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Assessment of Potential Probiotic Properties and Biotechnological Activities of Lactobacillus Strains Isolated from Traditional Algerian Fermented Wheat ELHAMOUM

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ABSTRACT

Background: The objective of the current study was to explore the *in vitro* probiotic and technological characteristics of lactic acid bacteria (LAB) isolated from Algerian fermented wheat.

Methods: Three LAB strains were selected and identified using 16S rRNA gene sequencing, *in vitro* assessments of probiotic properties included resistance to simulated gastric and intestinal conditions, auto-aggregation and antimicrobial susceptibility against common pathogens. Furthermore, the strains were evaluated for their biotechnological properties.

Results: The results of 16S rRNA gene sequencing showed that two of them were *Levilactobacillus brevis* (LAn91 and LAn110) and LAn12a was *Lactiplantibacillus pentosus*. The tolerance of all strains to gastric acid and bile salts was more than 90%; they showed a high auto-aggregation capacity and a variable antagonistic property against pathogenic strains. Strain LAn12a presented the best biotechnological characteristics (proteolytic, acidifying and lactose hydrolysis), while the LAn91 and LAn110 strains were not able to acidify milk. All the strains exhibited a survival count in fermented milk exceeding 7 log CFU/ml during storage at 4°C on day 21, which surpasses the requirement of 6 log CFU/ml to provide health benefits. These bacteria can also be employed for the creation of functional foods and to validate their suitability as probiotic starter cultures.

Key words: Biotechnological properties, Lactobacillus, Probiotic, Traditional fermented wheat.

INTRODUCTION

Algeria is known for its production of a wide variety of fermented foods and beverages, among them Elhamoum, a traditional Algerian couscous derived from the indigenous fermentation of wheat and produced in underground silos knowns as matmor (Merabti *et al*., 2015). Studying the probiotics in fermented foods can lead to new probiotic isolates that are beneficial for health (Angmo *et al*., 2016). Probiotics are typically defined as living microorganisms that provide a wide range of health benefits by forming a symbiosis with the gastrointestinal microflora (Verón *et al*., 2017). They can survive under gastrointestinal conditions and are generally regarded as safe (GRAS). In addition, it is important for these microbial strains to exhibit desirable patterns of antibiotic resistance and susceptibility, be hostile to pathogenic microbes and possess physiological functions that are beneficial to host health. In the context of food applications, it is imperative that LAB strains exhibit the requisite technological attributes (Rönkä *et al*., 2003). Therefore, for practical utilization as functional food products, probiotic functions and bioactive characteristics are essential (Yu *et al*., 2019).

The bacteria most frequently used as probiotics are Lactobacillus because of their significant role as desirable parts of the gut microflora (Berg 1998). They are widely used to improve the health of humans and animals (Lin *et al*., 2020). Furthermore, the fermentation of milk sugar lactose is among the main functions of Lactobacillus; and the claimed health benefit (Kechagia *et al*., 2013). This clarifies the positive rapport between probiotics and lactose intolerance by optimizing global gastrointestinal transit time (Marteau *et al*., 2002).

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Due to the importance of probiotics, the present study was carried out to isolate and identify candidate LAB strains with probiotic and technological properties from Algerian fermented wheat.

A series of tests were conducted to assess the suitability of isolates as supplementary strains in dairy products. The tolerance strains to low pH and bile salts were tested *in vitro*. Antimicrobial properties against pathogens and auto-aggregation were all investigated. Lactose hydrolysis and biotechnological performances were also evaluated.

MATERIALS AND METHODS

Isolation of LAB

The study was conducted at the laboratories of the Agronomy Higher School and Abdelhamid Ibn Badis University in Mostaganem, Algeria, from January to April 2022. Two samples of traditional fermented wheat (elhamoum) from the western Algerian region were used to isolate LAB strains. One gram of each sample was mixed and homogenized with 9 mL of sterilized phosphate-buffered saline (PBS). 100 µl of each serially diluted suspension was inoculated onto Man-Rogosa Sharpe (MRS) agar plates to isolate the potential LAB strains, incubation was carried out at 35°C. under aerobic conditions. After 48 hours, colonies were selected based on their distinct morphological characteristics to undergo purification through multiple subcultures until obtaining pure colonies, which were subsequently stored in glycerol at -80°C.

