Original scientific paper

UDK: 637.131.2

Technological, molecular, and safety characterization of autochthonous lactic acid bacteria in Moroccan *Jben* cheese: Towards the development of a native starter culture

DOI: 10.15567/mljekarstvo.2025.0304

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Received: 20.11.2024. **Accepted:** 15.06.2025.

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Abstract

The present study involved the technological, molecular, and safety characterization of lactic acid bacteria isolated from artisanal Moroccan *Jben* cheese. A total of eighty-nine Gram-positive, catalase-negative isolates were initially assessed for acidifying and proteolytic activity, with positive strains screened for additional traits, including citrate utilization, gas production, lipolytic activity, aroma and diacetyl production, and antibacterial activity. Species identification was achieved via species-specific PCR and partial 16S rRNA gene sequencing. It was revealed that sixty-seven strains (75.3 %) displayed at least two technological properties (acidification and proteolysis) with most classified as *Enterococcus* (67.2 %), *Lactococcus* (25.4 %), and, to a lesser extent, *Lactiplantibacillus* (6 %) and *Leuconostoc* (1.5 %) species. None of the isolates utilised citrate or exhibited lipolytic activity. Only *Leuconostoc mesenteroides* produced gas. Pleasing aromas were detected in 62.7 % of strains, while 26.9 % emitted sulphurous notes. Diacetyl production was observed in 79.1 % of the isolates, with *Lactiplantibacillus* and *Enterococcus* identified as the primary producers. Only *Enterococcus* strains (60 %) demonstrated antilisterial activity. For safety purposes, all *Enterococcus faecalis* strains, as well as some *Enterococcus faecium* strains that did not exhibit antibacterial activity, were excluded from the collection. The remaining strains (n=25) underwent autolysis and further safety testing. Autolysis patterns varied by strain, with some *Lactococcus* species exhibiting good autolytic potential. Safety assessments indicated partial haemolysis in some *Enterococcus faecium* strains, while antibiotic resistance analysis revealed higher resistance in *Lactiplantibacillus plantarum* and *Leuconostoc mesenteroides*, primarily of intrinsic origin. This study identified multiple strains of *Lactococcus lactis* with promising technological and safety profile, suggesting their suitability as effective starters for *Jben* cheese production.

Keywords: artisanal cheese; lactic acid bacteria; technological properties; antibiotic resistance; antimicrobial activity

Introduction

Despite the widespread availability of industrial cheeses nowadays, the artisanal *Jben* cheese remains highly valued and commonly enjoyed by Moroccans. *Jben* is a soft, white cheese made from unpasteurised cow and/or goat milk, produced without any starter cultures. The milk is coagulated using commercial or vegetable rennet, specifically an aqueous extract of *Cynara cardunculus* flowers. The curd is drained for a few hours, and the resulting cheese is traditionally sold in braided palm-leaf moulds (El Galiou et al., 2015). However, the production of Moroccan *Jben* cheese faces several challenges, including a lack of standardization and the absence of regulatory requirements, leading to variations in quality and safety (El Galiou et al., 2015; Azzouz et al., 2024).

To achieve a more consistent quality in *Jben* production, milk pasteurisation can be employed to eliminate undesirable and harmful microbiota. However, the cheeses made with pasteurised milk are tasteless and not as flavourful as those made with raw milk. An alternative approach is to incorporate native starter strains into *Jben* production, which can restore its distinctive flavour, prevent defects, and enhance its safety.

The microorganisms used in cheesemaking include primary cultures, which drive both acidification and ripening, and adjunct cultures, which act only during the ripening process. In the case of artisanal, unripened cheeses, only starter cultures are used. The starter cultures belong principally to lactic acid bacteria (LAB), commonly *Lactococcus*, *Leuconostoc*, and *Streptococcus*. Their major role is to utilise the glucose present in milk and produce lactic acid, resulting in milk acidification and curd formation. To date, the most common mesophilic strain used in cheesemaking is *Lc. lactis* (Cogan, 2014), which has also been identified as the predominant species among the bacterial populations in *Jben* cheese (Azzouz et al., 2024).

The key technological criteria to consider when screening starter cultures include effective acidification (the ability to reduce milk pH below 5.3 within 6 hours at 30 °C) (Cachon et al., 2002) and proteolytic activity, indicated by halo diameters between 15 mm and 21 mm (Vuillemard et al., 1986). Additional desirable properties include the production of pleasant aromas, compatibility with co-starters, phage resistance, salt tolerance, and autolysis (Broome et al., 2011).

In this study, the aim was to select native LAB from *Jben* cheese with promising technological and safety traits for potential use as starter cultures in Moroccan *Jben* production.

Material and methods

Isolation, purification and pre-identification of lactic acid bacteria

Fifteen samples of artisanal *Jben* cheese were collected from various farms, weekly markets, and dairies in the city of Tangier and its surrounding areas (Dayedaate, Bni Harchen, Oued Ras). Cheeses were made from goat and/or cow raw milk without the use of starter cultures to ensure the isolation of indigenous bacteria. Ten grams from each sample were homogenised with 90 mL of sterile 0.9 % (w/v) physiological solution using a stomacher Lab-Blender 400 (Gemini BV, Overijssel, Netherlands) for 3 minutes at a rattling speed of 3 times/sec. The stock solutions were serially diluted and evenly spread on MRS agar plates (Biokar, Beauvais, France) and M17 agar plates (Biokar, Beauvais, France). The plates were then incubated for 48 hours at 37 °C. Colonies were selected based on their size, colour, and shape, and were subcultured until they were purified. Isolated colonies were initially screened by Gram staining and catalase test. The isolates were conserved at -20 °C in 25 % (v/v) glycerol and MRS broth for subsequent analysis.

