Investigate the metabolites of Lactobacillus delbrueckii subsp. lactis isolated from local dairy (Yogurt) products in Iraq

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Abstracts:

Background and Objective: The production of different primary and secondary metabolites of probiotics,whose biological effects, metabolites of lactic acid bacteria offer therapeutic benefits in the prevention and treatment of various diseases, also responsible for the development of flavor, aroma and texture in food products in addition to their inhibitory activity for some pathogens such as fungi and pathogenic bacteria,The study aims to investigate the metabolites produced by Lactobacillus delbrueckii subsp.lactis as probiotics, its metabolites using Gc-Mass. Materials and Methods**:** Yogurt samples were collected from the local markets in Iraq for four local dairy products,The bacteria weresisolated from the dairy products using the Lactobacillus MRSsagar, Isolated species of dairy products were diagnosed based on phenotypic and microscopic characteristics,The isolate Lactobacillus delbrueckii subsp.lactis was chosen to study its metabolites using Gc-Mass. Results**:** Eight bacterial isolates were isolated and purified from local dairy products (L.delbrueckii subsp.lactis, Brevibacillus sp, B.paralicheniformis, B.adius ,Paenibacillus lautus ,B.licheniformis ,Bacillus spp and L.rhamnosus).The results of Gc-Mass analysis for supernatant of L.delbrueckii subsp.lactis showed that lactobacilli produce many diagnosed metabolic compounds,It produced thirty compounds including some organic acids, amino acids and alcohols. Conclusion**:** We have come to isolate and diagnose bacterial species(L.delbrueckii subsp.lactis, Brevibacillus sp, B.paralicheniformis, B.adius ,Paenibacillus lautus ,B.licheniformis .Bacillus spp and L.rhamnosus)from local dairy products in the local markets. The results of Gc-Mass analysis for supernatant of Lactobacillus delbrueckii subsp.lactis showed that lactobacilli produce many diagnosed metabolic compounds, it produced thirty compounds including some organic acids, amino acids and alcohols, which have an effective role in enhancing the flavor of food products.

Keywordss: Dairy productss, GC-Mass, Lactobacillus delbrueckii subsp.lactis.

Introductions: The term lactic acid bacteria is used for souring-milk which includes bacterial groups with the ability to produce Lactic acid, classified according to specific traits ,It includeds the form and naturesof fermentations and growths at criticals temperaturess (10-45)^{O}C **(1)** . Lactic acid is one of the organic acids having wide usesin manysfields, e.g. food industry, beverages industry, pharmaceutical industry, chemical industry and medicine **(2).** Lactic acids bacteria ares industrially importants microorganismss recognizeds for their fermentatives ability mostlys in their probiotics benefits as well as lactics acid productions for various applicationss. Neverthelesss, lactic acid fermentation often sufferss end-products

inhibitions which decreasess the cell growthsrate. The inhibition of lactics acids is dues to the solubilitysof the undissociatedslactic acid withinsthe cytoplasmic membranesand insolubility of dissociatedslactate, which causes acidification of cytoplasm and failures of protons motive forcess. This phenomenons influencess the transmembranes pH gradient and decreases the amount of energy availables for cell growths. In generals, the restrictions imposeds by lactic acid on its fermentations can be avoided by extractives fermentation techniquess which can alsosbe exploited for product recoverys**(3)** The application of fed-batch fermentation is another traditional method for improving biomass yield. Feed-batch fermentation systems can be classed in general based on feeding modes such as constant feeding, exponential feeding, intermittent additions, and optimal feeding with or without feedback control.**(4,5)**

A number of 21 Lactobacillus species strains were isolated from Turkish kefir sample in order to identify the best acceptable strains based on metabolic activities and probiotic qualities. Lactic acid, acetic acid, hydrogen peroxide (H2O2), enzyme activities, and acetaldehyde synthesis in Lactobacillus spp. were all assessed. Strain studies yielded varying amounts of organic acid; nonetheless, lactic acid levels ranged from (1.7 to 11.4 mg/mL.) All of the strains produced hydrogen peroxide. The L. bulgaricus Z14L strain had no proteolytic activity, whereas the L. caseis Z6L strain had the highest levels of proteolytic activity (0.16 mg/mL). Lactobacillus strains produced acetaldehyde concentrations ranging from 0.88 to 3.52 micro g/mL **(6)**.

