Studies on bio-functional activity of traditional Lassi

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Lassi is considered as digestive, nutritive and useful in gastrointestinal ailments. In the present study, the survey of traditional lassi from nearby villages of Karnal city was conducted with respect to method of preparation of lassi and the collected samples were analyzed for their physicochemical and bio-functional properties like antioxidant activity, angiotensin-converting enzyme (ACE)-inhibitory activity and caseinophosphopeptides (CPPs) content of traditional Lassi. The acidity, pH, total solids and protein content were 0.56±0.03 (% lactic acid), 3.99 ± 0.06, 5.16± 0.16% and 2.26 ± 0.07% in village sample. The lassi contents mainly strains of Lactobacillus and Lactococcus were 7.97 and 8.14 cfu/ml of lassi. Some samples showed the presence of yeast and mold and coliform. The average ACE inhibitory activity (IC50), antioxidant activity and caseinophosphopeptides (CPPs) content of village samples were 152.53 ± 9.12 (µg/ml), 0.11 ± 0.01 µM TEAC / mg of protein and 0.50 ± 0.05 (mg/ml), respectively. The more antioxidant activity and ACE inhibition was found in simmering treatment than boiling whereas caseinophosphopeptides was observed more in boiling condition (0.56 mg/ml).

Keywords: ACE-Angiotensin converting enzyme, CPPs-caseinophosphopeptides, LAB-Lactic acid bacteria, HHL-hippuryl-L-histidyl-L-leucine, Lassi

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India is diversified country, famous for its tradition, culture in each field because of agro-climatic zone, language, etc. having heritage of traditional fermented food, significantly known for their taste and texture, nutritional and therapeutic value. Some food products of particular region have specialty due to their manufacturing skill, use of special ingredient and recipe are found to be more appreciable in market than comparable products. The significance of traditional knowledge, their combination with scientific know how and the demand from consumers for variety and health conscious have prompted to look for variation in products such as lassi. Lassi is considered as digestive, nutritive and useful in gastrointestinal ailments. It is estimated that approximately 2144 million kg of lassi is being produced in India annually. The composition of lassi varies considerably depending on the method of production, the type of milk used, extent of dilution during churning, and efficiency of fat removal. The composition of lassi is: water 96.2%, fat 0.8%, protein 1.29%, lactose 1.2%, lactic acid 0.44%, ash 0.4%, calcium 0.6% and phosphorus 0.04%. Lassi finds mention in ancient Indian scriptures along with its precursor dahi. Since ancient times, it has been used either with sugar (powdered sugar) or with black salt and zira. The health benefits of lassi are the result of biologically active components that are present in native milk and also, due to their suitably modulated activities produced through the action of lactic acid bacteria, recognition of the immense therapeutic and nutritional value and used for the treatment for diarrhea, dysentery, chronic specific and non specific colitis, piles and jaundice. This practice has the scientific basis as the lassi contains beneficial microorganisms which attach on the intestinal surface and further multiply there. Besides, checking or control the growth of harmful organism it has nutritive properties in the form of vitamins, minerals, amino acids/peptides, etc. The curd from cow milk is considered “Vatanshak” and blood purifier. Lassi is very low in fat but it has large amount of beneficial bacteria or their breakup products in the form of amino acids, peptides, vitamins, minerals, etc. which are useful in human and animal health. Lassi (Butter milk) is known as

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“Tridoshnashak” and found useful in piles and other gastrointestinal disorders. The present study was conducted to correlate the bio-functional properties like antioxidant and ACE inhibitory activity and caseinophosphopeptides (CPPs) content of this traditional product to its health benefits.

Materials and methods

Collection of samples
The traditional butter milk (lassi) samples were collected from the nearby villages located at 25 km away from Karnal (Haryana, India) at early morning and evaluated for physicochemical, microbiological and bio-functional properties. Fifty ml supernatant after centrifugation (1000 rpm/10 min) of each sample was taken and store at 20°C temperature for further study. Data were analysed and expressed as range, mean values with standard error of mean by using SYSTAT software package.

