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# Technological Interest and Antimicrobial Activity of Lactic Acid Bacteria Isolated from Date Paste of the *Ghers* Variety (Bechar, South-West of Algeria)

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## ABSTRACT

**Background:** Dates are high nutritional value fruits. They are the basis of many traditional products and compose several oases by-products. Among these products, date paste represents a form of conservation widely practiced in Algeria to valorize common date's surplus production for commercial purposes.

**Methods:** First, microbiological analysis was carried out to isolate lactic acid bacteria (LAB) from date's paste and microbial contaminants from both date fruit by-products according to standard microbiological methods before investigating the technological characteristics attributed to the isolated LAB. Second, the antimicrobial effect was evaluated through three different techniques.

**Result:** Nine microbial contaminants were isolated from date paste and date extract, while eight LAB strains were isolated from date paste, which belong to the Streptococci and *Leuconostoc* strains. The SLM1 strain revealed a good acidifying power going up to 5.13 g/L of lactic acid within 6 hours of incubation compared to the other LAB strains. A moderate to strong antibacterial effect ranging up to a 20 mm zone of inhibition was revealed against bacterial contaminants. In decreasing order, the antifungal effect was significant against *Penicillium* sp, *Aspergillus niger* and *A. flavus*. The isolated lactic strains of streptococci could be good candidates for food preservation by extending the shelf life of foods and improving their nutraceutical and hygienic quality.


**Key words:** Antimicrobial effect, Date extract, Date paste, Food preservation, LAB, Technological properties.

## INTRODUCTION

The Algerian palm grove is rich with an important range of date varieties that constitute the main source of income for some twenty of these regions and are characterized by very interesting aptitudes for processing and conservation due to the diversification of their morphological and composition characteristics. The date palm sector has experienced remarkable developments in Algeria in recent years with more than 18.7 million palm trees (Laouar *et al*., 2021). According to the data reported by Fleck (2023), Algeria is the fourth-largest producer of dates in the world, with a total production of 1.2 million tons of dates recorded in 2022. An average of 60000 tons of the total production is made up of waste from palm groves, which undergo a selective sorting where the major part 90% is transformed into date paste using the *Ghers* as well as other varieties. Dates can be used as a raw material in the development of many by-products, but due to their rich composition and physiochemical properties, various factors can contribute to the contamination of dates (Benyagoub, 2011).

Through this nutrient-rich composition, we thought to characterize the microflora it contains by studying the nature of potential interactions between LAB strains and microbial contaminants, as well as the biochemical and technological characteristics of the isolated LAB strains.

## MATERIALS AND METHODS

All experiments were carried out at the University of Bechar (Algeria), for six months from February to July 2022.

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### Sampling

Two products were analyzed. The first one was a local *Ghers* variety date paste made without any treatment or additive, except that the dates were pitted and mixed until the paste was obtained. The second one was a date extract prepared at the laboratory scale as described by Benyagoub *et al.* (2011).

### Isolation and enumeration of LAB stains and microbial contaminants

From a stock solution, a series of dilutions from date paste and date extract was prepared using buffered peptone water (BPW) as a diluent (Benyagoub *et al*., 2022) to isolate bacterial contaminants from date paste and date extract, as well as LAB strains from date paste.

According to Benyagoub (2023a) and Bendada *et al.* (2022), the microbial isolation was performed as follows:

Total and fecal coliforms were isolated on MacConkey agar at 44°C for 24 to 48 hours.

Staphylococci were isolated on Baird Parker agar at 37°C for 24 hours.

Fungal flora was isolated on acidified Potato Dextrose Agar medium at 25°C for 3 to 5 days.

The isolation of LAB strains was carried out after an enrichment step on media suitable for their growth. M17 agar was used for the isolation of mesophilic and thermophilic streptococci at 30 and 45°C, respectively and MSE agar for the isolation of *Leuconostoc* species in a microbiological jar. For LAB strains, only Gram-positive and catalase-negative bacteria were retained.

### Microbial identification

### Microbial contaminants of date paste and date extract

Through phenotypic and biochemical tests, bacterial contaminants isolated from date paste and date extract were identified according to the techniques described by Guiraud (2003); Benyagoub *et al.* (2018). While the isolated molds were identified using the microculture technique (Garcia de Lomas *et al.,* 1981).

### LAB strains

Several tests have made it possible to identify LAB isolated from date paste according to microbiological standards as described by Benyagoub (2022).

