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PROTECTIVE EFFECT OF *LACTOBACILLUS ACIDOPHILUS* AND *SACCHAROMYCES CEREVISIAE* AGAINST MULTI-DRUG RESISTANT *SALMONELLA ENTERICA* SEROVAR TYPHIMURIUM *IN VITRO* AND *IN VIVO*

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ABSTRACT

It was aimed to investigate anti-bacterial effects of two probiotics (*Lactobacillus acidophilus* and *Saccharomyces cerevisiae*) against MDR isolate of *S.* Typhimurium *in vitro* and *in vivo* by using the mouse as an animal model of the infection. Accordingly, five groups of mice were established. In group I, mice received probiotic for 7 successive days, challenged with *S.* Typhimurium on day 8, and dissected on days 14 and 21. Group II was similarly treated, but the probiotic continued for 14 days. Group III was given a probiotic only; group IV was challenged with *S.* Typhimurium, while group V was controls. By using the probiotic filtrates at one-fold, two-fold and three-fold concentration, it was found that the three-fold filtrate recorded the highest inhibition zone for both probiotics (*S. cerevisiae* and *L. acidophilus*), which were 25.0 ± 1.0 and 31.0 ± 1.0 mm, respectively. Also, both probiotics were effective in reducing *S.* Typhimurium colony forming units per plate (CFU/plate) in liver and spleen. In liver, mice in group IV showed a count of 224.4 ± 62.7 CFU/plate, which was significantly higher than any CFU count in groups of *L. acidophilus* and *S. cerevisiae*. Group II mice recorded better results than group I mice, and a lowest count was observed at day 21, which was 21.6 ± 7.9 and 27.8 ± 10.0 CFU/plate, respectively for *L. acidophilus* and *S. cerevisiae*. The results strongly suggest the anti-*Salmonella* effects of both probiotics.

KEYWORDS: *Lactobacillus acidophilus*, *Saccharomyces cerevisiae*, *S.* Typhimurium.

INTRODUCTION

Salmonella enterica serovar Typhimurium (*S.* Typhimurium) is a major cause of food-borne illness, causing intestinal inflammation and diarrhea. [1] A major concern of *S.* Typhimurium for physicians in developing countries is their antibiotic resistance, and multidrug-resistant (MRD) strains continue to be a worldwide health challenge, and probiotics may have the promise. [2]

Probiotics are live microorganisms, which when administered in adequate amounts confer a health benefit in the host, and currently numerous organisms have met the criteria established by the World Health Organization (WHO) for a probiotic. [3] They are suggested to impact metabolism, endocrine system, proper gut development, and regulation of the immune system, and by modifying the microbial community within the gut, it is possible to prevent or treat gut-associated infections. [4] Therefore, new strategies devised the use of probiotics as an alternative therapy for treatment and prevention of bacterial gastrointestinal infections, and there is a growing interest in probiotics as a safe therapeutic agent through their ability to alleviate food allergies, enhance non-specific and specific immune responses, suppress intestinal infections, and have anti-carcinogenic activity. [5,6] It has also been evident that probiotics can be effective in the prevention and/or treatment of diarrheal diseases caused by *S.* Typhimurium. The suggested mechanism by which probiotics might exert their protective or therapeutic effect against enteric pathogens include non-immune mechanisms, such as stabilization of the gut mucosal barrier, increasing the secretion of mucus, improving gut motility, and therefore interfering with their ability to colonize and infect the mucosa; competing for nutrients; secreting specific low molecular weight anti-microbial substances, and influencing the composition and activity of the gut microbiota. [7]

The present study aimed to investigate the anti-bacterial effects of two probiotics (*Lactobacillus acidophilus* and *Saccharomyces cerevisiae*) against MDR isolate of *S.* Typhimurium *in vitro* and *in vivo* by using the mouse as an animal model of the infection.

