Use of lactic acid in Preserving local cheeses which Product from Microbial extracted Buffalo milk and identified by HPLC technology

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Abstract

The study aimed to extract lactic acid from alternative and cheap sources rich in carbon and nitrogen sources by using lactic acid bacteria and using the produced acid as a preservative in dairy products such as soft cheese and extending its shelf life. Two ideal isolates *L. paracasei* and *L. helvaticus* were selected based on phenotypic and biochemical examinations, 16S rRNA test and the isolates recorded lactic acid production of 64.52 and 53.08 $mg.l^{-1}$ respectively, using optimum conditions 7% molasses, 4% inoculum volume, at pH 6.9, at 38°C, 40% Whey Medium for 48 Hours. Lactic acid was used in different concentrations (0.5, 0.7, 0.9%) in preserving local soft cheese made from buffalo milk for 15 and 30 days at a temperature of 4 °C. Where the concentration of 0.9% recorded a significant superiority over the rest of the concentrations by registering the lowest microbial number at the end of preservation at 30 days, as the sample was completely free of aerobic bacteria compared to the control sample, which amounted to 122×10^3 cells/gm cheese, while the number of psychotropic bacteria reached 89×10^5 cells/g of cheese compared to a control sample that recorded 160×10^5 cells/gm of cheese.The results of the sensory evaluation showed a significant superiority of the sample of soft cheese to which lactic acid was added at a concentration of 0.9%, compared to the control sample, by recording the highest scores through the characteristics of color, smell, taste, and general acceptance.

Keywords: *Lactobacillus paracasei*, optimum conditions, Lactic acid production, isolates.

Introduction

Lactic acid is considered one of the organic acids of a therapeutic nature in various medical, pharmaceutical and cosmetic fields as a result of its possession of the ideal functional structure of carboxyl and hydroxyl groups that have an effective role with other compounds(15), as well as its catalytic role in the activity of lactic acid bacteria that have therapeutic and health effectiveness in some food products, including milk and dairy products and many foodstuffs (17).

As a result of the various studies and related to lactic acid production, it was found that low-cost materials are obtained from available sources throughout the year(18), in addition to the increasing demand for this important acid, which prompted researchers to intensify efforts and studies in developing production and extraction methods at a low cost (16) .Waste sugar cane and date juice such as molasses, maize decomposition and whey leftover from the cheese industry were used as sources of carbon and nitrogen as alternatives to the production medium due to its low cost and high yield of lactic acid. The lactic acid produced from the fermentation process is diagnosed using highly efficient techniques such as high-performance liquid chromatography (HPLC), and it is one of the approved methods in the diagnosis of lactic acid by calculating the retention time as a standard for the standard lactic acid, and the alternative medium. HPLC technology is a good and ideal way to get accurate results. Where a monochromatic light beam (a beam consisting of only one wavelength) was shown into the sample, this technique illuminates a beam containing many frequencies of light simultaneously and measures how much of that beam is absorbed by the sample. After that the beam is modified to contain a different set of frequencies, giving a specialized data set. This process is rapidly repeated for many times over a short period of time. Finally, the results were analyzed using a computer(13).

Materials and methods

Three samples were prepared from different sources of milk products such as raw milk, yogurt and curd with appropriate decimal dilutions in order to grow lactic acid bacteria and choose the best bacterial isolate in production of lactic acid during the research stages.

Appropriate concentrations of alternative lactic acid production medium (molasses) fortified with whey and other nutrients were prepared in the laboratory, and compared with standard medium MRS Broth.

Colonies with a different appearance (based on color, shape and size) were extracted from the MRS Broth and purified by streaking on a fresh MRS Broth plate. The purification process was repeated until single colonies with distinct appearance were obtained. The pure isolates were tested for Gram and catalase reactions. Cell morphology was observed under the microscope. The isolates that were Gram positive and catalase negative

were taken as presumptive LAB. The LAB isolates were stored at -20 °C in MRS broth containing 20% (v/v) glycerol until required for further tests(1).

