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Antimutagenic Activity of *Lactobacillus* spp. Isolated from Fresh Vegetables against Sodium Azide and 2-Nitrofluorene

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***Lactobacillus* spp. as a gastrointestinal tract normal flora has positive effects on human health with maintenance of microbial equilibrium. Many researchers have showed that *Lactobacillus* spp. can be effective to decrease cancer risk. The main objective of this study is evaluation of antimutagenic effects of fresh vegetables isolated *Lactobacillus spp.* on mutagenic and carcinogenic agents. 65 samples were collected by wet swabs in spring season and were enriched in Man-Rogosa-Sharpe medium (MRS) broth and isolated by growing on MRS agar medium. From 65 samples, as many as 20 gram positive, catalase negative, non spore bacilli were isolated in first phase. The samples were tested for tolerance against acidic conditions (pH: 2.5), tolerance against bile salts, tolerance samples identified through biochemical and sugar fermentation tests and verified according to Bergey's Manual. Antimutagenic effects were evaluated using bacterial culture supernatant against of sodium azide and 2-nitrofluorene by Ames test. *Lactobacillus plantarum*, *Lactobacillus casei* and *Lactobacillus brevis* produced the highest tolerance against acid and bile salts were isolated from fresh vegetables. *Lactobacillus* species could inhibit mutagenic agents activity until 40%, that is very good antimutagenic activity. The results showed that, *Lactobacillus* spp. can help to maintenance human health significantly. They can decrease absorption of mutagenic agents in the body by changing gut flora.**

Key words: *Lactobacillus* spp., Ames test, fresh vegetables, *Salmonella typhimurium* TA100.

Lactic acid bacteria (LAB) comprise a large and diverse group of gram positive, non-spore forming bacteria, catalase negative, able to produce lactic acid as the main end-product of the fermentation of carbohydrates. LAB have been isolated from specific habitats, including dairy products, plants, meat products, sewage, manure humans and animals[1]. They can decrease gut infection, colon cancer, cholesterol level, also they stimulate immune system[2-3]. Cancer is one of the most important deaths causing in the world and many factors as chemicals, rays, viruses and genetic factors may influence it. Cancers in many organs almost are developed because of genetic mutation[4-5]. Any action for removing, inhibiting and inactivating of mutagen substances is valuable. Today, bacteria are being used for the assessment of antimutagenic activities of different compounds in a short-time with excellent results[6]. One of the methods used for assessing the mutation prevention properties of a compound in bacteria is the Ames test. The Ames test is widely used as a simple and rapid in vitro method for detecting the mutagenicity of a variety of chemicals. The test is also used for predicting possible carcinogenicity from the results of mutagenicity testing. This test plays a critical role in new drug development for human use[7-8]. The

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Salmonella strains used in the test have different mutations in various genes in the histidine operon, each of these mutations is designed to be responsive to mutagens that act via different mechanisms[8-9]. In a comparative study, it was concluded that systems exploiting *Salmonella typhimurium* TA100 in the assays are most capable in identifying the mutagenic capacity of different chemicals. On the other hand, mouse hepatic homogenate, containing microsomal enzymes including cytochrome P450 has anticancer properties. Cytochrome P450 in liver S_9 fraction plays an important role in activating promutagens to proximate and ultimate mutagens. Rat and human liver P450 involved in the activation of some chemical carcinogens have different isoforms. Many researchers suggested that use of *Lactobacillus spp.* decrease the risk of cancer[7-8-9]. In this study, it was evaluated the effect of *Lactobacillus spp.* isolated from fresh vegetables against mutagenic and carcinogenic substances sodium azide and 2-nitrofluorene using Ames test (*Salmonella typhimurium* TA100) in presence and absence of liver microsome extract (S_9).

