Immune Enhancing Effect of *Lactobacillus acidophilus* and *Lactobacillus gasseri* in Mice Infected with *Salmonella typhimurium*

التأثـــير المناعـــي المحفز لبكتريا حـــامض اللاكـتيك في الفئران المخـتبرية والمـصابة تجريبياً ببكـتريا الســـالمونيلا

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Abstract

This study was carried out to assess the protective immune effect of mixed culture of *Lactobacillus acidophilus* and *Lactobacillus gasseri* in mice infected with *Salmonella typhimurium*. Parameters of evaluation were total and absolute count of leukocyte and phagocytosis. Fifteen albino mice divided into five groups and designated as follows: CG used as negative control, SG was infected with 0.1 ml *Salmonella typhimurium* 2.5×10^7 cfu/ml and used as a positive control, AC was treated with 0.1ml *Lactobacillus acidophilus* 1×10^9 cfu/ml and infected with *Salmonella typhimurium* 2.5×10^7 cfu/ml, GG was doused with *Lactobacillus gasseri* and infected with *Salmonella typhimurium*, AG was fed with 0.1 ml mixed culture of *Lactobacillus acidophilus* and *Lactobacillus gasseri* 1×10^9 cfu/ml and infected with *Salmonella typhimurium* 2.5×10^7 cfu/ml. Results indicated that mice treated with viable *Lb.acidophilus* and *Lb.gasseri* showed a significant protective immune effect compared with positive and negative control, while mice fed with mixed culture of *Lb.acidophilus* and *Lb.gasseri* exhibited less protective effect against *Salmonella typhimurium* compared with groups fed with monoculture of *Lactobacillus.*

Key words: Immune effect, *Lactobacillus, Salmonella typhimurium*

المستخلص

اجريت الدراسه لتقييم التأثير المناعي لبكتريا حامض اللاكتيك *Lb acidophilus* و بكتريا *LbgasseriL* على الفئران المصابة ببكتريا السالمونيلا *Salmonella typhimurium*. درس التأثير المعزز المناعي الناتج من بكتريا *Lb acidophilus* وبكتريا *Lactobacillus gasseri* من خلال دراسة التعداد الكلي والتفريقي لكريات الدم البيضاء وعملية البلعمة داخل الجسم الحي على 15 فأرة بيضاء والتي تم تقسيمها عشوائيا الى خمسة مجاميع 1،2،3،4،5 تضمنت كل مجموعة على ثلاثة فئران متساوية بالاعمار والاحجام. عدت المجموعة (1) التي تم تغذيتها على العليقة الاساس (مجموعة سيطرة سلبية)، اما المجموعة (2)، ثم اصابتها ب 0.1 مل من بكتريا *S.typhimurium* بعدد خلايا 2.5×10^7cfu/ml، بينما تم تغذية مجموعة (3) ب0.1 مل من بكتريا *Lb acidophilus* بعدد خلايا 1×10^9cfu/ml, وتم اصابتها ب0.1 مل من بكتريا *S.typhimurium* بعدد خلايا 2.5×10^7cfu/ml. المجموعة (4) فقد تم تغذيتها ب0.1 مل من بكتريا *gasseriLb* بعدد خلايا 1×10^9cfu/ml، ثم اصابتها ب- 0.1 مل من بكتريا *S.typhimurium* بعدد خلايا 2.5×10^7cfu/ml. اما المجموعة (5) فقد تم تغذيتها ب -0.1 مل من المزروع البكتيري المزيج من *Lb acidophilus* و بكتريا *gasseriLb* الحية بعدد خلايا 1×10^9cfu/ml، ثم اصابتها ب 0.1 مل من بكتريا *S.typhimurium* بعدد خلايا 2.5×10^7cfu/ml. اذ اظهرت النتائج انالتأثير المعزز المناعي في الفئران التي جرعت عتر احادية ببكتريا *Lb acidophilus* وبكتريا *gasseriLb* كان فعالا مقارنة مع مجموعة السيطرة بينما اظهرت الفئران المعاملة ببكتريا *Lbacidophilus* وبكتريا *gasseriLb* الثنائية العترة انخفاضا معنويا في التعداد الكلي والتفريقي لكريات الدم البيضاء وعملية البلعمة.

الكلمات المفتاحية: التأثير المناعي، بكتريا حامض اللاكتيك، الســـالمونيلا

Introduction

Probiotics are nonpathogenic organisms (yeast or bacteria, especially lactic acid bacteria) in foods that can exert a positive influence on the host's health [1].

