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Characterization and identification of lactic acid bacteria isolated from traditional cheese (*Klila*) prepared from cow's milk

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Various types of fermented dairy products exist worldwide. Their nature depends on the type of milk used, pretreatment, fermentation conditions and subsequent treatment. The fermentation of milk primarily involves lactic acid bacteria (LAB). Among these, the *Klila* is a hard variety cheese made by using the traditional procedures in the home, without using a starter culture. The different samples of traditional cheese (*Klila*) studied were collected from the rural area of the province of Djelfa. Isolates were phenotypically characterized by their capability to ferment different carbohydrates and additional biochemical tests. 132 lactic acid bacterial strains were isolated, purified and identified to all belong to the genus, *Lactobacillus,* their proportion were *Lactobacillus plantarum* (18.94%), *Lactobacillus casei* (18.18%), *Lactobacillus fermentum* (21.97%), *Lactobacillus acidophilus* (12.88%), *Lactobacillus brevis* (14.39%), *Lactobacillus alimentarus* (03.03%), *Lactobacillus intestinalis* (06.06%) and *Lactobacillus helveticus* (04.56%). These lactic acid bacteria were isolated against *Staphylococcus aureus*. Isolates *L. fermentum, L. intestinalis* and *L. acidophilus* were selected for their strong bactericidal activity against *S. aureus*.

Key words: *Klila*, lactic acid bacteria, identification, characteristics, *Lactobacillus, Staphylococcus aureus*.

INTRODUCTION

Milk is the lacteal secretion obtained by the complete milking of mammals. Due to its high nutritional value for human beings, it is a significant food of nutrition for immense population on earth. When temperature is suitable for growth of microorganisms, the milk appears as an excellent medium for their growth. The milk is contaminated very easily if it is handled carelessly and produced unhygienic-cally, resulting in its early spoilage (Bahanullah et al., 2013). Fermented milk is a dairy product providing the human diet with nutritious compounds of varied flavors, aromas and textures. These products are based on the metabolic activity of lactic acid bacteria which ferment sugars, especially glucose and galactose, to produce lactic acid and aroma substances that give typical flavors and tastes to fermented products. Several types of fermented milk products have been reported to exist through-out the world. The most popular ones in North African are *Jben, Lben, Klila* and *Raib* (Mechai and Kiran, 2008). The name

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"cheese" is reserved to fermented product or not obtained by coagulating milk, cream, skim milk, or a mixture of them, followed by draining. The cheese is made either by the traditional method in the rural environment and traditionally or by the semi-industrial or industrial methods which remains limited (Rhiat et al., 2013).

In Algeria, many traditional dairy products are not identified and studied; several types of cheeses are classified and identified in different parts of our country. Among these different types, we mentioned the following names *Mechouna* cheeses, *Bouhezza*, *Madeghissa*, *Klila, Djben Takammerite*, *Aoules*, *Igounanes* and *Takammerite*. In a variety of ecological niches, micro-organisms compete with each other for survival and through evolution from unique flora. In some food ecosystems, lactic acid bacteria constitute the dominant microbiota of these bacteria widely distributed in the nature and occurring naturally as indigenous micro-biota in raw milk as Gram-positive bacteria that play an important role in many foods and feed fermentations. These organisms are able to produce antimicrobial com-pounds against competing microbiota, including food-borne spoilage and pathogenic bacteria. In this group are included representatives of the genus *Lactobacillus, Enterococcus, Bifidobacterium, Aerococcus, Leuconostoc, Pediococcus, Streptococcus, Carnobacterium, Oenococcus, Weissella, Tetragenococcus, Vagococcus* and *Lactococcus* (Dortu and Thonart, 2009). The lactic acid fermentation, which these bacteria carry out, has long been known and applied by humans for making different food-stuffs. For many centuries, lactic acid bacteria have served to provide an effective form of natural preservation. In addition, they strongly determine the flavor, texture and, frequently, the nutritional value of food and feed products. They could be isolated from soil, water, plant, silage, waste product, and also from the intestinal tract of animals and humans. Since decades, by these processes, the application of well-studied starter cultures was established. They should possess stable fermentation characteristics and should be resistant to bacteriophages (Lee, 1996). The “wild” isolate, in biotechnological aspect are perceived as bacteriocin producers and probiotics (Tserovska et al., 2002). This study was aimed at the isolation and taxonomic determination of large number of lactic acid bacteria from traditional fermented milk (*Klila)* prepared from cow's milk and characterization of different groups of microbiota, acidifying power and antimicrobial producer bacteria using classical methods.

