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A STUDY ON THE EFFECT OF TWO PROBIOTIC STRAINS (*B. bifidum* and *Lac. rhamnosus*) IN THE PROTECTION OF KIDNEY FROM TOXICITY OF A CARCINOGENIC SUBSTANCE

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Abstract

The aim of this research is to study the effect of two probiotic strains (*Bifidobacterium bifidum* and *Lactobacillus rhamnosus)* which are used in a large scale to protect kidney tissue from a carcinogenic substance. 20 male albino male mice (8-10 weeks old) with an average initial body weight of 20 ± 3gm were used. Four groups were prepared, control was fed on a standard diet, the other groups were fed on basal diet containing 15 g yogurt with *Lac. rhamnosus* and *B. bifidum* as probiotic strains for 2 weeks (group A) and the second group was still fed the same diet for further 2weeks (group B), the third one was fed on basal diet containing 15 g yogurt with probiotic strains for 4 weeks but after further 2 weeks was treated orally by one dose of toxic mutagen (3amino-1 methyle-5H-pyrido(4-3-b)indole) (0.25/mouse group C). The results proved that feeding on probiotic bacteria increased the immune activity and reduced the toxic effects of the carcinogenic substance on the kidney tissues and kept the kidney functioning. .

Key words: Probiotic, kidney, *B. bifidum* and *Lac. rhamnosus.*, cancer

INTRODUCTION

Probiotics are dietary supplements containing potentially beneficial bacteria or yeast, with lactic acid bacteria (LAB) as the most common microbes used. LAB have been used in the food industry for many years, because they are able to convert sugars (including lactose) and other carbohydrates into lactic acid. This not only provides the characteristic sour taste of fermented dairy foods such as yogurt, but acts as a preservative, by lowering the pH and creating fewer opportunities for spoilage organisms to grow.

Probiotic bacterial cultures are intended to assist the body's naturally occurring gut flora to restablish them. They are sometimes recommended by doctors and, more frequently, by nutritionists, after a course of antibiotics, or as part of the treatment for gut related candidiasis. Claims are made that probiotics strengthen the immune system (Sanders, 2000).

In Japan and Europe, many products contain probiotics, prebiotics and symbiotics to promote health and well being are available. Probiotics can be defined as a live microbial food supplement that affects the host animal beneficially by improving its intestinal microbial balance (Fuller, 1989). A prebiotic is defined as " a nondigestible food ingredient that beneficially affects the host by selectively stimulating the growth and/or activity of one or a limited number of bacteria in the colon" (Gibson and Roberfroid, 1995).

Interest is growing in the U.S. for products containing probiotics such as *Lactobacillus acidophilus* and *L. bifidus*, which are expected to achieve mainstream appeal within the next few years (Slone, 1999). For decades, it has been believed that the consumption of fermented dairy products produce an array of health benefits.

Many beneficial effects of probiotic strains were observed in many studies, prevention of pathogen colonization (Slone, 1999 & Rayes, and Mehanna, 2007) , elevation of lactose tolerance (Jiang, et al., 1996), prevention of cancer (Mclntosh et al., 1999 &Rayes et al. 2006). Immunostimulation (Aattouri et al., 2002) anticholesterolic effect (Bertazzoni et al., 2001), growth enhancement of farm animals (Baird, 1977) and stimulation of immunomdulatory cells (Rolfe, 2000).

The aim of this research is studying the protective effect of two probiotic strains (B. bifidum and Lac. rhamnosus) which are used in large scale, on the toxic effect of the carcinogenic mutagen 3-amino-1 methyl-5H-pyrido(4-3-b)indole.

MATERIAL AND METHODS

I. Bacterial Strains: *Lactobacillus delbreuckii* ssp. *bulgaricus* and *Streptococcus thermophilus* (used in manufacture of yogurt) obtained from Chr. Hansen, Lab-Denmark. *Lactobacillus rhamnosus* and *Bifidobacterium bifidum*

were obtained from Friendly Bacteria Unit – National research Center – Cairo - Egypt.

