STUDY THE INH INHIBITORY EFFECT OF L*actobacillus acidophilus* ISOLATED FROM YOGHURT AS PROBIOTICS ON C*andida albicans* GROWTH IN VITRO AND IN VIVO

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ABSTRACT

This research was designed to study the inhibitory effect of *Lactobacillus acidophilus* which has been isolated from yoghurt as probiotics in reduction of *Candida albicans* growth *in vitro* and *in vivo*. The results showed that the Minimum Inhibitory Concentrations and Minimum Fungicidal Concentrations were 60% and 70% respectively, also the inhibition zone of *L. acidophilus* against *C. albicans* reached to 26mm in solid medium .The out come of *in vivo* study (histological examination) clarified that *C. albicans* caused clinical pathological effect in mice tissue organs (liver , intestine, stomach , kidney) when administrated orally by 1.5x 10^8 cfu \ml *C. albicans* that effect decrease by orally inoculated with same dose of *L. acidophilus*.The results reflect the ability of *L. acidophilus* to reduce certain clinical pathological change in mice organs, with promising encourage to use the *L. acidophilus* as biotherapeutic agents against *C. albicana*s infections.

Key words: *Lactobacillus acidophilus*, Candida *albicans*, Yoghurt

دراسة التأثير التثبيطي للعصيات اللبنية المعزولة من اللبن الرائب كمعززات حيوية للحد من نمو خميرة *Candida albicans* خارج و داخل الجسم الحي

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الخلاصة

تضمن البحث دراسة التأثير التثبيطي للعصيات اللبنية المعزولة من اللبن الرائب كمعززات حيوية للحد من نمو خميرة *Candida albicans* خارج و داخل الجسم الحي. أوضحت النتائج إن التركيز المثبط الأدنى والتركيز القاتل الأدنى كان 60 %و70% على التوالي بينما وصل القطر التثبيطي للعصيات اللبنية ضد هذه الخميرة إلى 26 مليلتر على الوسط الصلب . بينت النتائج عند إجراء الدراسة داخل الجسم الحي(الفحص النسيجي) أن خميرة *C. albicans* سببت تأثيرات مرضية واضحة في أنسجة الأعضاء (كبد، أمعاء، معدة، كلية) عندما جرعت الفئران بمقدار 0.1 *مليلتر* ($1.5x\ 10^8$ خلية حية) تناقص هذا التاثير عند تجريع الفئران بذات الجرعة من العصيات اللبنية، مما يعكس قابلية العصيات اللبنية في التقليل من التغيرات المرضية في أنسجة الفئران وهذه النتائج تشجع على استعمال هذه العصيات كعلاجات حيوية للحد من التأثيرات المرضية لخميرة *C. albicans*.

INTRODUCTION

Candida albicans is a virulent strain of yeast which naturally present in every body (1), often within areas of mucous membrane such as the inside of the mouth, on moist skin, vagina, intestines, lungs, on or under the fingers and toenails (2). Some common conditions that *Candida* overgrowth is responsible for include thrush, vaginal yeast infections and even diaper rash(3) *Candidiasis or Yeast Hypersensitivity* occurs when the yeast overgrowth becomes a systemic problem(4) When the immune system is suppressed, the yeast can multiply rapidly, penetrate the intestinal lining and move into the bloodstream,Yeast population is controlled by probiotic or "friendly" bacteria(5,6).The term Probiotic literally means "for life", is used to describe organisms that are used medicinally including bacteria such as *Lactobacillus* which naturally present in small intestine and vagina and have been shown to inhabit unhealthy organisms and play an important role in maintaining human health (7).The primary role of *Lactobacillus* is to reinforce protective mucosal surfaces and prevent the entrance and attachment of harmful microorganisms and allergens(8). Strains of probiotics, like *Lactobacillus acidophilus* are of high value for protection against pathogenic yeast infections of the vagina, intestinal and oral cavity, and can be described as one of the body's primary defense mechanisms against *Candida* (9). Typically, probiotics are consumed as part of cultured foods such as acidophilus milk, yogurt (10). The topical application of yogurt products has been reported to control yeast and bacterial infections, and the ingestion of these preparations has been recommended to reduce the symptoms of sore mouth caused by *Candida* infections (11). Research has shown that *L. acidophilus* was the most popular species of probiotic bacteria, produces substances that slow or prevent the growth of *Candida* (12). This research was designed to study the inhibitory effect of *L .acidophilus* which isolated from yoghurt as probiotics in reduction of *C. albicans* growth *in vitro* and *in vivo*.

