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Effect of Homogenization on Protein, Fat Structure and *in vitro* Protein Digestibility in Milk

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ABSTRACT

Background: Superiority of homogenized milk over non-homogenized is always a debatable subject. In this study 3 different samples of non-homogenized bovine milk samples (NH1, NH2 are market samples and NH3 is unpasteurized milk obtained from local dairy farm) and homogenized milk samples (H1, H2 and H3 milk samples are market samples), were analysed.

Methods: These samples were analysed to understand their digestive behaviour by studying fat and protein distribution, clot formation, pH variation and digestibility of protein in both unboiled and boiled condition.

Result: In boiled and unboiled conditions of non-homogenized and homogenized milk samples, clot formed are different after treatment with digestive enzymes. This difference is also observed in pH study of non-homogenized milk because of structure of coagulum. In clot study, boiled and unboiled milk did not show much difference. Clot weight decreased as compared to 0-time point as gastric digestion progressed. Difference was found between boiled and unboiled sample for protein digestion in case of non- homogenized milk, although not much difference is observed in homogenized milk except for H1. Protein digestion of H2 homogenized mixed milk was found to be superior over rest of the sample.

Key words: Clot structure, Gastric digestion, Heat treatment, *In vitro* protein digestibility, Non-homogenized and Homogenized milk.

INTRODUCTION

Milk being perishable food product, its quality is paramount; hence, it must be stored and transported accordingly. In market different forms of milk is available based on processing (homogenized and non-homogenized), source of milk (Bovine milk, mixed milk, goat milk, buffalo milk and camel milk), fat content (toned milk, double toned milk, skim milk). All these packed milk are pasteurized to improve shelf life. As explained by Lucey, 2015 there are four different ways by which raw milk can be contaminated and this has severe health hazards, hence pasteurization plays very important role. In homogenization process, hot pasteurized milk is forced between a valve needle and seat. This process reduces cream separation rate as this reduces fat globule size. Because of homogenization process shelf life of the milk increases as milk emulsion is more stable after processing. Homogenized milk is further passed through intensive heat treatment to preserve product from microorganisms. Effect of homogenization is enhanced by intensive heat treatment by improving organization of whole milk and health properties (Van Lieshout *et al.* 2019). High pressure application on milk is an effective method for inactivation of microorganisms; this process can inactivate food borne pathogens also. Rennet or coagulation of milk is also improved with high pressure application without changing quality characteristics like taste, flavor, vitamins and nutrients. High pressure application also improves sensory quality (Trujillo *et al.* 2002).

Hypothesis of our study is that, in terms of *in vitro* protein digestibility homogenized milk is superior to non-homogenized milk and *in vitro* protein digestibility of mixed milk (combination of Bovine and Buffalo milk) is superior to bovine milk. Availability of amino acids in food is determined by digestibility of protein present in food source, even though nutritive value of protein is evaluated by amino acid profile (Hsu *et al.* 1977). Food proteins have both nutritional and functional properties. Nutritional value of protein is determined by amino acid content and utilization of specific amino acids upon digestion and absorption. On the other hand, functional properties of protein are associated with physiochemical and sensory properties of foods (Thomas *et al.* 2011).

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MATERIALS AND METHODS

Materials

Three different types of Non-Homogenized (NH) milk samples were procured. NH1 is non homogenized, toned and pasteurized bovine milk, NH2 is non homogenized and pasteurized bovine milk and NH3 is non-homogenized and non-pasteurized raw bovine milk. Similarly three different types of Homogenized milk (H) samples were procured. H1 and H3 are homogenized, pasteurized and toned bovine

milk, whereas H2 is homogenized, toned and pasteurized mixed milk (combination of bovine and buffalo milk). NH1, NH2 and H1 are procured from Bangalore supermarket. H2 and H3 are procured from Kolkata super market, NH3 is procured from local raw milk supplier at Bangalore. Both non- homogenized and homogenized milk are studied as unboiled and boiled samples. For preparation of boiled sample, milk is heated until the temperature reaches 98°C (Approximately 5-7 minutes). Milk samples were brought back to 37°C before initiating digestion process.