### Antibiotic susceptibility testing for LAB strains and bacterial contaminants

The study of antibiotic susceptibility was evaluated using the disk diffusion method on Mueller-Hinton agar. The bacterial inhibition zone was measured and then interpreted based on the recommendations of the CLSI (2018) for bacterial contaminants (Benyagoub *et al*. 2020; Benyagoub, 2023b) and based on the EFSA (2012) for LAB strains.

### Technological characteristics of LAB strains

In this study, we targeted the following technological properties: Heat resistance test, sugar fermentation test, acidifying power, proteolytic, lipolytic and amylolytic activity (Meyers *et al.* 1996; Benyagoub, 2022).

### Antimicrobial effect of LAB strains

The antibacterial effect was assessed using the spot test on agar as described by Fleming *et al.* (1975). However, the antifungal effect was estimated by two methods. First, the mycelial disc method by measuring the radial growth of the mycelial disc expressed in percentage (%). Second, the biomass method in which fungal biomass was weighed and the antifungal effect was assessed based on the fungal biomass rate (%) compared to the control as described by Benyagoub (2022).

## RESULTS AND DISCUSSION

### Microbial contaminants of date paste and date extract

Through microbial isolation, selective culture media, as well as biochemical tests, the obtained results made it possible to isolate and identify the following contaminants: *Citrobacter* sp, *Cronobacter sakazakii*, *Staphylococcus* sp (1 and 2), *Enterococcus* sp (Table 1, Fig 1).

The isolated molds have mainly consisted of three species: *Aspergillus niger*, *Aspergillus flavus* and *Penicillium* sp (Fig 1).

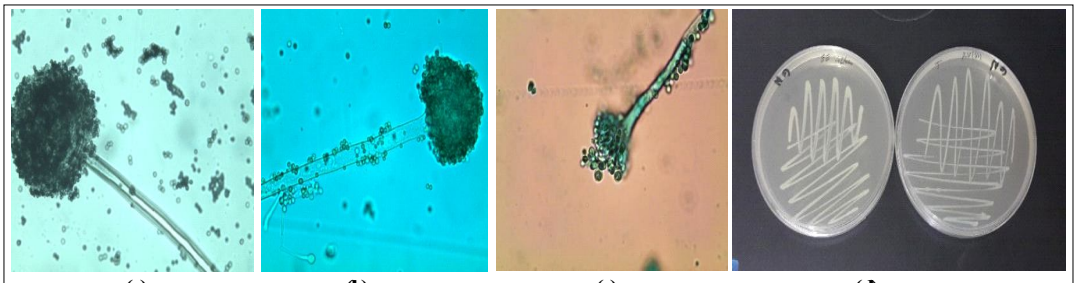
### Antibiotic susceptibility testing (AST) for bacterial contaminants

AST results for bacterial contaminants are presented in Table 2 and Fig 2.

### LAB strains

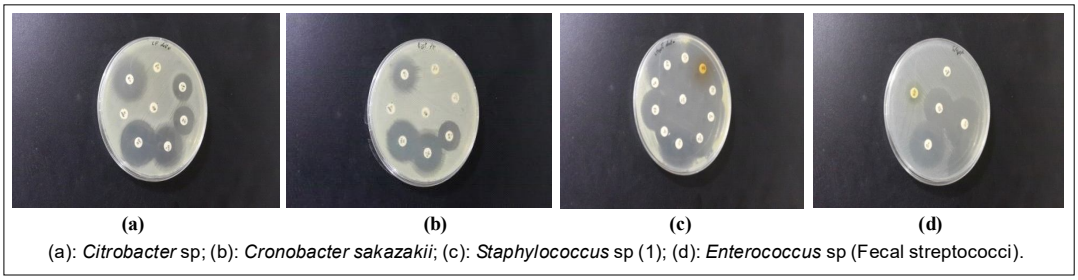
Eight (8) LAB were isolated from the date paste (Table 3). Colonies of LAB strains have a whitish color and smooth textures (Fig 3).

The physiological characteristics of LAB strains are presented in Table 4.




(a): *A. flavus*; (b): *A. niger*; (c): *Penicillium* sp; (d): Bacterial contaminants on nutrient agar.

**Fig 1:** Macro- and microscopic aspects of microbial contaminants.




(a): *Citrobacter* sp; (b): *Cronobacter sakazakii*; (c): *Staphylococcus* sp (1); (d): *Enterococcus* sp (Fecal streptococci).

**Fig 2:** Sensitivity test results.




(a): SLT 1, (b): SLM 3.