Technological characterization

All isolated strains (n=89) were tested for acidifying and proteolytic activity; however, only those showing both traits were selected for additional testing.

Acidification

Acidifying activity was evaluated using 100 mL of UHT whole milk with 1 % (v/v) of LAB precultures. The milk cultures were incubated at 37 °C and the evolution of pH (Jaouani et al., 2015) and acidity was monitored after 2, 6 and 24 hours (Jaouani et al., 2015). The pH was measured using a pH meter (Hanna Instruments, Woonsocket, Italy) and the lactic acid was titrated using 0.1 N NaOH (Labkem, Barcelona, Spain) (IDF, 1995).

Proteolysis

The protocol of Fguiri et al. (2016) was used to assess the proteolytic activity, with some modifications. The assay was carried out using the well diffusion method where nutrient agar (Biokar, Beauvais, France) was supplemented with 10 % (w/v) skimmed milk (Nestlé, Barcelona, Spain). Fifty microliters of actively growing cells were then inoculated into the wells, and the plates were incubated at 37 °C for 24 hours.

Gas production and citrate utilization

The gas production assay was performed according to Nikita and Develop (2012). Citrate utilization was assessed using the protocol established by Anagnostopoulos et al. (2018) using the *Salmonella* Typhimurium ATCC 14028 strain as a positive control.

Lipolysis

The lipolytic activity was determined according to the protocol of Buffa et al. (2005) with some modifications. The test was carried by well diffusion method using cream fat agar (nutrient agar supplemented with 1 % (v/v) pasteurised milk cream, 38 % fat). Fifty microliters of strains from the exponential phase were then transferred into the wells, and the plates were incubated at 37 °C. The halos' formation around the wells was measured and documented daily for three days.

Aroma assessement

The production of pleasant aromas was assessed by inoculating 1 % of isolates from the lag phase into 100 mL of UHT whole milk. The cultures were then incubated at 37 °C for 24 h. The flasks were manually shaken for a few seconds, and the aromas were perceived by smell using the "sniffing assay" (Garabal et al., 2008).

Diacetyl-acetoin production

The production of diacetyl-acetoin was screened according to Ribeiro et al. (2013) with a few modifications. Briefly, 0.5 mL of milk-grown strains were added to tubes along with 0.5 mL of 1 % (w/v) α-naphtol (Oxford Lab Fine Chem LLP, Maharashtra, India) and 0.5 mL of 16 % (w/v) KOH (Sigma Aldrich, St Louis, USA). The mixture was further incubated at 30 °C for 10 min. The presence of diacetyl was confirmed by the appearance of a pink ring in the medium.

Antagonism test

The antibacterial activity was assessed by the well diffusion method against the following bacteria: *Listeria monocytogenes* (ATCC19144), *S.* Typhimurium (ATCC14028), *Escherichia coli* (ATCC25922) and *Staphylococcus aureus* (B1). Suspensions of pathogenic bacteria (0.5 MacFarland) were spread over the surface of Muller-Hinton agar plates (Biokar, Beauvais, France). Afterwards, 50 µL of overnight grown LAB cultures were deposited on the wells. The inhibition halos around the wells were measured after 24 hours of incubation at 37 °C (Oldak et al., 2020).

Molecular identification

DNA extraction and species-specific PCR

The extraction of genomic DNA was carried out according to Linares-Morales et al. (2020). *E. faecalis* strains were identified by species-specific PCR with the primers ddlE1 (Forward: 5'-ATC AAG TAC AGT TAG TCT-3') and ddlE2 (Reverse: 5'-ACG ATT CAA AGC TAA CTG-3'), as described by Dutka-Malen et al. (1995). The reaction mix contained 5 µL of 5× buffer (Bioline, USA), 0.5 µM of primers (Biolegio, Nijmegen, Netherlands), 0.5 µL of Taq polymerase (Bioline, USA), 3 µL of DNA and 7.5 µL of milliQ water. The amplification conditions were as follows: an initial denaturation at 94 °C for 2 min, followed by 35 cycles, which consisted of three steps: denaturation at 94 °C for 1 min, annealing at 55 °C for 1 min and an extension step at 72 °C for 1 min. The final extension was carried out at 72 °C for 10 min. The product size was determined using 1 % (w/v) agarose (Bioline, USA) gel, visualised under a Quantum-ST4-3020/WL/20M gel documentation system (Vilber Lourmat, Marne-la-Vallée, France).

16S rRNA partial sequencing

The remaining strains (other than *E. faecalis*) were identified through partial sequencing of the 16S rRNA gene. The primers used for the amplification of the V3 region were V3f (Forward: 5'- CCT ACG GGA GGC AGC AG -3') and V3r (Reverse: 5'- ATT ACC GCG GCT GCT GG -3') (Muyzer et al., 1993). The PCR reaction mix was composed of 5 µL of 5× buffer (Bioline, USA), 0.3 µM of primers (Biolegio, Nijmegen, Netherlands), 0.5 µL of Taq polymerase (Bioline, USA), 2 µL of DNA and 16 µL of milliQ water. The amplification conditions were those described by Lorbeg et al. (2014) but with an annealing temperature of 60 °C. PCR products were migrated for 30 min at 100 V in a 1 % (w/v) agarose gel. After the purification steps, the PCR products were sequenced by a 3130xl genetic analyser (Applied Biosystems, USA). The sequences were searched in BLAST of the National Centre of Biotechnology Information (NCBI) database and aligned by the BioEdit software version 7.2.5 (Hall, 1999).

Autolytic activity

After molecular identification, all *E. faecalis* strains were excluded, along with some *E. faecium* strains that did not display antibacterial activity. The remaining 25 isolates were subsequently evaluated for autolysis and safety.

