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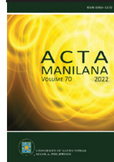

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Technological potential of indigenous lactic isolates of an Algerian artisanal cheese: J'ben *Elgafs*

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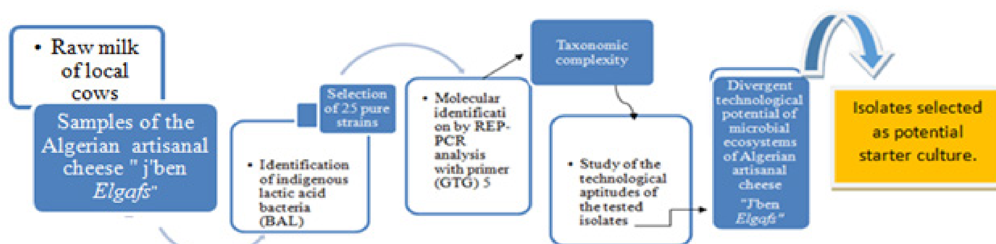
Graphical Abstract



Abstract

This study was carried out to identify indigenous lactic acid bacteria from artisan cheese made from raw milk of indigenous cows in order to select potential starter cultures. Nine cheese samples prepared during the periods of milk production which is relative to the abundance of grass in the rangelands, i.e. three samples per season, in summer and autumn 2019 (medium season), in winter (low season) and in spring 2020 (high season), are produced according to traditional techniques and without exogenous seeding. 25 strains are presumptively identified using the reaction REP-PCR with primer (GTG) 5. The results are then confirmed by sequencing the 16S rDNA. Molecular identification reveals the presence of the species *Lactococcus lactis*, *Enterococcus faecalis*, *Leuconostoc mesenteroides*, and finally the isolates of *Lactobacillus (Lb)* represented by *Lb. casei*, *Lb. fermentum* and *Lb. acidophilus*. Coded isolates of *Lactococcus lactis* (STRD4) and *Enterococcus faecalis* (SMRB1) show good acidification capacity and the ability to produce antimicrobial compounds, while coded isolates of *Lb. acidophilus* (SA3) have good proteolytic capacity and produce exopolysaccharides, and the coded isolates (SHWI2, SHWI3) belonging to the species *Leuconostoc mesenteroides*, use citrate and produce diacetyl and acetoin. These strains appear to be interesting candidates for addition to the starter culture. This study provided a better understanding of the role of indigenous LAB in the quality of artisanal cheeses and the possibility of using the selected isolates as a potential starter culture for cheese production under controlled conditions.

Keywords: J'ben *Elgafs*; terroir; lactic bacteria; REP-PCR; starter cultures

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Introduction

The production and processing of local cow milk can be a cost-effective alternative to dairy products through its composition, taste, texture and natural and healthy aspects when ingested. The indigenous cattle population is often known for its hardiness due to its resistance to harsh climatic conditions and diseases [1]. The quality of artisanal cheese is proportionally linked to the environment, the production area and its traditional culture. J'ben *Elgafs* belongs to the group of ripened cheeses of the region of Boussaâda, in the East of Algeria. Traditionally, it is made from cow's milk and then matured and preserved in an esparto grass layer until consumption [2]. It is characterized by a soft and homogeneous texture. In addition, the autochthonous character of the lactic acid bacteria (LAB) represents a natural reservoir of microbial cultures that contains various genetic information. The use of commercial cultures of LAB and pasteurized milk for industrial cheese production has resulted in a loss of taste and diversity of the dairy microflora [3]. The objective of the first stage of this study is to isolate, identify and characterize the LAB of the j'bens *Elgafs* made from raw milk of local cows. Of the 194 isolated strains, only 25 isolates were analyzed and identified by REP-PCR with primer (GTG) 5 and their technological characteristics are determined. All LAB isolates with the best technological characteristics are selected as potential candidates for the production of starter cultures.