Pathogenic strains indicators were used to study the antagonistic activity including *Escherichia coli* ATCC 8739, *Staphylococcus aureus* ATCC 6538, *Pseudomonas aeroginosa* ATCC 9027, *Bacillus subtilis* ATCC 6633, *Klebsiella pneumonia* ATCC 18833, *Salmonella typhimurium* ATCC 13311.

II. Animals: twenty male albino mice (8-10 wk old) with an average initial body weight of 20 ± 3 gm were used. Animals were placed in individual metallic cages and housed in a room that was maintained at a constant temperature of 22° ±2°C, a relative humidity of 60 ±5%. Mice were housed on a 12-h light: dark schedule, with free access to water and mouse standard diet containing (g/100 g): 64 starch, 23 protein, 3.5 fat, 5 fiber, 1 vitamin mixture and 3 salt mixtures.

Four groups were prepared, the first was a control group feeding on standard diet, other groups were feeding on basal diet containing 15 g yogurt with *Lactobacillus rhamnosus* and *Bifidobacterium bifidum* as probiotic strains for 2 weeks (group A) and the second group was still fed the same diet further 2 weeks (group B), the third one (group C) fed on basal diet containing 15 g yogurt with probiotic strains for 4 weeks but after further 2 weeks was treated orally by one dose of toxic mutagen 3Amino-1 methyl-5H-pyrido(4-3-b)indole which was imported from a scientific center in USA (0.25/mouse).

The fecal samples were collected at the beginning of the experiment, before two weeks and at the end of the experiment. On the last day of the experimental period, the mice were killed by carbon dioxide and dissected to process their kidney for light microscopy.

III- Study of the Strains for Probiotic Properties:

1. Bile tolerance

In order to assess bile salt tolerance of bacteria, the isolates of bacteria spp. strains, were incubated in MRS broth (pH 7.0) plus 0.05% L-cysteine at 37°C for 24 hrs under anaerobic conditions. MRS broth was supplemented with 0.3% (w/v) Oxgall (Sigma, USA, pH 7.0). All bacteria were inoculated as 30 µl volume and

incubated at 37°C for 3 hrs. Then, bacteria were spread onto BL agar plates (Pacher and Kneifel, 1996) and anaerobically incubated at 37°C for 48 hrs to confirm the survival of bacteria. If colonies were formed on the BL media, they were decided as the bacteria to have bile salt tolerance (De Smet *et al.*, 1995 and Kimoto *et al.*, 1999).

2. Growth at low pH

To assess low pH tolerance, the first isolates, bacteria were grown in MRS plus 0.05% L. cysteine media and harvested by centrifugation (5000 rpm for 10 mm at 4°C). The pellet was resuspended in the same volume of the same media adjusted to pH 1, 2 or 3 with 10% (wt/vol.) HC1. Control cultures at pH 7 were included. Resuspended cells were incubated at optimum temperature for 3 hrs. After incubation, viable counts were determined by spread onto BL media to discriminate the survival of bacteria and anaerobically incubated at 37°C for 48 hrs. If the colonies were formed on the BL media after 48hrs incubation, they were confirmed as the bacteria to have low pH tolerance (Kimoto *et al.*, 1999).

3. Antibacterial activities of the strains of isolated bacteria spp.

Antimicrobial effects of presumptive strains of bacteria spp. on *E. coli*, *S. aureus*, *P. aeroginosa*, *B. subtilis*, *K. pneumonia* and *S. typhimurium* were determined by the agar diffusion method (Fleming *et al.*, 1985). For the detection of antibacterial activity of the strains of bacteria spp., MRS plus 0.05% L. cysteine was used. Ten milliliters of broth was inoculated with each strain of bacteria spp. and were incubated at 37° C for 48 hrs. After incubation, a cell-free solution was obtained by centrifuging (6000 x g for 15 min) the culture, followed by filtration of the supernatant through a 0.2 μm pore size (Schleicher & Schuell, Germany) cellulose acetate filter. Some supernatants were neutralized with 1 N NaOH to pH 6.5, and the inhibitory effect of the hydrogen peroxide was eliminated by the addition of catalase (5 mg/ml). Unneutralized (general inhibitory effect) and neutralized (bacteriocin and bacteriocin-like metabolites) supernatants of the strains of bacteria spp. were checked for their antibacterial activity against the pathogenic bacteria in inoculated nutrient agar (Schillinger and Lucke, 1989 and Reinheimer *et al.*, 1990). Then 100 ml of cell free supernatants were filled in 8-mm diameter sealed wells cut in the nutrient agar. Once solidified, the dishes were stored for 2 hrs in a refrigerator. The inoculated plates were incubated for 24 hrs

at 37° C, and the diameter of the inhibition zone was measured with calipers in millimeters (Harris *et al.*, 1989).

