# Inhibitory effects of Probiotic on growth and adhesion of some gram negative pathogenic bacteria

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**Abstract:**

This work focused on three important human pathogens; *Escherichia coli, Salmonella typhi , Klebsiella pneumonae*. Results showed that there is several virulence factors in this bacteria such as capsule, enzymes, motility and fimbriae. *Escherichia coli* included fimbriae type I, II and III . *Salmonella typhi* contained only type II and III. While *Klebsiella pneumonae* included type I and III. Probiotic preparations from *Lactobacillus acidophilus* appeared to affect the bacterial growth and adhesion. Bacterial growth was inhibited by using stock lactic acid bacteria filtrate and 50:50 diluted filtrate. Higher inhibition zones were recorded during the use of stock filtrate of the probiotic on the pathogenic bacterial isolates. Bacterial adhesion to epithelial cells was inhibited also by using the probiotic. In the case of *Escherichia coli*, the adhesion was reduced from 59-61 to 24-21 and 33-30 bacterium/cell by using the stock and diluted probiotic respectively. In the case of *Salmonella typhi* reduction of bacterial adherence was also observed from 55-53 to 11-13 and 16-14bacterium/cell by using the stock and diluted probiotic respectively. While in the case of *Klebsiella pneumonae* from 44-46 to 8-9 and 14-10 bacterium/cell by using the stock and diluted probiotic respectively. This results explained that the bacterial adhesion is a crucial step in the colonization and pathogenesis of bacteria, which can be inhibited by using probiotic preparations.

**Introduction :**

The ability of successful pathogens to survive in an immunologically hostile environment is provided by large armamentarium of virulence mechanisms, which includes bacterial evade, neutralize or counter the host defense systems, but also manipulate host homeostasis and normal cell functions(1). Virulence factors of bacteria appears as various means of adhesion ,colonization , multiplication and spreading structures such as adhesins, fimbriae, bacterial endotoxin, enzymes ,exotoxins, capsule ,slime and some times motility (2, 3). Adhesion of bacteria to human tissue surfaces and implanted biomaterial surfaces is an important step in the pathogenesis and infection. Bacterial attachment to mucosal surfaces activates the production and release of pro-inflammatory cytokines which can cause both local and systemic inflammation, this process is enhanced mainly by the presence of capsule and fimbriae(4).

Fimbriae (or pili) are a group of rigid, straight, filamentous appendages on bacterial surface and are often no more than 4 to 7 nm in diameter and from 0.2 up to 20 nm length. Fimbriae composed from protein called pilin, the filamentous nature of fimbriae may mediate the adhesion by adhesins associated with fimbriae (3, 5).

Probiotics (prebiotics) is a dietary adjuvant that beneficially affects the host physiology by modulating mucosal and systemic immunity, as well as improving nutritional and microbial balance in human intestinal tract.Currently probiotic preparations include different species of LAB (Lactic acid bacteria) mainly(6) . The therapeutic effects of lactic acid bacteria include ; improvement of nutritional quality of food and feed, metabolic stimuli of vitamin synthesis, and enzyme production, stabilization of gut microflora and competitive exclusion of enteric pathogens ,enhancing the innate host defenses by production of antimicrobial substances, reduction of serum cholesterol by assimilation mechanism, decrease risk of colon cancer by detoxification of carcinogens ,and tumor suppression by modulation of cell mediated immunity (7). LAB making large proportion from normal flora of gut. And demonstrate a wide spectrum of antimicrobial characteristics, including acid and bile resistance, antimicrobial systems(ex: bacteriocin, lactic acid, peroxide) and adhesion to various types of pathogens ( 8 ).

*Lactobacillus acidophilus* has superior capability of producing lactic acid which is antimicrobial and helps the body protection from harmful bacteria adhering the intestinal mucosa. This bacteria can inhibit the activities of adherence and proliferation of pathogenic bacteria by several ways, such as decreasing luminal pH , rendering specific nutrients

unavailable to pathogens, decreasing the radix potential of the luminal environment, and producing hydrogen peroxide under anaerobic conditions and producing inhibitory compounds such as bacteriocin (6, 7). This work was carried in an attempt to investigate the inhibitory rule of probiotic on bacterial growth and adhesion.

