# Effect of Antioxidative Lactic Acid Bacteria on Rats Fed a Diet Deficient in Vitamin E

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## ABSTRACT

Lactic acid bacteria, including *Bifidobacterium*, with antioxidative activity were selected by in vitro screening. The effect of the antioxidative activity was investigated by in vivo experiments using rats that were deficient in vitamin E.

In the first stage of screening, 570 strains were examined; intracellular cell-free extracts of 19 strains (16 lactobacilli, 2 streptococci, and 1 lactococci) had antioxidative activity as determined by an assay using rat liver microsomes and thiobarbituric acid. In the second stage of screening, 7 strains of lactobacilli showed over 70% inhibition of oxidation activity. The highest activity was obtained by heterofermentative *Lactobacillus* sp. SBT 2028.

The effect of two strains, *Lactobacillus* sp. SBT 2028 and *Lactobacillus casei* ssp. *rhamnosus* SBT 2257, was evaluated for improvement of the condition of rats fed a diet deficient in vitamin E. Intracellular cell-free extracts of those two strains were also used for in vivo experiments. Hemolysis of red blood cells was inhibited in rats that were administered the extract of *Lactobacillus* sp. SBT 2028, which proved that the extract improved the vitamin E deficiency status. Antioxidative activity of an extract from *L. casei* ssp. *rhamnosus* SBT 2257 determined by hemolysis was relatively weak compared with the activity of *Lactobacillus* sp. SBT 2028 extract.

(**Key words**: lactic acid bacteria, antioxidative effect, vitamin E-deficient diet)

**Abbreviation key**: **A532** = absorbance at wavelength of 532 nm, **A540** = absorbance at wavelength of 540 nm, **IE** = intracellular cell-free extract, **MS** = microsome, **RBC** = red blood cells, **TBA** = thiobarbituric acid.

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## INTRODUCTION

Many published reports (1, 4, 5) concern the relationship between peroxidants and age-related human disorders. Peroxidants accumulated in the human body were reported to cause disorders, such as cancer, atherosclerosis, hypertension, and amyloidosis. Those disorders were named "free radical disease" by Harman (7). The human body has an inherently antioxidative system (i.e., superoxide dismutase, glutathione peroxidase, and uric acid) to protect itself from damage caused by peroxidants (4, 5). However, antioxidative substances lower the risk of oxygen stress on the human body. Ingestion of antioxidants in food or with food is most desirable. Vitamin E is the most common and safest antioxidative substance currently available (18).

Lactic acid bacteria and bifidobacteria have been studied for possible beneficial physiological effects, such as resistance to pathogens, immune-potentiating activity, and antitumor activity (8), based on the well-known theory of Eli Metchnikoff. However, an antioxidative effect of lactic acid bacteria has not been reported.

In this study, lactic acid bacteria, including *Bifidobacterium*, were selected according to antioxidative activity of their intracellular cell-free extract (**IE**), determined by in vitro rat liver microsome (**MS**)-thiobarbituric acid (**TBA**) assay. The IE from two selected strains were evaluated by in vivo experiments using rats in a status of vitamin E deficiency.

## MATERIALS AND METHODS

### Microorganisms and Preparation of IE

*Lactobacillus* (231 strains), *Streptococcus* (65 strains), *Lactococcus* (144 strains), and

*Leuconostocs* (5 strains) were cultured for 16 h at 37°C in MRS broth (Oxoid, Unipath Ltd., Basingstoke, England). *Bifidobacterium* (125 strains) was cultured for 16 h at 37°C in Gifu anaerobic medium broth (Nissui Pharmacy Ltd., Tokyo, Japan). The cells were harvested by centrifugation at 10,000 rpm for 10 min, followed by saline washes; 10 mg of wet cells were disrupted by an ultrasonic disrupter (model UR-200P; Tomy Seiko, Tokyo, Japan). Sonication was performed for 20 min at 0°C. The disrupted cells were centrifuged at 10,000 rpm for 10 min to obtain IE. The IE solutions were used for rat liver MS-TBA assay.