Evaluation of physico-chemical properties of lassi collected from villages

a) pH
The pH of lassi was determined electrometrically with the mains operated pH meter (PHAN, Labindia, India).

b) Titratable acidity
Titratable acidity of lassi samples was determined by the method described in IS:SP:18 (Part XI, 1981).

c) Protein estimation
Protein content in the lassi was determined according to the method of Lowry et al.

d) Total solid and moisture
Total solid (TS) content was determined by the gravimetric method as described in AOAC (2000).

Microbiological analysis of lassi samples collected from villages

The lassi samples were examined for the Lactobacilli count, Lactococcus count, coliform count, yeast and mold count and colony forming units were counted and expressed as log cfu/gm.

a) Lactobacilli count
Enumeration of Lactobacilli count of lassi was done using pour plate method employing MRS agar. The prepared plates were incubated at 37°C for 48-72 hrs.

b) Lactococci count
Enumeration of Lactococcus of lassi was done using pour plate method employing M 17 agar as a medium agar. Plates were incubated aerobically at 30°C for 24 hrs.

c) Coliform count
Enumeration of Coliform count of lassi was done using pour plate method described by Houghtby et al. employing Violet Red Bile Agar (pH 7.4±0.1) and were incubated at 37°C for 48 hrs.

d) Yeast and mold count
Lassi was ascertained for yeast and mold counts as suggested by Marshall using Potato Dextrose Agar and incubated at 30°C for 3-5 days, pH of media was adjusted to 3.5±0.1 using tartaric acid solution.

Bio-functional properties of lassi samples collected from villages

a) Preparation of samples
The supernatants of lassi samples were obtained by adjusting pH to 4.6 and centrifugation at 10,000 rpm for 10 min at 4°C (Kubota centrifuge, Tokyo, Japan). The supernatant was collected, filter sterilized (0.22 µm membrane filter, Millipore). This was used for assessing antioxidant activity, ACE inhibitory activity and caseinophosphopeptides (CPP) content.

b) Antioxidant activity (ABTS method)
Radical scavenging activity of the lassi samples was determined by ABTS method. 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 was compared to that of the trolox standard, and the result is expressed in terms of trolox equivalent antioxidant capacity (TEAC), i.e. µmol of Trolox equivalence/ mg of the protein. Based on the % Inhibition of absorbance of sample, trolox equivalent was determined from standard curve using following equation:

\[ y = 0.046x + 3.314 \]

where;

\[ y: \text{is the } \% \text{ inhibition} = \frac{[(A \, 734 \, \text{nm}_{\text{control}} - A \, 734 \, \text{nm}_{\text{sample}})]}{A \, 734 \, \text{nm}_{\text{control}}} X 100 \]

x is the µM concentration of trolox

c) 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 & Cheung as modified by Hernandez-Ledesma et al. The hippuric acid liberated by the ACE was extracted with 1.5 ml ethyl acetate by centrifugation at 3000 gm for 10 min then, heat evaporated at 95°C for 10 min, redissolved in 1 ml distilled water and measured spectrophotometrically at optical density of 228 nm. The extent of inhibition was calculated as follows:

\[
\frac{(B-A)}{(B-C)} \times 100
\]

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 (IC50), and 1 unit of ACE inhibitory activity was expressed as the potency showing 50% ACE inhibition under these conditions.

d) Caseinophosphopeptides (CPPs) content of Lassi

Caseinophosphopeptides (cpps) content in the lassi was determined according to the method of Adamson & Reynolds by adding calcium chloride at 0.5% level to the supernatant and allowing it for 10 minutes at room temperature. Add Ethanol 50% (V/V). The precipitate was collected after centrifugation at 5400 rpm for 10 min. The content of CPPs in precipitate was measured spectrophotometrically at 660 nm as described by Lowry et al.

