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Phenotypic, genotypic, and technological characterization of new lactic acid bacterium isolated from traditional dairy product in Djelfa, Algeria

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Abstract

A total of 33 lactic acid bacteria (LAB) were isolated from 5 samples of traditional dairy product (Jben) collected from various livestock farms in Oued El Malha, Djelfa, the Ouled Naïl Range of north-central Algeria. The taxonomic study of the strain B04 using a multiphase approach based on morphology, physiology, molecular (16S rRNA) and phylogenetic analyses allowed correlating this strain to *Enterococcaceae*. The difference of the 16S rRNA gene sequence of the isolate toward the most closely related genus *Enterococcus* was more than 6 % suggesting that the strain B04 represents a new genus. The results of the evaluation of some physiological tests indicated that B04 exhibited good biological activities including acidifying, proteolytic and bacterial inhibition. The strain B04 was antagonistic toward *Staphylococcus aureus* ATCC 6538, *Escherichia coli* ATCC 25922, and *Bacillus cereus* ATCC 10876.

Introduction

Dairy products and milk, as commonly consumed products, are rigorously investigated for their biochemical and physiological functions (Moussaid *et al.* 2021). Traditional dairy products are made by means of ancient artisanal processes, using milk or mixtures of milk from different animal species (Guetouache and Guessas 2018). Several types of fermented milk products have been reported throughout the world. These products differ in their taste and their consistency. In North of African countries, the most popular dairy products are Jben, Klila, Zebda, Rayeb, Lben, Bouhezza, Medghissa, and Takammarit. Traditional dairy products are cultural goods that deserve to be studied, characterized, and protected (Guetouache and Guessas 2019; Leksir *et al.* 2019). "Jben" cheese is

manufactured with the raw goat or sheep milk, voluntarily acidified and coagulated by coagulating enzymes of plant origin from wild thorny plant, cardoon flowers, pumpkin seeds, or artichoke (Ouadghiri *et al.* 2005). After a moderately heating (55 – 75 °C) of "Lben" to avoid degradation during the storage phase, the coagulum obtained is called "Klila", which is consumed as a fresh cheese (Benlahcen *et al.* 2017; Adewumi 2019). The fresh butter obtained after churning "Rayeb" (curdled milk) is called "Zebda". By shaking "Rayeb" added with a quantity of warm water (40 – 50 °C) the fat globules agglomerates and lipid globules appear on the surface and are recovered at the end of the churn. "Zebda" is furthermore produced in Middle Eastern countries and is known as "Zobdeh" (FAO 1990).

In traditional fermented dairy foods, the lactic acid bacteria (LAB) microflora plays an essential role giving out the dairy products' particular features and native flavors (Zuo *et al.* 2014). In addition to their role in food fermentation, LABs can improve nutritional value, texture, shelf life, flavor, and safety through the production of bioactive substances (Viana de Souza *at al.* 2017). LAB-controlled fermentation is also considered a next-generation strategy for functional foods (Moslehishad *et al.* 2013). Many LABs can be used as a starter culture for cheese fermentation due to its ability to release lipases, proteases, or β-galactosidases to produce a unique flavor, texture, and aroma (Juan *et al.* 2016). In cheese fermentation, the most important characteristics and functions of LABs are: (1) the transformation of lactose in milk toward small molecular monosaccharides, glucose and galactose, which boost the cheese flavor genesis (Blaya *et al.* 2018); (2) the destruction of proteins into free amino acids and peptides in cheese, and (3) the dissolution of lipids into fatty acids (Konkit and Kim 2016). LAB belongs to Gram-positive bacteria which also include aerobic and facultative anaerobic, acid-tolerant, non-sporulating, either rod-shaped or spherical, bacteria. As a commercial starter cultures LABs must have acidification, proteolytic, autolytic, and antimicrobial activities, and be resistant to bacteriophages. In this group are included the various major genera namely: *Aerococcus*, *Alloiococcus*, *Enterococcus*, *Lactobacillus*, *Lactococcus*, *Leuconostoc*, *Oenococcus*, *Pediococcus*, *Streptococcus*, *Carnobacterium*, *Symbiobacterium*, *Vagococcus*, *Tetragenococcus*, *Weissella*, *Atopobium*, and *Bifidobacterium* (Gutiérrez-Cortés *et al.* 2017). It is well known that traditional dairy foods production is based on the metabolic activity of LABs to ferment milk sugars, especially glucose and galactose, so to produce lactic acid and aroma substances that give typical flavors and tastes to fermented products. Moreover, LABs end products provide an effective form of natural preservation of many widely used dairy products such as, antimicrobial metabolites so called bacteriocins, which are considered safe and natural preservatives, with great potential to be used on their own, or synergistically with other methods in food preservation (Marshall 2005). In recent years, many of LABs, isolated from different fermented food systems in distinct parts of the world have been experimented for their probiotic potentiality and capacity to produce industrially relevant substances (Ranadheera *et al.* 2012). Then, the aim of the present work was to isolate and characterize lactic acid bacteria from different traditional dairy products (Jben, Klila, and Zebda) and to characterize the different groups of microflora, acidifying power and antimicrobial producing bacteria using classical and molecular methods.

