DRF-462
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RESEARCH ARTICLE Asian Journal of Dairy and Food Research

Assessment of the Antibacterial Potential and Safety Profile of Lactic Acid Bacteria Strains Isolated from Algerian Raw Milk

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10.18805/ajdfr.DRF-462

ABSTRACT

Background: Raw milk harbors most lactic acid bacteria. Research in this field is a hot topic with the aim of using them for probiotic and/or technological applications.

Methods: Lactic acid bacteria were isolated on MRS and M17 media. Antibacterial activity was tested by the direct method based on spots against four food-borne pathogens. Isolates demonstrating high antibiosis activity were selected following a statistical analysis (Two-way-ANOVA) and identified by MALDI-TOF MS. The safety profile was assessed by sensitivity to antibiotics, hemolytic activity, the ability to produce gelatinase and DNase and finally, the aptitude of lactic strains to form biogenic amines.

Result: A total of 172 strains were isolated, of which 103 were presumed to be lactic acid bacteria. The results of the antibacterial activity demonstrated that 82 strains exerted an antagonistic effect against all reference strains and 12 of these showed significant antibiosis activity. Selected strains were identified as *Lactococcus lactis ssp lactis* (50%) and *Enterococcus faecium* (50%). All strains of *Lactococcus lactis ssp lactis* (LMMDR33, LMMDR06, LMMDR22, LMMDR26, LMMDR05 and LMMDR23) and strains LMMDR27, LMMDR16 and LVBBR13 of *Enterococcus faecium* demonstrated a good safety profile. They were retained for valorization.

Key words: Algerian raw milk, Antibacterial activity, Lactic acid bacteria, MALDI-TOF MS, Safety assessment.

INTRODUCTION

Lactic acid bacteria (LAB) are a diverse group of microorganisms that play a crucial role in food fermentation and human health. These gram-positive, non-spore-forming bacteria are characterized by their ability to produce lactic acid as a primary metabolic end-product (Ludwig *et al.,* 2015). LAB comprise various genera, including *Lactobacillus*, *Lactococcus*, *Enterococcus and Streptococcus,* among others (Vos *et al.,* 2011). Their ubiquitous presence in fermented foods, particularly dairy products, has made them a subject of intense research in recent years.

Raw milk serves as a rich reservoir for diverse LAB species, but their diversity can be influenced by various factors, including animal species, geographical location and farming practices (Tilocca *et al.,* 2020). In Algeria, where traditional dairy products play a significant role in the local diet, the characterization of indigenous LAB from raw milk is of particular interest.

The potential applications of LAB extend beyond their traditional role in food fermentation. Recent studies have highlighted their promising probiotic properties and their ability to produce antimicrobial compounds. The antibacterial activity of LAB is primarily attributed to the production of organic acids, hydrogen peroxide and antimicrobial peptides known as bacteriocins (Reis *et al.,* 2012). These compounds can inhibit the growth of various foodborne pathogens, making LAB potential bio-preservatives (Madi and Boushaba, 2017). While the probiotic potential of LAB is well-documented, concerns remain regarding the safety of certain strains, particularly those belonging to the *Enterococcus* genus (Avci and Tuncer, 2017). Key safety considerations include antibiotic resistance, hemolytic activity and the production of biogenic amines (Sonei *et al.,* 2024).

The advent of advanced identification techniques, such as Matrix-Assisted Laser Desorption/Ionization Time-of-Flight Mass Spectrometry (MALDI-TOF MS), has greatly facilitated the accurate identification of LAB species (Bourassa and Butler-Wu, 2015). This technology allows

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How to cite this article: Hammadeche, M.A., Madi, N., Aoues, K., Kaced, A. and Doumandji, A. (2025). Assessment of the Antibacterial Potential and Safety Profile of Lactic Acid Bacteria Strains Isolated from Algerian Raw Milk. Asian Journal of Dairy and Food Research. 1-9. doi: 10.18805/ajdfr.DRF-462.

Submitted: 26-09-2024 **Accepted:** 07-04-2025 **Online:** 22-05-2025

for rapid and reliable identification, which is essential for characterizing newly isolated strains.