Phenotypic and biochemical identification

A preliminary identification of the isolated genera was conducted using cell morphology followed by a series of classic tests, such as Gram staining, catalase reaction, gas from glucose, H2S production, motility mannitol, production of acetoin, NaCl tolerance and growth at different temperature.

Identification of the finally selected isolates by 16S rRNA sequencing

The GF-1 Nucleic Acid Extraction Kit (Vivantis Technologies Sdn Bhd, Selangor DE, Malaysia) was used to extract the bacterial genomic DNA according to the manufacturer's instructions. PCR amplification was performed using the universal primers of 16S rRNA gene (27F: 5′- AGA GTT TGA TCC TGG CTC AG- 3′ and 1492R 5′- CCG TCA ATT CCT TTG AGT TT- 3′) added to 25 µl of master mix (1.25 U Taq DNA Polymerase (Solis Biodyne, Estonia) and 3 µl of DNA template. The temperatures used for the PCR cycles were 94°C (2 min), 94°C (1 min), 55°C (1 min) and 72°C (1 min). The amplification was repeated in 30 cycles, followed by a final extension at 72°C (7 minutes). 1.5% of agarose gel (Sigma-Aldrich, USA) was used to separate the PCR product. BigDye Terminator v3.1 (Applied Biosystems) was used for the sequencing. The generated sequences were identified by comparison with those deposited in the GenBank DNA database using the BLAST system (http://blast.ncbi.nlm.nih.gov) and then submitted to NCBI (http://www.blast.ncbi.nlm.nih.gov).

Probiotic properties

Tolerance to gastric acidity and bile salts

Each sample was used in triplicate to investigate the tolerance of the isolated strains to gastrointestinal conditions using the method of Cizeikiene and Jagelaviciute (2021) with some modifications. After an overnight incubation, 1 ml of a young culture ($OD_{600\ nm}$ = 1) of each sample was inoculated into 10 ml of MRS broth previously adjusted to pH 2, 4 and 6.2 (control) using 37% HCl. For the bile salt tolerance MRS broth supplemented with 0.3% (w/v) of bile salts was freshly prepared and inoculated with 1% of the overnight active suspensions (OD_{600} nm= 1). The determination of the survival rate, expressed as the number of viable cells after 3 hours of incubation at 35°C, was performed by plating an aliquot of 100 µL from serial 10-fold dilutions on MRS agar at 0 and 3 h of incubation. The plates were incubated at 35°C for 48 h and the survival rate of bacteria was calculated using Equation 1:

$$\% \text{ survival} = \frac{\log \text{ CFU/ml of viable survived cells at t = 3 h}}{\log \text{ CFU/ml of intial viable inoculated cells}} \times 100 \quad \text{Eq...1}$$

Auto-aggregation assay

Auto-aggregation ability was carried out using the spectrophotometric method described by Sakoui *et al*. (2022) with little modification. Overnight-grown *LAB* strains cultures were centrifuged (6000 g, 15 min, 4°C). Cell pellets were washed twice with PBS buffer and used to inoculate the same buffer ($OD_{600\ nm}$ = 1). From each cell suspension, 4 ml was taken and vortexed for 10s, then 1 ml of the upper layer was collected at 0, 3 and 24 hours of incubation at 35°C in order to measure the OD at 600 nm. The percentage of auto-aggregation was assessed using Equation 2.

$$\text{Autagg \%} = 1 - \frac{At}{A0} \times 100 \quad \text{Eq...2}$$

Where:

A0= Represents absorbance of mixture at.

t=0= At represents absorbance of mixture at time t of incubation.