MATERIALS AND METHODS

Samples collection

The various samples of dry *Klila* were processed by traditional procedure in the home. The different samples of traditional cheese (*Klila*) studied were collected from the rural area of the province of Djelfa. They were transported to the laboratory under refrigeration (4°C) and analyzed immediately; the pH measurement of the samples (sample preparation was carried out by dissolving 5 g of *Klila* in 25 ml of distilled water with a neutral pH) was performed by a pH meter with an Orion Research type combination electrode and previously calibrated with buffer solutions at pH 4 and 7. 10 ml of sample was transferred to a small beaker and 5 drops of phenol-phthalein was added to 1% indicator. The sample was titrated with 0.1 N NaOH. Note that the sample should be just barely pink (Rhiat et al., 2013).

Microbiological analysis

Microbiological analysis was performed for controlled traditional *Klila* to search: total aerobic mesophilic flora (FAMT) enumerated on PCA agar (plate count agar), incubated for 24 h at 30°C. The search for total coliforms wassought on désoxycolate citrate agar (DCL) incubated for 24 h at 37°C for total coliforms and at 44°C for fecal coliforms, fecal streptococci were counted on sodium azide after incubation for 48 h at 37°C. Staphylococci were counted on Chapman medium containing a high concentration of NaCl (75%) tolerated only by staphylococci, incubation at 37°C for 24 to 48 h (Labioui et al., 2009; Bouzaid et al., 2012), for Salmonella, a pre-enrichment on selenite-cysteine medium was provided for 12 h at 37°C, followed by an enrichment on bouillon of tetrathionate for 24 h at 37°C, then the enumeration and isolation were carried out on SS medium (*Salmonella-Shigella*) after 24 h of incubation at 37°C and the sulphitoreductor-clostridia were counted in the culture medium reinforced *Clostridium* agar in tubes to promote anaerobic conditions, with thermic treatment for 10 min at 80°C to activate the spores of clostridia: they can persist in a latent form in milk, germinate as soon as conditions are favorable and secrete toxic substances. The tubes are incubated for 48 h at 37°C. Only black colonies are counted. The microbiological analysis is performed in three steps: preparation of dilutions, seeding in the culture medium and enumeration of microorganisms. A count of lactic acid bacteria was responsible for the fermentation and acidification of milk, they were counted on the MRS agar (De Man et al., 1960) and incubated for 48 h at 30°C and the counts of yeasts and moulds were determined using potato dextrose agar (PDA), acidified with 10% tartaric acid to pH 3.5 by incubating at 30°C for 3-5 days.

Study of lactic microfloras

Isolation and purification of lactic acid bacteria

Each 25 g sample was aseptically weighed and homogenized by adding 225 ml of physiological saline solution for first dilution (10^{-1}). Next dilutions (10^{-2} to 10^{-7}) were made in 0.85% sterile saline. For lactic acid bacteria (LAB) isolation, 1 ml of the appropriate dilutions was plated on MRS and GM17 agar medium, respectively. The plates were incubated at 30 and 45°C for 72 h under aerobic and anaerobic conditions. Further decimal dilutions were prepared from this homogenized mixture (Kivanç et al., 2011; Terzic et al., 2014). The 0.1 ml from each dilution was then sub cultured, in duplicate, into the M17 and MRS agars used for isolating lactic acid bacteria. To prevent the growing of yeasts, the media were then supplemented with 100 mg/l of cycloheximide before being incubated at the appropriate temperatures for 2-3 days. The MRS agar plates were incubated anaerobically at 42, 35 and 30°C for three days, in order to provide an optimal temperature for growing thermophilic lactobacilli, mesophilic lactobacilli and Leuconostoc, respectively. M17 agar plates were also incubated aerobically at 30°C for 2 days, in order to set up an optimal temperature for growing lactococci. To perform the total counts, the higher dilutions were used (Azhari, 2011). Colonies were randomly selected and streak plating was then used to purify the isolates which were subsequently kept in two different conditions including at 4°C for MRS and M17 plates