The autolysis percentage was determined according to Bahy El-Din et al. (2002) with minor modifications. *Lactococcus* and *Enterococcus* strains were revived in Tryptic-Soy Broth (Biokar, Beauvais, France), while *Lb. plantarum* and *Leuconostoc* strains were cultured in MRS broth (Biokar, Beauvais, France). Cultures were centrifuged at 12,000 *g* for 5 min. Cell pellets were washed twice with (0.01 M, pH=5,5) potassium phosphate buffer (Sigma Aldrich, St Louis, USA) and resuspended in potassium phosphate buffer (0.05 M, pH=6) supplemented with 1 M NaCl. The optical density was adjusted between 0.6 and 0.8 at 650 nm. After the suspensions' incubation at -20 °C for 24 h and then at 37 °C for another 24 h, the optical density was remeasured. The percentage of autolysis was calculated as follows (Boutrou et al., 1998):

$$\%\ \mathrm{A} = \frac{(A0-At)*100}{A0}$$

Where A_0 is the optical density at t_0 and A_t is the optical density after incubation.

The results were interpreted by genus following the instructions of Ayad et al. (2004).

Safety evaluation

Haemolytic activity, gelatinase and DNase production

The haemolytic activity of the selected *Enterococcus* strains was investigated according to Pino et al. (2019). If clear or green zones appeared around the colonies, the haemolysis was interpreted as ß-haemolysis or α-haemolysis, respectively. DNase and gelatinase production were evaluated according to the methods described by Lavilla-Lerma et al. (2013).

Antibiotic resistance

The antibiotic susceptibility of the selected strains was tested by the disc diffusion method. In this study, nine different antibiotics were used: erythromycin (15 µg), gentamicin (10 µg), lincomycin (15 µg), norfloxacin (10 µg),

penicillin (10 µg), teicoplanin (30 µg), tetracycline (30 µg), trimethoprim-sulfamethoxazole (25 µg), and vancomycin (5 µg). All antibiotics were obtained from Oxoid (Basingstoke, UK).

For the analysis, 200 microliters of LAB precultures (1.5×10^8 cfu/mL) were spread on the surface of Muller-Hinton agar plates (Biokar, Beauvais, France). Antibiotic discs were placed aseptically with flamed forceps and the plates were incubated at 37 °C. After 24 hours of incubation, inhibition zones were interpreted according to the EUCAST (2021) and CLSI (2020) guidelines.

Detection of vancomycin genes

DNA was extracted using a quick technique described by Espinosa et al. (2013) with slight modifications. Briefly, one colony from a 24 h culture was placed in a PCR tube containing 50 µL of TE buffer (10 mM Tris-HCL, 1 mM EDTA). The tubes were then heated in a thermocycler at 100 °C for 5 min. The lysate was used directly for the PCR reaction.

The amplification of *vanA* and *vanB* genes was conducted using the following primer pairs: A1 (Forward: 5'-GGGAAAACGACAATTGC-3') and A2 (Reverse: 5'-GTACAATGCGGCCGTTA-3'), resulting in a 732 bp product size for the *vanA* gene; and B1 (Forward: 5'-ATGGGAAGCCGATAGTC-3') and B2 (Reverse: 5'-GATTTCGTTCCTCGACC-3'),yielding a 635 bp product size for *vanB* gene (Dutka-Malen et al., 1995). The PCR reactions were performed in a 25 µL reaction mixture containing 5 µL of buffer (Bioline, USA), 0.6 µM of primers (Biolegio, Nijmegen, Netherlands), 0.5 µL of dNTPs mix (Promega, Madison, USA), 0.5 µL of Taq polymerase (Bioline, USA) and 2 µL of DNA template. The PCR mixtures were subjected to the amplification conditions described by Asadpour and Ghazanfari (2019).

Lyophilisation

The lyophilisation was performed according to Hammes and Vogel (1995) with modifications. The strains were twice revived in MRS broth at 37 °C for 24 h (Biokar, Beauvais, France), then centrifuged at 3,000 g for 20 minutes. The resulting pellets were washed twice and resuspended in 1 mL of 0.1 % (w/v) peptone buffered water (Biokar, Beauvais, France). The suspensions were transferred to the lyophilisation ampoules containing 9 mL of skimmed milk (Nestlé, Barcelona, Spain) at 11 % (w/v) or sucrose (Labkem, Barcelona, Spain) at 10 % (w/v). The strains were prefrozen below -40 °C for 4 hours in a lyophilisation machine (Biobase, Jinan, China) and then vacuum-dried for 24 h.

Statistics

All analyses were performed in duplicate. Results were analysed using one-way ANOVA to evaluate statistical differences between means, employing Statgraphics 19 software (Statgraphics Technologies, Inc., 2020, The Plains, USA) with significance determined at p = 0.05.

Results and discussion

Technological properties

Acidifying activity

The identification of LAB isolates by specific PCR and partial 16S rRNA sequencing is presented in Table 1. The acidifying activity of the different LAB groups after 6 and 24 hours of incubation is presented in Table 2A.

