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The effect of *Lactobacillus acidophilus* on the clearance of pneumococcus pathogen from mice lung

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Abstract:

In the present study, we investigate the preventive effect of intraperitoneal inoculation of *Lactobacillus acidophilus* against intranasally challenge with *Streptococcus pneumonia* using a mouse experimental model.

Five days before challenge with Capsulated *S. pneumonia* the treated group injected intraperitoneally with 0.5 ml of heat –killed *Lactobacillus acidophilus*, while the positive control group injected with 0.5 ml of phosphate buffer saline. Mice were infected by dropping 25 µl of the inoculums containing 10^8 log- phase colony forming unit S.*pneumonia* in PBS into each nostril and allowing it to be inhaled by holding the mice in head-up vertical position for 2 min . Animals were sacrificed by cervical dislocation on day 1st, 2ed and 3rd day of infections.

The study showed that numbers of pneumococcal pathogen that recovered from lungs of positive control mice was increased from the first day of infection (Log 5.7±1.19) and the increasing rate continued in the next 2 days (Log 6.9±1.06 at 3rd day).In contrast the animal treated with *L. acidophilus* showed greater resistance to pneumococcal challenge, as their pathogen count in lung was significantly lower than those in positive control at same intervals (on day one was Log 2.5±0.65 to end as Log2±0.43 at third day), likewise the log mean number of bacteria found in blood stream of untreated animal increased steadily over the 3 days of the experiments starting with Log 3.3 ± 0.94 to Log 4.96 ± 0.7 at the last day, while the treated mice showed significant low level of Bacteremia gradually reaching to Log 0.5 ± 0.1 at the last, also the challenge with *S. pneumonia* increased the number of blood leukocytes in both experimental group of our study, but the pretreated mice with *L. acidophilus* present a significant higher value than positive control group. Also, the neutrophils were the principle cell population responsible for the increasing leukocyte of both study groups, followed by lymphocytes cells.

These results showed that administration of intraperitoneal *L. acidophilus* may increases the clearance rate of *S. pneumoniae* from lung and prevent the dissemination of pneumococci into blood and that may due to activation of immune system by increasing the total count and efficacy of leukocytes especially neutrophils and lymphocytes.

الخلاصة:

تم التحري عن التأثير الوقائي للتلقيح داخل الغشاء البريتوني لبكتريا *Lactobacillus acidophilus* ضد اصابات الجهاز التنفسي الناتجة عن بكتريا *Streptococcus pneumonia* .

حقنت فئران المجموعة الأختبارية لمدة خمسة ايام بـ 0.5 مل من عالق بكتريا الـ *L. acidophilus* المقتولة بالحرارة، وحقنت مجموعة السيطرة الموجبة بـ 0.5 مل من دارئ الفوسفات. أحدثت الأصابة ببكتريا *S.pneumonia* بتقطير25µl من العالق البكتيري (الحاوي على 10^8 من الخلايا المكونة للمستعمرات في الطور اللوغارتميي) في كل فتحة من فتحات الأنف ولتسهيل استنشاق اللقاح تم مسك الفئران بشكل عمودي لمدة دقيقتان،قتلت الفئران في الأيام 3,2,1 بواسطة الخلع العنقي (cervical dislocation) .

أظهرت نتائج هذة الدراسة أن أعداد بكتريا ال *S. pneumonia* المسترجعة من انسجة الرئة لحيونات السيطرة الموجبة زادت منذ اليوم الاول (Log 5.7±1.19) للاصابة، واستمرت الزيادة بالتقدم خلال اليومين اللاحقين حتى وصلت الى (Log 6.9±1.06) في اليوم الثالث، وعلى العكس من ذلك فأن الحيوانات التي عوملت ببكتريا ال *L. acidophilus* أظهرت مقاومة ملحوضة للاصابة بالبكتريا المرضية وان تعداد البكتريا في الرئة كان منخفضا بصورة معنوية عن حيوانات السيطرة الموجبة (Log2±0.43 في اليوم الاول الـى Log 2.7±0.65 في اليوم الثالث).

كذلك كان لوغارتم معدل الاعداد للبكتريا المسترجعة من الدم لحيوانات السيطرة متزايدا بصورة متواترة خلال الثلاثة ايام من التجربة أبتدا بـ 0.94 ± 3.3 Log وأنتهاء بـ 0.7 ± 4.96 Log في اليوم الثالث ,فيما اظهرت الحيوانات المعاملة مستويات معنوية واطئة من تجرثم الدم وبشكل تدريجي لتبلغ 0.1 ± 0.5 Log في اليوم الاخير.