The 16S rRNA target region was amplified using DreamTaqTM DNA polymerase (Thermo Scientific TM, Waltham, MA,USA) and the primers 16S-27F(sequence5′-AGAGTTTGATCM TGGCTCAG-3′)and16S-1492R(sequence 5′-CGGTTACCTTGTTACGATACGAC TT-3′). Likewise for the second isolate the primers 16S-27F (sequence 5′-CGAGTTTGAGCTTGGCTCTT-3′)and 16S-1492R(sequence5′-AGGTTACCTT TATACGAGACAG- 3′). Polymerase chain reaction (PCR) products were gel extracted (Zymo Research, Zymoclean TM Gel DNA Recovery kit), and sequenced in the forward and reverse directions on the ABI PRISMTM 3500 x l Genetic Analyzer. Purified sequencing products (Zymo Research, ZR-96 DNA Sequencing Clean-upTM kit) were analyzed using CLC Main Workbench 7 followed by a BLAST search on the database of the JAPAN National Center for Biotechnology Information. The API 50 CH kit was used to identify the two isolates by the 16S rRNA method(2). The two isolates recorded the production of its amount as L1 and M2 59.52 mg/L and 54.39 mg/L respectively was diagnosed genetically by the previous method, and it was the best bacterial isolate in terms of lactic acid production from molasses media and standard media(3).

The amount of lactic acid produced from the molasses and MRS Broth medium was estimated using the standard curve of lactic acid, then the absorbance of the samples was measured by using Spectrophotometer UV with a wavelength of 560 nm through lactic acid constriction that standard and produced(30). After the end of the fermentation period, a centrifugation is done at a speed of 4000 cycles/min for 15 minutes for the obtained solution with the addition of some chemicals as a modification of some previous studies to make the standard curve of lactic acid in order to measure and estimate the lactic acid in the alternative medium (molasses) as it is shown (Table1).

Table 1. The values of standard lactic acid and distilled water

Sample	Lactic acid Cons ($ml.l^{-1}$)	Final Volume (ml)	Water volume (ml)	Lactic acid volume (ml)
1	0.0	1.0	1.0	0,0
2	0.5	1.0	0.95	0.5
3	10.0	1.0	0.90	0.10
4	15.0	1.0	0.85	0.15
5	20.0	1.0	0.80	0.20
6	25.0	1.0	0.75	0.25
7	30.0	1.0	0.70	0.30

Add 100 µl of NaOH (1 N) to 200 µl of each concentration of the concentrations prepared in the table above and placed in sealed tubes, then the sealed tubes are incubated at a temperature of 38 C for 25-30 minutes, then add 2 ml of concentrated H_2SO_4 at a concentration of 70% for each tube separately and the tubes are sealed tightly, then the tubes are placed in a boiling water bath at 100 ° C for 5-7 minutes, then the tubes were cooled in an ice water bath and 20 microliters of $CuSO_4.5H_2O$ (aqueous copper sulfate) are added with continuous mixing, after that 40 microliters of bromophenol reagent (2%) were added to each tube separately and close the tubes tightly, the tubes are placed in a shaking incubator at a speed of 150 rpm for 20-30 minutes, After that, a Spectrophotometer is used at a wavelength of 560 nm to read the absorption for each concentration fig(1) (the process is repeated for three times)(5).

