

## Using *Lactobacillus* as probiotic to inhibit growth and adhesion of *Proteus mirabilis* causing urinary tract infection

استخدام بكتيريا *Lactobacillus* كـ probiotic لمنع نمو و التصاق بكتيريا *Proteus mirabilis* المسببة لالتهاب المجاري البولية

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### Abstract

The aim of the study was to use lactic acid bacteria (LAB), as probiotic, to treat growth and adhesion property of *Proteus mirabilis* isolated from patients suffering from urinary tract infection (UTI). For this purpose, one *P. mirabilis* isolate (P.M.9) was selected out of 9 isolates obtained from 150 urine specimens. Due to its resistance to 11 antibiotics tested, this isolate was treated with three-fold concentrated filtrates of two lactobacillus isolates (as probiotic). Results after treatment, showed that the filtrates exhibited significant inhibitory effect against the pathogenic P.M.9 and its adhesion property especially when only an average of 3-10 bacteria /cell were adhered to each epithelial cell compared to 44-55 bacteria/cell.

### المستخلص

هدفت الدراسة الى امكانية استخدام بكتيريا حامض اللاكتيك (LAB) Probiotic لمعالجة نمو و صفة التصاق بكتيريا *Proteus mirabilis* المعزولة من مرضى يعانون من التهاب المجاري البولية (UTI). لهذا الغرض اختيرت عزلة P.m.9 من مجموع 9 عزلات تم عزلها من 150 عينة ادراج مرضى التهاب المجاري البولية اعتمادا على مقاومتها 11 مضاد حيوي ، هذه العزلة عوملت بالحد المثبط الادنى لمستخلص رواشح بكتيريا حامض اللاكتيك ( Probiotic). اظهرت النتائج بعد المعاملة ان مستخلص بكتيريا حامض اللاكتيك يمتلك قابلية تثبيطية ضد البكتيريا الممرضة P.M.9 و صفتها الالتصاقية خصوصا وان معدل الالتصاق تراوح بين 3-10 بكتيريا/خلية مقارنة بمعدل الالتصاق قبل المعاملة والذي يتراوح بين 44-55 بكتيريا/خلية .

مستل من اطروحة ماجستير الباحث الثاني

**Introduction:**

During the last decades, microorganisms and their metabolic products were broadly used in treatment of various diseases and infections. Normal flora, such as lactic acid bacteria (LAB), found in the gastrointestinal tracts can produce different types of materials; organic acids, ammonia, hydrogen peroxide, diacetyl, bacteriocins and other compounds which had been used as inhibitory means against pathogenic bacteria. LAB were used to treat gastric disturbance, colon irritation, diarrhea and even colon carcinom [1]. One of the most common causes of UTI is *Proteus mirabilis* which have many various virulence factors, such as adhesion, swarming, urease, hemolysin and protease production that causing infection [2]. Adhesion is the initial step of *Proteus mirabilis* infection [3]. Investigations suggested that pili are the adherence element responsible for binding uropathogenic *Proteus mirabilis* to uroepithelium [4]. Adhesion involves complex interaction between pili and specific complex carbohydrate (as receptors) of host cell membrane [5]. It

was found that probiotic (LAB isolates) have inhibitory effect on the adherence of bacteria, and can alter some surface structures of gram negative bacteria without killing it [6].

The adherence of bacteria to biological surface is a complex process which often involves lock and key type interaction between bacterial attachment fimbriae and specific complex carbohydrate structures of the host cell membrane (receptors). It was also found that bacteria which are high adherence are more virulent than that of less adherent ability [7].

The word Probiotic is derived from the Greek and means (forlife). It was first used by [34]. Probiotic is a microbial dietary adjuvant that beneficially affects the host physiology by modulating mucosal and systemic immunity, as well as improving nutritional and microbial balance in the intestinal tract [8]. Currently probiotic preparations contain, *Lactobacillus acidophilus*, *Lactobacillus Plantarum*, *Lactobacillus casei*, *Lactobacillus fermentum*, *Lactobacillus lactis*,(9).