لوحظ من خلال النتائج أن الأصابة ب *S. pneumonia* أدت الى زيادة في عدد كريات الدم البيضاء لكلا المجموعتين .لكن الحيوانات المعاملة بـ *L. acidophilus* أظهرت زيادة معنوية عالية مقارنة بحيوانات السيطرة الموجبة.وجد من خلال العد التفريقي أن كريات الدم العدلة (Neutrophils) هي المسؤل الاول عن هذة الزيادة في كلا المجموعتين وتلتها في العدد كريات الدم اللمفاوية (Lymphocytes).

أشارت النتائج الى أن التلقيح داخل الغشاء البريتوني ببكتريا ال ***L. acidophilu***s ممكن أن يزيد من معدل أزالة بكتريا ***S.pneumonia*** من الرئة ويمكن ايضا أن يمنعها من الأنتشار الى الدم ,وقد يعزى ذلك الى تعزيز دورالجهاز المناعي بزيادة اعداد كريات الدم العدلة واللمفاوية وزيادة فعاليتها.

Introduction:

The upper airways of humans (nose and pharynx) may harbor potentially pathogenic bacteria (PPB) (*Staphylococcus aureus* is one of the most important, but *Streptococcus pneumoniae*, ß-hemolytic streptococci, and *Haemophilus influenzae* are also members of this group).

In patients carrying PPB, antiseptic regimens could be crucial for infection control after major operations or injuries of the head, nasal sinuses, or lungs. Such regimens may also be important for diabetic patients and persons receiving hemodialysis, in intensive care units, or with impaired immunity due to various other causes[1].

The widespread antibiotics usage exerts a selective pressure that acts as a driving force in the development of antibiotics resistance [2].

A multi-resistant strain becomes a serious global problem, which constrains scientists to search for new effective therapeutic agents. Alternative therapeutic options should be used as strategies to prevent the selective development of antibiotic resistant bacterial strains, restore a balanced microbial flora and enhance the defense mechanisms of the human body. These criteria are best fulfilled by live microorganisms which are naturally hosted by the human body [3].

The idea that guts microflora play a very important role in the maintenance of health and well-being is acquiring worldwide acceptance[4]. Lactic acid bacteria (LAB) are present in the intestine of most animals and the beneficial role played by these microorganisms in humans and other animals has been reported extensively [5,6].

Recently LAB have become an attractive option of modern medical practice and the attention has been paid to their health-promoting properties of particular importance are their probiotics properties and specially the ability to compete with pathogens in vivo [7].

The host defense against PPB is mainly the task of the immune system. In mammals, the mucosal surface area represents an extensive interface with the external environment through which pathogens mainly initiate infections. The mucosal surfaces of the upper airways are well equipped with an immune system that reacts to some extent independently of the immune system, and these surfaces are functionally linked to other mucosal surfaces [8].

Lactobacilli can directly promote the development of the gut mucosal barrier by the stimulation of the immune response. Since immune mechanisms at different mucosal sites interact through the common mucosal immune system, LAB could also play an important role in disseminating effecter cells to lung and other mucosal sites [9]. Clinical trials have demonstrated that probiotics may decrease the incidence of respiratory tract infections [10].

In this study we evaluate the role of intraperitoneal injection of heat killed *L. acidophilus* as a probiotic bacteria on clearance rate of *S. pneumoniae* from mice lung and dissemination of pneumoccoal in the blood.

Material and Methods:

Animals:

Male 6 week-old Swiss blab\c mice were obtained from Pharmaceutical Control department of the Ministry of Health, they were housed in plastic cages at room temperature and fed mice chow and water ad libitum. Mice divided into 3 groups consisting of 5 mice, in addition to the 3 positive control groups [11].

1 - *Lactobacillus acidophilus* (bacteria were obtained from department of biology college of science, AL-Mustansiriya university) ,and cultured on De man Rogsa sharp medium (MRS) at 37˚C for 48hrs.washed with distilled water ,killed at 100C˚ for 30 min, and suspended in phosphate buffer saline (PBS) at desired concentration just before use.
2 - Capsulated *S. pneumonia* was isolated from respiratory tract of patient in central child hood hospital, Baghdad. *S. pneumonia* was first grown on blood agar for 18 hrs; freshly grown colonies were suspended in brain hart infusion broth and incubated at 37˚C overnight. The pathogens harvested through centrifugation at 3000 rpm for 10 min and then washed 3 times with sterile PBS. The infecting dose was chosen according to Julio *et al*. [9].

