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Antimutagenic activity of *Lactobacillus plantarum* KLAB21 isolated from *kimchi* Korean fermented vegetables

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Abstract

Antimutagenic activity of *Lactobacillus plantarum* KLAB21, isolated from Korean *kimchi*, was investigated against MNNG (*N*-methyl-*N'*-nitro-*N*-nitrosoguanidine), NQO (4-nitroquinoline-1-oxide), NPD (4-nitro-*O*-phenylenediamine) and aflatoxin B1 using *Salmonella typhimurium* strains TA100 and TA98. Although all the cell fractions including the culture supernatant, dry cells and cell-free extract exhibited antimutagenic activity against MNNG and NQO, the culture supernatant possessed the highest activity. The antimutagenic ratio of the culture supernatant was 98.4% against MNNG on strain TA100 and 57.3% against NQO on strain TA98. Its antimutagenic activity was reconfirmed by a *Bacillus subtilis* spore-rec assay. Levels of the antimutagenic ratios of other lactic acid bacteria originating from fermented milk ranged between 26.8 to 53% against MNNG and 28.5 to 43.4% against NQO. The antimutagenic activities of the strain KLAB21 against NPD were 72.6% on TA100 and 62.8% on TA98, and those against aflatoxin B1 were 82.5% on TA100 and 78.2% on TA98.

Introduction

Lactic acid bacteria are regarded as synonymous with the family Lactobacillaceae that consists of Gram-positive, non-endospore forming, carbohydrate-fermenting lactic acid producers that are acid-tolerant, facultatively anaerobic and catalase-negative bacteria. Typically they are non-motile and can be largely sub-divided into five genera; *Lactococcus*, *Streptococcus*, *Leuconostoc*, *Pediococcus* and *Lactobacillus* spp. (Kandler & Weiss 1986). The selection of certain strains of these bacteria and their use under controlled conditions has improved the quality and stability of certain food products such that they are now indispensable in the manufacture of many fermented products such as cheese, yoghurt, yakult, buttermilk, sour cream, sauerkraut, sausages, silage and pickles (Sandine *et al.* 1972).

There have been many reports on the beneficial functions of lactic acid bacteria for humans; their antimicrobial (Parente & Ricciardi 1999), anticancer (Adachi 1992) and antimutagenic (Hosono *et al.* 1990, Nishioka *et al.* 1989) activities as well as their effects on modulating the immune system (Perdigon *et al.* 1988), lowering cholesterol levels (Shun *et al.* 1989) and reducing lactose-intolerance (Alm 1982) have been shown. Currently, one of the most important factors in human longevity is the control of tumors. A variety of lactic acid bacteria originating from fermented milk have been reported to possess antitumor effects as well as antimutagenic activities. These include *Lactobacillus acidophilus, L. brevis, L. delbrueckii* subsp. *bulgaricus, L. casei* subsp. *casei, L. plantarum, Lactococcus lactis* subsp. *cremoris, L. lactis* subsp. *lactis, Streptococcus salivarius* subsp. *thermophilus and Leuconostoc mesenteroides* subsp. *mesenteroides* (Adachi 1992, Hosono *et al.* 1990, Nishioka *et al.* 1989). *L. plantarum* is also known to exhibit antitumor activity against mouse fibrosarcoma and ascite tumors (Sandine *et al.* 1972). However, its antimutagenic activity has not been studied thus far.

In a previous paper, the current authors isolated a lactic acid bacterium, *Lactobacillus plantarum* KLAB21, participating in the fermentation of Korean

kimchi (Rhee & Park 1999). Three different glycoproteins with antimutagenic activity against MNNG (*N*-methyl-*N*′-nitro-*N*-nitrosoguanidine) were purified and characterized (Rhee & Park 2001). In this study, the antimutagenic activity of *L. plantarum* KLAB21 against various mutagens was assayed and its activity was compared with those of other lactic acid bacteria originating from fermented milk products.