Results and discussion

Survey study of traditional lassi regarding its preparation

The survey regarding the preparation condition of lassi was conducted with respect to the heating time and temperature before culturing, rate of culturing, time and temperature of incubation, method of churning and hygiene practices. The condition of pre-heat treatment given to milk is as per Fig. 1. The results are presented in the Table 1.

From the survey study it has been observed that the lassi in villages nearby Karnal district is prepared by subjecting milk to the pre-heat treatment. These pre-heat treatments include either by heating the milk to 75-95°C for 1-10 hrs or boiling of milk for 2-10 minutes (Table 1 & Fig.1). During the preparation of lassi heat treatment given to milk ranged from pasteurization to boiling as mentioned by Aneja et al. But during its mechanized production the milk is heated 90°C for 15 minutes (Aneja et al.). Rate of addition of culture was similar as in case of the mechanized process, i.e. 1 - 2 %. The incubation temperature and time also have a wide range, i.e. 25 to 40°C for 8-12 hrs. The churning time and temperature also differed which may depend on the type of stirrer (churning device).

Physicochemical, microbiological and biofunctional properties of lassi samples collected from the villages

These samples were evaluated for their physicochemical properties, microbiological count and biofunctional properties.

Physicochemical properties of lassi samples collected from the villages

Total solid in both types of lassi samples prepared with simmering and boiling treatment was in the range 3.48 – 6.8 % (5.34±0.23) and 4.12 – 6.46 % (5.09±0.20), respectively (Table 2). Similarly, the average protein contents in the both samples were found to be 2.27 ± 0.12 and 2.25 ± 0.10 (Table 2). There is no significant difference between the per cent total solid and protein of both the samples. But the acidity of both type of samples differ significantly. The mean acidity was observed as 0.64±0.06 and 0.54±0.03 % of lactic acid for samples prepared with

<table>
<thead>
<tr>
<th>Treatments</th>
<th>Simmering (n = 9)</th>
<th>Boiling (n = 21)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Heating temperature (°C)</td>
<td>75-95 (86.11 ± 2.98)</td>
<td>boiling</td>
</tr>
<tr>
<td>Heating time (Min)</td>
<td>60-600 (393.33 ± 74.22)</td>
<td>2-10 (4.38 ± 0.49)</td>
</tr>
<tr>
<td>Addition of culture @ in per cent</td>
<td>0.7-2 (1.3 ± 0.18)</td>
<td>0.5-2.5 (1.62 ± 0.10)</td>
</tr>
<tr>
<td>Incubation Temp. (°C)</td>
<td>29-40 (34.33 ± 1.42)</td>
<td>25-40 (27.48 ± 1.27)</td>
</tr>
<tr>
<td>Incubation Time (Hrs)</td>
<td>9-11 (9.78 ± 0.22)</td>
<td>8-12 (10.05 ± 0.19)</td>
</tr>
<tr>
<td>Churning Temp. (°C)</td>
<td>25-36 (31.88 ± 0.64)</td>
<td>28-36 (33.09 ± 0.40)</td>
</tr>
<tr>
<td>Churning Time (Min.)</td>
<td>6-25 (13.66 ± 1.95)</td>
<td>19-28 (23.19 ± 0.57)</td>
</tr>
</tbody>
</table>

Table 1—Processing parameters of Lassi (butter milk) collected from villages
Simmering and boiling treatment, respectively (Table 2). This may be due to that during prolong heating lactose degrade to organic acid rather than lactic acid\(^1\). Secondly, the precipitation of calcium phosphate and dephosphorylation of casein also lead to increase in titratable acidity\(^1\). The variation in acidity and pH indicates the difference in rate of addition of starter culture, type of strains, incubation period and other processing methods practiced by the farmers. There is no significant difference in pH of both the samples (Table 2).