Experimental infection:

For five days before challenge with *S. pneumonia*, 0.5 ml of heat–killed LBA was injected intraperitoneally (IP) [11].

Mice were infected by dropping 25µl of the inoculum containing 10^8 Log-phase colony forming unit(CFU)of *S.pneumonia* in PBS into each nostril and allowing it to be inhaled by holding the mice in head-up vertical position for 2 min[9]. Mice were killed on day 1st, 2ed, and 3rd day of infection.

Determination of bacterial growth in lung homogenates and blood:

S. pneumonia was challenged to positive control mice (non-treated) and mice that had been treated with *L. acidophilus*. After challenge, mice were killed by cervical dislocation, lung were excised to determine the bacterial count by homogenization in 1ml sterile normal saline and plated in duplicate on blood agar. Bacteraemia was monitored by sampling the blood via cardiac puncture by a heparinized syringe and cultured in duplicate on blood agar. Result was reported as Log CFU/ml after 18h and 37C˚ incubation period as negative or positive haemocultured [12].

Determination of total and differential numbers of leukocytes in the blood:

The total numbers of leukocytes was determined with haemocytometer and differential cell count was performed by counting 200 cells in blood smears with Giemsa stain [13].

Statistical examination:

Results were expressed as the mean ± standard deviation (SD). The paired t-test was used to test for differences between the control group and the treated group. Significant difference was defined as $P < 0.05$.

Results and Discussion:

Probiotics are live microorganisms thought to be healthy for the host organism. According to the currently adopted definition by FAO/WHO, probiotics are: "Live microorganisms which when administered in adequate amounts confer a health benefit on the host[14].

The use of probiotics in the treatment of specific diseases has evolved into an extremely valuable option yet to be optimally used in clinical medicine [15].

This study concern on the protective effect of heat–killed *L. acidophilus* against *S.pneumonia* infections in male swiss mice with age of 6 weeks was studied. The heat-killed *L.acidophilus* was injected intraperitonally for five days before challenge with vital *S. pneumonia.*

The results showed that numbers of pneumococcal pathogen that recovered from lungs of positive control mice at the 1st day of infection was Log 5.7±1.19, the

increasing rate continued in the next 2 days to reach Log 6.9±1.06 at 3rd day. In contrast the animal treated with *L. acidophilus* showed greater resistance to pneumococcal challenge, as their pathogen count in lung was significantly lower than those in positive control at same intervals (on day one was Log 2.5±0.65 to end as Log2±0.43 at third day (Fig-1).

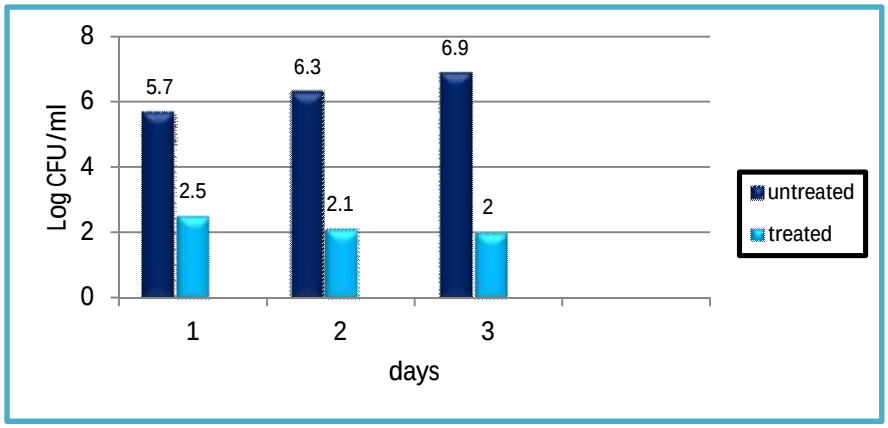


Fig-1: Log mean numbers of bacteria that recovered from mice lungs

The Log mean number of bacteria found in blood stream of untreated animal was increased steadily over the 3 days of the experiments starting with Log 3.3 ± 0.94 to Log 4.96 ± 0.7 at the last day, while the treated mice showed significant low level of Bacteremia gradually reaching to Log 0.5 ± 0.1 at the last day (Fig-2).

