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Identification of Indigenous *Lactobacillus fermentum* by 16S rRNA Gene Sequencing Isolated from Two Different Ecosystems: Newborn Infants and Bee's Intestine

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ABSTRACT

Background: The consumption of functional ferments that have functional characteristic sand contributes enormously to the safety and quality of food by offering several nutritional, technological and health properties. *Lactobacillus fermentum (Lb. fermentum*) is a Gram-positive bacterium belonging to the genus *Lactobacillus* and many are said to have the effect of preventing gastro-intestinal infections and improving immunological response. Additionally, *Lb. fermentum* produces diverse and potent antimicrobial peptides which can be applied as alternatives to antibiotics.

Methods: In this study, samples were isolated from two different ecological niches, the first was feces of newborn infants (0-3 months) and the second from bee's intestine, from south of Algeria : Ghardaïa 32°29′00″ North, 3°41′00″ East. Two indigenous lactic acid bacteria were isolated and evaluated for their probiotic properties. The isolate identification by sequencing 16S r DNA was performed using the universal primers 27F and 1492R. The NCBI Gen Bank database was used to have similarity of species.

Result: The cultures showed good survival in simulated transit fluids as well as high acid and bile tolerance. The isolates also demonstrated antibacterial activity against *Escherichia coli* and *Staphylococcus aureus*, the inhibition zone was measured between 10 mm and 20 mm of diameter respectively. Comparing the sequences of the isolates with the Gen Bank database (NCBI), a high percentage with species of *Lactobacillus fermentum* was shown.

Key words: Bee's intestine, Ecology, *Lactobacillus*, Newborn feces, PCR.

INTRODUCTION

Due to their involvement in multiple processes, including fermentation, aromatization, CO_2 production, proteolysis and bio-conservation, *Lactobacillus* species are the most well-liked among lactic flora. In addition, strains of *Lactobacillus* are widely recognized for their probiotic qualities (Ait Abdeslam *et al.,* 2019). Lactic bacteria are characterized natives trains with potential probiotic health benefits to humans. Dairy products containing *Bifidobacteria* have potential benefits for infants and adults. Effect is generally related to inhibition of pathogens and restoration of normal intestinal flora (Narayanan and Subramanian, 2023). The genus *Lactobacillus* was proposed by Beijerinckin in 1901 and includes Gram-positive, fermentative, facultative anaerobic and non-spore-forming micro-organisms. The genus is classified in the phylum Firmicutes, class *Bacilli, order Lactobacillales,* family *Lactobacillaceae*, which contains the genera: Lactobacillus, *Paralactobacillus* and *Pediococcus* (Zheng *et al.,* 2020).

It was in 1994 at the University of Tartu in Estonia that *Lactobacillus fermentum* ME-3 of the *Lactobacillus* genus was isolated from the faces of healthy children. During its cultivation, it was possible to show that the ME-3 strain had various interesting properties. In vitro, strain ME-3 has been shown to be able to reduce inflammatory processes, stimulate antioxidant defenses and synthesize nitric oxide (Naghmouchi *et al*., 2020). Other analyzes have demonstrated an anti-microbial effect, in particular against Gram-negative bacteria (*e.g. Staphylococcus aureus*, *Salmonella typhimurium* or *E. coli*). The metabolic activity of probiotic cultures participates in the formation of biological active peptides from milk proteins such as casein and whey

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(Prashant Bachanti *et al*., 2018). The aim of this study wasto isolate strain of *Lactobacillus fermentum* from two ecology strains and study their technological properties to select probiotic trains.

MATERIALS AND METHODS

Origin of samples

Samples have been isolated aseptically from two different ecological niches: newborn infants aged (0-3 months) and bee's intestine, the hive of bees is in Ghardaia, located in the northern part of the Algerian Sahara, 32°29′00″ nord, 3°41′00″ East. The sample collection has been carried out in October 2022.

Culture media

Isolation of bacteria from bee's intestine was done under strict sanitary conditions by separating the abdominal part and Grin digitizing forceps and Pasteur pipette and then dissolved in physiological saline (Guerzou *et al.,* 2017). From the stool, one gram was suspended in a tube containing 9 ml of physiological water. Plates were incubated at 37°C for 24 h/48 h. Typical colonies were selected and gram coloration was done.

Phenotypic characterization

After purification by successive planting, further identification tests were performed by using the following physiological and biochemical tests (catalase, oxydase, nitrate reductase, CO_2 formation from glucose and growth in a salt medium) and the fermentation of sugar is examined on MRS medium containing bromocresol purple as pH indicator. 2% of various sugars (Müller, 1990).

