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In-vivo studies of antioxidant activity of fermented milk (Lassi) by *Lactobacillus acidophilus* and standard dahi culture

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Abstract
The oxidative state of animal tissues represents balance between the action of pro-oxidant compounds and the endogenous antioxidant defense systems. Functional food act as health promoting properties through bio-functional agents like enzyme, vitamin and bioactive peptides, etc are the major dietry factors with the ability to act as antioxidants produced during food processing. Nutritional antioxidants play an important role in cellular antioxidative defense mechanisms. In this study, two sets of traditional Lassi were produced: the first one (L_0) using Mixed Dahi culture *NCDC-167(BD4)* and the second (L_1) with the same Dahi culture mixed with *Lactobacillus acidophilus* NCDC-15 as an adjunct culture. Total 64 Swiss albino male mice (25-30 g) were maintained for 15 and 30 days and feed with basal diet, basal diet + oxidized oil, basal diet +oxidized oil + control Lassi (L_0) and basal diet + oxidized oil + Lassi-L_1. The in-vivo antioxidant activity of Lassi was checked in RBC lysate and liver homogenate for antioxidative enzymes, viz., Catalase, Superoxide dismutase (SOD), Glutathione peroxidase (GSHPx) and lipid hydroperoxides. It has been observed from the results that the level of the antioxidant enzymes SOD, catalase, GSHPx increases in mice fed with Lassi-L_0 and Lassi-L_1 as compared to that of fed with oxidized oil. The effect of feeding Lassi-L_1 has been observed more as compared to Lassi-L_0 on the antioxidant enzymes activities.

Keywords: RBC lysate, liver homogenate, antioxidant activity, catalase, superoxide dismutase (SOD), glutathione peroxidase (GSHPx) and lipid hydroperoxides

Introduction
It is accepted that milk proteins may act as precursor of biologically active peptides such as antihypertensive, antioxidant and mineral binding (Meisel, 2004) [25]. These peptides are inactive within the proteins and can be released by enzymatic hydrolysis or fermentation with proteolytic lactic acid bacteria. The fermentation with proteolytic lactic acid bacteria can be used as a strategy to generate bioactive peptides in the fermented products. For that suitable starter cultures has to be selected which can produce bioactive peptides as well as maintaining the products quality. Fermented milk products in addition to providing a source of both energy and nutrition, are also a source of bio-functional peptides that may impart improved health benefits when ingested (FitzGerald and Murray, 2006) [7]. Reactive oxygen species (ROS) can cause damage in proteins and mutation in DNA, oxidation of membrane phospholipids and modification of low density lipoproteins (LDL) (Alaiz *et al*., 1994) [2]. The antioxidant defense systems has the ability to remove the reactive oxygen species through enzymatic antioxidants like superoxide dismutase and glutathione peroxidase and non enzymatic antioxidants such as proteins and peptides, vitamins, trace elements, coenzymes and cofactors. These defense system are not always effective enough to totally prevent the damage, and therefore, food supplements containing antioxidants may be used to help the human to reduce oxidative damage (Zommara *et al*., 1998 and Kullisaar *et al*., 2003) [40, 21]. Recently it has been described by few researchers that milk protein hydrolysates and peptides released after hydrolysis posses antioxidant activities. Nutritional antioxidants play an important role in cellular antioxidative defense mechanisms (Chow, 1988) [5]. Vitamin E and C, ß-carotenoids, selenium, copper and zinc are the major dietry factors with the ability to act as antioxidants (Capel, 1988) [4]. Among the protective endogenous factors the enzyme superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GSHpx) comprise the prominant cellular antioxidant defense system. SOD, CAT GSHpx and GR (Glutathione Reductase) constitute mutually supportive team of defense against reactive oxygen species (ROS) and preventing lipid peroxidation (Eaton and Barnmler, 1999) [6]. SOD is a metalloprotein and is the first enzyme involved in the antioxidant defense by lowering the steady rate of superoxide radicals (O2-). Decreased activity of SOD leads to increased production of free radicals eg. superoxide anion combine