In this study, 67.4 % of the isolates successfully reduced the milk's pH from 6.6 to 5.3 within 6 hours, and 71.9 % lowered the pH to below 4.8 after 24 h, meeting the criterion established by Cachon et al. (2002) for good acidifiers (Table 2B). Interestingly, despite being members of nonstarter lactic acid bacteria (NSLAB), *Lb. plantarum* strains exhibited the highest pH reductions (ΔpH 2.7-2.8) after 24 hours of

Table 1. Isolates identification by specific PCR and partial 16S sequencing

Species identification	Isolate code	Identity (%)	Accession number of closest relative
Lc. lactis	5.12	100	NR_040955.1
	7.10	100	NR_040955.1
	8.2	100	NR_040955.1
	8.10	100	NR_040955.1
	9.1	100	NR_040955.1
	12.1	100	NR_040955.1
	12.11	100	NR_040955.1
	12.12	100	NR_040955.1
	13.2	100	NR_040955.1
	13.3	100	NR_040955.1
	13.4	100	NR_040955.1
	13.8	100	NR_040955.1
	14.3	100	NR_040955.1
	15.4	100	NR_040955.1
	15.7	100	NR_040955.1
	16.3	100	NR_040955.1
	17.6	100	NR_040955.1
Lb. plantarum	14.5	100	NR_113338.1
	14.6	100	NR_113338.1
	18.1	100	NR_113338.1
	18.6	100	NR_113338.1
E. faecium	4.1	100	NR_042054.1
	4.2	100	NR_115764.1
	4.3	100	NR_115764.1
	4.4	100	NR_115764.1
	7.12	100	NR_115764.1
	10.1	100	NR_042054.1
	10.2	100	NR_115764.1
	10.3	100	NR_115764.1
	13.7	100	NR_115764.1
	15.2	100	NR_115764.1
	16.1	100	NR_115764.1
L. mesenteroides	17.5	100	NR_113912.1
E. faecalis (n=34)	Identified by species-specific PCR		

Table 2. A. Acidifying activity of LAB strains isolated from Moroccan Jben cheese

LAB groups	Number of isolates	Acidifying activity			
		pH after 6 h		pH after 24 h	
		Mean	Range	Mean	Range
E. faecalis *E. faecium*	34 11	5.0±0.2 4.9±0.2	4.4-5.7 4.7-5.2	4.4±0.2 4.4±0.2	4.1-4.7 4.0-4.6
Lactococcus lactis	17	5.0±0.3	4.6-5.6	4.6±0.1	4.4-4.8
Lb. plantarum	4	4.7±0.2	4.4-4.9	3.9±0.1	3.8-3.9
Leuconostoc mesenteroides	1	6.0±0.0	6.0	5.2±0.0	5.2

The mean values presented in the table are calculated from two replicates for each sample.

B. Percentage of isolated strains meeting desired thresholds in acidifying activity

Thresholds	Acidifying activity	
	pH≤5.3 in 6 h	pH≤4.8 in 24 h
Total isolates (n=89)	67.4 %	71.9 %

Table 3. LAB strains isolated from Jben cheese exhibiting notable technological properties

Strain	pH (after 6 h)	pH (after 24 h)	LA production (°D) (after 24 h)	Proteolytic activity (mm)	Diacetyl production	Aroma production	Gas production
Lb. plantarum 18.1	4.4±0.0^{a}	3.8±0.0^{a}	120.2±1.9^{g}	16±0.0^{e}	+	Yogurt/butter	No
Lb. plantarum 18.6	4.7±0.0^{b}	3.8±0.0^{a}	117.9±3.8^{g}	19.5±0.7^{f}	+	Yogurt	No
Lb. plantarum 14.5	4.9±0.0^{c}	3.9±0.0^{b}	111.6±3.8^{f}	18.5±0.7^{f}	+	Yogurt/ Sulphur	No
Lb. plantarum 14.6	4.7±0.0^{b}	3.9±0.0^{b}	111.6±3.8^{f}	19.0±0.0^{f}	+	Fermented milk	No
E. faecalis 12.2	4.4±0.0^{a}	4.1±0.0^{c}	72.0±0.0^{e}	11.5±0.7^{c}	-	Yogurt	No
E. faecium 13.7	5.2±0.0^{f}	4.6±0.1^{e}	67.1±0.6^{d}	13.0±0.0^{d}	+	Yogurt/Vanilla	No
E. faecium 15.2	5.1±0.0^{e}	4.6±0.0^{f}	63.5±0.6^{cd}	16.0±0.0^{e}	+	Yogurt	No
E. faecium 16.1	5.2±0.0^{f}	4.6±0.0^{f}	59.4±2.5^{bc}	15.0±0.0^{e}	+	Yogurt/Vanilla	No
Lc. lactis 12.1	5.0±0.0^{d}	4.7±0.0^{g}	57.6±0.0^{b}	11.0±1.4^{bc}	-	Yogurt/Vanilla	No
Lc. lactis 5.12	5.1±0.0^{e}	4.7±0.0^{g}	60.7±0.6^{bc}	11.5±0.0^{c}	+	Yogurt/Vanilla	No
Lc. lactis 16.3	4.9±0.0^{c}	4.7±0.0^{g}	56.7±1.3^{b}	12.0±0.0^{cd}	+	Yogurt	No
Lc. lactis 12.11	4.7±0.0^{b}	4.5±0.0^{d}	67.1±0.6^{d}	13.0±0.0^{d}	-	Yogurt	No
Lc. lactis 14.3	5.6±0.0^{g}	4.7±0.0^{g}	59.8±0.6^{bc}	10.0±0.0^{ab}	+	Yogurt/Butter	No
Ln. mesenteroides 17.5	6.0±0.0^{h}	5.2±0.0^{h}	45.5±0.6^{a}	9.5±0.7^{a}	-	Cooked milk	Yes

Significant differences (p<0.05) were observed among strains for pH at 6 hours, pH at 24 hours, lactic acid production at 24 hours, and proteolytic activity. Means with different superscripts in the same column indicate significant difference. LA= Lactic acid; -= no production; +=low production; ++ =moderate production; +++=high production; ++++ =very high production

incubation. In particular, the strains 18.1 and 18.6 achieved a ΔpH of 2.8, with Dornic acidity levels of 120.2 °D and 117.9 °D, respectively (Table 3). These values were comparable to those of *Lb. plantarum* strains isolated from Serpa PDO cheese (Araújo-Rodrigues et al., 2021) and were significantly higher than the results reported by Fguiri et al. (2016). *Enterococcus* isolates also demonstrated good acidifying properties by lowering the pH below 4.8 after 24 hours (Table 2A), aligning with the findings of Albayrak and Duran (2021).

