ANTIOXIDATIVE AND HYPOLDLIPIDEMIC EFFECT OF LACTOBACILLUS CASEI SSP CASEI (BIODEFENSIVE PROPERTIES OF LACTOBACILLI)

SUMAN KAPILA, VIBHA, P. R. SINHA

ABSTRACT

BACKGROUND: A positive correlation between an individual’s cholesterol level and development of CHD has been suggested. Low levels of high-density lipoprotein cholesterol (HDL-C) and high levels of low-density lipoprotein cholesterol (LDL-C) are important risk factors and oxidation of LDL has been implicated as an initiating mechanism of atherosclerosis. AIM: Attempts are being made worldwide for the search of effective antioxidants that can prevent oxidation of LDL. Role of fermented milk and culture containing dairy products as effective antioxidants and their potential hypolipidemic effect is the focus of research. Keeping this in view, the various lactobacilli cultures were screened for their in vitro antioxidative activity. Lactobacillus casei ssp casei showing maximum antioxidative activity was selected for carrying out in vivo studies. SETTINGS AND DESIGN: Six groups of Wistar albino rats were fed on diets containing 20% fresh or oxidized soybean oil supplemented with 5% lyophilized culture or fermented milk prepared using L. casei ssp casei for a period of 90 days. The plasma was separated in different lipoprotein fractions and analyzed for cholesterol content and thiobarbituric acid reactive substances (TBARS). RESULTS: The cholesterol levels were lower in plasma of groups fed on fermented milk by 2-11% and by 15-25% in groups fed on lyophilized culture as compared to group fed on skim milk. The levels of TBARS were lower in the LDL fraction of plasma in rats fed on fermented milk or culture than the control group fed on skim milk. CONCLUSIONS: The results depict the cholesterol-lowering and antioxidative potential of Lactobacillus casei ssp casei for their application as dietary adjunct.

Key words: Hyperlipidemia, lactobacilli, lipoproteins, TBARS, vitamin E

The enormous potential of good quality food in promoting and maintaining health has set the tone of research in the area of food for health worldwide. The scientific validation of many conventional fermented foods like yoghurt has served as a health link between user and the producer. The lactobacilli are important inhabitants of the intestinal tract of man and animals. Considerable evidence has implicated lactobacilli in a number of potentially beneficial roles, viz, immunostimulation, pathogen exclusion, production of bioactive materials, anticarcinogenic activity, deconjugation of bile acids, etc. In the face of growing opportunities for functional foods, dietary adjuncts and health-related products, it is prudent to understand the activities of various lactobacilli under in vivo conditions.

Atherosclerosis is primarily a disease of the large arteries and is the major cause of heart disease, stroke and death, both in developed and developing countries. Although epidemiological studies have revealed several risk factors associated with atherosclerosis, including hyperlipidemia, the incidence of atherosclerotic heart disease is increased in patients with hypercholesterolemia. Regulation of the serum cholesterol level is important to prevent atherosclerosis, as it has been shown that atherosclerosis could be suppressed by controlling the level of cholesterol in the serum. The hypolipidemic and oxidative processes of LDL are considered important to the pathogenesis of atherosclerosis. Therefore, by preventing the oxidation of LDL, it may be possible to reduce the incidence of atherosclerosis. It has not been confirmed whether lactobacilli actually prevent the oxidation of LDL, although few workers have reported these bacteria to have antioxidative properties. Such properties would suggest that the oxidation of LDL could be inhibited by the consumption of lactobacilli. Keeping this in view, the present investigation was undertaken with the objective to determine the cholesterol-lowering and preventive effect of Lactobacillus sp. on the oxidation of lipoproteins.

MATERIALS AND METHODS

1. 1-diphenyl-2-picrylhydrazyl (DPPH), vitamin E, thiobarbituric acid (TBA), Butylated Hydroxyl Toluene (BHT) and NADPH were procured from Sigma (St. Louis, MO, USA). The other chemicals used were from Sisco Research Laboratories, Hi-media and Loba (Mumbai, India).