IV-Preparation of Yoghurt

Fresh cow milk (8.6% solid not fat, 3.3% fat) was heated to 90 °C for 10 minutes, and then cooled to 40°C. Yoghurt starter (*Str. thermophilus* + *L. bulgaricus)* was inoculated at 2% level (volume/volume). And it was inoculated with 2% *Lactobacillus rhamnosus* and 5% *Bifidobacterium bifidum*.

V – Analysis

1. Fermented milk: *B. bifidum* was determined according to Blanchette *et al.* (1996) using modified MRS agar (oxoid) plus 0.05% cysteine HC1 and incubated at 37°C for 24 hours under anaerobic conditions BBL Gas Pak, Becton Dickinson, Cockeysville MA, USA). *Lac. rhamnosus* was counted on LC agar (Ramakanth and Nagendera, 1998). Plates were incubated anaerobically at 37 °C for 72 hrs.

2. Fecal microbial analysis: All fecal samples were collected fresh by gently squeezing the rectal area of the mice. The fecal pellets were immediately placed in tubes kept in anaerobic jars and the analysis was carried out within 30 to 60 min of collection. Anaerobic conditions were maintained as far as possible during the analysis. Following homogenization, a series of a 10-fold dilution of the samples was made in a prereduced sterile phosphate buffer. Triplicate plates were made for each sample. *B. bifidum* counted on MRS agar with L-cysteine and *Lac. rhamnosus* was counted on LC agar. Plates were incubated anaerobically in an anaerobic chamber at 37°C for 72 hrs.

3. Histopathological study: For histopathological study routine paraffin sections 7 µm thick were prepared and stained with haematoxylin and eosin (Drury and Walington, 1980).

RESULTES AND DISSCUSSION

1- Probiotic Properties:

Both the strains *Lactobacillus rhamnosus* and *Bifidobacterium bifidum* were tolerant to bile salt and pH 3.0, but not able to grow at pH 1.0 or 2.0 (the

colonies were formed on the BL media after 48 hrs incubation, they were confirmed as the bacteria which have low pH tolerance (Kimoto *et al.*, 1999).

Results in table (1) show antimicrobial activity of culture supernatants obtained from the two strains being tested against six pathogenic bacteria strains. The antimicrobial activity produced by *B. bifidum* and *Lac. rhamnosus* had inhibition effect on the growth of *Staph. aureus, P. aeroginosa, K. pneumonia, S. typhimurium, B. subtilis* and *E. coli* as shown in table (1). It was noticed that the highest effect of *B. bifidum* and *Lac. rhamnosus* was shown on *Staph. aureus* and *S. typhimurium* respectively and the lowest effect was noticed on *B. subtilis* and *K. pneumonia* respectively (Gibson and Wang, 1994 and Fujiwara *et al.*, 1997).

Table (1): Antimicrobial activity of culture supernatants of *B. bifidum* and *Lac. rhamnosus* strains (mm)

Strain	Pathogenic bacteria					
	E.coli	*K.pneumonia*	*P. aeroginosa*	*Stah. aureus*	*B. subtilis*	*S. typhimurium*
B. bifidum	6	6	6	8	4	5
Lac. rhamnosus	8	4	8	5	6	9

These results indicated that both strains have probiotic activity.

II. Fecal Microbial Analysis:

Fig (1) illustrates the count of *B. bifidum* in stool samples of mice which feeding on fermented milk containing such a strain. The results indicated that feeding on fermented milk that contains probiotic strains allow the strains to grow in track. The result shows that the average of the increase count of natural flora is low, while it is dramatically increase when the mice were feeding on it. The same figure shows that the mutagenic substance inhibited the growth of *B. bifidum* but still higher than the control.

