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RESEARCH ARTICLE

Effect of Probiotic Extracted from *Lactobacillus* Sp. Antagonist *Helicobacter pylori* of Human Stomach Ulcer

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Abstract

Total of (130) samples of stomach ulcer were collected from patients suffering from peptic ulcer who visited three different hospitals in Baghdad. After testing the sensitivity test against *Helicobacter pylori* against 10 antibiotics, the sensitivity test for 10 antibiotics demonstrated the efficacy of the antibiotic cephotaxim and rifampicin in the elimination of the bacteria. According to the results of the sensitivity test, one of the isolates of the *Helicobacter pylori 4 (*HpG 4) was chosen for being resistant to all antibiotics (except amoxicillin). Two isolates of *Lactobacillus acidophilus*, *Lactobacillus plantarum* were selected based on their high inhibitory activity against *Helicobacter pylori* growth bacteria for subsequent experiments .The minimum inhibitory concentration of Lactic Acid Bacteria concentrated filtrate was performed for three times .The concentrated filtrate showed the efficacy in minimizing and inhibiting the growth of pathogenic bacteria Helicobacter *pylori.* So the aim of the study was to discover the role of probiotics in the treatment of *Helicobacter pylori* infection and study of effect of probiotic on reducing *Helicobacter pylori* stomach ulcer as supporting and safer.

Keywords: *Human Stomach Ulcer; Probiotic Extracted; Lactic Acid Bacteria*

Introduction

Helicobacter pylori infection, a highly prevalent pathogen, is a major cause of chronic gastritis and peptic ulcer and a risk factor for gastric malignancies, antibiotics-based *Helicobacter pylori* eradication treatment is 90% effective, however, and it is expensive and causes side effects and antibiotic resistance [1]. Probiotics could present a low-cost, large-scale alternative solution to prevent or decrease *Helicobacter pylori* colonization; a literature search of the Medline database (1966-2006) has been performed selecting all in vitro, animal, and human fully published English-language studies dealing with *Helicobacter pylori* and probiotics.

Probiotics had an in vitro inhibitory effect on *Helicobacter pylori*, animal studies demonstrated that probiotic treatment is effective in reducing *Helicobacter pylori*-associated gastric inflammation [2].Seven of 9 human studies showed an improvement of *Helicobacter pylori* gastritis and decrease in *Helicobacter pylori* density after administration of probiotics. The addition of probiotics to standard antibiotic treatment improved *Helicobacter pylori* eradication rates [3]. Probiotic treatment reduced *Helicobacter pylori* therapy-associated side effects. No study could demonstrate the eradication of *Helicobacter pylori* infection by probiotic treatment. So long-term intake of products containing probiotic strains of probiotics have a favorable effect on *Helicobacter pylori* infection in humans, particularly by reducing the risk of developing disorders associated with high degrees of gastric inflammation, [4]. Wounds of stomach related framework is considered as the most famous infections around the world, which are identified with poor nourishment, thriving and sanitation conditions and additionally hereditary and natural effects.

Ulcers as irresistible maladies, which taint stomach related framework, had been found by [5] and viewed as another period with respect to the ideas and treatment of gastro duodenal. It was observed that Helicobacter pylori to be the fundamental driver of gastritis, peptic ulcers and gastric disease. Helicobacter pylori is considered as an effective pathogenic bacterium that instigates

gastritis in all discovered tainted patients and perceived as class-I cancer-causing agent that especially colonizes the gastric epithelial cells of human [6]. Helicobacter pylori have numerous harmful systems where the urease compound gives good microenvironment to the practicality of the microscopic organisms in the stomach corrosive medium by means of the creation of alkali at high movement and amount to ensure it, three fundamental gastric phenotypes have been distinguished and there are vary among perpetual and intense contamination which rely upon a few conditions [7].