and at -20°C for M17 and MRS broths supplemented by 20% glycerol for further use (Mathara et al., 2004). All isolates were examined for Gram reaction, production of catalase and oxidase activity. Gram-positive and catalase- and oxidase-negative isolates were stored for further analyses. Purification of the isolates was done by repeated pour plating technique using the same agar medium until pure cultures were obtained. Pure cultures were transferred and maintained on de Man Rogosa and Sharpe (MRS) agar stabs. Duplicate tubes of the isolates were prepared, one tube was stored in refrigerator as stock culture, and the other tube was used for identification studies (Neti and Erlinda, 2011).

Identification of lactic acid bacteria isolates

Isolates were identified using the following tests: ammonia production from arginine, CO_2 production from glucose, and growth at different temperatures (10, 15, 30, 37 and 45°C), growth at different pH values, and growth at different NaCl concentrations (Schillinger and Lucke, 1989). Each strain under examination was subcultured twice overnight in MRS broth. All strains were initially tested for Gram reaction, catalase production and spore formation (Harrigan and McCance, 1976). Cell morphology and colony characteristics on MRS agar were also examined, and a separation into phenotypic groups was undertaken. Only the Gram-positive, catalase-negative isolates were further identified. Growth at different temperatures was observed in MRS broth after incubation for 5 days at 15, 37 and 45°C. Hydrolysis of arginine was tested in M16BPC (Thomas, 1973). Growth in the presence of 4 and 6.5% NaCl performed in MRS broth for 5 days. Utilization of citrate was realized in Kempler and Mc Kay (1980) medium. Production of acetone from glucose was determined using Voges-Proskauer test (Samelis et al., 1994). To perform the biochemical tests, an MRS-BCP broth medium (BCP 0.17 g/l) was used. The carbon source was added to the sterile basal medium as filter sterilized solution to a final concentration of 1%. Carbohydrates utilization was assessed at the 24th and 48th h. All strains were tested for fermentation of the following 15 sugars: L-Arabinose, ribose, D-xylose, mannitol, sorbitol, cellobiose, maltose, lactose, melibiose, trehalose, mannose, rhamnose, esculine, sucrose and D-raffinose. To ensure anaerobic conditions, two drops of sterile liquid paraffine were placed in each tube after inoculation.

Kinetic of pH and acidity lactic acid production

The strains were initially grown on MRS broth and then in sterile reconstituted skim milk supplemented with yeast extract (0.3%) and glucose (0.2%) for two successive subcultures. Sterile reconstituted skim milk (100 ml) was inoculated with 1% of an 18 h preculture (Durlu et al., 2001) After gentle agitation, culture was divided into tube (10 ml/tube) and incubated at 30°C. At a regular interval time, samples were aseptically collected every 2 h. A volume of 1 ml culture samples was used for making suitable serial dilutions up to 10^{-8} by incorporating 1 ml into 9 ml of sterile saline water in sterile tubes. Enumeration of LAB was determined using selective media, MRS agar. The plates were incubated at 30°C for 48 h. After incubation, colonies were enumerated, recorded as colony forming units (cfu/ml). Only plates containing between 30 and 300 colonies were retained (Khedid et al., 2009). The generation time and growth rate were calculated in the exponential growth phase. The kinetics of the changes in pH and acidity were also followed by measuring pH and Dornic acidity. To measure Dornic acidity, we added 5 drops of alcoholic solution of 1% phenolphthalein as an indicator of the color change point in 10 ml of culture samples. We titrated sodium hydroxide N/9 (NaOH), until the sample changed color from white to light pink. The volume of NaOH sunk was recorded. The acidity was expressed in degrees Dornic (°D) (1°D = 0.1 g lactic acid/liter and acidity = volume of NaOH x 10) (Va´zquez et al., 2013). Titrable acidity of lactic acid was calculated according to FAO (1986).