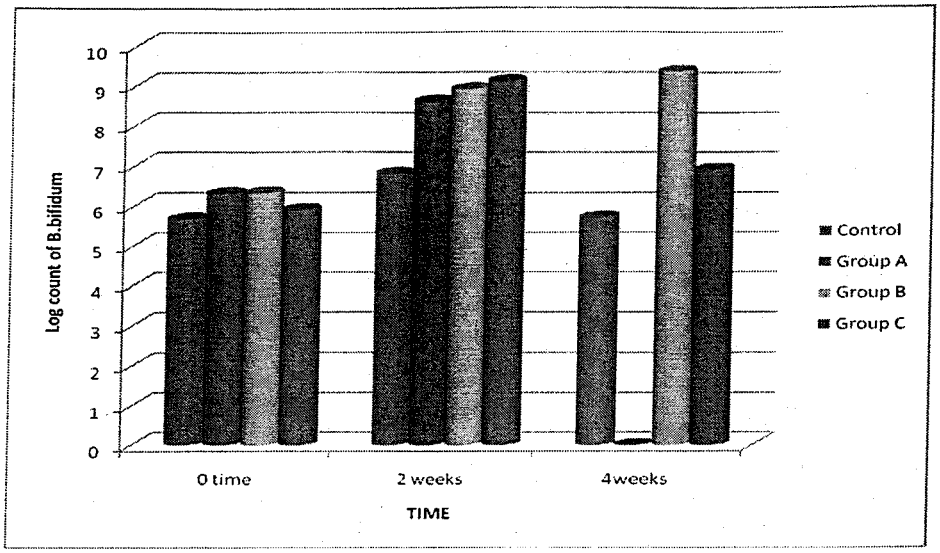

Fig. (1): Effect of feeding mice on yogurt contains *B. bifidum* through 4 weeks.

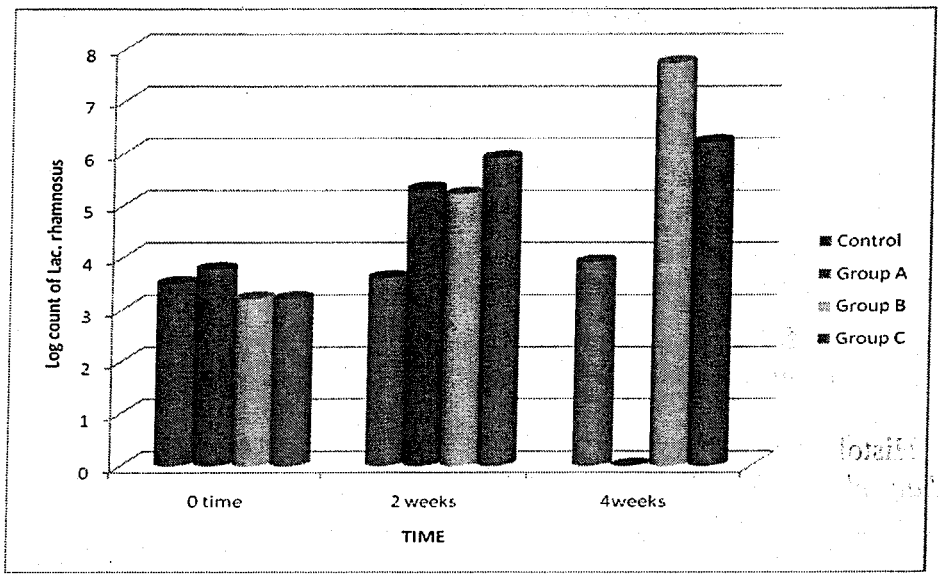

Fig. (2): Effect of feeding mice on yogurt containing *Lac. rhamnosus* through 4 weeks.

The same trend is noticed in Fig. 2. The count of *Lac. rhamnosus* in sharp increase when mice were feeding on fermented milk containing this strain. The bacterial count decreased in the group that was treated with mutagenic substance, the lowest value was detected in control.

III -Histological results:

Histological examination of the kidney sections from the control group showed the normal structure of the kidney of mammal (Figs:1,2&3).