The acidifying ability of the strains was also categorized based on pH reduction units after 6 hours of incubation resulting in four groups: "fast acid producers," which lowered the pH by more than 2 units; "medium acid producers," showing a decrease of 1.5 to 1.9 pH units; and "slow acid producers", resulting in a pH decline of 1 to 1.4 pH units. The largest group was that of "medium acid producers" comprising 52.8 % of the strains, and the smallest group was that of "fast acid producers" with 4.5 % of the strains. Regarding *Lactococcus* species, only 5.9 % of the isolates were classified as fast acidifiers, while 58.8 % were categorized as medium acidifiers. However, it is noteworthy that all *Lactococcus* strains, including the slow acidifiers, were capable of producing curd after 24 hours of incubation. The unique *Ln. mesenteroides* 17.5 strain, however, was classified as a slow acid producer and failed to produce a coagulum from whole milk, which is consistent with the results of Zarour et al. (2018).

Proteolytic activity

Proteolysis enhances both the flavor and texture of cheese, with LAB - particularly NSLAB - being the primary organisms responsible for this activity (Hutkins, 2006). In our study, all strains with acidifying properties also exhibited proteolytic activity (Table 4A). According to Vuillemard et al. (1986), strains are considered suitable for cheesemaking if they

produce proteolysis halos of 15-21 mm. In this study, 6.74 % of the isolates demonstrated proteolytic activity within this range (Table 4B), with *Lb. plantarum* isolates showing the largest proteolytic halos, surpassing those of other genera. Regarding *Enterococcus* isolates, most demonstrated low to moderate proteolytic activity, in line with the findings of Pisano et al. (2019). Similarly, the *Lactococcus* genus was classified as moderately proteolytic, while the *Leuconostoc* genus exhibited weak proteolytic activity, consistent with the results of Zarour et al. (2018).

Lipolytic activity

Lactic acid bacteria are generally considered to be weakly lipolytic. However, they can release small amounts of fatty acids in cheeses during extended ripening. Indeed, several lipases/esterases originating from strains of *Lactococcus* and *Lactobacillus* have been isolated and characterised (McSweeney, 2011). In the present study, none of the isolates exhibited lipolytic activity, consistent with findings by Câmara et al. (2019) and Yerlikaya (2019), who observed no lipolysis haloes in species from the *Enterococcus*, *Lactococcus*, *Lactobacillus*, and *Leuconostoc* genera when incubated in tributyrin agar.

Citrate utilization

Present in milk at low concentrations, citrate is metabolised by limited LAB species, primarily belonging to the genera *Leuconostoc*, *Lactococcus*, and *Lactobacillus*. This metabolic process not only yields CO_2, crucial for the formation of holes in certain cheeses, but also produces diacetyl and acetate, essential aromatic compounds found in soft cheeses and fermented milks (Parente et al., 2017). However, in the present study, none of the strains was able to use citrate, which is consistent with the results obtained by Anagnostopoulos et al. (2018).

Gas production

Based on the gas production assay, all strains were homofermentative except for *Ln. mesenteroides* 17.5, which produced gas bubbles after incubation (Table 3). This observation aligns with established knowledge, as genera known for heterofermentative activity include Leuconostocs, Oenococci, Weissellas, and certain Lactobacilli (Axelsson, 2004).

Production of diacetyl-acetoin

Diacetyl is one of the most important volatile compounds found in cheese (Broadbent et al., 2011). Beyond its sensory attributes, diacetyl is also recognized for its antimicrobial properties against yeasts, Gram-negative, and Gram-positive bacteria (Molloy et al., 2011). In this study, all *Lb. plantarum* strains and the majority of *E. faecalis* (91.2 %) and *E. faecium* (90.9 %) isolates exhibited diacetyl production. This finding aligns with previous studies indicating that *Enterococcus* and *Lactobacillus* species are the primary diacetyl producers among lactic acid bacteria (Domingos-Lopes et al., 2017). Certain species of the genus *Leuconostoc*, such as *Ln. mesenteroides* subsp. *cremoris* and *Lc. lactis* cit+, are also known diacetyl producers (Marsili, 2011). However, our analysis of the isolate *Ln. mesenteroides* 17.5 (Table 3) revealed no diacetyl production, corroborating the findings of Saidi et al. (2020). Regarding the *Lactococcus* isolates, diacetyl production was observed in 47.1 % of strains. Although this percentage is lower compared to the *Lb. plantarum* and *Enterococcus* isolates, in this study, it is still higher than that reported by Perin et al. (2017), who observed diacetyl production in only one out of 23 *Lactococcus* strains.

Notably, although citric acid is a well-established precursor of diacetyl, our isolates were found to produce it through alternative metabolic pathways, as none could use citrate as their only carbon source. These findings are consistent with those reported by Saidi et al. (2020). These pathways likely involve pyruvate derived from sugar metabolism or the acetaldehyde route, bypassing direct citrate utilization (Escamilla-Hurtado et al., 1996).