**Fig 3:** Macroscopic appearances of LAB strains on M17 agar.

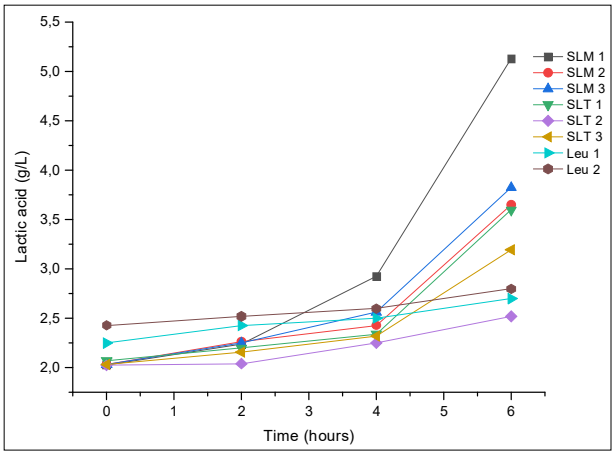



**Fig 4:** Evolution of the titratable acidity of the culture media.

## Technological interests of LAB strains

### Thermoresistance and sugar fermentation tests

The obtained results showed that all the isolated LAB strains were heat-resistant, mannitol (+) and were different from one strain to another in the fermentation of sugars (Table 5).

The data reported by Abekhti *et al.* (2021) confirmed the microbial diversity of 'Btana', a product similar to date paste, as follows: *Weissella paramesenteroides*, *W. cibaria*, *Leuconostoc citreum*, *L. pseudomesenteroides* and *Lactobacillus plantarum*. The isolated LAB strains have significant potential for sugar assimilation compared to the data reported by Abekhti *et al.* (2021), which fermented 7 carbohydrates. This could mean that the isolated species are adapted to the high-sugar environment of date paste.

### Acidifying power

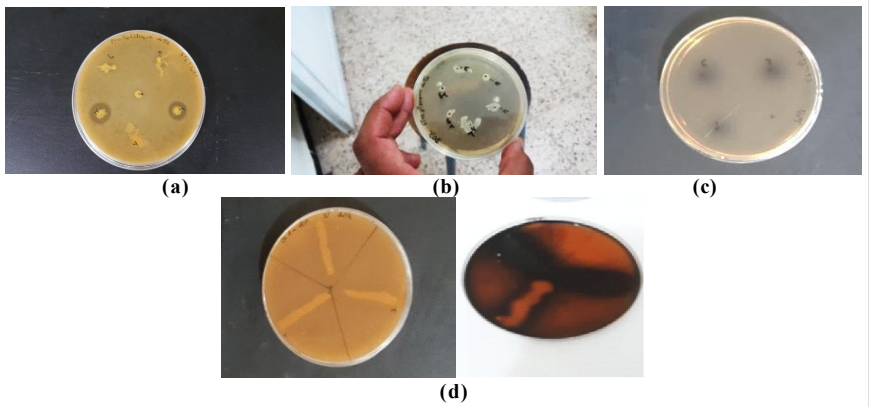
Fig 4 shows the evolution of titratable acidity of the culture media inoculated with the isolated LAB strains.

The results of the acidifying power study show that the isolated LAB strains have a strong acidifying capacity, in particular, the SLM 1 and SLM 3 strains, while the *Leuconostoc* strains have less acidifying power. Significant acidification caused by the production of lactic acid is one of the most sought-after technological properties. These metabolites are organic acids that modify the pH of the medium, contributing to the inhibition of microbial contaminants and even pathogenic ones. On the other hand, the production of acidity depends on several parameters such as the incubation temperature, the physiological state of the bacteria, the concentration of the inoculum and the medium composition (Benyagoub, 2023a).