Antibacterial activity

Determination of antimicrobial activity using spot test

A group of pathogens represented by *Escherichia coli* ATCC 25922, *Staphylococcus aureus* ATCC 25923, *Proteus mirabilis* 35659, *Bacillus subtills* ATCC 6633, *Bacillus cereus* ATCC 14579, *Klebsiella pneumonia* ATCC 700603 and *Pseudomonas aeruginosa* ATCC 27853 was employed to investigate the antibacterial activity of the isolated strains using the spot test method as described by Obioha *et al*. (2021).

Broth Inhibition assay

The spectrophotometric method of Araújo-Rodrigues *et al*. (2021) was used to study the growth of pathogens in the presence of cell-free supernatants (CFS) and pH-neutralized CFS from LAB strains in order to determine the source of inhibitory substances.

Technological characterization

Proteolytic and lipolytic activity

The extracellular proteolytic activity was assayed against casein using the method of Araújo-Rodrigues *et al*. (2021). While the production of extracellular lipases was tested by the presence of a clear halo around and under the strain's spots at the surface of MRS agar containing (1% v/v) Tween 80 and Tween 20 after incubation at 35°C for 24 h to 72 h (Merabti *et al*., 2019). Each test was performed in triplicate.

Exopolysaccharide production

The test of production of exopolysaccharide (EPS) was carried out according to Saif and Sakr, (2020) with a few modifications. Streaks of 48 h LAB strain cultures were performed on MRS agar plates supplemented with 10% of glucose and 0.008% of ruthenium red. The production of EPS is indicated by the presence of wight large and slimy colonies after 48 h of incubation at 35°C.

Acidification capacity and viability during stockage period

The ability of strains to survive and acidify milk was studied according to the method of Angmo *et al*. (2016). The fermentation test was conducted by inoculating sterilized (105°C for 5 minutes) skimmed milk (10% w/v) with 2% of an overnight LAB strains cultures, in triplicate. After 24 h of incubation at 35°C, the samples were stored at 4°C for 21 days. pH measure and strains viability assessments were performed at the end of fermentation and after 8, 15 and 21 days of the post-acidification period.

Lactose hydrolysis

The lactose hydrolysis capacity of LAB strains was assessed employing an enzymatic technique following the procedure outlined by Benbouziane *et al*. (2019), using kit K-LOLAC (Megazyme Int., Wicklow, Ireland) assay as per the manufacturer's instructions. This enzymatic assay allows the quantification of residual lactose in milk products. Succinctly, the K-LOLAC test quantifies the UV absorbance at 340 nm of generated NADPH, which is a product of several chain reactions from glucose. The latter is itself derived from the hydrolysis of lactose by the β-galactosidase of the kit. The LAB strains were inoculated in milk as described for the test of acidification capacity; assays were performed in triplicates.

Statistical analysis

The results of all tests were represented as the mean standard deviation of three independent experiments conducted. An ANOVA test using XLSTAT software was performed for quantitative parameters to determine the significant differences among the isolated strains ($p<0.05$).

RESULTS AND DISCUSSION

Phenotypic, biochemical and molecular identification

The probiotic, technological and protective characterization of three bacterial isolates from fermented wheat was carried out through this experimental study. Strains were characterized as gram-positive, catalase-negative, non-motile and rod-shaped bacteria that could grow at temperatures between 15°C and 40°C and at 3% NaCl concentration. Other biochemical and phenotypic characteristics of the isolates were summarized in Table 1.

Isolate LAn 12a was identified as *Lactiplantibacillus pentosus* based on the results of molecular identification 16S rDNA sequencing and BLAST analysis of its corresponding sequences on the NCBI website, whereas isolates LAn 91 and LAn110 were identified as *Levilactobacillus brevis*. Table 1 lists the GenBank accession numbers corresponding to the 16S rRNA gene sequences of the identified isolates.

Probiotic properties

A variation in the survival rate of the isolated bacteria under different acidic conditions was observed (Table 2). All strains grew well on the control medium with a pH of 6.2 (>100%), resulting in an increase in the initial number of cells and a maximum survival rate in *L. brevis* 91 (114.48%), followed by *L. pentosus* 12a (114.15%) and *L. brevis* 110 (104.15%). All of the isolates showed good growth at pH 4 with no significant differences from the control; however, their resistance to acidic conditions generally decreases significantly (p 0.05) in pH 2 by up to 98.16% (*L. pentosus* 12a), 98.01% (*L. brevis* 91) and 96.9% (*L. brevis* 110). This study produced comparable results to previous research (Angelescu *et al*., 2019) in which isolated bacteria showed a survival rate greater than 90%.

