Full Length Research Paper

Characterization of *Lactobacillus* strains isolated from Algerian children faeces for their probiotic properties

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Lactic acid bacteria termed probiotics have preventive as well as curative effects on several types of diarrhoea of different aetiologies. The main objective of this study was to screen lactobacilli strains having probiotic traits, isolated from Algerian healthy children faeces on the purpose of using them further in children diarrheal illnesses. One hundred and twenty (120) lactic acid bacteria isolates were selected from faecal samples of healthy Algerian children aged between five and ten years. Gram positive rods and catalase negative bacteria (52 isolates) were screened, *in vitro*, for their probiotic potential properties including ability to survive in simulated gastro-intestinal conditions, adherence to Caco-2 cells and their antimicrobial activity. The results show that only five strains resisted in simulated gastric juice at pH 1.5 and pepsin. Four of them were resistant to simulated intestinal conditions at pH 8 and pancreatin and have a good adherence. In the end, three of them were retained as they display interesting probiotic profiles characterized by a strong antimicrobial effect against some intestinal pathogenic bacteria. They were identified by 16S rDNA sequencing as *Lactobacillus plantarum* F12, *Lactobacillus brevis* G6 and *Lactobacillus paracasei* B13.

Key words: Probiotics*, Lactobacillus*, selection criteria, human origin.

INTRODUCTION

The digestive tract of the newborn infant is almost sterile at the time of birth.However, it is rapidly colonized by diverse groups of microorganisms. First**,** the infant digestive tract colonization by vaginal mother lactobacilli flora is realized (Song et al., 1999). Then, this flora is rapidly replaced by a new flora composed of lactobacilli and other microorganisms originating from food and environment (Tannock et al., 1990). Among intestinal biota, lactobacilli and bifidobacteria are the important organisms recognized for their health and nutritional benefits as probiotics (Rosenfeldt et al., 2002; Kesarcodi-Watson et al., 2008). Probiotics are defined as "live microorganisms that when ingested in adequate amounts, confer beneficial effects to the host by improving its intestinal microbial balance (Schrezenmeir and Vrese, 2001). Furthermore, for being selected, the

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probiotic strains must overcomesome barriers and perform some functions including, for example, human origin for Human use, resistance to gastrointestinal conditions such as gastric acidity and bile toxicity, the ability of adherence to the host's epithelial cells, antimicrobial activity against pathogens and the possession of a health-promoting effects on the host (Dunne et al., 2001; FAO/WHO, 2001). Probiotic strains should also have good technological properties (Parvez et al., 2006) and be safe without virulence factors, harmful activities and transmissible antibiotic resistances (Salyers et al., 2004). A number of clinical studies have demonstrated the efficacy of lactic acid bacteria (LAB) probiotics, particularly *Lactobacillus* strains, in the treatment and/or prevention of diarrhoea (Fuller, 1991; Rosenfeldt et al., 2002; Servin, 2004; Nomoto, 2005; Guo et al., 2010). In Algeria, as in some developing countries, despite declining cases, infectious diarrhoeas remain a major cause for infant mortality. This is because of a poor hygiene, a bad nutrition and sometimes a lack access to health structures (UNICEF/WHO, 2009).

According to the joint report of FAO/WHO (2001), probiotics can be an important means to reduce diarrhoeas in developing countries. Though the efficacy of probiotics needs to be demonstrated and controlled by clinical trials, their primary preselecting is based on *in vitro* tests (Dunne et al., 2001; FAO/WHO, 2002). This work aims to collect faeces from Algerian children cohabiting diverse rural areas in Jijel city (Eastern Algeria). The lactic acid bacteria were isolated from these samples, and screened for their probiotic traits, in order to use them further in child nutrition, to prevent diarrhoeas. The characterization of the screened bacteria was based on the study of several criteria, that is, the tolerance to simulated human gastric and intestinal juice, the adherence to Caco-2 cells and antimicrobial activity against some intestinal pathogens. The choice of different remote rural areas of Jijel city to collect faeces samples is not trivial; the objective was to target a diversity of strains based on the variability of natural food bowls.