Genomic DNA extraction, amplification, se-quencing and analysis of the 16S rRNA gene

Genotypic study was carried out in the laboratory DIAG Gene. ANGEE. France, in 2022.

Extraction of genomic DNA using FTA method

The commercial microlys is kit I cycler Biorad (Biorad, USA) was used for the extraction of DNA heeding the advice of the manufacturer. Centrifugation was used to extract the cells from fresh microbial cultures and aspiration was used to remove the supernatant. The Nano Drop spectrophotometer (Thermo Scientific, USA) is used to measure the amount and quality of DNA extracts.

PCR amplification and conditions

Amplification of the 16S r RNA gene from genomic DNA was carried out using the universal primers: 27f 5′-AGAGTTTGATCCTGGCTCAG-3′ and 1492R 5′-GGTTAC CTTGTTACGACTT-3′.

The amplification reaction was performed in a total volume of 25 µL consisting template (1 µL), 10× buffer (with Mg^{2+}) (2.5 µL), 2.5 mM each dNTP (1 µL), 10 µM F (0.5 µL), 10 µM R (0.5 µL) and enzyme (0.2 µL).

The MilliQwater was added for remaining of reaction and then performed in Biorad thermal cycler (Biorad, USA). PCR amplification reactions were carried out the following program: an initial denaturation at 95°C for 5 min, followed by 35 cycles of denaturation at 94°C for 30 s, annealing at 55°C for 45 s and extension at 72°C for 90 s. A final elongation was done at 72°C for 10 min (Bekenniche *et al.,* 2021).

The PCR amplicons of approximately 1500 pb was visualized in 1.5% aggarose gel electrophoresis under UV plate.

Studies of the technological properties of *Lactobacillus fermentum* strains

Before each experiment, the cultures are rejuvenated by incubation in an MRS- broth at 37°C for 24 h to obtain cultures at the end of the exponential phase. Indeed, according to the scientific literature, 18 to 20 hours of incubation are necessary to reach the end of the exponential phase, indicated by the appearance of disorders in the broth which testifies to the presence of a bacterial biomass: (-) (+).

Strain survival to the simulated gastrointestinal conditions

Bacteria was subjected to in vitro simulated gastric fluid and whose composition (%: P/V) is: 6.2 NaCl, 0.22 $CaCl_2$, 1.2 $NaHCO_3$, 2.2 KCl, 0.3 pepsin and pH 2(Kouadri Boudjelthia *et al.,* 2023). Distilled water solutions are adjusted to pH 2 with 1 M HCl. A distilled water at pH 6.5 was taken as control. The solutions are prepared in 10 ml tubes, sterilized and stored at room temperature before use. Subsequently, 1 ml of each young culture of the strain studied is transferred into the tubes containing the acid solutions. The incubation is carried out at 37°C, in anaerobic conditions for 180 minutes in order to simulate the survival of the bacteria in the acid conditions of the human stomach.

Enumeration of viable cells in simulated gastric pH solutions

One ml aliquot of each culture was taken to enumerate viable cells in acidic solutions after the predetermined incubation time and performed dilutions up to 10-8 in saline (0.9% of NaCl). Viable cells were counted with the agar plate method on MRS at the end of the time required for the process of converting the bolus into chyme in the stomach, which is 4 h of incubation at 37°C.

Resistance to bile salts

Different concentrations of bile were prepared: 0.2%, 0.3%, 0.4%, 0.5% in distilled water. The solutions were prepared in sterilized 9 ml tubes of each concentration and stored at room temperature before use. One ml of the young culture was transferred into the tubes containing the solutions of the bile salts, then carry out dilutions 10-1 up to 10-8. The last three dilutions are inoculated in MRS mass agar

followed by incubation at 37°C in anaerobic conditions for 48 h. The 10-1 tubes of strain are incubated at 37°C in anaerobic for 3 h, the count was also carried out after 3 hours of incubation. concentrations of bile were prepared 3%, 4% and 5% in MRS agar medium. The choice of bile concentration for (0.4%) was based on its physiological concentration in the duodenum (Sensoy, 2021).