Materials and methods

Sampling. This work is conducted in three dairy farms exploiting local cows in an area of western Algeria. Nine samples of j'ben prepared during the three dairy periods (medium, low and high), are produced according to traditional processes and analyzed at the LSTPA laboratory of Hassi Maméche in Mostaganem (Algeria) according to the usual standards. Each sample (curd, matured cheese at 5 days, 10 days and 15 days) corresponding to codes (S2, S3, S4 and S5) respectively is subjected to microbiological analysis.

Microbiological analysis. The stock solution is prepared by a tenth dilution [4]. One gram of each cheese sample is supplemented with 9 ml of sterile physiological water (0.9%). After homogenization of the samples, a double series (10^{-4} to 10^{-10}) of sequential decimal dilutions is performed. Using the pour plate technique, one ml aliquots of the dilutions are inoculated thoroughly onto MRS agar plates (Institut Pasteur, Algeria) for isolation of bacilli, MSE agar plates (Institut Pasteur, Algeria) for isolation and enumeration of *Leuconostoc* and M17 agar plates (Institut Pasteur, Algeria) for isolation of cocci. All plates are incubated under aerobic and anaerobic conditions for 2-5 days at 30, 37 and 45°C, respectively. Results are expressed in terms of the logarithm of colony format units per gram of cheese sample (log cfu/g).

Physiological and biochemical analysis. Preliminary identification and grouping of LAB is based on microscopic cell morphology and phenotypic properties, such as CO_2 production from Gibson-Abdelmalek medium consisting of (per litre): yeast extract (2.5 g), glucose (50 g), tomato juice (100 ml), milk (800 ml), ordinary agar (200 ml), growth at 30, 37 and 45°C and in 4 and 6. 5 NaCl broths, hydrolysis of L-arginine, acetoin production from glucose (VP test), and bile esculin agar (Institut Pasteur, Algeria) is

used according to [5] as a selective medium for the identification of *Enterococcus* strains. Exopolysaccharide (EPS) production is detected by the formation of large slimy colonies [6]. Diacetyl is indicated by the formation of a red ring after 24 hours in Clarck and Lubs medium (Institut Pasteur, Algeria). Classification of LAB isolates based on phenotypic characteristics is performed according to the criteria of Bergey's Manual of Determinative Bacteriology. The carbohydrate fermentation profiles of the selected strains are evaluated from 24 to 48 hours on MEVAG medium using the constituents for one litre: Meat extract (3g), KCl (5g), agar (3g) with the addition of phenol red (20mg) as pH indicator.

Molecular analysis of the selected isolates. The protocol adopted for the molecular identification of the 25 pure LAB strains selected on the basis of their phenotypic characteristics consisted of extracting the genomic DNA from the bacterial isolates [7], followed by determination of DNA concentration and purity by spectrophotometry (Thermo Scientific™ 840-210600), then DNA amplification by PCR using specific oligonucleotide primer sequences (GTG) 5 from Qbiogene Research Service Germany (October 2020), and finally by 1.2% agarose gel electrophoresis. The 16S ribosomal DNA sequences of the tested isolates obtained by REP-PCR are compared to those of the reference strains (ATCC) as shown in Table 1 and to those of the GeneBank database using the NCBI blast program.

Table 1. Sequence of specific primers used in PCR and reference strains according to their DNAr 16S genes.

Primer	Sequence	Sense	DNAr 16S Gene reference
Lactobacillus acidophilus	5'-GTAAATCTGTTGGTTCCGCT-3' 3'-ATGGCTGCTCGCGTCTTTAA-5'	sense antisense	ATCC 4356
Lactobacillus fermentum	5'-GCGACCAAAATCAATCAGGC-3' 3'-AAGTGTTATGGGCCTAGTCA-5'	sense antisense	ATCC 9338
Lactobacillus casei	5'-TGTTGAAATCAAGTGCAAGG-3' 3'-CGCACCACTTTTGCTTTAAT-5'	sense antisense	ATCC 393
Lactococcus lactis	5'-GCTGCCTCCCGTAGGAGTTG-3' 3'-TCGCCTCATGTAGGATCCAT-5'	sense antisense	ATCC 49032
Enterococcus faecalis	5'-TGTAGTTTGTCATCAACCAT-3' 3'-CCTTATGCGGTAGTCACCTC-5'	sense antisense	ATCC 14506
Leuconostoc mesenteroides	5'-CCGTTACCCCTAAACCCCGA-3' 3'-CAACTCCTTCAGACCACATG-5'	sense antisense	ATCC 19254