#### Rat Liver MS Preparation

Sprague-Dawley male rats (CLEA Japan, Tokyo, Japan), aged 6 to 10 wk, were used for preparing rat liver MS. The livers were removed, subsequently perfused via the portal vein before homogenization, and then washed with ice-cold 1.15% KCl. The livers were homogenized in 4 ml of 5 m*M* Tris-malate buffer (pH 7.4) containing 1.15% KCl by a Teflon® Potter-Elvehjem homogenizer (Iwaki Glass Co Ltd., Tokyo, Japan). The homogenate was centrifuged at 15,000 × *g* for 15 min. The supernatants then were centrifuged at 100,000 × *g* for 1 h, and the pellet was washed once with 5 m*M* Tris-malate buffer (pH 7.4). The MS were suspended in 1.15% KCl, and the final concentrations of the fractions were approximately 5 mg of protein/ml.

#### Screening Lactic Acid Bacteria with Rat Liver MS for Antioxidant Activity

The rat liver MS-TBA assay was as described by Kornbrust and Mavis (11, 12); 40 μl of MS (5 mg of protein/ml) were mixed with 840 μl of 50 m*M* Tris-malate buffer (pH 7.4), 40 μl of .125 μ*M* $FeSO_4$, and 40 μl of 1.5 m*M* NADPH; 40 μl of IE solution of lactic acid bacteria were incubated at 37°C for 30 min; .2% butylated hydroxytoluene (50 μl), 20% TCA (300 μl), and .05 *M* TBA (600 μl) were added rapidly to the MS mixtures to terminate peroxidation. The mixtures were centrifuged at 3000 rpm for 10 min. The supernatant was boiled for 20 min. The absorbance was measured at 532 nm ($A_{532}$). Percentage of inhibition of oxidation was defined as

$$\left[1 - \frac{A_{532}(\text{sample}) - A_{532}(\text{blank})}{A_{532}(\text{PBS}) - A_{532}(\text{blank})}\right] \times 100(\%).$$

Reaction of the blank sample was performed with 80 μl of water except for 1.25 μ*M* $FeSO_4$ (40 μl) and 1.5 m*M* NADPH (40 μl) as appropriate.

At the first stage of screening, the IE from 10 mg of wet cells were used. At the second stage of screening, IE from 19 strains that had high activity (Table 1) were diluted by .1.

#### Preparation of the Diet Deficient in Vitamin E

The diet deficient in vitamin E consisted of 15.3% corn starch, 20% vitamin-free casein (CLEA Japan), 5% of powdered filter paper, 3.5% mineral mixture AIN-76 (CLEA Japan), 50% granulated sugar, 1% vitamin mixture free of vitamin E (CLEA Japan), .2% choline chloride, and 5% stripped corn oil free of vitamin E. The diet was stored at –20°C until used.

The corn oil was prepared by the method of Mori et al. (16) as follows. Stripped corn oil was mixed with an equal volume of *n*-hexane. Two grams of granular activated charcoal (Cica-reagent, Tokyo, Japan) were added to 100 ml of corn oil and *n*-hexane and stirred at room temperature (22°C) for 1 h. The mixture was filtered to remove the activated carbon and then evaporated in vacuo to remove *n*-hexane.

#### Oral Administration of IE from Lactic Acid Bacteria

The rats were individually housed in the room kept at 22°C and provided with light for 12 h/d. The rats, weighing from 65 to 85 g, were divided into four groups of seven or eight rats each. Oral administration of IE was started when the diet deficient in vitamin E was fed; 2 ml of the IE were administered perorally once daily. The rats of the positive control group were administered dl-α-tocopherol (.4 mg) that was mixed with the cellulose powder (2 mg) and dispersed in 2 ml of water.

#### Peroxide Concentration of Rat Liver

Peroxide in rat liver was determined according to the method described by Masugi and Nakamura (13). Perfused rat liver (1 g) was

homogenized with 5 ml of .05 *M* phosphate buffer (pH 7.2). The concentration of malondialdehyde in rat liver was determined by the TBA assay.

#### Vitamin E Content in the Serum

Bloods were collected from rats in tubes and then centrifuged at 3000 rpm for 10 min. Concentration of serum vitamin E was determined by HPLC (2).