Aroma production

Before using lactic acid bacteria as starters or additives in cheese, their ability to produce pleasant flavours must be evaluated. In this study, 62.7 % of the isolates have been found to emit pleasing aromas, mostly reminiscent of the fresh smell of yoghurt and butter (Tables 3, 5 and 6). The majority of *E. faecalis* and *E. faecium* strains produced pleasant flavours, consistent with previous reports on the ability of *Enterococcus* strains to produce flavouring compounds in milk (Câmara et al., 2019). In the *Lactococcus* group, over half of the strains (52.9 %) produced sulphur aromas, a prevalent trait among *Lc. lactis* strains, as confirmed by the findings of Garabal et al. (2008). The 47.1 % remaining *Lactococcus* isolates produced a discernible yoghurt aroma. Interestingly, a noticeable yoghurt-vanilla flavour was detected in two *Lc. lactis* distinct strains: 5.12 and 12.1 (Table 3). The *Ln. mesenteroides* 17.5 strain produced a cooked milk flavor (Table 3), a characteristic previously observed in nearly half of the *Leuconostoc* isolates reported by Garabal et al. (2008). This result may be due to the weak acidifying and proteolytic activities of *Leuconostoc* species, which likely limit their ability to produce desirable aromas.

Table 4. A. *Proteolytic activity of LAB strains isolated from Moroccan Jben cheese*

LAB groups	Number of isolates	Proteolytic (mm)	
		Mean	Range
E. faecalis *E. faecium*	34 11	10.3±0.9 12±1.9	8.0-13.0 10.0-16.0
Lactococcus lactis	17	11.6±1.0	10.0-13.0
Lb. plantarum	4	18.3±1.6	16.0-19.5
Leuconostoc mesenteroides	1	9.5±0.7	9.5

The mean values presented in the table are calculated from two replicates for each sample.

B. *Percentage of isolated strains meeting desired thresholds in proteolytic activity*

	Proteolytic activity
Threshold	15 mm < H* < 21 mm
Total isolates (n=89)	6.74%

**Halo*

Antibacterial activity

The assessment of the antibacterial activity against four different pathogens - *L. monocytogenes* (ATCC19144), *S.* Typhimurium (ATCC14028), *E. coli* (ATCC25922), and *Staph. aureus* (B1) -revealed significant differences (p<0.05). Lactic acid bacteria produce various bioactive compounds with antimicrobial properties, including lactic acid, diacetyl, ethanol, fatty acids, and bacteriocins such as nisin (Molloy et al., 2011). In our study, noteworthy percentages of *E. faecalis* (70.6 %) and *E. faecium* (27.2 %) strains inhibited the growth of *L. monocytogenes* (ATCC19144) (Figure 1), aligning with the findings reported by Albayrak and Duran (2021). Among these, *E. faecium* 15.2 showed the largest inhibition zone diameter of 15 mm, followed closely by *E. faecalis* strains 9.3 and 1.13, which both exhibited diameters of 14 mm (Figure 1). However, isolates from *Lactococcus*, *Lactiplantibacillus*, and *Leuconostoc* genera showed no antibacterial activity.

Distribution of LAB Strains with Technological Properties in Jben Cheese

In this study, among the strains found to possess technological properties (75.3 %), the largest isolated group was *Enterococcus* (67.2 %), including *E. faecalis* (50.7 %) and *E. faecium* (16.4 %) isolates (Table 6). *Lactococcus lactis* strains came in second with a percentage of 25.4 %. *Lactiplantibacillus plantarum* and *Ln. mesenteroides* strains made up 6 % and 1.5 % of all isolates, respectively.

Autolysis

The autolysis test was exclusively conducted on candidate strains (n=25). Autolysis is the breakdown of bacterial cell walls by peptidoglycan hydrolases. This process enables the release of intracellular ripening enzymes that control the cheese's bitterness and proteolysis (Broome et al., 2011). In this study, the autolysis rates ranged from 6.3 % to 69.4 % (p=0.0000) (Figure 2) with most *Lc. lactis* strains exhibiting high levels of autolysis. Notably, the autolysis percentages of eight *Lactococcus* strains ranged from 39.4 % to 53.7%, exceeding the levels established by Ayad et al. (2004). According to the classification proposed by Ayad et al. (2004), our *Lb. plantarum* strains showed moderate autolytic percentages (41.7 %-69.4 %). Nevertheless, they were greater than those reported by Nieto-Arribas et al. (2009). Similarly, the *Enterococcus* strains demonstrated moderate autolysis (25.8-28.2 %), contrasting with the findings of Jaouani et al. (2015), who reported higher autolytic rates in *E. faecium* strains. Notably, the *Ln. mesenteroides* 17.5 strain exhibited higher autolytic activity (60.9%) compared to the rates reported by Seixas et al. (2018).

Table 5. *Aroma production by LAB strains isolated from Moroccan Jben cheese*

LAB groups	Fermented milk	Yoghurt	Yoghurt/ butter	Yoghurt /vanilla	Yoghurt/ sulphur	Sulphur	Cooked milk
E. faecalis *E. faecium*	-* -	22 4	2 1	- 2	3 1	7 3	- -
Lactococcus lactis	-	5	1	2	1	8	-
Lb. plantarum	1	1	1	-	1	-	-
Ln. mesenteroides	-	-	-	-	-	-	1