Materials and methods

Strains and bacterial culture

Lactobacillus plantarum KLAB21 used for the antimutagenic activity test has been previously isolated from Korean fermented vegetables, *kimchi*, and characterized (Rhee & Park 1999). The bacteria were cultured at 37 °C for 36 h with shaking at 150 rpm in MRS broth composed of 1% Bacto-peptone, 1% meat extract, 0.5% yeast extract, 2% glucose, 0.1% Tween 80, 0.5% sodium acetate, 0.2% tri-ammonium citrate, 0.2% K_2HPO_4, 0.07% $MgSO_4 \cdot 7H_2O$ and 0.05% $MnSO_4 \cdot 4H_2O$. *Salmonella typhimurium* strains TA100 (*his*G46, *rfa*, Δ*uvr*B) and TA98 (*his*D3052, *rfa*, Δ*uvr*B) were used for the test strains for the antimutagenic activity assay using the pre-incubation method (Maron & Ames 1983). For the antimutagenic test using a spore-rec assay, the *Bacillus subtilis* wild type strain H17 (*agr*A15, *trp*B3) and recombination-deficient mutant strain M45 (*agr*A15, *trp*B3, *rec*45) were used (Kada *et al.* 1985). *S. typhimurium* and *B. subtilis* cells were grown in the nutrient media composed of 0.8% nutrient and 0.5% NaCl in distilled water.

Mutagens for antimutagenic test

Mutagens used for the antimutagenic tests were MNNG, NQO (4-nitroquinoline-1-oxide), NPD (4-nitro-*O*-phenylenediamine) and aflatoxin B1. All the mutagens were purchased from Sigma (St. Louis, USA). NQO, NPD and aflatoxin B1 were all dissolved in dimethylsulfoxide, whereas MNNG was dissolved in distilled water. The final concentrations of MNNG, NPD and aflatoxin B1 used on *S. typhimurium* TA100 were 5, 15 and 1.0 μg per plate, respectively. The final concentrations of NQO, NPD and aflatoxin B1 used on *S. typhimurium* TA98 were 0.25, 2.5 and 1 μg per plate, respectively. For the antimutagenic test using a *B. subtilis* spore-rec assay, 10 μg of MNNG or 20 ng of NQO was used.

Preparation of bacterial cell fractions

After *Lactobacillus plantarum* KLAB21 cells were grown in the MRS media, the supernatant was obtained by centrifuging the culture broth at 25 000 × *g* for 10 min, which was then stored at 4 °C until being used as the culture supernatant fraction. The bacterial cells were washed with distilled water twice and then lyophilized to yield the dry cell fraction. To prepare the cell-free extract, cells were resuspended in distilled water (20 mg ml^{-1}) and sonicated at 60 μA at 4 °C for 3 h. The sonicated suspension was then centrifuged at 25 000 × *g* for 30 min to obtain the supernatant, which was used for the cell-free extract fraction.

Antimutagenic, mutagenic and toxic tests

The antimutagenic activity test was carried out using the *S. typhimurium* strains TA100 and TA98 as previously described (Maron & Ames 1983). For the antimutagenic activity test, 100 μl of each sample being tested, 50 μl of each mutagen solution, 100 μl of an overnight culture of *S. typhimurium* and 0.5 ml of a 0.2 M sodium phosphate buffer (pH 7) for MNNG, NQO and NPD or an S9 mix (Sigma) for aflatoxin B1 were mixed in glass, capped tubes. The mixture was then incubated at 37 °C for 30 min with agitation at 100 rpm in a shaking incubator. Following incubation, 3 ml of a molten top agar solution containing histidine and biotin was added, and the resulting mixtures were plated on a minimal glucose agar medium. After the plates were incubated at 37 °C for 2 days in the dark, the number of His^+ revertants per plate was counted. The antimutagenic activity was expressed as the percentage inhibition of His^+ reversion of the test strains; antimutagenic ratio (%) $= 100 \times [(A - B)/(A - C)]$, where A = number of His^+ revertants induced by a mutagen in the absence of a sample, B = number of His^+ revertants induced by a mutagen in the presence of a sample and C = number of spontaneous His^+ revertants in the absence of a mutagen. For the mutagenic test of the lactic acid bacteria, 50 μl of distilled water was used instead of the mutagen solution and all the other procedures were essentially the same as those described above. For the toxic tests, *S. typhimurium* cells mixed with an appropriate amount of sample were plated and grown on nutrient agar plates as reported previously (Maron & Ames