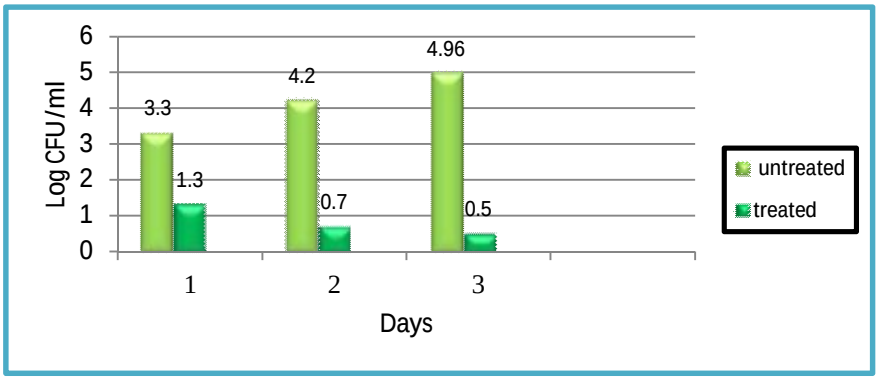


Fig-2: Log mean numbers of bacteria recovered from mice blood.

These finding are in agreement with Julio *et al*.[16] ,they found that pneumococcal colonization of lung and bacteremia were significantly greater in control group comparison with *L. casei* orally pretreated group, they found that the number of bacteria in lung and blood tended to decrease ($P<0.05$) during infection period and they suggest that the addition of *L. casei* to the diet has a beneficial effect because it accelerate the recovery of the innate immune response and improve the specific mechanisms against *S. pneumonia* respiratory infection in malnourished mice.

Other studies [9] found that the oral administration of yoghurt contains two type of lactic acid bacteria (*L. bulgaricus, L. thermophilus*) induced an early recovery of immunological parameters and accelerate the normalization of immune response against the pathogenic respiratory agents while Alvarez *et al*.[17] stated that the oral administration of *L.casei* or yoghurt

to young mice enhanced lung clearance of *pseudomonas aeruginosa.*

Nasally administration of live or heat killed *L. casei* prevent the dissemination of the pathogenic bacteria to the blood and induced its lung clearance [18].Rosa *et al.*[19] demonstrated that intranasal administration of *L. fermentum* isolated from pharynx of BALB/C mice was able to reduce pathogen count from all respiratory tract organs and that indicates the presence of Lactobacilli lead to decrease the adherence or faster clearance of *S. pneumonia* from respiratory tract. Marcela *et al.* [20] evaluate the effect of nasal administration of *L. lactis* to Swiss albino mice and showed that such treatment was able to increase the clearance rate of *S. pneumonia* from lung and prevent spreading the pathogen to the blood

and they suggest that the effect induced by the nasal inoculation of *L.lactis* could be explained by a decreased

adherence of *S.pneumoniae* to the respiratory epithelium, as it has been

reported that nasally given LAB could exclude competitively pneumococcal cells[21] Saito *et al.*[22] refer to a single intraperitonal injection of *L.casei* into mice led to nonspecific resistance against intraperitonal challenge with lethal dose of *pseudomonas aeruginosa.*

The challenge with *S. pneumonia* increased the number of blood leukocytes in both experimental group of our study, but the pretreated mice with *L. acidophilus* present a significant higher value (P<0.05) than positive control group (Fig-3).

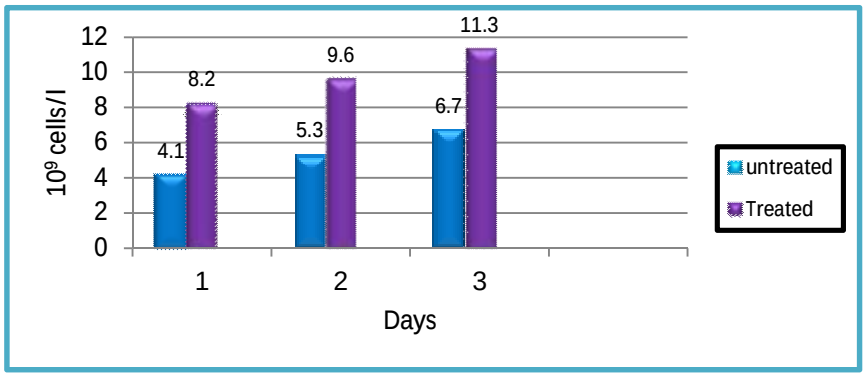
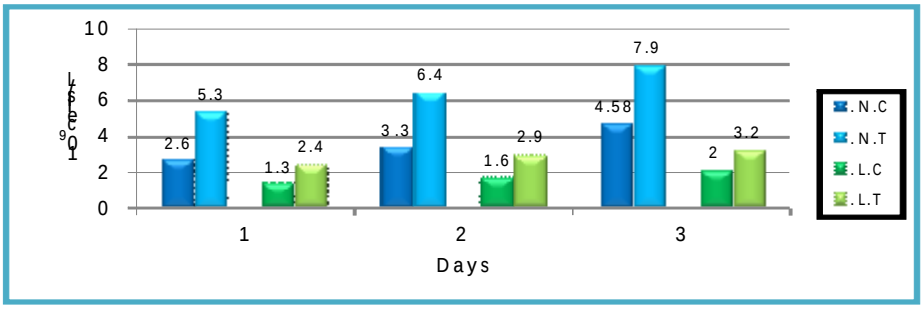