MATERIALS AND METHODS

Isolation and identification of lactic acid bacteria

In this study, the samples were recovered twice from healthy children faeces (five to ten years old). One gram of each faecal sample was mixed with nine ml of 0.9% of sterile peptone water, and decimal dilutions were prepared. Then, 0.1 ml of appropriate dilutions was spread on Man Rogosa Sharp (MRS) agar. After 48 to 72 h of plate incubation, the obtained bacterial colonies were purified several times on MRS agar. Preliminary identification of the isolated bacteria was carried out using microscopic observation, Gram staining, catalase reaction and API 50 CHL test strip (Biomerieux, France).

Survival ability under gastric conditions

The ability of bacteria to survive under gastric conditions was carried out according to (Huang and Adams, 2004) with slight modification. The simulated gastric juice was prepared (3 g/L of pepsin (EC 3.4.23.1, 7190 Merck) in 0.5% (w/v) NaCl), adjusted to pH1.5. Overnight cultures were prepared in MRS medium and 1 ml of each preculture strain was added to 9 ml of a simulated gastric juice and incubated at 37°C. One millilitre of culture was sampled at 0 and 2 h of incubation. The viable bacteria were determined by plating 100 µl of appropriate dilutions on MRS agar. The experiment was replicated three times.

Survival ability under intestinal conditions

The capacity of bacteria for surviving under intestinal conditions was carried out by using, simulated small intestinal juices (1 g/L pancreatin (EC 232-468-9, P7545 Sigma-Aldrich) in 0.5% NaCl), with and without 0.3% bile salts (Ox-gall, Sigma), and adjusted to pH 8. The total viable bacteria were determined, at 0 and 4h of incubation, as mentioned above (Huang and Adams, 2004).

In vitro adherence assay to Caco-2 cells

The ability of the strains to adhere to human cells was investigated in the Caco-2 intestinal cells following the procedure described by Sugimura et al. (2011) modified by Ren et al. (2012). Monolayers Caco-2 cells were grown in Dulbecco's Modified Eagle's Medium DMEM (Lonza BE12- 604F) supplemented with 10% heat-inactivated foetal bovine serum (Gibco 10270-106), penicillin (100 U ml^{-1}; Lonza) and streptomycin (100 mg ml^{-1}; Lonza), in 24-well (Cellstar 662- 160) tissue culture plates, at a concentration of 1.0 x 10^{5} cells ml^{-1}. The medium of Cells were replaced by fresh unsupplemented DMEM for 1h prior to the adhesion assay and then rinsed three times with DMEM. The cells were incubated with approximately 10[9] CFU ml^{-1}of LAB isolates. After 2 h of incubation at 37°C under 5% CO_2 atmosphere, the monolayers were washed three times with sterile phosphate buffer saline (pH 7.4). The adherent bacteria were detached by repeated pipetting. The counting of adherent LAB cells was done by performing a series of dilutions and plating on MRS agar. The assay was repeated three times for every strain; counts were performed in duplicates. The percentage of adhesion (CFU/100 cells) was estimated using the formula (the adhered LAB cells/the Caco-2 cells x 100%). On the other hand, the adherent bacteria to Caco-2 cells were fixed with methanol for 30 min, Gram stained (0.5% crystal violet) and observed by microscopic examination under oil immersion (x 100 magnification).

Antimicrobial activity

The capacity of the strains for inhibiting a representative group of intestinal pathogens was determined using the well diffusion method described by Hechard et al. (1990).The tested pathogens were *Escherichia coli* O111*, Escherichia coli* O55*, Salmonella typhimurium*, *Listeria monocytogenes*, *Bacillus cereus* and *Escherichia coli* ATCC 29522. Twenty millilitres of MRS agar at 45°C were vigorously mixed with 200 µl of an overnight culture of the indicator strain and poured into Petri dishes. Supernatants were prepared by centrifugation of the culture at 8000 x *g* for 10 min, filter sterilized through a 0.22 µm pore membrane (Sartorius, Germany), added to a sterile solution of catalase (1000 U ml^{-1}, Sigma) to eliminate the possible inhibition effect of hydrogen peroxide and neutralized to pH 6.5 with NaOH mol^{-1}. Wells were made and 50 µl of overnight cultures and cell freesupernatants were placed in each well. Experiments were performed in three independent experiments, and each assay was performed in triplicate.