**Aims of the Study**

- A- Isolation and identification of *Proteus* from patients suffering from UTI.
- B- Isolating the most antibiotics-resistant isolate of *Proteus mirabilis* to be used in the probiotic experiment.

C- Investigating the inhibitory effect of LAB isolates against the pathogenic *Proteus mirabilis*.

D- Determining the inhibitory effect of LAB filtrates on the adhesion property of *Proteus mirabilis* isolate.

### Materials and methods

#### Urine sample collection:

Mid stream urine samples specimen were collected in sterile tubes from patient of AL-Karama hospital and AL-Yarmoq hospitals in Baghdad. A total of 150 samples were aseptically collected and transported to the laboratory as fast as possible.

Two isolates of identified LAB were taken from Biotechnology Department/College of Science/Al-Nahrain University (*Lactobacillus plantarum* and *Lactobacillus acidophilus*).

#### Isolation of *P.M*:

One loopfull of undiluted urine sample was spread on blood agar and MacConkey agar plates. Plates were then incubated over night at 37°C. Single colonies which were non lactose fermenters, and gave negative reaction to oxidase test and making swarming on blood agar were transferred to blood and MacConkey agar. The process was repeated several times for purity before use for further diagnosis.

**Microscopic Examination of *Proteus mirabilis*:** A loopfull of *Proteus* isolates was fixed on a microscopic slide, then

stained by gram stain to examine cells shape, grouping, reaction and non-spore forming [10].

#### Api 20E Identification for *Proteus mirabilis* Isolates:

Identification was carried out by subculturing of selected colonies grown on MacConkey agar into Api 20E microtubes gallery. This system is designed for the performance of more than 20 standard biochemical tests from a single colony grown on plating medium. Each test in this minimized system is performed within a sterile plastic microtube which containing appropriate substrates and was fixed to an impermeable plastic strip (gallery) each gallery contain 20 microtubes including the biochemical test and sugar fermentation. Inoculation of the galleries was done with sterile pasture pipette and five ml of tap water dispensed into tray provide a humid atomsphere then incubated at 37 °C for 24 hr. After that reagent added for reading the galleries, each positive reaction was given a value 1,2 or 4 according to the position of the test in its group, so a value from 0 to 7 digit observed was then looked up in the

index and the identification is determined.

#### **Sensitivity of *Proteus mirabilis* to Antibiotics:**

Ten ml of nutrient broth were inoculated with each bacterial isolate, then incubated at 37 °C to log phase (O. D.<sub>600</sub> about 0.35) giving (1\*10<sup>8</sup>) cell / ml of broth. Then, 0.1 ml of the inoculated broth was transferred and spread 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 at 37°C for 18 hr in an inverted position. After incubation, the diameter of inhibition zones was measured by a ruler (mm). Results were recorded and compared according to the National Committee for Clinical Laboratory Standards [11].

#### **Bacterial Adhesion Test (12)...**

##### **Preparation of *Proteus mirabilis* Suspension:**

Ten milliliter of nutrient broth medium was inoculated with bacterial growth, the culture was then incubated at 37°C for over night (O. D.<sub>600</sub> about 0.4) giving (1\*10<sup>9</sup>) cell / ml. cultures of bacteria were washed twice with PBS and

centrifuged at 1000 rpm for 20 minutes and resuspending in PBS.

##### **Preparation of Epithelial Cells:**

Uroepithelial cells were isolated from the urine of some healthy females by centrifugation at 1000 rpm for 5 minutes then washed three times with PBS and centrifuged at 1000 rpm for 10 minutes before resuspended in PBS.

