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In-vitro and *in-vivo* bioavailability of iron and zinc from milk and dahi fortified with iron and zinc

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Abstract
Milk composed of many essential minerals required by the body and is one of the main nutrition for mankind especially for infants. The absorption of iron appears to be enhanced by fermentation, presumably because of the presence of organic acids, including lactic acid. Dahi is a fermented milk product obtained by lactic acid fermentation through the action of starter cultures viz., *Streptococcus lactis, Streptococcus diacetylactis* and *Streptococcus thermophilus* either singly or in combination with *Leuconostoc* species and *Lactobacillus* species with one or more of other optional ingredients such as sugar. It is one of the oldest popular foods of the world because of its nutritional and therapeutic value in the human diet. It is highly recommended to the lactose intolerants, because of the reduced lactose content. Bio-availability is preferably determined by *in-vivo* tests, but these are expensive, labor-intensive, time consuming. Human studies to study the bio-availability of minerals are proved to be costly affair and time consuming. As an alternative, *in-vitro* 34 methods can be used to predict bio-availability of nutrients from foodstuffs. The *in-vitro* methods that are currently in use simulate gastro-intestinal stimulation and then determine the amount of soluble mineral or the amount of mineral that has permeated a membrane of specific pore size (Jim Kling., 2001). In the current study, an attempt was made to fortify with iron and zinc by using ferrous lactate, zinc sulphate @ 40 mg/kg milk. From the present study, The bio-availability of iron from fortified samples was higher in case of *in-vivo* method than in *in-vitro* method (iron 8.52 versus 8.1percent for milk and 14.2 versus 14.02 percent for dahi). The bio-availability of zinc was higher in case of *in-vivo* method than in *in-vitro* method (zinc 2.71 versus 2.63 percent for milk and 5.25 versus 5.2 percent for dahi).

Keywords: Iron, zinc, *in-vitro* methods, bioavailability and *in-vivo* methods

Introduction
Fermented milk products reportedly have therapeutic, antihypercholesterolemic, anticarcinogenic and anticariogenic properties beyond their basic nutritive value. Dahi is the most important fermented milk product used in India from time immemorial. The popularity of dahi is not only due to its refreshing taste and palatability but also due to its scientifically proven role as a nutritious fermented milk product. In Indian system of medicine (Ayurveda), dahi has been strongly recommended for curing ailments like dyspepsia, dysentery and other gastrointestinal disorders. This product is also believed to improve appetite and vitality.

Iron as an essential trace element participates as catalyst in several metabolic reactions. As a component of hemoglobin, myoglobin, cytochrome and other proteins, iron plays an important role in the transport, storage and utilization of oxygen. It is also co-factor of many enzymes (Bates and Prentice, 1996) [1]

Micromineral especially zinc play a pivotal role in various physiological processes. It is the most important mineral for body metabolism, part of many enzymes like retinal dehydrogenase and alkaline phosphatase (Samy, 2010) [6]. Zinc is an essential nutrient for health and very important for growth, normal functioning of immune system and other physiological processes. It is also a component of the hormone insulin. Its deficiency results in growth retardation, delayed wound healing and hypogonadism.

Materials and Methods
The procedure for preparation of dahi by Khedkar *et al.* (2003) [5] was adopted with suitable modifications. Milk obtained from Student Experimental Dairy Plant (SEDP), of Karnataka Veterinary Animal and Fisheries Sciences University (KVAFSU), of Hebbal, Bengaluru-24. For standardization of cow milk (fat 3.5% and SNF 8.5%), Nandini cream and spray dried Nandini skim milk powder were used.

The standardized milk was preheated to 60 °C and homogenized (at 2500 psi and 500 psi). Then milk was heated to 95 °C for 15 min. During heating milk was stirred continuously with the help of stirrer to avoid formation of cream layer, cooled to 37 °C, divided into two equal portions: The first portion served as control. The second portion was fortified separately with zinc sulphate (Food grade: Merck Chemicals. Germany) at a level of 20, 30 and 40 mg zinc/ kg milk. Milk was inoculated with desirable proportion of starter culture (*Streptococcus lactis, streptococcus diacetylactis* along with the species of *Lactobacillus* such as *Lactobacillus bulgaricus* and *Streptococcus thermophilus* in the ratio of 1:1) at the level of 1%. Milk along with culture added was filled into clean polystyrene cups. The samples were incubated at 30±1 °C until curd formation/coagulation (17 hr). Thus, obtained dahi samples were stored at 5±1 °C until they are used for experiments.

pH of the sample was measured using a digital pH meter (Chemi line Pvt. Ltd.) at 25 °C. Acidity, protein, fat, total solids, ash and moisture content of dahi was estimated as per the standard procedure IS: SP 18 (Part XI) 1981. MSNF content in milk was computed by using ISI Lactometer by using the following formula

SNF (%) = 0.25 CLR+0.2F+0.35

Where,
CLR-Corrected Lactometer Reading
F-Fat (content in percent)

Prepared dahi samples were given to a panel of five judges for sensory evaluation. Each judge was supplied with standard score card of a total of 25 points for assessing the degree of firmness, whey separation, body and texture, flavour.