Technology analysis

Inhibitory activity. The technique consists of flooding the surface of the Mueller-Hinton medium (Institut Pasteur, Algeria) with a suspension of the indicator strain: *Escherichia coli* ATCC 25922, *Staphylococcus aureus* ATCC 25923 and *Pseudomonas aeruginosa* ATCC 27853. After an incubation period of approximately 18 hours at 30°C, 5 mm diameter wells were dug. Each well obtained received 20µl of young lactic acid bacteria suspension. The plates are incubated at 30°C for 24 hours. A net inhibition zone with a minimum radius of 2 mm is recorded as positive [8].

Proteolytic activity of lactic isolates. The bacteria analyzed, obtained from a young culture, are seeded on PCA-milk agar :casein peptone 5g, yeast extract 2.5g, glucose 1g supplemented with 10% sterile skim milk [9] and incubated for 18-24 hours at 30°C. Clear areas around the colony indicate caseinolytic activity.

Lipolytic activity. Sterile *Wattman™* (Cytiva 1001-090) paper discs are applied to the triglyceride agar (Institut Pasteur, Algeria). Each disc will receive 20µl of a young culture. The assembly is incubated at 30°C for 24 to 48 hours. Lipolysis results in opaque areas [10]. The diameter of these translucent zones is then measured.

Acetoin production. In Clarck and Lubs broth (Institut Pasteur, Algeria), the isolates to be tested are seeded and incubated at 30°C for 24 hours. After incubation and addition of 2-3 drops of VP1 and VP2 (ID-Biomérieux). A cherry red colour reveals a positive reaction [11].

Acidity kinetics. In order to determine the acidifying activity of lactic bacteria, two measurements are applied simultaneously:

- Monitoring the pH evolution of young bacterial cultures over time;
- Determination of the titratable acidity by soda (NaOH, N/9) in the presence of 1% phenolphthalein (a coloured pH indicator). After incubation at 30°C, growth is estimated by measurements taken at 2, 4, 6, 8 and 24 hour time intervals.

Results and Discussion

Microbiological evaluation. The study of the ecosystem of the samples from J'ben *Elgafs* allowed the identification of the most representative microbial species of this controlled production, so that each medium used grows the LAB differently (Figure 1). Indeed, on MRS agar, after one week (S2-S3), the number of viable cells increases and stabilizes almost until the end of maturation (S5) with an average of 6.31±0.01 log cfu/g. This rate is consistent with all the dairy seasons studied. The observations of [12] are in agreement with our observations. After one week of maturation, the flora of lactic acid isolated from the M17 medium reaches an average of 6.27 log cfu/g, compared to 5.95 log cfu/g at the end of maturation in the medium season. This also applies to the low and high seasons, where it decreases from 7.25 log cfu/g to 5.95 log cfu/g. Furthermore, [13] reported a similar evolution of cocci load on M17 medium in a traditional Italian raw milk cheese, as these bacteria are unable to compete with acidophilic bacteria and are less adapted to processing stresses, as revealed by [14]. On MSE medium, the number of viable cells after one week of maturation in the medium season is 5.31 log cfu/g and then increases to 6.36 log cfu/g at 15 days of maturation. This is the same for the other seasons, where the average number of viable cells in the low season is 6.26 log cfu/g and in the high season is 6.21± 0.04 during three weeks of maturation. On MSE agar, *Leuconostoc* grew more slowly and at lower values compared to the other MRS and M17 media (Figure 1) since upstream these microorganisms need the action of lactococci to grow.