### Amylolytic, proteolytic and lipolytic activity tests

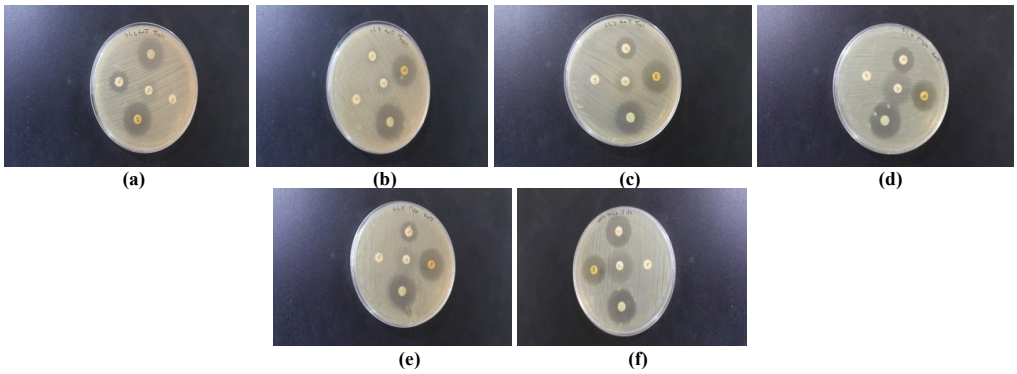
Table 6 and Fig 5 present the results of the amylolytic, proteolytic and lipolytic activity tests of the isolated LAB strains.

As part of industrial processes, LAB strains are challenged by various stressful conditions that are likely to affect their metabolic activities, including proteolysis (Savijoki *et al.* 2006). The obtained results show that the SLM 1 and SLM 3 strains exhibited proteolytic activity; these results were consistent with several studies (Ben Moussa *et al.*, 2008; Essid *et al.*, 2009).



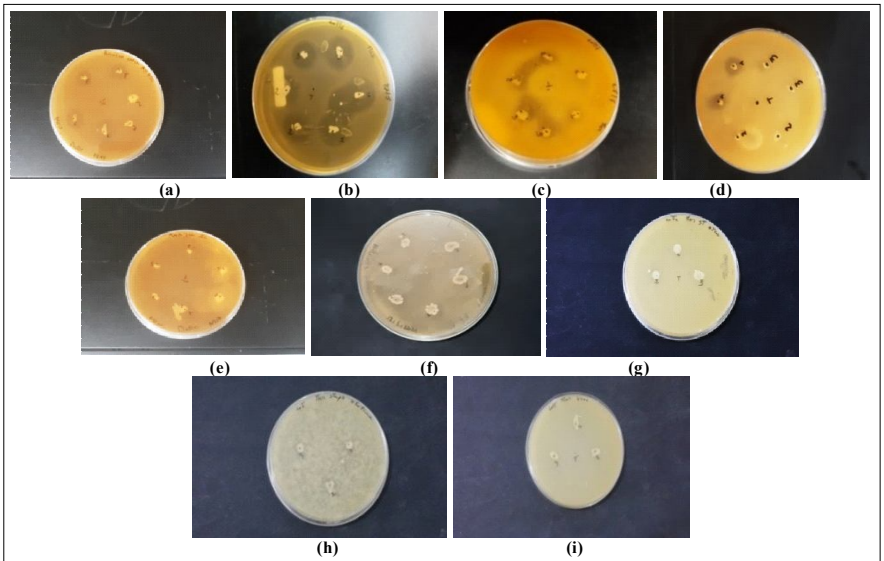

(a): Protease hydrolysis test (positive test for SLM 1 and SLM 3); (b): Search for lipase (negative for all tested LAB strains); (c): Esculinase test (positive test for SLM 1, SLM 2 and SLM 3 and negative test for Leu 1); (d): Search for amylase (positive test for SLM 1, SLM 2 and SLM3).

**Fig 5:** Technological ability of LAB strains isolated.




(a): SLT 1; (b): SLT 2; (c): SLT 3; (d): SLM 1; (e): SLM 2; (f): SLM 3.

**Fig 6:** Sensitivity test results.




(a): *B. cereus* ATCC 14579; (b): *Enterococcus* sp (F. Strep); (c): *Citrobacter* sp; (d): *Cronobacter sakazakii*; (e): *E. coli* ATCC 25922; (f): *Staphylococcus* sp (1); (g, h): *S. aureus* ATCC 25923; (i): *Staphylococcus* sp (2).