Lactic acid bacteria (LAB) such as *Lactobacillus* are important micro-organisms in a healthy human microbiotic environment and produces a number of antimicrobial substances, including organic acids, hydrogen peroxide, bacteriocins, and bacteriocin-like substances [2]. Bacteriocins or bacteriocin-like substances are peptides or proteins, which exhibit inhibitory activity against sensitive strains of bacteria [3].

Research using single probiotic strains has been reported earlier but at present probiotic combinations with possibly additional health benefits are being assessed prior to use in clinical studies. At present, only a few scientific reports on the effects of probiotic combinations are available [4].

Salmonella spp. is one of the principal causes of human food-borne infections and resulted in a variety of disease syndromes such as enteric fever, bacteriemia, focal infections and enterocolitis[5,6]. *Salmonella* species can adhere to and invade eukaryotic cells by using different types of fimbriae and numerous proteins [7]. The ability to invade mammalian cells is critical to initiate the infection [8]. An essential step in the pathogenesis of typhoid fevers in mice or humans is the establishment of a systemic infection after oral challenge. This systemic infection usually develops from foci established in the Peyer's patches of the small intestine soon after ingestion. Thus the virulence of typhoid bacteria depends on their ability to penetrate into the Peyer's patches, to survive and multiply therein, and subsequently to become disseminated and survive systemically [9].

Multidrug-resistant (MDR) strains of *Salmonella* are now encountered frequently and the rates of multidrug-resistance have increased considerably in recent years [10]. Even worse, some variants of *Salmonella* have developed multidrug-resistance as an integral part of the genetic material of the organism, and are therefore likely to retain their drug-resistant genes even when antimicrobial drugs are no longer used.

Probiotic LAB has recently been suggested to become a useful tool that could be used as a preventive substance instead of antibiotics. However, heavy use of antibiotic has become a major problem, since it results in drug-resistant bacteria, thus, alternative and non-pharmaceutical strategies for controlling enteropathogenic bacterial infection have been sought by oral challenge with such enter pathogens as *Salmonella typhimurium*, *Escherichia coli*, *Shigellasonnei* and *Listeria monocytogenes* [11]. This study was carried out to investigate the immunoprctotective effect of mono and mixed culture of *Lactobacillus acidophilus* and *Lactobacillus gasseri*.

Material and methods:

Bacterial isolates:

Lactobacillus acidophilus, *Lb. gasseri and Salmonella typhimuruim* were supplied by Immunology Lab. in the Department of Biotechnology, College of Science, Al-Nahrain University. *Lactobacillus acidophilus* previously isolated from vaginal swab, *Lb. gasseri*was previously isolated from red peach fruit and *Salmonella typhimuruim* previously isolated from patient's stool infected with Salmonellosis.

Mixed *Lactobacillus*culture

The *Lactobacillus* was grown in de Mann, Rogosa and Sharp (MRS) broth (Merck) media for 18 hr at a 37^0c. This activated culture was centrifuged at 2000 gat 4ºC and resuspended in phosphate-buffered saline with pH 7, in order to obtain 10^9colony forming units (cfu)/ml which were obtained by using McFarland's method. The culture of *Lb.acidophilus* and *Lb. gasseri* was adjusted to a cell density 10^9colony forming units (cfu)/ml. Two cultures were mixed at a ratio 1:1. 100µl of this suspension was administered to mice by gavage needle, before the challenge with the pathogenic bacteria [12].

Bacterial infection

Mice were challenged by the oral-gastric route with 0.1 ml of the bacterial suspension containing about 2.5×10^7cfu which was obtained by using McFarland's method [7].

Experimental Design

Fifteen albino male mice were randomly divided into five groups designated as CG, SG, AG, GG and AG. Each group consists of 3 mice, and subjected to the following treatments: GroupCG: This group was used as a negative control.GroupSG:This group was doused with 0.1ml of 2.5×10^7cfu/ml *Salmonella typhimurium* culture and use as positive control. GroupAG: This group was fed with 0.1ml of 10^9cfu/ml *Lactobacillus acidophilus* culture, and infected with 0.1ml of 2.5×10^7 cfu/ml culture of *Salmonella typhimurium*. GroupGG: This group was fed with 0.1ml of 10^9cfu/ml *Lactobacillus gasseri*culture, and doused with 0.1ml of 2.5×10^7 cfu/ml culture of *Salmonella typhimurium*. GroupAG: This group was fed

with 0.1ml of 10^9cfu/ml of mixed *Lactobacillus acidophilus* and *Lactobacillus gasseri* cultures, and doused with 0.1ml of 2.5×10^7 cfu/ml culture of *Salmonella typhimurium*.