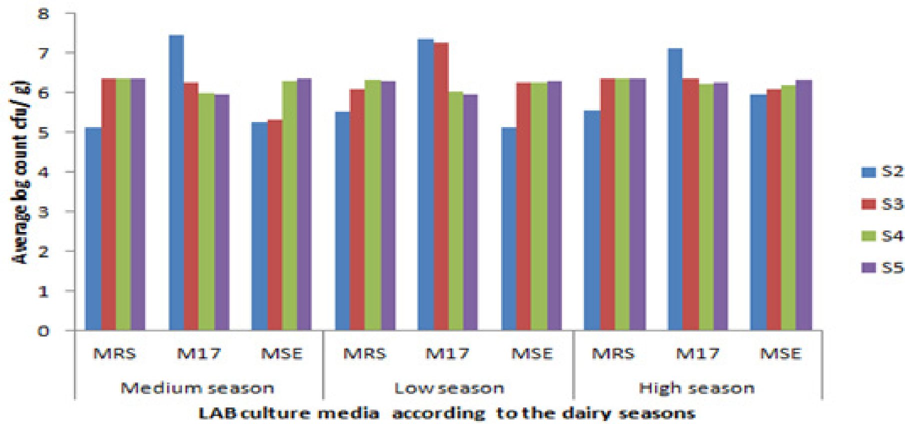


Figure 1. Evolution of the lactic flora on their culture media during the different stages of maturation for the three dairy seasons.

Identification of LAB isolated from J'ben *Elgafs***.** Preliminary identification of LAB based on cell morphology and phenotypic characteristics (Table 2) showed differences in microflora composition between cheese samples during maturation. The most numerous LAB groups in the fresh forms are *Enterococcus (En)*, *Leuconostoc (Ln)* and *Lactococcus (Lc)* while in the mature forms the most numerous LAB groups are *Enterococcus* and *Lactobacillus (Lb)*, which are the dominant populations while *Leuconostoc* and *Lactococcus* species are also present but in small percentage. The dominance of enterococci in our samples confirms once again their preferred host in milk derivatives in the Mediterranean basin as noted by several studies. In Moroccan j'ben according to [15], the number of cells counted varies according to the type of matrix, e.g. in fresh cheese, they are counted between 4 - 6 log cfu/g and in ripened cheese between 5 to 7 log cfu/g.

Table 2. Cultural and phenotypic characterization of tested strains.

Cell shape	**Cocci/ Rounds *(Lc)***				**Cocci/ Rounds *(En)***	**Cocci/ Ovoid *(Ln)***		**Rods *(Lb1)***	**Rods *(Lb2)***	**Rods *(Lb3)***
NH3 from arginine	+	+	+	-	-	-	+	-	+	-
Growth at Temperature (C°)										
25	+	+	+	+	+	+	+	+	+	+
37	+	+	+	+	+	+	±	+	+	+
45	-	-	-	-	+	-	-	+	+	-
Salt broth growth (Nacl %)										
4	+	+	+	+	+	+	+	+	+	+
6.5	-	-	-	-	+	-	-	+	+	-
Growth at pH										
4.5	-	-	-	-	±	-	-	+	+	+
6.5	+	+	-	-	+	+	+	±	±	+
9.6	-	-	-	-	+	+	-	-	-	-
Production of dex-trane from:										
Glucose	±	+	±	-	-	-	+	+	+	+
Sucrose	++	++	±	-	+	-	+	+	+	+

The REP-PCR approach is used to identify 25 LAB isolates by comparing the band profiles with those of appropriate reference strains (Table 1).

This molecular approach validates membership and similarity of the tested isolates, which are shown in Table 3. Examples of visualization of PCR products and dendrograms based on the statistical analysis of the REP-PCR are presented in Figures 2, 3 and 4. The results obtained from the REP-PCR analysis illustrate the differences in the composition of the LAB population throughout the maturation period. *Lactococcus* isolates coded STRD correspond to the species *Lc. lactis*; *Enterococcus* isolates coded SKAB, SMRB represent the species *En. faecalis*, the isolates of *Leuconostoc* coded SHWI represent the species *Ln. mesenteroides*, and finally *Lactobacillus* isolates coded SA9, (SA25, SA2) and (S1R, SA3) are representative of three species: *Lb. casei* ; *Lb. fermentum* and *Lb. acidophilus* respectively.