**Fig 7:** Photographic illustration of the antibacterial effect of LAB strains.

**Table 1:** Biochemical characteristics of bacterial contaminants.

| Tests \ Bacterial contaminants | | *Citrobacter* sp | *Staphylococcus* sp | Fecal streptococci (*Enterococcus* sp) | *Enterobacter* sp | *Cronobacter sakazakii* |
|---|---|---|---|---|---|---|
| Motility | | + | - | - | + | + |
| Gram stain | | Gram-negative bacteria | Gram-positive bacteria | Gram-positive bacteria | Gram-negative bacteria | Gram-negative bacteria |
| Catalase test | | + | + | - | + | + |
| Oxidase test | | - | - | - | - | - |
| TSI agar | Glu | + | / | / | + | + |
| | Lac | + | / | / | + | + |
| | Gas production | + | / | / | + | + |
| | Sucrose | + | / | / | + | + |
| | $H_2S$ | + | / | / | - | - |
| Mannitol motility test agar | | + | - | / | + | - |
| Indole test | | - | / | / | - | - |
| VP test | | - | + | / | + | + |
| RM test | | + | / | / | - | - |
| Simmons citrate agar | | + | / | / | + | + |
| Coagulase | | / | - | / | / | / |
| Hemolysis test | | / | / | γ-hemo | / | / |
| LDC | | + | / | / | - | - |
| Urease test | | - | / | / | - | - |
| API 20 E | | *Citrobacter* sp | / | / | *Enterobacter* sp | *Cronobacter sakazakii* |

TSI agar: Triple Sugar Iron Agar; Glu: Glucose; Lac: Lactose; VP: Voges-Proskauer test; RM: Methyl red test; LDC: Lysine decarboxylase test; (+): Positive reaction; (-): Negative reaction; γ-hemo: γ-hemolysis.

The lipolytic power is of great importance in the flavor development and the release of fatty acids but it is not very responsive to LAB compared to other groups of bacteria and this is the case of isolated SLM 2 strains, with an appreciable lipolytic power.

### Antibiotic susceptibility testing (AST) for LAB strains

AST results for LAB strains are presented in Table 7 and Fig 6.

The AST study is one of the important selection criteria for probiotics (Zhou *et al.,* 2005). Resistance remains a controversial condition; on the one hand, it is sought for in probiotics so that they are active even after treatment with antibiotics. On the other hand, there are risks that it might be transmitted to other bacteria, which could lead to the development of new pathogenic resistant bacteria (Salminen *et al.,* 1998).

The AST results agree with various reports that LAB strains are sensitive to major classes of antibiotics, such as penicillin G, amoxicillin β-lactam, cephalosporins, aminoglycoside, quinolone, imidazole, nitrofurantoin and fluoroquinolone (Halami *et al.,* 2000). According to Amalia *et al.* (2018), the bacteria isolated from fermented food are widely known to be safe for human consumption due to their safety to human health.

### Antimicrobial effect of LAB strains

The antibacterial effect results of LAB strains against bacterial contaminants are shown in Table 8 and Fig 7.

The antifungal effect of LAB strains against the isolated molds is presented in Fig 8, 9, 10 and 11.

The obtained results showed a significant rate of inhibition against *Penicillium* sp compared to the two other fungal species *A. niger* and *A. flavus* due to the effect of inhibitory agents secreted by lactic strains of streptococci. No antimicrobial effect of *Leuconostoc* strains has been detected. It may be related to their weak technological characteristics that do not allow the microorganism to dominate in the environment where it lives.