Experimental

Samples collection

Five samples of traditional dairy product (Jben) produced traditionally from sheep milk and without adding starter culture were collected from different area of the rural region (Oued El Malha) of Djelfa province (Fig. 1). Samples were brought to the laboratory at 4 °C in an icebox under sterile conditions, and stored in laboratory under refrigeration at 4 °C. All samples were analyzed immediately after delivery. The pH value of the different samples was measured using a Hanna Instruments HI 8424 Digital pH meter (Leighton Buzzard, UK). The pH probe was calibrated using standards of pH 4.0 and 5.6 (VWR International Ltd., Poole, UK) at 25 ºC.

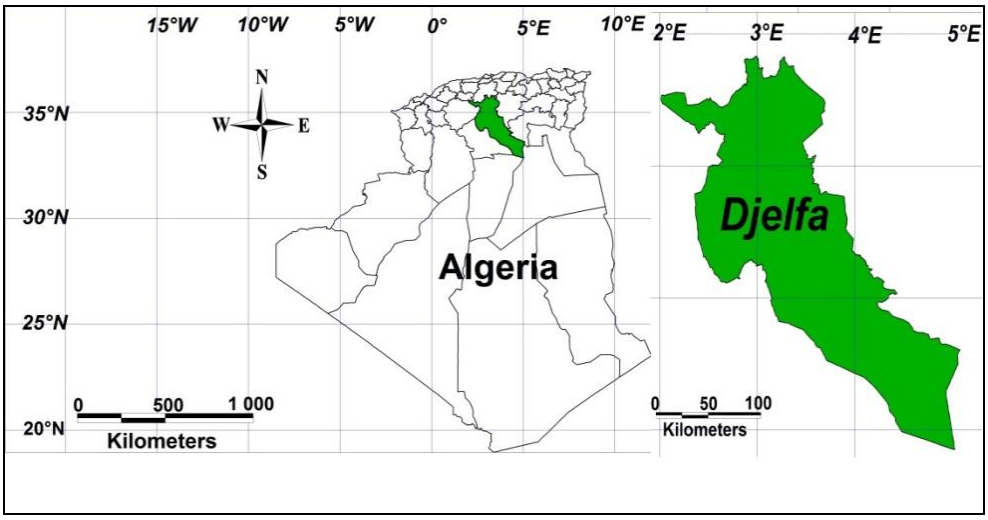

Fig. 1. The study area.

Isolation and phenotypic identification

Twenty-five grams of Jben sample were transferred to 225 mL of sterile 2 % sodium citrate solution, in an Erlenmeyer flask. Using a stomacher Laboratory Blender (Colwarth Stomacher 400C Seward Laboratory, UK) all the dairy samples were homogenized and decimal dilutions (10^{-1} to 10^{-5}) were prepared by mixing 10 mL with 90 mL of 0.1 % (w/v) sterile peptone water (Merck KGaA, Darmsadt, Germany), according to the International Dairy Federation (IDF) standard 122B (1992). Lactic acid bacteria were isolated and identified on three culture media: MRS agar (Merck KGaA, Darmstadt, Germany) a selective medium for counting and isolating lactobacilli, after incubation at 37 °C for 48 – 72 h, M17 agar (Merck KGaA, Darmstadt, Germany) a selective medium for counting and isolating lactococci after incubation at 30 °C for 48 – 72 h, and Eliker agar (Merck KGaA, Darmstadt, Germany) a selective medium for counting and isolating streptococci and lactobacilli after incubation at 37 °C for 48 h. For each batch of the fifteen dairy products samples, three colonies were taken randomly from MRS, M17, and Eliker agar plates. Consequently, a total of 135 colonies were gathered from each medium. All isolates were purified on MRS agar slant at 4 °C and sub-cultured every 4 weeks. Bacterial concentrations were calculated with the formula (Eq. 1):