#### Hemolysis Induced with Dialuric Acid

The red blood cells (**RBC**) were washed with PBS, and a 5% (vol/vol) suspension of RBC in .9% NaCl was prepared. Hemolysis was conducted by the method of Mino et al. (14). Crystalline dialuric acid (Tokyo Kasei Co., Ltd., Tokyo, Japan) was dissolved gently in .05 *M* phosphate buffer (pH 7.4) to prepare a .1% solution; .25 ml of RBC obtained from the rats fed a diet deficient in vitamin E was suspended in .2 ml of PBS, followed by preincubation. Then, .05 ml of dialuric acid (1 mg/ml in PBS) was added to the preincubated suspension and incubated at 37°C for 30 min. The negative control, sample A, was .05 ml of PBS. After 5 ml of PBS were added, the suspensions were centrifuged at 3000 rpm for 10 min. Color intensities of the supernatants were measured at absorbance at 540 nm ($A_{540}$). To induce complete hemolysis in a sample, 5 ml of distilled water were added instead of PBS to sample B.

The percentage of inhibition of hemolysis was calculated as follows:

$$\left[\frac{A_{540}(\text{sample}) - A_{540}(\text{sample A})}{A_{540}(\text{sample B}) - A_{540}(\text{sample A})}\right] \times 100(\%).$$

## RESULTS AND DISCUSSION

#### In Vitro Screening of Antioxidative Strains

Antioxidative activity of 570 strains of lactic acid bacteria, including *Bifidobacterium*, was determined by rat liver MS-TBA assay. The antioxidative activity was expressed as inhibition of peroxidant dependent on NADP and iron.

The results of the first stage of screening are shown in Figure 1. The IE of 19 strains, out of 570 strains tested, showed over 90% inhibition. Antioxidative activity occurred in 16 strains of *Lactobacillus*, two *Streptococcus salivarius* ssp. *thermophilus*, and one *Lactococcus lactis* in the first stage of screening. The IE of these 19 strains that showed 90% inhibition in the first stage of screening were diluted to .1 with PBS and examined for the inhibition to compare the antioxidative activity more precisely in the second stage of screening.

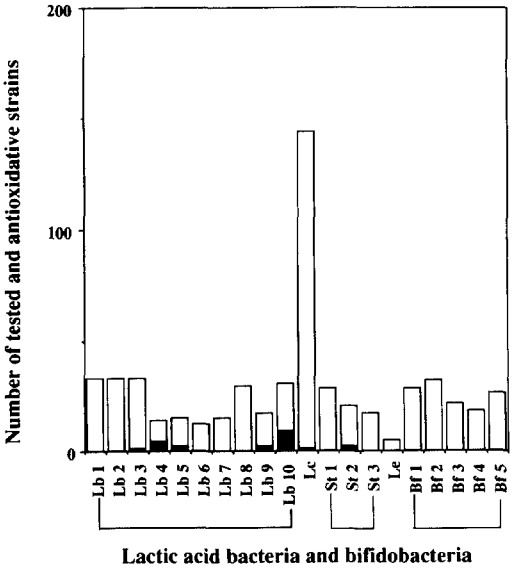


Figure 1. Antioxidative activity of intracellular cell-free extract of lactic acid bacteria and bifidobacteria at the first stage of in vitro screening. Bacteria identification: Lb 1, *Lactobacillus acidophilus*; Lb 2, *Lactobacillus delbruckii* ssp. *bulgaricus*; Lb 3, *Lactobacillus casei* ssp. *casei*; Lb 4, *Lactobacillus casei* ssp. *rhamnosus*; Lb 5, *Lactobacillus coryniformis*; Lb 6, *Lactobacillus crispatus*; Lb 7, *Lactobacillus helveticus*; Lb 8, *Lactobacillus jugurti*; Lb 9, *Lactobacillus lactis*; Lb 10, other *Lactobacillus* sp., Lc, *Lactococcus* sp.; St 1, *Streptococcus faecalis*; St 2, *Streptococcus salivarius* ssp. *thermophilus*; St 3, other *Streptococcus* sp.; Le, *Leuconostoc*; Bf 1, *Bifidobacterium animalis*; Bf 2, *Bifidobacterium adolescentis*; Bf 3, *Bifidobacterium bifidum*; Bf 4, *Bifidobacterium longum*; Bf 5, other *Bifidobacterium* sp. □ = Number of strains tested, ■ = number of strains with >90% inhibitory activity at the first stage of screening.

