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**Antimutagenic Effect of *Lactobacillus acidophilus* and
Lactobacillus bulgaricus Isolated from Iranian
Yoghurt on 2-Nitrofluorene**

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Abstract: The antimutagenic activity of *Lactobacillus bulgaricus* and *acidophilus* to potent mutagen, 2-nitrofluorene was examined by the modified Ames test using *Salmonella typhimurium TA100*. The results showed that the supernatants, heat killed cells and viable cells of *Lactobacilli*, preincubated with mutagenic factor such as 2-nitrofluorene, displayed characteristic antimutagenic activities. *L. bulgaricus* and *L. acidophilus* cells showed 98 and 64% antimutagenic activity against 2-nitrofluorene, respectively. Antimutagenic activities of *Lactobacilli* were higher than their supernatants. All the strains, heated for 15 min at 100°C, antimutagenic activities were reduced by 36-47%.

Key words: Antimutagenicity, *L. bulgaricus*, *L. acidophilus*, 2-nitrofluorene, Ames test

INTRODUCTION

The presence of mutagen contamination in food and environment is causative of several diseases including tumor (Wollowski *et al.*, 1999). It is important to control mutagenesis and carcinogenesis by identifying the hazardous agents and removing them from the environment. There are many reports that fermented milk products and Lactic acid bacteria inhibit the mutagenicity of a variety of chemical components (Asahara *et al.*, 2006; Hsieh and Chou, 2006). Epidemiological evidences indicate a negative correlation between the incidence of certain cancers and consumption of fermented milk products (Rachid *et al.*, 2002; Abdel-Gawad *et al.*, 2004; Femia *et al.*, 2002). Yoghurt and the lactic acid producing bacteria that it contains, have received much attention as potential cancer-preventing agents in the diet. Yoghurt has been reported to inhibit tumor progression and modulate the immune response and stimulate cellular apoptosis (Leblung and Perdigon, 2004; Rachid *et al.*, 2002). Yoghurt and extracts thereof have been shown to be antimutagenic against a range of mutagens and pro-mutagens in microbial and mammalian cell systems (Nadathur *et al.*, 1995; deMoreno de Le Blance and Perdigon, 2005). Certain epidemiological studies have suggested that consumption of yoghurt and other fermented milk products may reduce the incidence of colon or breast cancer (Parvez *et al.*, 2006; Norat and Riboli, 2003; Perdigon *et al.*, 2002; Rachid *et al.*, 2002). 2-nitrofluorene, widespread in the atmosphere, is a direct-acting mutagen and carcinogen (Paul *et al.*, 1994).

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In this study, we used a preincubation method, a modification of Ames test (Lo *et al.*, 2002) to investigate the microbiological and antimutagenic characteristics of *Lactobacilli*, isolated from Iranian yoghurt against 2-nitrofluorene as a potent mutagen.

MATERIALS AND METHODS

Bacterial Strain

Samples of Iranian commercial yoghurts were homogenized with Ringer solution by shaking for several minutes. 0.1 mL of this suspension was plated on the surface of MRS agar (Merk, Germany). After anaerobic incubation for 48 h at 37°C, the colonies were tested for catalase reaction. All *Lactobacilli* were rod shaped. The isolated strains were maintained as frozen cultures in MRS broth with 15% glycerol at 80°C. Growth at 15 and 45°C was tested in MRS broth after 2 and 5 days of incubation.

Assay for Antimutagenicity

The antimutagenicity of *Lactobacilli* was determined by measuring the extent of decrease in mutation induced by 2-nitrofluorene (Sigma LTD). A preincubation method reported by Maron and Ames (1983), which is a modification of the Ames test (Lo *et al.*, 2002) was used throughout this study. The following components were added to the sterile glass tubes: Overnight culture of *Salmonella typhimurium TA100* (0.1 mL), MRS culture of *Lactobacilli* (0.1 mL) and 2-nitrofluorene, (2 µg/plate). The entire mixture was incubated at 37°C with gentle shaking for 20 min. Then 2 mL of molten top agar (45°C) containing 0.05 mM histidine (Sigma LTD), 0.05 mM biotin (Sigma LTD) and 0.09 M NaCl was added to this mixture. This combined solution was then poured on a minimal glucose agar plate. The plate was then incubated at 37°C for 48 h to calculate revertants. Positive control consisted of *S. typhimurium TA100* and 2-nitrofluorene without MRS culture of *Lactobacilli* and spontaneous control consisted of *S. typhimurium TA100* and MRS medium. Each assay was performed in triplicate and the antimutagenicity was expressed as percentage of inhibition:

$$\text{Inhibition (\%)} = [(A-B)/(A-C)] \times 100$$

A = No. of his. revertants in the absence of *Lactobacilli* (positive control),

B = No. of his. revertants in the presence of *Lactobacilli*,

C = Spontaneous revertants (negative control).