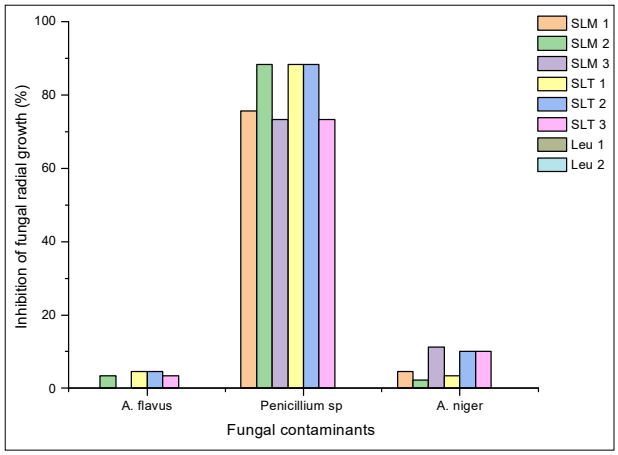



**Fig 8:** Qualitative antifungal effect of LAB strains against the isolated molds.

**Table 2:** AST results for bacterial contaminants and bacterial reference strains.

| Bacterial contaminants and bacterial reference strains | Antibiotics | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | C | AMP | AMX | AUG | FOX | IPM | AK | OFX | SXT | E | VA | CD | P |
| *Citrobacter* sp | S (24 mm) | R (6 mm) | / | R (6 mm) | S (18 mm) | R (6 mm) | S (18 mm) | S (24 mm) | / | / | / | / | / |
| *Enterobacter* sp | S (20 mm) | R (6 mm) | / | R (6 mm) | R (13 mm) | R (6 mm) | S (18 mm) | S (18 mm) | / | / | / | / | / |
| *Cronobacter sakazakii* | S (18 mm) | R (6 mm) | / | R (6 mm) | R (10 mm) | R (6 mm) | S (17 mm) | S (16 mm) | / | / | / | / | / |
| *Staphylococcus* sp (1) | / | / | / | / | S (24 mm) | / | / | / | S (30 mm) | S (32 mm) | S (32 mm) | S (26 mm) | S (29 mm) |
| *Staphylococcus* sp (2) | / | / | / | / | S (25 mm) | / | / | / | S (36 mm) | S (35 mm) | S (35 mm) | S (30 mm) | S (35 mm) |
| *Enterococcus* sp (F. Str.) | / | R (10 mm) | / | / | / | / | / | / | / | S (23 mm) | S (18 mm) | / | / |
| *S. aureus* ATCC 25923 | / | / | / | / | R (16 mm) | | / | / | S (30 mm) | S (30 mm) | S (15 mm) | S (28 mm) | R (10 mm) |
| *E. coli* ATCC 25922 | S (22 mm) | R (6 mm) | / | R (6 mm) | R (14 mm) | R (6 mm) | S (20 mm) | S (26 mm) | / | / | / | / | / |
| *B. cereus* ATCC 14579 | / | R (6 mm) | R (7 mm) | R(6 mm) | / | R(6 mm) | S (21 mm) | / | / | / | / | / | R (6 mm) |

C 30: Chloramphenicol; AMP 10: Ampicillin; AUG 30: Amoxicillin+clavulanic acid; FOX 30: Cefoxitin; IPM 10: Imipenem; AK 30: Amikacin; OFX 5: Ofloxacin; SXT 25: Co-trimoxazole; E 15: Erythromycin; VA 30: Vancomycin; CD 2: Clindamycin; P 10: Penicillin; mm: Zone of inhibition in millimeters.

We found that isolated lactic strains of streptococci have a good inhibitory power by synthesis of the inhibitor agents or by modification of the pH of the medium. This antibacterial activity affected both Gram-positive and Gram-negative bacteria, but no effect was revealed against the bacterial reference strains (*E. coli* and *S. aureus*), except for the SLM1 strain.

These results agree with the findings reported by Dubois *et al.* (1982); Hadef (2012), Elmoualdi *et al.* (2008) and Benyagoub (2022) who found that the broad-spectrum

**Table 3:** Microscopic and macroscopic appearance of LAB strains.

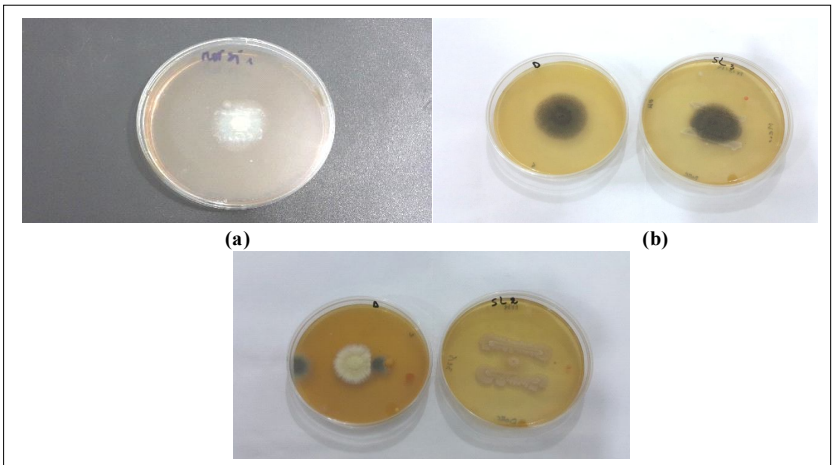
| Tests / LAB strains | Mobility | Cell arrangement mode | Gram-type | Catalase | Colony color |
|---|---|---|---|---|---|
| SLM 1 | (-) | Chains | Gram-positive bacteria | (-) | Whitish color |
| SLM 2 | | | | | |
| SLM 3 | | | | | |
| SLT 1 | | | | | |
| SLT 2 | | | | | |
| SLT 3 | | | | | |
| Leu 1 | | Pairs | | | |
| Leu 2 | | | | | |

SLM: Mesophilic streptococci; SLT: Thermophilic streptococci; Leu: *Leuconostoc*; (-): Negative reaction.

