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Antibacterial activity of some Lactic acid Bacteria isolated from Egyptian Dairy Products

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Abstract: The aim of the present study was planned to isolation and screens the antibacterial activity of bacteriocin producing lactic acid bacteria (LAB) isolated from local fermented dairy products against a few selected gram positive and gram negative bacteria in vitro. All isolates (75 strains) were characterized morphologically, physiologically and biochemically using API tests (BioMerieux, Lyon Company, France). API®50 CHL system was used for biochemical identification of lactobacilli (isolates grown on MRS agar).While, API®20 CH strep system was used for biochemical identification for streptococci (isolates grown on M17 agar). From seventy five isolated strains only eight strains were chosen as follows: *Lactobacillus rhamnosus* (1 strain), *Lactobacillus plantarum* (1strain), *Lactobacillus pentosus* (2 strains), *Pediococcus pentosaceus* (2 strains), *Lactobacillus brevis* (1 strain) and *Lactoccocus lactis* ssp. *Lactis* (1 strain).These chosen strains were proceeded to screen the antibacterial activity against five indicator pathogenic strains (*Bacillus cereus*, *Staphylococcus aureus*, *Escherichia. coli* 0157:H7, *Listeria monocytogenes* Type I and *Pseudomonas aeruginosa*). All isolates were observed to behave a good antagonistic activity against the tested indicator strains with differences in size of inhibition zone (mm) meanwhile *Pseudomonas aeruginosa* and *Listeria monocytogenes* Type I were not inhibited by the extract of *Pediococcus pentosaceus* NRC AM 1 and *Pediococcus pentosaceus* NRC AM 4 respectively. These obtained results revealed the possibility of using bacteriocins of LAB as food biopreservative to control food spoilage and pathogenic bacteria.

Key words: Lactic acid bacteria (LAB), Carbohydrate fermentation, dairy products, biochemical identification, antibacterial activity and indicator strains.

INTRODUCTION

In recent years, extensive work has been carried out on bacteriocin producing strains of lactic acid bacteria (LAB) for their potential use as biopreservatives in food industries[1]. The preservation of foods by natural and microbiological methods may be a satisfactory approach to solve economic losses due to microbial spoilage of raw materials and food products, as well as to reduce the incidence of food borne illnesses[2]. Lactic acid bacteria (LAB) are a heterogeneous group of Gram- positive, catalase negative rods and cocci usually non motile, non spore forming. These microorganisms are aerotolerant, microaerophilic or facultative anaerobic. They are mesophilic with optimal temperature of growth between 30 °C and 40°C, but some strains able to growth at the temperature lower than 5°C or higher than 45°C. They are protected from oxygen byproducts (e. g H_2O_2) because they have peroxidases. Lactic acid was their major end product of these microorganisms[3,4]. These microorganisms produce a number of antimicrobial metabolic end products such as organic acids, bactericidal proteins (bacteriocins), diacetyl, hydrogen peroxide and antibiotic like substance which help to extend the shelf life of food products[5-7]. The crucial of LAB importance is associated mainly with their physiological features such as substrate utilization, metabolic capabilities and probiotic properties. Their common occurrence in foods coupled with their long historical use contributes to their acceptance as GRAS (Generally Recognized As Safe) for food fermentation and human consumption[8,9].Isolation and classification of LAB genera were based on morphology, mode of sugar fermentations and growth at certain temperatures. Nowadays, LAB still play an important role in the majority of food fermentations and one of the most contributions of these microorganisms is the extended shelf life of fermented products. However, they also have beneficial influence on nutritional and sensory characteristics as well as on the standardization offend products[10]. Screening and characterization of new strains isolated from fermented raw milk products is highly interesting because these microfloras had a good technological functions potentially applicable in the dairy and food industries[11]. Also, lactic acid bacteria are wide spread in nature and great economic importance for dairy and other fermented food industries, predominate in the flora of milk and its products. These unique organisms possess a large number of metabolic activities and nutritional benefits responsible for their use as starter cultures, probiotics and dietary additives in the dairy industry[12]. Recently, there is a great need for special lactic acid starter cultures to enhance quality and organoleptic properties of cheese and other dairy products. So, many researches have been done to isolate and use of LAB for improving the quality of Egyptian dairy products[13].