##### **Adhesion Test:**

- A mixture of 0.2 ml of the bacterial suspension, 0.2 ml of the epithelial cells suspension and 0.1 ml of PBS was incubated at 37 °C for one hour.
- Unattached bacteria to uroepithelial cells were removed by centrifugation in PBS at 1000rpm for 10 minutes.
- The final pellet was resuspended in PBS then a drop was placed onto a microscope slide, air-dried fixed with methanol : acetic acid (3:1) and stained with methylene blue.
- The adherent bacteria to epithelial cells were observed by the compound light microscope.

Control of only epithelial cells was included.

##### **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 [13]. After incubation a cork porer (5mm) was used to withdraw discs of LAB growth and placed on surface of the nutrient agar that was inoculated (before) with 0.1 ml of pathogenic bacteria. After incubation, at 37°C for 24 hr, the inhibition zone around the disc was measured 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 with 1% of LAB culture, then incubated anaerobically at 37°C for different period of times (18, 24 and 48 hr) [14]. 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 NaOH 0.4 N (1ml), it was filtered through Millipore filter unit (0.22 µm).Then well diffusion method that mentioned by [15] was used; when nutrient agar plates which was

## Results and Discussion

### Isolation of *Proteus* Isolates:

One hundred and fifty midstream urine samples were collected from patients suffering from symptoms referred as urinary tract infection. It was found that 116 (77.3%) out of the total 150 samples

inoculated with 0.1ml of each pathogenic bacteria by a spreader. Then (5mm) wells were made by a cork porer. Each well was filled with the LAB filtrate, and then incubated at 37°C for (18, 24 and 48 hr).

**Effect of Concentrated Filtrate on Adhesion of *Proteus mirabilis*:**The minimum inhibitory concentration of the concentrated filtrate of LAB isolates was used to investigate the effect on adhesion property of *Proteus mirabilis*. For this purpose method described by [12] was applied which was mentioned previously with the addition of the following steps:

- Nutrient broth medium containing minimum inhibitory effect of concentrated filtrate was dispensed in sterile tubes and incubated with a loopfull of liquid culture of *Proteus mirabilis*, then incubated for 24 hr.
- Bacterial adhesion test was done as indicated previously. Adhesion free concentrated filtrate was prepared as control.

collected gave positive results on MacConkey agar and Blood agar. These results were near to those reported by [16] and [17] who found that the percentage of positive cultures of urine samples were (84%) and (83%)

respectively. But such results were in disagreement with those of [18] in Zymbaboy when found that percentage of positive culture of urine samples was (27%). The reason of the differences in percentage may be owed to differences in size and number of hospital surveyed as well as to the season and medications before sampling.

Results indicate that *Proteus* found in both sexes, but its percentage of isolation in male samples was higher (62.5%) when 10 isolates were belonged to them, while (37.5%) percentage of isolation in female when 6 isolates were belonged to them, this result agree with those reported by [19] who found that the percentage of isolation of *Proteus* in male was (63.5%) while it was (36.5%) in female. However, effect of vaginal fluid could be suggested as a killer agent in female which has low pH may act naturally as a selection pressure against *Proteus* [20].

#### **Identification of *Proteus mirabilis*:**

##### **Cultural Characteristics:**

In accordance to their pale colony appearance on MacConkey agar as non lactose fermenters, and swarming motility on blood agar after 24 hr incubation, the suspected isolates are considered to be as *Proteus* isolates which require more identification processes to be identified for species.

##### **Morphological Characteristics:**

microscopical examination, Gram staining examination, showed that cells of the suspected isolates appeared purple, non spore former, rods and motile.

Moreover, identification of the isolates was confirmed by using Api system (Api 20E) the findings obtained by the conventional biochemical tests.

##### **Antibiotics Sensitivity of *Proteus mirabilis*:**

The emergence of prevalence of antibiotic resistance strains is considered as a major therapeutic problem that could be explained by several hypothesis such as, the influence of excessive and or inappropriate antibiotic use, 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 lresistance[21].