Regarding bile salt tolerance, a significant decrease (p 0.05) was observed in the viability rate of the strains in

Table 1: Phenotypic and biochemical characteristics and molecular identification of the selected isolates.

Isolates	Biochemical and phenotypic identification							Molecular identification	
	Gr	Sh	Cat	H2S	CO_2	Mot	VP	Bacterial taxonomy	Accession number
LAn12a	+	Rod	-	-	-	-	-	*Lactiplantibacillus pentosus*	OP007318
LAn91	+	Rod	-	-	+	-	-	*Levilactobacillus brevis*	OP810992
LAn110	+	Rod	-	-	+	-	-	*Levilactobacillus brevis*	OP535162

Gr: Gram, Sh: Shape, Cat: Catalase, Mot: Motility, VP: Voges proskauer.

presence of 0.3% of bile salts compared to the control over the 3 h test period (Table 2), ranging from 114.82% (*L. brevis* 110) to 122.03% (*L. pentosus* 12a) in control and from 97.81% (*L. brevis* 91) to 98.88% (*L. brevis* 110) in bile salts. Our results were close to those found by Adesulu-Dahunsi *et al*. (2018). Shokryazdan *et al*. (2017) provided an explanation for this resistance to bile salts, indicating that bile salts, which disrupt the membrane structure of viable cells, undergo hydrolysis through the action of an enzyme known as bile salt hydrolase (BSH), produced by certain LAB probiotic strains.

This study found that during the incubation period, the auto-aggregation values increased significantly (Table 2). Although in the first three hours, the strains' behavior was difficult to distinguish, they exhibited an auto-aggregation rate that ranged from 17.61 to 38.05%; these characteristics support the findings reported by Ait Chait *et al*. (2021). After 24 hours, all bacterial isolates tested showed more than 90% aggregation. *L. brevis* 110 was found to be the best auto-aggregating strain among them, with 98.28% aggregation, followed by *L. pentosus* 12a (97.67%) and *L. brevis* 91 being the least auto-aggregative (94.25%), comparable finds are described by Kowsalya *et al*. (2022).

Antibacterial activity

One of the main advantages of probiotics is their antimicrobial activity, which is ensured by the production of various substances, such as short-chain fatty acids, bacteriocins and organic acids (Hojjati *et al*., 2020). With inhibition zones ranging from 20 to 48.33 mm, isolate *L. pentosus* 12a showed the greatest inhibitory activity against all the indicator strains (Table 3). In contrast, isolate *L. brevis* 91 had little inhibitory effect against the majority of the tested pathogens, with the exception of *E. coli* and *B. subtilis,* which were not susceptible. When the *L. brevis* 110 had the least impact on the growth of pathogens, it was only able to inhibit *B. cereus* and *K. pneumonia* with inhibition zones larger than 13 mm in diameter.

Acid CFS from the tested isolate was significantly active against all pathogens (Fig 1). The CFS of *L. pentosus* 12a had the highest antibacterial activity against all indicators evaluated, followed by that of *L. brevis* 91, while the CFS from *L. brevis* 110 had the minimal effect on the growth of pathogens. However, the neutralization of CFS to pH 6.5 eliminate the antibacterial activity of the three LAB strains which implies that the decrease in pH caused by organic acid production is the source of this significant inhibitory effect. A similar situation was described by Obioha *et al*. (2021).

Technological characterization

Isolates of LAB were tested for protease, lipolytic activities and EPS production. The results anticipated that only *L. pentosus* 12a was able to degrade casein in plate assays. Moussa *et al*. (2008) find that *Lactiplantibacillus pentosus* isolated from Tunisian raw milk has a high proteolytic character. As concerns lipolytic activity, bacterial strains did not show any extracellular lipolytic activity, a result that corroborated the data reported by Araújo-Rodrigues *et al*. (2021).