## Materials and methods

**Microbiology and biochemical test:**

Three important human pathogens include *Escherichia coli* , *Salmonella typhi* and *Klebsiella pneumonae* were chosen in this work. Three bacterial isolates for each genus ,were obtained from the biology department of college of science-Al-Qadisyia university. These isolates grown on culture media, then biochemical and microbiological aspects were documented, included capsule, hemolysis, lipase, gelatinase, oxidase, motility and catalase according to ( 10,11, 12 ).

**Fimbriae or Colonization factor antigen (CFA):**

Fimbriae type I, II and III were screened according to ( 3) .

**Probiotic and Bacterial adhesion test**

This test included some steps according to (13 ) as follow:

**Preparation of bacterial suspension:**

Ten milliliter of nutrient broth medium was inoculated with bacterial growth, the culture was then incubated at 37C˚ for over night ($O.D._{600}$ about 0.4) giving ($1*10^9$) cell/ml. Cultures of bacteria were washed twice with PBS and centrifuged at 1000g for 20 minutes and resuspended in PBS.

**Probiotic sensitivity assay**

This assay was curried by using three bacterial strains for each genus and two concentrations of the LAB filtrate against the bacterial strains, concentrations are stock filtrate or (concentration-1)and 1:1 dilution with normal saline (concentration-2), result was carried by measuring the minimum inhibition zone (12).

**Preparation of epithelial cell for probiotic assay :**

Uroepithelial cells were collected from the urine of some healthy females by centrifugation at 1000g for 10 minutes the washed three times with PBS and centrifuged at 100g for 10 minutes before resuspending in PBS.

**Adhesion Test:**

A mixture of 0.2 ml of the bacterial suspension, 0.2 ml of the epithelial cell 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 1000g for 10 minutes. The final pellet was resuspended in PBS then a drop of it was put onto a microscope slid, air dried fixed with methanol: acetic acid (3:1) and stained with methylen blue. The adherent bacteria to epithelial cells were observed by compound light microscope. Control of only epithelial cells was included (14).

## Results

Results obtained of this effort showed that the bacterial strains contained a group of virulence factors; all bacteria gave a positive test to gelatinase and lipase while Catalase was positive only in *Klebsiella pneumonae* , capsule found in *Escherichia coli* and *Klebsiella pneumonae* and absent in *Salmonella typhi*. Motility was observed in *E. coli* and *Salmonella typhi* but not in *Klebsiella pneumonae* as explained in table (1).

Table-1: Illustrate some biochemical tests for the bacteria.

| Catalase | Gelatinase | motility | Lipase | Capsule | Blood agar | Bacteria |
|---|---|---|---|---|---|---|
| - | + | + | + | + | β-Hemolysis | *Escherichia coli* |
| - | + | + | + | - | γ- Hemolysis | *Salmonella typhi* |
| + | + | - | + | + | γ- Hemolysis | *Klebsiella pneumonae* |

Results also demonstrate that the bacteria contained some types of fimbriae. Type III fimbriae was found in all bacteria under test, while type II was recorded in *Salmonella typhi* only and type I found in *Escherichia coli* and *Klebsiella pneumonae* while missing in *Salmonella typhi* as explain in table (2).

Table-2: Illustrate the fimbriae types of the bacteria.

| Fimbriae type III | Fimbriae type II | Fimbriae type I | Bacteria |
|---|---|---|---|
| + | - | + | *Escherichia coli* |
| + | + | - | *Salmonella typhi* |
| + | - | + | *Klebsiella pneumonae* |

The bacterial inhibition zone were estimated by using three isolates and two concentrations of the LAB probiotic, results showed that both *Klebsiella pneumonae* and *Salmonella typhi* were more sensitive than *Escherichia coli* especially when treated with stock filtrate or concentration (con. 1)of LAB filtrate ,as explained in table( 3).