Fig-3: Total WBC count in positive control group (untreated) and treated mice

The neutrophil was the principle population responsible for the increasing leukocyte of both study groups, followed by lymphocytes cells (Fig-4).



N.C.=Neutrophils of positive control mice

N.T. = Neutrophils of pretreated miceL.C. =

Lymphocytes of positive control mice

L.T. = Lymphocytes of pretreated mice

Fig-4: Neutrophils and lymphocytes counts of positive control and pretreated mice

In an uninfected lung, neutrophils are in the blood (with increased numbers marginated within the pulmonary capillaries compared with other blood vessels) but not the air spaces[23]. However, within hours of infection, neutrophils begin to appear in the interstitial compartments and air spaces of the lungs[24, 25].

Several studies demonstrated that phagocytic and microbial activity of neutrophils in blood and those recruited into the lung are great importance for the control of pneumococcal infections [26,27]. Also alveolar macrophage constitute the first line of phagocytic defense against infectious agent that evade the mechanical defense and gain access to the gas-exchange air ways[28], these macrophage are capable of generating numerous mediators that induce the recruitment of neutophils from pulmonary vasculature into the alveolar space. These recruited neutrophils provide auxiliary phagocytic capacities that are critical for the effective eradication of offending pathogens[28, 29, 30].

Our findings are goes with Julio *et al*. [18], they found the nasal administration of *L. casei* to malnourished mice increase the number of leukocytes and the peroxides activity of phagocytes cells in the blood and that would explain why low or no bacteria were detected in blood, they suggest that the treatment with probiotic bacteria exerts effects on hematopoiesis. Another study[16] showed that a progressive increase in white blood cells was observed in mice that receive orally *L. casie* in their diet after infection with *S. pneumonia* and the differential count indicate that this increasing phenomenon involved mainly neutrophil and they found that infection rapidly triggered the neutrophil recruitment into the alveoli of mice resulting in increased cell counts in broncho alveolar lavage (BAL). Also Salva *et al.*[31] reported that mice replete with *L. casei* and challenged with *S. pneumonia* had an elevated level of blood neutrophil and this elevation continue until the 5th day post infection.

Marcela *et al*.[20] refered to an increase in activation of macrophage observed in mice treated with *L. lactis* by nasal administration and that enhance the protective effect, this activation would induce more effective neutrophil recruitment[32,33]. Hidemura *et al*.[34] stated that impaired neutrophil recruitment into local inflammatory site can be restored by administration of *Bifidobacteruim longum* as a probiotic.

The protective effect of treatment with *Lactobacillus* against the pneumo-coccal colonization of lung, bacteremia and lung tissue injury was correlated with the stimulation of the systemic and respiratory immune response [18].

Pulmonary lymphocytes play an important role in lung defense against infection because they influence important events of inflammation and tissue repair, such as the recruitment of phagocytes from blood and fibroblast activation [35]. Salva *et al.*[31] demonstrated that the use of *L. casei* CRL 431, as a supplement in a repletion diet, induced an improvement in the production and maturation of myeloid and lymphoid cells. Perdigon *et al.* [36] showed that feeding with milk fermented with *L. casei*, *L. acidophilus* or a mixture of both produces a remarkable effect on the immunostimulation in the host and he found that macrophage and lymphocyte activation was much higher in cultures fermented with *L. casei* and *L. acidophilus* or both than in the controls or in the individual or mixed culture without a fermentation process. Such enhancement of the immune response might be due to the substances produced by these organisms during the fermentation process, such as some metabolites and casein peptides, bacterial enzymes (proteolytic and acteriolytic) that improve the digestibility of the milk constituents.

Conclusion:

These results from this study suggest that the previous intraperitoneal administration of heat–killed *L.*

acidophilus to penumococcal infected mice has a beneficial effect because it reduce significantly the colonization of pneumococcus pathogen in lung also increase the number of leukocytes mainly neutrophil and that may enhance the resistance of respiratory tract against the *S. pneumonia* infection.

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

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