with hydrogen peroxide in the presence of copper ion to four powerful hydroxyl radical (Ishikawa, 1993) [18]. This radical modifies protein and DNA, damages cellular membranes of mitochondria, nuclear envelop and endoplasmic reticulum (Hayashi *et. al*, 2005; Kawasaki *et al*., 2004 and Mimnaugh *et al*., 1985) [16, 19, 26]. CAT is a haemoprotein, localized in the peroxisome and catalyses the decomposition of H2O2 to water and oxygen. GSHpx is a selenoenzyme; present predominantly in liver and catalyses the reaction of hydroperoxides with reduced glutathione to form glutathione disulphide (GSSG) and the reduction product of the hydroperoxide (Venukumar and Latha, 2002) [35]. Increased activity of these antioxidant enzymes results in decreased formation of hydroxyl radical (OH-) (Gajanan et.al. 1997; Maiorino 1991) [19, 23]. Hydroperoxide have toxic effect both directly and through degradation to highly toxic hydroxyl radicals. They may also react with transition metal like iron and copper to form stable aldehydes such as malondialdehydes that damage cell membrane. Proteins are possibly the most immediate vehicle for inflicting oxidative damage on cell by the reactive oxygen species. Carbonyl groups are produced on protein side chains particularly the proline, arginine, lysine and threonine are oxidized. These are quite stable and can be used as marker for oxidative stress (Isabella *et al*., 2003) [17]. Furthermore, some antioxidant peptides were isolated from milk proteins by fermentation with lactic acid bacteria. Kudoh *et al* (2001) [20] identified one antioxidative peptide κ caseins (f96-106) in milk fermented with *Lactobacillus delbrueckii* subsp. *bulgaricus* (IF 013953). Virtanen *et al*. (2007) [37] showed that fermentation of milk with *lactobacillus jensenii* (ATCC 25258) and *lactobacillus acidophilus* (ATCC 4356) enhanced the antioxidant activity in the whey fraction. Similarly, Gupta *et al*., 2009 [14] demonstrated that the antioxidant activity of water soluble extract of cheddar cheese made with adjunct culture *Lactobacillus casei* ssp. *casei* NCDC 300 increased as compared to control cheese. The two antioxidant peptides originated from β-casein f (98-105) and αs_1-casein f (80-90) has been identified from the cheese made with adjunct culture (Gupta *et al*., 2010a) [13].

Materials and Methods

Milk and cultures

The buffalo milk was procured from NDRI., cattle yard and standard mixed Dahi culture *NCDC-167(BD$_4$)* with *Lactobacillus acidophilus* NCDC-15 were procured from the National Collection of Dairy Culture (NCDC), NDRI, Karnal.

Preparation of Lassi

The Lassi samples were prepared by using buffalo milk according to the procedures of Sukumar De (2004) [33] which slight modifications.

In-Vivo Antioxidant activity of Lassi

64 Swiss albino male mice (25-30 g) were obtained from the Small Animal House, NDRI, Karnal, India. They were maintained on standard pellet diet and tap water *ad libitum*. They were housed eight per cage and kept in polypropylene cages with stainless steel wire-bar lids using a rice husk as a bedding material under a 12 h light/dark cycle and room temperature 22–24^{0}C. Mice were then acclimatized to the environment for one-week prior to experiment. The present study was approved by the Institutional Animal Ethics Committee (IAEC), NDRI, Karnal, India. The in-vivo antioxidant activity of Lassi was checked in RBC lysate and liver homogenate for antioxidative enzymes, viz., Catalase, Superoxide dismutase (SOD), Glutathione peroxidase (GSHPx) and lipid hydroperoxides were analyzed as per Aebi (1984) [1]; Marklund and Marklund (1974) [24]; Lawrence and Burk (1976) [22] and Asakawa and Matsushita (1979) [3], respectively and lipid hydroperoxides of liver homogenate was analyzed Uchiyama and Mihara (1978) [34].