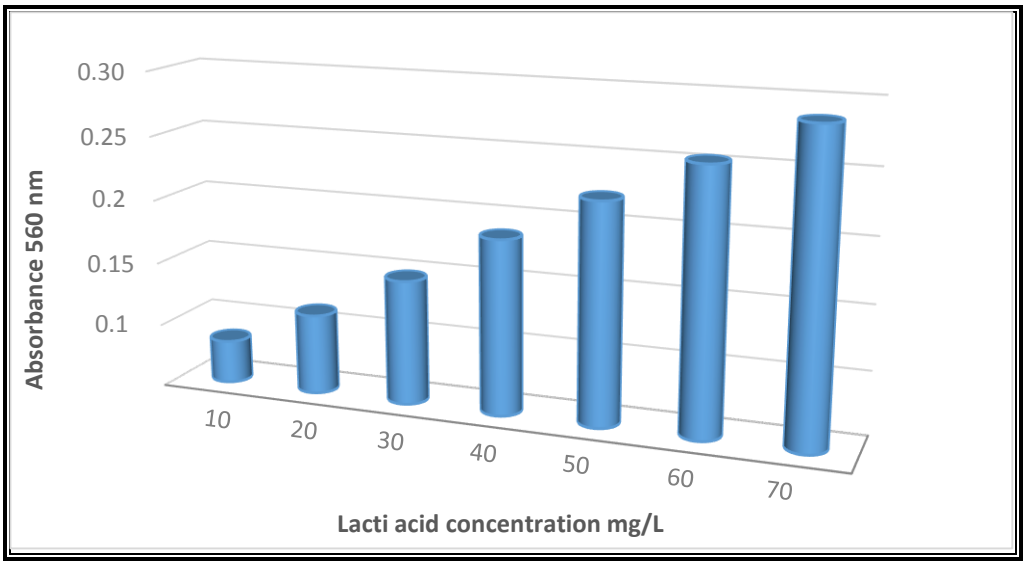

Figure 1. The concentration of lactic acid using Spectrophotometer

Its working principle is based on its mobile phase and the stationary phase. Shimadzu. LC - 2010AHT - LIQUID CHROMATOGRAPHY-IBC.QC.1.D2.01 was used. Located in the laboratories of the Iraqi Ministry of Science and Technology / Research and Development Authority / Ibn Al-Bitar Center, where 20 microliters of each of standard lactic acid, lactic acid produced from alternative medium, and lactic acid produced from MRS Broth were injected through a column Separator type Column 18 with dimensions (250×4mm NUCLEODUR/C18 Pyramid / 5µm) with phosphoric acid H_3PO_4 at a concentration of 2% as a mobile phase and silica as a stationary phase at a flow rate of 0.7 ml / min and at a temperature of 25 °C with a wavelength of 210 nm(6).

The samples to be diagnosed were prepared by weighing 0.1040 g of each sample and diluting it with deionized water with good mixing to homogenize

the mixture, then the samples were packed in vials for the HPLC device with a capacity of 20 microliters, after which they were placed in the device for the purpose of diagnosis, the column was injected and the glass in the device was filled with H_3PO_4, at a concentration of 2%, which is considered as a mobile phase with the presence of silica in another column as a stationary phase, then 20 microliters were taken from each sample separately and injected into the device in a designated location after establishing all the ideal conditions for diagnosis(7).

The selected bacterial such as L1 and M2 was used with molasses medium in the production of lactic acid after adjusting the ideal production conditions at a temperature of 38 °C, with pH (6.9), 7% molasses concentration, 40% whey, 4% inoculum volume, the speed of the shaking incubator 200 cycles/min with an incubation period 48 hours after the end of the fermentation process, and in order to obtain the filtrate containing lactic acid, a centrifugation process was carried out using the centrifuge at a speed of 4000 rpm for 15 minutes, after which the precipitate was removed and the required filtrate was obtained in order to conduct successive purification operations to obtain pure lactic acid. In order to achieve an ideal and highly efficient purification process and to obtain pure lactic acid, the ion exchange chromatography technique with two ion and cation exchangers were used (Amberlite IRA-92 and Amberlite IR-120 H), respectively, with pre-prepared solutions such as NaOH 0.85 M and $(NH_4)_2CO_3$ 3M to complete this process in an accurate and perfect manner(8).

The anion exchanger with a glass column (Amberlite IRA-92) with dimensions 1.315∈ was used according to the method of (8,9), with some modifications, by washing the column of the anion exchanger with 150 ml of deionized water several times to ensure all impurities and suspended compounds were removed, then the anion exchanger was placed in 250 ml of NaOH to ensure balancing of the purification and separation medium, then the NaOH base solution was filtered under vacuum and the column was re-washed several times with deionized water to complete the process of balancing the column medium, after which the column was filled with deionized water at a flow rate of 1.2 milliliters/min and left until the next day(9). Then, the column was washed with deionized water to ensure that all materials and compounds were released, and 50 ml of $(NH_4)_2CO_3$ (3M) was added in order to recover the largest amount of lactic acid, then all the separated and descending parts were collected from the column using filter paper (Wattman) if those parts were estimated at 7 milliliters/part for a flow rate of 1.2 milliliters/min(10).

The use of acation exchanger with an (Amberlite IR-120 H) column with dimensions 1.3∈14 and according to the method of(10), as the column is washed in the same way as the anion exchanger

with deionized water then the glass column is filled with a solution of HCl 1Molar 150 ml, then filtered and washed the column with deionized water for the process of balancing the cation exchanger, then the column is filled with distilled water and left for the next day at a flow rate of 1.2 milliliters/min, then the solution containing lactic acid obtained from the anion exchanger is passed onto the exchanger column Cationic Amberlite IR-120 H, then wash the column with deionized water, collect the separated fractions and estimate the amount of lactic acid in them(11).