1983). The effect of the sample on the growth of *S. typhimurium* cells was also investigated. The samples for the antimutagenic tests were used with the amounts which have neither mutagenic nor toxic effects on the *S. typhimurium* cells.

B. subtilis *spore-rec assay*

Spore-rec assay was carried out using the spores of *B. subtilis* H17 and the recombination-deficient M45 according to the method developed by Kada *et al.* (1985). Spores of *B. subtilis* H17 and M45 were spread on separate nutrient agar plates. Next, a filter paper disc (⌀ 8 mm), applied with an appropriate amount of the sample solution and 10 μl of MNNG (1 mg ml^{-1}) or NQO (2 μg ml^{-1}) solution, was placed on each agar plate. The plates were then incubated at 4 °C for 8 h and further incubated at 37 °C for 16 h. Thereafter, the diameter of the growth inhibition zones was measured. Antimutagenic activity of the culture supernatant was estimated based on the difference in the inhibition zone diameters between the two strains.

Desmutagenic test

Desmutagenic activity test was performed using the same procedure as the Ames test except for the following steps (Hosono *et al.* 1986). First, a mixture of 50 μl of each mutagen solution with 100 μl of an overnight culture of *S. typhimurium* in a glass cap tube was incubated at 37 °C for an appropriate period of time. Next, 100 μl of each sample being tested and 0.5 ml of a 0.2 M sodium phosphate buffer (pH 7) were added to the mixture. The mixture, then, was incubated at 37 °C for 30 min with agitation at 100 rpm in a shaking incubator. The other steps followed the same procedure as those used for the antimutagenic activity assay by the Ames test. The calculation of the desmutagenic activity was the same as that used in the Ames test. The activity was expressed as a desmutagenic ratio (%). All the data in the tables and figures represent the average of at least three trials that were performed in triplicate.

Results and discussion

Antimutagenic activity of L. plantarum *KLAB21*

When *L. plantarum* KLAB21 culture supernatant was tested for the antimutagenic activity against MNNG on *S. typhimurium* strain TA100 and against NQO on strain TA98, the activity increased against both the mutagens relative to the amount of culture supernatant used up to 100 μl per plate (Figure 1). The maximum antimutagenic ratio was obtained when 100 μl of the culture supernatant was added to the plate. When more than 100 μl of the culture supernatant was used, no significant increase in the activity was observed. When the bacterial culture supernatant was tested for its mutagenicity and toxicity on *S. typhimurium* strains TA100 and TA98, no increase in the viable counts relative to the amount of the culture supernatant used was observed in both tests.

