The prophylactic effect of heat- killed *Lactobacillus fermentum* against *Pseudomonas aeruginosa* infection in mice

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Abstract

The protective effect of heat – killed *Lactobacillus fermentum* against *Pseudomonas aeruginosa* infection in Swiss mice with age of 5 weeks . 0.5 mg of heat – killed *Lactobacillus fermentum* was injected in intraperitoneally (I.P.) 5 days before challenge with 0.2 ml of viable *P. aeruginosa* (10^8 cell/ ml).

Animals were sacrificed by Cervical disslocation after 12 h. from challenge dose. To follow bacterial growth in the peritoneal cavity of injected Mice , its contents were washed out with 5 ml of PBS .The number of colonies in 5 ml of harvested fluid was expressed as Log 10 CFU . Bacterial growth in the Spleen was determined by spreading the organ homogenates.

Survival of mice after intraperitoneal (I.P.) infection with *P. aeruginosa* was augmented in mice that had been pretreated I.P. with *L. fermentum* five days earlier. Mice became resistant to infection with *P. aeruginosa* after pretreatment with *L. fermentum* .

Growth of *P. aeruginosa* in the peritoneal cavity and spleen was markedly inhibited in *L. fermentum* pretreated mice, wherease such inhibition of bacterial growth was not observed in control group (mice don't treated with *L. fermentum*)

The mean number of peritoneal cells in control mice was 3 x 10^6 CFU and 60 % of these cells were macrophages . It was suggested that macrophages activated by *L. fermentum* were involved in the protection against *P. aeruginosa*.

التأثير الوقائي لبكتريا *Lactobacillus fermentum* المقتولة بالحرارة ضد الأصابة ببكتريا *Pseudomonas aeruginosa* في الفئران

درس التأثير الوقائي لبكتريا *Lactobacillus fermentum* ضد الأصابة ببكتريا *Pseudomonas aeruginosa* في الفئران ، اذ حقنت *L. fermentum* ملغم خلايا مقتولة بالحرارة من بكتريا 0.5 مجموعة من الفئران داخل الغشاء البريتوني ب 0.2 مل من 0.2 خلية / مل) داخل الغشاء البريتوني ، بينما حقنت مجموعة السيطرة ب 10^8 (*P. aeruginosa* مل خلايا حية لبكتريا 0.2ب مل دارئ الفوسفات .

تم قتل الفئران بعد 12 ساعة من حقنها ببكتريا *P . aeruginosa* وبعد قتل 5 ، حيث تم حقن 0.1 مل دارئ الفوسفات داخل الغشاء البريتوني مل على الأوساط الزرعية لحساب عدد الفأر أخذت محتويات البريتون والكبد والطحال ، تم عمل تخافيف من محتويات البريتون ، وزرع حسبت أعداد المستعمرات النامية .المستعمرات البكتيرية النامية ، كذلك تم مجانسة جزء من الطحال وزرع المزيج على الأوساط الزرعية وقورنت ألأعداد بمعاملة السيطرة ، كذلك تم حساب عدد الخلايا البلعمية (Macrophage) في محتويات البريتون .

أظهرت النتائج عدم تأثر الفئران المعاملة ببكتريا *L. fermentum* عند تعريضها لبكتريا *P. aeruginosa* خلافا للمجموعة التي عرضت *L. fermentum* فقط دون حقنها بالبكتريا الواقية ، مما يدل على التأثير الوقائي لبكتريا *P. aeruginos* في الحماية من الأصابة ببكتريا *P. aeruginosa*.

Introduction

Probiotics are usually bacterial components of the normal human intestinal flora, for example lactobacilli and bifidobacteria, that produce end products of metabolism of lactate and short chain fatty acids such as acetate and butyrate [1] . Lactic Acid Bacteria (LAB) are gram-positive bacteria with cell wall components such as peptidoglycan, polysaccharide, and teichoic acid, all of which have been shown to have immunostimulatory properties. In addition to cell wall components, immunostimulatory effects were observed with antigens originated from the cytoplasms of some strains of LAB [2]. Certain specific probiotic strains (for example, *Lactobacillus rhamnosus, L plantarum, L casei* and *L johnsonii*) have well defined and proven clinical effects for the treatment and/or prevention of diseases of intestinal and extraintestinal origin [1] , and have immunostimulatory properties, including modulation of cytokine production, increased phagocytic activity of polymorphs, adjuvant effects on specific humoral responses, T lymphocytic function, and NK activity [3][4]. Probiotic bacteria are shown to promote the endogeneous host defense mechanisms. In addition to the effects of probiotics on nonimmunologic gut defense, which is characterized by stabilization of the gut microflora , probiotic bacteria have been shown to enhance humoral immune responses and thereby promote the intestine's immunologic barrier. Moreover, probiotic bacteria have been shown to stimulate nonspecific host resistance to microbial pathogens ,and thereby aid in immune elimination, and to modulate the host's immune responses to potentially harmful antigens with a potential to down-regulate hypersensitivity reactions [5] .Oral administration of *Lactobacillus casei* and *Lactobacillus bulgaricus* activates the production of macrophages ,and administration of *L. casei* and *Lactobacillus acidophilus* activates phagocytosis in mice , Enhanced phagocytosis was also reported in humans by *L. acidophilus*[3]. De Simone et al [6] studied the influence of a yogurt-supplemented diet on the immunocompetence and survival of animals subsequently infected with *Salmonella typhimurium*, they reported that mice fed live LAB (*L. bulgaricus* and *Streptococcus thermophilus*)-containing yogurt for 7 and 14 day had a higher percentage of B lymphocytes than did mice fed a control diet supplemented with cow milk. In a similar experiment, Puri et al [7]showed that intestinal lymphocytes from mice fed live LAB-containing yogurt had a higher proliferative response to LPS than did mice fed milk after a challenge with *S. typhimurium*.Most studies investigated the effects of *Lactobacillus* on pathogenic bacteria *in vitro*, whereas very few studies have investigated the effects of heat – killed *Lactobacillus in vivo*. The aim of this work was to study the effect of I.P. injection of *Lactobacillus fermentum* on prevention of *P. aeruginosa* infection in mice.