MATERIALS AND METHODS

Isolation and identification of *C. albicans*

Two oral swabs for each patient were transported to the laboratory by inoculating them into a sterile tube containing 3.0ml of saturate transport medium (Sabourauds dextrose broth). One of the swabs was directly inoculated onto Sabourauds dextrose agar for microbiological investigation while the other was used for direct examination by wet mounted film and Gram stained for detection of yeasts. Inoculated plats were incubated for 24-72 hrs. The isolated colonies were identified by morphological feature and biochemical tests (sugar fermentation test and carbohydrate assimilation test). Stock culture was made by inoculating single colony of the isolated yeast into a slant of Sabourauds dextrose agar.

Identification of *C. albicans*:

The isolated yeasts were identified as described by Al-Thwani (13) and Nothan *et al*.(14).

Isolation of *L. acidophilus* from yoghurt

Lactobacillus acidophilus isolates were obtained from six samples of yoghurt from retail markets. The samples were inoculated in 10 ml MRS broth (Oxoid- England) then cultured onto MRS agar (Oxoid- England) of pH 5.2 and incubated at 37°C for 48 hs, followed by subculturing onto MRS agar of pH 4.3 at 37°C in a candle jar for 48 hs. *Lactobacilli* were identified on the basis of growth on selective MRS agar, colony

morphology, Gram staining, catalase activity, beside motility test and other biochemical testes Further identification of the species of the *Lactobacilli* was performed by carbohydrate fermentation test, growth at 15°C and 45°C in MRS broth according to Atlas *et al*. (15).

Determining inhibitory effect of *L. acidophilus* on *C. albicans*

MRS broth was inoculated with 1% of *L. acidophilus* isolates then incubated at 37°C for 18 hs. After incubation, the culture was centrifuged at 6000 rpm for 15 min (16), and filtered through millipor filter unit (0.22µm) .According to well diffusion method that mentioned by Lewus *et al*. (17). Sabourad agar plate was inoculated with 0.1ml of C. *albicans* by a spreader then, 5mm well was made by the cork borer. The well was filled with *L. acidophilus* supernatant, and then incubated at 37°C for 24 hr. Inhibition zone around the well were measured by (mm) and compared with that of control which contained MRS broth only.

Determining the Minimum Inhibitory Concentration (MIC) and Minimum Fungicidal Concentration (MFC) of *L. acidophilus* on *C. albicans*

This test determines the lowest concentration of *Lactobacillus acidophilus* supernatant recovered from vagina which minimizes (MIC) or prevents (MFC) the growth of *C. albicans* isolated from oral cavity. To evaluate MIC and MFC, the procedure was done according to Atlas *et al*. (15).

Laboratory animals

Twenty mice were obtained from biotechnology research center .Their age among (8-12) weeks and weighting (23-25) gm. They were divided into (4) groups and each group contains 5 mice as following:-

Negative control: The animals of this group not given any things

Positive control A: The animals of this group administrated orally by 1.5x 10^8 cfu \ml *L. acidophilus*.

Positive control B: The animals of this group administrated orally by 1.5x 10^8 cfu \ml *C. albicans*.

Challenge dose: The animals of this group administrated orally with same dose (1.5x 10^8 cfu \ml) of *L. acidophilus* and *C. albicans*. Laboratory animals was sacrified after (7) days as described by Oyetayo *et al*. (18).

Histological Examination.

Histological Examination was diagnosed under supervision of histopathologist .The samples which was fixed in (10%) formalin solution then was washed by tap water for few minutes and left in ethanol (50%) for (30 minutes) while (70%) ethanol was used to keep the samples for long time. The samples was transferred to (2.5% absolute ethanol +75% butanol) and left for (2hr). Paraffin wax sectioned in (4µm) thickness to be easier to use, then samples was stained with hematoxyline –eosin stain as described by Oyetayo *et al*. (18).

RESULTS AND DISCUSSION

Oral swabs were collected from ten children (1 – 5) years' old suffering from oral thrush. It was found that 7 out of the total 10 swabs gave positive for the microscopic examination. These results were near to those reported by Forbes *et al*. (19) who found that *Candida* species were frequently isolated from children patient. Moreover, Gorbach (20) proved that during childhood, 12 to 16% of children examined were carried *Candida*, and the proportion increased to 80% in adulthood.