Macroscopic investigation of kidney of animals which fed by basal diet of yogurt with *Lac. rhamnosus* and *B. bifidum* as probiotic strains for two weeks (group A) and those fed fermented milk for four weeks (group B) were enlarged in size than those of controls and were surrounded with masses of fats. Microscopical examination of the transverse sections of these groups showed that kidney's tissues were healthy and the cortex area exhibited a normal structure nearly similar to that of controls, but with a progressive increase in size of glumerular tuft ,Bowman's capsule space, cells and lumen of the renal tubules (Fig. 4&5). The sections of animals from (group B) showed some congested and dilated blood vessels (Fig. 6). Also, collecting tubules in sections of group B exhibited more luminal width filled with a strong eosinophilic substance and thinner walls (Fig. 7).

These results indicated increased physiological and metabolic activity in the kidney. Lindgren and Dobrogosz (1990) attributed these beneficial effects to that Probiotic bacteria produce bacteriocins that are proteinaceous anti-bactericidal proteins during lactic fermentations. These bacteriocins have been reported to be effective with closely related species of *mesophilic lactobacillus* and therefore comprise potential natural food preservatives (Daeschel, 1993).

Histological examination of kidney of mice fed on basal diet of yogurt with *Lac. rhamnosus* and *B. bifidum* as probiotic strains for two weeks and treated by a toxic mutagen at the day 15 of the experiment period (group C) revealed that the kidney was slightly enlarged surrounded by fats and the fibrous capsule was splited in some areas (Fig. 8).Some glumeruli were shrunken and atrophoid with necrotic cells. Also, many of the renal tubules appeared degenerated with epithelial debris, cell debris was also seen in the lumen of loop of henle and collecting tubules in addition to pyknotic nuclei.

These effects could be interpreted as nephrotoxic changes induced by the toxic mutagen Amyloid eosinophilic substance appeared initiated surrounding some of the convoluted tubules often appeared secondary to chronic diseases, often secondary to chronic diseases.The same results was recorded by Weater *et al.* (1985).

A number of the convoluted tubules appeared possessing degenerated cells while others appeared healthy with active nuclei, while the distal tubules appeared filled with eosinophilic secretory fluid (Fig. 9). The occurrence of healthy renal tubules filled with eosinophilic secretions indicates that the kidney is still active and functioning.

Few studies used toxic mutagens for induction of cancer Nagao and Sugimura (1993) mentioned that mutagens are carcerogenic heterocyclic amines (HCAs) which are mutagenic to microbes and eukaryotes and their precursors are creatine or creatinine, sugars and amino acids in meat and fish. These mutagens are genotoxic compounds, induced human colon carcinogenesis and should accordingly be avoided. Gold *et al.* (1993) studied the carcinogenic factors in food, They recorded that 79% of mutagenic and non-mutagenic carcinogens are positive in each species in at least one of the 8 most frequent target sites: liver, lung, mammary gland, stomach, vascular system and urinary bladder. Also, a recent research employed by a scientific team in Umm Al-Qura University (Rayes *et al.*, 2006) proved that oral administration of mutagens induce hepatic carcerogenesis in liver of mice. Synderwine *et al.* (1998) used the mutagen 2–amino 6H-Pridol(2-3-b)amine which is found in high concentrations in barbeared or grilled meat for induction of cancer in many tissues including isolated kidney. These observations were attributed to the improved case of the circulatory system in kidney of animals fed by basal diet of yogurt with *Lac. rhamnosus* and *B. bifidum* as probiotic strains and that the animal health was improved in animals of groups A&B and the pathogenic effects were modulated in animals of group C.

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LEGEND OF PHOTOS

Fig. 1: Kidney section of a control mouse showing the normal structure of the cortex (C) kidney. (HX-E) X100.

Fig. 2: Kidney section of a control mouse showing: Bowman's capsules containing glomerulus' corpuscles (G) and some renal tubules. (HX-E) X400.

Fig. 3: Kidney section of a control mouse showing Henle's loops (Hl) and collecting tubules (Ct) in the medulla. (HX-E) X400.

Fig. 4: Section of kidney of a mouse fed fermented milk 2 weeks (gpA) showing a Bowman's capsule (Bc) containing glomerulus and renal tubules. (HX-E) X400.

