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# Screening of lactobacillus cultures for their use in enhancing the biofunctional activities of fermented milk products

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**Abstract**
In the present study, twelve strains of lactic acid bacteria were procured from the National Collection of Dairy Culture (NCDC), NDRI, Karnal and screened for their ability to produce radical scavengers (antioxidant activity), ACE inhibitors (ACE inhibitory activity) and mineral binding substances (contents of Casein Phosphopeptides) in skim milk by inoculating 2 per cent of each culture and then incubated at 37°C for 10 h. Among all these cultures the *Lactobacillus acidophilus* NCDC-15 showed highest scavenging activity (0.65 µM of trolox/mg of protein) and minimum $IC_{50}$ value for ACE-inhibitory activity (28.9 ± 0.95 µg protein /ml). Whereas, L. *paracasei spp. paracasei* NCDC-63 showed highest Casein Phosphopeptides (CPPs) contents (2.81 ± 0.025 mg/ml). The proteolytic activity L. *rhamnosus* NCDC-24 showed maximum i.e. 202.47 ± 0.68 ug serine/ml. The correlation coefficients of the antioxidant activity, ACE inhibitory activity and Caseinophosphopeptide contents with proteolytic activity were 0.33, 0.02 and 0.38. The antioxidant activities, ACE inhibitory activities and Casein Phosphopeptides contents of all the cultures are strain specific but not related to the extent of proteolysis.

**Keywords:** ACE- Angiotensin converting enzyme, CPPs- Casein Phosphopeptides, LAB- Lactic acid bacteria, HHL- hippuryl-L-histidyl-L-leucine

## Introduction
Now a day, the consumers pay a lot of attention to the relation between food and health. As a result the market for foods with health promoting properties (functional foods) has shown a remarkable growth over the last decade [1]. Recently, the use of functional starter cultures in the manufacture of fermented food products is being explored. Such cultures can contribute to food safety and offer one or more organoleptic, technological, nutritional or health advantages. These cultures particularly the lactic acid bacteria (LAB) are able to produce antimicrobial substances, sugars, polymers, sweetness, aromatic compounds, useful enzymes or nutraceuticals. The group of lactic acid bacteria (LAB) occupies a central role in these processes, and has a long and safe history of application and consumption in the production of fermented foods and beverages [2].
The proteolytic system of LAB can contribute to the liberation of health enhancing bioactive peptides from milk. The latter may improve absorption in the intestinal tract, stimulate the immune system exert antihypertensive or antithrombotic effects and antioxidative activity or function as carriers for minerals especially calcium.
In the present study, twelve strains of lactic acid bacteria were screened for their ability to produce radical scavengers (antioxidant activity), ACE inhibitors (ACE inhibitory activity) and mineral binding substances (contents of Casein Phosphopeptides). Further it was also investigated whether the above mentioned activities can be correlated with proteolysis.

## Materials and Methods
Screening of *lactobacillus* cultures for antioxidant, ACE inhibitory activity and Casein Phosphopeptides (CPPs) contents

## Microbial Cultures
The standard *Lactobacillus* cultures (12) were procured from the National Collection of Dairy Culture (NCDC), NDRI, Karnal included:

**Proteolytic Strain of *lactobacillus* culture**
L. *delbrueckii* ssp. *lactis* NCDC-03
L. *helviticus* NCDC-05
L. *delbrueckii* ssp. *bulgaricus* NCDC -08
L. *acidophillus* NCDC-15
L. *casei* spp. *casei* NCDC-17
L. *rhamnosus* NCDC-24
L. *paracasei* spp. *paracasei* NCDC-63
L. *fermentum* NCDC-141
L. *helviticus* NCDC-288
L. *delbrueckii* ssp. *bulgaricus* NCDC-307
L. *paracasei* subsp. *paracasei* NCDC-401
L. *helviticus* NCDC-292

**Propagation and Maintenance of *Lactobacillus* Cultures**
All the *Lactobacillus* cultures were propagated in 10 ml sterile de Man-Rogosa-Sharpe (MRS) broth and maintained in litmus milk in refrigerator until use. These were periodically sub-cultured in the same medium once in a week. Each culture was activated by sub-culturing before use and purity was always ascertained by Gram's staining. One set of cultures was stored at -80°C in MRS broth containing 20% glycerol as a stock.

