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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, *p*H, 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 presences of yeast and mold and coliform. The average ACE inhibitiory activity (IC_{50}), antioxidant activity and caseinophosphopeptides (CPPs) content of village samples were 152.53 ± 9.12 (µg/ml), 0.11 ± 0.01µM 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[1]. 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%[2]. *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[3]. 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 considred "*Vatanashak*" 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[3].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 20^0C 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 physicho-chemical properties of *lassi* collected from villages

a) *p*H

The *p*H of *lassi* was determined electrometrically with the mains operated *p*H 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)[4].

c) Protein estimation

Protein content in the *lassi* was determined according to the method of Lowry *et al.*[5].

d) Total solid and moisture

Total solid (TS) content was determined by the gravimetric method as described in AOAC (2000)[6].

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[7]. The prepared plates were incubated at 37^0C 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[8]. Plates were incubated aerobically at 30^0C for 24 hrs.

***c) Coliform* count**

Enumeration of *Coliform* count of *lassi* was done using pour plate method described by Houghtby *et al.*[9] employing Violet Red Bile Agar (*p*H 7.4±0.1) and were incubated at 37^0C for 48 hrs.

d) Yeast and mold count

Lassi was ascertained for yeast and mold counts as suggested by Marshall[10] using Potato Dextrose Agar and incubated at 30^0C for 3-5 days, *p*H 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 *p*H to 4.6 and centrifugation at 10,000 rpm for 10 min at 4^0C (Kubota centrifuge, Tokyo, Japan). The supernatant was collected, filter sterilized (0.22 m 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[11,12]. 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: is the % inhibition = $[(A\ 734\ nm_{control} - A\ 734\ nm_{sample})/A\ 734\ nm_{control}] \times 100$

x is the M 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[13] as modified by Hernandez-Ledesma *et al.*[12].The hippuric acid liberated by the ACE was extracted with 1.5 ml ethyl acetate by centrifugation at 3000 gm for 10min then, heat evaporated at 95^0C 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) x 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

d) Caseinophosphopeptides (CPPs) content of *Lassi*

Caseinophosphopeptides (cpps) content in the *lassi* was determined according to the method of Adamson & Reynolds[14] 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.*[5].

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 preheat 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 includes either by heating the milk to 75-95^{0}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.*[15]. But during its mechanized production the milk is heated 90^0C for 15 minutes (Aneja *et al.*[15]). 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 ^{0}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 bio-functional 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 1—Processing parameters of *Lassi* (butter milk) collected from villages

Treatments	Simmering (n = 9) range (Mean)	Boiling (n = 21) range (Mean)
Heating temperature (^{0}C)	75 -95 (86.11 ± 2.98)	boiling
Heating time (Min)	60-600 (393.33 ± 74.22)	2-10 (4.38 ± 0.49)
Addition of culture @ in per cent	0.7-2 (1.3 ± 0.18)	0.5-2.5 (1.62 ± 0.10)
Incubation Temp.(^{o}C)	29-40 (34.33 ± 1.42)	25-40 (27.48 ± 1.27)
Incubation Time (Hrs)	9-11 (9.78 ± 0.22)	8-12 (10.05 ± 0.19)
Churning Temp.(^{o}C)	25-36 (31.88 ± 0.64)	28-36 (33.09 ± 0.40)
Churning Time (Min.)	6-25 (13.66 ± 1.95)	19-28 (23.19 ± 0.57)

n= (no of samples)

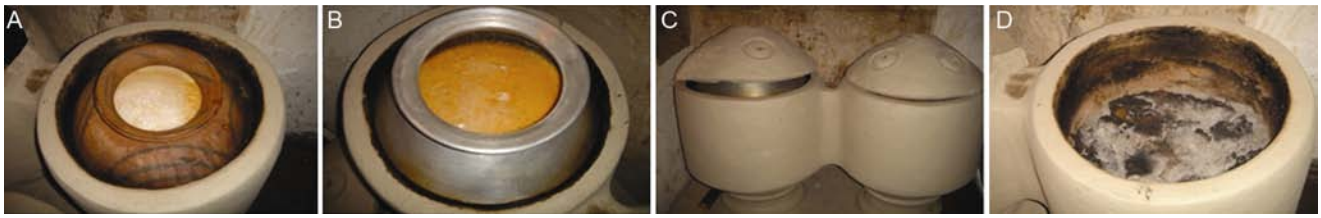

Fig. 1—Simmering of milk in earthen chamber, A- Earthen pot, B-Aluminum pot, C- Closed earthen chamber (*Hara*) and D- Open earthen chamber (*Hara*) at village level.

Table 2—Physicochemical properties of *Lassi* (butter milk) collected from villages

Treatments	T.S (%)	Moisture (%))	Protein (%)	Acidity (% of lactic acid)	*p*H
Simmering (n=9)	5.34±0.23[a]	94.66 ± 0.23[a]	2.27 ± 0.12[a]	0.64±0.06[a]	3.98 ±0.08[a]
	(3.48-6.8)	(93.2-96.52)	(2.25-6.55)	(0.32-0.82)	(3.24-4.7)
Boiling (n=21)	5.09±0.20[a]	94.91 ± 0.20[a]	2.25 ± 0.10[a]	0.54±0.03[b]	4.00±0.12[a]
	(4.12-6.46)	(93.54-95.88)	(3.80-5.83)	(0.36-0.94)	(3.41- 4.63)
Over all (n=30)	5.16± 0.16	94.84 ± 0.16	2.26 ± 0.07	0.56±0.03	3.99 ± 0.06
	(3.48-6.8)	(93.2-96.52)	(2.25-6.55)	(0.32-0.94)	(3.24-4.7)
CD	0.46	0.46	0.22	0.07	0.19

n= (no of samples) The values expressed as Means ± SE(range) and values with different small letters superscripts row wise differ significantly at 5% level of significance ($P \leq 0.05$)