MATERIALS AND METHODS

Isolation of Lactobacilli

65 samples were collected by wet swabs and kept in sterile tubes containing MRS broth medium (Merck; Germany) in spring season. The source of samples where fresh vegetables including cabbage and silage, the entire sample tubes where incubated at 37°C and 5% CO_2 conditions for 48 h, then sub cultured on Man-Rogosa-Sharpe medium (MRS) agar (Merck; Germany) for 48 h. Vegetables have rich microbial diversity. It is very time consuming to isolate and identify each of these bacteria. Thus, it is possible and practical to delete non tolerant strains by greatly acidifying the medium. To do so, the bacteria are transferred to a medium containing PBS (pH: 2.5) and washed before calculating survival rate of isolates in acidic medium, and determining population for each strain. Then, the tolerant bacteria were transferred to MRS broth medium containing 0.3% bile salt (Oxgall) and another medium was considered as control medium. Every half an hour, growth in both control medium and medium containing bile salt were recorded through measuring optical absorption in 600 nanometers wavelength using spectrophotometer[10-11-12].

Identification of lactobacilli

The isolated bacteria, which were tolerant to acid and bile salts, were examined for their morphologic characteristics and gram-positive, non spore bacilli were selected for biochemical tests, fermentation of sugars, growth in 45, 37 and 15°C and for producing NH_3 from arginine. Then, the present bacteria were identified as detailed as their species level and verified according to Bergey's Manual[13].

Preparation of cell-free supernatants lactobacilli

Strains to be tested for antimutagenic activity were incubated in MRS broth for 48 h at 37°C. Bacterial cells were removed by centrifuging the culture at 5000 g for 20 min at 4°C. The pH values of supernatants were adjusted to pH 6.5-7.0 by the addition of 1 N NaOH, the supernatants were membrane filtered (Millipore, 0.22µm) and stored at 4°C.

Ames test

Three plates (main plate containing *Lactobacillus spp.* with positive and negative control) were used synchronously. This test was carried out in the basis of described Ames test[8-9-14].

Bacterial strains

Histidine dependent strain of *S. typhimurium TA100*, developed by Dr. Ames of the University of California, Berkeley, USA, was cultured in a Nutrient broth (Merck; Germany). The overnight culture was used for strain identity confirmation.

Strain TA100 identity assays

Histidine requirement

The media conclude bacteria were incubated for 18h at 37ºC. Then, 0.1 ml of this media was added to histidine and biotion culture (minimal medium having a little histidine and biotin). Also, 0.1 ml *S. typhimurium* TA100 was added to biotin medium (minimal medium having biotin and lacking histidine) as control plate. All plates were incubated for 48h at 37ºC.

Rfa mutation

Sensitivity to crystal violet was tested. A 100 µl sample of the overnight bacterial culture was inoculated in 2 ml of melted and cooled top agar and spread over an agar nutrient plate. A disk dipped in crystal violet was later placed on this plate and after a18 h period, a bright zone was

observed around the disk, an indication of the lack of cell growth due to the Rfa mutation.

UVrB mutation

This test is used to confirm UV sensitivity. After culture the bacteria on plate, a half of one was covered with aluminum foil, and it was exposed to UV radiation 8 seconds. Then, the plate was incubated for 18 h at 37ºC.

R-factor assay

This test is used to show resistance factor against ampicillin. The absence of zone of growth inhibition around the disk was an indication of ampR and a proof for the presence of the R-factor in the bacterial strain.

Preparation of the rat microsomal liver enzyme (S_9) and mutagen substances

A broad range of carcinogenic agents require metabolic activation for recognition. In this investigation, 5 male rats (body weight~200g), were used. Rats were starved for 24 hours in order to get the titer of the liver enzymes to their highest levels. Animals were sacrificed by cervical dislocation and the livers were collected, homogenized in 0.15 M KCl. Livers were cut into pieces using sterile scissors and smashed prior to a 10 min centrifugation at 9000g. The supernatant (S_9) was stored at -80°C. The antimutagenic assay was performed in the presence and absence of S_9. Two chemical mutagen, sodium azide and 2-nitrofluorene were purchased from Sigma and Merck company.

Procedure in presence of liver microsome (S_9)

Sample

In this assay 0.1 ml of cultural supernatants lactobacilli mixed 0.1 ml of the overnight culture *S. Typhimurium* TA100 and 0.1 ml of our mutagenic substances including sodium azide and 2-nitrofluorene in test-tube containing 3ml top agar. Then, 0.1ml of histidine and biotin 0.5 mM solution and 0.5ml of liver microsome extract (S_9) were added. After were poured on glucose minimal medium and incubated for 24 h at 37ºC.