**Preparation of Cell Free Supernatant**
The fresh *Lactobacillus* cultures were inoculated into skim milk @ 2 percent and incubated at 37°C for 10 hours. After incubation, the supernatants of fermented skim milk was obtained by adjusting the pH to 4.6 and centrifugation at 10,000 rpm for 10 min at 4°C (Kubota centrifuge, Tokyo, Japan). The supernatant was collected, filtered through sterilized (0.22 µm membrane filter, Millipore). This supernatant was used for assessing antioxidant activity by ABTS method, ACE inhibitory activity, Casein Phosphopeptides (CPPs) content and protein content by Lowry's Method

**Biofunctional properties of skim milk fermented with *lactobacillus* cultures at 37ºC**
**Antioxidant activity (ABTS method)**
Free radical scavenging activity of the skim milk fermented with *lactobacillus* cultures were determined by ABTS method [3, 4] (Pellegrini *et al.*, 1999; Hernandez-Ledesma *et al.*, 2005). It is a spectrophotometric method based on the reduction of the cation radical of 2,2'-azinobis(3-ethylenebenzothiazoline-6-sulphonic acid) ($ABTS^{\bullet+}$),which is generated by the oxidation of ABTS with potassium persulphate prior to the addition of antioxidant. The antioxidant activity is determined by the reduction of the cation radical as the percentage inhibition of absorbance at 734nm. The absorbance of the reaction mixture of ABTS and antioxidant is compared to that of the trolox standard, and the result is expressed in terms of trolox equivalent antioxidant capacity (TEAC)
Based on the % Inhibition of absorbance of sample, trolox equivalent was determined from standard curve using following equation:

$$y = 0.046x + 3.314$$

**Where**
y: is the % inhibition = [(A 734 $nm_{control}$ – A 734 $nm_{sample}$)/A 734 $nm_{control}$] X 100
x is the µM concentration of trolox
The results were expressed as trolox equivalent antioxidant capacity (TEAC) values i.e. µmol of Trolox equivalence/ mg of the protein.

**Angiotensin converting enzyme (ACE) Inhibition assays**
The assay for the Angiotensin Converting Enzyme (ACE) inhibitory activity based on the liberation of hippuric acid from hippuryl-L-histidyl-L-leucine (HHL) catalyzed by ACE and measured by the method of Cushman and Cheung [5] as modified by Hernandez-Ledesma [4]. The hippuric acid liberated by the ACE was extracted with 1.5 ml ethyl acetate by centrifugation at 3000g for 10min then, heat evaporated at $95^0$C for 10min, redissolved in 1 ml distilled water and measured spectrophotometrically at optical density of 228 nm. The extent of inhibition was calculated as follows:

$$(B-A)/(B-C) \times 100$$

**Where**
A = absorbance in the presence of ACE and ACE inhibitory component,
B = absorbance without ACE inhibitory component, and
C = absorbance without ACE
Inhibition was expressed as the concentration of component that inhibits 50% of ACE activity ($IC_{50}$), and 1unit of ACE inhibitory activity was expressed as the potency showing 50% ACE inhibition under these conditions

**Casein Phosphopeptides (CPPs) content of *Lassi***
Casein Phosphopeptides (cpps) content in the supernatants of skim milk fermented by *lactobacillus* cultures determined out according to the method of Adamson and Reynolds [6]. By adding calcium chloride at 0.5% level to the supernatant and allow it for 10 minutes at room temperature. Add Ethanol 50% (V/V). The precipitate is collected by centrifugation at 5400 rpm for 10 min. The content of CPPs in precipitate was measured spectrophotometrically at 660 nm as per described by Lowry *et al.* [7].

**Results and Discussion**
**Screening of *lactobacilli* cultures for their biofunctional activity**
All biofunctional activities *viz*. antioxidant activity, ACE inhibitory activity and contents of Casein Phosphopeptides were evaluated in the skim milk fermented with twelve strains of lactobacilli as mentioned in the table No. 1. The lactobacilli were grown in skim milk by inoculating 2 per cent of each culture and then incubated at 37ºC for 10 h. The cell free supernatant of the fermented skim milk was prepared after centrifugation as mentioned in material and method section.