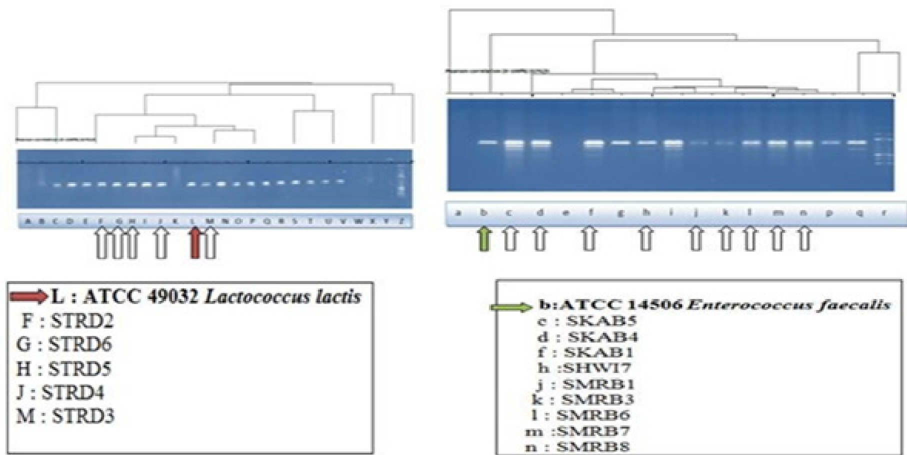


Figure 2. Dendrogram profiles of representative lactococci (left) and enterococci (right) and reference strains of (GTG) 5-PCR products.

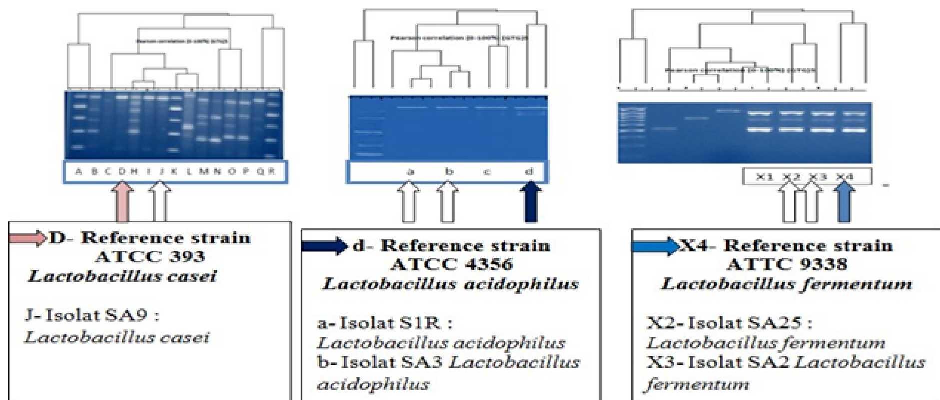
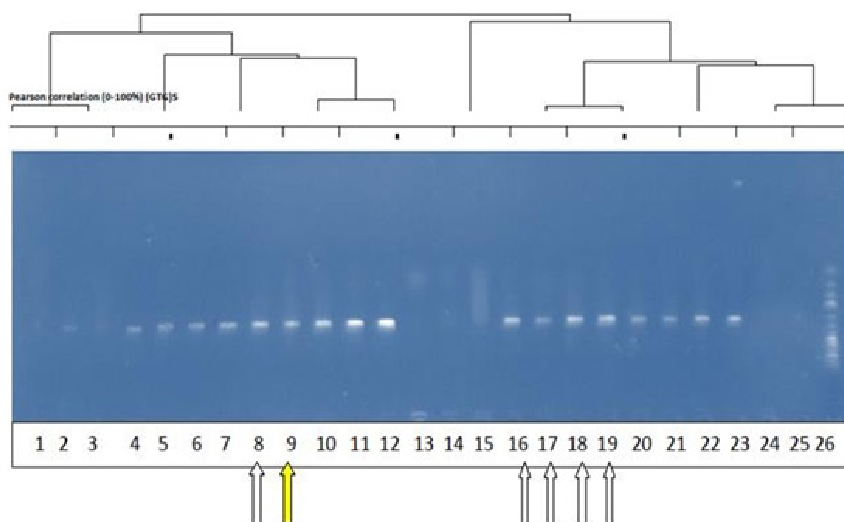


Figure 3. Dendrogram profiles of representative lactobacilli and reference strains of (GTG) 5-PCR products.