Positive control

The mixture of 0.1 ml of overnight cultured *S. typhimurium* TA100, 0.1 ml of mutagenic substances including sodium azide and 2-nitrofluorene were prepared and were poured in test-tube containing 3ml top agar. Then, 0.1ml of histidine and biotine 0.5 mM solution and 0.5ml of liver microsome extract (S_9) were added, after shaking for 3 minutes, the test-tube contents was poured on glucose minimal medium and incubated for 24h at 37ºC.

Negative control

The mixture of 0.1ml of overnight cultured *S. typhimurium* TA100, 0.1ml of DMSO, 0.1 ml of histidine and biotine 0.5 mM solution and 0.5 ml of liver microsome extract (S_9) were added to 3ml of top agar. After shaking for 3 minutes, it was poured on glucose minimal medium and incubated for 24h at 37ºC.

Procedure in absence of liver extract (S_9)

All the steps in this stage are the same as previous part. But, here, it was not used from liver microsome extract (S_9).

Inhibitory percentage calculation

The calculation percentage of inhibition was done according to the formula given by Ong *et al*.

Percentage inhibition = [1-T/M] ×100 where T is number of revertants per plate in presence of mutagen and test sample and M is number of revertants per plate in positive control. The number of spontaneous revertants was subtracted from numerator and denominator. The antimutagenic effect was considered moderate when the inhibitory effect was 25-40% and strong when more than 40%. Inhibitory effects of less than 25% was considered as weak and was not recognised as positive result[15]. Statistical analyses were performed using SPSS software.

RESULTS

In total, 20 (30.77%) presumptive lactobacillus strains were isolated from 65 samples were collected by wet swabs from fresh vegetables, All isolates were catalase negative, gram positive and oxidase negative rods producing no gas from glucose. From 20 gram positive rods, 3 strains *Lactobacillus plantarum*, *Lactobacillus casei* and *Lactobacillus brevis* produced the highest tolerance against acid and bile salts. Identified through biochemical and sugar fermentation tests and verified according to Bergey's Manual (Table 1).

In accordance with the *Salmonella typhimurium* TA100 strain genotype, the presence of colony in biotin-histidine medium and absence one in control biotin medium show that these

strains are dependent to histidine. The existence of inhibitory zone around the disk indicates that the bacteria do not grow and the Rfa mutation was occurred. This mutation can causes relative decreasing of lipopolysaccharide barriers and then, increase cell wall permeability for bigger molecules. If the inhibitory zone is not presence around the disk, the bacterium has R-factor plasmid and also, lack of growth in radiated culture region indicates that uvr B mutation was ocurred. According to the results, *L. Plantarum* was more effective than other bacteria with 54.82% inhibition of sodium azide effects and 50.50% inhibition of 2-nitrofloren effects in presence of liver microsame extract (S_9). Antimutagenic activity was increased significantly when there were S_9 (Table 2, 3).

Lactobacilli are normal inhabitants of gastrointestinal tract where they exert several health promoting effects such as prevention of intestinal infections, decrease of serum cholesterol levels, stimulation of immune response and reduction of colon cancer risk[16]. Cancer is considered as one of the main causes of mortality throughout the industrial world in the present century. The use of antimutagens and anticarcinogens in everyday life is the most effective procedure for preventing human cancer and genetic disease. Lactobacilli have important roles for inactivation of mutagenic substances and decreasing risk of cancer. The inhibitory mechanisms of mutagenic and carcinogenic substances by *Lactobacillus* spp. aren't completely known. But some mechanisms include; binding to mutagenic agent and prevention of their absorption by body, decomposition of them, decreasing the activities of some detrimental enzymes, inhibiting harmful bacteria in gastrointestinal, producing some special metabolites, decreasing bile acids and immune system stimulation[17]. Many studies confirm positive role of *Lactobacillus spp.* in decreasing mutagenic agent effects[18-19-20-21-22]. Hosono *et al*, were the first to report that milk fermented with *L. delbrueckii subsp. bulgaricus*, *Lactococcus lactis subsp. lactis* or *Enterococcus faecalis* exhibited an antimutagenic activity against NQO[18]. Lankaputhra and Shah, proved that *Lactobacillus spp.* has good activity in decreasing mutagenic substances[19]. Zobel *et al*, showed that, *L. acidophilus* and its culture extract prevented from