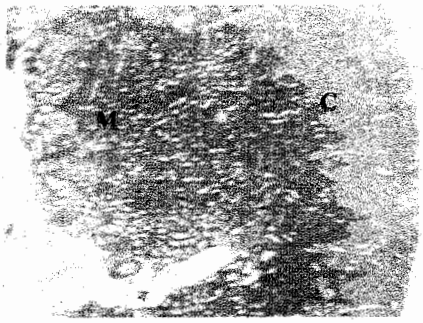
Fig. 5: Section of kidney of a mouse fed fermented milk 2 weeks (gp A) showing renal tubules in the medulla. (HX-E) X400.

Fig. 6: Kidney section of a mouse fed fermented milk 4 weeks (gp B) showing congested and dilated blood vessel (V) and Bowman's capsule (Bc) containing glomerulus. (HX-E) X400.

Fig. 7: kidney section of a mouse fed fermented milk 4 weeks (group B) showing the medulla containing wide Henle's loops (Hl) and collecting tubules (Ct) containing eosinophilic substance. (HX-E) X400.

Fig. 8: Kidney section in the cortex (C) of a mouse treated with the mutagenic subatance (gp C) illustrating the fibrous capsule (F) appeared splited in some areas. Also, congested dialated blood vessels (V) are noticed between the tubules. (HX-E) X100.

Fig. 9: Kidney section of a mouse treated with the mutagenic subatance (gp C) showing shrumken and atrophoid glumeruli (G) with necrotic cells. In addition, many of the renal tubules and loops of Henle appeared degenerated with epithelial debris, Amyloid eosinophilic substance (the arrows) appeared surrounding the convoluted tubules (C) while the distal tubules appeared filled with an eosinophilic secretory fluid (*). (HX-E) X400.



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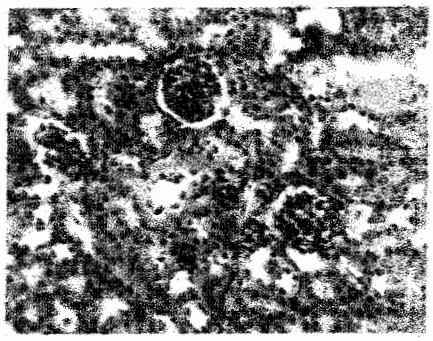

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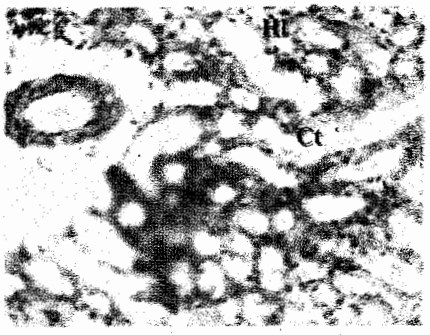

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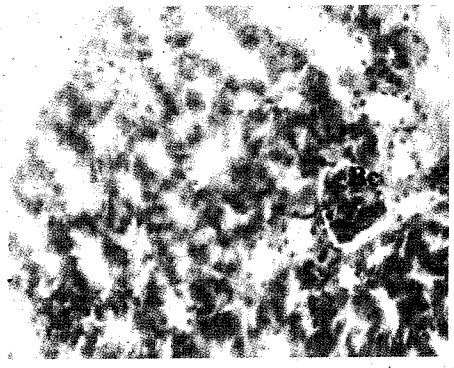

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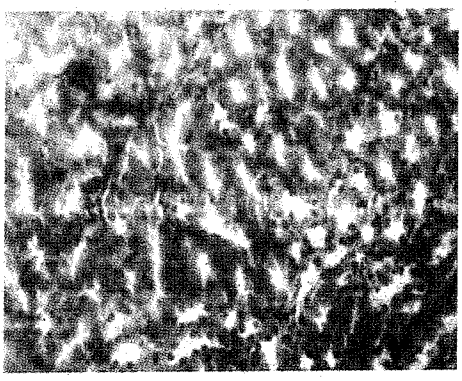

