Inhibition of Lipid Peroxidation by *Lactobacillus acidophilus* and *Bifidobacterium longum*

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The inhibition of lipid peroxidation by *Lactobacillus acidophilus* and *Bifidobacterium longum* was investigated using two lipid model systems. All eight strains, including six strains of *L. acidophilus* and two strains of *B. longum*, demonstrated an inhibitory effect on linoleic acid peroxidation. The inhibitory rates on linoleic acid peroxidation ranged from 33 to 46% when 1 mL of intracellular cell-free extract was tested. In the second model system, the cell membrane of osteoblast was used as the source for biological lipid. The results indicated that all strains were able to protect biological lipids from oxidation. The inhibition rates on cell membrane lipid peroxidation ranged from 22 to 37%. The effect of *L. acidophilus* and *B. longum* on inhibition of fluorescent tissue pigment accumulation was also obtained for osteoblastic cells. The inhibition rates on fluorescent tissue pigment accumulation ranged from 20 to 39%. The antioxidative effect of each milliliter of intracellular cell-free extract of *L. acidophilus* and *B. longum* was equivalent to 104−172 ppm of butylated hydroxytoluene (BHT). These results indicated that all strains demonstrated high antioxidative activity. The scavenging ability of lipid peroxidation products, *tert*-butyl hydroperoxide and malondialdehyde, was also evaluated. The results showed that *L. acidophilus* and *B. longum* were not able to scavenge the *tert*-butyl hydroperoxide. Nevertheless, malondialdehyde was scavenged well by these strains.

Keywords: *Lactobacillus acidophilus; Bifidobacterium longum; lipid peroxidation; antioxidation*

INTRODUCTION

Fermented dairy foods containing lactic acid bacteria have been consumed for centuries in the Western countries. These products are also becoming popular with consumers in other part of the world due to their benefits for human health. The potential health attributes of lactic acid bacteria include improvement of lactose intolerance, control of gastrointestinal infections, reduction of serum cholesterol, stimulation of immunological system, and anticarcinogenic actions (Fernandes and Shahani, 1989; Gilliland, 1990; Lin, 1995; Hitchins and McDonough, 1989; Sanders, 1993). Interest in the role of lactic acid bacteria in promoting human health goes back at least as far as 1908 when Metchnikoff suggested that consumption of milk fermented with lactobacilli would prolong life (Metchnikoff, 1908).

Health and life prolongation have been the focus of much research, although sickness and aging are natural phenomena of life. It has been discovered that oxidation and aging are closely related (Menken et al., 1986). Oxidative stress can damage biological molecules (Halliwell, 1994). Oxidative damage plays an important pathological role in human diseases. Cancer, emphysema, cirrhosis, atherosclerosis, and arthritis have all been correlated with oxidative damage (Halliwell and Gutteridge, 1984).

The antioxidative effect of lactic acid bacteria has been reported only recently (Ahotupa et al., 1996; Kaizu et al., 1993; Korpela et al., 1997; Sanders et al., 1995). There is not much data about the antioxidative ability of lactic acid bacteria available. *Lactobacillus acidophilus* and *Bifidobacterium* spp. have attracted a lot of attention among lactic acid bacteria for their potential role in promoting human health. Therefore, the objective of this study was to investigate the antioxidative effect of *L. acidophilus* and *B. longum* on the inhibition of lipid peroxidation using two lipid model systems. The ability of *L. acidophilus* and *B. longum* to scavenge the products of lipid peroxidation was also studied.

MATERIALS AND METHODS

Bacterial Strains. *Lactobacillus acidophilus* B, E, Farr, LA-1, N1, and 4356 and *Bifidobacterium longum* B6 and 15708 were obtained from our frozen stock culture collection. These strains were grown in MRS (Difco Laboratories, Detroit, MI) at 37 °C. All strains were serially transferred at least three times prior to use in these studies.