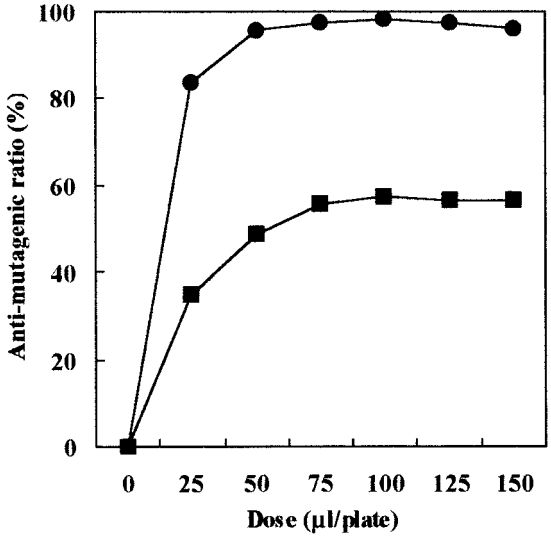

Fig. 1. Antimutagenic activities against MNNG and NQO relative to the amounts of *L. plantarum* KLAB21 culture supernatant. After the bacteria were grown at 37 °C for 36 h in MRS media, the antimutagenic activity of the culture supernatant was assayed against MNNG (●) on *S. typhimurium* TA100 and NQO (■) on *S. typhimurium* TA98. All the data in this figure and following figures and tables represent the average of at least three trials that were performed in triplicate and they were significantly different from the control at $P < 0.05$ levels.

These results suggest that the culture supernatant does not possess either mutagenicity or toxicity on *S. typhimurium* (Table 1). Many lactic acid bacteria such as *Lactobacillus*, *Lactococcus* and *Pediococcus* spp. secrete bacteriocins, which posess antibacterial activities against various bacterial strains, into the culture broth. Specifically, various types of bacteriocin including lactacin, sakacin, helveticin and curvacin are produced by a variety of *Lactobacillus* sp. including *L. acidophilus, L. sake, L. helveticus* and *L. curvatus* (Dodd & Gasson 1994). However, the culture supernatant of the strain KLAB21 up to 200 μl showed no

Table 1. Mutagenic and toxic tests of *L. plantarum* KLAB21 culture supernatant on *S. typhimurium* strains TA100 and TA98.

Dose (μl)	Mutagenic test (His^+ counts/plate)		Toxic test (c.f.u./plate)	
	TA100	TA98	TA100	TA98
0	78	37	1135	1192
25	76	37	1156	1201
50	85	36	1151	1194
75	82	38	1163	1196
100	79	35	1157	1203
125	89	39	1149	1198
150	92	36	1143	1205
175	87	33	1152	1209
200	81	38	1145	1201

toxic effect on *S. typhimurium* under the conditions of this study.

Antimutagenic activities of L. plantarum *KLAB21 cell fractions*

The bacterial cell fractions including the culture supernatant, dry cells and cell-free extract were prepared after *L. plantarum* KLAB21 were grown in MRS media for 36 h. And, 100 μl of culture supernatant, 1 mg of dry cells and 100 μl of cell-free extracts were tested for their antimutagenic activity against MNNG and NQO using *S. typhimurium* strains TA100 and TA98 (Table 2). Among them, the culture supernatant showed the highest antimutagenic activity. Its antimutagenic ratio was 98.4% against MNNG on *S. typhimurium* TA100 and 57.3% against NQO on *S. typhimurium* TA98 when 100 μl of the culture supernatant was used for the test. The other fractions, including dry cells and cell-free extracts, also exhibited a significant antimutagenic activity although lower than that of the culture supernatant. Their yield was very low compared with that of the culture supernatant, i.e., the yields of the dry cell and cell-free extracts were 1 and 50% of that of the culture supernatant, respectively (data not shown). Therefore, the total activity of the other fractions was considered as insignificant. Accordingly, this result suggests that the major antimutagenic substances in *L. plantarum* KLAB21 are of an extracellular type that is secreted into the culture medium. All the *L. plantarum* KLAB21 fractions were verified as not possessing any mutagenic or toxic effects on *S. typhimurium* strains TA98 and TA100 under the conditions of this study (data not shown).

The antimutagenic activity test of *L. plantarum* KLAB21 against MNNG and NQO was repeated by the spore-rec assay using the *B. subtilis* strains M45 and H17 (Table 3). Without the culture supernatant, MNNG and NQO exhibited 39 and 40 mm differences in the diameter of the inhibition zone between the two strains, respectively. However, when 100 μl of the culture supernatant was added, the differences between the two strains dramatically decreased, which were 4 mm in the case of MNNG and 2 mm in the case of NQO. These results also suggest that the culture supernatant of *L. plantarum* KLAB21 possesses a high antimutagenic activity. In contrast, the dry cell and cell-free extract fractions of *L. plantarum* KLAB21 produced no significant antimutagenic activity in the spore-rec assay.