Material and Methods

Animals :

20 Swiss Male mice were obtained from the College of Medicine/Baghdad University . Mice were used in experiments at 5 weeks of age and 25-30 gram weight. Mice were divsionated into 4 groups (5 mices of group): control , positive group , negative ,experiment group was treated with *L. fermentum* and *P. aeruginosa* .

Bacterial isolates :

1) *L. fermentum* **(** maintained from Department of Biology – College of Science - AL- Mustansiriya University) was cultured on De Man Regosa Sharpe medium (MRS) at 37 ˚C for 48 hrs , washed with distilled water , killed at 100 ˚C for 30 min, and suspended in phosphate buffered saline (PBS) at desired concentration just before use .
2) A clinical isolate of *P. aeruginosa* was isolated from wound infection , and identified according to [8]

Bacterial infection :

This experiment was conducted to test the protective effect of *L. fermentum* against *P. aeruginosa* in mice , 0.5 mg of heat – killed *L. fermentum* was injected in intraperitoneally (I.P.) 5 days before challenge with 0.2 ml of viable *P. aeruginosa* (10^8 cell/ ml). [9].

Determination of bacterial growth :

The challenge dose of bacteria was injected I.P. to negative group and experiment group mice that had been treated with *L. fermentum* 5 days earlier, but positive and experiment groups were injected with *P. aeruginosa* ,control group was lefted without injection with both bacteria. After the challenge , animals were sacrificed by Cervical disslocation . To follow bacterial growth in the peritoneal cavity , its contents were washed out with 5 ml of PBS ., and the fluid was diluted 10- fold with PBS. Each dilution 0.1 ml was spread on nutrient agar plates (containing 0.4% glucose) . The number of colonies in 5 ml of harvested fluid was expressed as Log 10 CFU . Bacterial growth in the Spleen was determined by spreading the organ homogenates.

Counting of WBCs in peritoneal cavity :

3) Smear specimens for differential counts were prepared for Giemsa staining and examined [10]

Statistical analysis

Statistical differences between the control group and the *L. fermentum* treated group were evaluated with the cumulative chi-square test ($P<0.05$ was considered significant).

Results and Disscussion

The number of the *P. aeruginosa* in the peritoneal cavity decreased gradually from 10^7 CFU to10^3 CFU by 12 h. after challenge in *L. fermentum* treated mice,because of *L. fermentum* was play a role in killed of *P. aeruginosa*(Figure -1) .

Figure (1) also shows that the spleen colonization levels were higher in nontreated mice (10^5 CFU) than in *L. fermentum* pretreated mice (10^3 CFU) at 12 h. Post inoculation. Results were indicated found significant variations among groups compared with control at $P<0.05$. Differential cell counts of peritoneal leukocytes were studied consecutively after treatment with *L. fermentum* . The mean number of peritoneal cells in nontreated control mice was 3 x 10^6 CFU and 60 % of these cells were macrophages (Figure- 2) . polymorphnuclear cells (PMNs) were characteristically increased in *L. fermentum* treated mice.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 *P. aeruginosa* and phagocytic activity of alveolar macrophages [11**].** Villena *et al* [12] found that pneumococcal colonization of lung and bacteremia were significantly greater in control group mice compared with the *L. casei* pretreated group. Although the number of bacteria in lungs and blood stream tended to decrease ($P < 0.05$) during infection in *L. casei* pretreated group mice, and they suggests that the addition of *L. casei* to the repletion diet has a beneficial effect because it accelerates the recovery of the innate immune response and improves the specific immune mechanisms against an *Streptococcus pneumoniae* respiratory infection in malnourished mice.

