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Antimutagenic activity of probiotic *Bifidobacterium* and *Lactobacillus* spp. by Ames test

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Abstract

Probiotics are live microbial food supplements that are considered to be beneficial for humans and animals. Many researchers have showed that probiotic bacteria can be effective to decrease cancer risk. In this study, Antimutagenic activities of four probiotic *Bifidobacterium* and *Lactobacillus* spp. against three potent mutagen acrylic amide, sodium azide and 2-nitrofluorene were examined by Ames test using *Salmonella typhimurium* TA100. Results showed *Bifidobacterium* and *Lactobacillus* spp. can inhibit mutagen agents unit 40%, That is very good antimutagenic activity. *Bifidobacterium bifidum* PTCC 1644 displayed the highest antimutagenic activity than other bacteria (inhibition 52.22% acrylic amide, 50.80% sodium azide and 48.66%, 2-nitrofluorene). Antimutagenic activity was increased significantly when there were liver microsome extract (S_9).

INTRODUCTION

Probiotics are living microorganisms which when ingested have beneficial effects on the equilibrium and the physiological functions of the human intestinal microflora [1]. Probiotics have been reported to play a therapeutic role by modulating immunity, lowering cholesterol, improving lactose tolerance and preventing some cancers [2, 3]. The strains with beneficial properties, which are potential sources of probiotics most frequently belong to the genera *Bifidobacterium* and *Lactobacillus* [4]. Probiotics are non-pathogenic, technologically suitable for industrial processes, acid fast, bile tolerant, adhere to the gut epithelial tissue and produce antimicrobial substances, including, organic acids, hydrogen peroxide and bacteriocins [5]. Humans are continually exposed to a variety of natural and artificial mutagens generated by industrial and environmental activities [6]. Each factor that causes removal, inhibition and inactivation of mutagen substances is rewarding. Today, bacteria are being used for the assessment of antimutagenic activities of different compounds in a short-time with excellent results [7]. One of the methods used for assessing the mutation prevention properties of a compound in bacteria is the Ames test. Ames test is a worldwide short-term bacterial reverse mutation test specifically designed for screening a variety of new chemical substances and drugs that can produce genetic damage that leads to gene mutations [8, 9]. The 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 [9, 10]. 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 [7]. Many researchers have suggested that use of probiotic bacteria decrease the risk of cancer [2, 6, 11, 12]. In this study, we evaluated the effect of *Bifidobacterium* and *Lactobacillus* spp. against mutagenic and carcinogenic substances acrylic amide, sodium azide and 2-nitrofluorene using Ames test (*Salmonella typhimurium* TA100) in presence and absence of liver microsome extract (S_9).

MATERIALS AND METHODS

Bacterial strains and growth conditions

Bifidobacterium bifidum PTCC 1644, *Bifidobacterium adolescentis* PTCC1536, *Lactobacillus plantarum* PTCC 1058, *Lactobacillus acidophilus* PTCC 1643 were obtained from collection center of fungi and bacteria, Tehran, Iran. *Salmonella typhimurium* TA100 supplied from Dr. Ames of the University of California, Berkeley, USA. All strains were grown in lactobacilli MRS broth (de Man, Rogosa and Sharpe) (Merck; Germany) supplemented with 0.05% cysteineHCl and incubated anaerobically in an anaerobic jar at 37ºC using an anaeropack system. 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 [2, 6]. *Salmonella Typhimurium* TA100 was cultured in a Nutrient broth (Merck; Germany). The overnight culture was used for strain identity confirmation [9, 10].

Strain TA100 identity assays

Histidine requirement

The media conclude overnight bacterial culture was 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 0.1 ml 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 an18 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 culturing 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

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. Three chemical mutagen acrylic amide, sodium azide and 2- nitrofluorene were purchased from Sigma and Merck Company and dissolved in dimethyl sulfoxide (DMSO), a final concentration of 5 µg/ml.

Antimutagenic activity assay

Antimutagenic properties of bacterial supernatants against acrylic amide, sodium azide and 2- nitrofluorene were evaluated by a pre-incubation method of Maron and Ames (1983) in presence and absence of liver microsome extract (S_9).

Procedure in presence of liver microsome (S_9)

Sample: In this assay 0.1 ml of bacterial cultural supernatants were mixed with 0.1 ml of the overnight culture *S. Typhimurium* TA100 and 0.1 ml of our mutagenic substances including acrylic amide, sodium azide and 2-nitrofluorene were added 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 acrylic amide, 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 content 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%. An inhibitory effect of less than 25% was considered as weak and was not recognized as positive result [13]. Statistical analyses were performed using SPSS software.