Lactic acid bacteria influences have been reported as adjuvant and immunoregulatory effects in adaptive immune responses, as well as strengthening of non-specific immunity. However, the bacterial component that causes these effects is frequently unknown. Lactobacillus bacteria have a wide range of cell surface architecture, which may influence the bacterial cell's physicochemical qualities as well as train-specific traits. Lactobacilli have a bilayer lipid cell membrane with embedded proteins attached to the cell wall, which is coated by a thick multilayered peptidoglycan sheet including lipo- and teichoic acids, pilis, and polysaccharides sheet. Polysaccharides can adhere to the cell surface like an outer capsule and are frequently covalently bonded to peptidoglycan Nacetylmuramic acids, or they can be freely connected. (**7**)

Materials and methods:

Sample Collection: 50 samples from Yogurt for each products have been collected from four Cities in Iraq. The samples when collected from these regions are placed in sterile polyethylene tubes and stored at 4oC until use.

Isolated of Lactobacillus from samples :1 ml of yoghurt samples were taken and spread on a Lactobacillus MRS medium and incubated under anaerobic conditions using candle jar at 37^{o}C for 48 hour.

Preservation of Bacterial Isolates: The isolates after definitive diagnosis were cultured on slant of brains heart infusion agar and incubated at 37^{o}C for 48 hr, under anaerobic conditions in an anaerobic jar. Isolation colonies were stored in the refrigerator at 4^{o}C and re-cultured monthly to maintain their viability and activity.

Microscopic examinations: The bacteria were stained with a gram stain to recognize the shape of the cell and their interaction with the dye

Biochemical tests : Included Catalase test ,Oxidase test ,Indol test,Methyl Red Test,Voges – Proskaurs Test and Citrate utilization (**8;9 ;10**)

Preparation Crude extract (CFS) for Lactobacillus sp. Cell free supernatant (CFS) is a crude extract of Lactobacillus delbrueckii subsp. lactis metabolic products that was generated by inoculating tubes with Lactobacillus MRS broth medium and incubating at 37° C for 5 days under anaerobic conditions. CFS was produced by centrifuging at 6000 for 15 minutes, then filtering the supernatant with Millipore (0.22) m microfilters according to the manufacturer's instruction (**11**). By adding 1M NaOH to the CFS, the pH was adjusted to 7.0 0.5. (**12**)

GC-Mass of supernatant to Lactobacillus sp.

Take 1 ml of the cell-free crude extract prepared in paragraph (2-7) for bacteria Lactobacillus delbrueckii subsp. lactis separately after incubation for 5 days to investigate the active compounds using GC-MS technique:

A- Analysis of the cell free supernatant (CF .)

According to the following conditions, the analysis was performed using the GCMS-QP2010 plus GC-MS system, which includes an automatic vehicle identification unit and a gas chromatograph paired to a mass spectrometer instrument, under the following conditions: In a Silica Separation Column, helium gas was employed as a carrier at a flow rate of 1 ml per minute.

The injected liquid has a volume of 0.5 ml and works in a split ratio (1:10), Total flow: (34.1) ml/min, Column flow: (1.00) ml/min, Linear speed: (36.1) cm/sec, Injection temperature: (220) CO, Pressure: (49.7) kPa, Total flow: (34.1) ml/min, Column flow: (1.00) ml/min, Linear speed: (36.1) cm/sec Flow rate for disinfection: (3) ml/min (30.0) Split Ratio The ionic source temperature is 200 °C, and the oven temperature is 40 CO, which is automatically programmed (equal temperature for 3 minutes) with a 10 mH/min increment and up to 220 mH for 5 minutes.

Using 70EV with a 0.5 sec interval and a 40-450 Dalton fission rate, mass spectrometry was performed. The chromatograph's total start-up time is 60 min.

B- Identification of ingredients

Components were discovered utilizing GC-MS interpretation and a comparison of the unknown component's spectrum of findings with a list of known components from the National Institute of Measurement and Technology (NIST) library, which has over 62,000 patterns. The test was carried out in the research facilities of Basra University's Food and Consumer Protection College of Agriculture..