Resistance to gastricjuice

Resistance to gastric juices was studied according to Thyrabonyanon *et al*. (2009). One ml of overnight culture (18 h) was suspended in 9 ml of gastric juice solution at pH 2. The viability was determined at 0h time and 3 hours of incubation on 450 µL of MRS agar medium (pH8 + bile salts (0.5%). The survival rate was calculated by the following formula:

$$\text{Survival rate (\%)} = \frac{\text{Log UFC at time } t_1}{\text{Log UFC at time } t_2} \times 100$$

Resistance to pancreatic enzymes

According to the method by Ouwehand *et al*. (2001) we took 1 ml from each tube and prepared cascade dilutions, 1 ml of young culture was suspended in the pancreatic solution. Strain suspension was inoculated into100 mg of pancreatic Trypsine solution freshly prepared and 100 mg of α-chymotrypsine solution freshly prepared. Viable cells were determined with the MRS agar plate count method after 5 h exposure to enzymes at 37°C. The survival rate was calculated using the previous formula of survival rate. The incubation times are respected for each treatment and the temperature was maintained at 37°C. Dilutions were carried out up, the 10-8 dilution which was inoculated in depth in an MRS medium at pH 7 and incubated at 37°C (Ouwehand *et al*., 2001).

Determination of biological activities of the strains

Preparation of active supernatant

The supernatant was obtained by centrifugation (10000 rpm, 4°C, 20 min) of a young culture incubated at 37°C. The supernatant was sterilized by filtration (0.45 µm) to remove residual cells. One ml of supernatant was retained as the untreated filtrate.

The supernatant obtained was divided into two volumes, the first was neutralized with 1N NaOH to obtain a pH of 6.50 and the second was left at the initial pH. In order to determine the protein nature of the inhibiting agent, 1 ml of supernatant was added with 1 mg/ml of proteinase k and trypsin. Then another incubation was carried out at a temperature of 100°C in or der to denature the proteolytic proteins (Tahara and Kanatani, 1996).

Technical

1 mL of the neutralized supernatant was mixed with 1mL of the enzyme solution (volume ratio 1:1) to have a final enzyme concentration of 1 mg/mL. Incubate for 2 hours at 37°C in a water bath to allow contact with the enzyme (Cocolin and Rantsiou, 2007). Load the wells with the following solutions: buffer, buffer with enzyme, supernatant without enzyme and supernatant with enzyme from dishes already seeded with the pathogenic strain. Allow to diffuse then incubate for 24 hours at 37°C.

Antimicrobial activity

The indicator bacteria *Escherichia coli* ATCC 25922, *Staphylococcus aureus* ATCC 33862, werecultured in a nutrient broth for 18 h, then culture (1% w/v) was added to the soft agar. A15 ml of the mixture was added into the petri dishes. A 50 µl of samples prepared rom the filtrate were added to each well of the dish. The agar plates were incubated at 37°C overnight for all pathogenic bacteria tested; the antimicrobial activity was recorded as a clear zone of inhibition around the wells.

RESULTS AND DISCUSSION

The colonies of *Lactobacillus* developed on the MRS medium are of the punctiform type, of whit is coloring, of regular outline. The strains develop in an anaerobic environment; do not produce gas from glucose, absence of urea indole and gelatinase activity as well.

Fermentation profile

The result of the fermentation profile shows that the LF1 strain isolated from bee intestines is characterized by are stricted fermentation of the sugars tested compared to the LF2 strain isolated from infant stool.

Genotypic identification

Sequences were analyzed by the National Center for Biotechnology Information (NCBI) database. BLAST results identified isolates LF1 and LF2 to *Lactobacillus fermentum at a* 99% of homology. LF1: *Lactobacillus fermentum* strain MH23 16S ribosomal RNA gene, partial sequence Sequence ID: MG027694.1 and LF2: *Lactobacillus fermentum* strain CAU1847 16S ribosomal RNA gene, partial sequence Sequence ID: MF424315.1.

Tolerance to low pH of the stomach expressed of survival

The loss of viability at pH 6 was negligible for all the strains tested, whose portion of viable cells after 3 hours of incubation was almost maximal. When exposed to pH 3, the LF2 strain maintained the largest portion of viable cells (Fig 1).

Resistance to gastric juices

The Following graph2 represents the results obtained by the strains isolated with respect to their resistance to gastric juices (Fig 2).

Resistance to pancreatic enzymes

Fig 3 represents the results obtained by the strains isolated with respect to their resistance to pancreatic enzymes.

Enumeration of viable cells in bile salt solutions

Table 1 represents the results Tolerance to bile salts expressed of survival.

