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ACTIVITY OF ANTI-LACTIC ACID BACTERIA ISOLATED FROM MILK AND MILK PRODUCTS AGAINST PATHOGENIC BACTERIA OF HUMANS

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ABSTRACT

The study included isolation and diagnosis of types of lactic acid bacteria from animal milk and local products, and the detection of the inhibitory ability of their metabolites against some types of pathological bacteria, where types of lactic acid bacteria were isolated from 25 samples that included fresh animal milk (cows, camels, goats) and a dairy product traditional Libyan (Laban Rayeb).126 isolates were obtained from lactic acid bacteria. They were identified on the basis of the common properties of the lactic acid bacteria based on the cultural, microscopic, and biochemical tests. The identity of some isolates was also confirmed by using the system: API 50CHL, Where possible Diagnosis of 4 isolates belonging to *Lb.fermentum* And 3 isolates belong to *Lb.bulgaricus*. And 2 isolates belong to *Lb.casei* And 2 isolates belong to *Lb. salivarius* And 2 isolates belong to *Lb. acidophilus* And 1 isolates belong to *Lb. plantarum* And1 isolates belong to Lb. lactis. The efficacy of the isolates filtrate in inhibiting the growth of some types of pathogenic bacteria was tested by using the test of disks saturated with the filtrate of the selected isolates. It was found that lactic acid bacteria filtrates have different inhibitory efficacy against some types of pathological bacteria, as the diameter of the inhibition zones ranged between 3-35 mm, Six of these isolates of the LAB which includes (Lb96, Lb5, Lb131, Lb151, Lb155 and Lb114) showed the highest inhibiting activity.

INTRODUCTION

Lactic acid bacteria (LAB) are widely found in many nutrient-rich media such as milk, meat, drink, vegetables, as well as in soil, lakes, and the intestinal tract of animals and humans.[1] Lactic acid bacteria are a group of Gram-positive, non-spore forming, cocci or rods, catalase-negative, and fastidious organisms, with high tolerance for low pH.[2,3] LAB are among the most important microbes which are used in food fermentations, as well as enhancing taste and texture in fermented food products.[2,4] They are characterized by the production of lactic acid as the main product from glucose and growth inhibition substances such as bacteriocins, hydrogen peroxide, diacyls, etc. which prevent the proliferation of food spoilage bacteria and pathogens.[5,6] The LAB group is currently classified in the phylum Firmicutes, class Bacilli, and order Latobacillales. LAB are classified based on cellular morphology, mode of glucose fermentation, range of growth temperature, and sugar utilization patterns.[7] LAB genera include *Lactobacillus, Lactococcus, Leuconostoc, Pediococcus, Streptococcus, Aerococcus, Alloiococcus, Carnobacterium, Dolosigranulum, Enterococcus, Oenococcus, Tetragenococcus, Vagococcus* and *Weissella*,[8,9] with Lactobacillus being the largest genus, including more than 100 species that are abundant in carbohydrate-rich substances.

The majority of Lactobacillus species have been isolated from the gastrointestinal tract of humans and animals. The second largest number of Lactobacillus species are from vegetables and their fermentation products.[10,11] The fermentation of sugar, which produces the acid in milk, which many bacteria have done for a long time, has been applied by humans to many food products, and it plays an essential and important role in the manufacture of fermentation and dairy products.[12] The antimicrobial effect of lactobacilli is primarily linked to the production of organic acids, such as lactic acid, acetic acid, propionic acid, and sometimes hydrogen peroxide, bacteriocins, and antimicrobial peptides (AMPs) with a variable range of action.[13,14] Lactic acid bacteria ferment carbohydrates to obtain energy, using endogenous carbon sources as the final electron acceptor instead of oxygen.[15] They are categorized into homofermentative and heterofermentative microorganisms, based on the products of the fermented carbohydrates. Homofermentative LAB mainly produce lactic acid from sugars, whereas heterofermentative LAB produce lactic acid, acetic acid or alcohol and carbon dioxide.[16,17] In addition, some species of LAB produce antimicrobial peptides known as bacteriocins. To date, several LAB isolates from the Lactobacillus genus and their bacteriocins have been applied in food preservation and in the control of human pathogens.[18] Due to their health benefits, some LAB are used as probiotics. Probiotics are organisms such as bacteria or yeast that improve human or animal health, and are available in

supplements and fermented foods such as yoghurt, or as nutritional supplements that contain live bacteria for building up the intestinal microbiota.[4] Several authors have documented the ability of various LAB to inhibit growth of pathogenic microorganisms, their ability to degrade mycotoxins, their probiotic capabilities, as well as antimicrobial activities of cell-free extracts of the LAB isolates from different sources.[19,20] While LAB have been used as starter cultures and bacteriocins as food preservatives, there is a need for more information on the inhibitory potential of LAB against human pathogenic bacteria, especially in the current era of antibiotic resistant pathogens.