In light of these considerations, this study aims to isolate and characterize LAB from Algerian raw milk, with a focus on their antibacterial potential against foodborne pathogens and their safety profile. By employing a combination of classical microbiological techniques and modern identification methods, we seek to identify promising LAB strains that could have potential applications in food preservation or as probiotics. This research contributes to the growing body of knowledge on indigenous LAB strains and their potential utilization in the food industry and healthcare sector.

MATERIALS AND METHODS

The experiment was carried out at the Hygiene Laboratory, the Microbiology department for quality control Laboratory and fraud repression and the Microbiology Laboratory of the public establishment specializing in organ and tissue transplantation in Blida, Algeria, from December 2021 to November 2023.

Sampling of raw milk

Raw milk was collected from various animals in north and south Algeria (Table 1). The animals' udders were cleaned, the first three jets discarded and milk collected in sterile glass bottles. Samples were transported to the laboratory in an ice box.

Isolation of LAB

Raw milk samples were incubated at 37°C and 44°C until coagulation (Fugl *et al.,* 2017). Dilutions (10^{-5} to 10^{-9}) were inoculated onto MRS (de Man *et al.,* 1960) and M17 (Terzaghi and Sandine, 1975) media (Table 2). Isolated colonies were purified by subculturing. Gram-positive, non-spore-forming, catalase and oxidase negative bacteria were presumed to be LAB (Ludwig *et al.,* 2015). Short-term storage was on agar slants at 4°C; long-term at -20°C in broth with 30% glycerol.

Table 1: Types of animals and geographical distribution of raw milk samples.

Animals	Regions	Sample identification
Camel	Boughezoul, Medea	LMBM
	Al-Mosran, Djelfa	LMMD
Cow	Chaiba, Berbissa, Tipaza	LVCB
	Ben Saleh, Blida	LVBB
Goat	Ben Saleh, Blida	LCBB
Sheep	Ben Saleh, Blida	LBBB

Screening for anti-bacterial activity

Reference strains

Four indicator strains were used: two Gram-positive (*Listeria monocytogenes* ATCC 7644, *Staphylococcus aureus* ATCC 25923) and two Gram-negative (*Escherichia coli* ATCC 8739, *Salmonella aboney* NCTC 6017). Strains were obtained from the Pasteur Institute of Algeria and the Frantz Fanon University Hospital Centre of Blida, Algeria. These represent common food contaminants causing foodborne illnesses.

Preparation of inoculum and antibacterial assay

Lactic isolates were reactivated in MRS or M17 broth and standardized to 10^8 CFU/mL in 0.9% NaCl. Indicator strains were cultured in TSB and TSA, then prepared at 10^6 CFU/mL in semi-solid TSA.

The direct spot method was used (Madi and Boushaba, 2017). 5 µL of each lactic isolate was spotted on MRS or M17 agar (5 spots/plate) and incubated at 37 or 44°C for 24 h. Plates were then overlaid with semi-solid TSA containing indicator strains (10^6 CFU/mL) and incubated at 37°C for 24 h. Inhibitions zones >2 mm were considered positive.

Identification by MALDI-TOF MS

Isolates with high antibiosis activity were identified using the Extended method on a MALDI-TOF MS (Bruker Daltonics). Results were compared with the BioTyper DB-5989 database. Identification reliability scores are shown in Table 3.

Safety assessment

Antibiogram

Antibiotic susceptibility was tested using the disk diffusion method (Sakoui *et al.,* 2022) on Mueller Hinton agar. The inoculum (0.5 McF) was swabbed on agar plates before applying antibiotic discs.

Table 2: Medium and culture condition for isolation of LAB strains.

