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LACTOBACILLUS SALIVARIUS BACTERIOCIN AND SUPERNATANT ACTIVITY AGAINST ENTAMOEBA HISTOLYTICA IN VITRO AND IN VIVO

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ABSTRACT: *Lactobacillus salivarius is a probiotic bacteria species found in the gastrointestinal tract and breaks and exert a range of therapeutic properties, so antiamoebic activity of Lactobacillus salivarius supernatant and bacteriocin were tested in vitro and in vivo on Entamoeba histolytica and using metronidazole as a reference amoebicidal agent. In vivo supernatant have a clear impact more than bacteriocin and metronidazole where led to the disappearance of parasite in the feces of infected mice after 9th day while bacteriocin and metronidazole were the 12th, 11th day respectively, the disappearance of the parasite after dosing while the results were expected to reverse invitro as it was observed that the effect was more of bacteriocin it leads to lower growth rate to (35 – 30.25×104 cell / ml) after 24 and 48hr while the supernatant and metronidazole were lower growth rate after 24 and 48hr (50 - 42×104 cell / ml), (44.5 - 40×104 cell / ml) respectively, compared to control group whose results were (55.5 – 60.75×104 cell / ml). Histological changes also explained that bacteriocin effects were clearly on intestinal tissue while the supernatant was safly and harmless.*
KEYWORDS: Lactobacillus, Lactobacillus salivarius, Entamoeba histolytica, bacteriocin, supernatant.

INTRODUCTION

A large number of population in Iraq are suffered from amoebiasis which caused by Entamoeba histolytica. E.histolytica is primarily of man infected and carrier humans forming there reservoir of infection (Al-Idrrise et al., 2008).

Dysentery is caused by the microbial infection in the gastrointestinal tract. Fever, vomiting, abdominal pain and diarrhea which often contain blood and pus are obvious symptoms (Rani, 2011).

Only limited numbers of drugs are a viable for the treatment of amoebiasis, among them metronidazole is used for many years, but indiscriminate use may cause drug resistance in future (Bansal et al., 2006; Sarkar et al., 2010).

Innovative approaches have been tried as alternative to antibiotics in treating gastrointestinal diseases and these include using live bio therapeutic agents Such as yeast (Saccharomyces.spp.) and bacterial isolates (Lactobacillus. Spp.) or fecal enamels (Fuller, 1992). Lactobacillus. Spp can form many barrier population for protect from pathogen by many mechanism such as adhesion to epithelial surfaces, self-aggregation and co-aggregation(Ocana and Nader-Macias,2002). Lactobacillus salivarius found in the mouth and small intestine has

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antimicrobial activities and breaks down proteinase and produces B vitamins, enzymes and Lactic acid. L. salivarius helps inhibits Salmonella and ulcer causing by bacteria H. pylori. Bacteriocins are proteinaceous, bactericidal substances synthesized by bacteria and usually have narrow spectrum of activity (Jack et al., 1995). McGoarty ,1993 reported the bacteriocin can inhibit wide range of both gram positive and negative bacteria as well as fungi amoebiasis is more common and both the efficacy, safety of probiotics which use to investigate the efficacy of bacteriocin and supernatant of L. salivarius for effection E. histolytica in vivo and in vitro.

MATERIALS AND METHODS

Samples

Collected stool samples from people suffering from diarrhea, from many laboratories analysis in Baghdad. Examined the samples and the work slide and make sure there is parasite Entamoeba histolytica cyst and Trophozoite. Placed improvised add it for the purpose of culturing and purification of parasite.

Purification of parasite

Added to the stool samples PBS (phosphate buffer saline) then mixed and passed through a layer of gauze for the purpose of removing the big minutes from the emulsion (Clark & Diamond, 2002).

Preparation culture media

Liver infusion agar medium prepared according to the present method Cleveland & Collier 1930 supplemented with 100VI/ml penicillin streptomycin sulphate 2mg/ml and Nystatine 2mg/ml (Taylor & Baker, 1968).