**Antioxidant activities of the skim milk fermented with *lactobacillus* cultures**
Antioxidative activity was evaluated by the ability of the cell-free extracts of the fermented skim milk to scavenge the $ABTS^+$ free radical and result expressed as trolox equivalent antioxidant capacity (TEAC) in µM of trolox/mg of protein in the supernatants. Among all these cultures the *Lactobacillus acidophilus* NCDC-15 showed highest scavenging activity (0.65 µM of trolox/mg of protein) followed by *Lactobacillus casei spp. casei* NCDC-17 (0.49 µM of trolox/mg of protein). Other cultures *Lactobacillus helveticus* NCDC-05, *Lactobacillus rhamnosus* NCDC-24, *Lactobacillus delbrueckii ssp. lactis* NCDC-03, *Lactobacillus helveticus*

NCDC-292 and NCDC-288, *Lactobacillus delbrueckii ssp. bulgaricus* NCDC-307, *Lactobacillus paracasei spp. paracasei* NCDC-63 and NCDC-401 and *Lactobacillus delbrueckii ssp. bulgaricus* NCDC-08 showed the antioxidant activity in decreasing order having range 0.43 – 0.10 µM of trolox/mg of protein or peptides, whereas *Lactobacillus fermentum* NCDC-141 showed least antioxidant activity as expressed in table 4.4. This indicates that the antioxidant activity of different species of Lactobacilli is strain specific [8, 9, 1] (Kudoh *et al*., 2001, Ramesh V, 2012 Aparna *et al*., 2010). Virtanen *et al*. [11] investigated the antioxidant activity of 25 lactic acid bacterial (LAB) strains by growing them in milk. They found that six strains of *Leuconostoc mesenteroides* ssp. *cremoris*, *Lactobacillus jensenii* (ATCC 25258) and *Lactobacillus acidophilus* (ATCC 4356) showed the highest radical scavenging activity as well as lipid peroxidation inhibition activity. The development of radical scavenging activity was connected to proteolysis with four strains. They concluded that the development of antioxidant activity was strains specific characteristics.

**Table 1:** Antioxidant activity, ACE inhibitory activity ($IC_{50}$ value), Casein Phosphopeptides contents and Proteolytic activity of the Skim Milk Fermented with 2% *Lactobacillus* Cultures at 37°C for 10h