Egyptian dairy products such as Karish cheese (skimmed milk cheese, Mish (pickled ripened Karish cheese), Zabady (yoghurt), Laban Rayeb (concentrated sour milk) and Kishk (wheat- based fermented milk) are a good valuable sources of LAB bacteria with new important industrial properties and genetic biodiversity[14,11]. The increasing consumer awareness of the risks derived not only from food-borne pathogens, but also from the artificial chemical preservatives used to control them has led to renewed interest in so-called ‘‘green technologies’’ including novel approaches for a minimal processing and exploitation of bacteriocins for biopreservation[15,16]. Furthermore, an increasing demand for safe foods, with low level of chemical additives, has increased the interest in replacing these compounds by natural products, which are not harmful to the host or the environment. Thus, biopreservation of food has emerged as an attractive and safe approach[17]. Also, food safety has become an international concern and grater attention is being drawn towards application of natural and safe metabolites of lactic acid bacteria in foods as biopreservatives. Therefore, the objective of the present study has been focused on the isolation and identification of multifunctional lactic acid bacteria and screened them for antibacterial activity against some pathogenic and food spoilage bacteria.

MATERIALS AND METHODS

1. Samples collection

A total of 20 samples of fermented dairy products samples were collected from different places in Egypt. Traditional fermented dairy products samples included Karish cheese (7 samples), Laban Rayeb (concentrated sour milk) (8 samples) and fermented goat's milk (5 samples). All samples were collected in sterile bags and transferred to the laboratory under aseptic cooled conditions.

2. Isolation, purification and growth conditions

For preparing to experiments ten grams of each dairy sample were homogenized with 90 ml of sterile sodium citrate solution (2 % w/v) for cheese samples and 90 ml of (0.85 % NaCl w/v) sterile physiological saline for fermented milk samples and homogenized well in a stomacher lab-blender (Stomacher 400, England) for 30 seconds then the resulting homogenate was serially diluted up to 10^{-8} using sterile physiological saline (0.85 % NaCl w/v)[18]. One milliliter from each dilution was plated onto petri sterile dishes after that; M17 and de Man-Rogosa-Sharpe (MRS) agar were poured. All plates were incubated at 37°C for 48-72 h (BBL Gas Pak Anaerobic System, MD, USA). Representative pure colonies were randomly selected from M17 and MRS agar plates. Selected colonies were examined with an optical microscope for gram stain reaction and tested for catalase production. The purified isolates classified as Gram-positive, catalase-negative rods or cocci were cultured on suitable media and were kept in sterile reconstituted skim milk (12.5 % w/ v) supplemented with 1% yeast extract and 25 - 30 % (w/v) glycerol then stored at -20°C in deep freezer until used.

3. Identification of isolates

After obtaining purified cultures, all the strains under examination were sub cultured twice overnight in MRS or M17 broth and were performed identified by morphological, biochemical and physiological tests according to[19,20] as follows**.**

3.1. Gram Staining

The gram stain reactions of the isolates were determined by light microscopy after gram staining. Lactic acid bacterial cultures were known to be gram positive so, it means that they give blue-purple color by gram staining.

3.2. Catalase Test

Fresh liquid cultures of isolates were used for catalase test by dropping 3 % hydrogen peroxide solution onto 1 ml of overnight cultures. The isolates, which did not give gas bubbles, were known as catalase negative.

3.3. Co_2 production from glucose

Isolates were grown in culture tubes containing MRS or M17 broth supplemented with glucose and inverted Durham tubes. The prepared tubes were inoculated with 1% overnight fresh cultures then, the test tubes were incubated for 48-72 hrs at 37°C, Gas accumulation in Durham tubes was taken as the evidence for CO_2 production from glucose.

3.4. Growth at different temperatures

Temperature test media, MRS and M17 broth containing bromocresol purple indicator, was prepared and transferred into tubes as 5 ml. Then 0.1 ml of overnight cultures inoculated to tubes and incubated for 5 days at 10 °C, 15°C, 30°C 37°C and 45 °C. During this incubation time at previous temperatures the growth of isolates were observed by the change of the color, from purple to yellow[20].