Culture of Entamoeba histolytica

After preparation of culture media added small amount (0.25ml) contain nearly of emulsified. Stool sample then incubated vertically at 37c^{o} for 48 hours (Mirelman et al., 1987). Trophozoites were chilled in an ice water bath for 7minute, then counted with haemocytometer.

Six tubes contained 5ml liver infusion agar for each substance, then added about 0.4ml from crud bacteriocin and supernatant while the sixth tube was used as control (cultures containing only parasites).All tubes were incubated at 37c^{o}, after (24 and 48 hr) trophozoites were counted by using haemocytometer in each 1ml according to (Al-Dujali , 1976) by use this equation:

$$(N1+N2+N3+N4) \times \frac{10}{n} \times 1000$$

Mortality rate:

Growth rate of the parasite tested was calculated from the trophozoite count per ml, mortality rate of E histolytica was obtained by use this equation (Ardalanet al., 2011).

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$$\text{mortality rate (\%)} = \left[\frac{\text{count/ml treated}}{\text{count/ml (untreated control)}} \times 100\right] - 100$$

Lactobacillus salivarius

Lactobacillus salivarius strain of fish intestinal origin, obtained from laboratory of Biology Department /AL-Mustansiriya University, Baghdad- Iraq. Morphological and biochemical characteristic as of the isolates were done as described by (Hammes& Vogel, 1995).

Preparation of cell free supernatant (CFS)

(CFS) was obtained from MRS broth cultures after 18hr incubation at 37c^{o} by centrifugation at 10000g for 10min at 4c^{o}. Supernatant was filtered through 0. 22-Mm pore size filters and concentrated (Lievin et al., 2000).

Crud Bacteriocin perperation

The supernatant which obtained in above step was adjusted to pH6.2 by adding 1N NaOH to remove the influence of organic acid (stren et al., 2006) the crud bacteriocin was precipitated with 80% ammonium sulphate saturation. The precipitate was dialysed against 20mM potassium phosphate buffer (pH7.0) for 12hr at 4c^{o} (Devi& Sumathy, 2013).

Experimental animals:

Thirty white albino mice, at eight weeks of age were obtained from the animal house at the College of Medicine University of Baghdad. Immunosuppression by injected with a drug dexamethasone dose (0.02ml/day), after five days the feces were examined by wet mount to make sure there are not any parasitic infection. Twenty four of them were inoculated with E.histolytica (1×104 Trophozoites) obtained from culture, and six mice were kept at the same environmental conditions as controls for histological sections, after 3-4 days the feces of each mice were examined. The infected mice kept in separate cage and divided into four groups:

Group 1: inoculated with 0.1ml from crud bacteriocin.

Group 2: inoculated with 0.1ml from supernatant

Group 3: inoculated with 0.1ml from metronidazole.

Group 4: only normal saline considered as control positive.

After starting the experiment, all the stools were checked by light microscopy on alternative days and numerate the number of parasite, after reading the slid, the number of parasites (Trophozoit and cyst) counted were multiplied by 1×10^{3} to obtain the number of parasite per gram of feces.

At the end of the experiment, mice were sacrificed and distal ileum and colon were removed from each mouse, fixed in formalin for studying histopathological changes.