Treatment with anti-infection agents was appeared to be the correct method to kill this bacterium, anyway in light of the development of anti-toxin obstruction in numerous nations it makes treatment troublesome, along these lines, the scan for better treatments against Helicobacter pylori is required, clinical examinations demonstrated that some probiotic strains can kill Helicobacter pylori infection so that when patients treated with probiotics diminished Helicobacter pylori , so probiotics used as helpful in the treating of Helicobacter pylori contamination [8].

Materials and Methods

Stomach Ulcer Sample Collection: *(Helicobacter pylori)*

A total of 130 stomach ulcer biopsy samples from male and female were collected for survey from (May 2017 to May 2018) from different hospitals in Baghdad governorate. A biopsy sample were spread on blood agar, MacConkey and columbia agar plates. Plates then were incubated over night under micro aerophillic condition. Single colonies which were non lactose fermenters, and gave negative reaction to oxidase test and growing on columbia were transferred to selective media with supplement of *Helicobacter pylori*. The process was repeated several times for purity before use for further diagnosis.

Biochemical Tests of Helicobacter Pylori Isolates

One presumptive *Helicobacter pylori* colony from each selective agar plate was sub cultured and tested by standard microbiological and biochemical procedures, differentiated at species level by Gram stain, oxidase and catalase test, hydrogen sulfide production and susceptibility to nalidixic acid by using a commercially available species differentiation kit API CAMPY.

Sensitivity of Helicobacter Pylori to Antibiotic

Ten ml of nutrient broth were inoculated by each bacterial isolate, and then incubated under micro aerophilic condition for 24 hr. to log phase (O. $D._{600}$ about 0.35) giving ($1*10^8$) cell / ml of broth. After that, 0.1 ml of the inoculated broth was transferred and spread by sterile cotton swab on Muller-Hinton agar plates surface in three different planes (by rotating the plate approximately 60˚ each time to obtain an even distribution of the inoculums).

The inoculated plates were then placed at room temperature for 30 minutes to allow absorption of excess moisture. With a sterile forceps the selected antibiotic disks were placed on the inoculated plates and incubated under micro aerophilic condition for 18 hr in an inverted position. After incubation, the diameter of inhibition zones was measured by a ruler (mm). Results were determined and compared according to the National Committee for Laboratory Standards [9].

Yoghurt Sample Collection: (*Lactobacillus*)

Yoghurt samples specimen were collected in sterile tubes under aseptic and cooled conditions from Baghdad markets from September/2017 to February/2018. A total of 35 samples were aseptically collected from local market .Serial dilutions of samples were made. From the last dilution, 1 ml was transferred to the poured MRS plates and incubates over night at 37˚C under anaerobic conditions using gas generating kit. After incubation, colonies were surrounded by inhibition zone and G +ve and catalase -ve were selected and transferred to MRS broth and incubated [10].

CHL 50 API System Identification for *Lactobacillus* isolates:

A suspension is made in the medium with the microorganism to be tested and each tube of the strip is then inoculated with the suspension. During incubation, the carbohydrates are fermented to acids which produce a decrease in the pH, detected by the change in color of the indicator.

The results makeup the biochemical profile which is used by the identification software to identify the strain.

Determining Inhibitory Effect of LAB

On Solid Medium (MRS Agar)

A culture of LAB previously grown in MRS broth was streaked on MRS agar, and then incubated under anaerobic conditions at 37˚C for 24 hr [11]. After incubation a cock porer (5mm) was used to withdraw discs of LAB growth and put on surface of the nutrient agar that was inoculated (before) with 0.1 ml of pathogenic bacteria. After incubate, at 37˚C for 24 hr, the inhibition zone around the disc was estimated in (mm). Same procedure was repeated by using different incubation times of LAB (18, 24, and 48 hr) to determine the optimum incubation time that gives greater inhibition effect.