Table 1. Survival ability of lactic acid bacteriaisolates in simulated gastric conditions.

| *Lactobacillus* strain | Code | Viable count (log CFU/ml) at time 0 and 2 h | | | |
		Simulated gastric juice	0 h	2 h	Survival cells (%)
L. paracasei subsp *paracasei*	A20	pH 1.5	8.45 ± 0.04	3.49 ± 0.03	41.30
		pH 1.5 and pepsin		2.01 ± 0.13	23.78
L. plantarum	F12	pH 1.5	9.65 ± 0.11	6.81 ± 0.01	70.56
		pH 1.5 and pepsin		5.92 ± 0.07	61.34
L. paracasei	B13	pH 1.5	8.77 ± 0.02	5.56 ± 0.03	63.39
		pH 1.5 and pepsin		4.79 ± 0.01	54.61
L. brevis	G6	pH 1.5	7.89 ± 0.00	4.75 ± 0.04	60.20
		pH 1.5 and pepsin		3.75 ± 0.02	47.52
L. fermentum	H2	pH 1.5	8.80 ± 0.13	5.35 ± 0.32	60.79
		pH 1.5 and pepsin		4.23 ± 0.04	48.06
L. acidophilus	8*	pH 1.5	8.37 ± 0.03	2.04 ± 0.01	24.37
		pH 1.5 and pepsin		<1	/
Lactobacillus spp. (9 strains)		Low growth at pH 1.5			
Lactobacillus spp. (37 strains)		No growth at pH 1.5			

Log CFU ml^{-1} values express the mean ± standard deviation; each data point is the average of repeated measurements from 03 independently replicated experiments, n = 3. P <0.05.

Identification of the potent probiotic strains by 16S rDNA analysis

The strains showing the best probiotic profiles were identified by the 16S rDNA analysis as described by Diop et al., (2008). Genomic DNA from MRS broth cultures were extracted using the Wizard® genomic DNA purification kit (Promega, Madison, USA), and used as a template for the amplification of 16S rRNA genes by the polymerase chain reaction (PCR). The primer pair 16SPO and 16SP6 was used. PCR products were resolved by electrophoresis in 1% (w/v) agarose gels and visualized by ethidium bromide staining and purified with Microcon YM-100 kit (Bedford, MA, USA). The BigDye Terminator sequence was performed using the Vector NTI (Version 8) software package (BD Biosciences, San Jose, USA). Sequences were BLAST in GenBank database (www.ncbi.nlm.nih.gov) for species assignment.

Statistical analysis

The data were calculated with mean values, and standard deviations (mean ± SD) were determined from triplicate trials. Statistical significance of the results was evaluated by ANOVA (analysis of variance). Statistical significance was attributed to (P<0.05).

RESULTS

Isolation and identification of lactic acid bacteria strains

One hundred and twenty (120) of LAB were selected and purified from seven samples collected twice from children faeces. Fifty two strains of them were retained for further work. They were rods, Gram positive, catalase negative and the fermentation profile obtained by the API50 CHLsystem, showed that they belonged to the *Lactobacillus genus* (data no shown). They were closely related to *L. fermentum* (10 strains), *L. casei* (eight strains), *L.gasseri* (eight strains), *L. salivarius* (five strains), *L. plantarum* (five strains), *L. vaginalis* (four strains) *L.paracasei* subsp*paracasei* (four strains), *L. brevis* (two strains), *L. reuteri* (two strains), *L. acidophilus* (2 strains) and *L. ruminis* (two strains).