The milk is examined sensory by color, taste, smell, and texture, making sure that it is free from impurities and dirt to ensure that it is not contaminated. Then the milk is pasteurized at a temperature of 63 C for 30 minutes. The milk is left to cool at a temperature of 30 C. Then rennet is added in an appropriate amount with continuous stirring to ensure the formation of a good clot and left. For a period of time, then it is cut regularly, after which the whey resulting from the formation of the coagulation is removed, after which salt is added according to demand, and the cheese is packed in appropriate molds and kept in refrigeration until use.

Soft cheese made from buffalo milk was prepared to test the microbial content after adding lactic acid at different concentrations (0.5, 0.7, 0.9)% and knowing the storage capacity of the cheese at a temperature of 4 °C and a period of preservation of 15 and 30 days.

Aerobic bacteria agar culture medium and Nutrient Agar culture medium were used and appropriate dilutions were made, then the microbes were counted separately using a bacterial counting device (29).

Results and Discussion

The results of isolating lactic acid bacteria showed obtaining four isolates of the genus *Lactobacillus spp*. It included two isolates from curdled milk and two isolates from raw milk, they were coded (L1, L2, M1, M2). The culture medium MRS Broth was used to grow lactic acid bacteria. It is considered a good and suitable medium for growth. It works on the growth of bacillus bacteria and reduces spherical bacteria as a result of the formation of a compound due to the decomposition of the compound Disodium B-Glycerophosphate, as a result of the decomposition of this compound, the pH becomes between (5.2-7.4) and the medium is suitable for the growth of lactic acid bacteria.

The primary screening of the four isolates obtained from the isolation processes and from different sources was carried out on the MRS Broth culture medium in order to diagnose these isolates which the starters were used for the manufacture of dairy milk products based on the following examinations:

The phenotypic examination of the growing colonies showed that they were white to a creamy glossy color, and they are cylindrical, rod-like, rod-like, or spherical. They may be a little spherical, and they may be single or clustered in the

form of chains(21), some of which have smooth convex edges, varying in size, as shown in figure(2) as these characteristics were identical to the studies. The results of the microscopic examinations revealed that all bacterial cells were positive for the Gram stain, as they appeared in violet color, in the form of long or short cylindrical or rosary chains as shown in the figure (3), and that they are immobile, and these results were consistent with the study(22).

Figure 2. *Lactobacillus* colonies on the MRS Broth

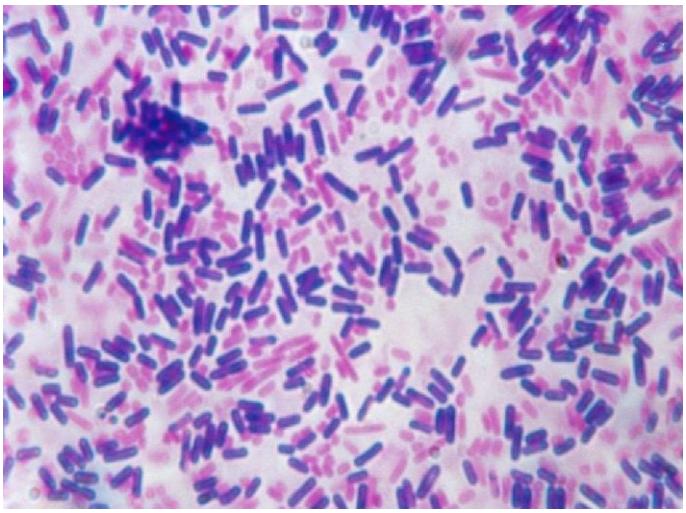

Figure 3. Gram-positive *Lactobacillus* cells

The results of the biochemical tests, as shown in tables(2) and(3), revealed that all four bacterial isolates do not produce catalase and oxidase enzyme, therefore, they are negative for the catalase and oxidase test, and the reason for this is that LAB does not have those enzymes that analyze hydrogen peroxide H_2O_2 To water and oxygen gas, which is the main cause of the appearance of bubbles when tested(23). The results also showed that the aforementioned bacterial isolates were unable to consume citrate and produce indole, while the isolates were positive

for the fermentation of carbohydrates as a carbon source through the production of gas, as they were able to ferment glucose, lactose and galactose, while they were unable to ferment xylose and arabinose and were positive in the whey hydrolysis test(24). The nitrogen source in the study, by the appearance of a transparent halo around the bacterial colonies, and it had the ability to grow in anaerobic conditions, in addition, it was able to grow at 37-41 °C, and it was able to grow at a pH number 5.1-7.7. It was unable to grow in high salt concentrations (5-7)% and was able to grow in concentrations (3-6)%, and all of these results were consistent with(25) It was unable to grow in high concentrations of salt (5-7)% and was able to grow in concentrations of (3-6)%. These results were consistent with (26), as it was shown that lactic acid bacteria do not grow at high salt concentrations of more than 8%. While the growth was appropriate at concentrations 3, 4, 5, 6, and 7.