Regarding EPS production, only the *L. pentosus* 12a strain was able to produce shiny, mucoid and white colonies on sucrose agar. Several factors affect the variations and production of EPS by LAB strains, such as the culture conditions, medium composition, age of the cell, type of LAB strains, pH and temperature (Ismail and Nampoothiri 2010).

Acidification capacity and viability during stockage period

L. pentosus 12a was able to decrease significantly the pH of milk after 24 h of incubation to pH 4.67 (Fig 2). Comparing

Table 2: Low pH and bile salt tolerance (%) and auto aggregation ability (%) of the strains.

Strains	Survival rate in % log CFU/ml					Auto-aggregation ability (%)	
	pH tolerance			Bile tolerance		3 h	24 h
	pH 6.2	pH 4	pH 2	Control	0.3 %		
L. pentosus 12a	114.15±1.72^{a}	111.37±1.96^{ab}	98.16±5.49^{cd}	122.03±2.25^{a}	98.35±0.96^{b}	38.05±2.34^{c}	97.67±1.52^{a}
L. brevis 91	114.48±5.04^{a}	109.78±0.85^{ab}	98.01±0.88^{cd}	118.68±1.2^{a}	97.81±1.37^{b}	25.62±1.81^{d}	94.25±0.34^{b}
L. brevis 110	104.15±3.72^{b}	104.32±1.31^{bc}	96.9±3.21^{d}	114.82±6.96^{a}	98.88±7^{b}	17.61±2.13^{e}	98.28±0.65^{a}

Values are means of replicate experiments; ± indicates standard deviation from the mean; for each column, different subscripts lowercase letters indicate significantly different at p<(0.05).

Table 3: Antimicrobial activity of the LAB isolates against indicator bacteria using spot test.