9 ATCC 19254 : ***Leuconostoc mesenteroides***

Figure 4. Dendrogram profiles of representative Leuconostocs and reference strains of (GTG) 5-PCR products.

The 16S ribosomal DNA sequences obtained by REP-PCR are compared to those obtained from reference strains (Table 1) and to those in the GeneBank database using the NCBI Blast program.(https://blast.ncbi.nlm.nih.gov/Blast.cgi).The result shows that the sequences obtained correspond to those of the reference species with similarity percentages of 100% (data not shown), therefore the tested isolates are assigned to the corresponding reference species (Table 3).

Comparing these results with those of [16] on a camembert type soft cheese produced with local cow's milk, it appears that lactic bacteria are, according to the same authors, the best adapted to the processing conditions of the experimental cheese paste. Thus, enterococci, heteroferment, lactococci and lactobacilli dominate during processing with a proteolytic activity favorable to the production of growth factors such as amino acids essential to the development of lactic microflora, subject to the participation of *Leuconostoc*.

Table 3. Molecular characterization of tested strains.

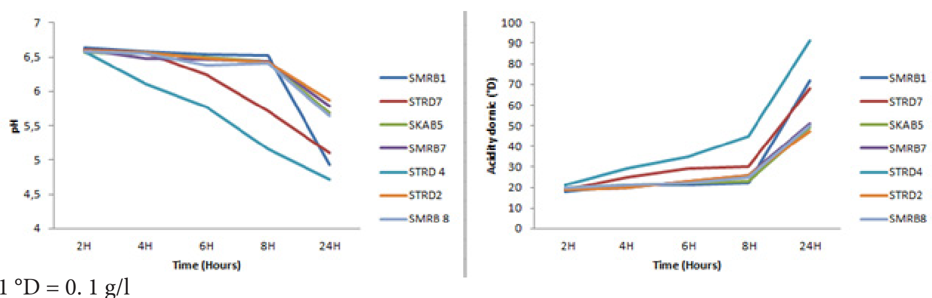
Cell shape number of isolates	Presumptive identification	Identification by REP-PCR	% of conformity
Cocci/Rounds *(Lc)* (6)	*Lactococcus*	*Lc. lactis*	100%
Cocci/Rounds *(En)* (9)	*Enterococcus*	*En. faecalis*	100%
Cocci/Ovoid *(Ln)* (5)	*Leuconostoc*	*Ln. mesenteroides*	100%
Rods (*Lb*1) (1)	*Lactobacillus*	*Lb. casei*	100%
Rods (*Lb*2) (2)	*Lactobacillus*	*Lb. fermentum*	100%
Rods (*Lb*3) (2)	*Lactobacillus*	*Lb. acidophilus*	100%

Characteristics of the lactic acid bacteria in J'ben *Elgafs*. Of the seven *Leuconostoc* isolates tested, only three strains (SHWI1, SHWI2 and SHWI3) are capable of using citrate (data not shown). *Leuconostoc* generate CO2 responsible for forming openings in cheese, and the use of citrate leads to the production of diacetyl, which is the main aromatic compound in fermented dairy products [17]. Five of the six lactobacilli tested are tolerant to 6.5% salt (Table 2). According to Table 3, and comparing their REP-PCR fingerprint of the appropriate reference strains (ATCC 4356), two strains are identified as *Lb. acidophilus*, the three appropriate strains (ATCC 9338), are identified as the species *Lb. fermentum* and only one strain identified as *Lb. casei* is appropriate to the reference strain (ATCC 393).