$$N = \Sigma C/[(n_1 + 0.1\ n_2)]\ d \quad (1)$$

where C is the sum of counted colonies; n_1 is the number of the plates for the first dilution ratio; n_2 is the number of the plates for the second dilution ratio; d is the first dilution ratio and expressed as colony forming unit per gram of sample ($CFU.g^{-1}$) (Abraha *et al.* 2017).

The selected isolates were purified by repeated seeding on the agar media, LAB strains were kept on MRS agar slant at 4 °C and sub-cultured every 4 weeks. Prior to use, LAB strains were activated in MRS broth at 30 °C for 24 h and sub-cultured in MRS agar at 30 °C for 24 h. The isolates were identified according to their morphological, physiological, and biochemical characteristics. All isolates were tested for morphology and catalase production and Gram stain (Norris *et al.* 1982). Catalase-negative and Gram-positive isolates were checked for gas production from glucose in MRS and M17 broth containing inverted Durham tubes (Song *et al.* 2018). Growth at different temperatures (4, 10, 37, and 45 °C), growth in different pH values (4.0, 6.5, and 9.6) and Growth in 10 % NaCl broth were tested for coccus- and bacillus-shaped isolates. Detection of arginine dihydrolase was tested in Bromocresol purple medium (Merck KGaA, Darmstadt, Germany) (Thomas 1973). Search for citratase was done in Kempler and McKay medium (Merck KGaA, Darmstadt, Germany) (Lee *et al.* 1986). The isolate was inoculated into Methyl Red Voges Proskauer medium (Merck KGaA, Darmstadt, Germany), and then was incubated for 24 h at 30 °C. A total of three drops of KOH and two drops of the alpha-

naphthol reagent were added to the medium. A positive result was interpreted as color change into the pink. All the strains were tested for fermentation of sugars. This is done by choosing the following sugars: glucose, arabinose, cellobiose, mannitol, melibiose, raffinose, ribose, lactose, rhamnose, sorbitol, xylose, trehalose, maltose, sucrose, galactose, and fructose by adding the specific sugar to the Man Rogosa and Sharp broth medium (MRS-BCP) (Merck KGaA, Darmstadt, Germany). After incubation, acid production was indicated by a change in color, and gas production was detected by observation of gas collection in the inverted Durham tubes (Thakur *et al.* 2017).

DNA extraction from pure cultures

LAB isolate sharing some of the screened properties was further identified by molecular biology. DNA extraction was performed according to Liu *et al.* 2000 by adding 500 µL of a lysis solution composed of 400 mM Tris-HCl (pH 8.0), 60 mM EDTA (pH 8.0), 150 mM NaCl, and 1 % sodium dodecyl sulfate (SDS). The colony fractions are well crushed with sterile cones and incubated at room temperature for 15 min. A volume of 150 µL of a pH 4.8 solution composed of 5 M potassium acetate and 11.5 % glacial acetic acid was added and vortex briefly and then centrifuged for one minute at 12,000 × g. A volume of 400 µL of supernatant are recovered and transferred to another sterile Eppendorf tube, then added with the same volume of isopropanol and mixed briefly by inversion, before being centrifuged for 2 min at 12,000 × g. The pellet is washed with 300 µL of 70 % ethanol by centrifugation for one minute at 12,000 × g. The DNA obtained (the pellet) was dried overnight at ambient temperature and resuspended in 40 µL of sterile double distilled water and stored at -20 °C before using.