Table-3: Illustrate the bacterial sensitivity to the probiotic filtrate of the LAB .

| **Bacteria** | **zone of inhibition ( mm)** | | | | | |
|---|---|---|---|---|---|---|
| | Isolate number 1 | | Isolate number 2 | | Isolate number 3 | |
| | Con. 1 | Con. 2 | Con.1 | Con.2 | Con.1 | Con.2 |
| *Escherichia coli* | 9 | 8 | 11.5 | 10 | 12.5 | 11 |
| *Salmonella typhi* | 13.5 | 11 | 14 | 13.5 | 13 | 11.5 |
| *Klebsiella pneumonae* | 14.5 | 12 | 13.5 | 13 | 12.5 | 11.5 |

Con.1= concentration 1 of LAB (stock bacterial filtrate)

Con.2= concentration 2 of LAB (1:1 of bacterial filtrate: normal saline)

Results of the effect of the probiotic reveled that, the bacterial strains adhesion was reduced by using the same concentrations of the LAB in comparison to control, *Escherichia coli* adhesion to Uroepithelial cells was reduced from 59-61 bacterium/ cell without probiotic to 33-30 and 24-21 bacterium/ cell by using con.2 and 1 of the probiotic respectively as explained in figure (1).

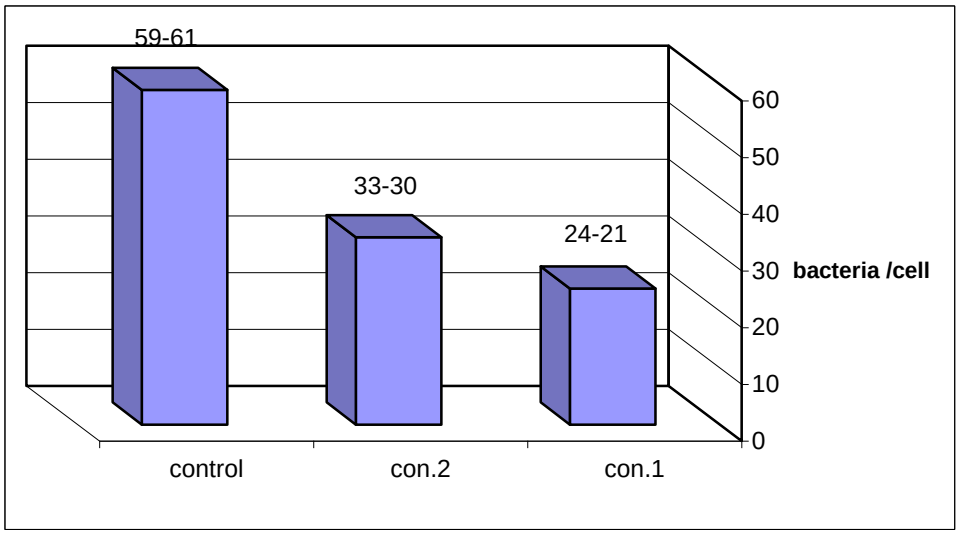


Figure-1 : Illustrate the inhibition of adhesion of *Escherichia coli* on the Uroepithelial cells.

Adhesion of *Salmonella typhi* to Uroepithelial cells was reduced from 55-53 bacterium/ cell without probiotic to 16-14 and 11-13 bacterium/ cell by using con.2 and 1 of the probiotic respectively as explained in figure (2).

