Using the *Lactobacillus gasseri* filtrate to protect the mice from the pathogenic bacteria *Aeromonas spp*.

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الخلاصة:

درس التأثير الوقائي لبكتريا المعزز الحيوي *Lactobacillus gasseri* ضد البكتريا المرضية *Aeromonas spp* داخل وخارج الجسم الحي، أذ اختبرت الفعالية التثبيطية لراشح بكتريا المعزز الحيوي بطريقة الأنتشار في الحفر ،وقد أظهر الراشح فعالية تثبيطية واضحة تجاه البكتريا المرضية اذ بلغ قطر منطقة التثبيط 16 مليمتر.

حقنت مجموعة من الفئران داخل الغشاء البريتوني ب 0.25 مليلتر من الراشح لمدة عشر أيام، بعدها حقنت ب 0.2 مليلتر من عالق خلايا حية لبكتريا *Aeromonas spp* (10^8 خلية / مل) داخل الغشاء البريتوني، بينما حقنت مجموعة السيطرة ب 0.25 مليلتر من دارئ الفوسفات الملحي المعقم.

تم قتل الفئران بعد 12 ساعة من حقنها بالبكتريا المرضية، أذ تم حقن 5 مل دارئ الفوسفات داخل الغشاء البريتوني وبعد قتل الفأر أخذت محتويات البريتون والكبد والطحال، تم عمل تخافيف من محتويات البريتون، وزرع 0.1 مل على الأوساط الزرعية لحساب عدد المستعمرات البكتيرية النامية، كذلك تم مجانسة جزء من الطحال وزرع المزيج على الأوساط الزرعية. حسبت أعداد المستعمرات النامية وقورنت ألأعداد بمعاملة السيطرة، كذلك تم حساب عدد الخلايا البلعمية (Macrophage) في محتويات البريتون، وأخذت عينات من الأعضاء مباشرة بعد القتل وثبتت في محلول الفورمالين (10%) لدراسة التغييرات النسجية المرضية.

أظهرت النتائج عدم تأثر الفئران المعاملة براشح بكتريا المعزز الحيوي بعد حقنها بالبكتريا المرضية خلافا للمجموعة التي حقنت بالبكتريا المرضية فقط دون حقنها براشح البكتريا الواقية، مما يدل على التأثير الوقائي لبكتريا *Lb.gasseri* في الحماية من الأصابة بالبكتريا المرضية *Aeromonas spp*، وأظهرت التغيرات المرضية وجود استجابة التهابية فضلا عن ظهور الأنسجة بمظهرها الطبيعي.

Abstract:

The protective effect of the Probiotic bacteria (*Lactobacillus gasseri*) against the pathogenic Bacteria (*Aeromonas spp*) was studied *in vivo* and *in*

vitro, the inhibitory effect of the Probiotic bacteria filtrate was tested with the Well diffusion method, the filtrate showed a clear inhibiting efficiency toward the pathogenic bacteria and the diameter of the inhibition zone was 16 mm.
A group of mice were injected intraperitoneally with (0.25 ml) of the filtrate for 10 days, then they were injected with (0.2 ml) of the *Aeromonas spp* bacteria living cells (10^8 cell\ml) intraperitoneally, while the control group were injected with 0.25 ml of PBS.

The mice were killed after 12 hours of injection with the pathogenic bacteria, they were injected with (5ml) of PBS intraperitoneally, the contents of the periton, Liver and the Spleen were taken after killing the mice, then a dilutions of the Periton contents were made and 0.1 ml was streaking on the media to calculate the number of the growing bacterial colonies, then a part of the Spleen was homogenized and streaking on the media, then the growing colonies were calculated and compared with the control mice, also the macrophage in the contents of the periton were counted, and a samples of the organs were taken directly after the killing and were putted in the formalin solution (10%) for study the histopathological changes.

The results shows that the mice who were injected with the probiotic bacteria wasn't effected when they were exposed to the pathogenic bacteria unlike the group that were only exposed to the pathogenic bacteria without being injected with the probiotoic bacteria, and that shows the protective effect of the (*Lactobacillus gasseri*) against the pathogenic bacteria (*Aeromonas spp*), and the pathogenic changes shows an inflammatory response and that the tissues were appeared in their natural form.

