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Original Research

Technological properties and antagonistic effect of lactic acid bacteria isolated from fermented goat milk flavoured with juniper leaves – a product from the southwest region of Algeria – against some microbial contaminants

Abstract

Lactic acid bacteria (LAB), as microorganisms that are naturally present in milk and dairy products, have interesting technological and antimicrobial properties. This study focussed on the antagonistic effect of LAB isolated from fermented goat milk naturally flavoured with Phoenician juniper leaves against some microbial contaminants, and some reference strains. First, microbial contaminants were isolated from dairy and meat products using selective media according to national standards. Second, a study of the technological properties of lactic strains isolated from goat milk flavoured with Phoenician juniper leaves was carried out, including an analysis of any antibacterial effects against microbial contaminants. This was established through spot tests on agar. Antifungal activity was established through mycelial radial growth on agar, and the evaluation of biomass on broth culture. Eleven microbial contaminants were isolated and identified: seven bacterial species – *Enterobacter sp* (1) and (2), *Salmonella sp*, *Pseudomonas sp*, *Staphylococcus sp*, *S. aureus*, *Enterococcus sp* (fecal streptococci); four fungal species – *Saccharomyces sp*, *Candida albicans*, *Penicillium sp*, and *Aspergillus niger*. Antibiotic susceptibility results showed that *Enterobacter sp* (1) and (2) and *Salmonella sp* strains were resistant to several antibiotics, namely ampicillin, amoxicillin/clavulanic

acid, cefoxitin, and imipenem. *Pseudomonas sp* was resistant to imipenem, fosfomycin and amikacin. *Staphylococcus sp*p and *S. aureus* strains were resistant to penicillin, oxacillin, and fosfomycin. *Enterococcus sp* was resistant to ampicillin and tetracycline. However, 13 isolates of Streptococcus were isolated from fermented and flavoured goat milk, each with different physiological and technological characteristics. Five strains (38.5%) showed good acidifying power and 13 strains (100%) revealed good proteolytic activity. High titratable acidity of 5.13 g/L of lactic acid was recorded for the thermophilic *Streptococcus sp* strain (St2). Although all LAB of streptococci species were resistant to penicillin and cotrimoxazole, they were susceptible to the majority of the tested antibiotics, and are therefore considered safe for use as probiotics. The antimicrobial effect results show that the isolated LAB strains have an antagonistic effect by inhibiting the growth of contaminating strains. This activity was important against the tested yeasts *Saccharomyces sp*, and *Candida albicans*. A greater antifungal action against *Penicillium sp* was also observed compared to the species A. niger, with a reduced rate of the fungal biomass which can go up to 90%. Regarding antibacterial action, Gram-positive bacterial contaminants, namely *Enterococcus sp* and the reference strain Bacillus cereus ATCC 14579 were the most susceptible among the tested bacteria, with zones of inhibition ranging from 14 mm to 22 mm. A medium to weak action was revealed against *Enterobacter sp* (1) and (2), *Pseudomonas sp*, *Salmonella sp*, and both isolated and reference *S. aureus* species. These results, obtained from a study of traditional practices of great indigenous wealth in the southwest regions of Algeria, constitute a perspective and platform for future investigation on the characteristics of microbial microflora in dairy products by helping in the selection of lactic strains of technological interest.

Elhassan Benyagoub [1,2*]

[1] Faculty of Life and Natural Sciences, Department of Biology, Mohammed Tahri University of Bechar, Algeria

[2] Archipel Laboratory, Mohammed Tahri University of Bechar, Algeria

*Corresponding author:
Elhassan Benyagoub

benyagoub.elhassan@gmail.com

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Introduction

Goat milk is nature's closest thing to perfect food. It contains proteins, vitamins, minerals, fatty acids, trace elements and enzymes that are easily absorbed and digested by the body [1]. Its high nutritional value and rich composition makes it a popular choice in the treatment of a variety of health-related issues, important for the prevention of cardiovascular disease, cancer, and allergy, has antimicrobial, antioxidant and anti-inflammatory properties, and is considered an immune-boosting and therefore, one of the most preferred dairy products worldwide recommended for infants, old and convalescent people [2, 3]. The composition and physicochemical properties of goat milk also make it a very favourable environment for the multiplication of microorganisms that are naturally present in milk, or which occur accidentally through handling [4, 5].

The microbial microflora of raw milk, which is mainly composed of lactic acid bacteria (LAB), forms a complex microbial ecosystem and plays an important role in the development of the organoleptic characteristics of fermented dairy products such as fermented milk and cheese [6].

From a hygienic point of view, LAB slow down the development of undesirable flora and improve food preservation by lowering the pH of the medium. They also produce several metabolites, which have an antimicrobial effect [7].

LAB belong to a group of beneficial bacteria with similar properties that produce lactic acid during the fermentation process. They are widespread and are also found in the human digestive system [8]. These microorganisms are non-pathogenic bacteria and account for the majority of GRAS (Generally Recognized As Safe) microorganisms by the US Food and Drug Administration (FDA). Nevertheless, some species of *Streptococcus* and *Enterococcus* are considered opportunistic pathogens [9].

There is still much to learn about the nature of the interaction between microflora during fermentation and about the molecules they secrete hence the study of bioactive compounds is a field of research that continues to rise. The present study fits within that context. It aims to investigate the *in vitro* antagonistic effect of lactic strains isolated from raw goat milk fermented and flavoured with Phoenicia juniper leaves – a product that is part of the traditional practice of the inhabitants of the southwest regions of Algeria – against some microbial contaminants.

Materials and methods

All chemicals and culture media used in this study were sourced from Liofilchem Diagnostici, Italy and Sigma-Aldrich, Switzerland. The bacterial reference strains ATCC were obtained from the Teaching Hospital Centre of Oran, Algeria. The study was carried out at Mohammed Tahri University of Bechar, Algeria.

Sampling

The goat milk sample used in this study was collected by hand milking goats in Bechar city under hygienic conditions and recovered in clean utensils. As per the rules of hygiene and asepsis recommended in microbiology, the udders of the goats were rinsed and washed with bleached water and the first jets of milk were discarded before sample collection.

An adequate amount of juniper leaves (**Fig. 1**) was added to the goat milk according to the traditions of the southwest regions of Algeria [5].

Figure 1
Juniperus phoenicea L. leaves

Isolation of microbial contaminants and LAB

The microbiological analyzes were carried out according to the standards established by the American Public Health Association (APHA) [10], and the Ministry of Trade and Export Promotion, Algeria (2016) [11].

From a stock solution, a dilution series was prepared for searching and isolating the following microorganisms:

- Staphylococci, group D streptococci, coliforms, *Salmonella sp*, and fungal flora yeasts and moulds.
- Lactic *streptococci* strains

After homogenization of the stock solution, 1 mL of the solution was taken using a sterile graduated pipette, and aseptically poured into a test tube containing 9 mL of buffered peptone water to have a 10^{-1} dilution solution, and similarly, successive decimal dilutions were carried out (up to 10^{-5}) [12].