Table 2. Biochemical tests for bacterial isolates

Symbol	Shape	Gram stain	Salinity tolerance	Whey analysis	Starch analysis	Indol	Oxidase	Catalase	Sugar fermentation
L1	Bcilli	+	+	+	+	-	-	-	+
L2	Bcilli	+	+	+	+	-	-	-	+
M1	Bcilli	+	+	+	+	-	-	-	+
M2	Bcilli	+	+	+	+	-	-	-	+

Table 3. The ability of bacterial isolates to The fermentation of sugars

Carbohydrate	M2	M1	L2	L1
Glucose	+	+	+	+
Lactose	+	+	+	+
Galactose	+	+	+	+
Xylose	-	-	-	-
Arabinose	-	-	-	-

Figure(4) showed Electrophoresis on 1.5% acarose gel. The diagnosis was confirmed by using an examination on the 16S rRNA gene to diagnose the highest isolate in terms of lactic acid production, as the polymerase chain reaction (PCR) test was performed and the DNA extracted for the selected isolate, and then the concentration and purity of the DNA were measured. The primers indicate the presence of the 16S rRNA gene by 100% in size (1500 bp), and the results were consistent and identical to the study(25). Bacterial strains isolated from dairy products during the emergence of bundles at (1500 bp) belonging to *Lactobacillus spp*. and that *Lactobacillus* spp.(ABST) is completely identical to *L.paracasei* (L1) and *L.helvaticus*(M2) registered earlier in the International Genbank, and through

the sequence of nitrogenous bases in the 16S rRNA gene amplification test and data analysis in the International Gene Bank NCBI, the ABST code was assigned to the *Lactobacillus bacterium spp*. It is a strain officially registered in the Japanese International Genome Bank(26).

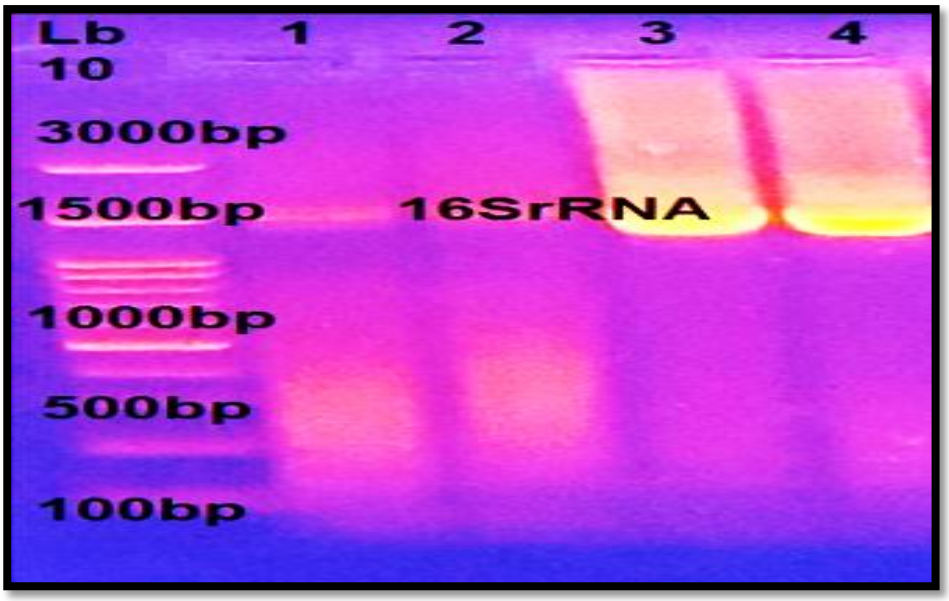

Figure 4. Electrophoresis on 1.5% acarose gel, voltage 100, voltage difference 80 ampere, for an hour and a half for investigation Determination of the 16S rRNA gene by PCR of isolates of *Lactobacillus spp* isolated from dairy products and Lb means Marker ladder 3000-100bp for *lactobacilli* and isolates (1-4) isolates of positive bacteria