Survival ability under gastric conditions

In this study, the effect of pH 1.5 was investigated with and without pepsin (0.3%) to test the ability of LAB to survive in simulated conditions of gastric juice. The results showed that pH 1.5 without pepsin, was critical forthe majority of isolates after 2 h of incubation. In fact, thirty seven isolates among the fifty two sited above, weretotally inhibited and nine manifested a low rate of growth. However, only six isolates were characterized by a good viability in low pH conditions (pH 1.5). The tolerance to simulated gastric conditions test (pepsin, pH 1.5) showed that five isolates of them supported such conditions with different degrees of viability. The highest survival percentage was observed with *L. plantarum* F12 (61.34%), followed by*L. paracasei* B13 (54.61%), *L. fermentum* H2 (48.06%), *L. brevis* G6 (47.52%) and *L. paracasei* subsp *paracasei* A20 (23.78%) (Table 1). The addition of pepsin to acid conditions causes a significant loss in isolates viability compared to the low acidity alone.

Survival ability under intestinal conditions

The most resistant LAB to simulated gastric conditions (F12, B13, H2, A20 and G6), obtained in the previous test, were assayed for their ability to survive in simulated intestinal conditions at pH 8, in the presence of pancreatin, with and without 0.3% of bile. The obtained results showed a decrease in growth of all tested LAB, depending on the strain, in comparison with their culture in the same conditions but without bile (Table 2). Four LAB tested had a

Table 2. Survival ability of lactic acid bacteria isolates in simulated intestinal conditions.

Strain	Code	Viable count (log CFU ml^{-1})			
		Absence of bile		Presence of 0.3% of bile	
		0 h	4 h	0 h	4 h
L. paracasei subsp. *paracasei*	A20	8.35±0.02	7.43±0.10	8.35±0.03	2.90±0.12
L. plantarum	F12	9.60±0.06	9.19±0.00	9.60±0.03	9.56±0.06
L. paracasei	B13	8.83±0.04	9.65±0.32	8.83±0.12	7.01±0.05
L. brevis	G6	7.91±0.09	7.97±0.08	7.91±0.10	6.89±0.00
L. fermentum	H2	8.82±0.01	6.54±0.02	8.82±0.07	<1

Log CFU ml^{-1} values express the mean ± standard deviation; each data point is the average of repeated measurements from 03 independently replicated experiments, "n = 3. P < 0.05.
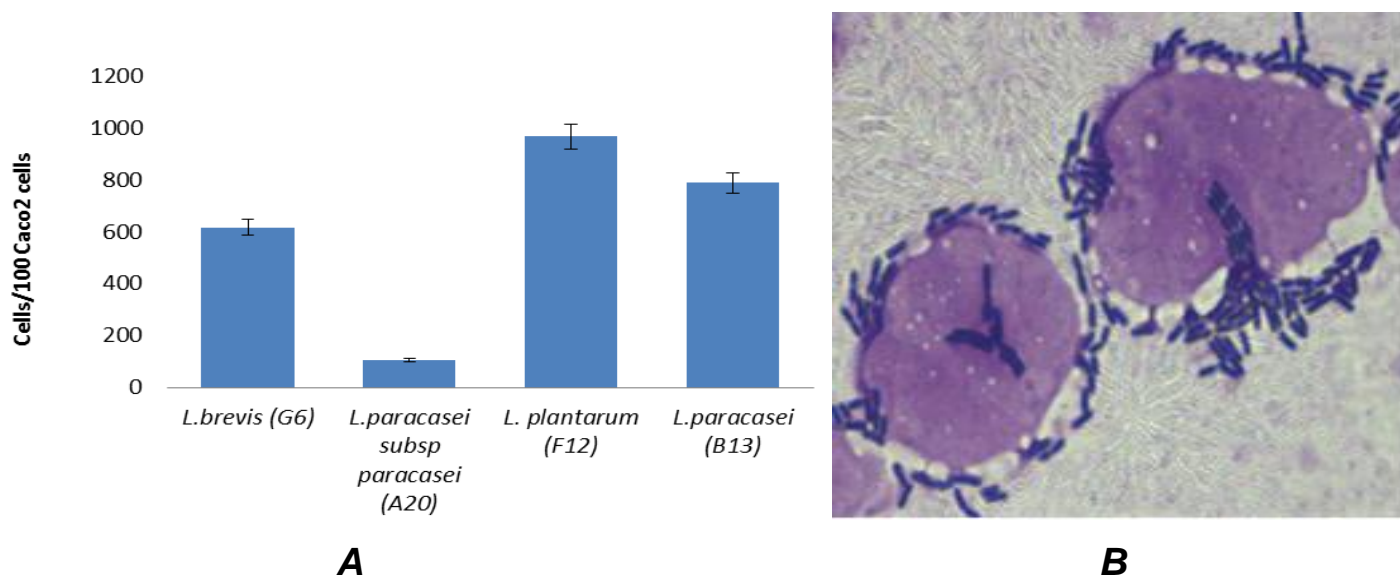
Figure 1. (A) Adhesion of Lactobacillus strains to Caco-2 cells after 2 h of incubation at 37°C in a 5% CO_2 atmosphere. **(B)** Adhesion image of L.plantarum (F12) to Caco-2 cells observed by microscope (x 100) after staining cells with crystal violet (0.5%). Values (cells/100 Caco-2 cells) express the mean ± standard deviation; each data point is the average of repeated measurements from 3 independently replicated experiments, "n = 3. P< 0.05.