Mice were fed with a single dose 0.1 ml of 10^9cfu/ml *Lactobacillus* culture daily by oral administration for 7 consecutive days. After 7 days treatment, at the 8thday of experiment period, each mouse was challenged with 0.1 ml *S. typhimurium* (2.5×10^7) by oral administration. After 6th day infection with *Salmonella*, mice were sacrificed by cervical dislocation and the blood samples of each mouse were collected to evaluate immunological parameters [3].

Total Leukocyte Count

Blood samples were collected by heart puncture using a disposable insulin syringe 1 ml precoated with heparin. The method of [14] was followed, in which, an aliquot of 0.02 ml blood was mixed with 0.38 ml of leucocyte diluents in a test tube, and left at room temperature for 5 minutes. A drop of the mixture was applied to the surface of Neubauer chamber under the cover slip, and the chamber was left for 3 minutes to settle the cells. The leucocytes were counted in 4 large squares (each with 16 small squares), and the total count of leucocytes was obtained using the following equation:

$$\textbf{Total Count (cell/cu.mm.blood)} = \left(\frac{\text{Number of Cells Counted}}{4}\right) \text{x } 20 \text{ x } 10$$

Absolute Count of Leukocytes

One drop of blood was smeared on a clean slide using another slide and left to dry at room temperature. The smear was stained with Leishman stain for 5 minutes and buffered for 10 minutes, and then washed with tap water. The slide was air-dried, and then examined under oil immersion lens (100X) [14]. At least 100 leucocytes were examined, and the percentage of each type was recorded, while the total count of each type was obtained using the following equation:

$$\textbf{Total Count (cell/cu.mm.blood)} = \left(\frac{\text{Percentage of Cells x Total Count}}{100}\right)$$

Phagoctic Index

Mice were anaesthetized with chloroform, and then injected intraperitoneially with 3 ml of normal warm saline 37°C, then the abdominal region was massaged for 3 minutes. After that mice dissected, and the peritoneal cells were collected with a pasture pipette and transferred to a clean test tube. The tube was centrifuged 2000 rpm/minutes for 5 minutes and cells were suspended in 1 ml of normal saline, counted and their number was adjusted to 10^6 cell /ml. Also, the cell viability was assessed using try pan blue stain. To carry out phagocytosis, 0.2 ml of cell suspension, 0.1 ml of heat-killed yeast suspension and 0.1 ml of human plasma AB were mixed in a test tube and incubated in a shaking water bath 37°C. After 30 minute incubations, smears were made and the slides were air-dried, and stained with Giemsa stain for 15 minutes. The slides were examined under oil immersion lens (100X), and at least 100 yeast-phagocytic and non-phagocytic cells were randomly counted. The phagocytic activity was expressed as a phagocytic index, which was calculated using the following equation [15]:

$$\textbf{Phagocytic Index (\%)} = \left(\frac{\text{Number of Phagocytic Cells}}{\text{Total Count}}\right) \text{x } 100$$

Statistical analysis

The values of the investigated parameters were given in terms of mean ± standard error, and differences between means were assessed by analysis of variance ANOVA and Duncan test, using the computer programme SPSS version 7.5. [16]

Results

Table (1): Total and absolute count of leukocyte (mean ± standard error) in mice fed with *Lacto bacilli* and infected with *S.typhimurium* and control groups

Groups	CG negative control	SG positive control	AC (*Lb.acidophillus*	*GC Lb.gasseri*	AG Mixed *Lactobacilli*
Total leukocyte count	7918 ± 366 a	3183 ± 320 a	9355 ± 669 a	10217 ± 377 a	5488 ± 478 b
Lymphocyte count	4960 ± 385 a	2210 ± 52 a	5304 ± 194 a	5600 ± 288 a	3844 ± 98 b
Neutrophile count	1966 ± 83 a	698 ± 85 a	2922±89 a	3420 ± 97 a	1010 ± 67 b
Monocyte count	849 ± 124 a	225 ± 24 a	889 ± 49 a	933 ± 79 a	566 ± 97 b
Eosinophile count	67 ± 42 a	30 ± 23 a	159 ± 43 a	178 ± 33 a	41 ± 31 b
Basophile count	76 ± 33 a	20 ± 12 a	81 ± 29 a	86 ± 32 a	27 ± 15 b

a: Significant difference (p≤0.05) as compared with Control.
b: Significant difference (p≤0.05) as compared with SG.