Preincubation of *Lactobacilli* Cultures with 2-Nitrofluorene

To evaluate the effect of preincubation of *Lactobacilli* cultures with 2-nitrofluorene, MRS culture of *Lactobacilli* (0.1 mL) and 2-nitrofluorene 2 µg/plate were initially mixed and incubated at 37°C for 20 min. Then 0.1 mL of *S. typhimurium TA100* was added to the mixture and the incubation was carried out at 37°C for another 20 min. Antimutagenicity of *Lactobacilli* was determined as mentioned above. Positive control consisted of *S. typhimurium TA100* and 2-nitrofluorene without *Lactobacilli* cultures and spontaneous control consisted of *S. typhimurium TA100* and MRS medium.

Preincubation of *Lactobacilli* Cultures with *S. Typhimurium TA100*

To determine the effect of preincubation of *Lactobacilli* cultures with *S. typhimurium TA100*, MRS culture of *Lactobacilli* (0.1 mL) and *S. typhimurium TA100* (0.1 mL) were mixed and incubated at 37°C for 20 min with gentle shaking. Then 2 µg/plate of 2-nitrofluorene was added to this mixture and the incubation was carried out at 37°C for another 20 min. Antimutagenicity was assayed as mentioned above.

Antimutagenicity of *Lactobacilli* Cells on 2-Nitrofluorene

Cell suspension in PBS (0.1 mL) and 2-nitrofluorene were mixed and preincubated for 20 min with gentle shaking, then *S. typhimurium* TA100 (01 mL) was added. The solution was then incubated at 37°C for 20 min with gentle shaking. Antimutagenicity assay was performed as mentioned above. Positive control consisted of *S. typhimurium* TA100 and 2-nitrofluorene without *Lactobacilli* and spontaneous control consisted of *S. typhimurium* TA100 and PBS.

Heat Treated Cells on 2-Nitrofluorene

Cell suspensions of *Lactobacilli* in PBS were heated in boiling water bath for 20 min. After heat treatment, the cells were vortexed for 5 min to break coagulum and plated in MRS culture to confirm the lethal effect of the heat treatment.

Statistical Analysis

The results are expressed as mean±standard deviation (n. as indicated). T-test was used to assess the statistical significance between the test groups and control group. The data were subjected to the Analysis of Variance (ANOVA). A significant difference ($p < 0.05$) was determined between the means.

RESULTS

Identification of *Lactobacillus* Isolated from Yoghurt

Fifteen *Lactobacillus* strains were isolated from 17 mild yoghurts and 1 bio-yoghurt. All the isolates were catalase negative rod, producing no gas from glucose. They were presumptively identified by determining the growth behavior at 15 and 45°C. Three strains were identified as *L. casei*, 6 strains as *L. acidophilus* and 6 strains as *L. bulgaricus*.

Inhibitory Effect of *Lactobacillus acidophilus* Cells on 2-Nitrofluorene

Antimutagenic activities of viable cells of *L. acidophilus* were detected by using modified Ames test. Inhibitory effect of viable cells of *L. acidophilus* was 64.45±9.50 on 2-nitrofluorene (2 µg/plate) (Table 1) while it was only 30.11±0.59 and 36.66±5.12 for the supernatant and heat killed cells of *L. acidophilus*, respectively ($p < 0.05$).

Inhibitory Effect of *Lactobacillus bulgaricus* Cells on 2-Nitrofluorene

The antimutagenic activities of the viable cells of *L. bulgaricus* were detected by modified Ames test. Viable cells of *L. bulgaricus* showed more than 97.99±0.88 inhibitory effect on 2-nitrofluorene (2 µg/plate) (Table 2) but supernatant and heat killed cells of *L. bulgaricus* only showed 34.11±6.9 and 47.75±5.85 antimutagenicity, respectively, which were significantly lower than those of the viable cells of *L. bulgaricus* ($p < 0.05$).

Table 1: Antimutagenicity of *Lactobacilli acidophilus* against 2-nitrofluorene

Subject	Revertant (CFU/Plate)	Inhibition (%)
Positive control (A)	437.66±70.68	-
Negative control (C)	114.00±15.39	-
Viable <i>L. acidophilus</i> +mutagen+TA100 (B)	228.33±48.04	64.45±9.50
Heat killed <i>L. acidophilus</i> +mutagen+TA100 (B)	317.33±33.62	36.66±5.12
Supematant of <i>L. acidophilus</i> +mutagen+TA100 (B)	340.00±51.41	30.11±0.59