Amplification and sequencing of 16S ribosomal DNA

The purified DNA was used as a template for 16S rRNA gene amplification on the automatic thermal cycler (BioRad MyCycler™ thermal cycler, Bio-Rad Laboratories, Inc., Hercules, USA). The primers of 16S rRNA gene were 27f (5'-AGAGTTTGATCCTGGCTCAG-3') and 1492r (5'-GGTTACCTTGTTACGACTT-3'). The amplification carried out in a volume of 50 µL of reaction mixture composed of 25 – 50 ng of genomic DNA, 0.5 µM of each primer 27f and 1492r, 1X PCR buffer containing $MgCl_2$ (10 mM Tris-HCl, pH 9.0, 50 mM KCl, 1.5 mM $MgCl_2$, 0.1 % Triton X-100, 0.2 $mg.mL^{-1}$ BSA), 200 µM of the mixture dNTPs and 1.5 U of Taq DNA polymerase. For amplification, the following conditions must be observed: initial denaturation of the DNA template at 98 °C for 4 min, 30 cycles of denaturation at 94 °C for 1 min, hybridization at 52 °C for 1 min, and extension at 72 °C for 2 min. At the end the reaction mixture was maintained at 72 °C for 10 min for final elongation and then cooled to 4 °C.

The size of the PCR product was determined by agarose gel electrophoresis in 0.8 % agarose gel containing ethidium bromide and visualized by UV light and documented by the Gel DOC XR+ Gel Documentation System (Bio-Rad Laboratories, Inc., Hercules, USA).

PCR products were submitted to Beckman Coulter, Inc. (High Wycombe, UK) for purification and sequencing. The same primers, sense: 27f and antisense: 1492r, were used for amplification.

Phylogenetic study

Once the sequences have been determined, the raw sequences obtained (800 to 1,100 bp) were processed using the MEGA 7.0 software (Molecular Evolutionary Genetics Analysis program, version 7.0) (Kumar *et al.* 2016) to build the sequence of 16S rRNA. In this treatment, the antisense sequence is inverted and then aligned with the sense sequence; the two sequences are then fused at the 3' end for the first and 5' for the second to have a single 16S rRNA sequence about 1,500 bp (depending on the quality and length of the initial sequences). The 16S rRNA sequence thus determined is compared, to BLAST, to the homologous sequences of reference species listed in the NCBI genomic library. This comparison was made through the Ez Biocloud server (Yoon *et al.* 2017) with the sequences of all validated standard

species of bacteria. The sequence of the bacterial strain is aligned with the reference sequences by the Clustal W tool (Larkin *et al.* 2007), and then the set of aligned data is used for phylogenetic analysis with the MEGA 7.0 software. The evolutionary distances were computed as described Jukes and Cantor (1969) and are in the units of the number of base substitutions per site. The algorithms for constructing topologies of phylogenetic trees are those of the neighbor-joining method (Saitou and Nei 1987). Statistical validation of established phylogenetic links is performed by the Bootstrap test, the values of which are based on the result of 1000 analyses (Felsenstein 1985).

Acidifying activity of isolates

Potentiometric (pH measurement) and titrimetric methods were used to determine acid production capacity (Villani *et al.* 2001). To do this, isolates from frozen stocks were activated in MRS and M17 broth for 24 h at 30 °C for lactococci and 37 °C for isolates of enterococci and lactobacilli and night cultures of 100 µL were inoculated in 10 mL of sterile UHT skimmed milk broths. After 3, 6, 9, and 24 h, 2 mL aliquots were removed aseptically and pH changes were recorded using a pH meter (Orion Model 250 A, Orion Research Inc., Boston, MA) and acidity titratable during incubations at 30 °C and determined 37 °C.

Proteolytic activity

The proteolytic activity of the strain B04 was tested using MRS agar containing 2 % skimmed milk. After incubation, the proteolytic strains would form the clearing zone around colonies. The zones were measured in mm (Jini *et al.* 2011). As for the proteolytic activity residual proteins concentrations in the culture were estimated by a Coomassie G-250 binding procedure (Bradford 1976).

Antibacterial activity

The agar well diffusion method was used to assess the antimicrobial activity of the isolate B04. Their antimicrobial properties were tested against four major pathogenic bacteria: *Bacillus cereus* ATCC 10876, *Staphylococcus aureus* ATCC 6538, *Pseudomonas aeruginosa* ATCC 27853, and *Escherichia coli* ATCC 25922. The pathogenic bacteria were seeded in MH agar medium and allowing them to solidify at 45 °C. Wells were prepared at the agar with a sterile cylinder 6 mm diameter. Fifty micro-liters of the filtered cell-free supernatant of test strain were separately placed into the wells. The plates, prepared in duplicate, were kept at 4 °C for 24 h into the agar and then incubated at 37 °C for 24 h. After incubation, the antimicrobial activity was determined by measuring the diameter of the inhibition zones around the well using caliper in mm clearing zone diameter around the well more than 1mm was measured as a positive result (Akabanda *et al.* 2014).