Two types of lactic acid producing bacterial isolates were used to compare them in terms of production, where *L.paracasei* and *L.helvaticus* were used on molasses medium. It was used as a source of carbon in addition to calcium carbonate ($CaCO_3$) using isolates of genetically determined bacteria (*L. paracasei* and *L. helvaticus*). The results obtained were the highest production of lactic acid from *L. casei* bacteria, which reached 64.52 mg/L and 53.08 mg/L. from *L. helvaticus* isolates. The reason for the difference and discrepancy in the obtained results was that the two isolates each have their own conditions, composition, and adaptation to the medium. A study confirmed that molasses contains good sugars such as glucose and lactose, in addition to some B vitamins such as B12. It also contains nutrients and important elements for the perpetuation of the growth and reproduction of bacteria(27).

A results of the diagnosis of lactic acid produced from the molasses and MRS Broth in the presence of *L. paracasei* and *L. helvaticus* and compared with standard lactic acid as shown in figures 5, 6 and 7 respectively, the appearance of more than one peak indicating other compounds in addition to lactic acid, as shown in

figure(5) and figure(6) that the peak with a duration of 5.269 minutes and 5.293 minutes is the retention time for lactic acid in MRS Broth and molasses media, respectively, compared with the retention time for standard lactic acid of 5.247 minutes in figure(7), in addition to being the highest peaks among other compounds as a result of the high amount of lactic acid(28). The apparent peaks for the rest of the compounds were uneven in terms of quantity and retention period as a result of the presence of some impurities or the appearance of these substances as by-products of the fermentation process in the production of lactic acid as they interfered with its compounds, which caused a variation in the retention time for each of the lactic acid produced from the alternative medium and the standard medium compared with the standard lactic acid sample, which showed only two peaks, being more pure and less retention period, and these results were consistent with when diagnosing lactic acid produced from the alternative medium of date juice fortified with urea solution using bacterial isolates *L. paracasei* and *L. helvaticus* through HPLC technology in the British national laboratories, the appearance of different peaks at a holding time of 5.3 minutes(4).