3.5. Growth at different concentrations of sodium chloride

The procedure of Vinderola *et al.*[40] was used to determine the tolerance of isolates to different concentrations of sodium chloride. Isolates were grown in MRS or M17 broth supplemented with different concentrations of NaCl (2, 4, 6, 8 and 10 %), all test tubes were incubated at 37°C for 48-72 h and the growths were monitored by measuring the optical density at 620 nm (OD_{620}) using spectrophotometer (UV-VIS spectrophotometer PD-303 UV Apel Co., LTD Japan). The viable counts (Log cfu/ml) of isolated strains were determined by using MRS or M17 agar (Oxoid). After anaerobically incubated at 37°C for 48 h the viability (%) was also calculated as the following equation: viability (%) = (Log cfu/ml after 24 h / initial Log cfu/ml) × 100according to Desai *et al*.[21].

3.6. Carbohydrate fermentation pattern of isolates

The purified isolated strains were characterized and identified by using API®50 CHL strips for lactobacilli (isolates grown on MRS agar) and API®20 CH strep for streptococci (isolates grown on MR17 agar) (BioMerieux, Lyon Company, France). The **API®** test streps were prepared as recommended by the kit supplier and scored after incubation for 24 and 48 hours at 37ºC. These fermentation profiles were facilitated by systematically comparing all results obtained for the isolates studied with information from the computer database program APILAB PLUS (BioMerieux, Lyon Company, France).

4. Inhibitory activity against food borne pathogens

Antibacterial activities of isolated lactic acid bacteria were assayed using cell-free neutralized supernatants (CFNS) against food borne pathogens by paper disc diffusion assay as described by Mabrouk *et al.* [22]. The plates were incubated aerobically at 37°C for 24 h. the antibacterial effect of the supernatant was evaluated by measurement the inhibition zone diameter around the discs. Each experiment was performed in three replicates.

5. Phenotypic safety assessment of isolates

Isolated strains were tested for hemolytic activity using Columbia agar (Oxoid) with addition of 5 % (v/v) whole human blood. The results were recorded after 48 h of anaerobic incubation at 37°C according to the method described by Marakoudakis *et al*. [23].

RESULTS AND DISCUSSION

Lactic acid bacteria were the predominant microbial group in fermented dairy products, which is playing an important role in fermentation processes. The LAB used in commercial starter cultures possesses numerous metabolic characteristics such as production of organic acids, aroma compounds, bacteriocins and exopolysaccharides. All of these essential activities contribute to the texture, flavour and frequently attributes of fermented products[24,25]. Chosen eight strains of lactic acid bacteria isolated strains were initially differentiated on the basis of their cultural and morphological studies after which they were subjected to various physiological and biochemical tests. According to the obtained results (Table 1) the isolates were gram positive rods or cocci, catalase negative, did not produce CO_2 and ammonia from glucose and arginine respectively. LAB species isolated were found to belong to genus *Lactobacillus* which included many species e.g.(*rhamnosus, plantarum* and *pentosus*) followed by genus *Pediococcus.*

- Growth at different temperatures

Isolates were showed moderate growth on different temperatures and luxuries growth was observed for all isolates at 30, 37ºC. Also, the strains *Pediococcus pentosaceus* NRC AM1 and *Pediococcus pentosaceus* NRC AM4 were grown well in all temperature degrees. On the other hand the data showed that strains *Lb. brevis* NRC AM2, *Lactoccocus lactis* ssp. *lactis* NRC AM3 and *Lb. pentosus* NRC AM5 were not able to grow in the degree of 45 ºC.

- Growth at pH

The growth of eight strains in different pH values were differences. All eight strains were grown well in pH values (4, 6 and 8) but only strains *Pediococcus pentosaceus* NRC AM1, *Lb. brevis* NRC AM2 and *Pediococcus pentosaceus* NRC AM4 were grown in pH 2.