Medium	Temperature (°C) /Duration (h)	Bacteria gender	Incubation conditions
MRS	44°C/24-48 h	Thermophilic *Lactobacillus*	Anaerobiosis
	37°C/24-72 h	Mesophilic *Lactobacillus*	
		Pediococcus	Aerobiosis
		Lactococcus	
		Enterococcus	
M17	44°C/72 h	*Streptococcus Thermophilus*	
		Enterococcus	
	37°C/24-72 h	*Lactococcus*	
		Enterococcus	

Antibiotics used for both *Lactococcus* and *Enterococcus*: Vancomycin (30 μg), Gentamicin (10 μg), Erythromycin (15 μg), Chloramphenicol (30 μg), Rifampicin (5 μg) and Tetracycline (10 μg).
For *Lactococcus* only: Amoxicillin (10 μg, 25 μg), Amoxicillin/ Clavulanic Acid (20/10 μg).
For *Enterococcus* only: Ciprofloxacin (5 μg).

Incubation was at 37°C for 24 h. Results were interpreted according to Jawan *et al.* (2021) for *Lactococcus* and CLSI (2020) for *Enterococcus*.

Hemolysis activity

Hemolytic activity was tested using the method of Colombo *et al.* (2020). Lactic strains were inoculated on TSA agar with 5% defibrinated horse blood and incubated at 37°C for 24 h. Results were interpreted as β (clear halo), α (green halo), or γ (no change) hemolysis.

Gelatinase production

Gelatinase activity was assessed following Sonei *et al.* (2024). Lactic isolates were spotted on nutrient agar with 3% gelatin, incubated at 37°C for 48 h, then flooded with saturated ammonium sulphate. A clear halo indicated positive gelatinase activity.

Table 3: Meaning of score values (standard sample).

Range	Description	Color
2.300-3.000	Highly probable species identification	Green
2.000-2.299	Secure genus identification, probable species identification	
1.700-1.999	Probable genus identification	Yellow
0.000-1.699	Not reliable identification	Red

DNase test

DNase activity was tested as per Barbosa *et al.* (2010). Lactic strains were spotted on DNase agar with 0.005% methyl green and incubated at 37°C for 48 h. A clear halo indicated DNA degradation.

Staphylococcus aureus ATCC 25923 served as a positive control for hemolytic, gelatinase and DNase activities.

Biogenic amine formation

Biogenic amine production was assessed using the method of Bover-Cid and Holzapfel, (1999). Lactic strains were sub-cultured in MRS broth supplemented with pyridoxal-5-phosphate and amino acids (tyrosine, histidine, lysine). Cultures were then spotted on decarboxylase medium with and without amino acids, incubated at 37°C for 4 days under aerobic and anaerobic conditions. A purple halo indicated positive results.

Statistical analysis

All tests were performed in duplicate. Data were treated using IBM SPSS Statistics v20. Two-way ANOVA ($\alpha = 0.05$) followed by Tukey's test were applied to select isolates with high antibiosis activity.

RESULTS AND DISCUSSION

Isolation and characterization of lactic acid bacteria

A total of 172 strains were isolated and purified from raw milk of various animals using MRS and M17 media. Table 4 summarizes the isolates per animal. After eliminating 69 non-lactic isolates based on positive catalase reactions, 103 isolates were presumed to be LAB. Macroscopically, the isolates were lenticular, white and varied in size between 1-4 mm. Microscopically, they were Gram-positive, appearing as cocci, rods, or coccobacilli, with differing