The results (average of 3 trials) are analyzed statistically for test of significance by using ANOVA as per SPSS10.0 software package and MS Excel 2007.

In-vitro bio-availability of minerals (Fe2+ and Zn2+) in Dahi

Dialysis method of Bosscher *et al*., (2001) [2] was employed to determine the bio-availability of minerals.

The prime steps involved in this method are as follows

Intraluminal Digestion Phase

10 g of sample was mixed with 80 ml water in a 250 ml beaker. The pH was adjusted to 2.0 by adding 6 M Hcl. The pH was checked after 15min and if necessary readjusted. Freshly prepared 16% pepsin solution (3 ml) was added and the sample was made up to 100 ml with distilled water. After mixing, the sample was incubated at 37 °C in a shaking water bath at 1200 Strokes /min for two hours, the gastric digests were stored in ice for 90 min.

Determination of Total Titratable Acidity

Titratable acidity was measured by taking homogenous pepsin digest (20 ml) at 20± 1 °C and five ml of freshly prepared 3:7 pancreatic bile mixture was added. The pH was adjusted to 7.5 with 0.5 M NaOH. After an equilibrium period time of 30min, the pH was checked and readjusted to original pH if necessary. The number of equivalents of 0.5 M NaOH required to titrate the amount of gastric digest to pH 7.5 was calculated.

Pancreatin Digestion

20 g homogenized pepsin digest was weighed into wide necked conical flasks, which was placed in a water bath at 37 °C for 5 min. Segments of dialysis tubing (MWCO 10-12 KDa) containing 25 g water and sodium bicarbonate being equivalents to the measured titratable acidity was placed into a wide necked conical flask were added to pepsin digest. Then seal the flask with aluminium foil and incubated in shaking water bath at 37 °C with continuous agitation (1200 strokes/min) until pH was about 5 (approximately 30 min). Afterwards 5g Pancreatin–bile extract mixture was added to digest, the digest was incubated in a shaking water 52 bath for another 2 hrs. at 37 °C. At the end of incubation period the pH was measured. The dialysis bags were rinsed with water, the volume of dialysate was noted down. Minerals content (Fe2+ and Zn2+) in the dialysate was estimated by means of Atomic Absorption Spectrophotometer (AAS).

Calculation

The availability of iron and zinc was calculated from the amount of element that passed through the dialysis membrane related to the total element content of the original food sample

Availability (%) = (D-B)/W*A X100

Where,
D= The total content of element in the dialysate (in mg)
B= The amount of micronutrient (mg) in the blank dialysate after digestion
W= Weight of food sample used for intestinal stage
A= Concentration of element in the food sample

In-vivo Studies to Ascertain the Extent of bio-availability of added minerals (Fe2+ and Zn2+)

In Dahi Bio-availability of added minerals as well as bioassay in dahi was conducted in albino rats. Thirty six male albino (Wister strain) rats aged between 5-6 weeks with a body weight ranging from 100-110 g were procured from the Indian Institute of Science, Bangalore. Animals were housed individually in a small animal house with 12-12 h light/dark cycle at an ambient temperature 20-25 °C. The rats were weighed and randomly assigned to six groups of six rats each. They were acclimatized to the experimental conditions for seven days. They were maintained in individual cages, food and water were given ad libidum for a period of 28 days. Gain in body weight was recorded at weekly intervals and All thirty rats were maintained under ideal standard laboratory hygienic conditions of care and maintenance.

Feeding schedule

Animals were divided into 6 groups, groups were segregated as:

1) Control group: Fed with ad libidum rat feed
2) Milk fed group: Fed with reconstituted non-fermented milk with 3.5% fat
3) Control dahi fed group: Fed with dahi
4) Iron fortified dahi fed group: Fed with iron fortified dahi
5) Zinc fortified dahi fed group: Fed with zinc fortified dahi
6) Iron and Zinc both fortified dahi fed group: Fed with iron and zinc fortified dahi

All the groups were provided with water ad libidum. And fortified dahi similarly along with control sample transformed

into pellets those pellets are fed to experimental animals (Dhar, 1959) [3].

Results and Discussion

In-vitro and *in-vivo* method for bio-availability of iron from milk and dahi

It is evident from the values of the table (1) that the *in-vitro* method of bioavailability of micro-mineral namely iron was compared with *in-vivo* method. It is clear from the values that the bio-availability which is expressed in terms of percentage was more from fortified samples than unfortified ones. Unfortified milk exhibited a bio-availability of iron to the tune of 7.01 percent whereas the bio-availability for fortified milk is 8.1. Similarly, unfortified dahi exhibited a bio-availability of iron to the tune of 10.5 percent whereas the bio-availability for fortified dahi is 14.02 percent. The maximum.