Microbial isolation was carried out as follows:

- Fecal coliforms were isolated using the pour plate technique on MacConkey agar medium. The plates were incubated at 44°C for 24 to 48 hours [13].
- Coagulase-positive Staphylococci (CoPS) were isolated by spread plate technique on Baird-Parker agar medium. The dishes were incubated at 37°C for 24 hours [14].
- The MPN (Most Probably Number) technique was used to detect and enumerate fecal streptococci in two steps. First, presumptive and confirmatory tests were performed on Rothe broth and EVA Litsky broth, respectively, where the tubes that were positive for Rothe broth were subcultured on EVA Litsky broth and incubated at 37°C for 24 hours. Second, streptococci isolation was carried out using a bile esculin azid (BEA) agar medium. The dishes were incubated at 37°C for 24 hours [15].
- A search for *Salmonella sp* was carried out in three stages [16]:
  1. Pre-enrichment on Buffered Peptone Water (BPW); **2.** selective enrichment on Rappaport Vassiliadis broth and Selenite Cystine broth; **3.** isolation on Hektoen Enteric agar and Salmonella Shigella agar. The plates were incubated at 37°C for 24 hours.
- Detection of yeasts and moulds was carried out by spread plate technique on OGA agar medium (Oxytetracycline-Glucose-Yeast Extract Agar). The plates were incubated at 25°C for three to five days [17].
- Isolation of LAB was carried out after an enrichment step on M17 broth. Then, M17 agar was used to isolate mesophilic and thermophilic streptococci at 30°C and 45°C respectively. Cycloheximide at a concentration of 1% (v/v) was added to the agar plate. The dishes were incubated in microaerophilic conditions using an anaerobic gas jar pack system to reduce oxygen levels [5, 18].

Purification of bacterial isolates was carried out by successive subculturing using nutrient agar and nutrient broth for bacterial contaminants – M17 broth, and M17 agar slant tubes for streptococci – until a pure culture was obtained. Purity of culture was confirmed by the macroscopic appearance. In addition, slides were prepared for microscopic observation using Gram staining for bacteria and lactophenol cotton blue (LPCB) staining for yeasts and moulds [19, 20, 21]. For LAB, only Gram-positive and catalase-negative strains were retained. Pure isolates were stored on M17 agar slant tubes at 4°C. Bacterial cultures were renewed every three weeks.

Identification of microbial isolates

Microbial contaminants

Pure isolates were identified using serial phenotypic and biochemical identification tests: a macroscopic examination of the colonies, microscopic examination of the fresh state,

Gram staining, oxidase test, the classic range identified through the IMViC test and the miniaturized API 20E range for Gram-negative bacteria. Coagulase test, catalase test, and esculin hydrolysis have been applied to Gram-positive bacteria in previous studies [22, 23, 24, 25], however, in the present study, the genus of the isolated moulds was identified by the microculture technique [26].

LAB

Pure lactic isolates were identified using a series of tests: catalase test; microscopic examination of the fresh state; Gram staining; test for gas production by fermentation of glucose, which makes it possible to differentiate homo-fermentative from hetero-fermentation LAB; growth test at different temperatures (10°C, 22°C and 45°C), which makes it possible to distinguish mesophilic from thermotolerant LAB; growth at different acid and alkaline pH (pH 4 and 9) [27]; growth in a medium in the presence of different NaCl concentrations (2%, 3% and 4%); the production of acetoin on MR-VP broth (Clark and Lubs medium), citrate test, and the esculin hydrolysis on esculin agar medium.

Antibiotic susceptibility testing

The multidrug resistance (MDR) of the isolated strains was determined by the agar diffusion method (disk method). The antibiotic susceptibility of bacteria was studied by using different discs impregnated with antibiotics placed equidistant on the surface of the Mueller-Hinton agar inoculated by the tested strain [28, 29]. After 18 to 24 hours of incubation at 37°C, the zone of inhibition was measured and qualified as susceptible (S), intermediate (I), or resistant (R) according to the recommendations given by Clinical and Laboratory Standards Institute (CLSI, 2018) [30]. In this study, the antibiotics tested against contaminating microorganisms (Liofilchem, Italy) were as follows: Vancomycin (VA 30 µg), Clindamycin (CD 2 µg), Ciprofloxacin (CIP 5 µg), Fusidic acid (FC 10 µg), Rifampicin (RD 30 µg), Chloramphenicol (C 30 µg), amoxicillin/clavulanic acid (AUG 30 µg), Colistin (CL 25 µg), Fosfomycin (FF 10 µg), Ofloxacin (OFX 5 µg), Amikacin (AK 30 µg), Nalidixic acid (NA 30 µg), Penicillin (P 10 µg), Erythromycin (E 15 µg), Tetracycline (TE 30 µg), Oxacillin (OX 1 µg), Ampicillin (AMP 10 µg), Cefoxitin (FOX 30 µg), and Sulfamethoxazole/Trimethoprim (SXT 25 µg).

The antibiotics tested against the isolated lactic acid strains were as follows: Amoxicillin (AMX 30 µg), Sulfamethoxazole/Trimethoprim (SXT 25 µg), Erythromycin (E 15 µg), Tetracycline (TE 30 µg), Penicillin (P 10 µg), Chloramphenicol (C 30 µg), Ciprofloxacin (CIP 10 µg), Gentamycin (CN 10 µg), Ofloxacin (OFX 5 µg), Pefloxacin (PEF 10 µg), Streptomycin (S 30 µg), and amoxicillin/clavulanic acid (AUG 30 µg).

For LAB, the Petri dishes were incubated under anaerobic conditions at 30°C for 24 hours, and under aerobic conditions for microbial contaminants at 37°C (18 to 24 hours).

Characterization of the technological potential of lactic strains

The present study targeted six technological characteristics: a heat resistance test at 60°C; twenty sugar assimilation tests – arabinose, galactose, sucrose, lactose, maltose, mannitol, melibiose, raffinose, rhamnose, ribose, amygdalin, inositol, sorbitol, starch, fructose, mannose, esculin, cellobiose, xylose, and sorbitol [31]; acidifying power by measuring pH and titratable acidity [32]; proteolytic (casein degradation, and gelatin hydrolysis) [33, 34]; lipolytic (polysorbate 20 and 80 hydrolysis) [35, 36], and amylolytic activity (starch degradation) [37].

In addition to the MDR test previously cited as one of the safety tests, a phenotypic haemolysis test was performed where the isolated lactic acid strains were streaked on 5% (w/v) blood agar, and, after incubation at 37°C for 24 to 48 hours, the Petri dishes were examined for the β, α, or γ-haemolysis character [38, 39].

Antagonistic effect of lactic acid isolates

Antibacterial effect

The antagonistic effects of the isolated LAB were evaluated by the spot test on agar. This method consists of cultivating two bacterial strains in the same medium in double layers. After 24 to 48 hours of incubation, a layer of Mueller-Hinton agar medium inoculated with the bacterial contaminant was poured over the first inoculated layer of the LAB [40, 41, 42, 43]. The inhibition of bacterial contaminant resulted in the formation of clear zones around the spot which was expressed in millimeters (mm).

Antifungal effect

Mycelial disc method: The lactic strains were inoculated in two streaks on M17 agar medium previously poured into Petri dishes, then incubated in anaerobiosis at 30°C for 48 hours. A mycelial disc was placed in the centre of the Petri dish and incubated at 25°C for seven days against a control [44]. The antifungal effect (AE) was estimated by measuring the radial growth of the mycelial disc expressed in millimeters (mm) according to the following equation:

$$AE\ (\%) = [(Dt - D) \div Dt] \times 100$$

Where:

Dt is the average mycelial growth diameter of the control, and D is the average mycelial growth diameter of the test one.

Biomass method. The M17 broth tubes were inoculated with the isolated lactic strains and incubated at 30°C for 24 hours in anaerobiosis. After incubation, the 50 mL flasks of M17 broth were inoculated with a spore load of the isolated moulds (10^5 spores/mL) as a test sample against a control one. The flasks were incubated again at 25°C for seven days [45].