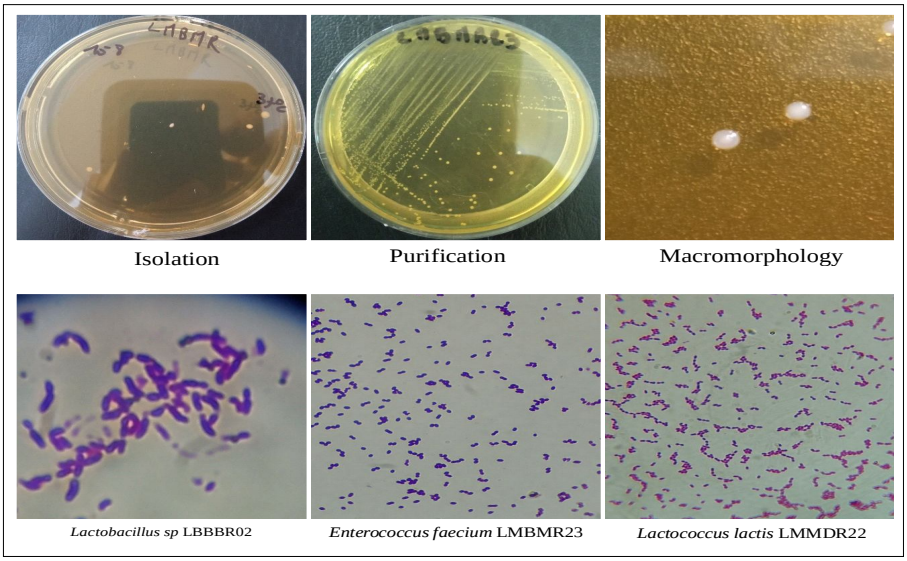


Fig 1: Morphology of lactic acid bacteria strains isolated from raw milk.

Table 4: Number and morphological characteristics of strains isolated from raw milk.

Milk origin	Isolate code		Number of isolates	Morphology						Preliminary tests	
				Macroscopically			Microscopically				
	From MRS	From M17		Shape	Color	Size (mm)	Gram	Shape	Arrangement	Catalase	Oxidase
Camel	LMMDR	LMMDM	33	Lenticular with regular contour	White	1-4	+	Rods Cocci Coccobacilli	Chains Pairs Singly	-	-
			12	Lenticular with irregular contour		Variable	+	Rods Cocci	Chains and grape-like-clusters	+	+/-
	LMBMR	LMBMM	30	Lenticular with regular contour		1-4	+	Rods Cocci Coccobacilli	Chains Pairs Singly	-	-
			13	Lenticular with irregular contour		>4	+	Rods	Chains Singly	+	+
Cow	LVBBR	LVBBM	35	Lenticular with regular contour		1-4	+	Rods Cocci Coccobacilli	Chains Pairs Singly	-	-
			07	Lenticular with regular contour		1-2	+	Cocci	Grape-like-clusters	+	-
	LVCBR	LVCBM	17	Lenticular with variable contour	White Yellow	Variable	+	Rods Cocci	Chains and Grape-like-clusters	+	+/-
Goat	LCBBR	LCBBM	01	Lenticular with regular contour	White	≈ 2	+	Coccobacilli	Pairs	-	-
			18			1-2	+	Cocci	Grape-like-clusters	+	-
Sheep	LBBBR	LBBBM	04			3-4	+	Rods	Chains	-	-
			02	Lenticular with irregular contour	Yellow	>4	+	Rods	Chains Singly	+	+

groupings among species. All isolates were non-spore-forming, catalase and oxidase negative, consistent with lactic bacteria traits (Ludwig *et al.,* 2015) (Fig 1 and Table 4).

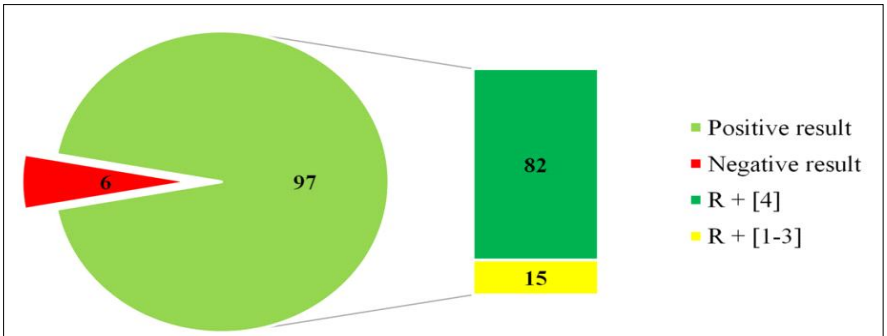
Raw milk's nutrient richness supports the growth of various microorganisms, especially LAB, including genera such as *Lactococcus*, *Lactobacillus*, *Pediococcus*, *Leuconostoc*, *Enterococcus* and *Streptococcus* (Bluma and Ciprovica, 2015). Our isolates aligned morphologically with these genera. Previous studies on LAB in raw milk and dairy products confirm this diversity (Bouchibane *et al.,* 2022; Madi and Boushaba, 2017).