RESULTS

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

Table 1. Antimutagenic properties of bacterial supernatants against acrylic amide

Revertant colony / Sampels	*S. typhimurium* / S_9^-		*S. typhimurium* / S_9^+	
	Revertants (CFU/plate)	Inhibition (%)	Revertants (CFU/plate)	Inhibition (%)
Positive control (acrylic amide)	368	-	473	-
Negative contorol (DMSO)	53	-	75	-
Bifidobacterium bifidum PTCC 1644	189	48.64	226	52.22
*Bifidobacterium adolescentis PTCC*1536	202	45.11	233	50.74
Lactobacillus plantarum PTCC 1058	218	40.76	249	47.36
Lactobacillus acidophilus PTCC 1643	208	43.48	238	49.68

Table 2. Antimutagenic properties of bacterial supernatants against sodium azide

Revertant colony / Sampels	*S. typhimurium* / S_9^-		*S. typhimurium* / S_9^+	
	Revertants (CFU/plate)	Inhibition (%)	Revertants (CFU/plate)	Inhibition (%)
Positive control (sodium azide)	396	-	498	-
Negative contorol (DMSO)	53	-	75	-
Bifidobacterium bifidum PTCC 1644	212	46.46	245	50.80
*Bifidobacterium adolescentis PTCC*1536	222	43.93	252	49.38
Lactobacillus plantarum PTCC 1058	245	38.13	273	45. 18
Lactobacillus acidophilus PTCC 1643	232	41.41	260	47.80

Table 3. Antimutagenic properties of bacterial supernatants against 2-nitrofluorene

Revertant colony / Sampels	*S. typhimurium* / S_9^-		*S. typhimurium* / S_9^+	
	Revertants (CFU/plate)	Inhibition (%)	Revertants (CFU/plate)	Inhibition (%)
Positive control (2-nitrofluorene)	423	-	561	-
Negative contorol (DMSO)	53	-	75	-
Bifidobacterium bifidum PTCC 1644	232	45.15	288	48.66
Bifidobacterium adolescentis PTCC 1536	248	41.37	306	45.45
Lactobacillus plantarum PTCC 1058	261	38.30	324	42.25
Lactobacillus acidophilus PTCC 1643	253	40.19	318	43.31

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 occurred. The bacterial supernatants were characterized with different patterns of antimutagenic activity against the three mutagens in presence and absence of liver microsome extract (S_9). All *Bifidobacterium and Lactobacillus spp.* exhibited can inhibit mutagen agents unit 40%, That is very good antimutagenic activity (Table 1, 2, 3). Antimutagenic activity was increased significantly when there were S9. *Bifidobacterium bifidum* PTCC 1644 displayed the highest antimutagenic activity (inhibition 52.22% acrylic amide, 50.80% sodium azide and 48.66%, 2-nitrofluorene) than other bacteria.

DISCUSSION

According to their antimutagenic mechanisms, antimutagens are classified as either desmutagens or bioantimutagens. Bioantimutagens inhibit the effects of mutagens by modulating cellular mutagenic processes, that is, by mainly acting on DNA replication and repair processes. Desmutagens, on the other hand, directly inhibit mutagens or their precursors by means of chemical or enzymatic inactivation [14]. Compared to other antimutagenic sources, namely eggs, seeds, and plants, bacteria have the advantage to be better defined genetically, easier to modify and maintain, and easier to scale up for production of large quantities [6]. Many studies confirm positive role of *Bifidobacterium* and *Lactobacillus* spp. in decreasing mutagenic agent effects. Hosono et al, reductases released by *L. bulgaricus* and *Streptococcus thermophilus* throughout milk fermentation abolished the mutagenic activities of 2-(2-(furyl-3-(5-

nitrofuryl))) acrylamid (AF2) and 4-nitroquinoline-1-oxide (4NQO) [15]. In study Hosoda et al. (1992) milk cultured with *Lactobacillus acidophilus* LA 106 (LA2) showed the highest inhibition of 77% against the mutagenicity of N-methyl-N'-nitro-N-nitrosoguanidine among the strains tested (*Lactobacillus*, *Streptococcus*, *Lactococcus*, and *Bifidobacterium*) [16]. Zobel et al, showed that, *L. acidophilus* and its culture extract prevented from DNA damage by MNNG [17]. Lankaputhra and Shah, found butyrate and acetate, fermented products of *Lactobacillus* and *Bifidobacterium*, exhibited strong antimutagenic activities against several mutagens and promutagens [11]. 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 [18]. Pei-Ren et al, evaluated the ability of Several Probiotic Bifidobacteria against Benzo[a]pyrene and Cells of *Bifidobacterium lactis* Bb-12 and *B. longum* CCRC 14634 showed higher antimutagenic activities than their supernatants [14]. 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. [6] In our study, *Bifidobacterium* and *Lactobacillus* spp. also showed good antimutagenic abilities. *Bifidobacterium bifidum* PTCC 1644 was more effective than other bacteria with inhibition of 52.22% acrylic amide, 50.80% sodium azide and 48.66%, 2-nitrofluorene in presence of liver microsame extract. When, it was used liver microsome extract in medium, more than 40% inhibition ability were observed. It was showed high antimutagenic effects using these bacteria.

CONCLUSION

This group of bacteria as gastrointestinal flora causes 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. *Bifidobacterium* and *Lactobacillus* spp. 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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