Preparation of Intracellular Cell-Free Extract. Cells of *L. acidophilus* and *B. longum* were harvested by centrifugation at 6000 rpm for 10 min after 18 h incubaction at 37 °C. The cell pellets were then quickly washed twice with deionized water. Cells were resuspended in deionized water followed by ultrasonic disruption (setting = 4; sonicator XL-2020; Heat System, Farmingdale, NY). Sonication was performed for three 1-min intervals in an ice bath. Cell debris was removed by centrifugation at 8000 rpm for 10 min, and the resulting supernatant was the intracellular cell-free extract.

Measurement of Linoleic Acid Peroxidation. Linoleic acid was chosen as the source for unsaturated fatty acid (Bertelsen et al., 1995). The analysis of the thiobarbituric acid (TBA) method was used for the measurement of lipid peroxidation and an Fe/ascorbate system was used for the catalysis of oxidation (Decker and Faraji, 1990). Twenty milliliters of linoleic acid emulsion was made up of 1 mL of linoleic acid, 0.2 mL of Tween 20, and 19.7 mL of deionized water. A sample of 0.5 mL of phosphate buffer solution (0.02 M, pH = 7.4), 1 mL of linoleic acid emulsion, 0.2 mL of $FeSO_4$ (0.01%), 0.2 mL

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of ascorbate (0.01%), and 0.4 or 0.8 mL of intracellular cell-free extract was mixed and incubated at 37 °C. After 12 h of incubation, 2 mL of the reaction solution was mixed with 0.2 mL of trichloroacetic acid (TCA; 4%), 2 mL of TBA (0.8%), and 0.2 mL of butylated hydroxytoluene (BHT; 0.4%). This mixture was incubated at 100 °C for 30 min and allowed to cool. Two milliliters of chloroform was then added for extraction. The extract was obtained, and the absorbance was measured at 532 nm. The percentage of inhibition of linoleic acid peroxidation was defined as follows: $[1 - A_{532}(\text{sample})/A_{532}(\text{blank})] \times 100\%$. The capability of intracellular cell-free extract inhibiting linoleic acid peroxidation is also expressed as the equivalent BHT with the same inhibitory effect.

Measurement of Cell Membrane Lipid Peroxidation and Fluorescent Pigment Accumulation. Osteoblastic cell cultures were used in this study and carried out as described by Umayahara et al. (1997). Cells were washed twice with Krebs buffer after cultivation. Oxidation was catalyzed by adding ferrous ions, and cells were incubated at 37 °C for 30 min. Cells were then washed again with Krebs buffer, and 1 mL of trypsin solution and 1.2 mL of TCA were added. The reaction solution was tested for fluorescent pigment accumulation and lipid peroxidation. For measurement of fluorescent tissue pigment accumulation, 1 mL of reaction solution was extracted with 2 mL of chloroform. The extract was analyzed with a fluorescent spectrophotometer (model 650-40; Hitachi, Tokyo, Japan) at Ex = 370 and Em = 430 (Ciuffi et al., 1991). For analysis of lipid peroxidation, the rest of the reaction mixture was treated with the same TBA method as mentioned previously for linoleic acid except that a fluorescent spectrophotometer was used for measurement (Ex = 515 and Em =550) (Aubourg, 1993). The inhibition rates of fluorescent tissue pigment accumulation and lipid peroxidation were calculated as follows: [1 − fluorescent value(sample)/fluorescent value(blank)] × 100%.

Scavenge of *tert*-Butyl Hydroperoxide and Malondialdehyde. *tert*-Butyl hydroperoxide was used as the source for the primary product of lipid peroxidation in this study. The method developed by Wolfe (1962) was used for analysis. *tert*-Butyl hydroperoxide solution and $TiCl_4$ stock solution were prepared at the concentrations of 4 mM and 10 mg/mL, respectively. A sample of 0.5 mL of *tert*-butyl hydroperoxide and 0.5 mL of intracellular cell-free extract was mixed and allowed to stand at 25 °C in the dark for 1 h. Two milliliters of $TiCl_4$ solution was then added, and the mixture was allowed to react at room temperature for 2 h. The upper phase was obtained, and the absorbance was read at 412 nm.