### Microbiological analysis of lassi samples collected from villages

The mean value of lactobacillus, lactococcus, yeast and mold and coliforms counts are presented (Table 3). Both Lactobacillus and Lactococcus were among the dominant microflora of the all most all the lassi samples. There was no significant difference in the Lactobacillus, Lactococcus and yeast and mold counts in both type of lassi. However, as predicted from the Fig. 2 the microbial population of the lassi samples collected from the villages showed different trends with respect to their Lactobacillus, Lactococcus, yeast and mold and coliforms counts. Out of the total 30, 20 samples had Lactococcus, nine samples had Lactobacillus and one sample had yeast and mold as predominant microflora. The yeast and mold were absent in eight samples while twenty samples were devoid coliform. The absence of coliform in most of the samples indicates maintenance of hygienic condition during preparation of this product at household level.

### Bio-functional properties of lassi samples collected from villages

Fermented milk products, in addition to providing both energy and nutrients, are an excellent source of bioactive peptides. These may be due to the antibacterial, anticancer, immunomodulatory, mineral
binding, opioid, antioxidative and antihypertensive peptides present in these products. Thus, the samples of lassi collected from the different villages were analyzed for their bio-functional characteristics which include antioxidative activity, ACE inhibitory activity and contents of mineral binding peptides, i.e. caseinophosphopeptides.

**Antioxidant activity**

The antioxidant activity of lassi samples was studied with ABTS. The samples were centrifuged and supernatant was filtered through 0.22 micron filter to remove the bacterial cell from the samples which contributes towards the antioxidant activity.\(^\text{17}\)

The antioxidant activity (Mean + SEM) of the lassi samples prepared with simmering pre-heat treatment was more than double when compared with the lassi prepared with boiling pre heat treatment, i.e. 0.19 ± 0.02 and 0.074 ± 0.01, respectively (Table 4). Similarly, when the antioxidative activities of all the samples were compared as presented in Fig. 3, the samples with simmering pre-heat treatment exhibited more antioxidant activity as compared with boiled one except the sample No. 15. This may be due to the released of –SH group and formation of some brown pigment during simmering which may also contribute towards antioxidative properties. Similar observations were made by Costa et al.\(^\text{18}\), who reported that heat treatment causes exposure of –SH group which increases the antioxidant properties. Taylor & Richardson\(^\text{19}\) and Tong et al.\(^\text{20}\), also reported that the antioxidant activity may increase as a consequence of thermal treatments, due to the protein unfolding and exposure of thiol group potentially acting as hydrogen donors. The difference in the antioxidant activity of all the samples (Fig. 3) of lassi may be attributed to the difference in the microflora of all the lassi samples (Fig. 2). Virtanen et al.\(^\text{21}\), also reported that the development of antioxidative activity was dependant on the strain used and in general increased during fermentation. Osuntoki & Korie\(^\text{22}\) compared the antioxidative activity of milk fermented with strains of Lactobacillus casei, Lactobacillus delbrueckii, Lactobacillus brevis, L. plantanum and L. fermentum isolated from indigenous Nigerian fermented foods. They concluded that the antioxidant activity in the fermented milk was strain dependant. Sabeena Fravin et al.\(^\text{23}\), found the antioxidant activity in the yoghurt fermented of milk by lactic acid bacteria and suggested that the higher oxidative stability of yoghurt might be due to antioxidant

![Fig. 2—Microbial count of villages lassi samples](image)

![Fig. 3—Antioxidant activity of supernatants of village lassi sample](image)