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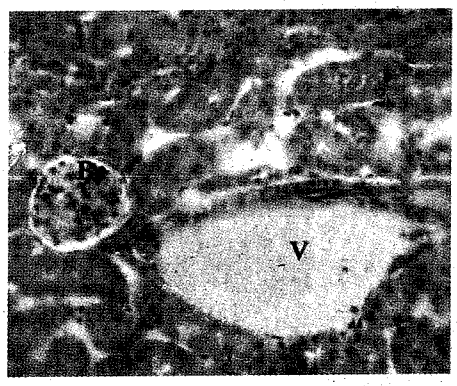

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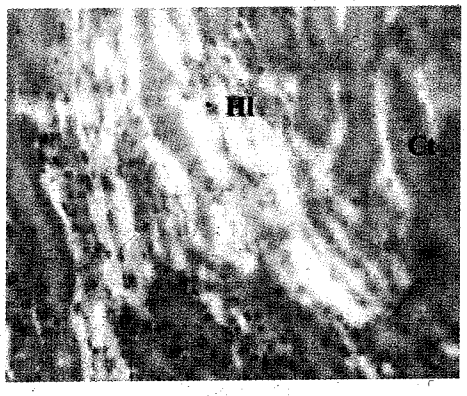

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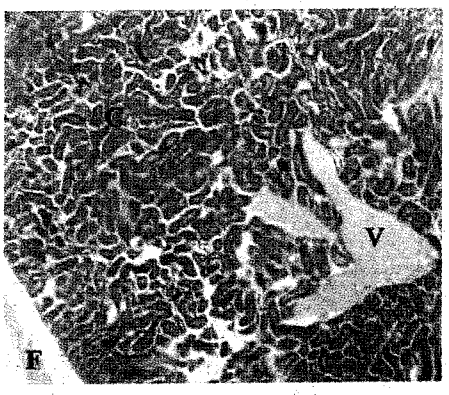

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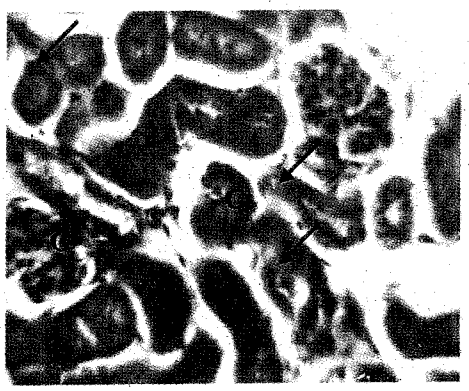

(Fig.9)

دراسة تأثير سلالتين من بكتريا البروبيوتك

(*B.BIFIDUM* AND *LAC. RHAMNOSUS*)

فى وقاية الكلية من التأثير السام لمادة مسرطنة

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تم اجراء البحث لدراسة نوعين من سلالات بكتريا البروبيوتك، والتى تستخدم على نطاق واسع (Bifidobacterium bifidum & Lactobacillus rhamnosus) فى حماية انسجة الكلية من التاثير السمى لمادة مسرطنة، تم استخدام عشرون فأر تجارب ذكور اعمارها عند بدء البحث بلغت من ٨-١٠ اسابيع ومتوسط اوزانها من ٢٠+ ٣ جم وقسمت الى اربع مجموعات المجموعة الاولى (ضابطة) تغذت على علف الفئران العادى والمجموعات الأخرى تغذت على وجبات تكونت من ١٥ جم من اللبن المتخمر (الزبادى) يحتوى على سلالتين من بكتريا البروبيوتك (B. bifidum & Lac. rhamnosus) ، المجموعة الثانية (a) تناولت وجبات اللبن المتخمر (الزبادى) لمدة اسبوعين، والمجموعة الثالثة (B) استمرت فى تناول وجبات الزبادى المحتوى على سلالتي البروبيوتك يوميا وذلك لمدة اسبوعين آخرين، اما المجموعة الثالثة (C) فقد تناولت وجبات الزبادى لمدة اربعة اسابيع ايضا ولكن اعطيت جرعة من مادة مسرطنة عن طريق الفم بعد اسبوعين من بدء التجربة وهى المادة المطفرة (الميوتاجين جرعة واحدة تتكون من ٢٥ . مجم لكل فأر) . اثبتت نتائج البحث ان تناول اللبن المتخمر المحتوى على سلالات البروبيوتك قد نشط الجهاز المناعى وقلل التاثيرات السمية للمادة المسرطنة على انسجة الكلية