The specific objectives of this study were (i) to isolate strains of LAB from fresh and fermented animal milk; (ii) to screen isolated LAB strains for their inhibition of selected human pathogenic bacteria; (iii) to identify the LAB strains selected

MATERIALS AND METHODS

Isolation and identification of LAB from fresh and fermented animal milk

Samples for isolation of LAB

The isolation material was fresh animal milk (Cow, Camel, Goats) obtained from different local farms and also traditional Libyan dairy products such as Laban Rayeb. (Table 1). The samples were collected in sterile containers and stored on ice until delivery to the laboratory. Once delivered to the laboratory, they were processed for isolation of LAB.

Table 1: Number of samples taken from each type of animal

Sample	Cow milk	Camel milk	Goats milk	fermented Cow milk	Fermented Goat milk
Number of Sample	5	5	5	5	5

Isolation and identification of lactic acid bacteria (LAB):

Samples were diluted to 10^{-1}, 10^{-2} and 10^{-3} using sterile peptone water. 1 ml aliquot of the samples and dilutions were plated into MRS agar (Man, Rogosa and Sharpe).[21] Plating was done within 3 – 6 hrs of arrival of the samples in the laboratory and incubated at 37°C for 48hrs. After incubation, individual colonies having different appearances were selected and subcultured from MRS agar plates and plating on MRS agar to give purified isolates.[22] The pure isolate selected were identified on the basis of their cultural morphological and biochemical characteristics.

Morphological Observation of Lactic Acid Bacteria Colonies

Observations of colony morphology was done after getting a pure culture in a petri dish. These observations were made visually including shape, color, edges, and the elevation of bacterial colonies. The surface of colonies can be seen from the side and the edge of the colony can be seen from the above.[23]

Morphological Observation of Lactic Acid Bacteria Cells (Gram Staining)

One of bacteria isolates was sampled aseptically and smeared on a slide and fixed several times over Bunsen burner flame. Then bacteria isolates were etched with two drops of crystal violet and allowed for 1 minute. Furthermore, isolates were washed with distilled water and air dried. Then, iodine was dropped on it and allowed to stand for 1 minute, and isolates were washed with distilled water and air dried. Bacteria isolates were mixed with drops of 95% alcohol slowly for 15- 20 seconds, then isolates were washed with distilled water and air dried. Isolates were then stained by safranin slowly for 30 seconds. Then, isolates were washed with distilled water and then air dried again. Bacteria isolates preparates were observed using a microscope. Observations included the color and shape of a bacterial cell. Gram-positive bacteria marked by the purple (or blue) color which indicates that the bacteria cell wall are capable of binding to crystal violet dye, whereas Gram negative bacteria is characterized by the formation of pink color indicates that the bacteria cell wall are not able to bind the crystal violet dye, and only stained by safranin dye (as comparative dye).

Motility Test

Hanging-drop wet method was performed as described by MacFaddin 2000. The slide was observed under a light microscope with 40x magnification to check the motility of the bacteria.[25]

Catalase Test

The catalase test is one of the most useful diagnostic tests for the recognition of bacteria due to their simplicity. To perform this test, a single isolated colony was streaked on a glass slide and one drop of 3 % (H2O2) hydrogen peroxide was added on to it. The effervescence of oxygen indicated the bacteria produce the catalase enzyme which converts H2O2 to water (H2O) and oxygen (O2).[23,24]

Classification of isolates studied using API 50CHL system:

Confirmation of the identification was based on the use of (API 50CHL) analytical profile index is a system, based on quick identification of clinically anaerobic bacteria.[26]

Preservation of Lactobacillus isolates

Isolates were grown in MRS broth medium with 5% inoculum and incubated at 37 ° C for (24-48) hours, then

kept in refrigerator temperature and activated every month.[27]

Pathogenic bacterial strains:

The pathogenic indicator strains used in this work were provided by the Laboratory of medical laboratory, technology Department at the Faculty of engineering and technology, university of sebha, Libya. They included both Gram- negative (Escherichia coli, Pseudomonas sp., Salmonella sp, Proteus sp, Klebsiella sp) and Gram-positive strains (Staphylococcus aureus, Streptococcus sp). Determination of anti-microbial activity Fifteen LAB Strains were selected for anti-microbial activity experiment

