching for new desirable strains in natural niches. Plants, foods, fermented products, animals and humans constitute natural ecological systems and good sources for LAB. To the best of our knowledge, antagonism of LAB against human bacterial gastrointestinal pathogens is the most important feature for selecting such a strain designed for the human gut.

Here, we described isolation of LAB from raw pollen grains showing antimicrobial activity against Gram-positive and Gram-negative pathogenic bacteria. The 54 LAB effective strains were characterized by means of phenotypic tests. The relationships among the phenotypically characterized strains of LAB were determined by cluster analysis. It is well known that pollen grains are sterile before anther opening. Flowers and their constituents are parts of the plant phylloplane. Stirling and Whittenburg [25] suggested that the LAB are not usually part of the normal microflora of the growing plant and indicated the role of insects in the spread of these organisms. LAB commonly found on fresh herbage and in silage have been investigated [26, 27]. Nilsson and Nilsson [28] were found that the predominant

LAB during silage fermentation were streptococci and lactobacilli, with *Lactobacillus plantarum* the species most frequently recovered. Other studies [29, 30] reported the occurrence of pediococci and lactobacilli on leaving or decayed plants. Lactobacilli commonly share the habitat phyllosphere with species of the genera *Leuconostoc*, *Pediococcus* and *Weissella*. Species frequently recovered from the leaves include *L. plantarum*, *L. paracasei*, *L. fermentum*, *L. brevis* and *L. buchneri* [16], which is in line with our results. Part of the accumulated information about the occurrence of lactobacilli (and other LAB members) on plants is derived from microbiological studies of the fermentation process. Thus, the microbial population upon initiation of the process is known for several plants (grasses, cabbage, silage raw materials, carrots and beets, olives and fruits such as grapes and pears, etc.). But scarce information about the occurrence of LAB on flowers and pollens is available in the literature. Indeed, we report in this work the occurrence of LAB in pollen grains and for the first time the isolation and characterization of several species belonging to LAB. The results reported here indicate that 54 isolates (25%) of the total antagonistic isolates (216 strains) inhibit indicator bacteria. Inhibition caused by hydrogen peroxide and organic acids was ruled out, as the producer strains were cultured anaerobically and the culture supernatant was neutralized (pH 6.5) before assaying the antimicrobial activity. This study indicates that the compound inhibiting the microbial growth in the neutralized cell-free supernatant was not organic acid or hydrogen peroxide commonly produced by many LAB [31].

As reported by Daeschel et al. [32], certain LAB protect plants by producing antagonistic compounds [12] contributing to inhibition of the plant pathogens *Xanthomonas campestris*, *Erwinia carotovora* and *Pseudomonas syringae*. Furthermore, LAB are well known for their antagonism towards other Gram-positive bacteria, especially taxonomically related species (*Listeria* spp., *Bacillus* spp. *Micrococcus* spp., etc.), and Gram-negative plant and animal pathogens, such as *Escherichia coli*, *Salmonella* spp., *Helicobacter pylori* and *Pseudomonas aeruginosa*. Contrary to what is believed, that LAB are more potent in inhibiting Gram-positive bacteria than Gram-negatives bacteria, which is claimed for the type of cell wall of the target microorganism, this study reveals that Gram-negative bacteria used in this study (*E. coli*, *Salmonella typhimurium*, *Pseudomonas aeruginosa*, and *Shigella* sp.) were susceptible to cell-free supernatants from tested LAB, especially the strains 9 (*Pediococcus pentosaceus* AH3433E), 12 (*Lactobacillus plantarum* BH1010), 14 (*Lactobacillus plantarum* BH0600), 16 (*Lactobacillus* sp. BH1811), 19 (*Pediococcus acidilactici* AH5255), 29 (*Lactobacillus plantarum* BH0800), 39 (*Lactobacillus plantarum* BH1411), 41 (*Lactobacillus plantarum* BH0104) and 47 (*Lactobacillus plantarum* BH0102). These results are in accordance with earlier results reported by Trias et al. [33], who showed that most LAB originating from fruits and vegetables displayed good antagonistic activity against foodborne pathogens, such as, *Listeria monocytogenes*, *Salmonella typhimurium* and *Escherichia coli*. Several indicator strains displayed different degrees of susceptibility towards antimicrobial compounds from a given producer strain; for example, strain 46 (*Lactobacillus* sp. BH2122) inhibits strongly *E. coli*, *Salmonella typhimurium* and moderately inhibits *Pseudomonas aeruginosa* but is inactive towards *Shigella* sp. The indicator strain *L. innocua* was used in this study instead of *L. monocytogenes*, as the two microorganisms show similar physiological properties with the difference that the former does not belong to the pathogenic species of *Listeria*. Moreover, some papers have reported a greater sensitivity of *L. monocytogenes* towards some antibacterial compounds than *L. innocua* [34, 35].