Table 1. Identification of lactobacilli on the basis of biochemical tests

Strains	Growth at			catalase	motility	NH_3 from arginine	Sugar fermentation										
	15 ºC only	45 ºC only	15 and 45 ºC only				Cellobiose	lactose	Mannitol	Raffinose	galactose	Melebiose	sucrose	Maltose	Mannose	Trehalose	Sorbitol	Asculin
L. Plantarum	+	-	-	-	-	+	+	+	+	+	+	+	+	+	+	+	+	+
+	+																	
L. casei	-	+	-	-	-	-	+	+	-	-	-	-	-	-	-	-	-	-
-	-																	
L. brevis	+	-	-	-	+	-	+	+	-	-	-	+	+	+	+	+	+	-
+	-																	

Table 2. Antimutagenic effect of lactobacilli supernatant against sodium azide

Revertant colony	*S.typhimurium* / S_9^-		*S.typhimurium* / S_9^+	
Sampels	Revertants (CFU/plate)	Inhibition (%)	Revertants (CFU/plate)	Inhibition (%)
Positive control(sodium azide)	398	-	498	-
Negative contorol(DMSO)	53	-	75	-
L. Plantarum	198	50.25	225	54.82
L. casei	200	49.74	236	52.61
L. brevis	207	47.99	245	50.80

Table 3. Antimutagenic effect of lactobacilli supernatant against 2-nitrofluorene

Revertant colony	*S.typhimurium* / S_9^-		*S.typhimurium* / S_9^+	
Sampels	Revertants (CFU/plate)	Inhibition (%)	Revertants (CFU/plate)	Inhibition (%)
Positive control (2-nitrofluorene)	426	-	596	-
Negative contorol (DMSO)	53	-	75	-
L. Plantarum	215	49.53	295	50.50
L. casei	232	45.54	310	47.99
L. brevis	241	43.43	318	46.64

DNA damage by MNNG[20]. Heui-dong and Chang-Ho showed that, *L. plantarum* KLAB 21 was isolated from Kimchi can inhibit four mutagenic and carcinogenic agents effects; Aflatoxin B1, NQO, MNNG and NPD. He used two salmonella strains TA100 and TA98. Results showed that the bacterial culture supernatant inhibited mutagenic effects of MNNG (98.4%) in presence of TA100 and NQO (57.3%) in presence of TA98[21]. Chalova *et al*, evaluated the ability of some probiotic bacterial supernatants to decrease the effects of two mutagenic substances benzo[a]pyrene and sodium azide in different growth phases and *Bifidobacterium adolecenti* ATCC 15703 had 48.7% inhibitory in Log phase duration, *L. plantarum* ATCC 8014 showed 59.37% inhibitory function on mutagenic substance benzo[a]pyrene, and *L. plantarum* ATCC 8014 had 54.64% inhibitory on mutagenic substance sodium azide in lag phase duration[22]. In our study, *Lactobacillus spp*. also showed good antimutagenic abilities. *L. plantarum* was more effective than other bacteria with 54.82% inhibition of sodium azide effects and 50.50% inhibition of 2-nitrofloren effects in presence of liver microsame extract. When, it was used liver microsome extract in medium, more than 45% inhibition ability were observed. It was showed high antimutagenic effects using these bacteria.

CONCLUSION

Lactobacillus spp. are found in dairy products, plants, meat products, sewage, manure humans and animals. These kinds of bacteria have positive effects on immune system by inhibition of pathogen attachment to epithelial cells, changing the receptor of bacterial toxins, producing antimicrobial substances such as acid, bacteriocins, fatty acid and aromatic compounds and competition for food. In this study, good impacts in inhibiting mutagenic agents were observed. This group of bacteria as gastrointestinal flora cause to decrease absorption of mutagenic and carcinogenic substance. At presence, with increasing of the antibiotic resistance and side effects of chemical drugs, it seems, we need to use alternative remedies. Lactobacilli and their produced metabolites can have therapeutic application in future and they can help to decrease absorption of mutagenic substances and elimination of detrimental bacteria in body.

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