In this study the effect of antibiotic on *Proteus mirabilis* was tested by using standard disk diffusion method, and results obtained were compared with those of NCCLs, (1991). Antibiotic resistance among *Proteus mirabilis* isolates varied according to the nature of the isolate or antibiotic. Among them no single antibiotic was resisted by all the isolates of *Proteus mirabilis* or sensitive to it. However, amikacin was the most

effective antibiotic when only one isolate (Pm4) of *Proteus mirabilis* resisted it, while all others were sensitive. Ciprofloxacin was the second highly effective antibiotic when all isolates, except two (Pm3 and Pm9), were except resistant to it. Gentamycin was the third except three (Pm1, Pm7 and Pm9), were resist to it. On the other hand, penicillin G was the least effective antibiotic because all isolate, except (Pm7), were sensitive to it.

Followed by amoxicillin, chloramphenicol and tetracycline which were sensitive by only two isolate each. The results reported in this investigation, that isolate *P. m* 9 was the strongest isolate which was resisted to almost all antibiotics, so it selected from other isolates to study the adhesion property and the inhibitory effect of LAB.

#### **Adhesion of *Proteus mirabilis*:**

Ability of *Proteus mirabilis* to adhere to uroepithelial cells is considered as an important virulence factor in pathogenesis of urinary tract infections. In this study, adherence property of *Proteus mirabilis* as well as how this property may be affected by LAB isolates was investigated. Adherence ability of *Proteus mirabilis* to uroepithelium which observed by viewing under oil-immersion of the compound light microscope which was

include the uroepithelium from healthy female and the infected uroepithelium (UEP), where the *P. m.* 9 appeared as rod and adhere to the uroepithelium (by pili), the results show that the highest number of adhering bacteria to UEP (rang from 45-55 bacteria/cell) recorded by isolate *P. m.* 9, this result almost in agreement with that recorded [22]who found that the highest number of mucous strain of *Pseudomonas aerogi nosa* adhere to the tracheal epithelium was 45 bacteria/cell, but disagreed with the result of [4] that the highest number of adherent of *Proteus mirabilis* to UEP was 29 bacteria/cell. *Proteus mirabilis* usually has fimbriae which are considered as adherence elements responsible for binding of uropathogenic *Proteus mirabilis* to uroepithelial cell [4]. [22] stated that mucoid strains of *Pseudomonas aeroginosa* contain two adherence elements namely fimbriae and alginate.

#### **Inhibitory Effect of LAB:**

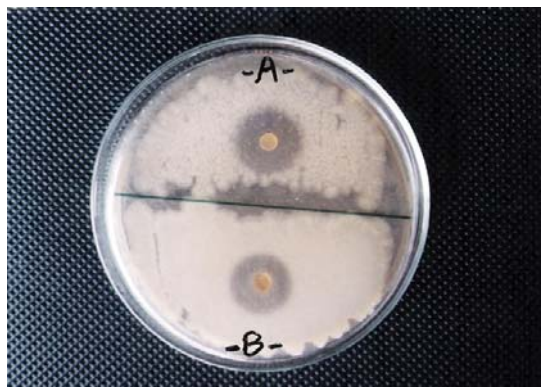
##### **On Solid Medium (MRS Agar):**

Propagating LAB isolates on MRS agar under anaerobic conditions was the most efficient method for production of their inhibitory metabolites against tested pathogenic bacteria. Despite that all LAB isolates, exhibited serious inhibitory effect on *Proteus mirabilis* isolates, an inhibitory effect of LAB

isolates *Lb. a.11* was the most effective against *P. m. 9*, where as *Lb. p.3* also have effect on *P. m. 9* but less than *Lb. a. 11*, when inhibitory zone reached 18.5 mm after 24 hr incubation time, while *Lb. p. 3* have 17mm after the same incubation time. Moreover, such LAB isolate (*Lb. a. 11*) was effective against *P. m. 9* it also gave highest inhibition zone against the other *P. m.* isolates for both incubation time (18 and 24 hr). Results showed that the general that almost all LAB isolates exhibited better inhibitory effect on *P. m.* isolates after incubation for 24 hr. Generally incubation period of (24 hr) resulted in production of more inhibitory effect by almost all LAB isolates against all *P. m.* isolates than the incubation period of 18 hr. However, when longer incubation periods (30 hr and more) were tested on