Filtration of the broth was carried out using filter paper, and the fungal biomass weighed against a control represented the antifungal effect 'B' estimated in percentage according to the following formula:

$$[B = B_1 - B_0]$$

Where:

B_0 is the weight of filter paper

B_1 is the weight of filter paper and fungal biomass after drying

Statistical analysis of data

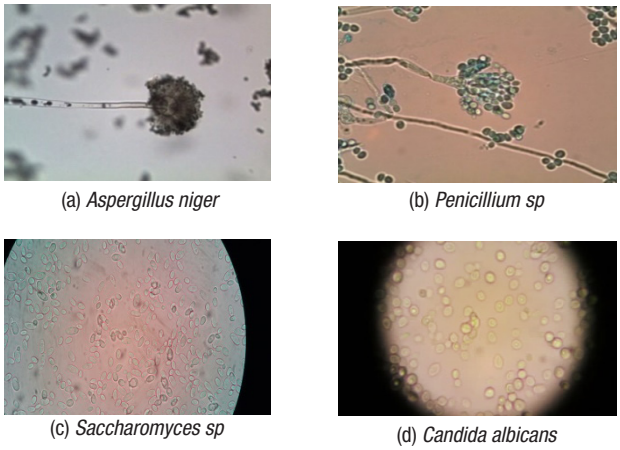
The different analyses were carried out three times to confirm the obtained results, where the mean value ± standard deviation (SD) was used for each parameter, on which the graphical presentations in the form of curves and histograms were plotted using the Origin Lab software (2018).

Results

Microbial contaminants

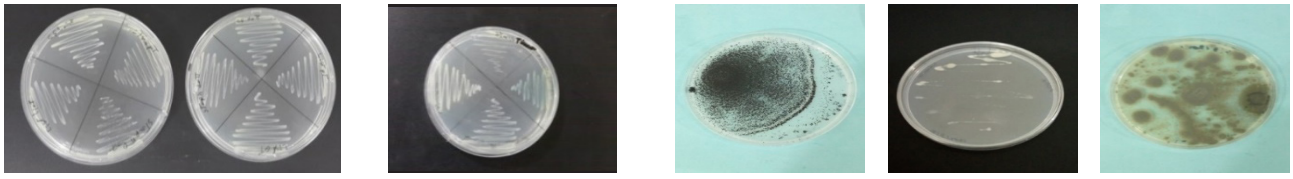
Isolation and identification of microbial contaminants

Microbial isolation using selective culture media in addition to biochemical tests made it possible to isolate and identify the following microbial contaminants: *Enterobacter sp* (1) and (2), *Salmonella sp*, *Staphylococcus aureus*, *Staphylococcus sp*, fecal streptococci (*Enterococcus sp*). However, the isolated moulds mainly consisted of four genera: *Saccharomyces sp*, *Candida albicans*, *Penicillium sp*, and *Aspergillus niger* (**Fig. 2** and **Fig. 3**). Yeasts are part of the specific flora of certain types of fermented milk and should there fore not be considered contamination organisms [46].



(a) *Aspergillus niger* (b) *Penicillium sp* (c) *Saccharomyces sp* (d) *Candida albicans*

Figure 2 Microscopic appearance of isolated mould contaminants



(a) *Bacterial contaminants* (b) *A. niger* (c) *Saccharomyces sp* (d) *Penicillium sp*

Figure 3 Macroscopic appearance of microbial contaminants isolated from fermented and flavoured goat milk

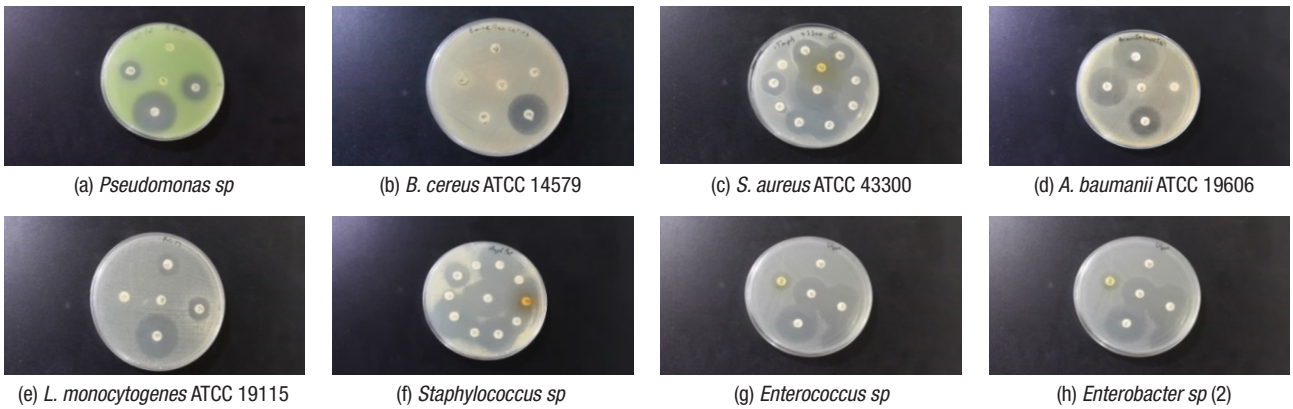
Antibiotic susceptibility test of bacterial contaminants

A study of the antibiotic susceptibility of bacterial contaminants made it possible to determine their antibiotic resistance profiles in accordance with the CLSI (2018) [30]. The results of the antibiotic resistance profile of the tested bacterial contaminants are given in **Table 1**.

The obtained results showed that *Enterobacter sp* (1) and (2) and *Salmonella sp* strains were resistant to several antibiotics, namely ampicillin, amoxicillin/clavulanic acid, cefoxitin, and imipenem, and susceptible to chloramphenicol, ofloxacin, and cotrimoxazole. *Pseudomonas sp* was resistant to imipenem, fosfomycin and amikacin. *Staphylococcus spp* strains were resistant to penicillin, oxacillin, and fosfomycin. *Enterococcus sp* (fecal streptococci) was susceptible to erythromycin, clindamycin, and vancomycin and resistant to ampicillin and tetracycline (**Fig. 4**).

Table 1 Multidrug resistant profiles of the tested uropathogenic agents and bacterial reference strains

Bacterial strains	Antibiotics
Enterobacter sp (1) and (2)	AMP-AUG-FOX-IPM and susceptible to C-AK-OFX-SXT
Pseudomonas sp	IPM-FF-AK and susceptible to CIP-OFX
Salmonella sp	AMP-AK-FOX-AUG-IPM and susceptible to C-OFX-SXT
Staphylococcus sp	P-FF-FOX-OX and susceptible to FC-AK-E-CD-VA-SXT-RD
Staphylococcus aureus	P-FF-FC-AK-FOX-OX-CD-E and susceptible to VA-SXT-RD
Enterococcus sp	AMP-TE and susceptible to E-VA-CD
A. baumanii ATCC 19606	CIP-IPM-FF and susceptible to AK-OFX
E. coli ATCC 25923	AMP-FOX-AUG-IPM and susceptible to C-AK-OFX-SXT-CS
P. aeruginosa ATCC 27853	IPM-FF and susceptible to CIP-AK-OFX
S. aureus ATCC 25922	P-FF-FOX-OX and susceptible to FC-VA-NA-SXT-RD
S. aureus ATCC 43300	P-FF-FC-VA-CD-NA-AK-FOX-OX and susceptible to SXT-RD
L. monocytogenes ATCC 19115	IPM-FF and susceptible to CIP-AK-OFX
Bacillus cereus ATCC 14579	P-AMP-AUG-IMP-AMX and susceptible to AK



(a) *Pseudomonas sp* (b) *B. cereus* ATCC 14579 (c) *S. aureus* ATCC 43300 (d) *A. baumanii* ATCC 19606

(e) *L. monocytogenes* ATCC 19115 (f) *Staphylococcus sp* (g) *Enterococcus sp* (h) *Enterobacter sp* (2)

Figure 4 Antibiotic susceptibility test of bacterial contaminants to antibiotics by the diffusion method on Mueller Hinton Agar

LAB

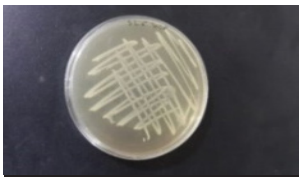
Isolation and phenotypic identification of LAB

Thirteen lactic acid strains were isolated from fermented and flavoured goat milk following screening for phenotypic characteristics (macro and microscopic), in addition to the catalase test, which was negative for all isolated lactic strains (**Table 2**). Colonies of lactic isolates were whitish in colour, and smooth in texture (**Fig. 5**).