Malondialdehyde was chosen as the representative for the secondary product of lipid peroxidation. Malondialdehyde was prepared from the hydrolysis of 1,1,3,3-tetraethyoxypropane (Jain and Hochsten, 1980). The malondialdehyde solution obtained from this preparation was then diluted to 0.0545 mM for use. One milliliter of malondialdehyde solution and 0.5 mL of intracellular cell-free extract were mixed. The mixture was allowed to react at 100 °C for 2 h. The procedure for malondialdehyde quantitative analysis was the same TBA method as mentioned previously for linoleic acid peroxidation.

RESULTS

Inhibition of Linoleic Acid Peroxidation by *L. acidophilus* and *B. longum*. As shown in Table 1, the *L. acidophilus* and *B. longum* strains tested all demonstrated an inhibitory effect on linoleic acid peroxidation. The inhibitory rates on linoleic acid peroxidation ranged from 33 to 46% when 1 mL of intracellular cell-free extract was added.

The capability of intracellular cell-free extract of *L. acidophilus* and *B. longum* to inhibit linoleic acid peroxidation is also expressed as the equivalent BHT, which was used as the standard for comparison of antioxidative activity in this study. The antioxidative effect of each milliliter of intracellular cell-free extract was equivalent to 104−172 ppm of BHT. This indicated that all strains tested demonstrated a high antioxidative activity for inhibiting lipid peroxidation.

Table 1. Inhibition of Linoleic Acid Peroxidation by Intracellular Cell-Free Extract of *L. acidophilus* and *B. longum*

strain	inhibition rate (%)[a,b]	equiv BHT (ppm)[b,c]
Lactobacillus acidophilus		
B	34.9	103.9
E	35.6	132.5
Farr	43.1	168.0
LA-1	39.4	133.8
N1	37.5	149.1
4356	46.3	168.4
Bifidobacterium longum		
B6	43.0	171.8
15708	33.1	133.9

[a] Inhibition rate of 1 mL of intracellular cell-free extract. [b] Data reported were the average of experiments repeated three times. [c] The capability of 1 mL of intracellular cell-free extract on linoleic acid peroxidation is expressed as the equivalent butylated hydroxytoluene (BHT) concentration with the same inhibitory effect.

Table 2. Inhibition of Osteoblastic Cell Membrane Lipid Peroxidation by Intracellular Cell-Free Extract of *L. acidophilus* and *B. longum*

strain	inhibition rate (%)[a,b]
Lactobacillus acidophilus	
B	24.6
E	22.1
Farr	36.8
LA-1	27.2
N1	23.3
4356	25.0
Bifidobacterium longum	
B6	27.4
15708	25.9

[a] Inhibition rate of 1 mL of intracellular cell-free extract. [b] Data reported were the average of experiments repeated three times.

Inhibition of Cell Membrane Lipid Peroxidation and Fluorescent Pigment Accumulation by *L. acidophilus* and *B. longum*. The cell membrane of osteoblast was used as the source for biological lipid in this study. The result indicated that all strains were able to protect biological lipids from oxidation. The inhibition rates on cell membrane lipid peroxidation ranged from 22 to 37% (Table 2).

The effect of *L. acidophilus* and *B. longum* on inhibition of fluorescent tissue pigment accumulation, which is used as an index of oxidative damage of an organism, was also obtained for osteoblastic cells. The results of fluorescent tissue pigment accumulation for iron-catalyzed peroxidation are shown in Table 3. Among these eight strains, *B. longum* B6 demonstrated the highest inhibition rate at 39%, and *L. acidophilus* B had the lowest rate at 20%.

Scavenge of *tert*-Butyl Hydroperoxide and Malondialdehyde. *tert*-Butyl hydroperoxide was used as the representative for hydroperoxides in this study. The results of scavenge of *tert*-butyl hydroperoxide by *L. acidophilus* and *B. longum* are shown in Figure 1. The absorbance values at 412 nm for *tert*-butyl hydroperoxide treated with intracellular cell-free extract of *L. acidophilus* and *B. longum* were not different from that of the control. This indicated that strains of *L. acidophilus* and *B. longum* tested were not capable of scavenging this primary product of lipid peroxidation.