| Name of Strains | Antioxidant activity (µM of Trolox/ mg of protein in supernatants) | ACE Inhibitory activity ($IC_{50}$ value in µg protein /ml) | Caseinophosphopeptide (mg/ml) | Proteolysis (ug serine/ml) |
|---|---|---|---|---|
| L. *delbrueckii* ssp. *lactis* NCDC-03 | 0.30 ±0.051$^{a}$ | 46.11 ± 0.20$^{ab}$ | 1.50 ± 0.052$^{a}$ | 95.23±0.59$^{a}$ |
| L. *helviticus* NCDC-05 | 0.43 ±0.041$^{b}$ | 41.34 ± 0.55$^{ab}$ | 1.91 ± 0.001$^{b}$ | 135.82±0.34$^{b}$ |
| L. *delbrueckii* ssp. *bulgaricus* NCDC -08 | 0.10 ±0.079$^{c}$ | 67.23 ± 0.56$^{c}$ | 2.32 ± 0.001$^{c}$ | 130.39± 0.40$^{c}$ |
| L. *acidophillus* NCDC-15 | 0.65 ±0.074$^{d}$ | 28.9 ± 0.95$^{a}$ | 0.54 ± 0.007$^{d}$ | 128.74± 0.25$^{d}$ |
| L. *casei* spp. *casei* NCDC-17 | 0.49 ±0.041$^{b}$ | 69.6 ± 0.99$^{c}$ | 2.67 ± 0.001$^{e}$ | 171.72± 0.14$^{e}$ |
| L. *rhamnosus* NCDC-24 | 0.34 ±0.043$^{a}$ | 62.43 ± 0.73$^{bc}$ | 2.72 ± 0.004$^{f}$ | 202.47± 0.68$^{f}$ |
| L. *paracasei* spp. *paracasei* NCDC-63 | 0.13 ±0.051$^{c}$ | 77.44 ± 0.82$^{c}$ | 2.81 ± 0.025$^{g}$ | 149.36± 0.52$^{g}$ |
| L. *fermentum* NCDC-141 | 0.08 ±0.062$^{c}$ | 104.52 ± 0.88$^{d}$ | 2.65 ± 0.000$^{e}$ | 94.59 ± 0.34$^{h}$ |
| L. *helviticus* NCDC-288 | 0.21 ±0.048$^{d}$ | 36.89 ± 0.37$^{ab}$ | 2.16 ± 0.004$^{h}$ | 128.57± 0.03$^{d}$ |
| L. *delbrueckii* ssp. *bulgaricus* NCDC-307 | 0.14 ±0.051$^{c}$ | 49.56 ± 0.92$^{bc}$ | 2.04 ± 0.001$^{i}$ | 110.62 ± 0.18$^{i}$ |
| L. *paracasei* subsp. *paracasei* NCDC-401 | 0.12 ±0.061$^{c}$ | 44.74 ± 0.54$^{ab}$ | 1.48 ± 0.000$^{a}$ | 132.48± 0.17$^{j}$ |
| L. *helviticus* NCDC-292 | 0.24 ±0.061$^{d}$ | 49.15 ± 5.73$^{b}$ | 2.33 ± 0.007$^{c}$ | 127.63± 0.06$^{k}$ |
| CD at 5% level | 0.06 | 10.04 | 0.03 | 0.52 |
| R value | 0.33 | 0.02 | 0.38 | |

The values are Mean ± SE, (n=3)

**ACE inhibititory activities of the skim milk fermented with *lactobacillus* cultures**

The ACE-inhibitory activity for the cell-free extracts of fermented skim milk prepared using twelve lactobacillus strains in skim milk medium was measured by using the method of Cushman and Cheung [5] as modified by Hernandez *et al*. [4] The method is based on the liberation of hippuric acid from hippuryl-L-histidyl-L-leucine (HHL) catalyzed by ACE and is expressed in table No. 1 as the concentration of component that inhibits 50% of ACE activity ($IC_{50}$). The minimum $IC_{50}$ value for ACE-inhibitory activity was shown in the supernatants of skim milk fermented with L. *acidophillus* NCDC-15 (28.9 ± 0.95 µg protein /ml) and maximum $IC_{50}$ value in case of L. *fermentum* NCDC-141 (104.52 ± 0.88 µg protein /ml). The $IC_{50}$ values of other ten lactobacillus strains i.e. L. *helviticus* NCDC-288, L. *helviticus* NCDC-05, L. *paracasei subsp. paracasei* NCDC-401, L. *delbrueckii ssp. lactis* NCDC-03, L. *helviticus* NCDC-292, L. *delbrueckii ssp. bulgaricus* NCDC-307, L. *rhamnosus* NCDC-24, L. *delbrueckii ssp. bulgaricus* NCDC -08, L. *casei spp. casei* NCDC-17 and *L. paracasei spp. paracasei* NCDC-63 were exhibited in the range of 35 - 78 µg protein /ml in the increasing order. The result presented in the table 1 indicated that the ACE-inhibitory activity is a characteristic of the strains.

Similar studies were demonstrated by Anne Pihlanto *et al.* [12], Smacchi and Gobbetti, [13] and Saito *et al*., [14] showing strain specific variation for ACE-inhibitory activity in fermentation of milk with different LAB species such as *Lactobacillus acidophilus, Lactobacillus casei, Lactobacillus helveticus, Lactobacillus jensenii, Lactobacillus reuteri, Lactobacillus rhamnosus, Lactococcus lactis ssp. lactis, Lactococcus raffinolactis, Leuconostoc mesenteroides ssp. cremoris, Lb. delbrueckii* subsp. *bulgaricus, Lactococcus lactis* subsp. *cremoris* FT4, bifidobacteria, and *Streptococcus thermophilus.*

**Casein Phosphopeptides contents in the skim milk fermented with *lactobacillus* cultures**

The separation of Casein Phosphopeptides (CPPs) was done according to the method of Adamson and Reynolds, [6] and the protein contents in precipitate was measured spectrophotometrically at 660 nm as per described by Lowry *et al.* [7] The Casein Phosphopeptides contents of the skim milk fermented with all twelve strains of *lactobacilli* were presented in table 1.