Results and Discussion

In the present study the effect of feeding the Lassi (L_1) on the antioxidative enzymes (AOE), level of TBARS and carbonyl proteins was studied in the RBC and liver lysate of the mice. For that the mice were fed with basal diet, basal diet + oxidized oil, basal diet + oxidized oil + Lassi (L0) and basal diet + oxidized oil + Lassi (L1).

The oxidative stress was developed in three groups of mice by feeding oxidized oil create (except control group) to free radicals. There was no significant difference ($P \leq 0.05$) observed in the weight of mice before feeding trial (table 1).

Table 1: Weight of mice groups fed with Lassi (L_0) made with standard Dahi culture (NCDC167) and Lassi (L_1) made with *Lactobacillus acidophilus* (NCDC15) as an adjunct culture.

Groups	Duration in days	Basal diet	Basal diet +Oxidized oil	Basal diet +Oxidized oil + Control Lassi (L0)	Basal diet + Oxidized oil + Lassi-*L1*	CD at 5 %
15 days	0	17.33±0.54^a	17.35±0.44 ^{a}	17.23±0.46^a	17.26 ± 0.53^a	4.21
	15	22.63±1.03^a	22.16±1.36 ^{a}	23.53±0.28^a	23.58 ± 0.49^a	
30 days	0	15.31±0.66^a	14.81±0.65^a	14.38±0.60^a	14.63±0.63^a	4.56
	30	21.95±1.03^a	21.62±1.18^a	22.25±0.44^a	26.54±0.85^b	

The values expressed as Means ± SEM for 8 mice per group. The values with different small letters superscripts column wise differ significantly at 5% level of significance ($P \leq 0.05$)

The weight of mice increased in all the groups after 15th and 30th day of feeding, but in the group fed with Lassi it increased significantly. Our results were similar to those of Hayam *et al.*, (1995) [15] and Nidhi, (2011) [28] which indicate that oxidized oil in diet impaired to some extent the animal growth.

Catalase activity was determined in RBC lysate and liver homogenate of the mice fed with Lassi as per method given by Aebi (1984) [1]. The catalase activity increased significantly in the RBC lysate in mice groups fed with both type of Lassi after 15 and 30 days feeding. After 30 days the catalase activity in mice was significantly higher in the group fed with Lassi -L_1 (72.30 ± 2.10 U/g Hb) as compared to the group fed with control Lassi-L_0 (64.83 ± 2.16 U/g Hb) (Table 2).

Table 2: Catalase activity in RBC lysate and Liver homogenate of mice fed Lassi (L_0) made with standard Dahi culture (NCDC167) and Lassi (L_1) made with *Lactobacillus acidophilus* (NCDC15) as an adjunct culture.

Group	Basal diet	Basal diet + Oxidized oil	Basal diet +Oxidized oil + Control Lassi (L_0)	Basal diet + Oxidized oil + Lassi-L_1
Blood				
15- days	55.12 ±3.24^a	58.28 ±2.73^a	78.45 ±3.36^b	84.01 ±2.27^b
30- days	57.08 ± 2.11^{ab}	55.02 ± 4.68^a	64.83 ± 2.16^b	72.30 ± 2.10^c
Liver				
15- days	138.10 ± 3.10^a	119.54 ± 1.75^b	179.25 ±7.26^c	225.92 ± 7.02^d
30- days	150.76 ± 4.60^a	121.77 ± 3.01^b	261.25 ±7.81^c	285.45 ±9.53^d

CD (15 days Blood) = 8.49; CD(30 days Blood)= 8.62
CD (15 days liver) = 15.51; CD (30days Liver) = 19.53
Catalase activity of RBC lysate is expressed in U/g Hb and of liver homogenate is in U/mg of protein.
The values expressed as Means ± SEM for 8 mice per group. The values with different small letters superscripts column wise differ significantly at 5% level of significance ($P \leq 0.05$)

Similarly the catalase activity in liver homogenate increased significantly after 15 and 30 days of feeding in the control Lassi-L_0 and Lassi-L_1 fed groups when compared with the groups fed with basal diet and oxidized oil. The catalase activity decreased significantly in the liver homogenate in group fed with oxidized oil when compared with basal diet group

It is predicted from the table 3 that the SOD activity in RBC lysate decreased significantly in the mice group fed with oxidized oil both after 15 and 30 days as compared to the group fed with basal diet. It further increased after 15 days feeding with control Lassi (594.49 ± 28.31 U/ g of Hb) but increased significantly in group fed with Lassi-L_1 (made with *Lactobacillus acidophilus* NCDC15) (670.00 ± 18.93 U/ g of Hb). After 30 days of feeding the SOD activities in both Lassi fed group were significantly higher than the basal diet group.