Introduction:

The early studies about the useful effects of the *Lactobacillus* that is conjugated with human begun more than 100 years ago, the scientist Lister was the first to isolate these bacteria from the milk in 1878 and after that there were a lot of studies to isolate these bacteria from the human and they were isolated from the digestive canal and the vagina [1] .
In 1908, Metchnikoff refers to the role of the *Lactobacillus* for treating many diseases and he proved that consuming the products which contain these bacteria will enhance the balance of the microorganisms in the digestive canal [2], in 1945 the dairy products were supported with these bacteria and it was noticed that consuming these products daily has an important role in the intestine's health and to protect it from the diseases [3].

Probiotics are defined as “live micro-organisms which when administered in adequate amounts confer a health benefit on the host” and include various species of lactobacilli and bifidobacteria [4].

At the start of the 20th century, probiotics were thought to beneficially affect the host by improving its intestinal microbial balance, thus inhibiting pathogens and toxin producing bacteria. Today, specific health effects are being investigated and documented including alleviation of chronic intestinal inflammatory diseases, prevention and treatment of pathogen-induced diarrhea, urogenital infections, and atopic diseases.[5,6]

Tuomola *etal*. [7] found out that the *Lactobacillus* and its filtrate has the ability to prevent the adhesion of the *E.coli* bacteria to the mucus membrane . probiotic bacteria works as a protection barrier to prevent the attachment of the pathogens like Typhoid and bacillary dysentery bacteria to the intestine walls and therefore pushing these pathogens outside the intestinal canal [8].

The probiotic bacteria has the ability to increase the efficiency of the useful enzymes like (galactosidase) (which reduce the lactose indigestion) and reduce the efficiency of the carcinogenic enzymes like (nitroreductase) [9], also it destroys the toxins produced by the pathogenic bacteria and inhibit the inner toxins' action [3] , also the probiotic bacteria has a positive side effects on the immune system because adding the *Lactobacillus* to the diary products will enhance the body immunity by stimulating the produce of the antibiotics, increase the Interferon and increasing the efficiency of the devouring and the natural killer cells [9] .

Aeromonas spp. has the ability to produce cellular toxins like (enterotoxins, haemolysin and phospholipase), its pathogenecity depends on attaching to the tissues and producing toxins, it can attach itself to the tissues because it has S-layer and cilium and it could have capsule [10]. *Aeromonas spp* causes large range of systematic and extra intestinal injuries,it also causes Gastroenteritis, Wound Infections and bacteremia [11] .

Most studies investigated the effects of *Lactobacillus* on pathogenic bacteria *in vitro*, whereas very few studies have investigated the effects of *Lactobacillus gasseri* filtrate *in vivo*.

The aim of this work was to study the effect of *Lactobacillus gasseri* filtrate on prevention of *Aeromonas spp.* infection in mice.

Materials and Methods

Bacterial isolates:

1- Lactic acid bacteria:

Lactobacillus gasseri was isolated from the infant's stool samples and after mixing these samples were streaked onto MRS agar contain 1% CaCo3. The plates were incubated at 37C for 24-72 h. under anaerobic conditions, then the isolated colonies were streaked onto SL agar and incubated at 37C for 24-48 h. , after that the growing colonies were transferred to MRS broth and incubated at 37 C for 24 h. [12].

The isolate was subjected to the microscopic and biochemical tests for the diagnosis as mentioned in [13,14]

2- Pathogenic bacteria:

Aeromonas spp isolates were provided from the labs of the Biology Department\Collage of Science\ Al-Mustanseriya University.

3- Preparing the *Lactobacillus gasseri* filtrate:

The filtrate was prepared by growing the isolate of the *Lactobacillus gasseri* in MRS broth at pH 6 and incubated at 37C for 24 h. under unaerobic conditions. After centrifugation at 6000 rpm for 10 min., *Lactobacillus gasseri* filtrate was sterilized by filtration (0.22 µm millipore filters) [15].