TABLE 1. Antioxidative activity of intracellular cell-free extract of lactic acid bacteria at the second stage of screening by rat liver microsome-thiobarbituric acid assay.

| Strain | Inhibition |
|---|---|
| | (%) |
| *Lactobacillus* sp. SBT 2028 | 91 |
| *Lactobacillus casei* ssp. *pseudoplantarum* SBT 0624 | 77 |
| *Lactobacillus fermentum* SBT 2558 | 76 |
| *Lactobacillus casei* ssp. *rhamnosus* SBT 2257 | 76 |
| *Lactobacillus casei* ssp. *rhamnosus* SBT 2254 | 76 |
| *Lactobacillus coryniformis* ssp. *tonquens* SBT 0023 | 72 |
| *Lactobacillus casei* ssp. *rhamnosus* SBT 2258 | 71 |
| *Lactococcus lactis* SBT 1224 | 64 |
| *Streptococcus salivarius* ssp. *thermophilus* SBT 1002 | 64 |
| *Lactobacillus casei* ssp. *casei* SBT 2210 | 63 |
| *Lactobacillus curvatrus* SBT 0092 | 60 |
| *Streptococcus salivarius* ssp. *thermophilus* SBT 1007 | 60 |
| *Lactobacillus casei* ssp. *casei* SBT 2202 | 59 |
| *Lactobacillus casei* ssp. *casei* SBT 2223 | 55 |
| *Lactobacillus casei* ssp. *rhamnosus* SBT 2259 | 53 |
| *Lactobacillus coryniformis* SBT 0808 | 52 |
| *Lactobacillus casei* ssp. *casei* SBT 2213 | 51 |
| *Lactobacillus casei* ssp. *casei* SBT 0848 | 51 |
| *Lactobacillus casei* ssp. *pseudoplantarum* SBT 0836 | 50 |

The 19 strains selected and the results of the second stage of screening are shown in Table 1. *Lactobacillus* sp. SBT 2028 obtained the highest antioxidative activity (91% inhibition) among the 19 strains. Others resulted in <80% inhibition.

*Lactobacillus* sp. SBT 2028 was Gram-positive, rod-shaped, nonspore-forming, catalase-negative, milk-coagulating, and gas-forming, as determined by the method of Mistuoka (15). *Lactobacillus* sp. SBT 2028 was characterized as heterofermentative *Lactobacillus*.

The inhibition by water as a control was 0%, and inhibition by a positive control sample that contained $10^{-4}$ *M* $\alpha$-tocopherol ranged from 80 to 90% throughout the first and second stages of screening. The rat liver MS-TBA assay was a suitable in vitro screening method and was in accordance with the in vivo peroxidation system. The peroxidation that is dependent on NADP and iron against rat liver MS simulated the microsomal NADP-cytochrome P-450 system (11). Therefore, this peroxidation reflected the peroxidation taking place in the liver. In the screening system mentioned, the inhibitory effect of IE from lactic acid bacteria on radical reaction for physiological membrane was evaluated, and the results were expected to show good agreement with the results of in vivo experiments.

### In Vivo Effect of IE on Rats Deficient in Vitamin E

Various experiments (3, 9, 10, 17) confirmed the antioxidative effect in vivo. Vitamin E is widely accepted as an antioxidant that protects against oxygen stress in vivo. A diet deficient in vitamin E weaken the rat's body defense system against oxygen stress, hemolysis, and accumulation of peroxidants in liver (14). During in vivo experiments, model rats fed a diet deficient in vitamin E were used to confirm the antioxidative effect of IE from lactic acid bacteria screened by in vitro experiments. *Lactobacillus* sp. SBT 2028, which showed the highest antioxidative activity in the first and second stage screenings, was used. Because *Lactobacillus* sp. SBT 2028 was heterofermentative, *L. casei* ssp. *rhamnosus* SBT 2257 was also selected for the in vivo experiment to represent homofermentative strains that are used for fermented milk products.

No differences occurred in body weight or feed intake of rat groups during the experiment (Table 2). Serum vitamin E concentration of rats administered water as control, IE of *Lactobacillus* sp. SBT 2028, and IE of *L. casei* ssp. *rhamnosus* SBT 2257 ranged from 1.0 to 1.4 $\mu$g/ml. No difference among them was significant (Figure 2). All rats except the group administered vitamin E were deficient in vitamin E throughout the experimental period.