In Liquid Medium (MRS Broth)

MRS broth was inoculated by 1% of LAB culture, then incubated an aerobically at 37˚C for different period of times (18, 24 and 48 hr) .After incubation the culture was centrifuged at 6000 rpm for 15 minutes, the supernatant was obtained. After adjusting the pH of the filtrate to 6.5 by using Noah (1ml), it was filtered through Millipore filter unit (0.22 µm).Then well diffusion method that mentioned by [12] was used; when nutrient agar plates which was inoculated with 0.1ml of each pathogenic bacteria by a spreader. Then (5mm) wells were made by a cock porer. Each well were filled with the LAB filtrate, and then incubated at 37˚C for (18, 24 and 48 hr).

The inhibition zone around the well was measured by (mm) and compared with that of the control which contained MRS broth without bacteria .The filtrate was concentrated by freeze-dryer and the well diffusion method was repeated to detect the effect of each concentrated filtrate against the pathogenic bacteria. Control was containing concentrated MRS broth without LAB.

Determination of Minimum Inhibitory Concentration for Concentrated Filtrate

Different concentrations of each concentrated filtrate were made in tubes containing sterile nutrient broth each. The concentrations were (1/9, 2/8, 3/7, 4/6, 5/5, 6/4, 7/3, 8/2 and 9/1) giving final volume 10ml in each tubes. Then each concentration was inoculated by 0.1ml culture previously grown in nutrient agar (*Helicobacter pylori*) and incubated at 37 ˚C for 24 hr. After incubation the growth of tubes was observed and minimum inhibitory concentration was determined as the lower concentration of the filtrate that gave no growth of *Helicobacter pylori* in the tubes.

Result and Discussion

Identification of *Helicobacter pylori*

The forty five isolation was diagnosed based on the tests used for this purpose, which is the form of colonies, the colour of a grams stain, the urease enzyme test and the enzyme test , oxidase and catalase and motility test and obtained (20) isolates were subjected to other tests as well as previous tests, which included test the growth in temperature (25) C and (42) C and the sensitivity test of the antibiotics cephalotoxin and nalidixic acid .CAMPY test and molecular diagnosis were made to 10 isolates of pathogenic bacteria.

CAMPI Api for Characterization of Helicobacter pylori

The API Campy strip consists of 20 micro tubes containing dehydrated substrates. It is made up of two parts. The first part of the strip (enzymatic and conventional tests) is inoculated with a dense suspension which rehydrates the substrates. During incubation (in micro aerobic conditions),metabolism produces color changes that are either spontaneous or revealed by the addition of reagents .The second part of the strip (assimilation or inhibition tests) is inoculated with a minimal medium and incubated in micro aerophilic conditions.

The bacteria grow if they are capable of utilizing the corresponding substrate or if they are resistant to the antibiotic tested .The reactions are read according to the Reading Table .The identification is obtained by consulting the profile list in the package insert or the Identification Table if necessary. The identification software can also be used, [13].

Sensitivity Test of Helicobacter Pylori for Antibiotics

The emergence of prevalence of antibiotic resistance strain is considered as a major therapeutic problem that could be explained by several hypothesis such as, the influence of excessive and /or in appropriate antibiotic use

[14], transmission of resistant isolates, among people, consumption of food from animals that had received antibiotics, and greater mobility of individual worldwide have also contributed to the extension of antibiotic resistance [15].In this study effect of *Helicobacter pylori* was tested by using standard disk diffusion method, and results obtained were compared with those of NCCLs, (1991). The results indicated that the local isolation of the *Helicobacter pylori* was 100% resistant for amoxicillin and 10% for antibiotics, rifampicin and cephotaxim, and 20% resistance to tetracyclin and doxycycline, six isolates resistant to erthromycin and clarthromycin 30% resistance, antibiotic resistance ratio gantamycin is 50%, and the resistance to ciprofloxacin is equal to 80%. Moreover, results indicated in Table (1) show the frequency of resistance *Helicobacter pylori* isolates to used antibiotics.

Table 1: Frequency of antibiotic resistance of *Helicobacter pylori* isolates.