Screening for antagonistic activity

The many methods described for the detection of isolates bacteriocin-producing lactic acid is based on the premise that these protein substances can diffuse into a solid culture medium or semi solid which was previously inoculated with a target strain (*Staphylococcus aureus* ATCC 65 38). The bacteriocin production inhibitor is detected by the power of the filtrate microorganism tested growth target seed. Isolates of lactic acid bacteria after culture on medium MRS at pH 6.8, incubation at 30°C were tested for their antibacterial activity following diffusion method agar TSA (Tryptic Soy Agar, Difco, Detroit, USA) (Barefoot and Klaenhammer, 1983). The supernatant containing the crude extract is collected by centrifugation bactériocinique adjusted to neutral pH of 6.5 to 7 with 10 M NaOH neutralizing the extract bactériocinique which eliminates the effect of organic acids. The extract was then filtered on millipore filters sterile 0.22 µ in diameter, the antimicrobial activity was determined for each selected isolate of *Lactobacillus*. Petri dishes were overlaid with 15 ml of molten agar (1%), inoculated with 30 µl of an overnight culture of the indicator microorganism, in which wells were formed. Wells, mm in diameter and of 30 µl in capacity, were formed by carving the agar with a cork borer. Afterwards, 30 µl of an overnight culture of the putative inhibitor strain were placed in each well. The plates were then incubated aerobically for 24 h at a temperature conducive for growth of the indicator microorganism and were subsequently examined for zones of inhibition. Inhibition was recorded as negative if no zone was observed around the agar well. Each antagonistic activity was related to the area (2 mm) of the inhibition zone displayed (Mathur and Singh, 2005).

RESULTS AND DISCUSSION

Physicochemical analysis

The pH range for traditional cheeses (*Klila*) was 3.8 to 4.8 with an average of 4.2. Titratable acidity of *Klila* samples varied from values as low as 68°D to values as high as 91°D. Mean titratable acidity value was 79.4°D; these values are almost similar to that reported by Rhiat et al. (Table 1).

Microbiological analysis

The coliforms and pathogenic microorganisms, *S. aureus* and *Salmonella* were not detected; Lactic acid bacteria are by far the major microbial group in traditional cheeses (*Klila*) products. The microbiological analysis showed an average of FAMT of about $2.1x10^3$, $1.5\ x10^3$, $2.6\ x10^3$, $2.1\ x\ 10^3$ and $2.8x10^3$ cfu/ml, respectively, in samples S2, S3, S4 and S5. However, the staphylococci were not detected. These values are almost similar to that found by Mennane et al. (2008). The total and fecal coliforms were found in sample S3 and S5, the levels of coliforms (total, fecal) found in two samples are lower than those reported by Hamama and EL Mouktafi (1990). We also noticed the absence of pathogenic flora especially for controlled products. But the burden of yeasts, $1.2x10^2$ to $1.2x10^2$ cfu/ml is a normal standard (Table 2).

LAB were enumerated in traditional cheeses (*Klila*) using usual media by the classic method. The presumptive lactic acid bacteria levels varied from $0.3x10^4$ to $4.2x10^4$ cfu/ml with an average of $2.2x10^3$.

Table 1. Results of physicochemical characteristics of traditional cheese (*Klila*).