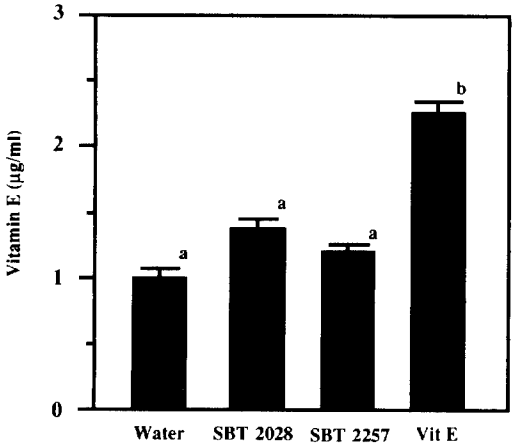


Figure 2. Mean (and SD) vitamin E concentration in the serum of rats that had been fed a diet deficient in vitamin E. Water = Water administered; SBT 2028 = intracellular cell-free extract (IE) from *Lactobacillus* sp. SBT 2028 administered; SBT 2257 = IE from *Lactobacillus casei* ssp. *rhamnosus* SBT 2257 administered; Vit E = vitamin E (.4 mg/d) administered. The differences among the groups were calculated by Tukey-Kramer's multiple comparison test. Bars with different letters are significantly different ($P < .01$).

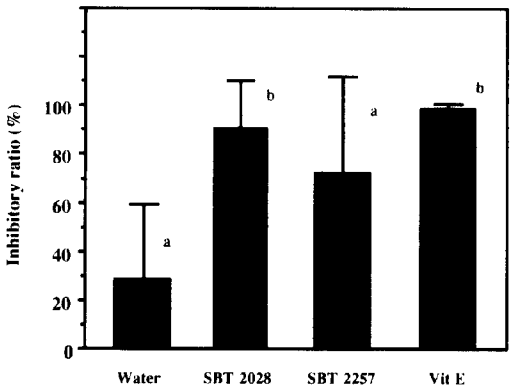


Figure 3. Mean (and SD) inhibitory effect of intracellular cell-free extract from lactic acid bacteria on hemolysis of red blood cells from rats fed a diet deficient in vitamin E. Water = Water administered; SBT 2028 = intracellular cell-free extract (IE) from *Lactobacillus* sp. SBT 2028 administered; SBT 2257 = IE from *Lactobacillus casei* ssp. *rhamnosus* SBT 2257 administered; Vit E = vitamin E (.4 mg/d) administered. The differences between water group as control were calculated by Dunnett's *t* test. Bars with different letters are significantly different ($P < .01$).

The results of in vivo experiments are given in Figures 3 and 4. Hemolysis was significantly suppressed ($P > .01$) in rats administered vitamin E and IE of *Lactobacillus* sp. SBT 2028 compared with that of the group receiving water (Figure 3). The IE from *L. casei* ssp. *rhamnosus* SBT 2257 also decreased hemolysis. However, the means of inhibitory effects were not significantly different ($P > .05$). The concentrations of peroxidants in rat livers are shown in Figure 4. Suppressions of peroxidant concentrations in rat liver by groups administered vitamin E or *Lactobacillus* sp. SBT 2028 IE were not statistically significant ($P > .05$), but peroxidant concentrations of both groups tended to be lower than those of the control group. Peroxidant concentrations of the group fed *L. casei* ssp. *rhamnosus* SBT 2257 were not different ($P > .05$) from those of the control group.

Body defense system of rats against oxygen stress was suppressed by a diet deficient in

TABLE 2. Food intake, body weight gain, and food efficiency during administration.

| | Food intake | | Body weight gained | | Food efficiency |
|---|---|---|---|---|---|
| | (g) | | | | (%) |
| | $\bar{X}$ | SD | $\bar{X}$ | SD | |
| Water | 941.8 | 40.7 | 299.1 | 25.9 | 3.16 |
| Vitamin E | 951.8 | 72.1 | 303.1 | 29.5 | 3.15 |
| SBT 2028[1] | 969.5 | 91.2 | 307.8 | 36.3 | 3.16 |
| SBT 2257[2] | 930.1 | 54.9 | 290.9 | 21.7 | 3.20 |

[1]*Lactobacillus* sp. SBT 2028.