The yeasts colonies appeared on Sabaroed dextrose agar as white, glossy, smooth and circular colonies, while on Corn meal agar as creamy and soft colonies, moreover colonies appear light to medium green on CHROM agar and appearance of growth layer at the surface of Sabourauds dextrose broth when cultured in it, as well as stained positive with Gram-stain, the cells appeared to be violet and oval shape, the other tests showed that this yeast had ability to form chalamydospore on Corn meal agar, and germ tube on human serum at 2 – 4 hrs and that confirmed that the isolates belong to *C.albicans* as mentioned by Cheryl *et al*. (5).

The results of sugar fermentation test and carbohydrate assimilation test showed that *C.albicans* isolates were able to ferment glucose and maltose during 24 hrs. but not lactose and sucrose ,as well as assimilation glucose, sucrose ,trehalose, but not lactose and raffenose. In this regard, Ping (3) insisted on such above tests to differentiate of *C. albicans* from other species. These results agree with Hawar(21) and Majeed (22). The susceptibility tests of isolates to different antifungal agents were conducted and it was found that all tested isolates were sensitive to Miconazol, Clotrimazole and Ketoconazole, while resistant to Nystatin ,Griseofulavin. When we compared present results with others it was found close similarity with AL-Maliky(23) and Liao *et al*. (24).

Antifungal treatment for *C.albicans* are limited to three compounds, mainly azoles, polyenes, and echinocandins. Azoles such as Miconazol act on ergosterol biosynthesis as fungistatic. Emergence of resistance to azoles is an increasing problem. Polyenes, such as nystatin, bind to membrane sterols, leading to membrane permeability, leakage, and cell death. These drugs had clinical drawbacks based on their toxicity. Echinocandins were a new class of antifungal agents that inhibit the synthesis of 1, 3-ß-D-glucan, a key component of the cell wall (25).

Yeasts are always present in the human body in small numbers and symptoms only appear with overgrowth (26). Several factors are associated with increased symptomatic infection, including uncontrolled diabetes and the disuse of antibiotics , other factors that may increase the incidence of yeast infection are occur in immunodeficiency cases and poor diets (25). Concentration of *Candida* was positively related to the presence of another enteric pathogen (26) also Odds (27) found that *C. albicans* was increased in cancer patients.

Identification of *L. acidophilus* bacteria

From a total of 6 yoghurt samples collected from Baghdad local markets, four isolates of *L.acidophilus* were obtained.

Suspected colonies appeared pale, round, convex, soft, mucoid and surrounded by a zone as a result of dissolving calcium carbonate. When parts of the colonies were examined microscopically cells appeared Gram positive bacilli, mainly grouped in chains containing 3-8 cells, and non–sporformers. Biochemical tests showed positive results to lactose fermentation, growth at 45 °C and 15 °C. Furthermore, curd formed in litmus milk, while gave negative result to catalase, oxidase, urease, motility and production of NH3 from arginine. These results come in concord with those obtained by Baron *et al*. (28). In order to differentiate the isolates of *L. acidophilus*, carbohydrates fermentation test was performed. Isolates which were unable to ferment both xylose and manitol but ferment other sugars were considered to be belonging to *L. acidophilus* (29).

Inhibitory effect of *L.acidophilus* on *C. albicans*

Well diffusion method was used to determine the inhibition activity of *L. acidophilus* against

C. albicans isolates .High inhibitory effect was obtained during using supernatant of *L.acidophilus*, the inhibition zones reached to 26 mm.

Such finding was confirmed by Tadao (29) who mention that MRS broth stimulated inhibitory effect against Gram positive and Gram negative bacteria ,similar results were also obtained by Kubba [30] who found that best inhibitory effect was gained when liquid media (MRS broth) was used to estimate the effect of *Lactobacillus* on pathogenic bacteria.

MIC and MFC of *L. acidophilus* against *C. albicans*

The result revealed that concentrations 10%, 20%, 30% ,40% and 50% had no effect on *C. albicans* isolates when clear growth was noticed, while concentration 60% led to minimized and sharp decrease in growth(MIC) of *C. albicans*, the concentration 70% inhibit (MFC) of *C. albicans* growth completely, similar result were recorded by Kubba (30) and Aziz (31) who found that the MIC of *Lactobacillus* concentration was 60% that minimized growth of *Proteus mirabilis* and *E.coli* also our results closely related with results of Al-Jeboury (32) who noticed that the MIC 60% minimized growth of *Staph.aureus*, *P. aeruginosa* and that agree with observation by Al- Yas (33) and Lateef (34) who found the MBC against growth of *H. pylori* and MFC of *C. albicans* was 70%.