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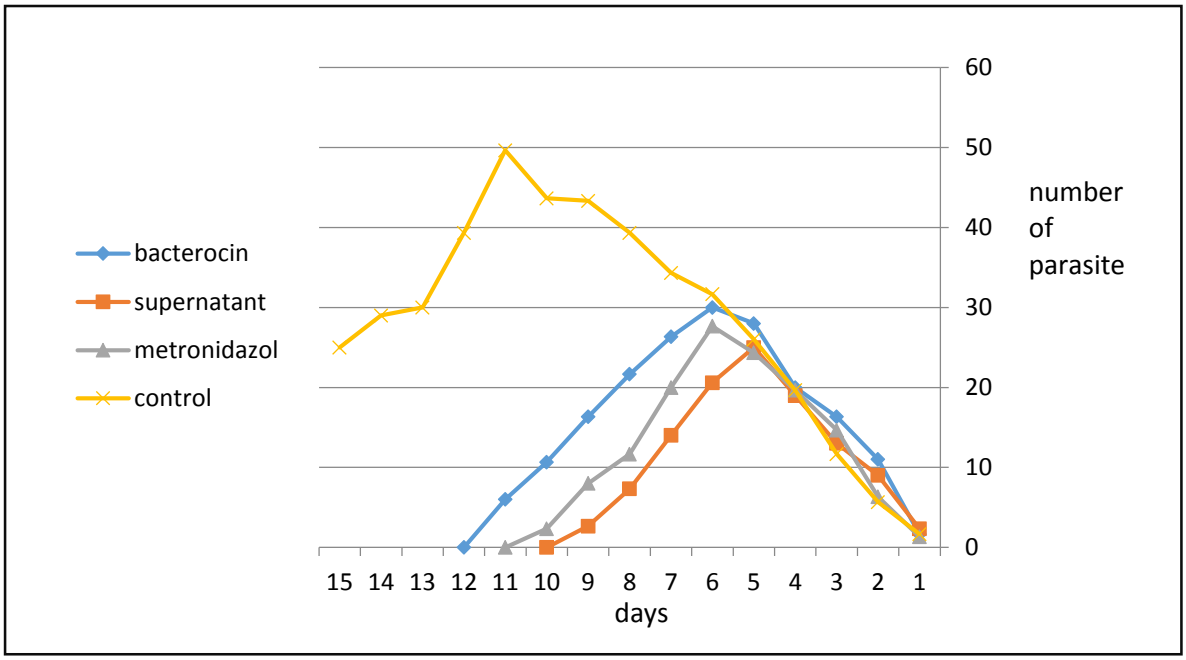
Statistical analysis:

The mean and standard deviation were calculated. T-test was used to determine p-values for the difference observed between the test sample and the control.

RESULTS

Antiparasitic activity of crud bacteriocin and supernatant for L. salivarius against E. histolytica was investigated in this research in both invivo and initro. The results of invivo study showed that the inoculum mice infected with E. histolytica with crud bacteriocin or supernatant of L. salivarius had influence clearly addressing mice, but the effectiveness of supernatant more than bacteriocin as observed in Fig(1),that the inoculated mice with (0.1ml) of the supernatant lead to the disappearance of the parasite from feces completely after (9th day) while their mice inoculated with (0.1 ml) from bacteriocin lead to the disappearance of the parasite after (11th day). The comparison with the effect of metronidazole which showed the influence of the two disappeared where the parasite in the feces of mice after 10days).

Fig. 1 :-Number of Entamoeba histolytica parasites in bacteriocin , supernatant, metronidazole and control groups.



While the result with in vitro difference with expected, was shown the bacteriocin clear impact on breeding parasite.The bacteriocin inhibited the growth of parasite were (36.93 , 50.20%) after (24 and 48hr) respectively, while the supernatant inhibited growth of parasite to (9.90, 30.86%) respectively compare with metronidazole the mortality rate were (19.81 and 34.1%) after the same incubated period (Table 1). On the other hand, the parasite growth rate in control, crud bacteriocin , supernatant and metronidazole after 24hr were (55.5, 35, 50 and 44.5×104) respectively , while after 48hr the growth rate increase in control group and reach to (60.75×104) while decrease in bacteriocin, flagyl and supernatant reach to (30.25, 40 and 42×104) respectively (Table 2).

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Table (1):- Effect of Lactobacillus salivarius bacteriocin, supernatant and metronidazole on Entamoeba histolytica trophozoit mortality rate (%).