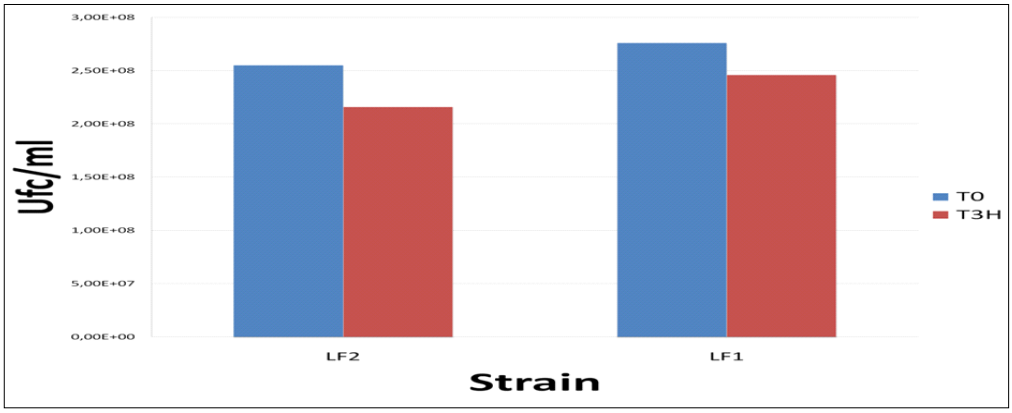


Fig 1: Tolerance to low pH of the stomach expressed of survival.

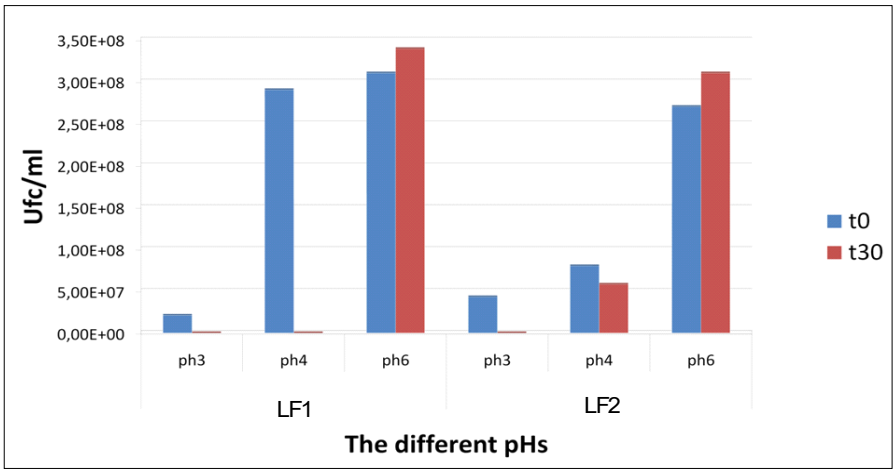


Fig 2: Results of strain resistance to gastric juice.

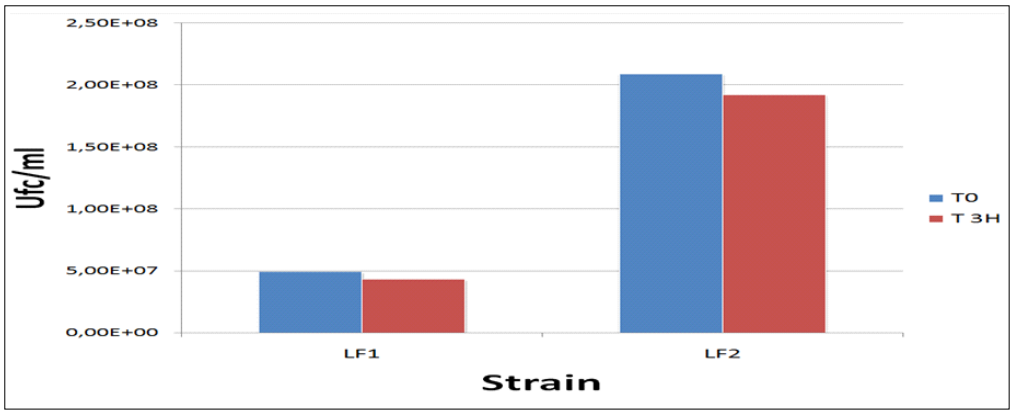


Fig 3: Resistance of strains to pancreatic enzymes.

Table 1: Tolerance to bile salt expressed of survival.

	0.2%	0.3%	0.4%	0.5%	3%	4%	5%
LF2	+	++	+++	+++	-	-	-
LF1	++	++	+++	+++	-	-	-

+: Weak growth, ++: Intermediate, +++: Strong, -: Zero growth.