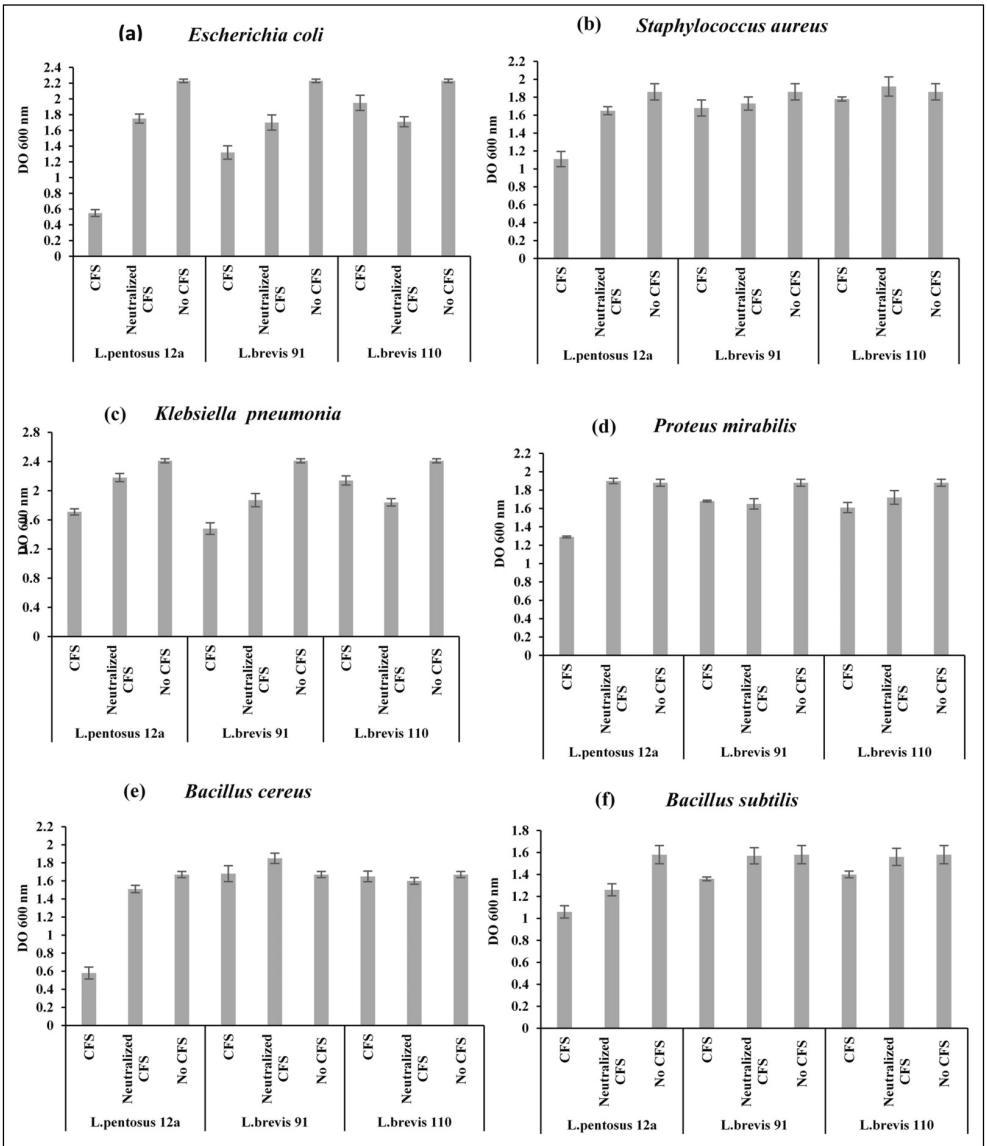
Isolates	Zone of inhibition (mm)						
	E. coli	*S. aureus*	*P. mirabilis*	*P. aeruginosa*	*B. subtilis*	*B. cereus*	*K. pneumonie*
L.pentosus 12a	22±2.65	27.67±2.08	26±1	28±2.65	20±1	28.33±1.53	25±1
L.brevis 91	-	26.67±3.06	18.67±1.53	27.33±1.53	-	26.33±3.51	18±1
L.brevis 110	-	11.33±1.53	7.33±0.58	-	-	13.67±0.58	13..33±3.06

values are means of triplicate experiments; ± indicates standard deviation from the mean; -: Absence of inhibition.

with the inoculation level, an increase of 3 logs in the viability rate of this isolate during 21 days of post acidification period was found; during this time, the pH of the milk products remained relatively constant. These technological features are promising for future application in fermented products such as yogurt and acidified milk. The literature shows that some strains of *Lactiplantibacillus pentosus* isolated from raw cereal material had a high acidification power (Merabti *et al*., 2019). However, *L. brevis* 91 and *L. brevis* 110 were not able to acidify milk during the 24 h fermentation time (pH 5.35 and 5.07, respectively); according to González *et al*. (2010), these findings may be due to the slow rate at which the lactose is metabolized by the Lactobacillus genus. Both strains survived the cold storage period, whereas the number of *L. brevis* 91 cells decreased by 1 log during the third week of storage. These results are in agreement with those of Rönkä *et al*. (2003) who found that *L. brevis* strains are not appropriate for use alone in the manufacturing of fermented dairy products.

Lactose hydrolysis

The organoleptic and nutritional characteristics of milk and its derivatives are attributed mainly by lactose hydrolysis using the enzymes β-D-galactosidase (EC 3.2.1.23) (Santos *et al*., 2015). In addition to their good technological performances, probiotics can reduce the symptoms of lactose intolerance through their B-galactosidase activity (Sakoui *et al*., 2022). The concentration of lactose used by



(a: *Escherichia coli,* b: *Staphylococcus aureus,* c: *Klebsiella pneumonia,* d: *Proteus mirabilis,* e: *Bacillus cereus* and f: *Bacillus subtilis)* using spectrophotometric method, statistics were performed by ANOVA test ($p<0.05$), Error bars are standard deviations with respect to the mean values of triplicate analyses.

Fig 1: Antimicrobial activity of the treated and non-treated CFS of LAB against different pathogenic bacteria.