Table 3: Superoxide dismutase activity in RBC lysate and liver homogenate of mice fed Lassi (L_0) made with standard Dahi culture (NCDC167) and Lassi (L_1) made with *Lactobacillus acidophilus* (NCDC15) as an adjunct culture.

Group	Basal diet	Basal diet + Oxidized oil	Basal diet +Oxidized oil + Control Lassi (L_0)	Basal diet + Oxidized oil + Lassi-L_1
Blood				
15- days	598.25 ± 19.33^a	431.11 ± 28.37^b	594.49 ± 28.31^a	670.00 ± 18.93^c
30- days	602.81 ± 28.44^a	489.36 ± 16.46^b	709.26 ± 35.90^c	726.64 ± 22.12^c
Liver				
15- days	5.82 ± 0.38^a	5.15 ± 0.26^a	6.27 ± 0.49^a	12.50 ± 0.70^c
30- days	5.95 ± 0.19^a	5.20 ± 0.47^a	7.81 ± 0.56^b	11.48 ± 0.51^c

CD (15 days Blood) = 70.03; CD (30 days Blood) = 77.41;
CD (15 days Liver) = 1.41; CD (30 days Liver) = 1.31
SOD activity of RBC lysate is expressed in U/ g of Hb and of Liver homogenate is in U/mg of protein
The values expressed as Means ± SEM for 8 mice per group. The values with different small letters superscripts column wise differ significantly at 5% level of significance ($P \leq 0.05$)

In liver the SOD activity of Lassi-L_1 group was significantly higher as compared to the other groups after 15 days of feeding. But after 30 days of feeding the activity increased in the group fed with control Lassi-L_0 (7.81 ± 0.56 U/ g of protein) while the Lassi –L_1 fed group showed highest activity (11.48 ± 0.51 U/ g of protein).

The glutathione peroxidase activity in RBC lysate and liver homogenate of mice fed with control diet and experimental diet have been presented in the table 4. The GSHPx activity decreased both in RBC lysate and liver homogenate of mice fed with oxidized oil as compared the mice fed with basal diet. Further feeding with Lassi there was significant increased in activity both in liver and blood. The GSHPx activity was highest in group fed with Lassi-L_1 prepared using *L. acidophilus* as an adjunct culture both in liver and blood (Table 4).

Table 4: Glutathione peroxidase activity in RBC lysate and liver homogenate of mice fed Lassi (L_0) made with standard Dahi culture (NCDC167) and Lassi (L_1) made with *Lactobacillus acidophilus* (NCDC15) as an adjunct culture.

Group	Basal diet	Basal diet + Oxidized oil	Basal diet +Oxidized oil + Control Lassi (L_0)	Basal diet + Oxidized oil + Lassi-L_1
Blood				
15- days	190.42 ± 1.41^a	167.50 ± 5.13^b	306.38 ± 9.54^c	357.64 ± 9.60^d
30- days	187.19 ± 2.42^a	181.10 ± 7.06^a	208.30 ± 9.05^{ab}	235.32 ± 9.54^c
Liver				
15- days	3.39 ± 0.31^a	3.12 ± 0.23^a	4.38 ± 0.20^b	6.30 ± 0.28^c
30- days	4.19 ± 0.32^a	3.67 ± 0.34^{ba}	4.58 ± 0.27^{ac}	5.55 ± 0.27^d

CD (15 day Blood) = 21.05; CD (30 day Blood)= 21.90
CD (15 day Liver) 0.75; CD (30 day Liver)= 0.87
Glutathione peroxidase activity of RBC lysate expressed in U/ g of Hb and of Liver homogenate is in U/mg of protein.
The values expressed as Means ± SEM for 8 mice per group. The values with different small letters superscripts column wise differ significantly at 5% level of significance ($P \leq 0.05$).