Bacterial cultures

Twelve strains of lactobacilli were screened for antioxidative activity. All the cultures were obtained from National Collection of Dairy Cultures (NCDC), National Dairy Research Institute, Karnal (Haryana), India. The list is as follows: L. acidophilus 14, L. acidophilus 15, Lactobacillus sp. L13, L. casei ssp. casei 19, L. casei ssp. casei 63, L. delbrueckii ssp. bulgaricus 4, L. delbrueckii ssp. bulgaricus 9, L. helveticus 6, L. fermentum 156, L. fermentum 155, L. fermentum 141 and L. plantarum 20. The cultures were maintained by subculturing fortnightly by inoculating into MRS broth and incubating for 18 to 20 h at 37°C.

Detection of antioxidative strain

For detection of antioxidative Lactobacillus strain, the method of Terahara was followed. The Lactobacillus strains were cultured by inoculating in skim milk (10% v/v) at 37°C for 18 to 20 h.

The supernatants were extracted twice with ether (5 ml). The resulting ethereal extract was evaporated to dryness and dissolved in methanol (0.2 ml) and used as a sample for the detection of radical scavengers by TLC. The extract (2 µl) was spotted on TLC plate
Using chloroform: methanol (8:2) as a developing solvent. The radical scavengers on the plate were visualized by DPPH, which got converted from purple to colorless upon reaction with radical scavengers.

**Antioxidative assay**

Two TBA (thiobarbituric acid) methods, based on the *in vitro* microsome (MS) and linoleic acid peroxidation assay, were followed for the screening of probiotic *Lactobacillus* sp. exhibiting antioxidative activity.

**MS-TBA assay**

Antioxidative activity of the intracellular cell-free extract (IE) of the *Lactobacillus* sp. was determined by *in vitro* MS-TBA assay.[10]

The intracellular extract was prepared by culturing lactobacilli for 18 h at 37°C in MRS broth. The cells were harvested by centrifugation at 12,000 × g for 10 min, followed by saline (0.9%) washing; 10 mg of wet cells were disintegrated by ultrasonic vibrations. The cell debris was removed by centrifugation at 12,000 × g for 10 min to obtain IE. The IE was used for TBA estimation. Rat liver MS was prepared by removing liver and working with ice-cold KCl (1.15%). The liver was homogenized in 4 ml of 5 mM Tris-maleate buffer (5 mM, pH 7.4) containing KCl (1.15%) by using homogenizer. The homogenate was centrifuged at 15,000 × g for 15 min. The supernatant was collected and recentrifuged at 100,000 × g for 1 h. The pellet obtained was washed once with Tris-maleate buffer (5 mM, pH 7.4). This was referred to as rat liver microsome (MS). The MS were suspended in KCl (1.15%) and the final concentration of the fraction was approximately 5 mg of protein/ml.

For rat liver MS-TBA, 40 µl of MS (5 mg protein/ml) was mixed with 840 µl of Tris-maleate buffer (50 mM, pH 7.4), 40 µl of FeSO₄ (1.25 µM), 40 µl of NADPH (1.5 mM) and 40 µl of IE solution of *Lactobacillus* and incubated at 37°C for 30 min. At the end of incubation, 50 µl BHT (0.2%), 300 µl TCA (20%) and 600 µl TBA (0.05 M) were added rapidly to the MS mixtures to terminate peroxidation and centrifuged at 1,200 x g for 20 min. The supernatant was collected and absorbance was measured at 534 and 570 nm. Percent inhibition of oxidation was defined as

\[
\text{Linoleic acid peroxidation assay} \\
1 - \frac{(A_{534} - A_{570}) \text{ Sample}}{(A_{534} - A_{570}) \text{ blank}} \times 100\%
\]