Screening for antibiosis activity

All 103 isolates were tested against *Listeria monocytogenes* ATCC 7644, *Staphylococcus aureus* ATCC 25923, *Escherichia coli* ATCC 8739 and *Salmonella abony* NCTC 6017. Six isolates showed no antibacterial activity against the pathogens (zone of inhibition ≤2 mm), while 97 isolates demonstrated antagonistic effects against at least one pathogen. Of these, 82 inhibited all pathogens and 15 inhibited up to three (Fig 2). Only isolates with broad-spectrum activity were statistically analyzed. Using two-

Table 5: MS MALDI-TOF identification results for selected isolates.

Organism	Isolate code	Score value
Enterococcus faecium	LMMDR27	1.85
	LMMDR16	2.066
	LMBMR28	2.27
	LMBMR23	2.221
	LVBBR25	2.159
	LVBBR13	2.157
Lactococcus lactis	LMMDR06	1.869
Lactococcus lactis ssp lactis	LMMDR33	2.113
	LMMDR22	2.307
	LMMDR26	2.274
	LMMDR05	2.203
	LMMDR23	2.289



Positive result: Inhibition zone diameter > 2 mm against at minimum one indicator strain.
Negative result: Inhibition zone diameter ≤2 mm.
R + [4]: Inhibition zone diameter > 2 mm against all indicator strains.
R + [1-3]: Inhibition zone diameter > 2 mm against a maximum 3 indicator strains.

Fig 2: Distribution of lactic isolates by antibiosis capacity against the pathogens tested.

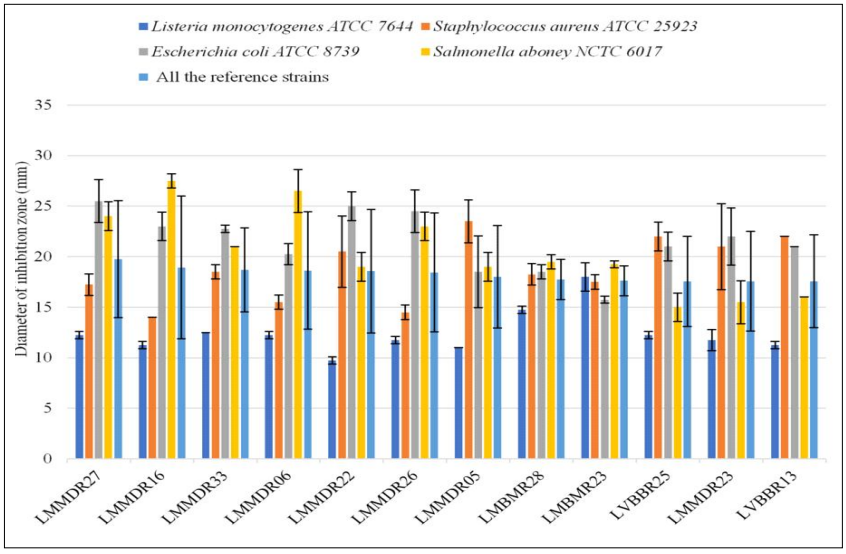


Fig 3: Graphical representation of inhibition zones for lactic isolates against indicator strains.