MATERIALS AND METHODS

Multi-drug resistant *S.* Typhimurium isolate

The MDR *S.* Typhimurium was previously isolated, characterized and identified in our laboratory. The isolate was resistance to ampicillin, amoxicillin/clavulanic acid and nalidixic acid, and its details were recently published. [8]

Anti-*Salmonella* Effects of Probiotics *in Vitro*

S. cerevisiae and *L. acidophilus* were supplied by Biotechnology Department, College of Science, Al-Nahrain University as stock samples. After thawing the sample, 1 ml of *S. cerevisiae* stock was inoculated into 99 ml of Sabouraud dextrose broth, and incubated at 28°C for 48 hours, while for *L. acidophilus*, 1 ml of the stock was inoculated into 99 ml of MRS broth, and incubated anaerobically at 37°C for 48 hours. [9] After the end of an incubation, the broth was centrifuged at 5000 rpm for 15 minutes to obtain cell-free culture solution, and the suspension was filtrated using Millipore filter (0.22 µm) to obtain filtrate, which was concentrated by evaporating 100 ml of it in a vacuum oven at 40-45°C to obtain one-fold filtrate (50 ml) that was tested for its anti-microbial activity. By using Izgü and Altinbay method, [10] 5 mm diameter wells were made in a nutrient agar plate that were already spread with 100 µl of *S.* Typhimurium isolate from a previous overnight culture. Then, each well was filled with 50 µl of the filtrate to be tested for the anti-microbial activity, which was based on three replicates, and in addition a blank well was filled with a broth, as a negative control. The plate was incubated at 37°C for 24 hours. After incubation, the inhibition zone diameters were measured in millimeter (mm). The one-fold filtrate was further evaporated to 25 ml (two-folds), and to 12.5 ml (three-folds), and the filtrates were tested for their anti-microbial activity in a similar manner.

Anti-*Salmonella* effects of Probiotics *in Vivo*

The two probiotics (*L. acidophilus* and *S. cerevesiae*) were tested *in vivo* for their anti-microbial effects against *S.* Typhimurium by using BALB/c male mice as a model for the infection. The experimental design was adopted from De Moreno De Leblanc *et al*. with minor modifications. [7] For each probiotic, the mice were distributed into 5 groups, and each group was kept in a separate plastic cage for the entire period of experiment. Group I (12 mice) received a probiotic for 7 successive days, and on day 8 was challenged with *S.* Typhimurium. On days 14 and 21 were dissected (6 mice for each period) for laboratory evaluation. In this design, the preventive effect of probiotics was assessed. Group II included

12 mice that received a probiotic for 14 successive days, and on day 8 was challenged with *S.* Typhimurium. On days 14 and 21 were also dissected (6 mice for each period). In this design, the treatment effect of probiotics was assessed. Group III (6 mice) received a probiotic for 7 successive days, and dissected on day 8 (a probiotic group). Group IV (6 mice) was challenged with *S.* Typhimurium on day 1, and dissected on day 8 (a pathogen group). Finally, group V (6 mice) were left untreated (a control group).

The two probiotics was grown as previously described in the "anti-*Salmonella* effects of probiotics *in vitro*". The cells were harvested by centrifugation at 5000 rpm for 15 minutes, washed three times with fresh sterilized PBS and then re-suspended in non-fat milk diluted with sterile distilled water to reach the concentration 10% (v/v). Each of the probiotics was administered to the mice as a drinking solution (10% non-fat milk) to reach a concentration of 1×10^8 CFU/ml, and mice had a free access to it (*ad libitum*), but it was changed every 24 hours with a fresh diluted milk supplemented with the probiotic. For the pathogen, 200 µl from overnight culture of *S.* Typhimurium isolate was placed in a test tube containing 5 ml of sterile brain heart infusion broth before incubation for 24 hours. Concentration of *Salmonella* culture was adjusted to 1×10^8 CFU/ml in PBS. Each mouse was challenged with 100 µl of 1×10^8 CFU/ml of *S.* Typhimurium given by gavage.

Bacterial Load in Liver and Spleen

At the end of each experiment, the mice were sacrificed by cervical dislocation and dissected by using sterile instruments after cleaning the abdominal area with alcohol. Then, a longitudinal incision was made and the two organs (liver and spleen**)** were removed. After cutting the organs into two pieces, a loopfull from each of them was diluted in 10 ml of sterile saline. An aliquot of the later solution (100 µl) were spread onto the surface of MacConkey agar plate, which by then was incubated at 37°C for 24 hours. After incubation, the plate was inspected for the formation of bacterial colonies, which were scored as number of colony forming unit per plate (CFU/plate).