Statistical analysis

Statistical data analysis was conducted using the Microsoft Excel 2007 (Microsoft Corporation, Redmond, WA, USA). All determinations were represented as mean values ± standard deviation of triplicate experiments.

Results and Discussions

Physicochemical analysis

The pH of samples was from 4.20 ± 0.39 to 5.72 ± 0.57. These values are similar to those found by Rhiat *et al.* (2013).

Isolation and identification of LAB

This study was carried out for main purpose to isolate and characterizes of large number of lactic acid bacteria from traditional dairy product (Jben) and characterization of different groups of microbiota. The results obtained from the present study demonstrated that there is diversity of lactic acid bacteria; with 65 lactic acid bacteria belonging to the genus *Lactobacillus* (33.85 %), *Lactococcus* (32.31 %), *Leuconostoc lactis* (23.07 %), *Enterococcus* (4.62 %). These bacteria have good biochemical and biotechnological properties, which puts it at the forefront of industrial microbiology. After enumeration on the appropriate culture

media, MRS, M17, MSE, and Elliker, the results are as follows: 2.2 x 10^4 to 2.1 x 10^6 CFU.mL^{-1}, 1.1 x 10^2 to 1.7 x 10^3 CFU.mL^{-1}, 2.0 x 10 to 1.8 x 10^2 CFU.mL^{-1}, and 2.2 x 10 to 2.8 x 10^4 CFU.mL^{-1}, respectively. All isolates were Gram-positive and catalase negative bacteria; among the isolated lactic acid strains, one strain named B04 was purified and identified (Fig. 2, 3); its characteristics are shown in Table 1.

Table 1. Morphological, physiological, and biochemical characteristics of the isolated LAB strain B04.

Characteristics	Strain B04
Gram staining	+
Catalase test	-
Motility	-
Gas production from glucose	-
Hydrolysis of:	
ADH	V
Citrate	-
Growth at different temperature (°C):	
04	+
10	+
37	DR
45	DR
Growth at different pH:	
4.0	+
6.5	-
9.6	+
Growth in the presence of 10% NaCl	+
Sugars fermentation:	
Arabinose	-
Cellobiose	+
Mannitol	+
Melibiose	+
Raffinose	+
Ribose	+
Lactose	+
Rhamnose	+
Sorbitol	DR
Xylose	-
Trehalose	+
Maltose	+
Sucrose	+
Galactose	+
Fructose	+

(+) – Positive reaction, (-) – Negative reaction, V – variable, DR – delayed reaction, ADH – alcohol dehydrogenase.

After extraction and amplification of the 16S rRNA of the isolate B04, the corresponding sequence is determined, and then aligned and compared with the sequences of the bacteria contained in the genomic databases deposited at the GenBank. Among the isolates selected in this work, the almost complete 16S rRNA gene sequence (1,500 bp) of the isolate B04 was determined and deposited in the GenBank data library under the accession numbers KY746350.1. The BLAST performed for the isolate B04 shows its proximity to the species *Enterococcus lactis* (BT159^T), which appeared to be the closest, with a similarity of 91.22 %. Neighbor-joining phylogenetic analysis shows the relationship between the isolate B04 and the typical strains of the closest species of the genus *Enterococcus* (Fig. 4). The similarity rate between 16S rRNA sequence is less than the critical threshold for qualifying an isolate as a new species that is 98.65 % (Kim *et al.* 2014) and a new genus that is 94 % similar to 16S rRNA sequences (Yarza *et al.* 2008). These results indicate that the strain B04 belongs to a new genus. The confirmation of these results can be possible with whole genome sequencing then digital DNA-DNA hybridization and further chemotaxonomic analyses.