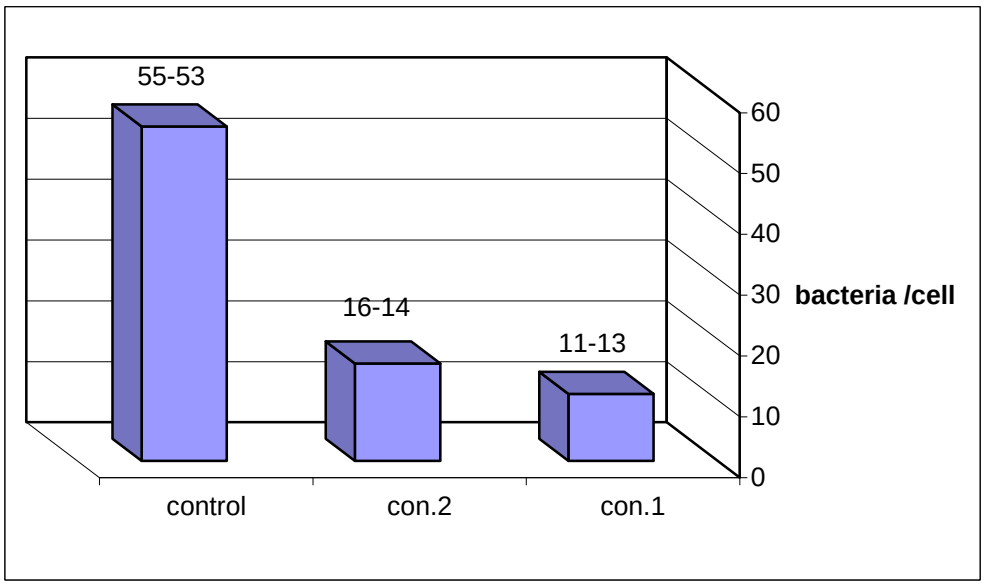


Figure-2 : Illustrate the inhibition of adhesion of *Salmonella typhi* on the Uroepithelial cells.

Also the adhesion of *Klebsiella pneumonae* to Uroepithelial cells was reduced from 44-46 bacterium/ cell without probiotic to 14-10 and 8-9 bacterium/ cell by using con.2 and 1 of the probiotic respectively as explained in figure (3).

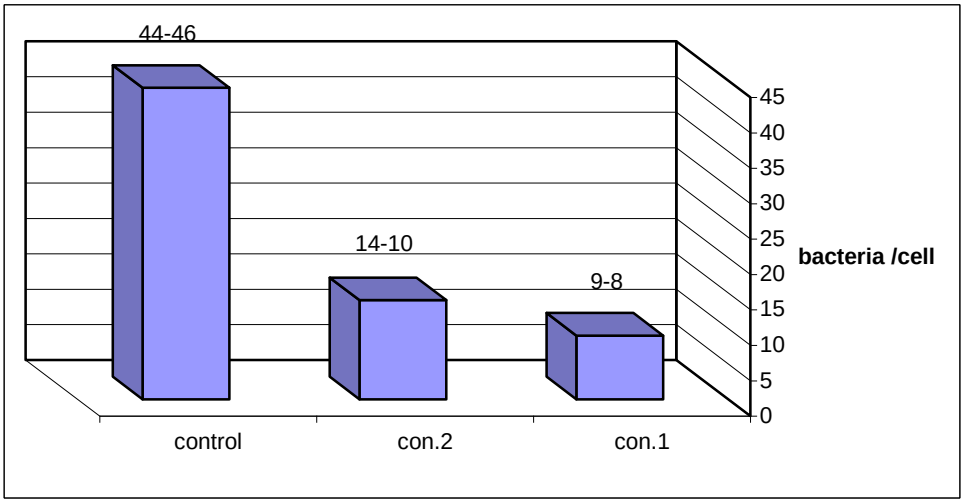


Figure-3 : Illustrate the inhibition of adhesion of *Klebsiella pneumonae* on the Uroepithelial cells.

**Discussion:**

Results of this work revealed that pathogenic bacteria must contain suitable virulence factors which enable this bacteria to adhere and colonize the body surfaces and cells. Although both motile and nonmotile species form biofilms, in motile species, the ability to move using flagella or pili is generally required for efficient cell-to-surface attachment (15) . The production of extracellular polysaccharide molecules is a common feature of many bacteria, these structures termed as capsule or alternatively, may comprise an amorphous slime layer (16). The polysaccharide capsule represents the outer most layer of the cell, that mediates the interactions between the bacterium and it's immediate environment. It's importance in promoting the formation of biofilms and stimulate interspecies coaggregation, thereby enhancing the bacterial colonization. It has been known that the expression of a capsule is an essential virulence factor. In invasive bacterial infections, the interactions between capsule and the host's immune system may be vital in deciding the outcome of an infection, and may also used as permeable barrier against some harmful agents such as antibiotics (17). In the case of *E. coli* , studies using gontobiotic rats have demonstrate that expression of K antigen (capsule) enhances the persistence of the bacteria in the large intestine and converse a selective advantage in colonization of rats(15). Also enteropathogenic *E. coli* appear to have predilection for human ileum and supposing that the first step of adherence to the host cells is thought to involve bundle-forming pili (4).

In the case of expression of *S. typhi* capsular polysaccharide, the capsule offer the protection from environmental insults and host non specific immune response facilitating the invasion, by mediating the interaction between the bacteria and the mucus surrounding host epithelial cells(2, 16 ).