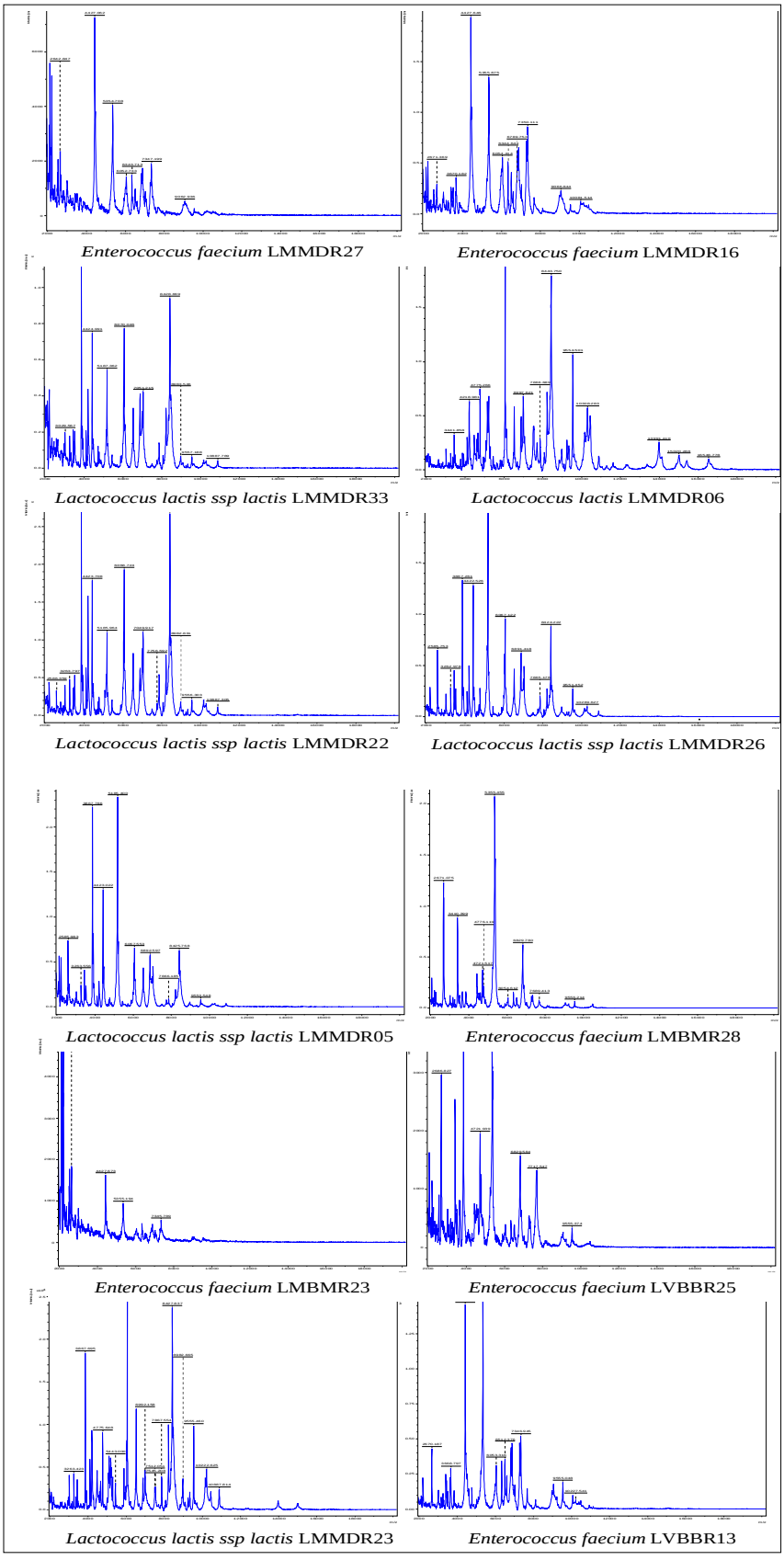


Fig 4: Appropriate spectra generated by MS MALDI-TOF for each species of selected strains.

way ANOVA (α = 0.05) and Tukey's post hoc test. Inhibition zones ranged from 8.25±0.35 mm (LCBBR04 vs. *Listeria*) to 27.5±0.71 mm (LMMDR16 vs. *Salmonella*). 12 isolates were selected for further study, with inhibition zones averaging between 19.75±5.79 mm (LMMDR27) and 17.56 ± 4.59 mm (LVBBR13) (Fig 3).

LAB ability to inhibit pathogens is critical for probiotic selection. Our potent isolates, identified as *Lactococcus lactis ssp. lactis* and *Enterococcus faecium*, have demonstrated broad antibacterial effects, consistent with prior studies (Cavicchioli *et al.,* 2015; El-Ghaish *et al.,* 2017). The production of antimicrobial metabolites like organic acids, hydrogen peroxide and bacteriocins likely drives their antagonistic effects, which are valuable in food preservation and combating antibiotic-resistant bacteria (Fernandes and Jobby, 2022; Rajasekhar *et al.,* 2022).