Physicochemical characteristics	Sample				
	S1	S2	S3	S4	S5
pH	3.8	3.9	4.2	4.3	4.8
°D (Dornic acidity)	68	71	79	88	91

Lactic acid bacteria counts found in traditional cheeses (*Klila*) were low as compared to LAB levels already reported in other types of traditional dairy product such as Jben and cow's milk (Khedid et al., 2009; Labioui et al., 2009) (Table 2).

Study of lactic microbiota

A total of 132 isolates from traditional cheese (*Klila*) isolates were Gram-positive, catalase-negative, non spore-forming and short rod or cocobasilli shaped. These isolates were selected for identification and antagonism analysis, the results of the isolation and identification of the standard physiological and biochemical tests (Table 3) identified the isolates as 18.94% isolates of *Lactobacillus plantarum*, 18.18% isolates of *Lactobacillus casei,* 21.97% isolates of *Lactobacillus fermentum,* 12.88% isolates of *Lactobacillus acidophilus,* 14.39% isolates of *Lactobacillus brevis,* 03.03% isolates of *Lactobacillus alimentarus,* 06.06% isolates of *Lactobacillus intestinalis* and 04.56% isolates of *Lactobacillus helveticus.* We have divided the Lactobacilli group into three subgroups according to Orla-jensen (1919) and Moreik (2011) as follows: *L. plantarum, L. alimentarus* and *L. casei* subsp. *casei* are mesophilic facultative hetero-fermentative, *Lactobacillus helveticus, L. acidophilus, L. intestinalis* and *L. fermentum* which are thermophilic obligate homo-fermentative and *L. brevis* which are mesophilic obligate hetero-fermentative (Azadnia and Khan, 2009).

The morphological, biochemical and physiological characterization of the isolates revealed that all the isolates that produced highest lactic acid among each group are *L. acidophilus, L. fermentum* and *L. plantarium.* All isolates fermented the same carbohydrates; Olarte et al. (2000) noted that the presence of *L. plantarum* in the cheese (Cameros) from goat's milk decreased the number of the enterbacteria and fecal coliforms in the final product (Table 3).

Kinetics of acidification and growth evolution

The variation of acidification was monitored for all isolates, as shown in Figure 1. The diminution of pH of the milk is due to the production of lactic acids from lactose fermentation (Thomson et al., 1994). The amount of lactic acid varies according the isolates and their capacity and the rate of degradation of the lactose. Thus, according to their ability of acidification, the strains were divided as follows: highly acidifying isolates (include GM91 and GM14) that coagulate milk before 18 h of incubation, low acidifying isolates (strains GM33 and GM88) that coagulate milk after 18 h of incubation and the remaining isolates coagulate milk after 18 to 24 h of incubation.

The initial pH of skim milk was 6.2 to 6.4 for all the tested isolates. Then, the pH decreases with time to reach 3-3.4 in highly proteolyses isolates. Regarding the acidity, we noteed that after 2 h of incubation, the amount of lactic acid was measured (15-22°D) for all our isolates. The acidity increases with the time in a variable way to arrive until 74°D after 24 h with isolate GM14 and up 31°D with the isolate GM11. The acidity produced can reach 63 and 74°D for thermophilic and mesophilic isolates, respectively.

Antibacterial activity

The antimicrobial activity of *Klila* isolated from lactic acid bacteria were detected using the method of well diffusion test on the basis of their ability to inhibit the growth of the indicator isolate *S. aureus* ATCC 65 38. Based on the results, a total of 5 different traditional *Klila* samples analyzed. 09 bacteria inhibitor of production, which are alleged to constitute lactic acid bacteria were isolated (Table 4).

Five of these isolates producing inhibitor were selected for further study on the basis of their relatively broad antimicrobial spectrum (Figure 2). The sensitivity of the antibacterial substances produced by lactic acid bacteria in α-chymotrypsin, pepsin, catalase, and the lipase was determined in reproducible and controlled conditions indicated in Table 5.