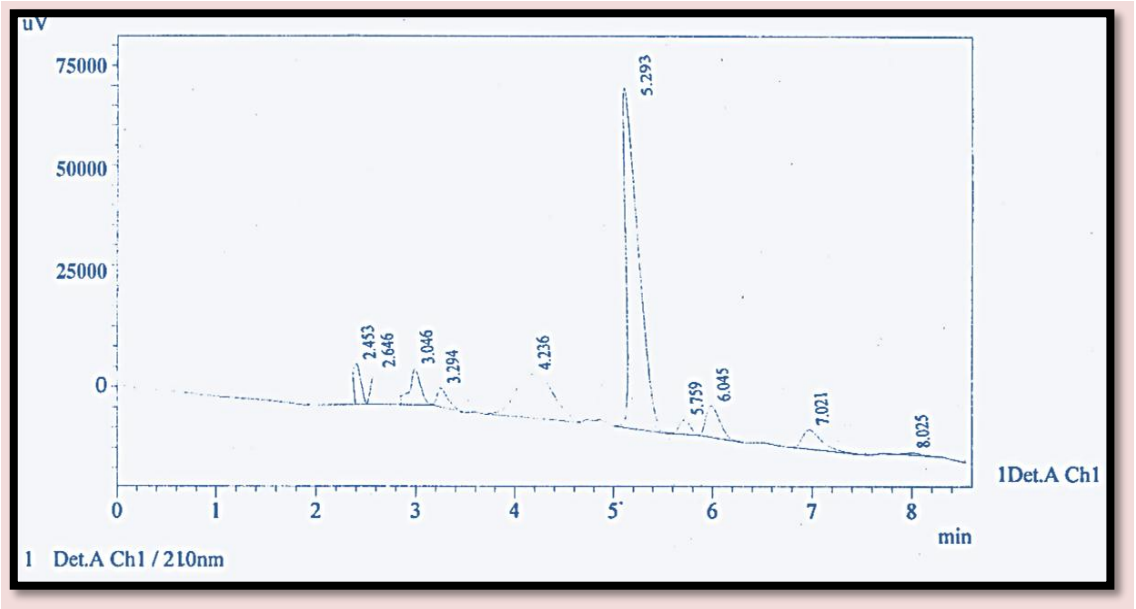

Figure 5. Diagnosis of lactic acid produced from the molasses using HPLC

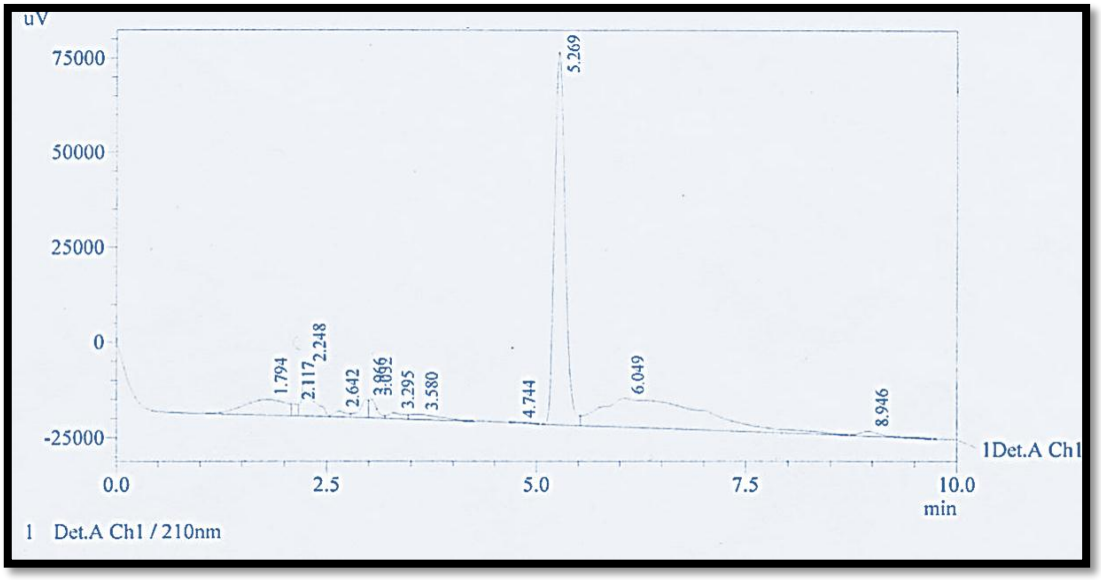

Figure 6. Diagnosis of lactic acid produced from MRS Broth using HPLC

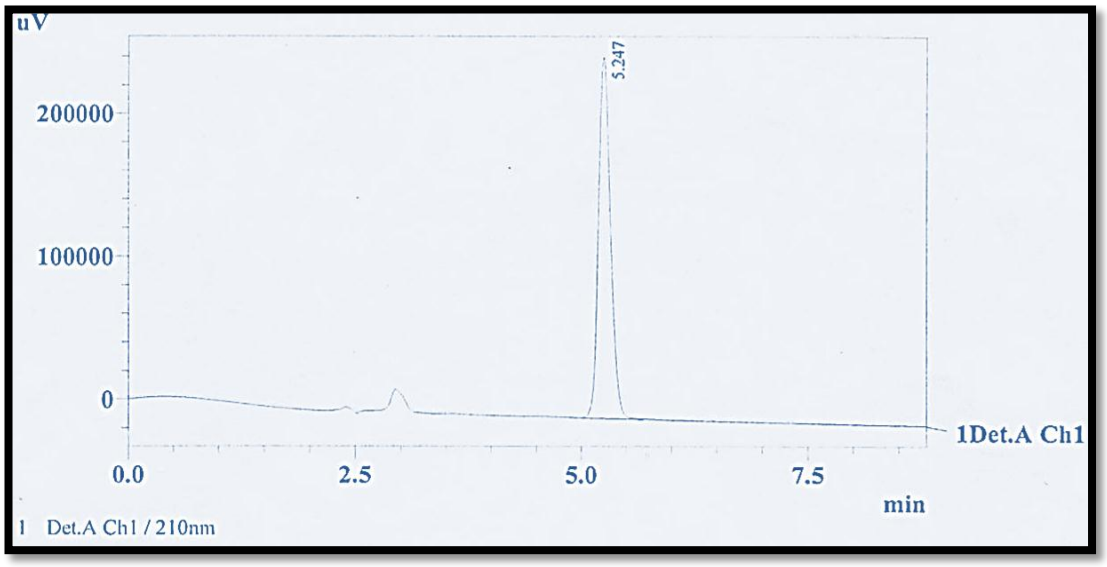

Figure 7. Diagnosis of lactic acid standard using HPLC

After washing the Amberlite IRA-92 column with deionized water in order to get rid of all impurities and suspended parts to obtain an ideal result from the purification process, the filtrate was injected into the column and deionized water was added inside the anion exchanger for the purpose of the separation process, and after the descent substances and impurities removed from the filtrate and the acid remains in the column(30). A recovery process was carried out for lactic acid from the parts removed from the anion exchanger if they contained an amount of acid using ammonium carbonate $(NH_4)_2CO_3$. As a result, a quantity of lactic acid was obtained from within those parts in addition to an amount the acid contained in the exchanger column, as the amount of acid was estimated at 59.84 $mg.l^{-1}$ and a purity of (91.66)%(14).

A results of the secondary purification by the Amberlite IR-120H cation exchanger for the lactic acid obtained through the primary purification by the Amberlite IRA-92 anion exchanger, where the acid was placed in the cation exchanger and the purification process was carried out to obtain pure lactic acid after removing all impurities and associated materials from the first purification process. The amount of acid in this process is (64.52 $mg.l^{-1}$) and the purity is (92.84)%. The results also came in agreement with who used Amberlite IR-120H cation exchanger and gives an amount of lactic acid amounted to 59.72 $m.l^{-1}$ and a purity of (93.25)%(14).

Table (4) shows that the concentration was significantly superior to 0.9% compared to the rest of the concentrations, as the results showed that the numbers 98, 90 and 85×10^5 cells/gm of cheese of psychotropic bacteria were recorded at 30 days of preservation for concentrations of lactic acid 0.5, 0.7, and 0.9%, respectively, compared with the control sample, which recorded 155×10^5 cells/gm of cheese. While the sample recorded On the 15 day 84, 60 and 48×10^3 cells/gm of aerobic bacteria respectively, compared to the control treatment, which amounted to 149×10^3 cells/gm. As we notice the continuous decrease in the number of bacteria when the concentration is increased due to the increase in the number of lactic acid bacteria and their exploitation of the nutrients inside the medium, and thus the medium is not suitable for other bacteria.