Statistical Analysis

Data were given as mean ± standard deviation (S.D.) and differences between means were assessed by ANOVA (analysis of variance) followed by either LSD (least significant difference) or Duncan test. The analyses were carried out using the software SPSS version 13.0 (statistical package for social sciences).

RESULTS AND DISCUSSION

Anti-*Salmonella* Effects of Probiotics *in Vitro*

It was found that the inhibition zone was fold- and probiotic-dependent. The three-fold filtrate recorded the highest inhibition zone for both probiotics (*S. cerevisiae* and *L. acidophilus*), which were 25.0 ± 1.0 and 31.0 ± 1.0 mm, respectively, and they were significantly different from the other fold filtrates (one-fold: 7.3 ± 0.6 and 10.0 ±1.0; two-fold: 12.0 ± 1.0 and 16.0 ± 1.0, respectively). In addition, *L. acidophilus* was better than *S. cerevisiae* in recording a significantly larger inhibition zones in the investigated folds of filtrates (Figure 1).

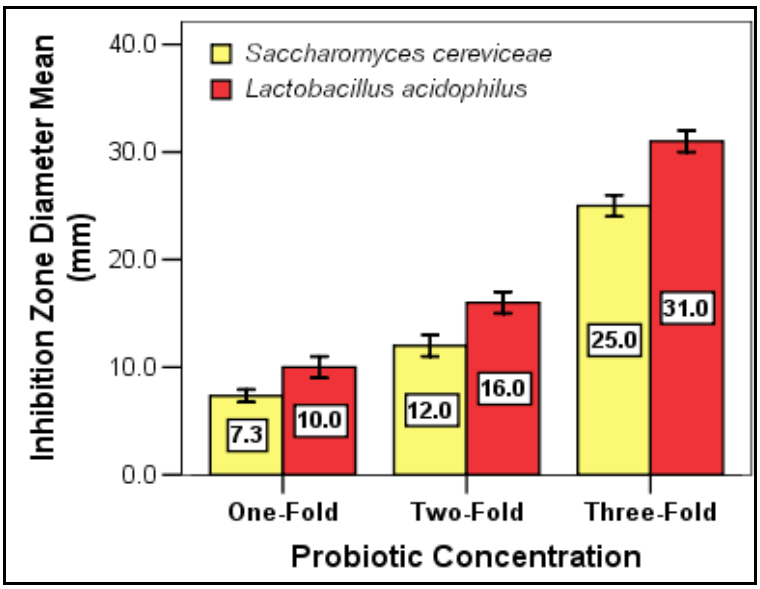

Figure 1: Inhibition zone diameter of *S. cerevisiae* and *L. acidophilus* filtrates against growth of *S.* Typhimurium.

These results suggest that filtrates of both probiotics (*S. cerevisiae* and *L. acidophilus*) were effective in limiting the growth of *S.* Typhimurium, which was resistant to three antibiotics. With respect to *S. cerevisiae*, antagonism of microorganisms by this yeast has been attributed primarily to competition for nutrients, pH changes in the medium as a result of growth-coupled ion exchange or organic acid production, secretion of anti-bacterial compounds and release of anti-microbial compounds such as killer toxins or mycocins, which are extracellular proteins or glycoproteins that disrupt cell membrane function. [11] The well known mechanisms of the killer toxin are the interruption of cell division by blocking the DNA synthesis, inhibition of synthesis of the cell wall component β-1,3-glucan, [10] and ion leakage caused by the formation of channels on the cytoplasmic membrane. [12]