The scavenging ability of *L. acidophilus* and *B. longum* for malondialdehyde is presented in Figure 2.

Table 3. Inhibitory Effect of Intracellular Cell-Free Extract of *L. acidophilus* and *B. longum* on the Accumulation of Fluorescent Tissue Pigment of Osteoblasts

strain	inhibition rate (%)[a,b]
Lactobacillus acidophilus	
B	20.4
E	28.0
Farr	34.3
LA-1	33.5
N1	30.2
4356	29.9
Bifidobacterium longum	
B6	38.8
15708	29.6

[a] Inhibition rate of 1 mL of intracellular cell-free extract. [b] Data reported were the average of experiments repeated three times.

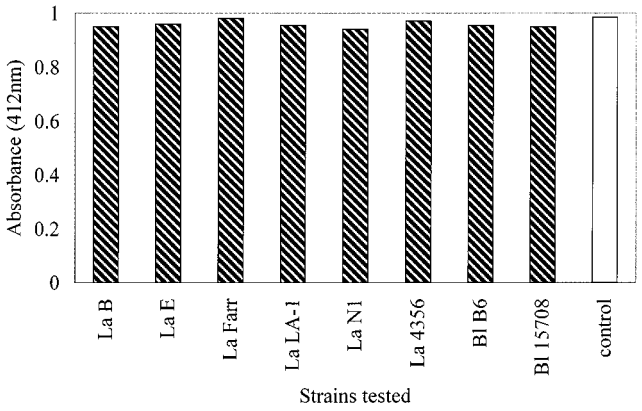

Figure 1. Scavenging of *tert*-butyl hydroperoxide by *L. acidophilus* and *B. longum*.

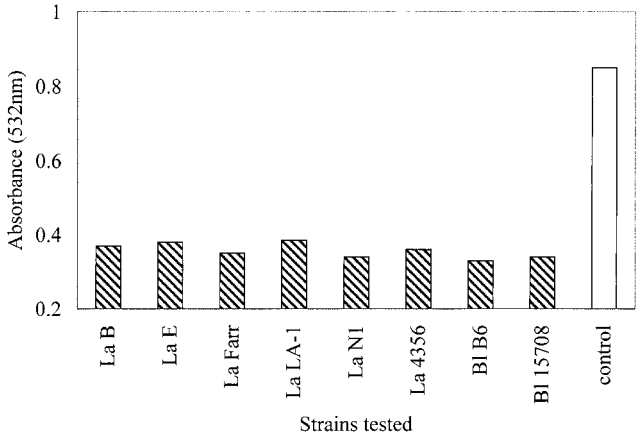

Figure 2. Scavenging of malondialdehyde by *L. acidophilus* and *B. longum*.

All eight strains showed the ability to scavenge malondialdehyde. These strains had similar malondialdehyde scavenging abilities in the range of 2.29−2.62 μM/mL of intracellular cell-free extract (Table 4).

DISCUSSION

Oxidative metabolism is essential to many living organisms for the production of energy to fuel biological processes. Nevertheless, oxidative damage plays a significant pathological role in human diseases. It is well-established that oxygen-centered free radicals and other reactive oxygen species are continuously produced in vivo (Halliwell and Chirico, 1993). A wide variety of reactive oxygen species can be formed in the human body and in food systems. Although humans and other organisms possess antioxidant defense and repair systems that have evolved to protect them against oxidative damage, these systems are not effective enough to totally eliminate the damage (Simic, 1988). Therefore, the use of antioxidant supplements or foods containing antioxidants may help the human body reduce oxidative damage.

Table 4. Scavenging of Malondialdehyde by Intracellular Cell-Free Extract of *L. acidophilus* and *B. longum*

strain	malondialdehyde (μM) scavenged[a,b]
Lactobacillus acidophilus	
B	2.36
E	2.32
Farr	2.43
LA-1	2.29
N1	2.62
4356	2.40
Bifidobacterium longum	
B6	2.51
15708	2.47

[a] Amount of malondialdehyde scavenged by 1 mL of intracellular cell-free extract. [b] Data reported were the average of experiments repeated three times.