Antibiotic	Symbol	Resistant isolates	
		Number	Percentage (%)
–β-lactam penicillin			
Amoxicillin	AMX	20	100%
–Cephalosporins			
Cephotaxim	CTX	2	10%
– Aminoglycosides			
Gentamycin	GM	9	50%
– Tetracyclines			
Tetracycline	TE	4	20%
– Quinolons			
Ciprofloxacin	CIP	16	80%
– Others			
Metroidazole	MT	16	80%
Clarthromycin	CLR	6	30%
Rifampcin	RA	2	10%
Arythromycin	E	6	30%
Ducoxy cycline	Do	4	20%

Identification of Lactic Acid Bacteria

Colonies of LAB on MRS agar were pale, round shape, soft, mucoid, convex and surrounded by inhibition zone as a result of dissolving calcium carbonate *Lactobacillus* appeared blue, bacilli, mainly grouped in long to chain containing (3-8 cells), non-spore formers.

API 50 CHL Systems

Fermentation of carbohydrates was determined using API 50 CHL, a standardized system, consisting of 50 biochemical tests for the study of carbohydrate metabolism by microorganisms. API 50 CH is used in conjunction with API 50 CHL medium for the identification of *Lactobacillus* and related genera strips according to the manufacturer's instructions (Biomerieux, Marcy l' Etoile, France) [16].

10 ml of pure water was dispensed into the incubation box with the strip placed in the incubation box, after the bacterial cultures had been introduced into the API 50 CHL system in API 50 CHL medium (5 ml), in concentration 2 McFarland. The set-up system was then incubated at appropriate temperature of 35°C for 48 h, after the wells were filled with the bacterial suspensions by the line mark with the addition of mineral oil.

Inhibitory Effect of LAB

On Solid Medium (MRS Agar)

Propagating LAB isolates on MRS agar under anaerobic conditions was the most efficient method for production their inhibitory metabolites against tested pathogenic bacteria. Despite that all LAB isolates, exhibited serious inhibitory effect on *Helicobacter pylori* isolates, an inhibitory effect of LAB isolates *lactobacillus acidophillus* was the most effect against *Helicobacter pylori*, where as *lactobacillus plantarum* also have effect on *Helicobacter pylori* but less than *lactobacillus acidophillus*, when inhibitory zone reached 18.5 mm after 24 hr incubation time, while *lactobacillus plantarum* have 17 mm after the same incubation time.

Moreover, such LAB isolate (*lactobacillus acidophillus*) was only effective against *Helicobacter pylori* it also gave highest inhibition zone against the other *Helicobacter pylori* isolates for both incubation times (18

and 24 hr). Results shown in Figure (1) LAB isolate exhibited better inhibitory effect on *Helicobacter pylori* isolates after incubation for 24 hr.