With respect to *L. acidophilus*, the observed inhibitory effect might be due to the production of inhibitory compounds, especially organic acids and bacteriocins, as well as, some lactobacilli play this protective role by producing compounds such as hydrogen peroxide, lactic acid and biosurfactants, which inhibit the growth of potential pathogens. [13] In addition, it has been demonstrated that all tested lactobacilli isolates produced biofilm on polystyrene surface in all media tested with different degrees, but *L. acidophilus* showed the highest biofilm formation in Rogosa medium. [14] It has also been justified that the antagonist activity of lactic acid bacteria against diarrheal causing bacteria might be due to its ability to produce organic acids that can lower the pH, in addition to competition on the nutrients with the pathogenic bacteria. [15]

The results also showed that the inhibition zone diameter was increased by increasing the filtrate concentration and this was perhaps due to the presence of the inhibitory compounds secreted in the growth medium, which become more lethal by increasing the concentration. In agreement with such findings, it has been found that the death of tested bacteria was increased by increasing the concentration of *L. acidophilus* filtrate due to the increased concentration of inhibitory compounds especially the bacteriocins. [16] In addition, Sreekumar and Hosono found that lactic acid bacterial filtrates showed no inhibitory effects against *E. coli*, but such effect was developed upon increasing the concentration folds of the filtrates. [17]

Bacterial Load in Liver and Spleen

Both probiotics were effective in reducing *S.* Typhimurium colony forming units per plate (CFU/plate) in liver and spleen, although some differences were observed. In liver, mice challenged with the pathogen but not treated (group IV) showed a count of 224.4 ± 62.7 CFU/plate, which was significantly higher than any CFU count in groups of *L. acidophilus* or *S. cerevisiae*. Probiotic-treated groups, which received a continuous treatment with a probiotic (group II) recorded better results than group I mice, which received the probiotic 7 days pre- *S.* Typhimurium challenge. The lowest count of CFU was observed in group II at day 21, which was 21.6 ± 7.9 and 27.8 ± 10.0 CFU/plate, respectively for *L. acidophilus* and *S. cerevisiae*, and the reduction was almost 90% compared to group IV. Although, no significant difference was observed between the groups of *L. acidophilus* and *S. cerevisiae*, the former probiotic was better than the latter in reducing the CFU count in all investigated groups (Table 1).

Table 1: Bacterial load in liver of mice treated with probiotics (*L. acidophilus* or *S. cerevisiae*) and infected with *S.* Typhimurium.

Groups	Assessment Day	Mean ± S.D. (Colony Forming Unit/Plate)		P ≤
		L. acidophilus	*S. cerevisiae*	
I	14	78.1 ± 14.7^{B}	88.6 ± 15.5^{B}	N.S.
	21	96.8 ± 10.6^{B}	115.3 ± 36.9^{B}	N.S.
II	14	38.3 ± 13.6^{C}	64.6 ± 18.9^{B}	N.S.
	21	21.6 ± 7.9^{C}	27.8 ± 10.0^{C}	N.S.
III	8	No Growth		
IV	8	224.4 ± 62.7^{A}	224.4 ± 62.7^{A}	
V	8	No Growth		

Different superscript letters: Significant difference ($P \leq 0.05$) between means of columns.

For spleen, almost similar results were obtained, but group II at 14 days of *L. acidophilus* showed a significantly lower count ($P \leq 0.001$) than the corresponding group in *S. cerevisiae* (21.6 ± 6.6 *vs.* 79.6 ± 12.1 CFU/plate). However, in group II at 21 days, the CFU of *Salmonella* was also significantly decreased; moreover it was also approximated in *L. acidophilus* or *S. cerevisiae* treated mice (24.5 ± 7.8 and 23.8 ± 13.9 CFU/plate, respectively), and there was no significant difference between the two means. The reduction in this group was almost 90%. Accordingly, a continuous treatment with the probiotics recorded better results at 21 days of the treatment (Table 2).

Table 2: Bacterial load in spleen of mice treated with probiotics (*L. acidophilus* or *S. cerevisiae*) and infected with *S.* Typhimurium.