L. fermentum may become a potent and useful macrophage activator in experimental studies and clinical trials. The immunostimulatory effects of LAB have not been fully determined . Some studies, however, showed no difference in immunogenicity between viable and non viable bacteria [2].

The use of probiotics (live viable microbial organisms) in the treatment of specific diseases has evolved into an extremely valuable option yet to be optimally used in clinical medicine [13]. Probiotics have been shown to have immunomodulating properties and enhance the mucosal barrier [14]

A limited number of animal studies were conducted on the effect of LAB on macrophages. Goulet *et al* [15] 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. Perdigon *et al* [3] showed that feeding milk (100 µg protein/d)

fermented with *L. casei* and *L. acidophilus*, or both for 8 d. increased the *in vitro* and *in vivo* phagocytic activity of peritoneal macrophages . .

Other studies in which reconstituted lyophilized LAB were administered orally or intraperitoneally showed enhancement of macrophage activation by LAB [16] .

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.

Further experiments are required to establish the mechanism by which *L. fermentum* isolate affects *P. aeruginosa* pathogenicity . In the future , the immunological aspects of the protective role of *L. fermentum* should be studied .

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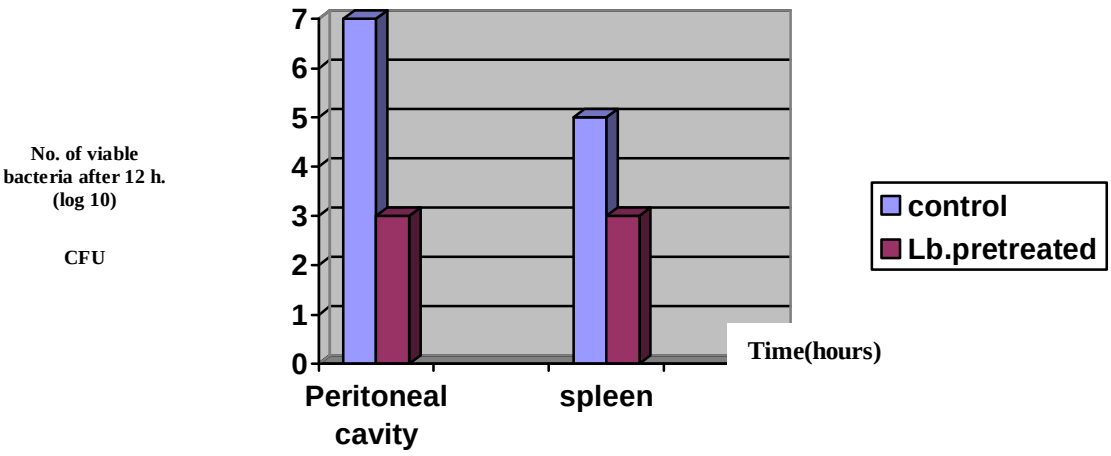
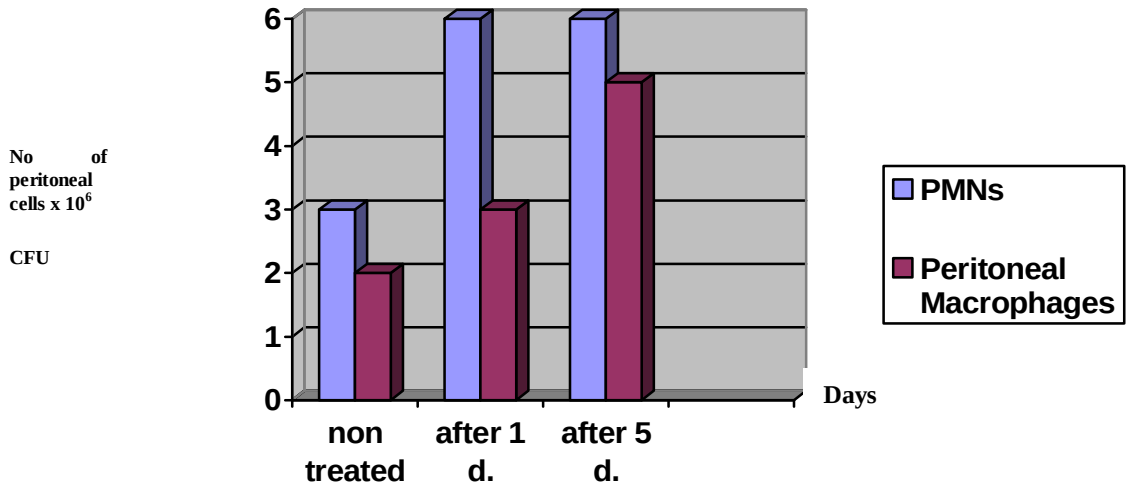


Figure(1). Total no. of *P. aeruginosa* in the peritoneal cavity and spleen after 12 h.



Figure(2) . Number of peritoneal exudates cells in mice treated with *L. fermentum* .