Out of these cultures the L. *paracasei spp. paracasei* NCDC-63 showed highest Casein Phosphopeptides (CPPs) contents (2.81 ± 0.025 mg/ml) (table 1) followed by L. *rhamnosus* NCDC-24 (2.72 ± 0.004 mg/ml). The least CPPs content was observed in supernatants of skim milk fermented with L. *acidophillus* NCDC-15. Other cultures, L. *helviticus* NCDC-292, L. *casei spp. casei* NCDC-17, L. *fermentum* NCDC-141, L. *delbrueckii ssp. bulgaricus* NCDC-307, L. *delbrueckii ssp. bulgaricus* NCDC -08 and *L. delbrueckii ssp. lactis* NCDC-03 showed the CPPs contents more than 2 mg/ml of supernatants (Table 1). Corsetti *et al*., [15] prepared Casein Phosphopeptides using partially purified proteinase from *Lactobacillus helveticus* PR4.

The proteolytic activity of all the twelve cultures were presented in table 1. It was determined by OPA (o-phthaldialdehyde) method of Church *et al.* [16] and expressed in term of ug serine/ml. It has been observed from the table 1 that L. *rhamnosus* NCDC-24 showed maximum proteolytic activity i.e. 202.47 ± 0.68 ug serine/ml followed by L. *casei* spp. *casei* NCDC-17 (171.72 ± 0.14 ug serine/ml). The least proteolytic activity was shown by L. *delbrueckii* ssp. *lactis* NCDC-03 and L. *fermentum* NCDC-141 i.e. 95.23 ± 0.59 and

94.59 ± 0.34 ug serine/ml. All the cultures showed different proteolytic activity. It means all the *lactobacillus* cultures have differ in their proteolytic systems.

The correlation coefficients of the antioxidant activity, ACE inhibitory activity and caseinophosphopeptide contents with proteolytic activity were 0.33, 0.02 and 0.38 as presented in the table 1. It has been observed from the table 1 that all the biological activities of fermented skim milk do not have correlation with proteolytic activities of all the twelve *lactobacilli* used. This indicates that antioxidant activities, ACE inhibitory activities and Casein Phosphopeptides contents of all the cultures are strain specific but not related to the extent of proteolysis. Nielsen *et al.*, [17] reported the similar results with respect to ACE inhibitory activities and the total peptides contents. They established that the ACE inhibitory activity correlated non linearly with the total peptide peak area, due to the presence of both active and inactive peptides. This is probably due to differences between species and strains with respect to the ratio of peptides that inhibit ACE and peptides that do not.

## Conclusion

The *Lactobacillus acidophilus* NCDC-15 showed highest radical scavenging activity (0.65 µM of trolox/mg of protein) and minimum $IC_{50}$ value for ACE-inhibitory activity (28.9 ± 0.95 µg protein /ml). Whereas, L. *paracasei spp. paracasei* NCDC-63 showed highest Casein Phosphopeptides (CPPs) contents (2.81 ± 0.025 mg/ml). The proteolytic activity L. *rhamnosus* NCDC-24 showed maximum i.e. 202.47 ± 0.68 ug serine/ml. The correlation coefficients of the antioxidant activity, ACE inhibitory activity and caseinophosphopeptide contents with proteolytic activity were 0.33, 0.02 and 0.38. The antioxidant activities, ACE inhibitory activities and Casein Phosphopeptides contents of all the cultures are strain specific but not related to the extent of proteolysis. Starter cultures not only play an essential part in the manufacture of fermented dairy products like preservation, aroma production and digestibility but act as a health promoting though production of different biofunctional peptides.

The studied strains with their distinct functional properties to produce the antioxidative, ACE inhibitory and mineral binding (CPPS) peptides may be used for the development of new health promoting fermented dairy products.

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