The antioxidative activity of *Lactobacillus* sp. was assayed by using thiobarbituric acid (TBA) method based on inhibition of linoleic acid peroxidation by intracellular cell-free extract. Linoleic acid was chosen as the source of unsaturated fatty acid.[11] The TBA method was used for measurement of lipid peroxidation[12] and Fe-ascorbate system was used for the catalysis of oxidation.[13]

One hundred µl of linoleic acid was emulsified with 0.2 ml of Tween 20 and 19.7 ml of distilled water. Phosphate buffer solution (0.02 M, pH 7.4) was mixed with 1 ml of linoleic acid emulsion, 0.2 ml of FeSO₄ (0.01%), 0.2 ml of ascorbate (0.01%) and 0.4 or 0.8 ml of intracellular cell-free extract and incubated at 37°C. Distilled water was substituted for the norms of Institutional Animal Ethics Committee. The animals were about 8 weeks old and their body weight ranged between 78.57 and 94.50 g. Forty-eight adult male rats were divided into six groups of eight rats each and maintained for a period of 90 days [Table 1]. Composition of the diets has been described in Table 2. Oxidized oil was prepared by heating fresh soybean oil at 60°C for 15 days (peroxide value 91 mEq).

Blood was withdrawn from the orbital venous plexus under anesthesia and collected in heparin-coated vials. Plasma was prepared from the blood by centrifugation (1,500 x g, 10 min).

**Determination of vitamin E levels in plasma by HPLC**

The level of vitamin E in the plasma was determined according to the method of Chawla and Kaur.[16] Briefly, 0.5 ml of plasma was deproteinized with an equal volume of 95% ethanol containing 5% ascorbic acid. Three extractions were carried out using 2 ml petroleum ether each time, to maximally extract the vitamin and pooled in amber glass tube. HPLC analysis was carried out in reverse
phase C18 Spherisorb 5 μ 0.052, 4.6 x 250 mm column with 5 cm guard column (Waters, USA). Mobile phase used was methanol: 
H₂O: 96: 4. Elution was carried out at the rate of 1 ml/min and the vitamin E peak was detected with the help of UV detector (Dual and Absorbance detector) at 290 nm.

**Ultracentrifugation of plasma for separation of lipoproteins**

A portion of plasma obtained after centrifugation of blood was recentrifuged at 15,000 xg for 10 min, for removing all cellular debris and supernatant was collected. The method of Dieb-Rotheneder et al.[6] was followed for the separation of lipoproteins by a single 20-hour run with a discontinuous density gradient.

Preparation of discontinuous density gradient Plasma (up to 1.5 ml) adjusted to a density of 1.22 g/ml with solid KBr was layered at the bottom of a centrifuge tube (total volume 5.0 ml) and then overlaid with KBr density solutions of 1.08 g/ml (1.25 ml), 1.05 g/ml (1.25 ml) and 1.0 g/ml (to fill the tube). All the KBr density solutions (1.08 g/ml, 1.05 g/ml and 1.0 g/ml) contained EDTA (200 μg/ml). All were prepared in phosphate buffer (pH 7.4). All density solutions were purged with nitrogen before use.

**Determination of cholesterol and TBARS**

Density gradient tubes were centrifuged in Hitachi Ultracentrifuge using rotor SW at 100,000 xg for 10°C for 20 h. After centrifugation, the separated lipoprotein fractions were distributed in 10 different tubes, each containing 500 μl aliquot and cholesterol and HDL cholesterol levels were estimated using Autopak Kit (Bayer Diagnostic, India). To 300 μl of lipoprotein fraction, added 3 ml of HCl (0.05 N) and 1 ml of TBA (0.67%) and the reaction mixture was heated for 30 min in a boiling water bath. After the mixture had been cooled in an ice bath, TBARS in the reaction mixture were extracted with 4 ml of n-butanol. The absorbance of butanol phase was measured at 535 nm. The levels of TBARS were expressed as malondialdehyde equivalents/ mg of cholesterol.