Desmutagenic activity

Desmutagenic activity of the culture supernatant of *L. plantarum* KLAB21 against MNNG and NQO was assayed for 50 min at 10-min intervals (Table 4). This culture supernatant also possessed a significant desmutagenic activity against both mutagens although its desmutagenic activity dramatically decreased with an increase in the incubation time of the mixture of each mutagen with the *S. typhimurium* cells. After 10 min of incubation, the desmutagenic activities of the culture supernatant were 90.2% against MNNG on *S. typhimurium* TA100 and 55.6% against NQO on *S. typhimurium* TA98. However, the activities decreased to 72.5% against MNNG on the strain TA100 and 22.7% against NQO on the strain TA98 after 50 min of incubation.

Table 2. Antimutagenic activities of *L. plantarum* KLAB21 cell fractions against MNNG on *S. typhimurium* TA100 and NQO on *S. typhimurium* TA98.

Fraction	Dose/plate	MNNG		NQO	
		His^+ counts	AR (%)	His^+ counts	AR (%)
Culture supernatant	100 μl	156	98.4	97	57.3
Dry cell	1 mg	186	95.8	109	50.6
Cell-free extracts	100 μl	247	90.6	199	38.2
+ Control	–	1303	–	199	–
− Control	–	137	–	21	–

The AR (antimutagenic ratio, %) represents the inhibition ratio (%) of His^+ reversion of *S. typhimurium* strains TA100 or TA98 in the presence of antimutagenic substances. The + and − controls indicate the number of His^+ counts in the presence and absence of a mutagen, respectively.

Table 3. Antimutagenic activities of *L. plantarum* KLAB21 cell fractions against MNNG and NQO in the *B. subtilis* spore-rec assay.

Fraction	Dose/disc	Inhibition zone (mm)					
		MNNG			NQO		
		M45	H17	Difference	M45	H17	Difference
Culture supernatant (μl)	0	39	0	39	40	0	40
	50	32	24	8	31	27	4
	100	35	31	4	35	33	2
	150	38	34	4	39	36	3
Dry cell (mg)	0	39	0	39	40	0	40
	0.5	24	0	24	33	0	33
	1.0	25	0	25	10	0	10
	1.5	25	0	25	10	0	10
Cell-free extract (μl)	0	39	0	39	40	0	40
	50	26	0	26	32	14	18
	100	26	10	16	24	14	10
	150	30	10	20	23	11	12

The antimutagenic activity was estimated based on the difference in the inhibition zone diameters between *B. subtilis* strains M45 and H17 in the presence of antimutagenic substances by the method of Kada *et al.* (1985). Smaller number in the difference represents stronger antimutagenic activity.

Comparison of antimutagenic activity of L. plantarum *KLAB21 with other lactic acid bacteria*

Antimutagenic activities of other lactic acid bacteria originating from fermented milk, such as *Lactobacillus plantarum* IAM261, *L. delbrueckii* subsp. *bulgaricus* IFO3533, *L. acidophilus* IFO3205, *L. brevis* IFO3029, *Lactococcus lactis* subsp. *lactis* IFO1254 and *Streptococcus salivarious* subsp. *thermophilus* IAM1390 were tested against MNNG on *S. typhimurium* TA100 and NQO *S. typhimurium* TA98. Their activities were then compared with that of *L. plantarum* KLAB21 (Figure 2). The antimutagenic ratios of the other lactic acid bacteria ranged between 26.8 to 53% against MNNG on strain TA100 and 28.5 to 43.4% against NQO on strain TA98. As a result, their activities were found to be a lot lower than those of *L. plantarum* KLAB21 isolated from Korean *kimchi*. A similar experiment to that in Figure 2A was performed using NPD (Figure 2B) and aflatoxin B1 (Figure 2C) as the mutagens on the *S. typhimurium* strains TA100 and TA98. *L. plantarum* KLAB21 also exhibited high antimutagenic activities against these mutagens as well with antimutagenic ratios of 72.6% on TA100 and 62.8% on TA98 against NPD as well as 82.5% on TA100 and 78.2% on TA98 against aflatoxin B1. However, the antimutagenic ratio levels of the other lactic acid bacteria originating from fermented