some isolates result in no difference in inhibition zone were recorded or, in some times lower. *Lb. p.* have also inhibitory effect against tested isolates and its effect increased after (24 hr) incubation (24 hr) and this was it could be due to lactocidin which produce from *Lb. p.* [23] and due to plantaracin which was active against the tested isolates [24]. While the *Lb. a.* have the highest inhibitory effect after (24 hr) incubation and this was due to acidophilin produce from *Lb. a.* [25].

LAB have an inhibitory effect against gram negative and positive bacteria [26]. [27] stated that LAB has a high inhibitory effect against enteropathogenic bacteria. So the inhibitory effect of *Lb. p 3* and *Lb. a. 11* against *P. m. 9* have the highest inhibitory effect



**Plate (2):** Inhibitory effect of *Lactobacillus plantarum 3* and *Lactobacillus acidophilus 11* against *Proteus mirabilis 9* isolate (MRS agar). A- *Lactobacillus acidophilus 11* after 24 hr. of incubation on MRS agar giving zone diameter of (18.5) mm. B- *Lactobacillus plantarum 3* 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 *Proteus mirabilis*. Well diffusion method was used by filling the wells which made in nutrient agar plates which is cultured the *P. m. 9* with the filtrate of two LAB isolates (*Lb. p. 3*, *Lb. a. 11*). Selection of these two isolates depended on their ability in production best inhibitory effect. Maximum inhibition zone diameters reached 20mm which is highest than that recorded by solid medium, this may be due to the existence in MRS broth exhibited a wide spectrum inhibitory effect against gram 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 [28]. To study the effect of incubation time period in the liquid medium the two isolates of LAB were grown for (18, 24 , 48) hr. Incubation period of 24 hr gave the best inhibitory effect by *Lb. p. 3* the inhibition zone diameter reached to 18 mm against tested *Proteus mirabilis* isolates. Increasing incubation period to 48 hr resulted in least inhibitory effect for *Lb. p. 3* isolates. *Lb. a.11* also gave optimum inhibitory effect after 24hr incubation and not after 48 hr, the reason

for such two LAB isolates may be that the inhibitory materials (acidophilin, plantaracin) loose activity when secreted outside the cells after increasing the incubation time. The investigation also includes the inhibitory effect of concentrated filtrates on tested isolate. The filtrates of *Lb. p. 3* and *Lb. a. 11* were concentrated three fold by using freeze-dryer. The first and second fold of concentrated filtrates of *Lb. p. 3* have zone diameter (20,21)mm, respectively against *P. m. 9* while first and second fold of concentrated filtrates of *Lb. a. 11* have zone diameter (21,23)mm, respectively against *P. m. 9*, while the third fold has the highest inhibitory effect after 24 hr incubation because all the inhibitory substances was concentrated, zone diameter of *Lb. p. 3* against *P. m. 9* reached to 27mm and *Lb. a. 11* against *P. m. 9* have zone diameter reached to 30mm. Incubation time of 18hr and 48hr gave inhibitory effect less than effect after 24hr incubation so [29] stated that there is a relationship between the diameter of inhibition zone and the concentration of inhibitory substances. On the other hand [30] mentioned that death of tested bacteria increased by the increasing of inhibitory substances like bacteriocin and acidophilin and plantaracin of LAB.