Inhibitor compounds produced by strains inhibitors showed different patterns of sensitivity. All were completely inactivated by α-chymotrypsin alone which was resistant to pepsin (isolate GM11), whereas the compounds produced by GM91 and GM14 isolates were inactivated after treatment with the lipase, indicating that these substances can have inhibitory lipid moiety in their chemical composition. The inhibitory compounds produced by the three isolates showed great resilience to thermal treatments.

In another way, bacteriocin has proved stable over a wide pH range with all peptides, now some antimicrobial activity in the pH range from pH 4-7. According to Allouche et al. (2010), recent bacteriocin is very sensitive to pH. Its stability was detected at a pH range of 3.5 to 6.5. In this study, bacteriocin produced by isolates GM91 and GM14 had the same profile and were active at pH values 4-6 (Table 5). In a similar study, the work of Zamfir et al. (1999) reported that the bacteriocin produced by *L. acidophilus* develop a positive activity against *S. aureus*.

Table 2. Results of microbiological analysis (cfu/ml) of traditional cheeses (*Klila*).

Microbiological analysis	Sample					
	S1	S2	S3	S4	S5	M
Yeast 10^2	1.2	1.5	2.0	2.2	1.3	1.64
Total aerobic mesophilic 10^3	2.1	1.5	2.6	2.1	2.8	2.22
Total coliforms 10^3	0.0	0.0	2.0	0.0	2.5	2.25
Fecal coliforms 10^3	0.0	0.0	1.2	0.0	2.5	1.85
Staphylococci 10^3	0.0	0.0	0.0	0.0	0.0	0.00
Lactic microflora 10^4	2.1	3.5	0.8	4.2	0.3	2.18

Table 3. Morphological, cultural, physiological characteristics of isolated isolates.

Species	GM14	GM91	GM62	GM12	GM33	GM88	GM67	GM11
Gas from glucose	+	+	+	-	-	-	+	+
Motility	-	-	-	-	-	-	-	-
Hydrolysis of								
ADH	-	+	+	-	-	-	-	-
Citrate	+	+	-	+	-	-	+	+
Growth at different temperature (°C)								
15	-	-	+	+	+	-	+	-
30	+	+	+	+	+	+	+	+
45	+	+	-	-	-	+	+	+
Growth at different pH								
6.5	+	-	-	+	+	-	+	-
9.6	-	-	-	-	-	-	-	-
Growth in the presence of NaCl								
4%	+	-	-	+	+	+	+	+
6.5	+	+	+	+	+	+	+	+
9.6	-	-	-	-	+	-	-	-
Sugar fermentation								
Arabinose	+	+	+	-	-	-	-	-
Cellobiose	+	+	-	+	+	-	+	-
Mannitol	-	-	-	+	-	-	+	+
Mannose	+	+	-	+	+	+	+	-
Melebiose	+	+	+	+	-	-	-	+
Raffinose	+	+	+	+	-	-	-	+
Ribose	-	+	+	+	+	-	+	-
Lactose	+	+	+	+	+	+	-	+
Rhamnose	-	-	-	+	-	-	-	-
Sorbitol	-	+	-	+	+	+	-	+
Xylose	-	-	+	-	-	-	-	-
Tehalose	-	-	-	+	+	+	+	-
Maltose	+	+	+	+	+	-	+	+
Esculine	-	-	+	+	+	+	+	-
Sucrose	+	+	-	+	+	-	-	+

GM12: *Lactobacillus plantarium*, GM33: *Lactobacillus alimentarus*, GM67: *Lactobacillus casei* subsp. *casei,* GM88: *Lactobacillus helveticus*, GM14: *Lactobacillus acidophilus*, GM11: *Lactobacillus intestinalis*, GM91: *Lactobacillus fermentum*, GM62: *Lactobacillus brevis.*