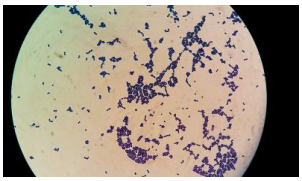
Physiological characterization of lactic strains

To provide health benefits, probiotics need to overcome the physical and chemical barriers that make testing their growth under different environmental conditions so important. The physiological and biochemical characteristics of the lactic acid strains are presented in **Table 3.** For safety reasons, the haemolysis test was performed where none of the tested lactic acid strains formed a halo and therefore have no haemolysis character.

(a) S8



(b) S5



(c) Microspopic appearance of lactic streptococci (Gram-positive cocci)

Figure 5 Macroscopic and microscopic appearance of LAB on M17 agar

Table 2 Microscopic and macroscopic appearance of isolated lactic strains

LAB	Mobility	Cell arrangement mode	Gram-type	Catalase	Form	Macroscopic appearance
S1	nonmotile bacteria	chains	positive	negative	cocci	circular, whitish, raised and smooth colonies
S2	nonmotile bacteria	chains	positive	negative	cocci	
S3	nonmotile bacteria	chains	positive	negative	cocci	
S4	nonmotile bacteria	chains	positive	negative	cocci	
S5	nonmotile bacteria	chains	positive	negative	cocci	
S6	nonmotile bacteria	chains	positive	negative	cocci	
S7	nonmotile bacteria	chains	positive	negative	cocci	
S8	nonmotile bacteria	chains	positive	negative	cocci	
St1	nonmotile bacteria	chains	positive	negative	cocci	
St2	nonmotile bacteria	chains	positive	negative	cocci	
S9	nonmotile bacteria	chains	positive	negative	cocci	
S10	nonmotile bacteria	chains	positive	negative	cocci	
S11	nonmotile bacteria	chains	positive	negative	cocci	

LAB: Lactic acid bacteria; **S:** Mesophilic strains of Streptococci; **St:** Thermophilic strains of Streptococci

Table 3 Physiological and biochemical characteristics of isolated lactic acid strains

Lactic acid bacteria	Catalase	Metabolic types	pH			T(°C)			NaCl (%)				Thermo resistance test	VP test	Esculin hydrolysis	ADH test	Haemolysis test	Citrate testing
			4	7	9	10	22	45	2	3	4	9						
S1	-	Homo-F	+	+	+	-	+	+	+	+	+	-	+	-	+	+	y-h	-
S2	-	Hetero-F	+	+	+	-	+	+	+	+	+	+	+	-	+	+	y-h	-
S3	-	Hetero-F	+	+	+	-	+	+	+	+	+	-	+	-	+	+	y-h	-
S4	-	Hetero-F	+	+	+	-	+	+	+	+	+	+	+	-	+	+	y-h	-
S5	-	Hetero-F	+	+	+	-	+	+	+	+	+	+	+	-	+	+	y-h	-
S6	-	Hetero-F	+	+	+	-	+	+	+	+	+	+	+	-	+	+	y-h	-
S7	-	Hetero-F	+	+	+	-	+	+	+	+	+	+	+	-	+	+	y-h	-
S8	-	Hetero-F	+	+	+	-	+	+	+	+	+	+	+	-	+	+	y-h	-
St1	-	Homo-F	+	+	+	-	+	+	+	+	+	+	+	-	+	+	y-h	-
St2	-	Hetero-F	-	+	+	-	+	+	+	+	+	+	+	-	-	+	y-h	-
S9	-	Hetero-F	-	+	+	+	+	+	+	+	+	-	+	-	+	+	y-h	-
S10	-	Homo-F	-	+	+	+	+	+	+	+	+	-	+	-	+	+	y-h	-
S11	-	Hetero-F	-	+	+	-	+	+	+	+	+	-	+	-	+	+	y-h	-

T(°C): Temperature in °C, **Hetero-F:** Hetero-fermentative, **Homo-F:** Homo-fermentative, **VP test:** Voges-Proskauer test, **y-h:** y-haemolysis, **ADH test:** Arginine dihydrolase test, (+): Positive reaction, (-): Negative reaction

Technological potential of isolated LAB

Fermentation of sugars

The fermentation results of the tested sugars are given in **Table 4** which were variable from one lactic acid strain to another.

Acidifying power

The acidifying power differs from one strain to another. **Fig. 6** and **Fig. 7** show the evolution of pH and titratable acidity during 24 hours of incubation, respectively.

Table 4 Metabolism of carbohydrates of lactic acid bacteria isolated from fermented and flavoured goat milk

Lactic acid bacteria	Fermentation of carbohydrates (sugars)																		
	Glucose	Inositol	Sorbitol	Rhamnose	Saccharose	Melilbiose	Amygdalin	Arabinose	Mannose	Maltose	Fructose	Sorbose	Lactose	Cellulose	Xylose	Raffinose	Galactose	Starch	Mannitol
S1	+	-	-	+	+	-	+	+	+	+	+	-	+	+	+	+	+	-	+
S2	+	-	-	+	+	-	+	+	+	+	+	-	+	+	+	+	+	-	+
S3	+	-	-	-	-	-	-	-	+	+	+	-	+	+	+	+	+	-	+
S4	+	-	+	-	+	-	+	+	+	+	+	-	+	+	+	+	+	-	+
S5	+	-	-	-	-	-	-	-	+	+	+	-	+	+	+	+	+	-	+
S6	+	-	-	-	+	-	+	+	+	+	+	-	+	+	+	+	+	-	+
S7	+	-	+	+	+	+	+	+	-	+	+	-	+	+	+	+	+	-	+
S8	+	-	+	-	+	-	+	+	+	+	+	-	+	+	+	+	+	-	+
St1	+	-	-	-	-	+	+	+	+	+	+	-	+	+	-	+	+	-	+
St2	+	-	-	+	+	+	+	+	+	+	+	-	+	-	-	+	+	-	+
S9	+	-	+	+	+	+	+	+	-	-	-	-	+	+	+	+	+	-	+
S10	+	-	+	+	+	+	+	+	+	-	-	-	+	+	+	+	+	-	+
S11	+	-	+	+	+	+	+	+	-	-	-	-	+	+	+	+	+	-	+

(+): Positive reaction; **(-):** Negative reaction (No fermentation)