Identification of selected lactic isolates

All 12 selected isolates exhibited a similar appearance macroscopically on MRS Agar. Microscopically, they appeared as cocci or coccobacilli, grouped singly, in pairs, or in short chains. Identification using MALDI-TOF MS allowed us to classify these isolates into two distinct species in equal proportions: *Lactococcus lactis ssp. lactis* (50%) and *Enterococcus faecium* (50%) (Table 5). The spectral profiles generated for each strain (Fig 4) were compared with those available in the database and score values that varied between species were recorded. Identification reliability was confirmed by a score greater than 2 for most strains. The degree of similarity between the identified strains was illustrated by constructing a dendrogram (Fig 5).

The predominance *of Lactococcus lactis ssp lactis* and *Enterococcus faecium* species in raw milk has been demonstrated by Cavicchioli *et al.* (2015) and Medjahed *et al.* (2021). In research conducted by Kadri *et al.* (2021), the *Firmicutes* phylum was dominated by *Lactococcus lactis ssp. lactis* and *Enterococcus faecium* species.

The probiotic and technological effect of *Enterococcus* and *Lactococcus* strains have been demonstrated in the study of Sonei *et al.* (2024) and Yerlikaya, (2019) respectively.

Safety aspects of identified strains

The results for this section are shown in Table 6.

Antibiotic susceptibility

The *Lactococcus lactis ssp. lactis* strains were sensitive to 77.78% of tested antibiotics but resistant to 22. 22%. Rifampicin Resistance of these strains has been documented in a previous study of Jawan *et al.* (2021).

The *Enterococcus faecium* strains were sensitive to 54.14% of antibiotics but resistant to 14.28%. Intermediate resistance was noted for Erythromycin (83.33%) and Ciprofloxacin (50%). To be useful in applications, *Enterococcus* strains must be sensitive to Vancomycin (Komprda *et al.,* 2010). Xiao *et al.* (2024) demonstrated resistance to Rifampicin, while Kim *et al.* (2018) reported resistance to Erythromycin and Ciprofloxacin at rates of 55.4% and 43.8%, respectively.

Hemolytic activity, DNase, Gelatinase and biogenic amines formation

All strains exhibited γ-hemolysis and tested negative for DNase and gelatinase. *Lactococcus lactis ssp. lactis*