<table>
<thead>
<tr>
<th>Treatments</th>
<th>Protein in supernatants (mg/ml)</th>
<th>Antioxidant (TEAC (µmol)/mg of protein)</th>
<th>ACE Inhibitory (IC(_{50}) µg/ml)</th>
<th>CPPs (mg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Simmering (n=9)</td>
<td>3.85 ± 0.60 (^\text{a}) (2.49 – 8.46)</td>
<td>0.19 ± 0.02 (^\text{a}) (0.10 - 0.30)</td>
<td>75.93 ± 2.94 (^\text{a}) (40.11 - 158.12)</td>
<td>0.36 ± 0.06 (^\text{a}) (0.11 - 0.61)</td>
</tr>
<tr>
<td>Boiling (n=21)</td>
<td>2.82± 0.11 (^\text{b}) (2.08 – 4.00)</td>
<td>0.074 ± 0.01 (^\text{b}) (0.02 - 0.13)</td>
<td>185.36 ± 3.43 (^\text{b}) (38.81 - 453.27)</td>
<td>0.56 ± 0.06 (^\text{b}) (0.13 - 1.13)</td>
</tr>
<tr>
<td>Over all (n=30)</td>
<td>3.13 ± 0.21 (2.08 – 8.46)</td>
<td>0.11 ± 0.01 (0.02 - 0.30)</td>
<td>152.53 ± 9.12 (38.81 - 453.27)</td>
<td>0.50 ± 0.05 (0.11 - 1.13)</td>
</tr>
<tr>
<td>CD at 5 %</td>
<td>0.56</td>
<td>0.04</td>
<td>49.28</td>
<td>0.13</td>
</tr>
</tbody>
</table>

\(n = \) (no of samples) The values expressed as Means ± SE and values with different small letters superscripts Column wise differ significantly at 5% level of significance (\(P \leq 0.05\)).
peptides released during the fermentation of milk by lactic acid bacteria and/or by the lower oxygen content of yoghurt, which subsequently reduces the oxidative stress of fish oil incorporated in the yoghurt.

**Angiotensin converting enzyme (ACE) inhibitory activity**

In the preparation of fermented milk products during fermentation of milk, the cell wall associated proteinases of lactic acid bacteria hydrolyse casein in to large peptides, which are then transported into the cell and broken down by intracellular peptidases, resulting in a range of peptides. The ACE-inhibitory activity of the lassi obtained from villages was measured in the form of percent inhibition and IC50 values by the method of Cushman & Cheung as modified by Hernandez-Ledesma et al.

The ACE-inhibitory activities (IC50 values) of lassi samples ranged from 38.81 - 453.27 µg/ml (Fig. 4). As predicted in Fig. 4 the ACE-inhibitory activity (IC50 values) of the lassi samples collected from villages and prepared by simmering pre heat treatment (range 40.11 - 158.12 µg/ml) was more as compared to the samples with boiling as pre heat treatment (range 38.81 - 453.27 µg/ml). Because more the IC50 values less will be the ACE inhibitory activity and vice-versa.

Similarly, the mean ACE-inhibitory activity was more in case of simmering heat treatment as compared to boiling (Table 4). This indicates that the ACE-inhibitory activity of lassi also effected by the intensity of pre-heat treatment. Similar observations were made by Gul & Sibel. Their result showed that the 30 min. heat treatment caused a decreased in ACE inhibitory activity while with 50 min. heat treatment ACE inhibitory activity increased. The degree of heat treatment of milk is also having greater significance during the manufacture of fermented milk products as it influences the metabolic activity of different starter cultures. Krishna & Shankar noted maximum metabolic activities of L. lactis subsp. lactis, L. lactis subsp. diacetylactis, L. lactis subsp. cremoris, S. thermophilus, L. delbrueckii subsp. bulgaricus and combined L. lactis subsp. lactis and L. lactis subsp. cremoris in cow milk heated for 30 min at 80°C, 80–90°C, 70°C, 70–90°C, 121°C and 90°C, respectively. The enhanced metabolic activity may lead to enhanced production of proteolytic enzymes, which further degrade the milk proteins in to ACE inhibitory peptides. The sample No. 15 showed minimum inhibitory concentration as compared to other samples which may be attributed to the strains of starter culture used in the preparation of lassi. These results were supported by the finding of Anne who reported that ACE inhibitory activities of the milk fermented with 25 different lactic acid bacteria differed significantly and depended on the strains used. The lassi samples collected from the different villages differed in the microfloras which further determine different ACE inhibitory activities (Figs. 2 & 4).