In the search for antioxidants, inhibition of lipid peroxidation is commonly utilized for analysis. Unsaturated fatty acids such as linoleic acid (Wanasundrara et al., 1994), methyl linoleate (Bertelsen et al., 1995), and arachidonic acid (Husain et al., 1987) are typically used. The antioxidative activity of *L. acidophilus* and *B. longum* was measured based on the inhibition of linoleic acid peroxidation in this study. A second model system for the inhibition of lipid peroxidation using osteoblastic cells was also investigated. Cell membrane lipids, which are mainly composed of phospholipids, are very similar even when cells are from different tissues. Therefore, the protection of *L. acidophilus* and *B. longum* against oxidative damage to biological lipids using osteoblastic cells as the model was identified. Tables 1 and 2 demonstrate that the intracellular cell-free extract of all eight strains of *L. acidophilus* and *B. longum* had an inhibitory effect on lipid peroxidation for both model systems.

In the fluorescent tissue pigment study, pigment accumulation is a function of the aging process and of in vivo oxidative damage (Menken et al., 1986). Table 3 indicates that all eight strains tested possess antioxidative ability for the inhibition of fluorescent tissue pigment accumulation. This result further confirms the antioxidative effect of *L. acidophilus* and *B. longum*.

Lipid peroxidation may result in toxic compounds. Hydroperoxides are the primary initial products of lipid autoxidation, which is the major reaction involved in the oxidative deterioration of lipids. Hydroperoxides are potentially toxic and capable of damaging DNA (Baker and He, 1991; Shertzer et al., 1992; Vessey et al., 1992). Malondialdehyde is a secondary product of lipid peroxidation. It is a highly reactive substance that can cause deterioration of biological molecules such as proteins and DNA (Aubourg, 1993; Gardner, 1975). The effect of malondialdehyde on cultured mammalian cells has also been studied. Cells exposed to malondialdehyde exhibited altered morphology, cytoplasmic vacuolization, and a marked reduction of protein-synthesizing capability (Bird and Draper, 1980). Malondialdehyde at low concentration (μM) was scavenged by all eight strains of *L. acidophilus* and *B. longum* although *tert*-butyl hydroperoxide, which was used as the source of hydro-

peroxide in this study, was not scavenged by these strains. The capability for scavenging malondialdehyde by *L. acidophilus* and *B. longum* may provide an effective protection against this toxic product of lipid peroxidation.

The antioxidative activity of *L. acidophilus* and *B. longum* was also expressed as the equivalent BHT. Data given in Table 1 show that all strains tested had an antioxidative capability equivalent to 104−172 ppm of BHT, which is much higher than the concentration usually used in food. In general, the total concentration of authorized antioxidants must not exceed 0.02 wt % based on the fat content of the food. Butylated hydroxytoluene is one of the most widely used chemicals to retard oxidation. It has found widespread commercial use in the food industry. At present, some controversy surrounds the use of this synthetic antioxidant.

Various antioxidants have been reported. Antioxidants from natural sources are likely to be more desirable since there are doubts about the safety and long-term effects on health of synthetic antioxidants. The general public's concern with chemical additives and food safety has triggered a continuing search for antioxidants that occur naturally in food. Sanders et al. (1995) reported that *Lactococcus* demonstrates antioxidative superoxide dismutase enzyme activity. Also, Korpela et al. (1997) found an antioxidative effect on vascular endothelial cells. Besides the long history of consumption that proves the safety of consuming *L. acidophilus*, *B. longum*, and other lactic acid bacteria, lactic cultures have been reported to have health-promoting characteristics (Fernandes and Shahani, 1989; Gilliland, 1990; Lin, 1995; Hitchins and McDonough, 1989; Sanders, 1993). Consumption of *L. acidophilus*, *B. longum*, and other lactic acid bacteria-containing foods may be recommended as healthful. In addition, these microorganisms are potential candidates for the production of functional foods or of natural antioxidant supplements.

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