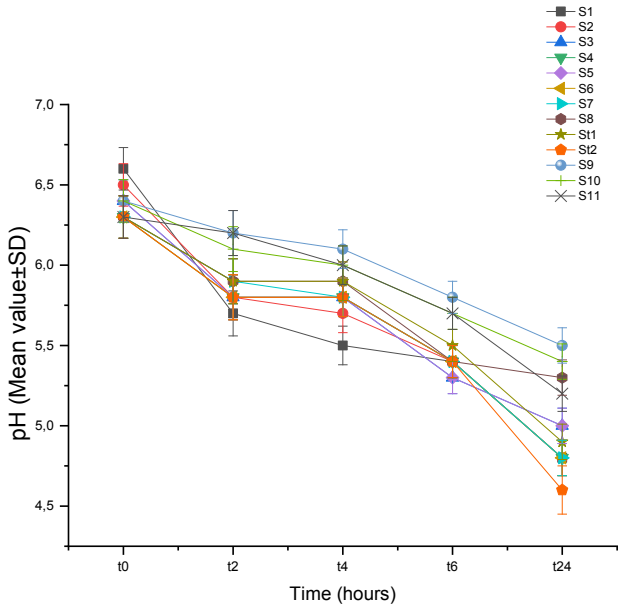


Figure 6 pH kinetics of the medium as a function of time

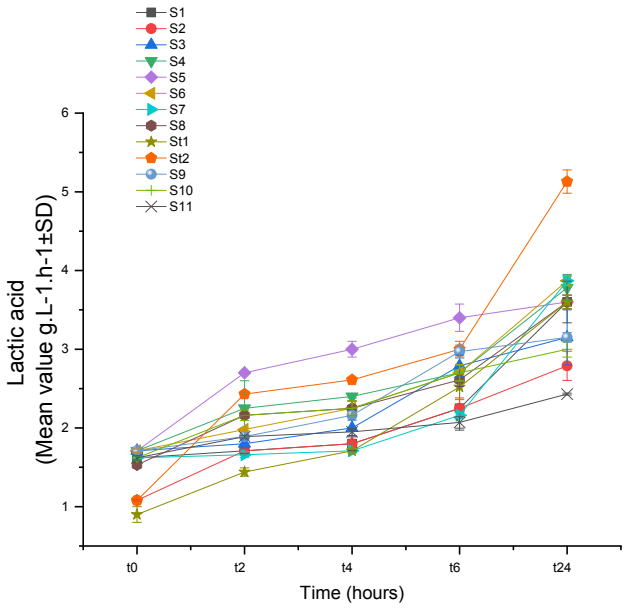


Figure 7 Titratable acidity kinetics of the medium as a function of time ($g.L^{-1}.h^{-1}$)

The obtained results showed a different acidifying power from one strain to another where the lactic acid strains S4, S5 and St2 have an interesting acidifying power from a technological point of view, where after six hours of incubation, these strains developed a titratable acidity of 2.6 g/L, 3.4 g/L and 3 g/L of lactic acid, respectively.

Amylolytic, proteolytic and lipolytic activity

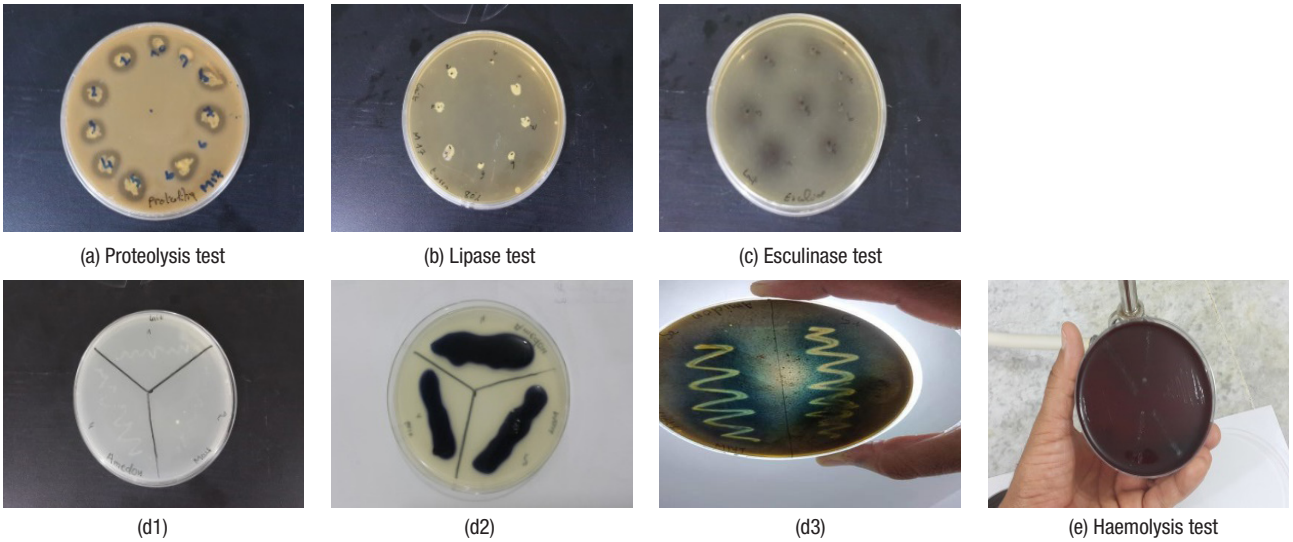
Table 5 presents the results of the amylolytic, proteolytic and lipolytic activity of the lactic acid strains isolated from fermented and flavoured goat milk.

Results on the physiological and technological characteristics results of the isolated lactic strains showed that all the tested streptococci strains were producers of protease and gelatinase. However, none of the strains showed either lipolytic or amylolytic activity, except for the S5 Streptococci strain that was able to hydrolyze polysorbate 20 and 80, and starch so it has a positive lipolytic and amylolytic reaction, respectively (**Fig. 8**).

Table 5 Technological suitability of lactic acid strains isolated from fermented and flavoured goat milk

LAB Technol. A	S1	S2	S3	S4	S5	S6	S7	S8	St1	St2	S9	S10	S11
Proteolytic activity (mm)	+ (14)	+ (13)	+ (10)	+ (13)	+ (14)	+ (13)	+ (15)	+ (11)	+ (5)	+ (10)	+ (5)	+ (5)	+ (5)
Gelatin hydrolysis	+	+	+	+	+	+	+	+	+	+	+	+	+
Lipolytic activity	-	-	-	-	+	-	-	-	-	-	-	-	-
Amylolytic activity	-	-	-	-	+	-	-	-	-	-	-	-	-

Technol. A: Technological ability, **LAB:** Lactic acid bacteria, **S:** Mesophilic Streptococci, **St:** Thermophilic Streptococci, (+): Positive reaction, (-): Negative reaction



(a) Proteolysis test (b) Lipase test (c) Esculinase test

(d1) (d2) (d3) (e) Haemolysis test

Amylase test (d1 before the addition of iodine-potassium iodide solution, (d2 and d3 after the addition of iodine-potassium iodide solution)

Figure 8 Technological ability of isolated lactic acid strains

Susceptibility test of isolated LAB

The susceptibility test results proved that the tested lactic strains were qualified as resistant to the action of penicillin and cotrimoxazole, and susceptible to the action of ampicillin, with variation from one strain to another for erythromycin, and tetracycline action (see **Table 6** and **Fig. 9**).