Mice exerted a significant variation in phagocytic index between control groups and different groups of mice treated with *Lb.acidophillus*, *Lb.gasseri* anda mixed culture of *Lb.acidophillus* and *Lb.gasseri* Table (2)

Table (2): Phagocytic index of peritoneal cells (mean ± standard error) in mice fed with *Lactobacilli* and infected with *S.typhimurium* and control groups

Groups	Mean ± Standard Error	Statistical evaluation
(negative control)CG	47.51 ± 1.26	a
(positive control)SG	49.6 ± 1.3	a
(*Lb.acidophillus*)AC	55.50 ± 3.50	a
(*Lb.gasseri*)GC	44.00 ± 3.0	b
(Mixed *Lactobacilli*)AG	53.50 ± 1.50	a

a: Significant difference (p≤0.05) as compared with Control.
b: No Significant difference (p≥0.05) as compared with Control

Discussion

Results showed that mice fed with monoculture of *Lactobacillus acidophilus* and *Lactobacillus gasseri* exhibited a statistically increase in total and absolute count of leukocyte and phagocytic index compared with positive and negative control. These results come in agreement with previous results obtained by [17]who stated that human *Lb. acidophilus* strain LB produces an antibacterial activity effective *in vitro* against Gram-negative and Gram-positive pathogens, *in vitro* against an enter invasive pathogen which adheres to and enters cultured human enterocytic cells, and *in vivo* in the *S. typhimurium*-infected-mouse model believe that the component(s) secreted by *Lb. acidophilus* LB that supports the antimicrobial activity could contain an unusual acidic amino acid present in a novel peptidic agent.

Many investigators reported that protective effect *of Lactobacillus* species against *Salmonella typhimurium* infection in mice might be due to their ability to adhere to intestinal mucus and this property was proved to be correlated with successful and long time *in vivo* colonization of gastrointestinal tract [18]. Potentially genes of *Lb.acidophilus* involved in adhesion, the complete genome sequence of *Lb.acidophilus* was analyzed and results showed that three genes were involved in *Lb. acidophilus*adhesion to intestinal cells which were, fibronectin-binding protein (FpbA), a mucin-binding protein(Mub), and a surface layer protein (SlpA). It was proven that the genes encoding FbpA, Mub, and SlpA contribute to the ability of *Lb.acidophilus* to adhere to intestinal cells *in vitro* [19]. A significant decrease in adhesion was observed in the fibronectin-binding protein mutant 76% and the mucin-binding protein mutant 65%. A surface layer protein mutant also showed reduction in adhesion ability 84%, but the effect of this mutation is likely due to the loss of multiple surface proteins that may be embedded in the S-layer [20]. *Lb. acidophilus* is more effective at inducing T-helper-1cytokines while *L. salivarius* induces a more anti-inflammatory response[21]. Another study revealed that mice fed with *L*b. *acidophilus* or *L*b. *paracasei* also enhanced the secretion of anti-inflammatory cytokine (IL-10) and pro-inflammatory cytokine (IFN-γ). The results of this study suggest that *Lb. acidophilus* and *Lb. paracasei* were able to enhance specific gut and systemic immune responses in mice [22].The same result was obtained by [23] who found that several strains of *Lb.gasseri* showed a wide inhibitory activity against the tested bacteria. Gassericin A produced by *Lb. gasseri* LA39 was one of the most widely active bacteriocins. It was bactericidal without causing cell lysis. Mice fed with LAB exhibited a significant increased in IFN-γ level and enhanced phagocyte function, and this caused a significant increase 66–100% in the phagocytic activity of (monocytes and polymorph nuclear cells) of mice fed with LAB compared with the control [24]. Results in this study demonstrated that mice fed with mixed culture of *Lb. acidophilus* and *Lb. gasseri* caused a statistically decrease in total and absolute count of leukocyte compared with negative control but this result was significantly higher when compared with positive control. A different observation was found by [25] who reported that feeding milk fermented with a mixture of *Lactobacillus casei* and *Lactobacillus acidophilus* exhibited a protective effect against *Salmonella typhimurium* infection in mice. The mixed culture of probiotic strains could increase the protective and treatment effects against *Salmonella typhimurium* infection and that they are more effective than using the individual probiotic strain [26]. This result may be due to the probiotic combination and the pathogen tested, indicating a very high specificity and requiring identification of the pathogens or related microbiota aberrancies involved in the probiotic target population. This result agreed with who reported that the ability to inhibit the adhesion of pathogens appears to be dependent in both, the probiotic combination and the pathogen tested and the displacement of pre-adhered pathogens was also found to be probiotic combination and pathogen dependent [27] a significant increase in total and absolute count and phagocytic index was ain mice treated with monoculture and mixedculture of *Lactobacillus acidophilus* and *Lactobacillus gasseri*.

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