4- Estimating the inhibitory effect of *Lb. gasseri* filtrate against *Aeromonas spp* (*in vitro*):

Inhibitory effect of *Lb. gasseri* filtrate against *Aeromonas spp*. Was tested by using agar diffusing assay according to Gupta *etal*.[16]. Pathogenic bacteria culture was plated on fresh Nutrient agar plates,and wells were prepared into the agar by using sterile Pasteur pipettes. 50 ul aliquts of *Lb. gasseri* filterate was suspended in the agar wells. Plates were incubated for 24h. at 37 C , and the diameters of inhibition zones around the wells were measured.

5- Testing the inhibitory effect of the *Lb. gasseri* filtrate *In vivo*:

To study the inhibitory effect of *Lb. gasseri* filtrate against *Aeromonas spp. in vivo* , a white male Swiss mice age between (6-7 weeks) weight between (15-20 gram) were used and they were given by the National Center of Medicine Supervising\Baghdad, the first group were injected intraperitoneally daily with (0.25 ml) filtrate for 10 days, then they were injected with (0.2 ml) of *Aeromonas spp*. 10^8 cell\ml intraperitoneally, the second group were injected with (0.25 ml) sterile PBS for 10 days, then they were injected with (0.2 ml) *Aeromonas spp*. (10^8 cell\ml) intraperitoneally and they were used as positive control, the third group were injected with (0.25 ml) sterile PBS for 10 days and were used as negative control.

6- Estimating the Viable cells of *Aeromonas spp* in the mice tissues:

The Liver and the Spleen of the experimented mice were collected and washed eight time with PBS, then were mixed with the stirrer for 2 min., the Viable cells of the bacteria was estimated by using the plate count method by transferring (0.1 ml) from every sample to plates containing MacConkey's Agar medium, the plates were incubated at 37C for 24 h.

7- Counting of WBCs in peritoneal cavity:

Smear specimens for differential counts were prepared for Giemsa staining and examined. [17].

8-The Histopathological test:

A samples of the organs (Liver and Spleen) were taken directly after the killing and were putted in the formalin solution (10%), then the tissue slides preparation procedures were followed, the samples passed through escalating

series of Alcohol, then with Zylol, covered with Paraffin, sliced (4-6 microliter thickness) with the Microtome and dyed with hematoxylin and eosin according to (Harris hematoxylin and eosin H&E) method [18].

Results and Discussion:

The inhibitory effect of the probiotic bacteria (*Lactobacillus gasseri*) against the pathogenic bacteria (*Aeromonas spp.*) was tested with the Well Diffusion method, *Lb.gasseri* filtrate shows a clear inhibitory effect and the inhibition zone diameter was 16 mm. (figure-1), and after comparing this result with the other results the filtrate growing in liquid MRS medium shows a wide inhibitory efficiency against the pathogenic bacteria like *E. coli* and *Klebsiella spp*[16], Dunne *etal*. [19] found that the *Lactobacillus* filtrate has a inhibitory effect against Gram negative pathogenic bacteria especially *E. coli* and *Salmonella*, Al Dulaimy [20] found that the lactic acid bacteria has a clear efficiency against dysentery bacteria like *Shigella flexneri*, Entropathogenic *E. coli* and *Salmonella typhimurium*, and the inhibition zone diameter was higher than 17 mm.