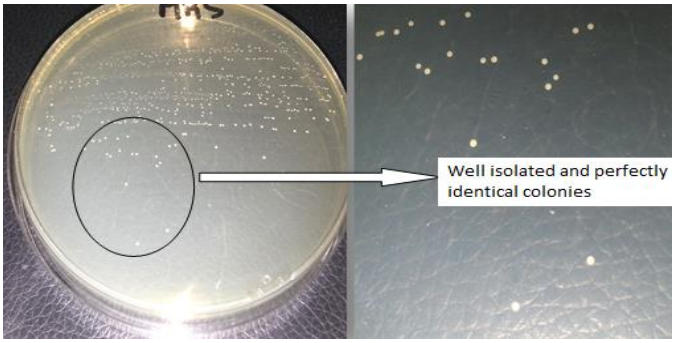

Fig. 2. Macroscopic appearance of colonies of a lactic acid bacterium B04 after 48 h of incubation at 30 °C, seeded by streaks on MRS Agar medium.

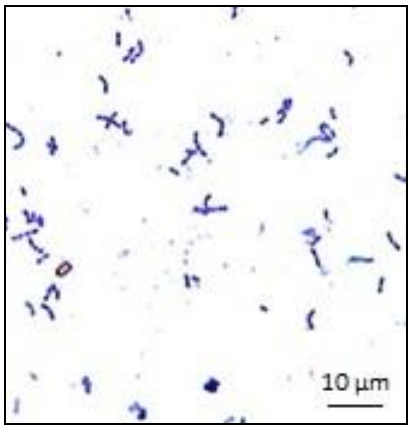

Fig. 3. Microscopic image showing the appearance of Gram-positive stain of the strain B04.

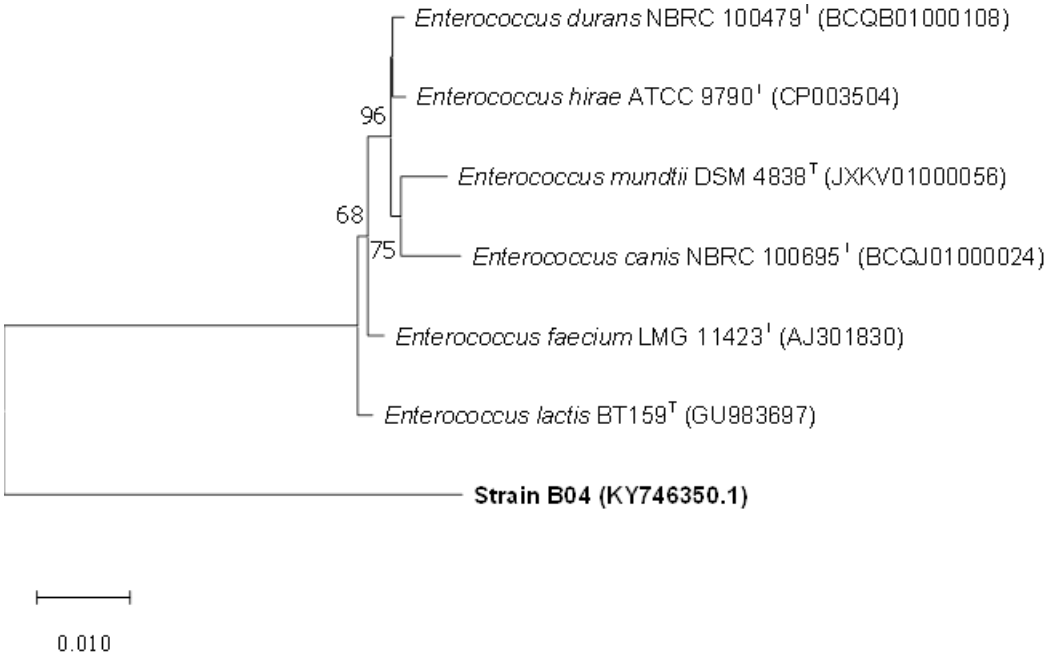

Fig. 4. Phylogenic tree based on 16S rRNA gene sequences showing the position of the strain B04 and its related species of the genus *Enterococcus*. The optimal tree is shown. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1,000 replicates) are shown next to the branches. Numbers at nodes indicate percentages of bootstrap support based on neighbor-joining analysis of 1,000 resampled datasets. Bar, 0.010 substitutions per nucleotide position. This analysis involved 7 nucleotide sequences. All ambiguous positions were removed for each sequence pair (pairwise deletion option). Numbers in the parentheses indicate the GenBank accession numbers.