Bio-availability of iron from fortified dahi was observed (14.02) whereas the bio-availability from fortified milk is only 50 percent (8.1) when compared to dahi. Similarly, in *in-vivo* method also, the unfortified milk exhibited lesser bio-availability when compared with fortified samples. For unfortified milk the bio-availability of iron is 7.05 percent whereas the bio-availability for fortified milk is 8.52. The corresponding values for, unfortified dahi were 10.62 percent and the bioavailability for fortified dahi is 14.2 percent. The maximum bio-availability of iron from fortified dahi was observed (14.2) whereas, the bio-availability from fortified milk is only 40 percent when compared to dahi. From the results it is very much clear that the bio-availability of iron from fortified samples was higher in case of *in-vivo* method than in *in-vitro* method (iron 8.52 versus 8.1 percent for milk and 14.2 versus 14.02 percent for dahi).

In-vitro and *in-vivo* method for bio-availability of zinc from milk and dahi

It is evident from the values of the table (2) that *in-vitro* method of bio-availability of micro-mineral namely zinc was compared with *in-vivo* method. It is clear from the values that the bio-availability which is expressed in terms of percentage with respect to both *in-vitro* and *in-vivo* method was more from fortified samples than unfortified ones. Unfortified milk exhibited a bio-availability of zinc to the tune of 1.30 percent whereas the bio-availability for fortified milk is 2.63. Similarly, unfortified dahi exhibited a bio-availability of zinc to the tune of 3.68 percent whereas, the bio-availability for fortified dahi is 5.2 percent. The maximum bio-availability of zinc from fortified dahi was observed (5.2) whereas the bio-availability from fortified milk is less (2.63) when compared to dahi. Similarly, in *in-vivo* method also, the unfortified milk exhibited lesser bio-availability of zinc when compared with fortified samples. For unfortified milk the bio-availability of zinc is 1.36 percent whereas the bio-availability for fortified milk is 2.71. The matching values for unfortified dahi were 3.75 percent whereas the bio-availability for fortified dahi is 5.25 percent. The maximum bio-availability of zinc from fortified dahi was observed (5.25) whereas the bio-availability from fortified milk is less (2.71) when compared to dahi. From the results it is very much clear that the bio-availability of zinc was higher in case of *in-vivo* method than *in-vitro* method (zinc 2.71 versus 2.63 percent for milk and 5.25 versus 5.2 percent for dahi.

Table 1: *In-vitro* and *in-vivo* method for bio-availability of iron from milk and dahi

Type of sample	Bio-availability			
	***In-vitro* method**		***In-vivo* method**	
	Iron			
	Total iron (mg/100g)	**Bio-availability (%)**	**Total iron (mg/100g)**	**Bio-availability (%)**
Milk- Unfortified	0.02	7.01	0.02	7.05
Dahi –Unfortified	0.10	10.5	0.10	10.62
M1	4.02	8.10	4.02	8.52
D1	4.10	14.02	4.10	14.2
CD ($P \leq 0.05$)	0.08	0.12	0.08	0.09

All values are average of triplicates
M1: Fortified milk with iron (40 mg/kg)
D1: Fortified dahi with iron (40 mg/kg)

Table 2: *In-vitro* and *in-vivo* method for bio-availability of Zinc from milk and dahi

Type of sample	Bio-availability			
	***In-vitro* method**		***In-vivo* method**	
	Zinc			
	Total Zinc (mg/100g) Total iron (mg/100g)	**Bio-availability (%)**	**Total zinc (mg/100g) Total iron (mg/100g)**	**Bio-availability (%)**
Milk- Unfortified	0.39	1.30	0.39	1.36
Dahi –Unfortified	0.60	3.68	0.60	3.75
M1	4.39	2.63	4.39	2.71
D1	4.60	5.20	4.60	5.25
CD ($P \leq 0.05$)	0.12	0.17	0.12	0.2

All values are average of triplicates
M1: Fortified milk with zinc (40 mg/kg)
D1: Fortified dahi with zinc (40 mg/kg)

Conclusion

Dahi made by using 1percent starter culture showed uniform body and texture, optimum acidity with no whey separation. The increase in starter culture level resulted in increased acidity reflecting, increased sourness. Hence, dahi made by using 1% starter culture was employed for experimental

studies. The sensory score revealed that possibility of making good quality dahi by fortifying milk with food grade ferrous lactate and food grade zinc sulphate and dahi made from such fortified milk respectively at a level of 40 mg/kg milk, the resultant dahi was not differing than the control upon organoleptic properties. Over and above, this level of fortification the dahi samples exhibited metallic flavour. Hence, dahi fortified with ferrous lactate and zinc sulphate at an optimum level of 40 mg/kg is employed for the study. Fortification of dahi with iron and zinc salts at this rate had no noticeable effect on physico-chemical properties. It is very much clear from the findings that the bio-availability of iron was more in dahi fortified with iron. Similarly zinc bio-availability also more in dahi fortified with zinc compared to control and dahi fortified with both iron and zinc. Calcium bio-availability was more in dahi fortified with both iron and zinc compared to dahi fortified with iron or zinc and control diet. Thus, indicating that both iron and zinc salts can be effectively fortified into dahi for enhancement of bio-availability of these micro-minerals.

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