Results :

The results showed that 8 bacterial isolates were isolated and purified from local dairy products sold in Al-Diwaniyah markets. The initial diagnosis showed that all isolates are Gram-positive (**Fig. 1**), and most of the isolates are negative for catalase , oxidase , methyl red test, Voges - Proskaur test and Indol test

Eight types of bacteria were isolated from local dairy products in Iraq such as (Lactobacillus delbrueckii subsp.lactis, Brevibacillus sp, Bacillus paralicheniformis, Bacillus adius, Paenibacillus lautus, Bacillus licheniformis, Bacillus spp and Lacticaseibosillus rhamnosus). **(Fig. 2)**. This result is similar to was found(**14**) if the species was isolated from different dairy products. The results showed the highest occurrence rate of isolate Lactobacillus delbrueckii subsp.lactis from dairy products traded in the markets of cities (Diwaniyah, Najaf, Karbala and Bayel), where the percentage reached the percentage (44%, 42%, 34% and 30%), respectively**, (Fig .2),** followed by isolate Brevibacillus sp, which recorded percentages (16% ,20%,18% and 20%) respectively, and then Lacticaseibosillus rhamnosus which recorded percentages (12%,18%,16% and 20%) respectively. Lactobacillus delbrueckii subsp.lactis was selected to detect the metabolic products produced by it

The results of Gc-Mass analysis of the supernatant from Lactobacillus delbrueckii subsp.lactis showed that lactobacilli produce many of the diagnosed metabolic compounds, and they produce thirty compounds including some organic acids, amino acids and alcohols **(Fig. 3) (Table 1)**

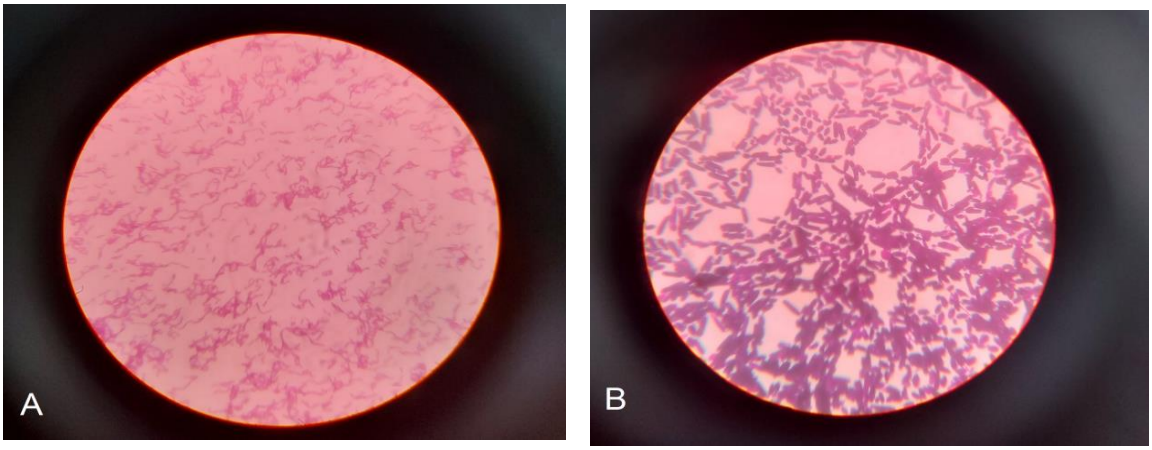

Figure (1): Lactobacillus cells stained with Gram stains under a light microscope (A) Lacticaseibacillus rhamnosus ,(B) Lactobacillus delbrueckii subsp.lactis