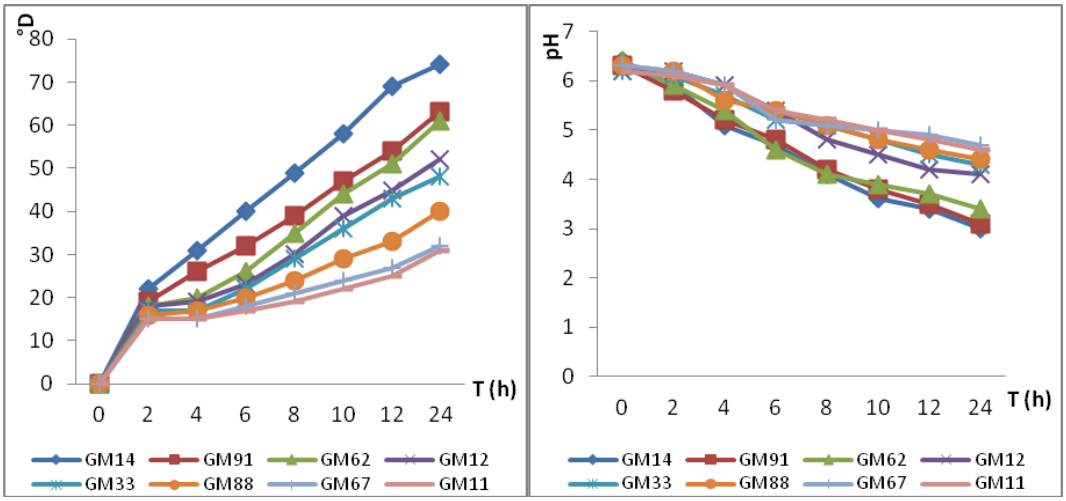

Figure 1. Kinetics of degree dornic acidity of the different isolates in milk medium.

Table 4. Antagonism of *Staphylococcus aureus* ATCC 65 38 by *Lactobacillus* isolates using agar diffusion method.

Statistical analysis	Isolates test								
	GM14	GM91	GM62	GM11	GM12	GM67	GM60	GM03	GM46
Mean	9.950	9.150	9.275	6.800	6.050	3.825	2.575	2.850	2.425
SD	0.9883	0.3109	0.2630	0.4830	0.2380	0.4500	0.4113	0.5066	0.4573
SE	0.9883	0.3109	0.2630	0.4830	0.2380	0.4500	0.4113	0.5066	0.4573

SD: Standard deviation; SE: standard error.

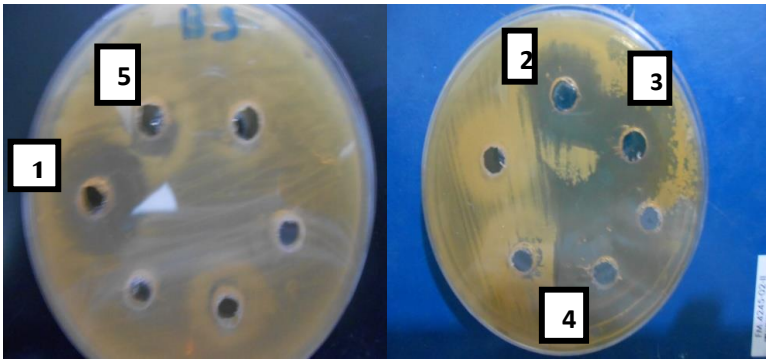

Figure 2. Inhibition of *S. aureus* ATCC 65 38 by the cell-free supernatants of the five producing isolates using the agar well-diffusion assay: 1: GM11, 2: GM62, 3: GM91, 4: GM14 and 5: GM12.

Table 5. Action of proteolytic enzymes, pH and heat treatment on the antimicrobial activity of crude extracts against the growth of *Staphylococcus aureus* ATCC 65 38.

Crude extracts	Enzymes			pH			Heat treatment °C/20 min	
	α-Chymotrypsin	Lipase	Pepsin	3	5	7	100	120
GM14	-	-	-	+	+	+	+	+
GM91	-	-	-	+	+	+	+	+
GM62	-	-	-	-	+	+	+	-
GM11	-	-	+	-	+	+	-	-
GM12	-	+	-	-	-	+	-	-

Conflict of interests

The authors have not declared any conflict of interest.

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