Lactobacillus is a natural resistance factor against potential pathogenic microorganisms, by producing autogenic regulation factors e.g. organic acids, hydrogen peroxide and bacteriocins (35).

In recent years, the use of *Lactobacilli* as biotherapeutic agents has received wider attention and several studies provide evidence supporting the ability of *Lactobacilli* to prevent infection (36). Although many commercially available *Lactobacillus* products can be found in healthy food stores, their reliability is questionable and there is only little evidence proving their efficacy (35). Other drawbacks of the currently available products include poor product, viability, and possible contamination with other organisms (36) .Therefore current research efforts are directed towards preparing safe and effective *Lactobacillus* preparations; this involves the careful selection of strains with specific properties shown to be important in the interference of pathogenic adhesion (37).

Histological Diagnosis

Histopathological changes of the organs by *C. albicans* with or without *L.actobacillus* were detected in different organs in administrated mice as following:-

-From positive control A which administrated with *L. actobacillus* only liver sections shows normal appearance in hypatocyte as illustrated in Figure (1).

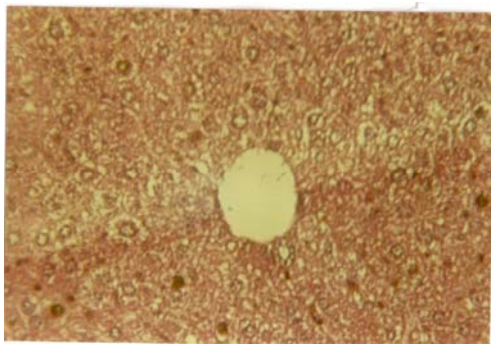

Figure (1): Normal liver tissue from control group(A) of mice. (hematoxyline –eosin stain 40x).

-Liver sections from positive control(B) which administrated with *C. albicans* only, which showed degenerative changes in addition to an irregular conditions in arrangement in liver cells, increase kuffer cell and there are areas of congestion distributed within the degenerative cells, filtration of inflammatory cell such as monocytes and netrophils, the presence of necrotic foci surrounded by inflammatory cell in great number and present of heamosidrin in liver sinuses and congestion in central vein as shown in Figure (2)

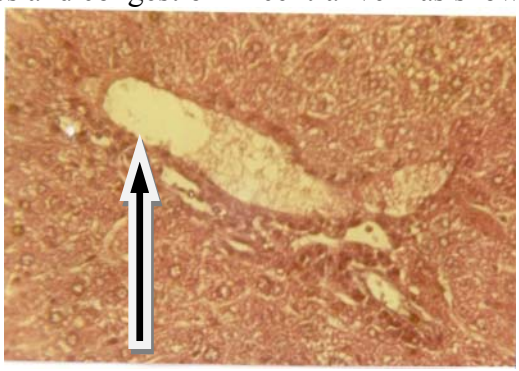

Figure (2):Histopathological sectioning of mice liver tissue from control(B) showed congestion and necrosis in liver cells (hematoxyline –eosin stain 40x)

-In liver sections from animals of challenge dose shows normal appearance in hypatocyte and decrease in the lesion it was noticed as a slight congestion in central vein and a few filtration of inflammatory cell around it and in the portal area,but the rest the liver tissue looks healthy as illustrated in Figure (3)

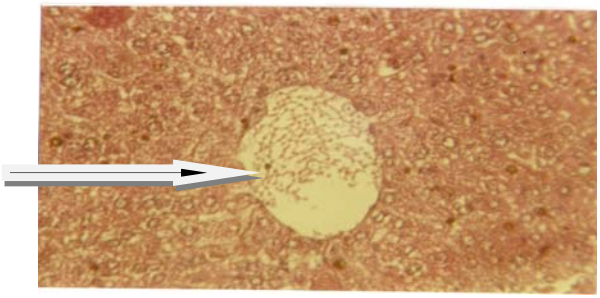

Figure (3):Histopathological sectioning of mice liver tissue from animals of challenge dose showed a slight congestion in central vein and a few filtration of inflammatory cell, but the rest liver tissue looks healthy (hematoxyline –eosin stain 40x).

-Kidney sections were taken from positive control (A) looks like normal organ as shown in Figure (4).