**Statistical analysis**

Each result is expressed as the mean ± SEM. One-way ANOVA was used to examine the difference between groups.

**RESULTS**

**Radical scavenging (RS) activity**

Out of the 12 strains of lactobacilli screened, 8 strains of lactobacilli showed radical scavenging potential on TLC plates [Figure 1]. Similar kind of detection method was followed by Terahara.[4] They showed that L. delbrueckii ssp. bulgaricus 2003 exhibited radical scavenging potential. Likewise, DPPH method was used for screening of antioxidants in marine bacteria from fish and shellfish and 112 bacterial isolates producing antioxidants obtained.[13]

**Measurement of radical-scavenging activity: MS-TBA assay**

Antioxidative activity of the intracellular cell-free extract (IE) of the Lactobacillus sp. determined by in vitro MS-TBA assay - results are shown in Table 3.

Maximum antioxidative activity in terms of percent inhibition of oxidation was observed in L. casei ssp. casei 19 (76.82%), followed by L. acidophilus 14 (62.21%), Lactobacillus sp. L13 (59.25%), L. casei ssp. casei 63 (53.30%), L. helveticus 6 (52.70%) and L. delbrueckii ssp. bulgaricus 4 (52.64%). All other remaining strains exhibited less than 50% activity.

**Linoleic acid peroxidation assay**

The inhibition of linoleic peroxidation followed similar trend [Table 3] as observed in MS-TBA assay, i.e., maximum in L. casei ssp. casei 19 (72.04%), followed by L. acidophilus 14 (51.74%), Lactobacillus sp. L13 (51.38%) and rest of all the strains showed less than 50% inhibition.

Likewise, the antioxidative activity of 570 strains of lactic acid bacteria by TBA assay was studied and observed to be as high as 91% activity in Lactobacillus sp. SBT-2028 and 77% in L. casei ssp. pseudoplanatarum SBT 0624.[10] They have also reported 51 to 63% antioxidative activity in different strains of L. casei ssp. casei. Similarly, the radical-scavenging ability of yoghurt organisms based on linoleic peroxidation assay was examined. Streptococcus thermophilus and L. delbrueckii ssp. bulgaricus demonstrated an antioxidative effect on the inhibition of linoleic acid peroxidation.[13] In another study, both intact cells and intracellular cell-free extract of B. longum and L. acidophilus (10⁶ cfu/ml) inhibited linoleic acid peroxidation by 28 to 48%.[13]

**In vivo studies**

There was no variation in the body weight among the rats of the six groups, indicating that the type of diet did not have any effect on the body weight. Among the lipid profile, there was no variation in the cholesterol levels in the VLDL fraction in groups fed fresh oil/oxidized oil supplemented with skim milk or fermented milk or culture. Whereas levels
of LDL cholesterol were significantly (P<0.001) higher in groups fed on fresh oil or oxidized oil supplemented with skim milk in comparison to groups fed on fresh/oxidized oil supplemented with fermented milk or culture. In HDL fraction, levels of cholesterol were higher by 13-29% in groups fed on fresh oil/oxidized oil supplemented with fermented milk in comparison to groups supplemented with skim milk [Table 4].

Levels of TBARS in VLDL of rats fed on fresh/oxidized oil supplemented with fermented milk or culture are shown in Figure 2. Results indicated maximum levels of TBARS in groups FS (17.05 nmoles MDA/mg cholesterol) and OS (25 nmoles MDA/mg cholesterol) which were fed on 20% fresh or oxidized oil supplemented with skim milk, whereas groups fed on fermented milk or culture showed lower values of TBARS ranging from 10.54 to 19.60 nmoles MDA/mg cholesterol. Similarly in LDL fraction, TBARS levels were more in groups fed on fresh or oxidized oil supplemented with skim milk. Among oxidized oil groups, there was decline in TBARS by 55% in group fed on culture in comparison to skim milk fed group.