[2]*Lactobacillus casei* ssp. *casei* SBT 2257.

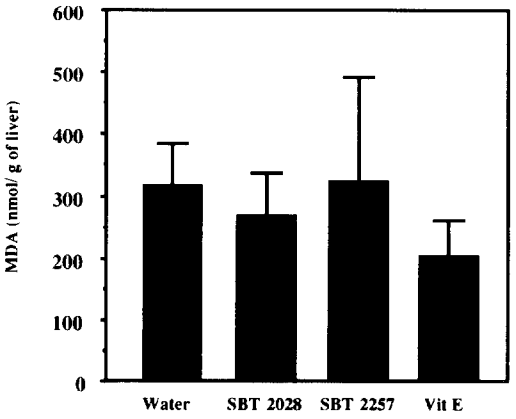


Figure 4. Mean (and SD) effect of intracellular cell-free extract form lactic acid bacteria on peroxidant accumulation in livers from rats fed a diet deficient in vitamin E. Water = Water administered; SBT 2028 = intracellular cell-free extract (IE) from *Lactobacillus* sp. SBT 2028 administered; SBT 2257 = IE from *Lactobacillus casei* ssp. *rhamnosus* SBT 2257 administered; Vit E = vitamin E (.4 mg/d) administered. The differences among the groups were calculated by Tukey-Kramer's multiple comparison test.

vitamin E in the in vivo experiment (6, 19). At dissection, the concentrations of vitamin E in serum of all rat groups except the positive control group (administered vitamin E) were low (Table 2). *Lactobacillus* sp. SBT 2028 suppressed hemolysis and decreased peroxidant concentration in the liver, which meant that *Lactobacillus* sp. SBT 2028 could improve vitamin E deficiency status and lower the risk from oxygen stress. At present, no reports have been made about the synthesis of vitamin E by *Lactobacillus*. The concentration of vitamin E in blood at dissection (Figure 2) also suggested that the antioxidative effect of *Lactobacillus* sp. SBT 2028 was not caused by vitamin E synthesis. Also, TLC analysis did not detect vitamin E in the culture of *Lactobacillus* sp. SBT 2028 (data not shown).

From these results of the experiments using the diet deficient in vitamin E, the ability of *Lactobacillus* sp. SBT 2028 to suppress hemolysis, a condition of vitamin E deficiency, was confirmed. However, the decrease of peroxidant concentration in rat liver was not clearly observed. The result of hemolysis did not agree with the result of peroxidant accumulation in rat liver. Hemolysis is susceptible to vitamin E deficiency status, and its response is all or nothing at the critical level of endogenous vitamin E feeding as reported by Mino et al. (14). All rats of the water group showed hemolysis, but only one out of seven rats showed hemolysis in the group administered *Lactobacillus* sp. SBT 2028 IE. Because the amount of vitamin E given to the positive control group (administered vitamin E perorally) was about .1 of the recommended daily intake, the same type of result also occurred in the group administered vitamin E. At dissection, the serum vitamin E concentrations of the rat group deficient in vitamin E were close to the critical vitamin E concentrations (range 1.0 to 1.3 $\mu$g/ml; Figure 2) given by Mino et al. (14), which may have been the reason that the results of the hemolysis and perioxidant accumulation did not agree.

## CONCLUSIONS

The IE from *Lactobacillus* sp. SBT 2028 acted as a substitute for vitamin E, and IE improved vitamin E deficiency status of rats. The IE from *Lactobacillus* sp. SBT 2028 can potentially decrease the risk of oxygen stress. The concentration of vitamin E in serum of rats administered IE from *Lactobacillus* sp. SBT 2028 at dissection was not increased compared with that from the group administered vitamin E; antioxidative activity of IE of *Lactobacillus* sp. SBT 2028 was not caused by vitamin E synthesis of this lactic acid bacteria. Also, vitamin E in the culture of *Lactobacillus* sp. SBT 2028 was not detected by TLC (not shown). Substances showing antioxidative activity of IE from *Lactobacillus* sp. SBT 2028 were not identified.

The precise mechanism of antioxidative IE from *Lactobacillus* sp. SBT 2028 is difficult to identify. We are currently trying to identify the chemical nature of the substances in order to elucidate the mechanisms of the antioxidative effect in this strain.

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