Malondialdehyde is the most abundant individual aldehyde resulting from lipid peroxidation (LP) breakdown in biological systems and is commonly used as an indirect index of LP (Sorg, 2004) [30]. The lipid peroxidation level measured as TBARS levels and expressed as increased Malondialdehyde (MDA) formation. MDA is a marker of

oxidative lipid damage and it is major oxidative product of peroxidised polyunsaturated fatty acids (Zhang *et al*., 2004) [39].

The levels of TBARS increased significantly in groups fed with oxidized oil (293.13 ± 8.94 and 311.89 ± 6.76 nM MDA/g of Hb) as compared to the control group (224.12 ± 11.30 and 258.49 ± 14.17 nM MDA/g of Hb) in 15 and 30 days of feeding period (table 5). Further the TBARS levels in RBC lysate of the mice fed with control Lassi-L_0 and Lassi-L_1 were significantly decreased after 15 days and 30 days of feeding when compared to the oxidized oil and control fed group (table 4.20).

Table 5: Level of TBARS in RBC lysate and liver homogenate of mice fed Lassi (L_0) made with standard Dahi culture (NCDC167) and Lassi (L_1) made with *Lactobacillus acidophilus* (NCDC15) as an adjunct culture

Group	Basal diet	Basal diet + Oxidized oil	Basal diet +Oxidized oil + Control Lassi (L_0)	Basal diet + Oxidized oil + Lassi-L_1
Blood				
15- days	224.12 ± 11.30^a	293.13 ± 8.94^b	190.03 ± 4.09^c	176.20 ± 7.95^{dc}
30- days	258.49 ± 14.17^a	311.89 ± 6.76^b	214.90 ± 2.22^c	185.05 ± 8.55^c
Liver				
15- days	6.56 ± 0.26^a	11.10 ± 0.75^b	7.31 ± 0.22^a	6.33 ± 0.25^a
30- days	7.05 ± 0.80^a	11.75 ± 0.60^b	7.69 ± 0.31^a	6.69 ± 0.44^a

CD (15 days Blood) = 24.56; CD (30 days Blood) = 38.42
CD (15 days Liver) = 1.25; CD (30 days Liver) = 1.64
TBARS value of RBC lysate expressed in nMoles Malonaldehyde/g of Hb and of liver homogenate is in µMoles Malonaldehyde/g of protein.
The values expressed as Means ± SEM for 6 mice per group. The values with different small letters superscripts column wise differ significantly at 5% level of significance (P≤0.05).

In liver homogenate the levels of TBARS in the group fed with basal diet was much lower as compared to the group fed with oxidized oil both after 15 and 30 days. The level of TBARS in both groups differ significantly ($P \leq 0.05$). Whereas the group fed with Lassi L_0 and Lassi-L_1 showed almost same levels of TBARS after 15 and 30 days of feeding as that of control diet fed group.

The protein carbonyls are the marker of protein oxidation (Stadtman, 1991) [32]. It is depicted from the table 6 that when mice were fed with oxidized oil the protein carbonyl level in both blood and liver increases significantly as compared to control group. But on further in group fed with Lassi-L_0 and Lassi-L_1, the level of protein carbonyl reached as that of control group except in the liver homogenate after 15 days feeding of both the Lassi samples significant decrease in the protein carbonyl was observed as compared to control and oxidized oil fed group.