Among fresh oil group in LDL fraction, there was insignificant decline in TBARS in rats fed on fermented milk or culture when compared to control group fed on skim milk. However, in respect of HDL fraction, there were not many variations in the levels of TBARS between the different groups [Figure 2].

The effect of feeding different experimental diets on vitamin E levels in plasma of rats has been shown in Figure 3. The results clearly showed that vitamin E levels were less in groups fed oxidized oil as compared to fresh oil fed groups and supplementing diets with fermented milk or culture resulted in increased levels of vitamin E.

**DISCUSSION**

Our aim was to check the antioxidative and hypocholesterolemic effect of lactobacillli. Results clearly depict the antioxidative nature of *L. casei* ssp *casei* under in vivo condition as observed in MS-TBA and linoleic acid peroxidation assay. The inhibition of peroxidation was more than 70% in both experiments. The culture (*L. casei* ssp *casei*) was used for carrying out *in vivo* studies in order to ascertain whether the similar preventive effect of culture on oxidation can be observed under *in vivo* condition. Therefore, in order to induce oxidative stress and hyperlipidemia, rats were fed diet containing 20% fresh or oxidized soybean oil. The effect of supplementing diet with fermented milk or culture on oxidative stress and cholesterol level was observed. The levels of LDL cholesterol were significantly lower in groups fed on fresh oil or oxidized oil supplemented with fermented milk or culture in comparison to groups fed on diet supplemented with skim milk. In addition, feeding of fermented milk resulted in increased HDL cholesterol level in experimental rats by 14-29%. Atherogenic index expressed as the ratio of LDL/HDL [Table 4] was significantly lowered by 1.6 to 2.1 times in rats fed on fermented milk or culture. Similar observations have been made on feeding lactobacillus strain NTU101,102.
Lactobacillus reuti and Lactobacillus gasseri to hamsters, mice and rats respectively.\[4,20,21\]

The oxidation of LDL plays an important role in the pathogenesis of atherosclerosis and the antioxidants in the foods like tea, wine, vegetables inhibit this oxidation.\[8,22\] Similarly in the present study, reduction in levels of TBARS in LDL fraction on consumption of L. casei ssp casei may exhibit prevention of oxidation of LDL. The effect was very prominent in oxidized oil group; there was significant (55%) reduction in TBARS in LDL fraction in group fed on culture in comparison to control, which were given skim milk. The levels of TBARS in the LDL fraction obtained from rats fed on oxidized soybean oil were higher than that in the rats fed on fresh soybean oil. Similar observation has been reported by other workers also.\[4,20,21\] Not only was there a reduction in TBARS levels, but levels of total plasma cholesterol were also less in groups fed on lyophilized culture, thereby exhibiting the hypocholesterolemic effect of L. casei ssp casei. Hypcholesterolemic effects of lactic cultures have been reported earlier by several workers.\[4,20,21\] Furthermore, the levels of vitamin E in the plasma were also affected by the diet; it was lower in rats, as expected, which were fed on oxidized oil than in the rats fed on fresh soybean oil and higher in the experimental rats fed on culture than in the rats fed on skim milk, because feeding of fat-rich diet increases the oxidative stress and thus increases the requirements of antioxidants like vitamin E. These results show that the antioxidative ability of the diet containing the L. casei ssp casei and its fermented product (Figure 3) was stronger than that in the diet containing the skim milk powder. Therefore, the antioxidant present in the culture of L. casei ssp casei had an antioxidative effect similar to that of vitamin E. It is rather difficult to compare the antioxidative ability of lactobacilli with that of other antioxidants because of their different characteristics. In respect of antioxidant in the culture of L. casei ssp casei, further studies are still needed to prove its clear role in prevention of LDL oxidation and to determine its chemical nature and characteristics. Attempts are now being made to use this potential culture in combination with other cultures for preparation of dahi for human consumption and to carry out further clinical studies.

REFERENCES


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