**Indicates that no strain produced this aroma*

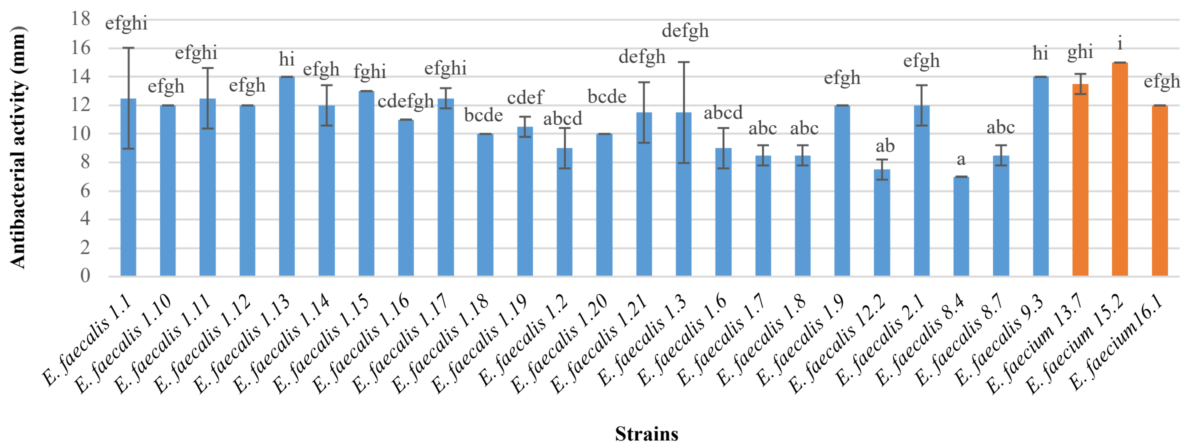

Figure 1. *The antibacterial activity of Enterococcus strains against L. monocytogenes (ATCC19144). Means with different superscripts differ significantly (p<0.05)*

Safety assessment

Screening of haemolysis, gelatinase, DNAse Activity

In this study, four virulence determinants - haemolysis, gelatinase activity, DNase activity, and antibiotic resistance - were evaluated in *E. faecium* strains (Table 7). None of the isolates showed β-haemolysis, gelatinase or DNase activity. However, the strains 15.2 and 13.7 showed partial haemolysis (α- haemolysis) and were then excluded from the collection.

Antibiotic resistance

All of the *Lc. lactis* strains isolated in this study demonstrated susceptibility to the tested antibiotics, with the exception of two *Lc. lactis* isolates, which exhibited resistance to sulfamethoxazole-trimethoprim (Table 8). *Enterococcus faecium* strains showed susceptibility to all antibiotics except erythromycin, for which they displayed intermediate susceptibility, in line with previous research (Nasiri and Hanifian, 2022). Furthermore, the selected *E. faecium* strain 16.1, chosen based on its technological activity and absence of virulence traits, was tested for the presence of *vanA* and *vanB* genes and was found to be negative for both. The *Lb. plantarum* isolates demonstrated resistance to vancomycin, teicoplanin, norfloxacin, and tetracycline. This genus is known for its intrinsic resistance to teicoplanin, vancomycin, and norfloxacin (Liong et al., 2009). In contrast, resistance to tetracycline appears to be acquired, although it is frequently observed in *L. plantarum* strains isolated from dairy products (Kamarinou et al., 2022). The *Ln. mesenteroides* 17.5, in turn, exhibited a high level of resistance among the isolated strains, demonstrating resistance to vancomycin, teicoplanin, norfloxacin, and trimethoprim-sulfamethoxazole.

Table 6. Prevalence of technologically active LAB strains isolated from Moroccan Jben cheese and their distribution in some technological properties

Species	Strain prevalence	Pleasant aroma production	Diacetyl-acetoin production	Antilisterial activity
Lc. lactis	25	47.1 %	47.1 %	0 %
Lb. plantarum	6 %	75.0 %	100 %	0 %
E. faecalis	50.7 %	70.6 %	91.2 %	70.6 %
E. faecium	16.4 %	63.6 %	90.9 %	27.2 %
Ln. mesenteroides	1.5 %	0 %	0 %	0 %
Total isolates (n=67)	100 %	62.7 %	79.1 %	50.6 %

Table 7. Safety aspects of selected E. faecium strains isolated from Moroccan Jben cheese

Criteria	***E. faecium* 16.1**	***E. faecium* 13.7**	***E. faecium* 15.2**
Haemolysis	No haemolysis	Partial haemolysis	Partial haemolysis
Gelatinase	Absence	Absence	Absence
DNase	Absence	Absence	Absence
vanA and *vanB* genes	Absence	ND*	ND*

**ND - not determined*

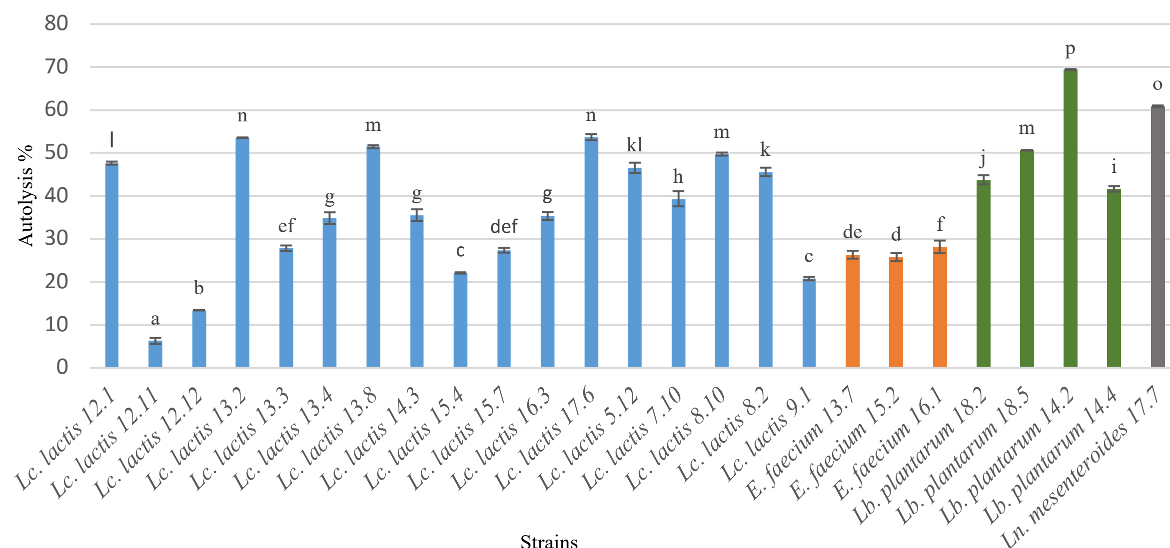

Figure 2. The autolytic activity of selected lactic acid bacteria. Means with different superscripts differ significantly ($p<0.05$)