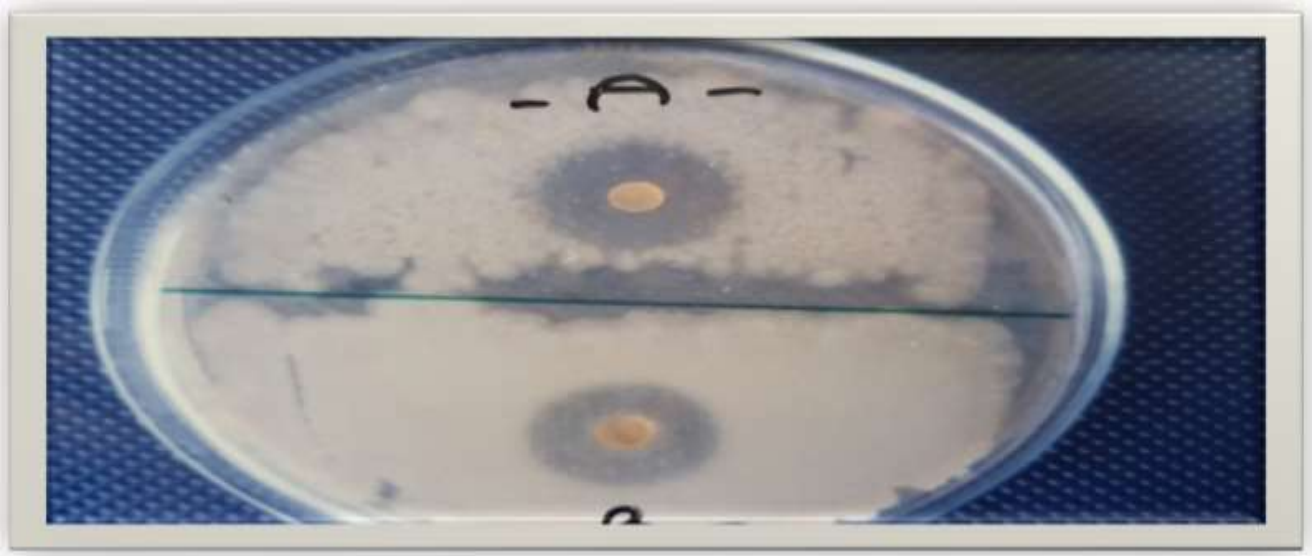

Figure 1: Inhibitory effect of *Lactobacillus plantarum* and *Lactobacillus acidophilus* against *Helicobacter pylori* isolate on solid medium (MRS agar) *A-* *Lactobacillus acidophilus* after 24 hr. of incubation on MRS agar giving zone diameter of (18.5) mm. ***B-*** *Lactobacillus plantarum* after 24 hr of incubation on MRS agar giving zone diameter of (17) mm

In Liquid Medium (MRS Broth)

Inhibitory effect of LAB isolates grown in MRS broth was evaluated against the tested isolates of *Helicobacter pylori*. Well diffusion method was used by filling the wells with made in nutrient agar which is cultured by the *Helicobacter pylori* with the filtrate of two LAB isolates (*Lactobacillus plantarum* and *Lactobacillus acidophilus*). Selection of such two isolates depended on their ability in production best inhibitory effect.

Maximum inhibition zone diameters reached (20) mm which is a highest than that recorded by solid medium, this may be due to the existence in MRS broth exhibited a wide spectrum inhibitory effect against positive bacteria (*Staphylococcus aureus, Bacillus subtilis*) and gram negative bacteria (*E. coli, Klebsiella spp., Proteus spp.*) when the inhibition zone diameter ranged between (13-19) mm [17].

To study the effect of incubation time period in the liquid medium the two isolates of LAB were grown for (18, 24 and 48) hr. Incubation period of 24 hr of gave the best inhibitory effect *Lactobacillus plantarum* reached to (18 mm) against tested *Helicobacter pylori* isolates. Increasing incubation period to 48 hr resulted in least inhibitory effect for *Lactobacillus plantarum* and isolates.

Lactobacillus acidophilus gave its optimum inhibitory effect after 24 hr incubation not 48 hr, the reason for such two LAB isolates due to that the inhibitory material (aciophilin, plantaracin) loose its activity when secreted outside the cells while increase in incubation time. Results shown in Figure (2) and (3) show the inhibitory effect of *Lactobacillus plantarum* and *Lactobacillus acidophilus* respectively against the tested isolate *Helicobacter pylori* liquid media for different incubation time (18, 24 and 48) hr.

Figure 2: Inhibitory effect of *Lactobacillus plantarum* against *Helicobacter pylori* isolate in liquid medium (MRS broth) A– *Lactobacillus plantarum* filtrate after 18 hr. incubation in MRS broth giving zone diameter (12) mm. B– *Lactobacillus plantarum* filtrate after 48 hr. incubation in MRS broth giving zone diameter (15) mm. C– *Lactobacillus plantarum* filtrate after 24 hr. incubation in MRS broth gibing zone diameter (18) mm