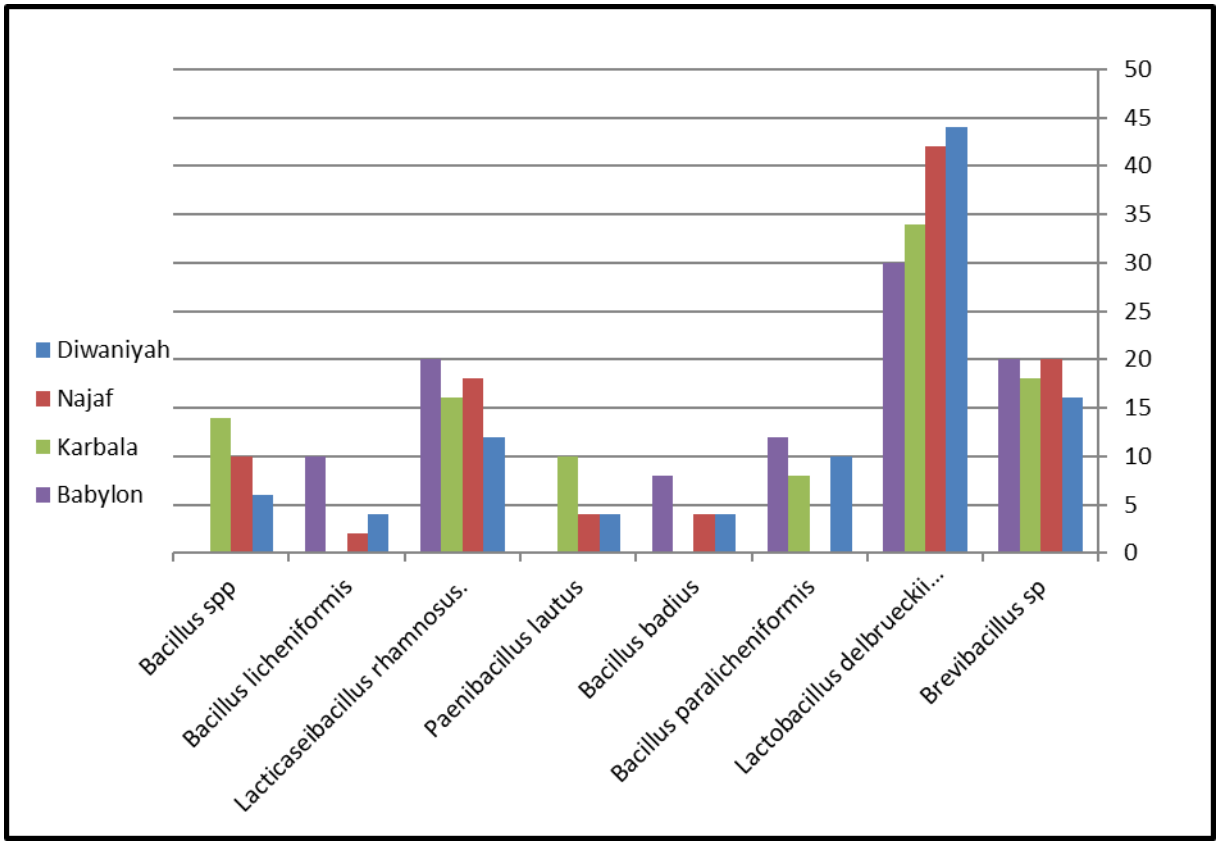

Figure (2): Percentage of bacterial species isolated from local dairy products

chromatogram of GC-

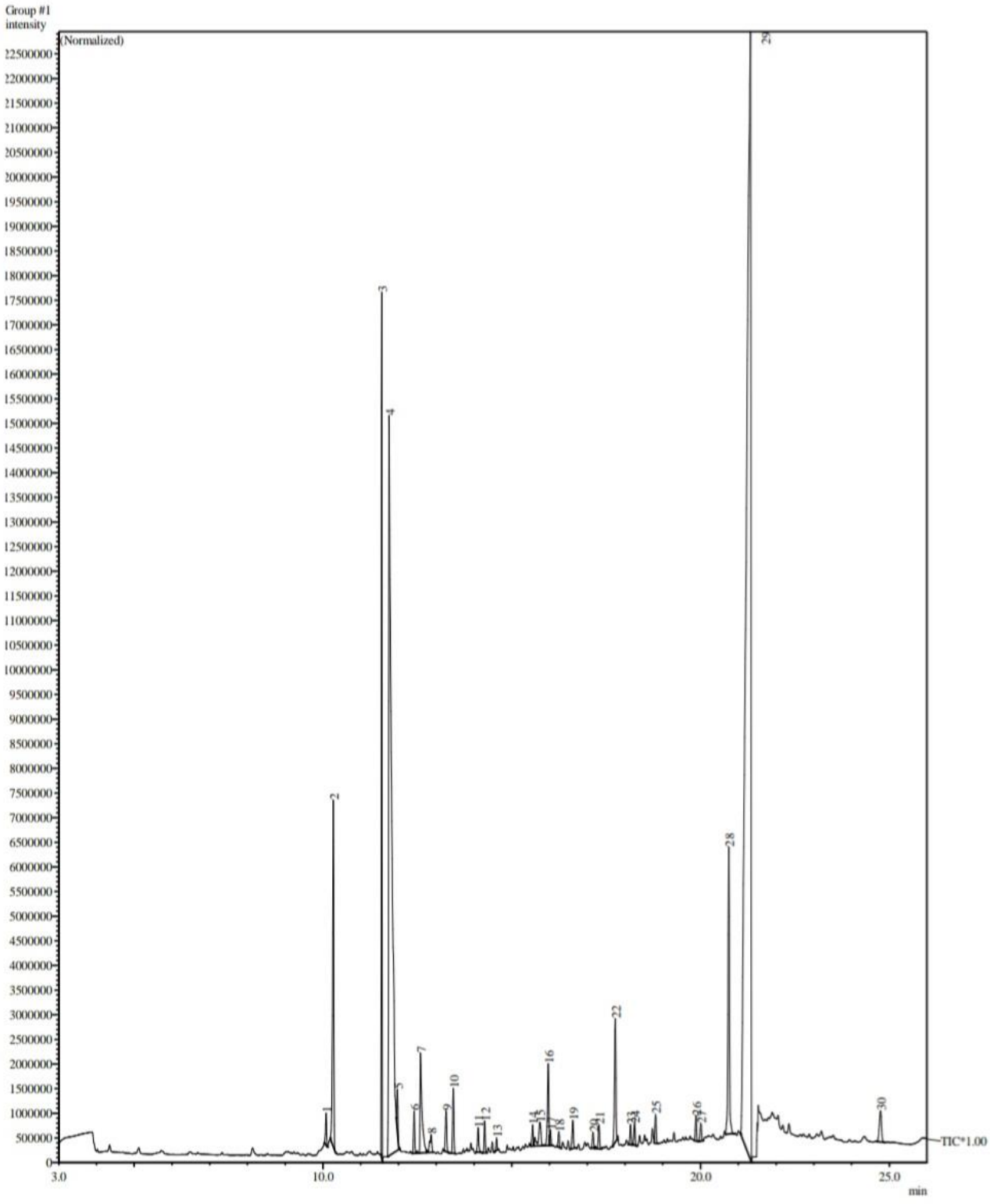

Figure (3):: The GC-Mass chromatogram of crude extraction of Lactobacillus delbrueckii subsp.lactis

Table1: peak report TIC bacterial extracte of isolate Lactobacillus delbrueckii subsp.lactis

Peak #	R.Time	Area	Area %	Name
1	10.080	1320400	0.36	
2	10.274	15638244	4.20	2- Propanone , 1- hydroxy-
3	11.556	21853474	5.88	Acetic acid
4	11.754	80575815	21.67	Acetic acid
5	11.972	1326250	0.36	2- Propanone , 1- (acetyloxy)
6	12.413	1855923	0.50	Ethanone , 1- (2- furanyl)
7	12.584	7190235	1.93	Formic acid
8	12.864	1420907	0.38	Propanoic acid
9	13.260	2038995	0.55	2- Furancarboxaldehyde , 5- methyl -
10	13.452	2919236	0.78	Benzene , (2- chloroethyl) -
11	14.111	1257372	0.34	Butanoic acid , 4- hydroxy-
12	14.285	1227677	0.33	2- Furanmethanol
13	14.597	558427	0.15	Benzene , (3- chloropropyl) -