Table (1): Morphological, physiological and biochemical properties of the isolated lactic acid bacterial strains**.**

Characristics		isolated strains							
		1	2	3	4	5	6	7	8
Morphology		Cocci	rods	Cocci	Cocci	rods	rods	rods	rods
Growth at different temperatures (^{o}C)	10	+	-	+	+	-	-	-	-
	15	+	+	+	+	-	+	+	-
	30	+	+	+	++	+	+	++	++
	37	+	+	+	+	+	+	++	++
	45	+	-	-	+	-	+	±	+
Growth at pH	2	+	±	-	+	-	-	-	-
	4	+	+	+	+	±	±	+	+
	6	+	+	+	+	+	+	+	+
	8	+	+	+	+	+	+	+	+
NH3 from Arg.		+	+	+	+	-	-	-	-
Catalase		-	-	-	-	-	-	-	-
Gram stain		+	+	+	+	+	+	+	+
Co_2 from glucose		-	+	-	-	-	-	-	-
Growth at NaCl	2	+	+	+	+	+	+	+	+
	4	+	+	+	+	+	+	+	+
	6	+	+	+	±	+	+	+	+
	8	±	±	-	-	+	+	±	+
	10	-	-	-	-	+	-	-	+
Hemolysis*		-	-	-	-	-	-	-	-

* (+): growth, (±): weak growth, (++) high growth, (-): no growth
* In the test of hemolysis the sign (-) mean no blood hemolysis

1- *Pediococcus pentosaceus* NRC AM1 2- *Lb. brevis* NRC AM2
3- *Lactoccocus lactis* ssp. *lactis* NRC AM3 4- *Pediococcus pentosaceus* NRC AM4
5- *Lb. pentosus* NRC AM5 6- *Lb. rhamnosus* NRC AM6
7- *Lb. plantarum* NRC AM7 8- *Lb. pentosus* NRC AM8

- Phenotypic safety assessment

As shown in table one at the latest row phenotypic safety assessment of all isolated and identified lactic acid strains were tested in Columbia blood agar but there is no hemolytic activity were observed. The haemolytic reactions were recorded by observation of a clear zone around the colonies (β-hemolysis), a partial hydrolysis and greening zone (α-haemolysis) or no reaction (γ-haemolysis). The results of our study showed that, no haemolytic activities (γ-haemolysis) were observed for all lactic acid isolates. Thus, these isolates have not exhibit any pathogenicity and regarded as safe organisms. These results are in the harmony with the earlier and many reports which are revealed that, LAB do not exhibit haemolysis and those obtained by (Osmanagaoglu *et al*.[26], Gao *et al* [27], Malek *et al*.[28], Vidhyasagar and Jeevaratnam[29]) they reported that lactic acid starters are generally regarded as safe because they do not harm the host. No haemolytic activity was observed for any of lactic acid strains on blood agar. These results were interesting for a potential use isolated strains in dairy products *i.e*. fermented milks, different varieties of cheese and milk beverages.

Table (2): Carbohydrate fermentation patterns of the isolated lactic acid bacterial strains

	isolated strains							
Carbohydrates	1	2	3	4	5	6	7	8
L- Arabinose	+	+	+	+	+	+	+	+
D- Arabinose	-	-	-	-	-	-	-	-
D- Cellibiose	+	+	-	+	+	+	+	+
Esculin	+	+	+	+	+	+	+	+
D-Galactose	+	+	ND	+	+	+	+	+
Inulin	-	+	-	-	-	-	-	-
D- Lactose	-	+	+	-	+	+	+	+
D-Maltose	+	-	-	-	+	+	-	+
D- Manitole	-	+	+	-	+	+	+	+
D- Mannose	+	+	ND	+	+	+	+	+
Raffinose	-	+	-	-	-	-	+	+
L- rhamnose	+	-	ND	+	+	+	+	-
D-Mellibiose	-	-	ND	-	-	+	-	+
D- Ribose	+	+	+	+	+	+	+	+
Salicin	+	+	ND	+	-	+	+	+
D-Sorbitol	-	+	-	-	+	+	+	+
D- Sucrose	-	+	ND	-	+	+	-	+
D- Fructose	+	+	ND	+	+	+	+	+
D- Trehalose	+	+	-	+	+	+	+	+
D-Xylose	+	+	ND	+	+	+	+	-
Glycerol	-	-	ND	-	-	-	-	-
L-Xylose	-	-	ND	-	-	-	-	-
Erythritol	-	-	ND	-	-	-	-	-
L- Sorbose	-	-	ND	-	-	-	+	-
D- Glucose	+	+	ND	+	+	+	+	+