**Caseinophosphopeptides contents**

Caseinophosphopeptides (CPPs) are mineral binding peptides, fragments of casein, generated in fermented milk products due to the proteolytic system of lactic acid bacteria. Fermented milk develops various new constituents while undergoing fermentation and casenophosphopeptides among these constituents. CPPs have always been a widely studied peptides group in dentistry. These peptides also have been researched in the area of sports, medicine, remineralisation, immunoenhancement and immunomodulation. The CPPs contents were higher in the lassi prepared from milk given boiling treatment (0.56 ± 0.06 mg/ml) than the lassi prepared with simmering heat treatment to milk before incubation(0.36 ± 0.06mg/ml) (Table 4).

The total soluble protein which may contain the different peptides in both types of lassi has also been present in the Table 4. The CPPs contents of simmering heat treatment samples were approximately 1/10 of the total soluble proteins while CPPs contents of boiled milk lassi samples were founded approximately 1/5 of the total soluble proteins in the supernatants (total peptides).

Similar results are depicted in the Fig. 5, where the CPPs contents of individual samples are presented. Some exceptions were there which may be due to the different microbiological strains used for lassi
Caseinophosphopeptides content in supernatants of milk can serve as an innovative and useful tool to generate the bioactive peptides in fermented milk. Traditional knowledge of fermentation is easy and cost-effective method to increase the bioactive peptides in fermented milk products. The possibilities of designing new dietary products depend on the traditional knowledge with its holistic and systematic approach supported by research finding. The decrease in bioactive peptides content in simmering heat treated samples may be due to the dephosphorylation of casein which produces less quantity of phosphopeptides on fermentation. Because in simmering treatment, the milk was heated at high temperature for long time as compared than the boiling treatment.

Traditional significance of study

Bioactive peptides found in traditional lassi have various physiological activities such as antihypertensive and antioxidative and it also contain caseinophosphopeptides (CPPs). Its application has only become available in the last few decades, it is now possible for researchers to custom produce fermented foods with not only specific flavor and other nutritional properties, but that also impart that benefit consumers for functional, health imparting characteristics. The question of what kinds of bioactive foods are beneficial and desirable as food constituents or as drugs should be carefully examined. The possibilities of designing new dietary products and drugs to help reduce or control diet-related chronic disease look promising. ACE inhibitory peptides, immunomodulating peptides, and caseinophosphophopeptides are the most favourite bioactive peptides for application to foodstuffs formulated to provide specific health benefits. ACE inhibitory activity and caseinophosphopeptides. As the more protein content in supernatants of lassi given simmering treatment indicated that more peptides were formed in it due to the partially denaturation of milk protein, resulted in more proteolysis and could be reason for released of more peptides during fermentation. Science from last few decades the researchers are focusing for the development of food having medicinal properties along with nutrition carrier like Yakut in Japan.

Conclusion

The traditional knowledge with its holistic and systematic approach supported by research finding can serve as an innovative and useful tool for the development of functional drinks like Lassi. The functional properties of traditional lassi depend on type of culture, incubation period and temperature and pretreatment of milk like heating. The significant differences (P>0.05) were observed between lassi samples prepared by giving pre heat treatment to milk, i.e. simmering and boiling treatment before incubation for antioxidant activity, ACE Inhibition activity and caseinophosphopeptides. As the more protein content in supernatants of lassi given simmering treatment indicated that more peptides were formed in it due to the partially denaturation of milk protein, resulted in more proteolysis and could be reason for released of more peptides during fermentation. Science from last few decades the researchers are focusing for the development of food having medicinal properties along with nutrition carrier like Yakut in Japan.

Acknowledgement

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References