**Plate (5):** Inhibitory effect of concentrated filtrates for *Lactobacillus plantarum* 3 and *Lactobacillus acidophilus* 11 against *Proteus mirabilis* 9 isolate. A– Control (contain concentrated MRS). B– *Lactobacillus acidophilus* 11 concentrated filtrate after 18 hr. incubation in MRS broth resulted zone of inhibition with (25) mm diameter. C– *Lactobacillus plantarum* 3 concentrated filtrate after 18 hr. incubation in MRS broth resulted zone of inhibition with (23) mm diameter. D– *Lactobacillus acidophilus* 11 concentrated filtrate after 24hr. incubation in MRS broth resulted zone of inhibition with (30) mm diameter. E– *Lactobacillus plantarum* 3 concentrated filtrate after 24 hr. incubation in MRS broth resulted zone of inhibition with (27) mm diameter

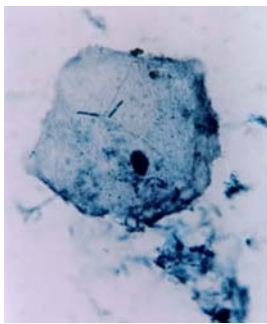
#### **Adhesion Inhibition by LAB Filtrates:**

The initial step in the infection of host cells by *Proteus mirabilis* to the host cell is the adhesion so interference with adhesion process cause a prevention of infection. Effect of concentrated filtrates of LAB against adhesion property of *Proteus mirabilis* was investigated using [12]. Results obtained showed that the three-fold concentrated filtrated of LAB isolates (*Lb. p.* 3 and *Lb. a.*11) against tested *P. m.*9 was observed to minimizing adhesion of *P. m.* 9 to the uroepithelial cells reaching an average of (3-10) bacteria/cell. It was clearly observed that the adhesion of *P. m.* 9 to UEPCs was clearly minimized. This may be due to the effect of the inhibitory

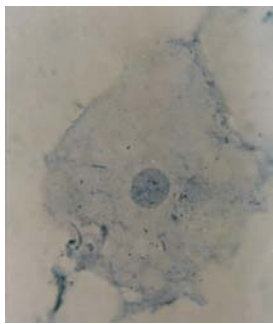
substances found in the filtrates of the LAB isolates and to the acidic pH which affect growth of the gram negative bacteria by altering some surface structures (like pili), leading to prevent bacterial cells from adhesion to UEPCs with out killing the bacteria [6]. Some authors were reported partial and complete inactivation of adherence of several gram negative uropathogenes. they investigated the inhibitory effect produced by *Lactobacillus casei* on *E. coli* (which is also uropathogenic), and found that the inhibitory effect was not caused by the bacteriophage or hydrogen peroxide but due to the coaggregation of *E. coli* and *Lb. c.* in urine which was occurred after

20hr at 37°C. The prevalence of inhibitory-producing LAB on the uropathogenes and the ability of LAB to interact closely with the uropathogenes seem to constitute an important host defense mechanism against infection [31]. [32] reported that precoating of LAB strains reduced the binding of uropathogenic coagulase-negative

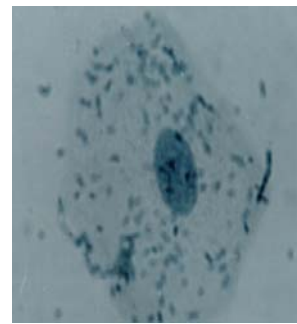
*Staphylococci* and *E. coli* to 8 bacteria/cell, So biosurfactant surlactin as released by *Lactobacillus* isolates may open the way to the development of anti-adhesive biologic coating against *Enterococcus faecalis*, they reported a decrease in the percent of adhering *Enterococcus* which was reach to approximately 70%.



-A-



-B-



-C-

**Microscopical examination of adhesion of *Proteus mirabilis* cells to uroepithelium cells (1000x). A: after treating *Proteus mirabilis* (*P. m. 9*) with three-fold filtrate of probiotic LAB (isolate *Lb. p. 3*), B: *Proteus mirabilis* (*P.m.9*) with probiotic treating three-fold filtrate of LAB (isolate *Lb. a. 11*), C: Adhesion of *Proteus mirabilis* (isolate *P. m. 9*).**

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