Table 4. Desmutagenic activity of *L. plantarum* KLAB21 culture supernatant against MNNG on *S. typhimurium* TA100 and NQO on *S. typhimurium* TA98.

Time (min)	MNNG		NQO	
	His^+ counts	Desmutagenic ratio (%)	His^+ counts	Desmutagenic ratio (%)
0	93	97.8	113	59.4
10	179	90.2	121	55.6
20	210	87.5	138	47.3
30	283	81.1	155	39.1
40	351	75.1	172	30.9
50	380	72.5	189	22.7
+ Control	1204	–	236	
− Control	68	–	29	

Desmutagenic activity test was performed using *S. typhimurium* strains TA100 and TA98 by the method of Hosono *et al.* (1986). A mixture of each mutagen and an overnight culture of *S. typhimurium* incubated at 37 °C for an appropriate period of time is shown in the table. Then, each sample being tested was added to the mixture. The other steps followed the same procedure as those used for the antimutagenic activity assay by the Ames test (Maron & Ames 1975). The desmutagenic ratio (%) represents the inhibition ratio (%) of His^+ reversion of *S. typhimurium* strains TA100 or TA98 in the presence of antimutagenic substances. The + and − controls indicate the number of His^+ counts in the presence and absence of a mutagen, respectively.

milk were found to be much lower, ranging between 24.4 to 44.9% on TA100 and 21.9 to 46.7% on TA98 against NPD and 5.1 to 33.1% on TA100 and 4.7 to 28.7% on TA98 against aflatoxin B1.

Accordingly, *Lactobacillus plantarum* KLAB21, isolated from the Korean traditional fermented vegetable, *kimchi*, possesses a strong antimutagenic activity against various mutagens such as MNNG, NQO, NPD and aflatoxin B1 (Figures 1 and 2, Tables 2 and 3). The majority of the activity in *L. plantarum* KLAB21 was exhibited in the culture supernatant fraction, thereby suggesting that its antimutagenic substance is of an extracellular type (Table 2). Plus, this antimutagenic activity was confirmed using the *B. subtilis* spore-rec assay (Table 3).

Concerning the antimutagenic activity of lactic acid bacteria, Hosono *et al.* (1986) 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. The lactic acid bacteria that display antimutagenic activity on *S. typhimurium* now include *L. delbrueckii* subsp. *bulgaricus, L. helveticus, L. lactis* subsp. *lactis* and *Streptococcus salivarious* subsp. *thermophilus* (Nishioka *et al.* 1989). Although their activities vary depending on the type of mutagen and the culture medium in which they are grown, they all exhibited strong antimutagenic activities against NQO and AF-2 [2-(2-furyl)-3-(5-nitro-2-furyl)-acrylamide] (Nishioka *et al.* 1989).

In Korea, *kimchi*, a traditional vegetable pickle fermented by lactic acid bacteria, has been widely consumed for thousands of years. Korean *kimchi* extract displays antimutagenic activity with *S. typhimurium* (Park *et al.* 1995). The antimutagenic activity of raw vegetables and their particular components have also been previously reported (Carmeron *et al.* 1979). Recently, the minor ingredients for *kimchi*, including garlic and pepper, were also found to exhibit antimutagenic activity (Kim *et al.* 1997). However, until now little is known about the antimutagenic activity of the lactic acid bacteria that participates in the fermentation of *kimchi*. This study argues that the lactic acid bacteria *L. plantarum* KLAB21 participating in the fermentation of Korean *kimchi* (Rhee & Park 1999) possesses a strong antimutagenic activity against MNNG, NQO, NPD and aflatoxin B1. Its activity was higher than those of various lactic acid bacteria originating from fermented milk; *L. plantarum* IAM261, *L delbrueckii* subsp. *bulgaricus* IFO3533, *L. acidophilus* IFO3205, *L. brevis* IFO3029, *L. lactis* subsp. *lactis* IFO1254 and *S. salivarious* subsp.*thermophilus* IAM1390 (Figure 2).