The results are presented as mean±SD for three plates., Inhibition (%) = [(A-B) / (A-C)] × 100%, A= revertants TA100 colonies in the presence of 2-nitrofluorene (positive control), B= revertant TA100 colonies in the presence of *Lactobacillus acidophilus* + 2-nitrofluorene C= Spontaneous revertant colonies of TA100

Table 2: Antimutagenicity of *Lactobacilli bulgaricus* against 2-nitrofluorene

Subject	Revertant (CFU/Plate)	Inhibition (%)
Positive control (A)	437.66±70.68	-
Negative control (C)	114.00±15.39	-
Viable <i>L. bulgaricus</i> +mutagen+TA100 (B)	120.33±14.36	97.99±0.88
Heat killed <i>L. bulgaricus</i> +mutagen+TA100 (B)	281.66±37.2	47.75±5.87
Supernatant of <i>L. bulgaricus</i> +mutagen+TA100 (B)	325.00±36.37	34.11±6.9

The results are presented as mean±SD for three plates, Inhibition % = [(A-B)/ (A-C)] ×100%, A= revertants TA100 colonies in the presence of 2-nitrofluorene (positive control), B= revertant TA100 colonies in the presence of *Lactobacillus bulgaricus* + 2-nitrofluorene, C= spontaneous revertant colonies of TA100

DISCUSSION

The role of diet on the etiology of cancer has been receiving increasing attention in the last 20 years (Le Blung and Perdigon, 2004; Brady *et al.*, 2000). Studies on the conditions that enhance or decrease activation or inactivation of mutagens are important to control their risk to man.

Yoghurt and the lactic acid producing bacteria that it contains, have received much attention as potential cancer-preventing agents in diet.

In this study the inhibitory effect of *L. acidophilus* and *L. bulgaricus* strains of yoghurt origin against the 2-nitrofluorene was evaluated *in vitro* by modified Ames test (Lo, 2002). 2-nitrofluorene is a direct-acting mutagen and carcinogen and its mutagenicity has been proved on experimental animals. Paul *et al.* (1994) showed the nitroreduction of 2-nitrofluorene (2-NF) by a human microflora in female Wistar rats, while our finding showed the number of revertants was significantly reduced by incubation of *L. acidophilus* and *L. bulgaricus*.

We isolated *Lactobacillus* and tested them directly as viable bacteria in culture, which means that antimutagenic activities were exerted by yoghurt bacteria. It seems that these antimutagenic activities are dependent on the survival of LAB in yoghurt and in the intestinal tract.

Administration of LAB can also decrease DNA damage induced carcinogenesis (Pool Zoble *et al.*, 1996). The mechanisms by which LAB exerts protective effects on DNA damage and tumorigenesis have not been yet elucidated.

In present study, viable cells of *L. acidophilus* showed antimutagenicity more than 64% but *L. bulgaricus* cells showed significantly (97%) higher antimutagenicity than *L. acidophilus*. It has been well established that various lactic acid bacterial strains originating from fermented milk possess antimutagenic activities (Rhee and Park, 2001; Haza *et al.*, 2005). In humans, a decrease in fecal mutagenicity has been revealed after the consumption of *L. acidophilus* fermented milk together with fried meat (Lidbeck *et al.*, 1992). *L. acidophilus* feeding has been shown to decrease the incidence of colon tumors in the rats challenge with the colon carcinogen DMH (Goldin and Gorbach, 1980). *L. acidophilus* is expected to be the main *Lactobacillus* species involved in the production of mild and probiotic yoghurt. Milk products fermented by *L. bulgaricus* and *S. thermophilus* were shown to exhibit dose dependent antimutagenic activity against a number of direct acting and indirect acting mutagens. Aceton extracts of yoghurt fermented by *L. bulgaricus* and *S. thermophilus* have been shown to be antimutagenic via metabolite fractions such as palmitic acid (Nadathur, 1995, 1996). Wollwoski (1999) has found that, oral application of *L. bulgaricus* in rats could prevent DMH induced DNA break in colon *in vivo*.

Inactive milk constituents may be converted into anti-mutagens by fermenting organisms, or milk may lack such precursors but serve as a neutral medium for the organisms that ordinarily produce antimutagens during the fermentative growth.

We also investigated supernatant and heat killed cells of *Lactobacilli* for their antimutagenic abilities. Our findings showed that the antimutagenic activities of the heat killed cells significantly decreased (Table 1 and 2), but Oata Zhang (1990) has been shown that heat killed cells of lactic acid bacteria have higher binding activities than viable bacterial cells.

In conclusion, present results demonstrated that viable *L. acidophilus* and *L. bulgaricus* isolated from yoghurt, exhibited strong antimutagenic activity against 2-nitrofluorene *in vitro*, but heat killed cell bacteria has shown weaker antimutagenic activity, which means that these antimutagenic activities are dependent on the survival of LAB in yoghurt and in the intestinal tract.

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