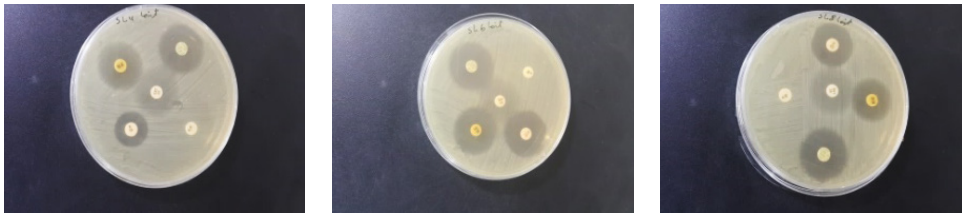
Antagonistic effect of lactic strains against microbial contaminants

Antibacterial effect

Results on the antagonistic effect of lactic strains against bacterial contaminants isolated from goat milk are shown in **Table 7** and **Fig. 10.**

Table 6 Multidrug resistant profiles of *Streptococcus spp* isolated from fermented goat milk

LAB	Antibiotics
S1	TE-P-SXT and susceptible to TE-AMX-C-CIP-AUG-OFX-RA-CN-VA-S
S2	TE-P-SXT-AUG-FOS; Intermediate to E and susceptible to AMX-C-CIP-VA-S- RA-OFX-CN
S3	P-SXT; Intermediate to TE and susceptible to E-AMX-
S4	P-SXT-AUG-SXT-FOS; Intermediate to E-TE and susceptible to AMX-C-CIP-VA-S-RA-OFX-CN
S5	P-SXT; Intermediate to E-TE and susceptible to AMX
S6	P-SXT; Intermediate to CN and susceptible to E-TE-AMX-VA-AUG-E-TE-C-CIP-RA
S7	P-SXT; Intermediate to E and susceptible to TE-AMX
S8	P-SXT-AUG-FOS; Intermediate to E-TE and Susceptible to AMX-C-CIP-VA-S-RA-OFX-CN
St1	E-P-SXT-AUG-OFX-FOS; Intermediate to RA and susceptible to TE- AMX-C-CIP-VA-S-CN
St2	P-SXT; Intermediate to E-TE-OFX and susceptible to AMX-C-CIP-RA-VA-FOS-AUG-CN
S9	SXT-P-OFX-FOS; Intermediate to E and susceptible AMX-VA-S- CIP-AUG-RA-GEN-C-TE-CN
S10	P-E-SXT and susceptible to AMX-VA-S-CIP-AUG-RA-CN-OFX-C-TE
S11	TE-P-E-SXT and susceptible to AMX-VA-S-CIP-AUG-RA-CN-OFX-C-FOS



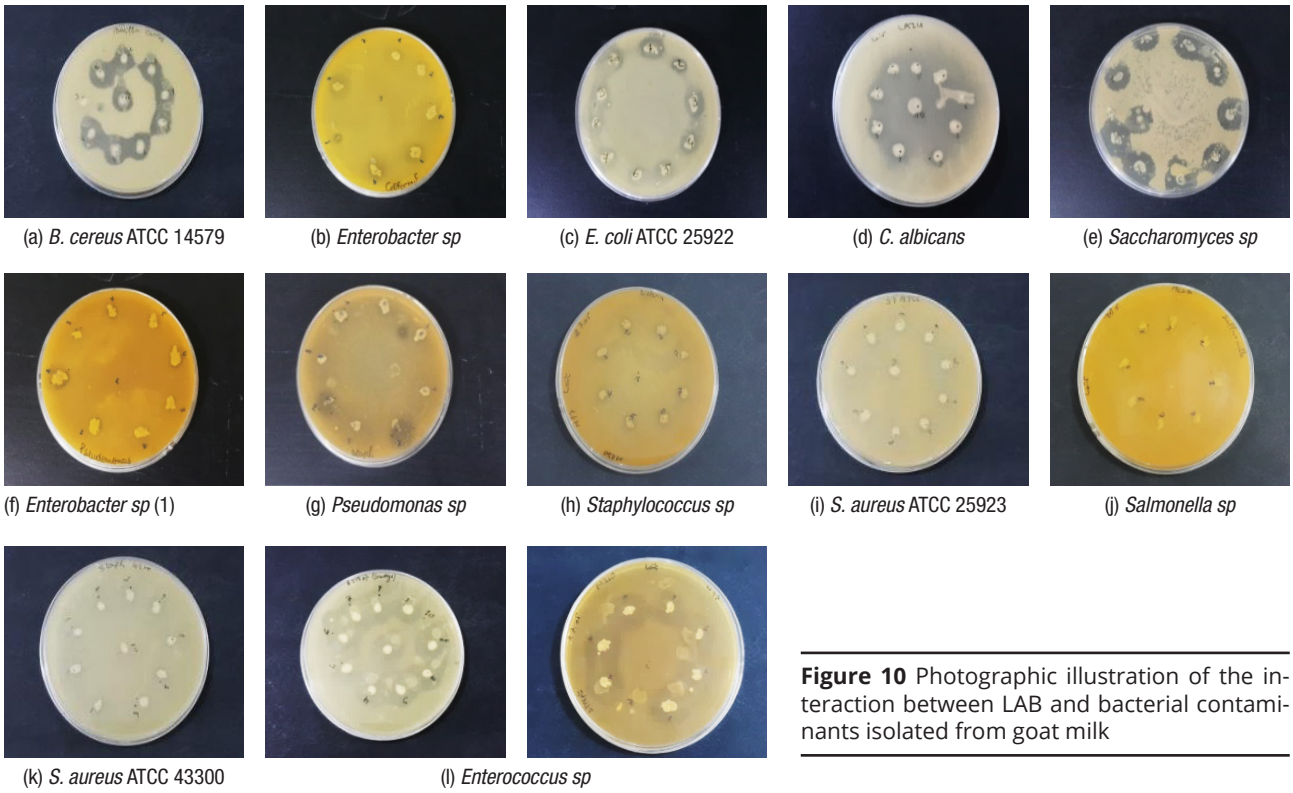
(a) S4 (b) S6 (c) S8

Figure 9 Susceptibility test of lactic acid strains using the disc diffusion method on MH agar

Table 7 Antagonistic effect of lactic acid bacteria strains against bacterial contaminants isolated from goat milk

	Lactic acid bacteria (zones of inhibition in mm)												
M. contaminants	**S1**	**S2**	**S3**	**S4**	**S5**	**S6**	**S7**	**S8**	**St1**	**St2**	**S9**	**S10**	**S11**
Enterobacter sp (1)	07	07	07	07	07	07	07	07	07	07	06	06	06
Enterobacter sp (2)	11	10	**11.5**	10	06	**14**	04	06	06	06	06	06	06
P. aeruginosa	08	**12.5**	6.5	08	08	**10.5**	06	08	06	06	06	06	06
Salmonella sp	07	07	07	07	07	07	07	06	7.75	10	06	06	06
Staphylococcus sp	07	07	07	07	08.25	07	07	07	07	07	06	06	06
Staphylococcus aureus	06	06	9.5	06	**14.5**	06	**11.5**	06	06	06	06	06	06
Enterococcus sp	**20**	**17.5**	**18**	**18.5**	**18.5**	**15.5**	**17.5**	**17.5**	**15**	**22.5**	06	06	06
Saccharomyces sp	**13.5**	**15.5**	**17.5**	**16.5**	**15**	**18**	**17.5**	**17.5**	06	**16.5**	06	06	06
Candida albicans	**22**	**16**	**10**	**14**	**12.5**	**10**	**12**	**16**	**20**	**15**	06	06	06
A. Baumanii ATCC 19606	07	06	06	06	06	06	06	07	06	06	06	06	06
E. coli ATCC 25922	**15.5**	09	**13.5**	9.5	**12**	**11**	**11**	**11**	**12.5**	**11.5**	06	06	06
P. aeruginosa ATCC 27853	06	06	06	06	06	06	06	06	06	06	06	06	06
S. aureus ATCC 25923	07	07	07	07	07	07	07	06	06	06	06	06	06
S. aureus ATCC 43300	8.5	09	09	06.5	**12.75**	07	07	9.5	08	07	06	06	06
L. monocytogenes ATCC 19606	11.5	07	08.5	06	**14**	**18.5**	**17.5**	**10.5**	06	06	06	06	06
Bacillus cereus ATCC 14579	**16.5**	**14.5**	**15.5**	**14**	**14**	**14.5**	**15.5**	**16**	06	**11.5**	06	06	06

M. contaminants: Microbial contaminants; **S (1 to 11):** Mesophilic LAB; **St (1 and 2):** Thermophilic LAB.



(a) *B. cereus* ATCC 14579 (b) *Enterobacter sp* (c) *E. coli* ATCC 25922 (d) *C. albicans* (e) *Saccharomyces sp*

(f) *Enterobacter sp* (1) (g) *Pseudomonas sp* (h) *Staphylococcus sp* (i) *S. aureus* ATCC 25923 (j) *Salmonella sp*

(k) *S. aureus* ATCC 43300 (l) *Enterococcus sp*

Figure 10 Photographic illustration of the interaction between LAB and bacterial contaminants isolated from goat milk

Qualitative antifungal effect

The antagonistic effect of lactic acid strains against mould contaminants isolated from goat milk was assessed by measuring fungal radial growth. Results are presented in **Fig. 11** and **Fig. 12.**

The qualitative antifungal effect results showed a significant inhibition rate against *Penicillium sp* species compared to *A. niger*, which reached up to a maximum inhibition rate of 55% under the effect of inhibitory agents secreted by lactic acid strains. No antifungal effects were revealed for the three species of streptococci (S9, S10, and S11) where the radial growth of moulds on the test plates was equivalent to or greater than those of the control.