Mortality rate (%)after 48hr	Mortality rate (%) after24hr	Groups
50.20 -	36.93 -	Bacteriocin
30.86 -	9.90 -	Suspandant
34.1 -	19.81 -	metronidazole

Table (2):- Effect of Lactobacillus salivarius bacteriocin and supernatant on Entamoeba histolytica trophozoit growth in compare with metronidazole.

After 48hr	Mean ± SD×104 of parasite growth after 24hr	Amount of addition substance	Groups
60.75 ± 1	55.5 ± 2	0.00	Control
30.25 ± 1.25	35 ± 4.72	0.1ml	Bacteriocin
42 ± 1.52	50 ± 1	0.1ml	supernatant
40 ± 1	44.5 ± 3.05	0.1ml	metronidazole

Histology

Besides crud bacteriocin and supernatant of L.salivarius on growth of E.histolytica in vivo and in vitro. Bacteriocin greatly affected on the intestinal cells Fig.(2and3) note bacteriocin led to an increase in the number of goblet cells and an increase in the size of lymph node and get necrosis, while the supernatant did not effect on the intestinal cells and appeared as normal, only a small increase in goblet cell count Fig(4 and 5).

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Figure (2). Large intestine of mice dosed with L.salivarius bacteriocin show increase in number of goblet cells.

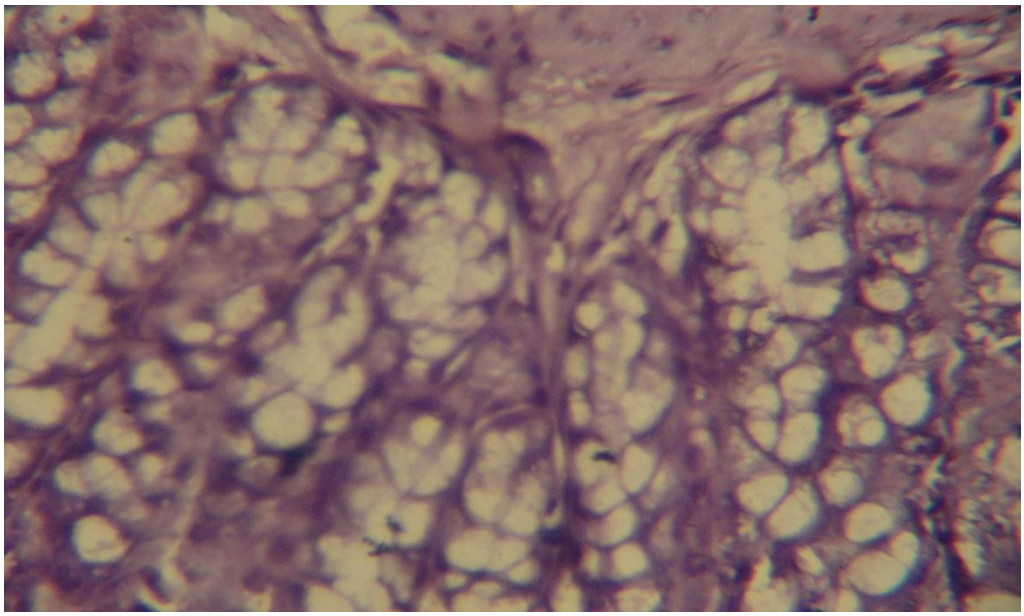

Figure (3). Large intestine of mice dosed with L.salivarius bacteriocin show an increase size of lymph gland.