ND = Not detected

1- *Pediococcus pentosaceus* NRC AM1 2- *Lb. brevis* NRC AM2
3- *Lactoccocus lactis* ssp. *lactis* NRC AM3 4- *Pediococcus pentosaceus* NRC AM4
5- *Lb. pentosus* NRC AM5 6- *Lb. rhamnosus* NRC AM6
7- *Lb. plantarum* NRC AM7 8- *Lb. pentosus* NRC AM8

Furthermore, carbohydrate fermentation of the isolated lactic acid bacterial strains which were determined by using 49 acidification tests API®50 CHL strips for lactobacilli and API®20 CH strep for streptococci. The tests were done according to the instruction of the manufacture and anaerobiosis in the inculated strips was obtained by overlaying with sterile paraffin oil and incubated at 37°C for 48 h. According to the carbohydrate fermentation the data in table (2) revealed that all the strains showed variation in their sugar fermentation patterns. After preliminary phenotypic characterization tests and interpretation of the API® database, the isolated strains could be successfully identified as *Lactobacillus rhamnosus* (1 strains), *Lactobacillus plantarum* (1 strains), *Lactobacillus pentosus* (2 strains), *Pediococcus pentosaceus* (2 strains), *Lactobacillus brevis* (1 strain) and *Lactoccocus lactis* ssp. *Lactis* (1 strain). These results were agreement with those obtained by several researches focused on the isolation and characterization of LAB from war milk and traditional fermented dairy products[30,24,25,31-34]. Survival (Log cfu/ ml) of isolated strains at various concentrations of sodium chloride at zero time and after incubation period at 37° C for 24 were presented in Table (3) and (4). The results showed that, the counts of all tested strains in MRS broth supplemented with different concentrations of NaCl at zero time nearly the same log counts but after incubation period at 37° C for 24 the counts were changed a lot because the high concentrations of NaCl had more effects on the survival of strains. The high survival (Log cfu/ ml) counts were recorded in control medium (MRS broth without NaCl) which ranges from 10.57 to 10.10 with *Lb. brevis* NRC AM2 and *Lactococcus lactis* ssp. *lactis* NRC AM3 respectively. The counts of all strains were decreased by increasing the concentrations of NaCl and one strain did not grow in the presence of 8 % NaCl (*Lb. brevis* NRC AM2). On the other hand four strains were able to grow in 10 % NaCl (*Lactococcus lactis* ssp. *lactis* NRC AM3, *Lb. pentosus* NRC AM5, *Lb. plantarum* NRC AM7 and *Lb. pentosus* NRC AM8). Furthermore, Table (5) showed that the growth of isolates measured as changes in OD_{620}

nm during incubation at 37^{o}C for 30 h. with increasing the concentrations of NaCl the growths were affected and only four previous strains still grow in the presence of 10 % NaCl. These four strains had a good resistance to high concentrations of NaCl[2433].

Table (3): Survival (Log cfu/ ml) of isolated strains tested in MRS medium supplemented with different concentrations of NaCl at zero time.

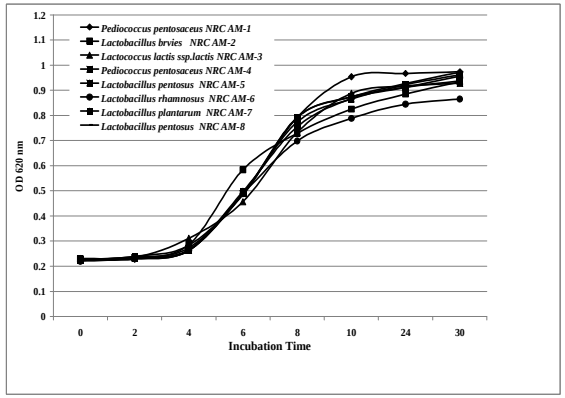
Isolated strains	NaCl concentrations %					
	control	2	4	6	8	10
1	8.24	8.35	8.29	8.23	8.10	8.11
2	8.18	8.32	8.10	8.95	8.22	8.14
3	8.83	8.24	8.37	8.29	8.35	8.64
4	8.51	8.30	8.26	8.95	8.16	8.10
5	8.77	8.37	8.34	8.51	8.17	8.85
6	8.67	8.33	8.19	8.36	8.97	8.11
7	8.39	8.22	8.11	8.40	8.13	8.10
8	8.45	8.41	8.20	8.32	8.15	8.28