14	15.552	1009218	0.27	2 (5H) - Furanone
15	15.743	2402595	0.65	4 (1H) - Pyridinone, tetrahydro - 1,3,5- trimethyl-
16	15.966	4308211	1.16	Tripropylene glycol monomethyl ether
17	16.025	613259	0.16	Acetic acid , 2- phenylethyl ester
18	16.246	721770	0.19	1 , 2- Cyclopentanedione , 3- methyl-
19	16.616	1402991	0.38	2 , 5- Dimethyl- 4- hydroxyl -3 (2H)-furanone
20	17.151	735988	0.20	Phenylethyl Alcohol
21	17.311	1234094	0.33	1, 4- Dioxan - 2 - ol
22	17.741	6339341	1.70	Maltol
23	18.147	887486	0.24	Phosphonic acid , (p-hydroxyphenyl)-
24	18.253	1006648	0.27	Furyl hydroxymethyl ketone
25	18.809	1352889	0.36	2 (3H) –Furanone , 5- acetyldihydro-
26	19.878	1140468	0.31	2- Hydroxy-gamma-butyrolactone
27	20.008	900109	0.24	3 , 5 –Octadien – 2 -one, (E,E)-
28	20.747	14838116	3.99	4H – Pyran - 4-one , 2 ,3- dihydro- 3 , 5-dihydroxy- 6-methyl-
29	21.321	191263125	51.43	Glycerin
30	24.768	2563284	0.69	2(3H)- Furanone, dihydro -4- hydroxyl -
		371902547	**100.00**	