Preparation of Cell Free Supernatant (CFS) of the LAB grown in the MRS broth

Cell free filtrates were prepared according to Kalalou et al., 2004. The LAB cultured on the MRS agar were inoculated each into 10 ml of MRS broth and incubated for 24±2 hours at 37^0C. After the incubation, the inocula was transferred into the centrifuge tubes and centrifuged at 10,000 rpm for 15 minutes. The solvent was then filtered using the syringe filter with a pore size of 0.02 µm and a disposable syringe.[28]

Antimicrobial activity test by disk assay method:

The disk assay method was used to determine the antimicrobial property of the LAB isolates. The pathogenic bacterial cultures of (*Escherichia coli*, *Pseudomonas* sp., *Salmonella* sp, *Proteus sp*, *Klebsiella sp*, *Staphylococcus aureus* and *Streptococcus* sp), were grown in Nutrient liquid medium at 37 °C. After 24 h of growth, each microorganism was inoculated on the surface of Mueller-Hinton agar plates. Subsequently, filter paper discs (5 mm in diameter) saturated with supernatant broths of selected isolated LAB were placed on the surface of each inoculated plate. The plates were incubated at 37 °C for 24 h. After this period, after incubation the plates were observed for a zone of inhibition (ZOI) around the filter paper discs. Results were considered positive if the diameter (mm) of the ZOI was greater than 1mm, controls were the solvents used for each extract and the phytochemicals and they showed no inhibitions in preliminary studies.[29]

RESULTS AND DISCUSSION

Isolation and Identification of Lactic acid bacteria:

25 samples of fresh animal milk (Cow, Camel, Goat) and traditional Libyan dairy products such as Laban Rayeb were used as an isolation source. Lactic acid bacteria were isolated from medium at 37°C under aerobic conditions. From approximately 200 isolates, 126 isolates remained at the end of the isolation (Table 1), these isolates were initially diagnosed based on the formation of a transparent aura around their colonies growing on a solid (MRS) medium containing calcium carbonate which dissolves with acid produced from bacteria. The MRS medium is well suited for the growth of lactobacilli isolated from milk because it contains all the nutrients needed for the growth of lactic acid bacteria. The isolated bacteria were observed by light microscope, it is clear that the bacteria were gram positive, rod shaped coccobacilli, occurring singly or in chains. The gram staining results indicated that the isolated bacteria could be identified as lactobacilli.[14] Hanging-drop wet method showed that the isolated bacteria were nonmotile. Therefore, the nonmotile behavior is a characteristic of Lactic acid bacteria.[30] Isolates were tested for catalase activity. They were all catalase negative.

Table 1. Origin and Number of isolates from fresh animal milk and traditional Libyan dairy products sample after screening for *Lactobacillus* spp.

Source of isolation	No. of isolates	No. of *Lactobacillus* spp.
Camel milk	83	64
Cow milk	36	19
Goat milk	39	22
Cow's Laban Rayeb	18	6
Goat's Laban Rayeb	24	15
Total	200	126

The identity of some of the isolates was further confirmed using the API 50CHL system. It was possible to diagnose 4 isolates belonging to *Lb.fermentum*, 3 isolates belonging to *Lb.bulgaricus*, 2 isolates belonging to *Lb . salivarius*, 2 isolates belonging to *Lb. casei*, 2 isolates belonging to *Lb. acidophilus*, 1 isolate belonging to *Lb. lactis* and 1 isolate belonging to *Lb. plantarum* as shown in Table (2) Lilia et al. (2002) reported that it is easy to isolate *Lb.fermentum* and *Lb.bulgaricus* from dairy products.[31] Also, these results are in line with Parvez et al (2006) who indicated that these types are found in dairy products and have importance in the vital strengthening of the natural flora in the digestive system as it was found to improve intestinal health, enhance the immune system, reduce symptoms of lactose intolerance syndrome and reduce the spread of allergies. It reduces the risk of spreading certain types of cancers such as stomach and intestinal cancer.[32]

Table 2. Isolate types of lactic acid bacteria and sources of isolation.