Table (4): Survival (Log cfu/ ml) of isolated strains tested in MRS medium supplemented with different concentrations of NaCl incubated at 37° C for 24 h.

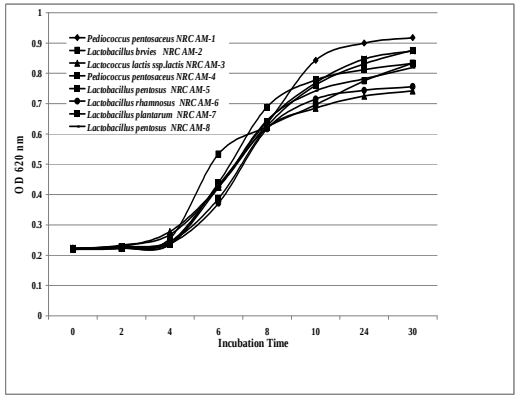
Isolated strains	NaCl concentrations %					
	Control (MRS without NaCl)	2	4	6	8	10
1	10.14	9.24	7.16	6.96	5.23	ND
2	10.10	9.16	7.51	5.25	ND	ND
3	10.57	9.24	8.18	7.10	ND	ND
4	10.33	8.18	6.27	5.11	4.46	ND
5	10.29	8.23	7.38	6.56	5.17	4.89
6	10.15	8.22	7.39	5.15	4.10	ND
7	10.28	9.14	8.46	6.20	5.13	4.00
8	10.32	9.25	8.13	7.45	6.15	5.48

1- *Pediococcus pentosaceus* NRC AM1 2- *Lb. brevis* NRC AM2
3- *Lactococcus lactis* ssp. *lactis* NRC AM3 4- *Pediococcus pentosaceus* NRC AM4
5- *Lb. pentosus* NRC AM5 6- *Lb. rhamnosus* NRC AM6
7- *Lb. plantarum* NRC AM7 8- *Lb. pentosus* NRC AM8

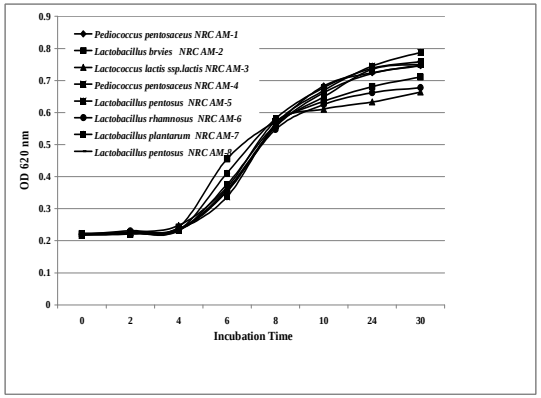
Fig. (5): Growth curves of isolated lactic acid bacteria in MRS broth with or without different concentration of NaCl measured as changes in OD_{620} nm during incubation at 37o C for 30 h.



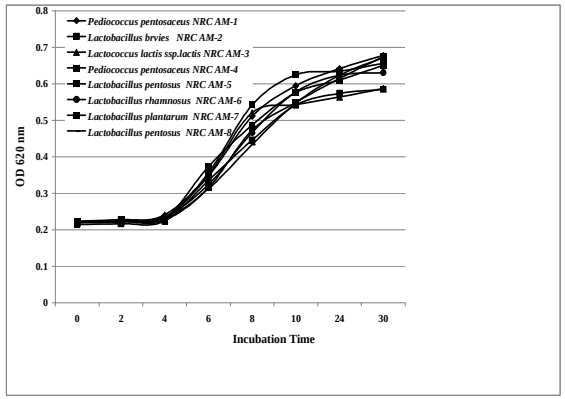
MRS broth without NaCl (Control)