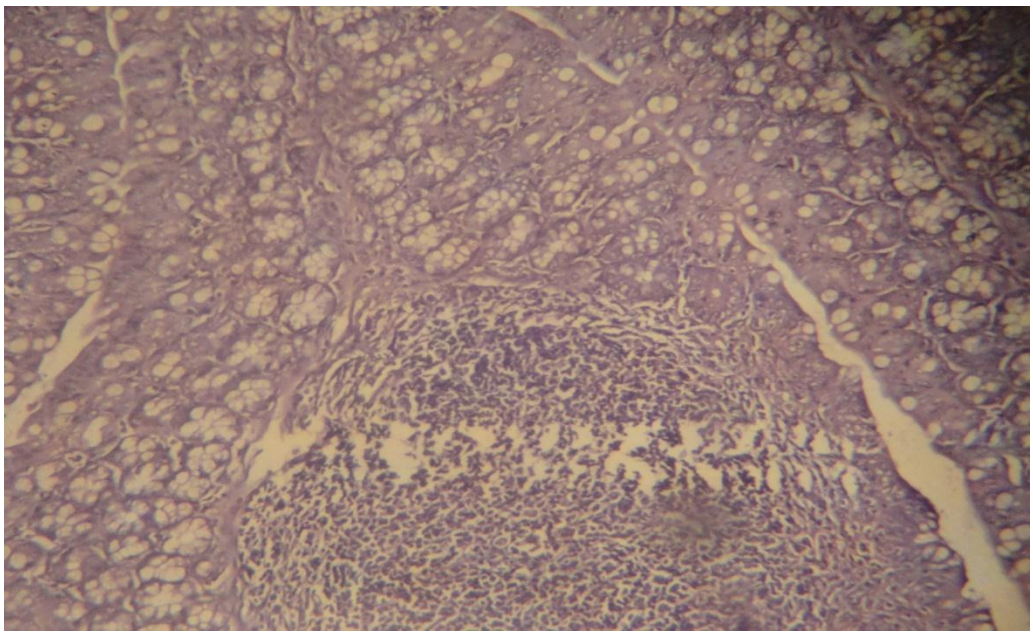

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Figure (4). Large intestine of mice dosed with L.salivarius supernatant show normal tissue.

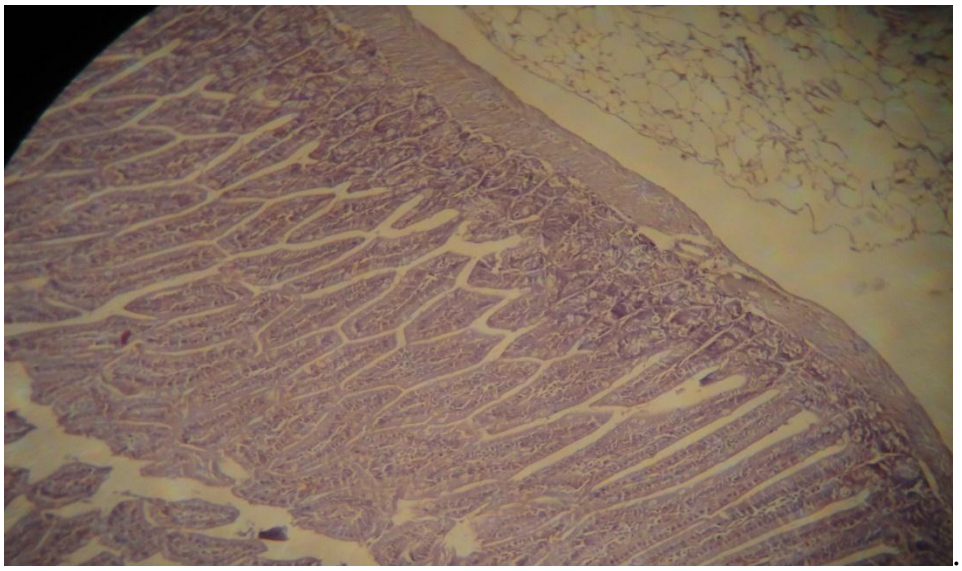
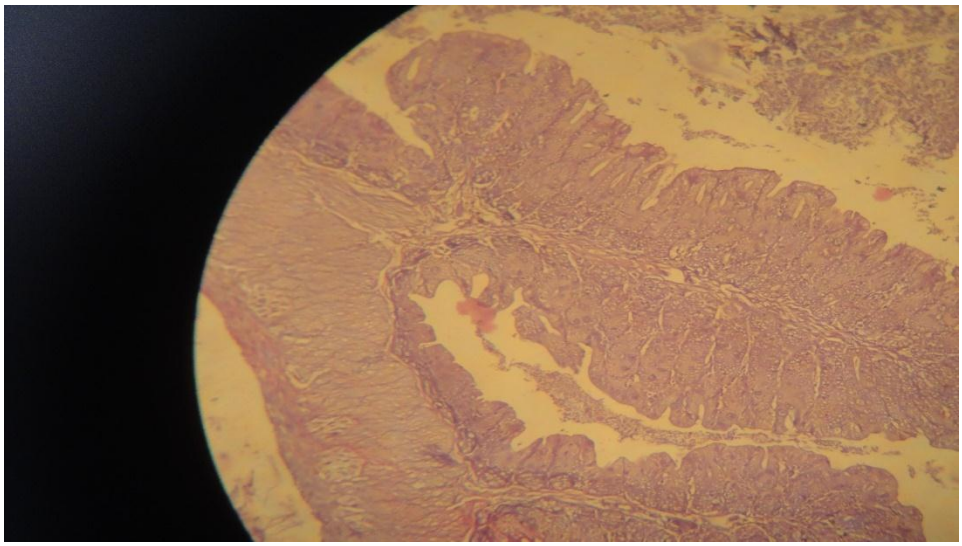