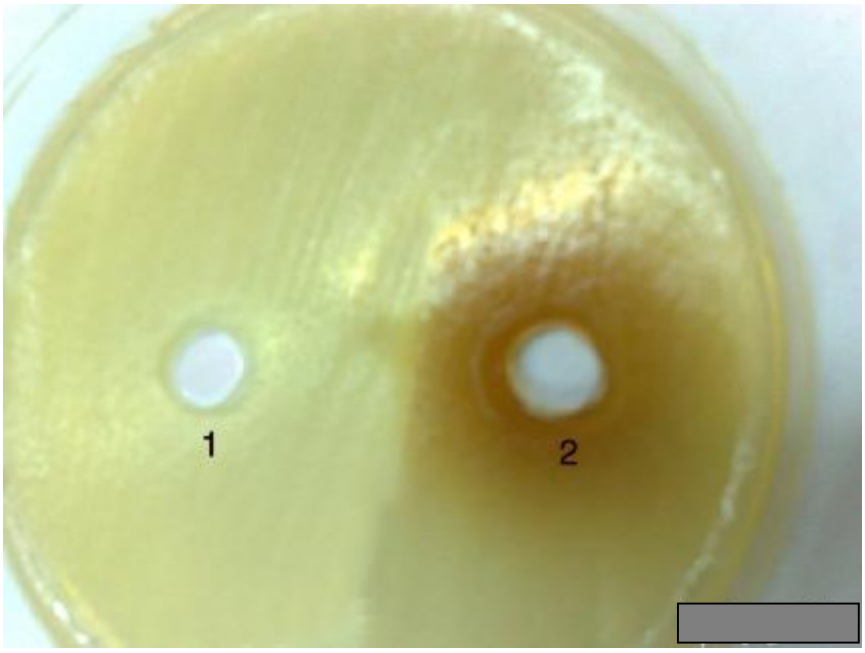

Figure 1: The inhibitory effect of *Lactobacillus gasseri* filtrate against *Aeromonas spp.*
1- control 2-*Lactobacillus gasseri* filtrate

The effect of *Lactobacillus gasseri* filtrate against *Aeromonas spp.* was tested in the mice *in vivo* , the mice were given the filtrate for 10 days before they were injected intraperitoneally with *Aeromonas spp*., 12 hours later the mice were killed, then the peritoneal liquid was taken and the numbers of the growing colonies were calculated and compared with the control, also the Macrophage numbers in the peritoneal contents were calculated, the results

shows no effects on the mice treated with the *Lactobacillus gasseri* filtrate when they were exposed to the pathogenic bacteria *Aeromonas spp*., unlike the group that were exposed only to the *Aeromonas spp*. bacteria without being injected with *Lactobacillus gasseri*.

The results of the living number calculation for the pathogenic bacteria in the Spleen and the peritoneal cavity shows that the living number of the *Aeromonas spp* in the peritoneal cavity was about 10^6 CFU by 12 hours after challenge in control mice and 10^3 CFU in the Spleen, the Neutrophil cell rate (PMNs) was 80 in the blood sample of the same group, and the numbers of the Monocyte cells was 2% while in the test group (treated previously with the probiotic bacteria) shows no *Aeromonas spp*. bacterial growth in the Spleen and the number of the Monocyte cells was 7% and the PMNs was 65 in the test group.

Our results shows the inhibitory effect of the *Lactobacillus gasseri* filtrate against the *Aeromonas spp*. bacteria when treating the mice with the filtrate before the infection therefore this filtrate will have an important role in the protection against bacterial infections and the reason for that might be the inhibiting materials in the filtrate that may inhibit the growth of the pathogenic bacteria.

These findings are in agreement with the previously reported result that showed that the administration of *Lactobacillus* or yogurt to young mice enhanced lung clearance of pathogenic bacteria and phagocytic activity of alveolar macrophages [21] ,and agreement with result of Bohan [22] who found the total count of WBC was increased significantly in mice group treated with *Lb. gasseri* and decreased significantly in mice group treated with pathogenic bacteria.Lievin *etal*., [23] shows that the *Lactobacillus gasseri* isolated from the human stool produce an active inhibiting materials against the bacterial infections inside and outside the living body. Goulet *et al.* [24] found that phagocytic activity of alveolar macrophages was significantly ($P < 0.05$) higher in mice fed milk fermented with *L. acidophilus* and *L.casei* than in control mice fed ultrahigh-temperature-treated milk. Other studies in which reconstituted lyophilized *Lactobacillus* were administered orally or intraperitoneally showed enhancement of macrophage activation by *Lactobacillus* [25].

These observations reviewed together suggest that specific immunomodulatory properties of probiotic bacteria should be characterized during the development of clinical applications for extended target populations. On the other hand, this study includes taking Liver and Spleen samples to study the histopathological changes, these results shows histological changes in the Spleen and the Liver when injecting the control group mice with the *Aeromonas spp*. , the microscopic examination of the histological sections in the Spleen shows many changes like few infiltration of the Neutrophil cells and congestion in the blood vessels (figure-2), the microscopic examination of the

Liver tissue shows a decay in the Liver tissue (figure-3), and when examining the histological sections of the Liver and the Spleen in the test group animals the microscopic examination for the Spleen histological sections shows large infiltration of the neutrophils cells and plasma, also the macrophage and the tissue returns to their normal condition (figure-4).