good tolerance to simulated intestinal juice after 4 h of exposure. *L. plantarum* F12 was the most resistant strain, followed by *L. paracasei* B13, *L. brevis* G6 and *L. paracasei* subsp *paracasei* A20. The strain *L. fermentum* H2 was completely inhibited.

In vitro adherence assay to Caco-2 cells

L. plantarum F12 *L. paracasei* B13, *L. brevis* G6 and *L. paracasei* subsp *paracasei* A20 were tested for their adherence to Caco-2 cells as they displayed high tolerance rates tosimulated gastro-intestinal juice. The results showed a difference in the abilities of this LAB to adhere to Caco-2 cells. However, 03 of them exhibited a good adherence. These were: *L. paracasei* B13, *L. brevis* G6, and particularly, F12 *L. plantarum* strain which presented the highest adherenceto Caco-2 cells (Figure 1).

Antimicrobial activity

The strains *L. paracasei* (B13), *L. brevis* (G6) and *L. plantarum*

(F12) which exhibited a good adherence to Coco-2 cells were tested for their antimicrobial activity.In this study, the overnight cultures of the three tested strains demonstrated a strong antagonist activity against pathogens but their neutralized supernatants at pH 6.5 and treated with catalase (NSC) were unable to inhibit any pathogenic Gram-negative microbe. Moreover, the NSC of three LAB isolates had a good antimicrobial activity against Gram-positive bacteria (Table 3).

Identification of the potential probiotic strains by 16S rDNA sequencing

Based on the results of our study, three strains were retained as they displayed a high potential probiotic profile. Their identification by API CH 50 system was confirmed by 16S rDNA sequencing. BLAST analysis of their corresponding 16S rDNA sequences, in GenBank database, showed that they were closely related to *L. plantarum* (F12), *L. brevis* (G6) and *L.paracasei* (B13) (Table 4).

Table 3. Antimicrobial activity of lactic acid bacteriaisolates against bacterial pathogens.