Figure (5). Large intestine of mice dosed with L.salivarius supernatant show asmall increase in number of goblet cells.



DISCUSSION

Lactobacilli exert their protective or therapeutic effect through production of antimicrobial compounds (Dodd & Gasson, 1994), reduction of gut pH by stimulating the lactic acid producing microflora (Langhendries et al., 1995), competition for binding of receptor sites that pathogen occupy (Kailasapathy & Chin, 2000) and competition with pathogens for available nutrients(Rolfe2000).

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Lactobacillus salivarius are the dominate microorganisms isolated from the vagina of healthy women (Redondo-Lopez et al., 1990; McGoarty, 1993). They interfere with colonization of pathogens by different mechanisms, such as the production of organic acid, H2O2 and bacteriocins (Baerheim et al., 1994; Hawes et al., 1996).

L. salivarius and L .plantarum can inhibit the growth of E. coli, S. typhimurium ,and C.perfringens by can ferment carbohydrates in poultry feed to produce pH levels and concentrations of lactic and acetic acid (Murry et al.,2004),also can L. salivarius ssp. facilitates the recovery of the inflamed tissue in the TNBS model of rat colitis(Peran et al.,2005). The term bactericidal substance is applied to antagonistic substances which are not completely defined or do not fit the typical criteria of bacteriocin (McGoarty, 1993).The bacteriocin that producing from lactic acid bacteria are widely used for the elaboration of probiotics for the gastrointestinal tract (Havenaar et al., 1992; Nader et al., 1993).

Bacteriocins may facilitate the introduction of a producer into an established niche, directly inhibit the invasion of competing strains or pathogens, or modulate the composition of the microbiota and influence the host immune system (Messaoudi et al., 1992).

Virginia et al., 1999 reported that the activity of bacteriocin at low pH is also important because the effect would be exerted in the vagina, where the pH is between 3.8 and 4.5. There are many facts relied upon to prove the effectiveness of the suspandant such as contain lactic acid, toxin and H_2O_2.

The ability of L. salivarius to produce toxic metabolites such as lactic acid, H2O2 and bacteriocin has been suggested as being responsible for their ability to inhibit other bacteria (Juven &Lindner,1992).Other facts such as host immunodulation (Hatcher&Lambrecht, 1993) also play a prominent role.

The report presented here showed that bacteriocin of L.salivarius has a better effect than supernatant on the E. histolytica growth in vitro, while the supernatant best protective against the E. histolytica in vivo.

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