In *K. pneumonae*, it has been shown that expression of a polysaccharide capsule is essential for the colonization of the large intestine of mice(4, 15). One interpretation of these data is that capsule is required for initial steps of colonization by interacting with the mucus layers and this step is vital for successful colonization in vivo(3). The concomitant increase in adhesion expression would have the net effect of enhancing bacteria-epithelial cells interactions essential for long-term colonization ( 17 ).

Lactic acid bacteria have an inhibitory effect against the gram negative and positive bacteria. Some investigators stated a high inhibitory effects of LAB against the enteropathogenic bacteria (6). While others (9) documented a significant inhibition of LAB on *Proteus mirabilis* , proposing the effect to the presence of active antimicrobial secretions

such as lactocidin , plantaracin and acidophilin, these results agreed with the results obtained in this work.

The obtained data reflect a remarkable decrease in the numbers of all tested adhered bacterial cells to the epithelium cells . This was due to the effect of inhibitory substances found in the filtrate of LAB isolates and the acidic pH which affect the growth of the gram negative bacteria by altering some surface structures (18,19). (20) investigated the effect of *Lactobacillus casei* on *E coli* and found that the inhibitory effect was not caused by bacteriophage of hydrogen peroxide but due to the aggregation of *E. coli* an LAB . (14) reported that precoating of LAB strains reduced the binding of uropathogenic coagulase negative *Staphylococci* and *E. coli* to 8 bacteria / cell . while (9) observed aclear reduction in adhesion of *Proteus mirabilis* after the treatment with LAB filtrates reaching to 3-8 bacteria/ cell. Others found that the 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 number of adhering *Enterococcus* reaching to approximately 70% ( 21 ).

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# تأثير **Probiotic** على نمو والتصاق بعض أنواع البكترية المرضية السالبة لصبغة غرام

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الخلاصة:

ركز هذا العمل على ثلاث أجناس بكتيرية مهمة ممرضة للإنسان . وهي *Escherichia coli* و *Salmonella typhi* و *Klebsiella pneumonae* . أوضحت النتائج احتواء هذه البكتيريا على العديد من عوامل الضراوة والاستيطان مثل المحفضة والإنزيمات والحركة والأهداب. أوضحت النتائج احتواء *Escherichia coli* على أهداب من النوع الأول والثاني والثالث، في حين احتوت *Salmonella typhi* على النوعين الثالث والثاني، أما *Klebsiella pneumonae* فقد احتوت على أهداب من النوع الأول والثالث فقط. كذلك بينت الدراسة بان probiotic المحضر من بكتريا *Lactobacillus acidophilus* قد اثر سلباً على نمو والتصاق البكتريا المرضية. إذ أن النمو البكتيري تم تثبيطه باستخدام راشح النمو البكتيري لهذا الجنس وأيضا لوحظت نتائج مشابهة عند استخدام الراشح المخفف 50:50، وقد كانت أكثر مناطق التثبيط لوحظت باستخدام راشح بكتريا حامض الحليب LAB غير المخفف على الأجناس المرضية المستخدمة في الدراسة. كذلك تم ملاحظة التثبيط الحاصل على قابلية التصاق البكتريا المرضية على الخلايا الطلائية باستخدام probiotic . ففي حالة بكتريا *Escherichia coli* انخفضت نسبة الخلايا الملتصقة من 59-61 بكتريا/خلية إلى 21–24 و 30–33 بكتريا /خلية عند استخدام المحلول المركز من الراشح(probiotic ) والمحلول المخفف منه على التوالي. وفي حالة *Salmonella typhi* كان الانخفاض في أعداد الخلايا الملتصقة من 53-55 بكتريا/خلية إلى 13-11 و 14-16 بكتريا/خلية عند استخدام المحلول المركز من الراشح والمحلول المخفف منه على التوالي. وكذلك بالنسبة *Klebsiella pneumonae* فقد كان الانخفاض في أعداد الخلايا الملتصقة من 44–46 بكتريا/خلية إلى 8–9 و 10–14 بكتريا/خلية عند استخدام المحلول المركز من الراشح والمحلول المخفف منه على التوالي.

هذه النتائج توضح أهمية التصاق البكتريا كخطوة أساسية في عملية الاستعمار والامراضية والتي يمكن أن تثبط باستخدام مستحضرات probiotic.