For technological characteristics in general, the acidifying activity of all isolates in milk was variable. All isolates reduce pH below 6.5 after 6 hours of incubation. Only the isolate (STRD4) belonging to *Lc. lactis* showed the greatest capacity for acidification. After 24 hours, the pH values of all isolates tested dropped visibly. When the isolates are compared to each other, the *En. faecalis* isolate (SMRB1) lowers the pH to 4.93 with a production of 7.2 g/l of lactic acid. This result moves it to second place in the ranking (Figure 5).

Diacetyl production, as indicated by the formation of the red ring, depends on the strain *Lc. lactis* isolates (STRD7 and STRD5) are very good acetoin producers, followed by *Leuconostoc* strains (SHWI1, SHWI2 and SHWI3) and all *Lactobacillus* except strain S2A (data not shown). All lactococci tested and almost all enterococci are bacteriocin producers, inhibiting the growth of indicator strains ATCC 25922, ATCC 25923 and ATCC 27853 (data not shown), so it can be assumed that more than one agent is produced by these LAB. The highest proteolytic activity is demonstrated by *Leuconostoc* (SHWI6) and lactobacilli (SA2 and SA3), while the other groups of LAB show little or no casein degradation (data not shown). Many lactobacilli and *Leuconostoc* are EPS producers. These include, in order of importance, *Lb. casei* (SA9), *Lb. acidophillus* (S1R, SA3), *Lb. fermentum* (SA25, SA2) and *Ln. mesenteroides* (SHWI10).

These cultures can improve the sensory characteristics of dairy products, with smooth and creamy products being of considerable interest to consumers. Therefore, the production of EPS is an important characteristic to consider in the selection of ferments [18].



1 °D = 0. 1 g/l

Figure 5. Evaluation and comparison of the pH and the amount of lactic acid produced by lactococci coded: STRD and enterococci coded: SMRB and SKAB after 24 hours.

Conclusion

Quantitative evaluation of the lactic microflora of the experimental samples of j'bens *Elgafs*, for each dairy season and ripening stage, reveals an inherent microbial profile, both in terms of dominant species and quantity. The phenotypic characterization of the identified isolates provided a clear indication of the species diversity of the microbiology of J'ben *Elgafs* prepared with raw milk from local cows in the study area. Thus, the cultural and phenotypic identification shows that the most numerous BAL in the fresh forms (S2-S3) are *Enterococcus* and *Lactococcus* while in the mature forms (S5) the most numerous are *Enterococcus* and *Lactobacillus*, subject to the participation of *Leuconostoc*s. The *Lactococcus* regress with the maturation of the j'bens because they are less well adapted to the processing conditions, whereas *Enterococcus* are found in a punctual manner and are in the majority at the end of maturation (S5). The prevalence of *Enterococcus* once again attests to their host of choice in milk derivatives in the Mediterranean basin. Molecular identification of the 25 strains selected in this study by REP-PCR analysis reveals a taxonomic complexity in J'ben *Elgafs*: *Lc. lactis* (6), *En. faecalis* (9), *Ln. mesenteroides* (5), *Lb. casei* (1), *Lb. fermentum* (2) and *Lb. acidophilus* (2). The technological abilities measured for all tested strains have made it possible to detect isolates with divergent technological potential. The *Lc. lactis* isolates (STRD4, STRD7) and lactobacilli (S2A, SA2) isolated from J'ben *Elgafs* show potentially important properties for practical application as mixed starter cultures, while the *Ln. mesenteroides* isolates (SHWI6, SHWI3) serve as secondary cultures. Overall, all tested strains exhibit variable antagonistic activities against the indicator strains tested. Maintaining and managing the microbial ecosystems of local cow's milk in Algeria will strengthen the link with the terroir by safeguarding the organoleptic characteristics of raw milk cheeses and ensuring food safety and wholesomeness for consumers.

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Conflict of Interest

The authors declare no conflict of interest.

Author Contributions

All authors have read and agreed to the final version of the manuscript.

Institutional Review Board Statement

Not applicable.

Informed Consent Statement

Not applicable.

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