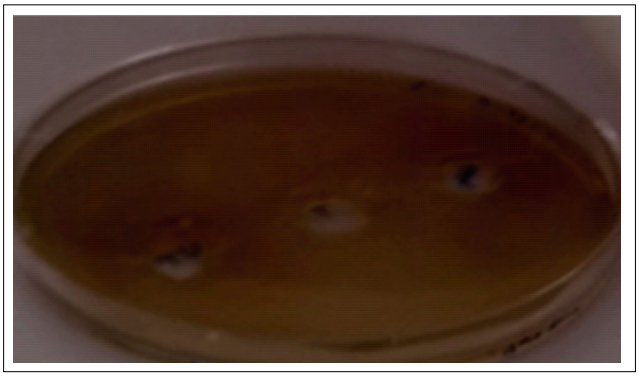


Fig 4: The diameter zone of inhibition mesured with *E. coli* and *S. aureus.*

Antibacterial activity

The test of disk diffusion determined the diameters of the zones of inhibition in antimicrobial activity of the strains. The inhibitory activity persisted during the incubation of the strains with the native supernatants from the cultures of the LF1 and LF2 strains in the test and persisted less when not using the treated supernatants. The diameter zone of inhibition was mesured with *E. coli* and *S. aureus* at 10 and 20 mm respectively (Fig 4).

All strains exhibited good tolerance to the *in vitro* tests of simulated host gastrointestinal conditions and good resistance against digestive enzymes (Trypsine and α chymotrypsine).

The DNA identification at laboratory DIAGE Gene permitted the classified species according with universel sequences DNA at *Lactobacillus fermentum* genes.

Technological characterization of the strains studied

The effect of pH on microbial growth and bile resistance acts on the enzymatic activity of the membrane, the permeability and the bioavailability of certain nutrients. Our results were similar to studies that have been done on the probiotic activity of *Lactobacillus fermentum* strains that were screened for mucin adhesion, resistance to low pH and bile, auto aggregation, hydrophobicity and survival in-*in vitro*-environment (Kanwal *et al*., 2019)

The results obtained showed good resistance to pancreatic enzymes as well as gastric juices and this is a good indication that our strains have a probiotic character. The cultures showed a high tolerance to acid and to bile as well as survival in simulated transit fluids; no results agree with the results of Archer *et al*. (2015) on the probiotic attributes of *Lactobacillus fermentum* isolated from human feces which revealed good viability under the stimulated condition of gastric stomach.

As part of this work, we have tried to make our contribution to the study of lactic acid bacteria isolated from two different ecosystems, stools of newborn infants which is an ecological niche very rich in microorganisms which provide nutritional and health benefits. Very important to humans as well as from the digestive tracts of bees where work has shown the diversification of the digestive microbiota of the latter.

According to the results obtained in work of Kouadri Boudjelthia *et al.* (2023) with 450 µg of enzymes added, our study reveled good resistance against digestive enzymes added at a concentration of 100 mg. It is clear that the two strains of *lactobacillus fermentum* tested had a good inhibitory activity against the pathogenic germs *Escherichia coli* and *Staphylococcus aureus* with diameters of the inhibition zones ranging from 10 to 20 mm respectively. Radja *et al*. (2022) obtained an antimicrobial activity with inhibition zone diameters ranged from 5±0.02 to 21±2.12 mm of *Lactobacillus* isolated from honey.

These results are in agreement with those found by Falah *et al*. (2019) on the evaluation of the adhesion and anti-infective properties of the 4-17 strain of the probiotic *Lactobacillus fermentum* against *Escherichia coli* responsible for urinary tract infection in the man. The antimicrobial is an important criterion for the selection of probiotics.

On the other hand, a weak inhibition was noted for the supernatants neutralized at pH 6.5 and the supernatants treated with trypsin and proteinase k; which proves that the enzymes have partially degraded the inhibiting agent. This result may suggest that the inhibitory agent is of a protein nature, emphasizing the probiotic characteristics of *lactobacillus fermentum* such as the production of bacteriocin as well as the role of lactic acid production in the prevention of infections. Gastrointestinal disorders revealed by several I authors (Strompfova *et al.,* 2018; Naghmouchi *et al.,* 2019; Thumu *et al.,* 2020).

CONCLUSION

Probiotics are a major research topic. During this work we were interested in the isolation of *Lactobacillus fermentum* from two different origins stool of infants and intestine of bee where we chose to study bacterial properties which are used in priority during the selection of strains of probiotic interest, including technological characters and biological characters: resistance to gastrointestinal conditions, tolerance to bile, gastric juice and antibacterial activity, making them very good candidates for technological applications. After several phenotypic and genotypic identifications, the 2 strains demonstrated a very high acidifying power with quite significant resistance and tolerance to bile and pancreatic enzymes as well as an antagonistic effect *via* the pathogenic bacteria tested *Escherichia coli* and *Staphylococcus aureus.*

These characteristics support more research to improve these strains' probiotic status so they can be employed in the food business us probiotic product.

Conflict of interest

The authors declare no conflict of interest.

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