It is well known that the presence of lactobacilli is important for maintenance of the intestinal microbial ecosystem [36]. They have been shown to possess inhibitory activity toward the growth of pathogenic bacteria such as *Listeria monocytogenes* [37, 38], *Escherichia coli* and *Salmonella* spp. [39, 40]. This inhibition could be due to the production of inhibitory compounds such as organic acids, hydrogen peroxide, and bacteriocins. Our results agreed with the latter statements; therefore, our isolates are strong candidates for clinical use during gut treatment regimes. Furthermore, a major advantage of using LAB as biocontrol agents is that they are considered GRAS (generally recognized as safe) and usually comply with all recommendations for food and drug products [41]. Moreover, LAB are natural colonizers of fresh plant products and have been previously described as good antagonists of several bacteria and fungi [42, 43].

Siezen et al. [44] hypothesized that the fermentative profile reflects the original habitat and that lactose utilization is less prevalent in plant isolates with respect to those from cheese and the human gastrointestinal tract. Indeed, lactose was fermented by most isolates in the present study, except isolates of *Pediococcus* from clusters I and E and isolates of *Lactobacillus* sp. from cluster A. The inability of plant-related LAB to ferment lactose was presumably due to the relatively recent acquisitions, via horizontal gene transfer and subsequent

natural selection, of lactose metabolic genes, which are often plasmid encoded in dairy and human strains [45]. Contrary to the findings of Cagno et al. [46], who studied *Lactobacillus plantarum* from vegetables and fruits, all *Lactobacillus plantarum* isolates of this study used this carbon source.

Overall, all isolates fermented maltose and cellobiose except *Pediococcus acidilactici* AH5255 (cluster E) and *Pediococcus pentosaceus* AH3433, AH3433A, AH3433B, AH3433C, AH3433D and AH3433E (cluster I). Strain AH7777 (*Pediococcus pentosaceus*; cluster I) fermented cellobiose but not maltose. Also, cellobiose was not fermented by *Lactobacillus fermentum* BH0400, *Lactobacillus* sp. BH0500 (cluster D), members of cluster H (*Lactobacillus fermentum* BH2722, and BH2422) and members of clusters B and G. Arabinose, glucose, fructose, mannose, mannitol, B-gentiobiose, melezitose, melibiose, saccharose and trehalose were variously fermented. These carbon sources correspond mainly to those prevalent in the plant kingdom [47]. Similar phenotypic profiles were found for *Lactobacillus plantarum* isolated from Thai fermented fruits and vegetables [45, 47, 48]. Glycosides such as amygdalin were not used only by isolates of clusters H, D, B and C, salicin was not used only by eight isolates belonging to cluster A, (*Lactobacillus* sp. BH0700, 0701, 0702, 0703, 0704, 0705, 0706 and 0707). On the other hand, arbutin was used by isolates of all clusters. These glycosides are typically found in vegetables.

Starch is a reserve polysaccharide in pollen grains, and starch-hydrolyzing strains belong to the clusters E, F, and H. According to the fermentation profile, it seems that the isolates assimilate variously a panel of carbohydrates that reflects their enzymatic and genetic potentials. Furthermore, these traits were shared with LAB of dairy or animal origin. Based on the limited number of tests used, phenotypic profiles did not cluster the isolates according to the original habitat. This was probably because the studied isolates were obtained from raw pollen samples having a very similar chemical composition. It could also have been because the pollen residing LAB were of animal as well as plant origin. Nevertheless, phenotypic profiling was useful to understand the manifestations of environmental adaptation, which will be reflected in the technological processes.

From the different phenotypic clusters, ten selected isolates (displaying a good assimilation activity) were identified by means of 16S rRNA gene sequencing. It is well known that the species *Lactobacillus plantarum*, *Lactobacillus pentosus* and *Lactobacillus paraplantarum* are genotypically closely related and show highly similar phenotypes. In the present results, misidentification of some isolates to defined species was encountered. More molecular techniques should be used for determination of the taxonomic status of these strains. The occurrence of *Weissella cibaria*, *Lactobacillus ingluviei* and *Lactobacillus acidipiscis* in pollen grains is in accordance with the ubiquity of these bacteria in nature [24]. In fact, insects, honeybees, soil, water and animal and human feces are main microbial contamination sources of pollens [4, 5]. From another point of view, misidentification of some isolates by phenotypic traits is probably due in part to the limited characters used for this purpose, or by the similarity of the metabolic patterns expressed by the isolates, even if they belong to different genotypic ranks. As reported elsewhere [19], physiological and biochemical criteria used for LAB strain identification are often ambiguous because most of the bacteria have very similar nutritional requirements and grow under similar environmental conditions. Therefore, a clear identification to species level by simple phenotypic tests may be troublesome and inaccurate. Molecular methods used for discrimination of LAB strains to genus and species level are more efficient than phenotypic approaches.

A preliminary study on LAB associated with pollens having remarkable antimicrobial activity is reported here. The uses of these strains for biocontrol or bio-preservation purposes should be evaluated. Phenotypic traits do not reveal the real taxonomic position of some isolates; therefore, exploitation of other molecular methods for exact identification of these bacteria is of great scientific and practical interest. In addition, the search for new LAB exhibiting a wider spectrum of antimicrobial activities from pollen grains that can be used in human health, agriculture and the food industry is of great importance. Furthermore, studies on other biological and biotechnological criteria of these isolates as well as their safety aspects are necessary. Finally, it seems that pollens and possibly other beehive products are predominant sources for isolation of LAB with potent applications.

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