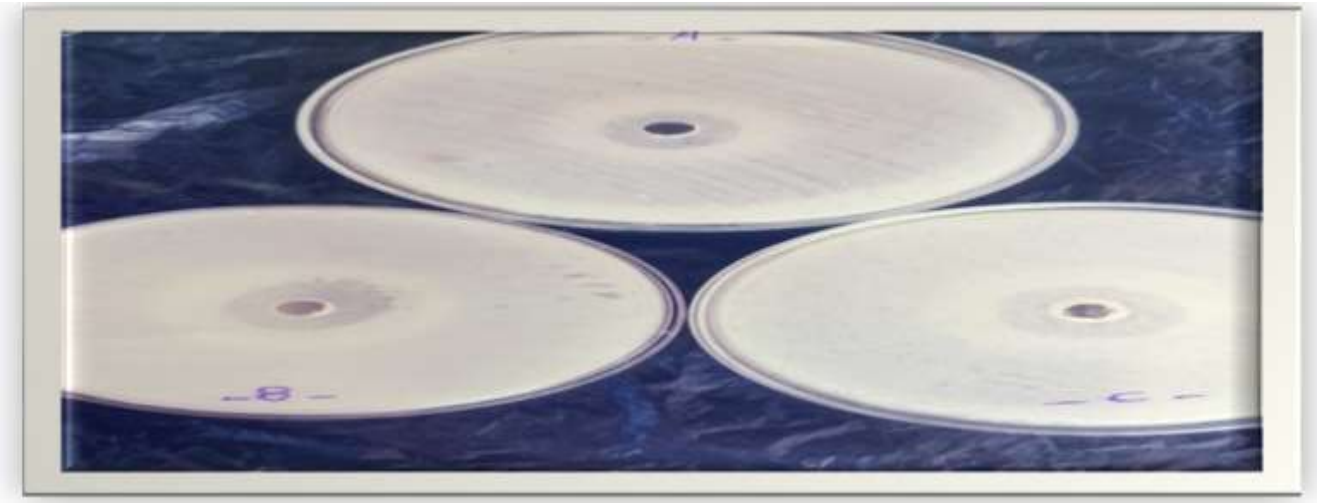

Figure 3: Inhibitory effect of *Lactobacillus acidophilus* against *Helicobacter pylori* isolate in liquid medium (MRS broth) A-*Lactobacillus acidophilus* filtrate after 18 hr. incubation in MRS broth giving zone diameter (15) mm. B-*Lactobacillus acidophilus* filtrate after 48 hr. incubation in MRS broth giving zone diameter (16) mm. C-*Lactobacillus acidophilus* 24 hr. filtrate after incubation in MRS broth gibing zone diameter (20) mm

Minimum Inhibitory Concentration of LAB Filtrates against *Helicobacter pylori*: Determining MIC, s of LAB against *Helicobacter pylori* Growth

To determine MIC,s of the filtrates of LAB isolates which inhibit or minimize *Helicobacter pylori*, serial dilutions were prepared from the three-fold filtrates of isolates *Lactobacillus acidophilus* and *Lactobacillus plantarum*. Result in Table (2) show that diluents 1:9 and 2:8 (Filtrate: medium) had no effect on *Helicobacter pylori* when clear growth of this test bacteria was observed. Diluents 3:7 and 4:6 of filtrates led to minimize growth of the test bacteria. Filtrate of *Lactobacillus plantarum* in the dilutions 7:3 and above also caused total inhibition for the test bacteria when growth of isolate *Helicobacter pylori* was completely inhibited by such diluents. On the other hand diluents 6:4 and above of *Lactobacillus acidophilus* filtrate completely inhibited growth of *Helicobacter pylori* isolate. Depending on the just mentioned findings, diluents 6:4 of *Lactobacillus plantarum* and 5:5 of *Lactobacillus acidophilus* were selected and recorded as the MIC, s of the two LAB filtrates against growth of the test bacterial isolate *Helicobacter pylori*.

Table 2: Minimum Inhibitory Concentrations (MIC) of Lactic Acid Bacteria (LAB) against *Helicobacter pylori* isolate

MIC / LAB isolate	1:9	2:8	3:7	4:6	5:5	6:4	7:3	8:2	9:1
Lacto. plantarum	+	+	+	+	+	+	–	–	–
Lacto. acidophilus	+	+	+	+	+	–	–	–	–

+ = growth-= no growth

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