Table 8. Antibiotic resistance profile of selected LAB strains isolated from Moroccan Jben cheese

LAB groups	TEC	VAN	TET	NOR	GEN	L	SXT	PEN	ERY	Number of isolates
E. faecium	(3)S	(3)S	(3)S	(3)S	(3)S	(3)S	(3)S	(3)S	(2)I	3
Lc. lactis	(17)S	(17)S	(17)S	(17)S	(17)S	(17)S	(2)R	(17)S	(17)S	17
Lb. plantarum	(4)R	(4)R	(4)R	(4)R	4(S)	4(S)	4(S)	4(S)	4(S)	4
Ln. mesenteroides	(1)R	(1)R	(1)S	(1)R	(1)S	(1)S	(1)R	(1)S	(1)S	1

S = Susceptible; R = Resistant; I = Intermediate; TEC = teicoplanin, VAN = vancomycin, TET = tetracycline, NOR = norfloxacin, GEN = gentamicin, L = lincomycin, SXT = trimethoprim-sulfamethoxazole, PEN = penicillin, ERY = erythromycin.

While *Leuconostoc* species possess intrinsic and non-transferable resistance to vancomycin and teicoplanin (Dicks et al., 2011), the resistance to fluoroquinolones and trimethoprim-sulfamethoxazole is likely acquired but is relatively common among *Ln. mesenteroides* strains (Zarour and Benmechernene, 2012).

Conclusion

The characterization of lactic acid bacteria strains isolated from artisanal Moroccan cheese has provided valuable insights into their technological properties, diversity, and safety aspects. It was determined that *Jben* cheese boasts a rich diversity of lactic acid bacteria, including *Lactococcus*, *Enterococcus*, *Lactiplantibacillus*, and *Leuconostoc* species, all of which possess important technological properties. However, the study also uncovered a significant level of resistance among the *Ln. mesenteroides* and *Lb. plantarum* strains, with a notable proportion being of intrinsic origin.

Most importantly, this study identified multiple strains of *Lactococcus lactis* with promising technological and safety profile, suggesting their suitability as effective starters for *Jben* cheese production. Future research should focus on conducting in-depth investigations into the virulence traits of these strains, as well as evaluating their practical applications and long-term performance in cheese production.

Funding

This work was supported by the Academy Hassan II of Sciences and Techniques.

Tehnološka, molekularna i sigurnosna karakterizacija autohtonih bakterija mliječne kiseline u marokanskom *Jben* siru: u sklopu razvoja izvorne starter kulture

Sažetak

Ovo istraživanje uključivalo je tehnološku, molekularnu i sigurnosnu karakterizaciju bakterija mliječne kiseline izoliranih iz tradicionalnog marokanskog *Jben* sira. Zakiseljavanje i proteolitička aktivnost procijenjena je za osamdeset i devet gram-pozitivnih, katalaza-negativnih izolata. Kod pozitivnih sojeva istražena su dodatna svojstva: iskorištavanje citrata, proizvodnja plina, lipolitička aktivnost, proizvodnja arome i diacetila te antibakterijska aktivnost. Identifikacija vrste postignuta je PCR-om specifičnom za vrstu i djelomičnim sekvenciranjem gena 16S rRNA. Otkriveno je da je šezdeset sedam sojeva (75,3 %) pokazalo najmanje dva tehnološka svojstva (zakiseljavanje i proteoliza), a većina ih je klasificirana kao *Enterococcus* (67,2 %), *Lactococcus* (25,4 %) i u manjoj mjeri *Lactiplantibacillus* (6 %) te *Leuconostoc* (1,5 %). Nijedan od izolata nije sadržavao citrat niti je pokazao lipolitičku aktivnost. Samo je *Leuconostoc mesenteroides* proizvodio plin. Ugodne arome utvrđene su kod 62,7 % sojeva, dok je 26,9 % proizvodilo sumporne note. Proizvodnja diacetila uočena je u 79,1 % izolata, pri čemu su *Lactiplantibacillus* i *Enterococcus* identificirani kao primarni proizvođači. Samo su *Enterococcus* sojevi (60 %) pokazali antilisterijsko djelovanje. Iz sigurnosnih razloga iz zbirke su isključeni svi sojevi *Enterococcus faecalis*, kao i neki sojevi *Enterococcus faecium* koji nisu pokazali antibakterijsko djelovanje. Preostali sojevi (n=25) podvrgnuti su autolizi i daljnjem ispitivanju sigurnosti. Obrasci autolize varirali su ovisno o soju, a neke *Lactococcus* vrste pokazuju dobar autolitički potencijal. Procjene sigurnosti ukazale su na djelomičnu hemolizu kod nekih sojeva *Enterococcus faecium*, dok je analiza rezistencije na antibiotike pokazala veću rezistenciju kod *Lactiplantibacillus plantarum* i *Leuconostoc mesenteroides*, prvenstveno intrinzičnog podrijetla. Ova je studija identificirala višestruke sojeve *Lactococcus lactis* s obećavajućim tehnološkim i sigurnosnim profilom, što ukazuje na njihovu prikladnost kao učinkovite startere za proizvodnju *Jben* sira.

Ključne riječi: tradicionalni sir; bakterije mliječne kiseline; tehnološka svojstva; otpornost na antibiotike; antimikrobno djelovanje

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