All these results shows that the lactic acid bacteria has an effective role in reducing the infection spread because these bacteria stimulate the immune system and when eating the diary products containing these bacteria it will cause the increase of the lymphatic cells and will also stimulate cells to produce a lot of cytokines like (IL-12, IL-2, IL-1), activate and increase the ability of the Macrophages to swallow and kill the germs, also producing some factors that increase the lymphatic aromas[26].

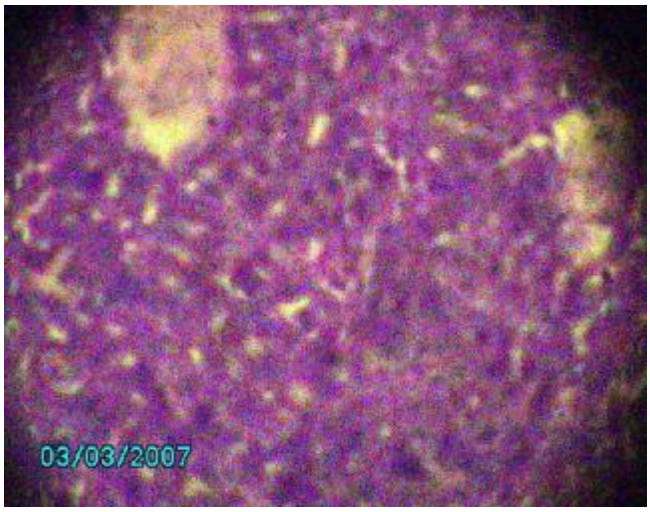

Figure-2: Section of the Spleen from the second group mice (H and E) 100X

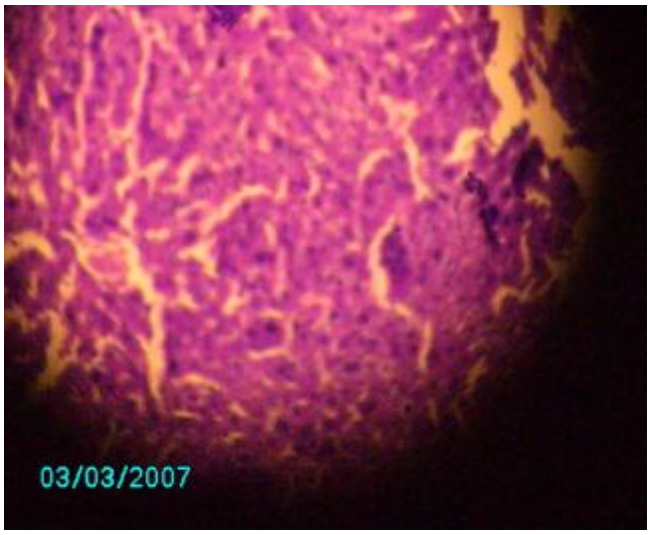

Figure-3: Section of the Liver from the second group mice (H and E) 100X

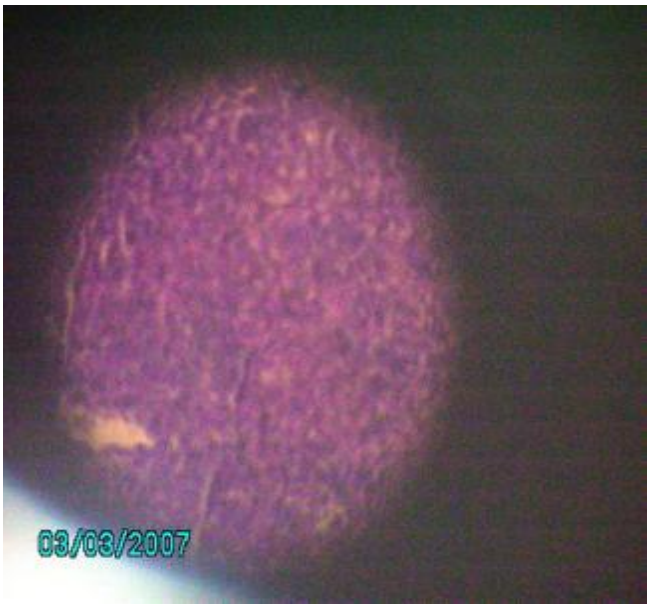

Figure-4: Section of the Spleen from the first group mice (H and E) 100X

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