(30). It was observed that the cheese sample was empty at the end of the preservation period of aerobic bacteria due to the lack of growth sources and the oxygen required for its growth, as well as the low number of psychotropic bacteria, the reason for this is due to the increase in the number of lactic acid bacteria in the growth medium, which led to an increase in the acidity rate and limiting the growth of these microorganisms(4).

Table 4. Microbial tests of soft cheese preserved with lactic acid

Treatments	Preservation periods/day	psychotropic bacteria $\times10^5$ cells/gm	aerobic bacteria $\times10^3$ cells/gm
Control 0.0 %	15	101	149
	30	155	113
Lactic acid 0.5 %	15	83	84
	30	98	0
Lactic acid 0.7 %	15	77	60
	30	90	0
Lactic acid 0.9 %	15	72	48
	30	85	0
LSD		1.257	0.736

A results of the sensory evaluation as shown in Table(5) showed a significant superiority of soft cheese preserved in different concentrations of lactic acid and at different times compared with the control treatment through the

characteristics of color, flavor, taste and general acceptance using statistical analysis at the level of probability ($p<0.05$), as it gave the highest values and reached 9.1 , 8.5, 8.4, 8.8, while the control treatment recorded 7.6, 7.1, 6.9, 7.8, and this was confirmed by previous studies.

Table 5. Results of sensory evaluation of soft cheese with control treatment

Adjectives approved in sensory evaluation				Sample name
General admission	Taste	Smile	Color	
7.8 b	6.9 b	7.1 b	7.6 b	Treatment (Control)
8.8 a	8.4 a	8.5 a	9.1 a	Soft cheese with 0.9% lactic acid

Conclusion

Through the results, lactic acid was found to be a highly effective compound in the preservation process of dairy products, including local soft cheese, by reducing the numbers of both aerobic and psychotropic bacteria throughout the preservation period. Which leads to the ability of lactic acid to possess active elements and compounds in its structural build and that works to interact and overlap with medium compounds and Creating a good environment for beneficial bacteria and other microorganisms.

Conflict of interest

The authors have no conflict of interest.

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