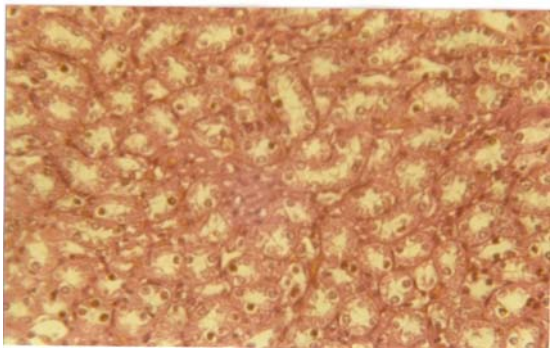

Figure (4):Histopathological section showed normal kidney tissue from mice of control (A) (hematoxyline –eosin stain 40x).

While in positive control (B) sections revealed marked congestion with distributed area of degenerative tubules of kidney beside rare glomeruli degenerative changes and bleeding as appeared in Figure (5)

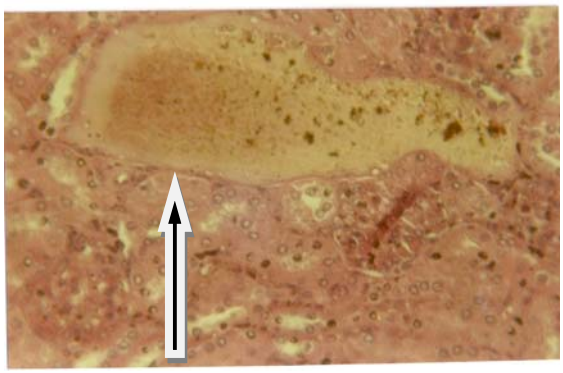

Figure (5): Histopathological section of kidney tissue from mice of control (B) showed degenerative changes and bleeding (hematoxyline –eosin stain 40 xs).

-Mild degenerative changes in the renal tubules appeared in kidney sections taken from animal's ademenstrated with challenge dose this may be due to the effect of probiotic bacteria which protect the kidney from *C. albicans* as Figure (6) illustrated.

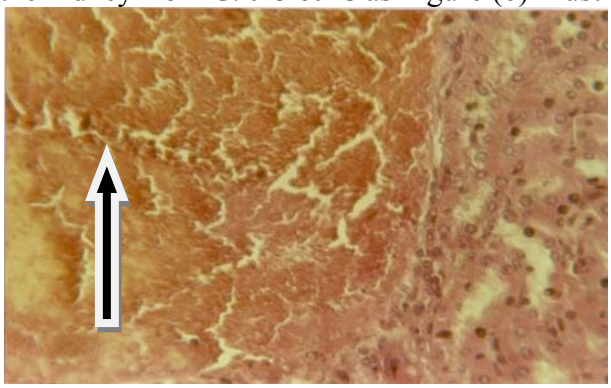

Figure (6):Histopathological section of kidney tissue from animals of challenge dose showed mild degenerative changes (hematoxyline –eosin stain 40x).

-Intestine sections from positive control (A) looks like normal organ as shown in Figure (7).

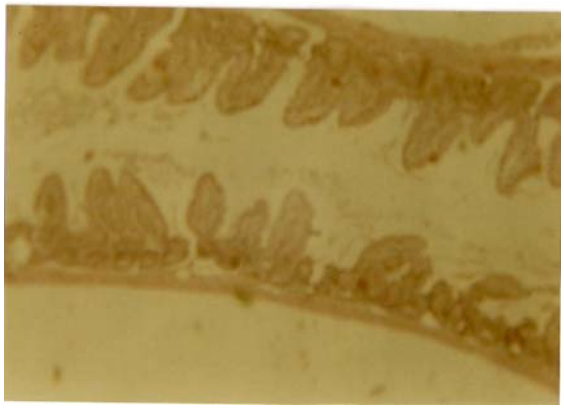

Figure (7):Histopathological section showed normal mice intestine tissue from control (A) (hematoxyline –eosin stain 40x).