**Table 4:** Physiological characteristics of LAB strains.

| LAB strains | Metabolic types | Thermo-resistance test | T | | | pH | | | NaCl | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | 10°C | 22°C | 45°C | 4 | 7 | 9 | 2% | 3% | 4% | 9% |
| SLM 1 | Hetero-F | + | + | + | + | + | + | + | + | + | + | + |
| SLM 2 | Hetero-F | + | + | + | + | + | + | + | + | + | + | + |
| SLM 3 | Homo-F | + | + | + | + | + | + | + | + | + | + | + |
| SLT 1 | Hetero-F | + | - | + | + | - | + | + | + | + | + | + |
| SLT 2 | Hetero-F | + | - | + | + | + | + | + | + | + | + | + |
| SLT 3 | Hetero-F | + | - | + | + | - | + | + | + | + | + | + |
| Leu 1 | Hetero-F | + | + | + | + | - | + | + | + | + | + | - |
| Leu 2 | Hetero-F | + | + | + | + | - | + | + | + | - | - | - |

T: Temperature in (°C); Hetero-F: Hetero-fermentative; Homo-F: Homo-fermentative; SLM: Mesophilic Streptococci; SLT: Thermophilic Streptococci; Leu: *Leuconostoc*; (+): Positive bacterial culture; (-): No bacterial growth.



(a): SLM 2 strain against *Aspergillus flavus*; (b): SLM 3 strain against *Aspergillus niger*; (c): SLM 2 strain against *Penicillium* sp.

**Fig 9:** Mycelial disc method.

**Table 5:** Sugar fermentation tests.

| LAB strains | Sugars | | | | | | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Arabinose | Galactose | Sucrose | Lactose | Maltose | Melibiose | Raffinose | Rhamnose | Amygdalin | Glucose | Inositol | Sorbitol | Starch | Fructose | Mannose | Esculine | Cellobiose | Xylose | Mannitol | Sorbose |
| SLM 1 | + | + | + | + | + | + | + | + | + | + | - | - | + | + | + | + | + | + | + | - |
| SLM 2 | + | + | + | + | + | + | + | + | + | + | - | - | + | + | + | + | + | + | + | - |
| SLM 3 | + | + | + | + | + | + | + | + | + | + | - | - | + | + | + | + | + | + | + | - |
| SLT 1 | + | + | - | + | + | + | + | - | + | + | - | - | + | + | + | + | + | + | + | - |
| SLT 2 | + | + | + | + | + | + | + | + | + | + | - | - | + | + | + | + | + | + | + | - |
| SLT 3 | + | + | - | + | + | + | + | - | + | + | - | - | + | + | + | + | + | + | + | - |
| Leu 1 | + | + | + | - | - | + | + | + | - | + | + | + | - | - | - | - | - | - | - | - |
| Leu 2 | + | - | - | - | - | + | + | - | + | + | - | - | - | - | - | - | - | + | + | - |

(+): Positive reaction; (-): Negative reaction.

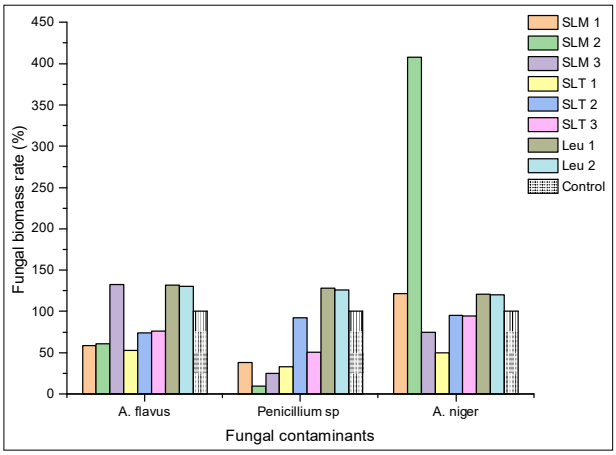



**Fig 10:** Quantitative antifungal effect of LAB strains against the isolated molds.



**Fig 11:** Fungal biomass method.

antimicrobial activity of LAB strains isolated from different biotopes varies from one microorganism to another. Also, Mameche-Doumandji (2008) proved that lactic acid cocci have a high inhibitory activity on Gram-negative bacteria compared to that obtained against Gram-positive ones. The study carried out by Castellano *et al.* (2007) showed that substances with antimicrobial effects may have a greater possibility of targeting Gram-negative pathogenic strains if the outer membrane has been destabilized by the presence of another obstacle, such as organic acids, chelating agents, or other agents.