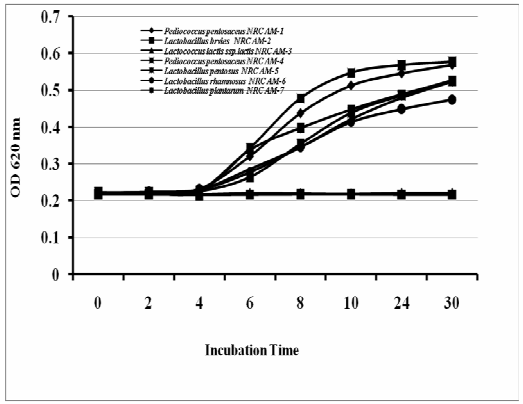
MRS broth with 2 % NaCl



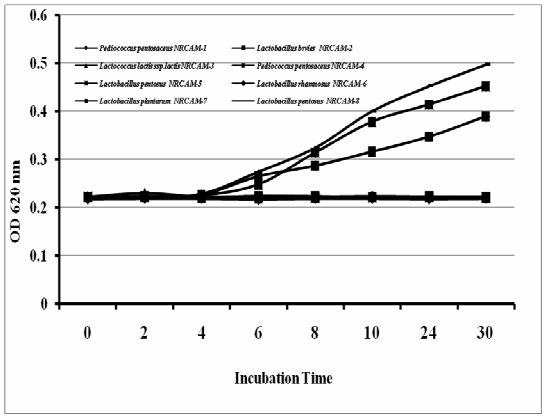
MRS broth with 4 % NaCl



MRS broth with 6 % NaCl



MRS broth with 8 % NaCl

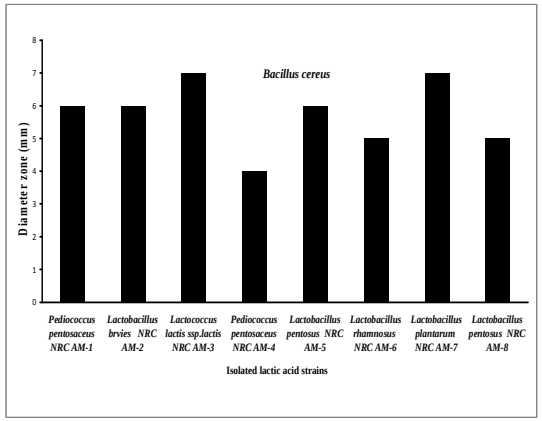


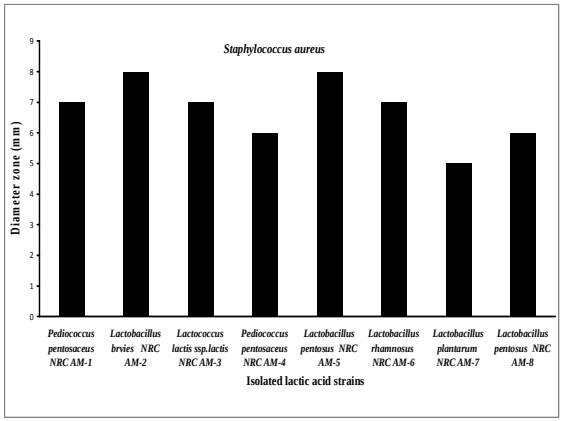
MRS broth with 10 % NaCl

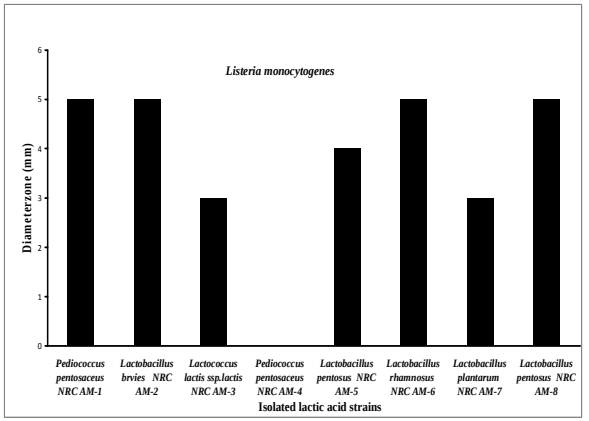
- Inhibitory activity against food borne pathogens