Table 6: Level of protein carbonyls in RBC lysate and liver homogenate of mice fed Lassi (L_0) made with standard Dahi culture (NCDC167) and Lassi (L_1) made with *Lactobacillus acidophilus* (NCDC15) as an adjunct culture

Group	Basal diet	Basal diet + Oxidized oil	Basal diet +Oxidized oil + Control Lassi (L_0)	Basal diet + Oxidized oil + Lassi (L_1)
Blood				
15- days	72.96 ± 1.92^a	90.60 ± 5.82^b	74.60 ± 2.19^a	69.78 ± 1.34^a
30- days	79.14 ± 2.45^a	95.48 ± 3.85^b	82.50 ± 3.85^a	78.70 ± 2.23^a
Liver				
15- days	67.61 ± 4.82^a	84.70 ± 2.92^b	54.80 ± 4.07^c	57.56 ± 4.30^{ac}
30- days	64.60 ± 2.76^a	93.87 ± 3.78^b	65.37 ± 3.72^a	65.78 ± 4.53^a

CD (15 days Blood) = 9.61; CD(30 days Blood) = 9.23
CD (15 days Liver) = 11.83; CD(30 days Liver) = 10.86
Protein carbonil value of RBC lysate expressed in µMoles /g of Hb and of liver
Homogenate is in µMoles /g of protein. The values expressed as Means ± SEM
For 8 mice per group. The values with different small letters superscripts column
Wise differ significantly at 5% level of significance (P≤0.05).

It has been observed from the results that the level of the antioxidant enzymes SOD, catalase, GSHPx increases in mice fed with Lassi-L_0 and Lassi-L_1 as compared to that of fed with oxidized oil (Table 2, 3 and 4). It may be because of these products contains antioxidant peptides which form during fermentation process. The effect of feeding Lassi-L_1 has been observed more as compared to Lassi-L_0 on the antioxidant enzymes activities. Moreover as mentioned before the antioxidant activity of Lassi-L_1 was significantly higher than that of the Lassi-L_0. Similar observation was made by Nidhi (2011) [28], that whey protein hydrolysates (WPHs) elevate the antioxidant activities in mice. Because during hydrolysis of whey proteins the smaller peptides are formed which may contributes towards the antioxidant activity of WPHs. Pena-Ramon and Xiong (2001) [29] reported that hydrolysis of heat treated WPI with flavourzyme produces the most active antioxidant hydrolysates.

Further the effect of the various antioxidants containing diet on antioxidant enzymes has been studied by various workers in recent past. Nevin and Rajamohan (2006) [27] observed that virgin coconut oil with more unsaponificable components viz. vitamin E and polyphenols than copra oil exhibited increased levels of antioxidant enzymes and prevented the peroxidation of lipids in both *in vitro* and *in vivo* conditions. These results showed that virgin coconut oil is superior in antioxidant action than copra oil and groundnut oil.

The antioxidants present in tea extracts also elevated the activity of antioxidant enzymes. Green tea polyphenols significantly inhibited UVB (ultraviolet radiation with wavelengths between 290 and 320 nm) induced decrease in epidermal catalase and glutathione reductase. Oral administration of black or green tea extract resulted in increased serum SOD activity in mice exposed to carcinogen, 3-methyleolanthrene. Rats provided with green tea extract exhibited attenuation of ethanol-associated decrease in serum

and liver SOD as well as GSHPx and CAT activities (Frei and Higdon, 2003) [8].

In the present study the level of lipid peroxidation decreased in the group fed with the Lassi-L_0 and Lassi-L_1 (Table 5). This may be because of the higher level of GSHPx due to feeding of Lassi-L_0 and Lassi-L_1 (Table 4). The biological function of GSHPx is to reduce lipid hydroperoxides conversion to their corresponding alcohols and reduce free hydrogen peroxide reaction (Verma *et al*., 2007) [36]. In our experiment the GSHPx activity was significantly increased in mice fed with Lassi samples. The result in agreement with many researchers (Gultekin *et al*., 2001; Zama *et al*., 2007; Gendy *et al*., 2010) [12, 38, 11].

The mice fed with oxidized oil showed increased protein carbonyl in our study as compared to control. This may be because of presence of reactive oxygen species (ROS) in the system when mice fed with oxidized oil. These ROS react with peoteins and introduce a carboxyl groups in the proteins (Garibaldi *et al*., 1994; Stadtman and Levine, 2000) [10, 31].

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