Table 3—Microbial count of *Lassi* (butter milk) collected from villages

Treatments	Microbial count (cfu/ml)			
	Lactobacillus	*Lactococcus*	Yeast & Mold	E. Coli
Simmering (n= 9)	8.01 ± 0.11[a]	8.10 ± 0.104[a]	2.12 ± 0.718[a]	1.52±0.488[a]
Boiling (n=21)	7.95 ± 0.107[a]	8.15 ± 0.043[a]	2.94 ± 0.29[a]	0.36±0.197[b]
Over all (n=30)	7.97 ± 0.081	8.14 ± 0.043	2.69 ± 0.291	0.71± 0.220
CD at 5 %	0.24	0.13	0.83	0.57

n= (no of samples) The values expressed as Means ± SE and values with different small letters superscripts row wise differ significantly at 5% level of significance (P≤0.05)

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[16]. Secondly, the precipitation of calcium phosphate and dephosphorylation of casein also lead to increase in titratable acidity[16]. The variation in acidity and *p*H 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 *p*H 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. caseinophoshopeptides.

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[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.*[18], who reported that heat treatment causes exposure of –SH group which increases the antioxidant properties. Taylor & Richardson[19] and Tong *et al.*[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.*[21], also reported that the development of antioxidative activity was dependant on the strain used and in general increased during fermentation. Osuntoki & Korie[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.*[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

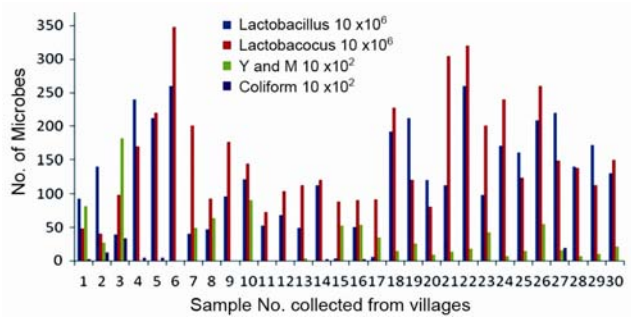

Fig. 2—Microbial count of villages *lassi* samples

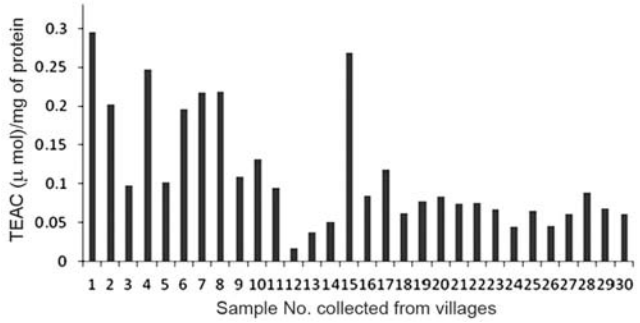

Fig. 3—Antioxidant activity of supernatants of village *lassi* sample

Table 4—Bio-functional properties of traditional *Lassi* collected from villages

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

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≤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 IC_{50} values by the method of Cushman & Cheung as modified by Hernandez-Ledesma *et al.*

The ACE-inhibitory activities (IC_{50} values) of *lassi* samples ranged from 38.81 - 453.27 μg/ml (Fig. 4). As predicted in Fig. 4 the ACE-inhibitory activity (IC_{50} 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 IC_{50} 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[24]. 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

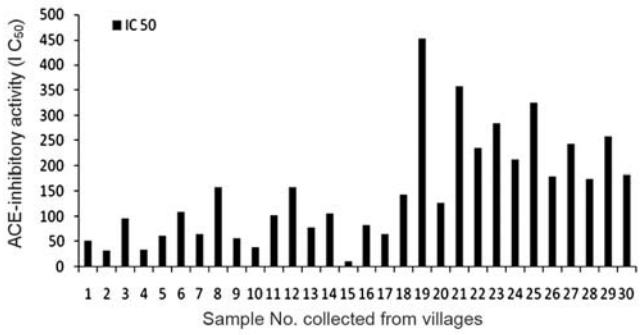

Fig. 4—ACE-inhibitory activity of supernatants of village *lassi* samples

it influences the metabolic activity of different starter cultures. Krishna & Shankar[25] 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 80^0C, 80–90^0C, 70^0C, 70–90^0C, 121^0C and 90^0C, 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[26] 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*

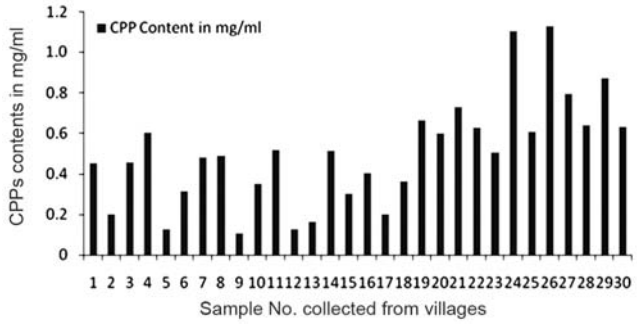

Fig. 5—Caseinophosphopeptides content in supernatants of village *lassi* samples

preparation by different farmers (Fig. 2).The decrease in CPPs contents in simmering heat treated samples may be due to the dephosphorylation of casein which produces less quantity of phosphopeptides on fermentation[19]. 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 decade, 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. The results suggest that the bioactive peptides in fermented milk products can be increased using controlled fermentation and proteolytic starter strain. Fermentation is easy and cost effective method to generate the bioactive peptides in fermented milk products.

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.

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