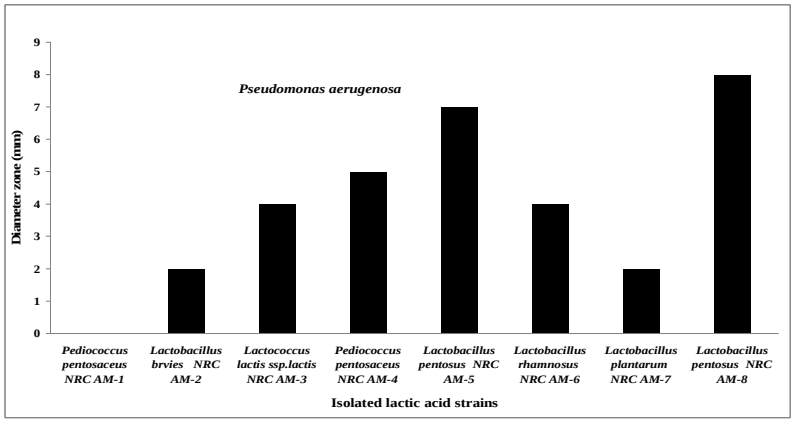
Antibacterial activities of isolated lactic acid bacterial strains tested against 5 indicator pathogenic strains are presented in Figure (6) the results showed that, there are differences were found between 8 lactic acid bacterial isolated strains against indicator pathogenic bacteria. All indicator pathogenic strains were inhibited by the supernatant of isolated lactic acid bacterial strains except *Listeria monocytogenes* Type I not affected by the supernatant of *Pediococcus pentosaceus* NRC AM4 and *Pseudomonas aeruginosa* not affected by the extract of *Pediococcus pentosaceus* NRC AM1.On the other hand, all isolated lactic acid bacterial strains showed a good inhibitory activities toward 5 indicator pathogenic strains. The diameter zones are included between 2 to 10 mm and the biggest clear zones were recorded by the extracts of *Pediococcus pentosaceus* NRC AM1 and *Pediococcus pentosaceus* NRC AM4 with *Escherichia coli* 0157:H7, but the lowest clear zones were obtained with the extracts of *Lb. brevis* NRC AM2 and *Lb. plantarum* NRC AM7 against *Pseudomonas aeruginosa.* Most extracts of isolated lactic acid bacterial strains were had a good inhibitory activities toward Gram positive and Gram negative pathogenic indicator strains. These results are in agreement with the literature data and scientific reports which mentioned that bacteriocins of lactic acid bacteria have a good broad spectrum activity against pathogens and food spoilage bacteria[35-39].

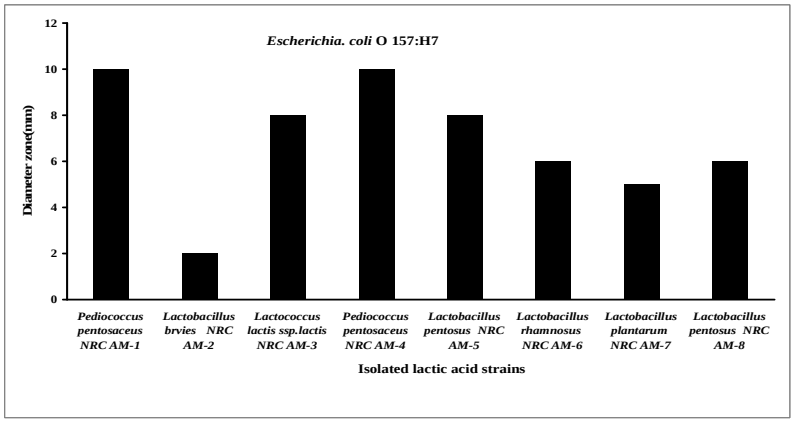
Fig. (6): Antibacterial activities of 8 selected isolated lactic acid strains against food pathogens











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