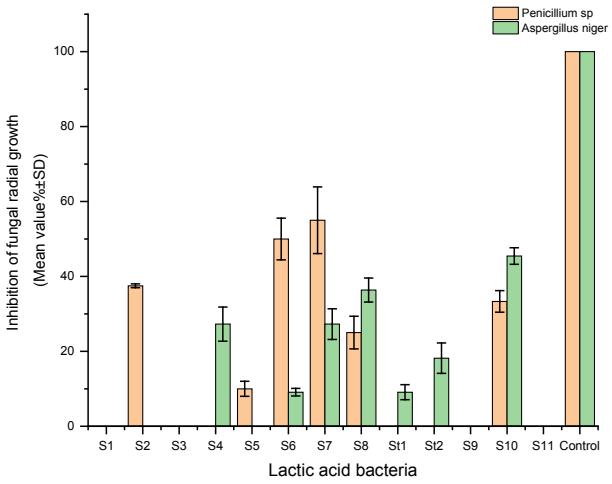
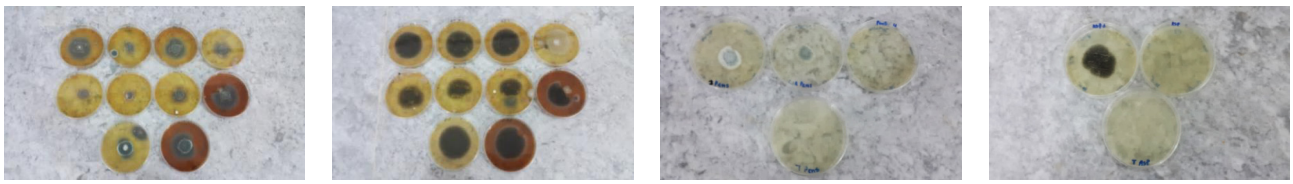


Figure 11 Qualitative antifungal effect of lactic acid bacteria against fungal contaminants isolated from goat milk.



(a) *Streptococcus spp* (LAB strains from S1 to S8, St1, and St2) vs. *Penicillium sp*

(b) *Streptococcus spp* (LAB strains from S1 to S8, St1, and St2) vs. *Aspergillus niger*

(c) *Streptococcus spp* (S9, S10, and S11) vs. *Penicillium* sp

(d) *Streptococcus spp* (S9, S10, and S11) vs. *Aspergillus niger*

Figure 12 Photographic illustration of the interaction between LAB and fungal contaminants isolated from goat milk (radial growth method)

Quantitative antifungal effect

Fungal biomass rate was used to measure quantitative antagonistic effect of lactic acid strains against fungal contaminants isolated from goat milk. Results are presented in **Fig. 13** and **Fig. 14**.

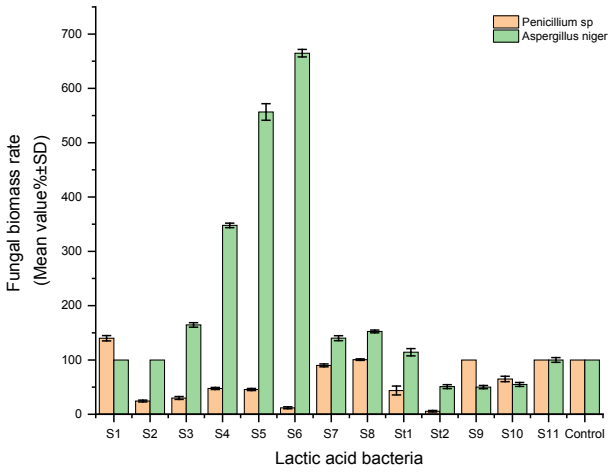
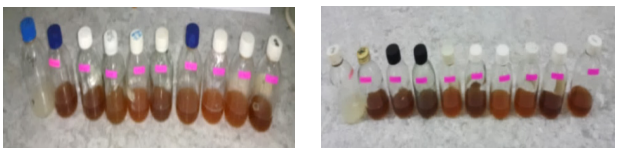


Figure 13 Quantitative antifungal effect of lactic acid bacteria against fungal contaminants isolated from goat milk

The results given in **Fig. 12** illustrate a significant antifungal effect of lactic acid strains of streptococci against *Penicillium sp*, compared to *A. niger.* The fungal biomass reduction rate varied from 5.5% to 90% for S2, S3, S4, S5, S6, S7, St1, and St2 strains against *Penicillium sp*. Both the S9 and S10 species showed antifungal activity (higher for the S10 strain) – expressed by the reduction of fungal biomass – from 50% to 65% against the two isolated fungal species. No action was revealed for the S11 strain against the two tested fungal species.



(a) *Streptococcus spp* (LAB) vs. *Aspergillus niger*

(b) *Streptococcus spp* (LAB) vs. *Penicillium sp*

Figure 14 Photographic illustration of the interaction between lactic acid bacteria and fungal contaminants isolated from goat milk (fungal biomass method)

Discussion

The inhibition of pathogenic microorganisms, namely *Escherichia coli*, *Staphylococcus aureus*, *Salmonella spp*, and *Listeria monocytogenes*, as well as food contamination flora, are considered a major concern, especially in the processing of food products including dairy products [47]. Within the framework of this study, we have tried to bring our contribution to the study of the antagonistic effect of LAB isolated from a fermented and flavoured goat milk against some microbial contaminants.

The antibiotic susceptibility test results of microbial contaminants showed that *Enterobacter sp* (1) and (2) and *E. coli* strains were resistant to several antibiotics, namely ampicillin, amoxicillin/clavulanic acid, cefoxitin, and imipenem. *Pseudomonas sp* was resistant to imipenem, fosfomycin and amikacin. *Staphylococcus spp* species were resistant to penicillin, oxacillin, and fosfomycin. *Enterococcus sp*, as fecal streptococci, was resistant to ampicillin and tetracycline. According to Mayrhofer *et al* (2007) [48], the widespread use of antimicrobial treatments has led to resistant populations of microorganisms in several habitats.

Through the analyses carried out in this study, we were able to isolate thirteen lactic acid isolates, which were all Gram-positive cocci, catalase-negative, and non-sporulated.

The study of antibiotic susceptibility tests is an important criterion for selecting probiotics [49]. Resistance remains a controversial condition – on the one hand, it is sought in probiotics to be active even after antibiotic treatment, and on the other hand, it is feared that it is transmitted to other undesirable bacteria, leading to the development of new antibiotic-resistant pathogenic microbes [50].

However, the EFSA (European Food Safety Authority) clearly states that any strain, even if it has a qualified presumption of safety status (QPS), must be free of acquired and transmis-

sible clinically important antibiotic resistance genes. This is to prevent antibiotic resistance genes (dangerous horizontal gene transfer) from being transferred from harmless LAB to dangerous pathogens.