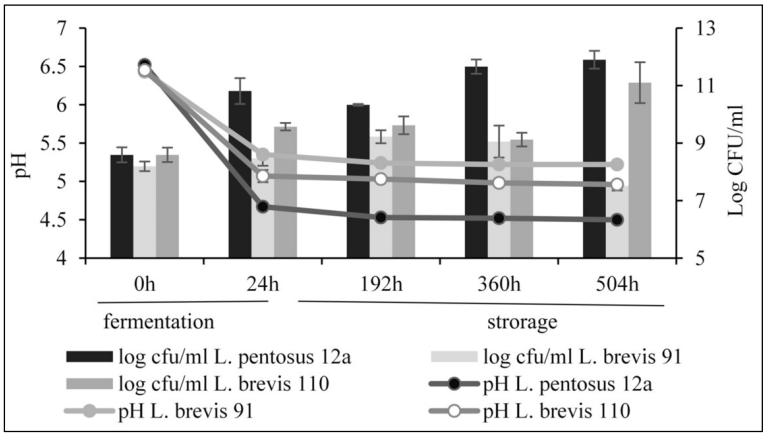


Fig 2: pH and viable count of LAB isolates during milk fermentation (24 h at 35°C) and cold storage (21 days at 4°C).

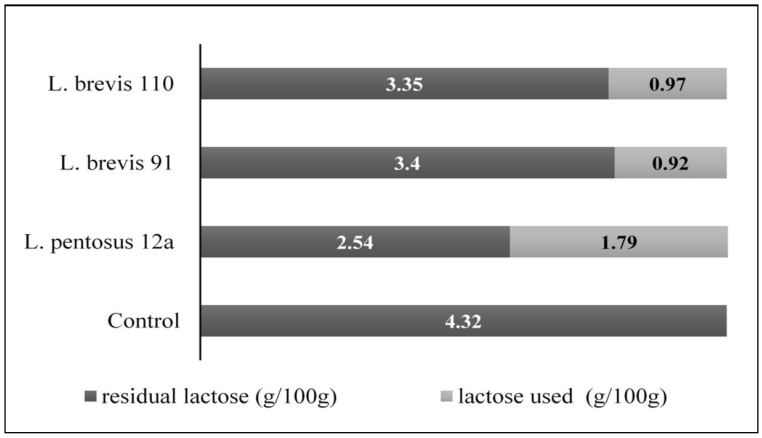


Fig 3: Amount of used and residual lactose for different LAB isolates using enzymatic method with k-lolac kit.

each strain is presented in Fig 3. A significant difference (p 0.05) was observed in the use of lactose by the LAB isolates compared to the control. In this study. *L. brevis* 91 and *L. brevis* 110 strains were the ones that used the least amount of lactose (0.92 and 0.97 g/100 g, respectively). In their study, Martinez *et al*. (2013) demonstrated that *L. brevis* strains have typically been characterized by a limited capacity for lactose utilization. While the amount of lactose consumed by the isolate *L. pentosus 12a* was higher (1.79 g/100 g). Maischberger *et al*. (2010) previously described very efficient growth of *L. pentosus* on lactose, implying a strong β-galactosidase activity.

CONCLUSION

The Isolation of LAB strains from previously unexplored traditional foods and beverages has contributed to the development of research in the probiotics field, addressing the growing demand for products with health benefits beyond nutrition. This research aimed to isolate LAB strains for use as a probiotic from Algerian traditional fermented wheat.

The findings of this study indicate that the three isolated strains exhibit significant probiotic potential, meeting all the prerequisites for probiotic use. These include gastrointestinal resistance, antimicrobial activity and auto-aggregation capacity. None of the isolates presented high acidifying activity, as would be expected in the *Lactobacillus* species. By integrating these bioactive characteristics with additional technological attributes, the *L. pentosus* strain 12a presents itself as a highly potential candidate for use as supplementary cultures, aimed at enhancing the quality and functional attributes of dairy products.

Conflict of interest

I declare on behalf of all authors that there are no conflicts of interest.

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