Physiological and antibacterial properties of LAB isolated from traditional dairy products

The diminution of pH of the milk is due to the production of lactic acid from lactose fermentation (Marroki *et al.* 2011). The lactic acid values of analyzed samples showed that the isolate B04 was highly acidifying isolate that coagulate milk before 18 h of incubation and the remaining isolates coagulate milk after 18 to 24 h of incubation. The amount of lactic acid was measured (15 – 25 °C). The acidity increases with the time in a variable way to arrive until 75.00 ± 02.11 °D (°D: Dornic degree; this result represents the mean ± standard deviation of three replicates) after 24 h with isolate B04.

It has been found that LAB B04 isolate presented antimicrobial activity against *Bacillus cereus* ATCC 10876, *Staphylococcus aureus* ATCC 6538 and *Escherichia coli* ATCC 25922. The strain LAB B04 was completely inactivated by α-chymotrypsin and pepsin. These results suggest that the biochemical nature of the molecule produced is peptidic. The inhibitory compounds produced by the isolate showed great resilience to thermal treatments. The bacteriocin has proved stable over a wide pH range with all peptides, now some antimicrobial activity in the pH ranges from pH 4.0 – 7.0. Bacteriocin is very sensitive to pH its stability was detected at a pH range of 3.5 to 6.5. In this study, bacteriocin produced by the strain B04 had the same profile and was active at pH values 4.0 to 6.0. Based on the results, it can be concluded that the bacteriocin produced develops a positive activity against *Staphylococcus aureus* ATCC 6538 and *Escherichia coli* ATCC 25922 (Table 2, Fig. 5).

Table 2. Antibacterial activity of lactic acid bacteria strains against some pathogenic bacteria.

	Diameter of inhibition zone [mm] on pathogenic strains			
Strain	*S. aureus*	*E. coli*	*B. cereus*	*P. aeruginosa*
B04	17.33 ± 3.06	9.33 ± 2.52	9.67 ± 1.15	0

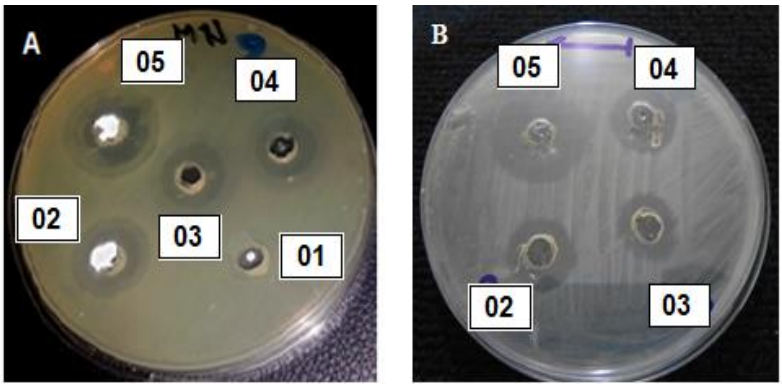

Fig. 5. Antibacterial activity of strain B04 compared to four species of LABs against (**A**) *Escherichia coli* ATCC 25922 and (**B**) *Staphylococcus aureus* ATCC 6538 by the diffusion method on Mueller-Hinton agar after 24 h of incubation at 30 °C in the darkness. 1 – *Lactobacillus brevis*, 2 – *Lactobacillus intestinalis*, 3 – *Leuconostoc lactis*, 4 – Strain B04, 5 – *Lactobacillus plantarum.*

Conclusions

The selected strain of lactic acid bacterium B04 has high acidifying power and proteolytic activity and a strong bactericidal effect against pathogenic strain *Staphylococcus aureus* ATCC 6538 and a medium effect against *Bacillus cereus* ATCC 10876 Gram-negative pathogenic strain *Escherichia coli* ATCC 25922. Based on these results, it can be concluded that traditional dairy products are cultural goods that deserve to be studied, characterized, and protected. Furthermore, it would be interesting to enhance the physicochemical and biological investigations for the transformation of milk into different dairy products through the intervention of controlled industrial fermentations.

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Conflict of interest

The authors declare that they have no conflict of interest.

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