Groups	Assessment Day	Mean ± S.D. (Colony Forming Unit/Plate)*		P ≤
		L. acidophilus	*S. cerevisiae*	
I	14	64.6 ± 13.3^{C}	78.1 ± 16.1^{B}	N.S.
	21	108.6 ± 24.8^{B}	84.6 ± 15.6^{B}	N.S.
II	14	21.6 ± 6.6^{D}	79.6 ± 12.1^{B}	0.001
	21	24.5 ± 7.8^{D}	23.8 ± 13.9^{B}	N.S.
III	8	No Growth		
IV	8	206.6 ± 66.9^{A}	206.6 ± 66.9^{A}	
V	8	No Growth		

Different superscript letters: Significant difference ($P \leq 0.05$) between means of columns.

The presented results suggest that *L. acidophilus* and *S. cerevisiae* were effective in controlling the growth of *S.* Typhimurium *in vivo*, and such results came to confirm the *in vitro* findings, and *L. acidophilus* was better than *S. cerevisiae* in controlling the infection. Several mechanisms of *Lactobacillus* have been suggested to inhibit *Salmonella* invasion. For instance, some metabolic products such as lactic acid and/or bacteriocins produced by *Lactobacillus* may inhibit the growth of pathogenic bacteria. Furthermore, *Lactobacillus*

strains that maintain adhesive properties and the ability to colonize the human gastrointestinal tract may hinder the association or invasion between the epithelial cells and the pathogenic bacteria.[18] In a further study, the adhesion of five *Lactobacillus* strains to colonize Caco-2 cells (a cell line model of the intestinal barrier) was investigated and their results suggested the competitive exclusion of adhesion of *S.* Typhimurium. This was explained by the consideration that the entry of *Salmonella* into a given environment can be prevented if the space is already occupied by probiotic organisms that are better suited to establishing and maintaining themselves in the environment or those that excrete substances that inhibit the growth of *Salmonella*. [19]

In the present study, a consumption of *L. acidophilus*, especially after a continuous treatment (group II at 21 days) was associated with higher antagonistic effects against the MDR *S.* Typhimurium isolate and such findings were favored by Vesterlund *et al*. [20] In addition, it has also been found that the probiotic *L. acidophilus* decreased the translocation of *S.* Typhimurium. [21] Other researchers indicated that *Lactobacillus* may possess immune-enhancing properties or bacteriocin that can reduce the occurrence of infection by pathogens. [22,23] *L. acidophilus* also produces lactic acid as the major metabolic end-product of carbohydrate fermentation, and the resultant pH may be sufficiently low to inhibit the growth of other microorganisms including the most common human and animal pathogens. [24] Therefore, *Lactobacillus* has been the most commonly studied organisms for their probiotic properties in controlling *Salmonella* infections. [25]

With respect to *S. cerevisiae*, the results were also encouraging, and such probiotic was also able to control *S.* Typhimurium efficiently, although it was resistant to three antibiotics. In agreement with such theme, it has been shown that *S. cerevisiae* was able to colonize and survive in the gastrointestinal tract of germ-free and conventional mice and to protect them against experimental infections with *S.* Typhimurium and *Clostridium difficile*. [26] Additional findings revealed that *S. cerevisiae* was able to reduce the translocation of *S. Typhimurium* and to stimulate the immune system in mice, and at the histological level, *S. cerevisiae* conferred protection to intestine and liver tissues, and promoted an increase in the number of Kupffer cells after experimental infection with *S. Typhimurium*.[27] Further data demonstrated that this yeast protected against bacterial translocation, preserved gut barrier integrity, and stimulated the immune system in a murine model of intestinal obstruction. [28] In a more recent investigation, other *Saccharomyces* species (*S. boulardii*) was investigated in a mouse

model of *S.* Enteritidis infection, which included pretreatment with *S. boulardii*, to reveal the protection mechanisms of *S. boulardii* against *S.* Enteritidis infection, including the translocation of *S.* Enteritidis to the liver 10 days after *S.* Enteritidis challenge, and the colonization of *S.* Enteritidis and the formation of hepatic tissue lesions in mice after *S.* Enteritidis challenge on the 10th day. Their results revealed that compared to *S.* Enteritidis infection in mice, *S. boulardii* decreased *S.* Enteritidis translocation to the liver by 96%. [29]

CONCLUSIONS

L. acidophilus and *S. cerevisiae* were an effective agent in controlling bacterial growth of MDR *S. Typhimurium in vitro* and *in vivo.*

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