The toxic effect of lactic acid bacteria includes a reduction of intracellular pH and dissipation of the membrane's potential (Kos *et al.* 2010). On the one hand, LAB strains have less antifungal activity against *A. flavus* and *A. niger* and on the other hand, *Penicillium* sp was sensitive to the effect exhibited by the isolated lactic strains of streptococci. These results are already found by Magnusson *et al.* (2003) and Sathe *et al.* (2007). Lactic strains of streptococci include a set of heterogeneous species whose common trait is that they can synthesize antibacterial substances.

These results show that dates have native LAB strains able to delay or stop the growth of microbial contaminants.

## CONCLUSION

The results of this study suggest that lactic acid bacteria isolated from date paste can play the role of a barrier flora

**Table 6:** Technological suitability of isolated LAB strains.

| LAB / Technol. A. | | SLM 1 | SLM 2 | SLM 3 | SLT 1 | SLT 2 | SLT 3 | Leu 1 | Leu 2 |
|---|---|---|---|---|---|---|---|---|---|
| Amylolytic activity | | + | + | + | - | - | - | - | - |
| Proteolytic activity | | + (13 mm) | - | + (12 mm) | - | - | - | - | - |
| Lipolytic activity | Tween 20 | - | - | - | - | - | - | - | - |
| | Tween 80 | - | + | - | - | - | - | - | - |

Technol. A.: Technological ability; SLM: Mesophilic streptococci; SLT: Thermophilic streptococci; Leu: *Leuconostoc* sp.

**Table 7:** AST results for LAB strains.

| LAB strains | Antibiotics | | | | |
|---|---|---|---|---|---|
| | AMX | SXT | E | TE | P |
| SLM 1 | S (26 mm) | S (26 mm) | S (15 mm) | S (24 mm) | R (6 mm) |
| SLM 2 | S (24 mm) | R (6 mm) | S (13 mm) | S (24 mm) | R (6 mm) |
| SLM 3 | S (26 mm) | S (24 mm) | S (25 mm) | S (25 mm) | R (6 mm) |
| SLT 1 | S (23 mm) | R (6 mm) | S (15 mm) | S (25 mm) | R (6 mm) |
| SLT 2 | S (25 mm) | R (6 mm) | R (6 mm) | S (23 mm) | R (6 mm) |
| SLT 3 | S (25 mm) | R (6 mm) | S (14 mm) | S (25 mm) | R (6 mm) |
| Leu 1 | S (24 mm) | S (15 mm) | S (13 mm) | S (22 mm) | R (6 mm) |
| Leu 2 | S (21 mm) | S (14 mm) | S (14 mm) | S (20 mm) | R (6 mm) |

S: Sensitive; R: Resistant; AMX 25: Amoxicillin; SXT 25: Co-trimoxazole; E 15: Erythromycin; TE 30: Tetracycline; P 10: Penicillin.

**Table 8:** Antibacterial effect of LAB strains against bacterial contaminants and bacterial reference strains.

| Bact. strains | LAB strains (Zones of inhibition in mm) | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | SLM 1 | SLM 2 | SLM 3 | SLT 1 | SLT 2 | SLT 3 | Leu 1 | Leu 2 |
| *Citrobacter* sp | 11 | 09 | 10 | 08 | 11 | 10 | - | - |
| *Enterobacter* sp | 07 | 09 | 07 | 10 | 09 | 09 | - | - |
| *Cronobacter sakazakii* | 08 | 07 | 07 | 08 | 10 | 13 | - | - |
| *Staphylococcus* sp (1) | 10 | - | - | 10 | - | - | - | - |
| *Staphylococcus* sp (2) | - | - | - | 08 | 10 | 11 | - | - |
| *Enterococcus* sp (F. Str.) | 20 | 12 | 20 | 07 | 14 | 10 | - | - |
| *S. aureus* ATCC 25923 | 07 | - | - | - | - | - | - | - |
| *E. coli* ATCC 25922 | - | - | - | - | - | - | - | - |
| *B. cereus* ATCC 14579 | 13 | - | 12 | 15 | - | 12 | - | - |

Bact. strains: Bacterial strains; *S. aureus*: *Staphylococcus aureus*; *E. coli*: *Escherichia coli*; SLM: Mesophilic Streptococci; SLT: Thermophilic Streptococci; Leu: *Leuconostoc*; (-): Negative antibacterial effect; F. Str.: Fecal streptococci.

towards microbial contaminants and therefore participate in the preservation of date fruit by-products.

**Source of support**

Nil.

**Conflict of interest**

All authors declare that they have no conflict of interest.

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