Discussion:

Which have an effective role in enhancing the flavor of metabolic products in addition to their inhibitory role for pathogenic microorganisms (bacteria, Fungi) (**13,14,15**). In addition, some studies have proven that these compounds have anti-cancer and antioxidant action , and production of short-chain fatty acids by LAB and probiotics, which are also actors in human nutrition. The mechanisms of how these microbial outgrowths, produced by these bacterial strains, are produced,(**16**)

These products are to be used as tools to restore energy intakes either by improving ATP production from foods or by fermenting sugars(**17**) . The results also showed the recurrence of the appearance of some compounds in different concentrations and in different periods of time, such as the compound (Acetic acid) appeared per minute (11.556 and 11,754) and in two different concentrations (5.88 and 21.67), and attributed the reason for the appearance of compounds at two different times and with different concentrations is the degree of similarity of the compounds, as it is difficult for the Gc-mass device to distinguish them, so the high concentration that appears in the results is the real diagnosis of the compounds.

The lactobacilli bacteria's anti-inflammatory, anti-cancer, anti-cholesterolemic, and anti-diarrheal properties are used as food additives and flavorings in this study, demonstrating its huge potential in the food and pharmaceutical industries. Several researchers have observed comparable metabolic products in several lactic acid bacteria when bacteria influence human metabolism, such as probiotics or meals fermented by the bacteria involved (LAB). (**18**), Organic acids such as Lactic, Actic, and Formic acid, as well as volatile compounds such as benzoic acid, dodecanoic acid, dodecamethyl cyclohexasiloxane, isopropyl myristate, tetradecanoic acid, 1-hexadecanol, and hexadeconoic acid, contribute to the scent and flavor. The chemicals present, as well as the metabolites emitted by Lactobacillus plantarum, were investigated. Hexadecane, hexadeconic acid, dodecanal, and fatty acids have antifungal and antioxidant properties. (**19,20**). Propionic acid shares physical properties with both smaller carboxylic acids like formic and acetic acids and larger fatty acids like linoleic acid. It is miscible with water, however it can be extracted from the solution using salt. It, like acetic and formic acids, is made up of hydrogen-bonded pairs of molecules in both the liquid and vapor forms. (**21**) In biotechnological propionic acid manufacturing, Propionibacterium isolates are frequently employed. Large-scale generation of propionic acid by Propionibacteria, on the other hand, encounters issues including end-product inhibition and the creation of by-products during cell development (acetic acid and succinic acid). Cell immobilization techniques, which allow for easy recovery and reuse of cell biomass while also improving microbe stress tolerance, are one strategy to increase fermentation productivity and yield. (**22**). The US food and drug Administration considers succinic acid to be safe as a food ingredient and dietary supplement.

The results showed that the lactobacilli supernatant contains a high concentration of glycerin, Glycerin is one of the compounds from which lactic acid can be produced. It was mentioned (**23**) that lactic acid was concentrated and produced using a low concentration of elemental glycerol. It is also an acidity regulator in industry, cosmetics, as a skin moisturizer,

and in pharmaceutical applications in the synthesis of many medicines and medical drugs, such as the manufacture of surgical threads and solutions used in dialysis (**24**).

Conclusions : In conclusion, we have come to isolate and diagnose bacterial species (Lactobacillus delbrueckii subsp.lactis, Brevibacillus sp ,, Bacillus paralicheniformis, Bacillus adius ,Paenibacillus lautus , ,Bacillus licheniformis .Bacillus spp and Lacticaseibacillus rhamnosus) .from local dairy products traded in the local markets. The results of Gc-Mass analysis for supernatant of Lactobacillus delbrueckii subsp.lactis showed that lactobacilli produce many diagnosed metabolic compounds, it produced thirty compounds including some organic acids, amino acids and alcohols , which have an effective role in enhancing the flavor of metabolic products in addition to their inhibitory role for pathogenic microorganisms (bacteria, Fungi) . In addition, some studies have proven that these compounds have anti-cancer and antioxidant action , and production of short-chain fatty acids by LAB and probiotics, which are also actors in human nutrition. The mechanisms of how these microbial outgrowths, produced by these bacterial strains, are produced

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Ethical Approval: Ethical approval to perform this study was obtainedby Department of Biology, Faculty of Education, Al-Qadisiyah University, Iraq

Conflict of Interest: The authors declared no competing interests.

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