Indicator bacteria	L. plantarum F12		L. brevis G6		L. paracasei B13	
	Overnight	Supernatant	Overnight	Supernatant	Overnight	Supernatant
Escherichia coli ATCC 29522	++	–	++	–	+++	–
Escherichia coli O11	+++	–	+	–	+++	–
Escherichia coli O51	+++	–	++	–	+++	–
Salmonella typhimurium	++	–	++	–	+++	–
Listeria monocytogenes	+++	++	+++	+++	+++	++
Bacillus cereus	++	++	++	++	+++	++

All indicators bacteria were tested for inhibition of growth microbes' growth inhibition . Tested overnight cultures and supernatants were treated with catalase and neutralized to pH 6.5. Pathogen growth inhibition was determined by measuring inhibition zones . No inhibition (–); diameter between 0 and 3mm (weak, +); diameter between 3 and 6 mm (good,++) and diameter larger than 6 mm (strong,+++). Each data point is the average of repeated measurements from 3 independently replicated experiments, n = 3. P< 0.05.

Table 4. Comparison between API and 16S rDNA identification of the potential probiotic strains.

Isolate	API CHL identification	Percent of identification	Identification by 16S rDNA		
			Identification	Sequence number access	Percent of match
CWBI\B-1515 (B13)	Lactobacillus paracasei	99.3	Lactobacillus paracasei	JQ436724	98% L.paracasei DQ462440*
CWBI/B-1542 (G6)	Lactobacillus brevis	91.3	Lactobacillus brevis	JQ436725	98% L.brevis APO12167*
CWBI/B-1543 (F12)	Lactobacillus plantarum	97.2	Lactobacillus plantarum	JQ436723	95% L.plantarumKF171888*

* Strains from GeneBank used for comparison with our strains.

DISCUSSION

The initial screening of strains using *in vitro* methods remains a useful preliminary step in the detection of probiotic candidates, despite the difficulties encountered to characterize reliable probiotic strains in this way (Campana et al., 2012). In this work, fifty two isolates were selected among one hundred twenty LAB, screened from faecal samples of healthy children aged between five and ten years. The selected bacteria were rods, Gram positive, catalase negative and non-motile. Their identification according to API test revealed its appurtenance to *Lactobacillus* genus. They were tested, then, for their probiotic properties. In fact, human origin, resistance to simulated GI conditions, adherence to Caco-2 cells and antimicrobial activity against pathogens are the *in vitro*, tests suggested for preselction of the probiotic candidates in human use (FAO/WHO, 2002).

During fasting, the stomach pH can drop as low as 1.5 (Draser et al., 1969) and can dramatically affect bacterial growth. In the present study, among fifty two strains only six showed interesting viability at pH1.5 after 2 h of exposure. Previous reports indicated that pH (1.5-2) is the lethal acidity value for LAB isolated from human GI tract (Lankaputhra and Shah, 1995; Khalil et al., 2007; Xiaodong et al., 2009; Kirtzalidou et al., 2011). In the stomach, in addition to low pH, the probiotics are, also, subjected to the stress of proteolytic enzyme, pepsin

(Holzapfel et al., 1998). In the current study, the most resistant LAB (6 isolates) to pH 1.5 were tested for their tolerance to simulated gastric juice. At pH 1.5 with 0.3% pepsin, only five isolates were able to survive. The high survival rates to these conditions observed with the strains: *L. plantarum* F12, *L. paracasei* B13, *L. brevis* G6, *L. fermentum* H2 and *L. paracasei* subsp *paracasei* A20. This makes them, may be, able to transit the stomach and to reach the intestinal level.

One of the roles of bile acids is the inhibition of the proliferation of bacteria in the upper part of the digestive system. The mean intestinal bile concentration is believed to be 0.3% w/v, in addition, the transit time is suggested to be 4 h (Parasad et al., 1998). In this study, the most resistant strains (F12, B13, H2, A20 and G6) to simulated gastric juice were tested for their ability to survive in simulated intestinal juice. Four of the tested LAB had a good tolerance to simulated intestinal juice after 4 h of exposure and their growth decreased, in comparison with their culture in the same conditions but without bile. These findings were conforming to those of Haller et al. (2001), Vamanu and Vamanu (2010). The most resistant of them was the strain *L. plantarum* F12 followed by *L. paracasei* B13, *L. brevis* G6 and *L. paracasei* subsp *paracasei* A20. The strain *L. fermentum* H2 was completely inhibited.