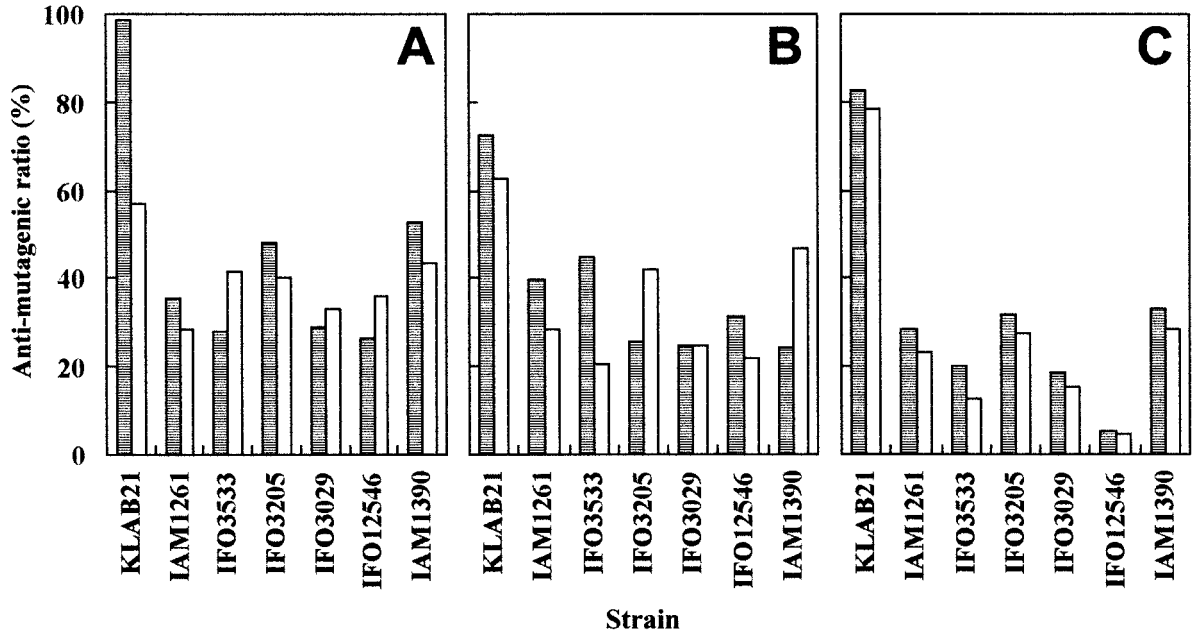

Fig. 2. Comparison in the antimutagenic activity of *L. plantarum* KLAB21 with a variety of lactic acid bacteria originating from fermented milk. The antimutagenic activity of *L. plantarum* KLAB21 culture supernatant was assayed against MNNG on *S. typhimurium* TA100 (▤) and NQO on *S. typhimurium* TA98 (□) (A). Its antimutagenic activity against NPD (B) and aflatoxin B1 (C) on both TA100 (▤) and TA98 (□) strains was assayed as well. And, they were compared with those of *L. plantarum* IAM261, *L delbrueckii* subsp. *bulgaricus* IFO3533, *L. acidophilus* IFO3205, *L. brevis* IFO3029, *L. lactis* subsp. *lactis* IFO1254 and *S. salivarious* subsp. *thermophilus* IAM1390.

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