Such a transfer of genes could, in the worst-case scenario, make infectious diseases impossible to treat due to the multi-resistance of pathogens [38]. Antibiotic susceptibility results are in agreement with various reports indicating that LAB are normally resistant to major types of antibiotics, such as G penicillin, amoxicillin β-lactam, cephalosporins, aminoglycoside, quinolone, imidazole, nitrofurantoin, and fluoroquinolone [51]. Otherwise, resistant lactic acid strains may act as a potential reservoir for antibiotic resistance genes, which may be a result of a higher antibiotic selection pressure environment. Regarding the possible use of these as probiotics [52], the high presence of antibiotic resistance genes in these species reduces the probiotic potential of the candidate strain, according to the evaluation guidelines of the European Food Safety Authority (FEEDAP Panel, 2005) [53]. However, the use of antibiotic-resistant bacteria could be beneficial, where the high sensitivity of probiotics to antibiotics prevents stable colonization of the gut, thus ensuring only non-significant and transient effects [54].

The proteolytic activity results of the LAB isolates shows that all strains have a significant ability to degrade milk caseins and to hydrolyze gelatin – the tested strains synthesized caseinases and gelatinase enzymes, respectively, and this is in agreement with the criteria given by Vuillemard *et al* (1986) [55], for a strain to be proteolytic, a clear zone around the colony must be greater than 5 mm. In comparison to these data, all isolated LAB of streptococci strains were revealed to be proteolytic – the diameters of the proteolysis zones ranged from 5 mm to 15 mm.

These results confirm that the proteolytic character of isolated LAB, as has been reported in several studies, i.e. that LAB are unable to synthesize several amino acids, but due to a complex bacterial proteolytic system, they are nevertheless well adapted to an environment that is rich in proteins and low in free amino acids such as milk.

Lipolytic power is of great importance in the development of flavour and for the release of fatty acids but it is not very responsive in lactic acid bacteria compared to other groups of bacteria [56, 57], and this was shown to be the case for the isolated LAB strains, even though most of them share the same property of not having an appreciable lipolytic power, except for the S5 streptococci strain. In general, LAB are considered weakly lipolytic compared to other bacterial species such as *Pseudomonas spp*, *Acetobacter spp*, or *Flavobacterium spp* [36, 57, 58]. However, Karam *et al* (2012) [59] suggested that the presence of LAB at high loads in cheeses and under favourable conditions can lead to the production of a significant amount of free fatty acids, probably due to an adaptation to these conditions. Strains with lipolytic activity can synthesize extracellular lipases which transform lipids into fatty acids, revealed by the presence of an opaque zone around the bacterial colony.

The ability of LAB strains to ferment carbohydrates was also tested during fermentation using different sugars. All tested LAB strains were able to ferment hexose sugars like glucose, lactose, and sucrose. The isolates were unable to ferment inositol, sorbose, and starch. Most tested LAB strains were able to ferment arabinose, amygdalin, mannose, maltose, fructose, and xylose.

The antibacterial activity results showed that the isolated cocci LAB have an inhibitory activity ranging from 9 mm to 15.5 mm against *E. coli* ATCC 25922, and an activity characterized by a zone of inhibition of 7 mm – from 15.5 mm to 27.5 mm against *S. aureus* ATCC 25923 and *B. cereus* ATCC 14579, respectively.

These results corroborate the data re-

ported by Belarbi (2011) [47] who found that LAB cocci, mainly *Lactococcus*, *Leuconostoc*, and *Streptococcus* isolated from raw goat and cow milk have antagonistic activity against *E. coli* (0 mm to 24 mm) and *S. aureus* (0 mm to 16 mm). The obtained results in this study were more important than those obtained by Baafou and Bourdache (2017) [60] who studied the antagonistic effect of LAB isolated from camel milk against some pathogenic bacteria.

The antimicrobial effect is due to the difference in the composition of the cell envelope between Gram-positive and Gram-negative bacteria. However, some studies have suggested that a change in the permeability properties of the outer membrane following certain stress conditions can make Gram-negative bacteria susceptible to bacteriocins as inhibitory agents [61].

The majority of isolated streptococci strains show the potential to inhibit the growth of isolated microbial contaminants. Within these LAB, several have a high antibacterial potential, showing the broadest spectrum of antibacterial activity (20 mm) against group D fecal streptococci (*Enterococcus sp*) and an activity characterized by a zone of inhibition ranging from 4 mm to 14 mm against *Enterobacter sp* (2).

Several studies have shown the inhibitory activity of LAB against pathogenic bacteria following the release of substances of a protein nature: bacteriocins [42, 62].

According to Dortu and Thonart (2009) [63], LAB represent interesting properties in the preservation of foodstuffs by their ability to regulate the microflora in fermented products and to inhibit the growth of pathogenic microorganisms.

In addition, Mameche-Doumandji (2008) [8] reported that lactic cocci have a high inhibitory activity on Gram-negative bacteria (25 mm) compared to that obtained against Gram-positive (23 mm).

According to several authors namely, Leonard (2013) [9]; Dortu and Thonart (2009) [63]; Suvorov *et al* (2019) [64], the bactericidal effect of lactic strains can be attributed to various factors such as nutritional competition and space as well as the production of a set of metabolites with antimicrobial properties. These metabolites are organic acids (mainly lactic acid which modifies the pH of the medium and can have an influence on acid-sensitive pathogenic bacteria): hydrogen peroxide which inhibits bacteria that do not have defenses against oxidative stress; carbon dioxide; diacetyl reuterin; and bacteriocins. Bacteriocins as enzymes deplete other bacteria of essential metabolites, and according to Marroki *et al* (2011) [65]; Woraprayote *et al* (2016) [24], the use of bacteriocins can effectively contribute to food safety, due to their potential use as natural food preservatives.

According to Suskovic *et al* (2010) [66], lactic acid causes deterioration by acidification, thus its inhibitory effect is mainly due to its form, which diffuses through the cell membrane to the cytosol making it more alkaline and interferes with essential metabolic functions. The toxic effect of lactic acid includes reduction of intracellular pH and dissipation of membrane potential.

Isolated lactic acid strains have also an antifungal effect against yeasts and moulds – relatively lower than what was revealed against bacteria – probably due to the ability of moulds to resist unfavourable environments by the enzymatic equipment they possess.

Conclusion

LAB serve many primary functions in food – for fermentation, to improve the nutritional and organoleptic quality of food and also to contribute to food preservation (shelf life) through their ability to produce antibacterial compounds. This study focused on the production of antibacterial compounds of LAB and the antagonistic effect of lactic acid strains isolat-

ed from fermented and flavoured goat's milk against some microbial contaminants.

The study of the technological properties of the isolated lactic strains made it possible to draw the following conclusions:

The majority of the isolated strains present interesting technological characteristics (acidifying and proteolytic activities, γ-haemolysis, and low resistance to antibiotics).

The lactic acid isolates with a strong acidifying activity showed an interesting antimicrobial effect against foodstuffs' microbial contaminants by synthetizing inhibiting substances or by modifying the physicochemical characteristics of the medium, hence the interest of these probiotic strains and their possible metabolites to be used as a bio-preservative.

Traditional food practices and know-how may hide many secrets and beneficial advantages. This cultural reservoir of ancestoral knowledge is a promising field for investigation and should be valued and transmitted from one generation to the next.

Further research on lactic acid strains that are naturally selected by microbial interactions, and on the effect of Phoenician juniper is needed. This combination could potentially be a source of newly selected LAB used as a starter culture in the production of dairy products and other biotechnology applications.

Conflict of interests

The author declares that he has no conflict of interest.

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