NO	Type of bacteria	Source of isolation	Isolation number
1	*L. fermentum*	Camel milk	ES114, ES151, ES97
		cow milk	ES130
2	*L. acidophilus*	Laban Rayeb	ES150, ES131
3	*L. lactis*	cow Laban Rayeb	ES53
4	*L. casei*	Goat's Laban	ES122, ES199
5	*L. plantarum*	Goat milk	ES5
6	*L. bulgaricus*	Camel milk	ES96, ES116, ES155
7	*L. salivarius*	Camel milk	ES111, ES91

Antimicrobial Activity

The 15 isolates selected after isolation from milk and its derivatives were subjected to a study that reveals their ability to prevent the growth of pathogenic bacteria, through the development of isolates in MRS broth. The ability of the filtrate of each isolate to inhibit the pathogenic bacteria including *Escherichia coli, Pseudomonas sp., Salmonella sp, Proteus sp, Klebsiella sp, Staphylococcus aureus and Streptococcus sp*.by the disk examination method, the results showed that the filtrate of bacteria containing bacterial products possesses the ability to inhibit the growth of pathogenic bacteria used in the experiment. The pathogenic bacteria varied in their response to the filtrate of Lactobacillus, the diameter of inhibition zones (Table 3) showed that there are differences in the inhibitory activity of lactobacilli against the test bacteria, where the average diameter of inhibition zones around the pits ranged from 3-35 mm. Results indicate that isolate ES114 had the highest antimicrobial activity, against all the indicator pathogens tested except *Klebsiella sp* and *Staph aureus* amongst the 15 isolates. Its activity was highest against *Pseudomonas sp* with a ZOI of (35mm) and least for *E. coli* (3 mm) (Figure 1). Isolate ES155 also showed antimicrobial activity against all tested pathogens except *E. coli*, *Klebsiella sp* and *Proteus sp* with its activity being highest against *Streptococcus sp* (32 mm) and least against *Pseudomonas sp* (7mm). Isolate ES151 also showed antimicrobial activity against all tested pathogens except *Klebsiella sp* and *Staph aureus* with its activity being highest against *E. coli* (28 mm) and least against *Salmonella* and *Proteus sp* (3mm). Isolate ES131 showed greater activity against *E.coli* (34 mm) and least against *Salmonella* (3 mm). Isolate ES5 showed antimicrobial activity against 2 tested pathogens with its activity being highest against *Staph aureus* (25 mm) and least against *Proteus sp* (3mm). Isolate ES96 showed antimicrobial activity against 3 tested pathogens with its activity being highest against *Proteus sp* (20 mm) and least against *Staph aureus*(5mm).

Table (3): rates of inhibition zones diameters (mm) for filtrates of lactic acid bacteria isolates against test bacteria by disk examination method.

Isolates	Indicator Microorganisms						
	Diameter of inhibition zones(mm)						
No	*E. coli*	*Salmnella*	*Pseudomonas sp*	*Proteus sp*	*Klebsiella sp*	*Staph aureus*	*Strepto coccus sp*
ES114	3mm	5mm	35mm	5mm	0	0	9mm
ES151	28mm	3mm	7mm	3mm	0	0	15mm
ES150	0	0	0	0	13mm	10mm	0
ES131	34mm	3mm	5mm	0	0	0	0
ES96	14mm	0	0	20mm	0	5mm	0
ES130	0	0	0	3mm	0	0	15mm
ES199	0	0	0	0	10mm	0	0
ES97	0	0	3mm	11mm	0	0	0
ES116	0	18mm	0	15mm	0	0	0
ES122	0	0	0	5mm	0	0	0
ES5	0	0	0	3mm	0	25mm	0
ES53	0	0	0	7mm	0	3mm	0
ES155	0	12mm	7mm	0	0	19mm	32mm
ES91	3mm	0	0	5mm	16mm	0	0
ES111	5mm	0	11mm	0	0	0	3mm

Figure 1: Disk-diffusion method of of lactic acid bacteria isolates against test bacteria.

The current study shows the inhibitory ability of lactic acid bacteria filtrate towards some pathogenic bacteria, and the reason for this may be attributed to the production of lactic acid bacteria, organic and inorganic acids such as lactic and acetic acid, and that the inhibitory effect of these two acids is due to their non-disintegrating form through their ability to penetrate and hinder the transport of foodstuffs. In addition to being weak and partial organic acids that dissolve in aqueous solutions and possess the property of solubility in fats, which makes them rapidly and freely spread through the plasma membrane into the cytoplasm.[33] In addition to the role of lactic acid bacteria play in reducing pH, oxidation and reduction effort, and competition for nutrients, in addition to the production of antagonists and bacteriosins.[34] Also, the difference in the inhibition processes may be due to the fact that the production of inhibitory substances by the lactic acid bacteria needs to be received on the surfaces of the cells that affect them and then they perform their work by making holes in the cell membranes leading to the exit of small components from the cells and then death.[35]

By this study it was noted the filtrates of Lactobacillus bacteria isolated from fresh animal milk (Cow, Camel, Goat) and traditional Libyan dairy products possess an inhibitory ability towards the pathological bacterial species under study, and this opens the door for their use as biological enhancers for the treatment of some diseases.

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