By studying intestine sections from positive control (B) we found that there is reformation or shortness of intestinal villi and with inflammatory cell infiltration, odema in the muscular layer and increase in the numbers of Goblets cell and disintegration in mucus layer of the intestine as shown in Figure (8) in accordance with Pati *et al.* (38) who found that the intestinal sections was infected by enteropathogenes

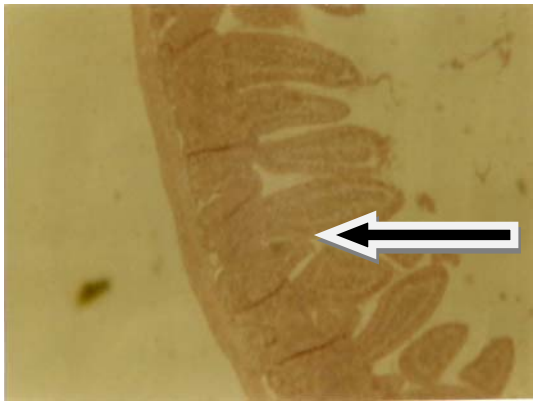

Figure (8): Histopathological section of mice intestine tissue from control (B) showed reformation or shortness of intestinal villi and with inflammatory cell infiltration. (hematoxyline –eosin stain 40x).

-Intestinal sections appeared no pathological lesions from animals of challenge dose, but a slight infiltration well be noticed in inflammatory cell of Lamina propira as figure (9) illustrated this is back to the various species of lactic acid bacteria exert antagonistic action against intestinal and food borne pathogens which are capable of preventing the adherence, establishment, replication pathogenic action of specific enteropathogenes (39).

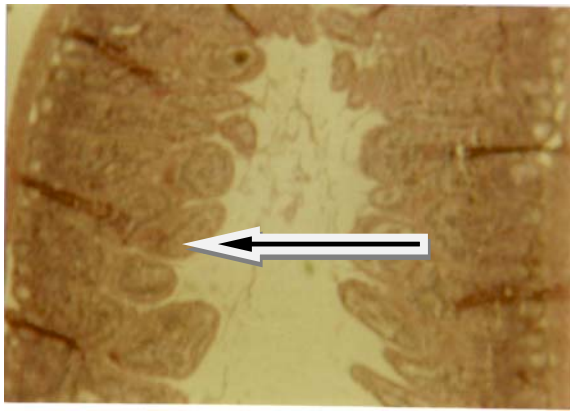

Figure (9): Histopathological section of mice intestine tissue from animals of challenge dose showed a slight infiltration of inflammatory cell (hematoxyline –eosin stain 40x).

-Stomach sections from positive control (A) showed no histological changes, also the same result appear in sections from positive control B and from animals of challenge dose, therefore, it looks like normal, because the environment in stomach is acidic, so the pathogens have no ability to live in this organ, so no lesion appeared as shown in Figure (10).

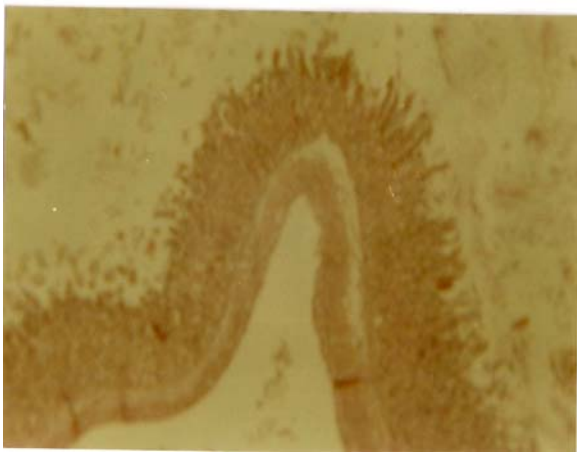

Figure (10):Histopathological sectioning showed normal mice stomach tissue (hematoxyline –eosin stain 40x).

This result was agreed with Doug *et al*. (40) when their study the effect of probiotic bacteria to reduce candidiasis infection in mice, found that the prolonged survival of mice, decreased severity of mucosal and systemic candidiasis, modulation of immune responses, decreased number of *C. albicans* in the alimentary tract, and reduced numbers of orogastric infections demonstrated not only that probiotic bacteria have biotherapeutic potential for prophylaxis against and therapy of this fungal disease but also that probiotic bacteria protect mice from candidiasis by a variety of immunologic (thymic and extrathymic) and nonimmunologic mechanisms in this model. The criteria for selection of effective probiotic strains have been proposed and should include verification of safety, colonization ability in the vagina and ability to reduce the pathogen count through competitive exclusion of adherence and inhibition of pathogen growth (41, 42). From previous outcome of *L.actobacillus*, which is used in this experiment and the result for healing the lesion which cause by *C. albicans* mainly in liver, intestine and kidney .We conclude that this bacteria can be used as probiotic for therapeutic of some pathogens, L.actobacillus may survive in the human gut were significant role of intestinal microflora to resist the disease.

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