The ability of probiotic bacteria to adhere to the intestinal epithelium is prerequisite for probiotic microorganisms

to be effective (Collado et al., 2005; Xiaodong et al., 2009). It was reported that adhered probiotic cells interact with the immunomodulatory cells of mucosal immune system, such as leucocytes, in order to stimulate their phagocytic activity against pathogens (Azacarate-Peril et al., 2009). In our study, four strains were tested for their adherence to Caco-2 cells as they showed high tolerance to simulated gastro-intestinal conditions. There was a significant difference (P<0.05) in their ability to adhere to Caco-2 cells, however, three of them exhibited a good adherence. They were *L. paracasei* B13, *L. brevis* G6 and particularly, the strain *L. plantarum* F12 which presented the highest adherence to Caco-2 cells, compared to that of *L. rhamnosus* GG reported by other studies (Thornton, 1996; Ren et al., 2012). The lowest adherence was observed with the strain *L. paracasei* subsp *paracasei* A20. Previous studies concluded that some *Lactobacillus* strains of human origin have good adherence to Caco-2 cells (Dunne et al., 2001; Campana et al., 2012). In our study, the high adherence of the strains B13, G6, and F12 to Caco-2 cells suggests their ability to colonize the human intestinal epithelium tissue and to be in competition with pathogens. They were tested for their antagonistic effect against a represent-tative group of intestinal pathogens bacteria.

Antibacterial activity is vital for the successful colonization of lactobacilli in the intestinal mucosa as they provide a barrier effect and defence against pathogens (Vaughan et al., 1999). According to several studies, the antimicrobial activity of LAB may be due to a number of factors such as decreased pH levels, competition for substrates, production of H_2O_2 and bactericidal or bacteriostatic substances, including bacteriocins (Servin, 2004). In our study, the overnight culture of the strains *L. plantarum* F12, *L. brevis* G6, *L. paracasei* B13 demon-strated a strong antagonist activity against pathogens. Moreover, their supernatants neutralized and treated with catalase were unable to inhibit any pathogenic Gram-negative tested. This suggests that antagonist activity of our LAB against Gram-negative rods is due to the com-bined action of low pH, organic acids and H_2O_2 or to one of them.

Moreover, we found that the NSC of the three tested strains has a good antimicrobial activity on Gram-positive bacteria, *Listeria monocytogenes* and *Bacillus cereus.* This is probably due to substances supposed bacteriocins. Our results go in accordance with the data reported by Dortu and Thonart (2009), where the authors reported that no bacteriocin produced by lactic acid bacteria with activity against Gram-negative bacteria has been described.

Owing to the wall structure of Gram negative bacteria, the bacteriocins can't cross the outer membrane to reach the inner membrane, the site of their activity. Similar results were obtained with the strains belonging to *L. paracasei*, *L. plantarum* and *L. brevis* (Vaughan et al., 1999; Tham et al., 2011; Rishi et al., 2011; Ren et al.,

2012; Russo et al., 2012).

The identification of probiotic strains by 16S rDNA sequencing was recommended by FAO/WHO (2002). In our study, the identification by API system, of the three potential screened probiotic strains, was confirmed by16S rDNA sequences analysis using BLAST analysis. They were *L. brevis* (G6) *L. paracasei* (B13) and *L. plantarum* (F12).These strains were registered in the GenBank database under access numbers respectively JQ436725, JQ436724 and JQ436723.

Conclusion

The faeces of healthy children living in Algerian rural areas seem to be a good source for isolating potential probiotic strains. In this study, we have selected three strains of *Lactobacillus* with high potential probiotic; *L. plantarum, L.brevis and L. paracasei.* All the isolate strains belong to the dominant lactobacilli of intestinal populations of healthy people and are usually recognized for their probiotics traits. Furthermore, their strong antimicrobial activity against the tested intestinal pathogens suggests their use in the prevention of bacterial childhood diarrhoea. However, further investigations, *in vitro,* and, *in vivo,* are needed to study their potential health benefits and their technological properties.

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