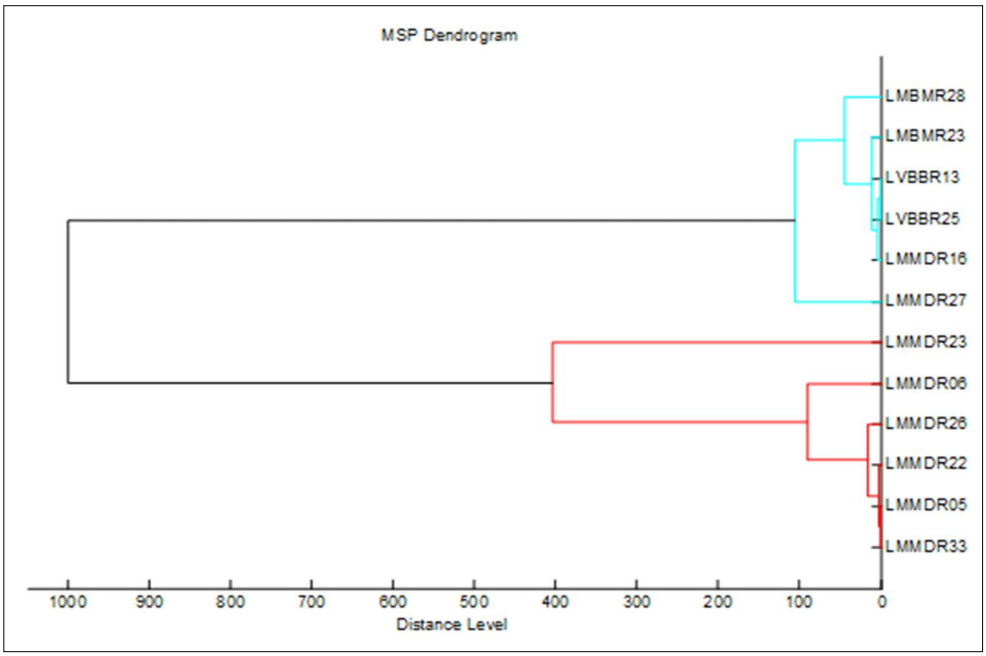


Fig 5: Dendrogram generated by MALDI-TOF MS for *Lactococcus lactis ssp lactis* (Red) and *Enterococcus faecium* (Blue) strains.

Table 6: Safety profile for *Lactococcus lactis ssp lactis* and *enterococcus faecium* strains.

Strains	Hemolysis	DNAse	Gelatinase	Biogenic amines			Tested antibiotics									
				Tyrosine	Histidine	Lysine	AX 10	AX 25	AMC 20/10	VA 30	GEN 10	E 15	C 30	RA 5	TE 10	CIP5
***Lactococcus lactis ssp lactis* strains**																
LMMDR33	γ	-	-	-	-	-	R	S	S	S	S	S	S	R	S	/
LMMDR06	γ	-	-	-	-	-	R	S	S	S	S	S	S	R	S	/
LMMDR22	γ	-	-	-	-	-	R	S	S	S	S	S	S	R	S	/
LMMDR26	γ	-	-	-	-	-	R	S	S	S	S	S	S	R	S	/
LMMDR05	γ	-	-	-	-	-	R	S	S	S	S	S	S	R	S	/
LMMDR23	γ	-	-	-	-	-	R	S	S	S	S	S	S	R	S	/
***Enterococcus faecium* strains**																
LMMDR27	γ	-	-	+	-	-	/	/	/	S	S	–	S	R	S	S
LMMDR16	γ	-	-	+	-	-	/	/	/	S	S	–	S	R	S	S
LMBMR28	γ	-	-	+	+	-	/	/	/	S	S	–	S	R	S	–
LMBMR23	γ	-	-	+	+	-	/	/	/	S	S	–	S	R	S	–
LVBBR25	γ	-	-	+	+	-	/	/	/	S	S	–	S	R	S	–
LVBBR13	γ	-	-	+	-	-	/	/	/	S	S	S	S	R	S	S

δ: No hemolysis; +: Positive result; -: Negative result; R: resistant; I: intermediate; S: sensitive.

strains were unable to degrade the tested amino acids, indicating the absence of decarboxylases responsible for the formation of tyramine, histamine and cadaverine. In fact, *Lactococcus* strains can be used to control biogenic amine formation, as demonstrated by Tabanelli *et al.* (2014).

Enterococcus faecium strains, on the other hand, formed tyramine but did not decarboxylate lysine. Histidine decarboxylase activity was observed in strains LMBMR28, LMBMR23 and LVBBR25. Tyramine production by *Enterococcus* strains has been well documented (Avci and Tuncer, 2017; Bover-Cid and Holzapfel, 1999), as well as histamine production (Komprda *et al.,* 2010). When comparing the safety profiles, LMMDR27, LMMDR16 and LVBBR13 had better safety profiles, with LVBBR13 being the safest.

CONCLUSION

This study has successfully isolated and characterized LAB from Algerian raw milk of various animal origins (camel, cow, goat and sheep), with a focus on their antibacterial potential and safety profile. Our findings contribute significantly to the growing body of knowledge on indigenous LAB strains and their potential applications in food preservation and probiotic development. For this purpose, 12 LAB strains demonstrating high antibiosis activity, were selected and precisely identified by MALDI-TOF MS. After assessing their safety aspect, all six *Lactococcus lactis ssp. lactis* strains (LMMDR33, LMMDR06, LMMDR22, LMMDR26, LMMDR05 and LMMDR23) and three *Enterococcus faecium* strains (LMMDR27, LMMDR16 and LVBBR13) were retained to be used for industrial applications (bio-preservatives) or as probiotics after additional tests.

Conflict of interest

There are no conflicts to declare.

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