Chemicals

Pepsin and Pancreatin enzymes were procured from Sigma Aldrich. Sodium hydroxide (NaOH) pellets, Hydrochloric acid (HCl), Boric acid (H_3BO_3), Copper sulphate Pentahydrate ($CuSO_4.5H_2O$), Sulphuric acid (H_2SO_4), Sodium di hydrogen phosphate (NaH_2PO_4), Disodium hydrogen phosphate (Na_2HPO_4) and sodium sulphate anhydrous (Na_2SO_4) were procured from Merck, India.

Bio digestibility

In vitro digestion for milk samples were carried out with reference to research article by Garrett 1999 with slight modification. Milk sample was distributed in 8 falcon tubes. To each tube 10ml of milk sample was added. These milk samples were treated with 12.5 ml of Pepsin in 0.03M HCl. These tubes were incubated at 37°C under shaking water bath at 150 rpm for 3 hours. First sample was withdrawn at 0 hour followed by sample withdrawal at every 1 hour.

Confocal imaging

Sample collected in the form of clot, at a time interval of 60 minutes was further studied using confocal microscope. Fat content of clot sample was studied using Nile red (Argon laser with an excitation line at 488 nm) and protein content by using Fast green dye (He-Ne laser with an excitation line at 633 nm). Small quantity of clot was smeared on glass slide and stained with Nile red and Fast green at 100 ug/ml concentration and 1:100 dilution. Changes in microstructure of clot on *in vitro* gastric digestion using Pepsin enzymes at different time interval was recorded.

Clot study

In vitro digested sample was withdrawn at one hour interval starting from 0 hour. Withdrawn sample was passed through Whatman filter paper number 1 to collect clot. Weight of the paper was recorded before collecting clot. After drying filter paper with clot for overnight, weight of dried filter paper with clot was recorded. Dried mass of clot was weighed to determine clot weight. Cone shaped whatman filter paper in which clots are collected were dried by keeping these filter paper on hot surface with the help of glass beaker.

pH study

The pH was recorded for the *in vitro* digested sample at every one hour including 0 hour to understand pH variation during digestion process. The pH of sample and enzyme mixture (undergoing gastric digestion) was recorded.

***In vitro* protein digestibility**

In vitro protein digestibility was determined according to the method of Akeson and Stahmann, (1964) with slight modification. 10 ml milk sample treated with 15 ml of Pepsin enzyme (1.5 mg) dissolved in 0.1 M HCl. Sample was incubated with enzyme at 37°C under shaking condtion, 150 rpm for 3 hours, suspension was neutralized with 7.5 ml of 0.2M NaOH. This step was followed by treatment of suspension with 7.5 ml of Pancreatin enzyme (4 mg) prepared in 0.2 M phosphate buffer (pH 8.0). Mixture was incubated for additional 4 hours at 37°C under shaking condition. After completion of 4 hours, sample was passed through whatman filter paper 1 to collect the undigested matrix. All the samples were studied in duplicates and 3 independent experiments were carried out. Protein content of sample treated with pepsin enzyme for 0-hour and undigested matrix collected through whatman filter paper 1 obtained after approximately 7 hours *in vitro* digestion was estimated by Gerhardt Vapodest 50S method (IS: 7219-1973). Percentage of protein digestibility was calculated as:

$$\% \text{ Protein digestibility} = \frac{(\text{Total nitrogen in 0-hour sample} - \text{Total nitrogen in 7 hour digested sample})}{\text{Total nitrogen in 0-hour sample}} \times 100$$

Statistical analysis

Data are presented as mean ± standard deviation (n=3). To determine the statistical differences analysis of variance (ANOVA) was performed. Two-way ANOVA was utilized for evaluating the differences. P values <0.05 were considered as significant.

RESULTS AND DISCUSSION

Confocal study

From confocal images Fig 1, it can be seen that in all 'as such' milk samples free protein has surrounded fat globule and dispersed in aqueous phase. In case of homogenized milk sample, refer Fig 2, protein were found in both aqueous and interfacial layer. This is because homogenization process has disrupted the native milk fat globule membrane, which in turn has increased the interfacial area leading to the adsorption of casein and whey protein to the fat globule surface. Also as discussed by Garcia *et al*. (2013), in homogenized milk size of fat globule has reduced by 13 or 20 fold, which is very evident in Fig 2 confocal images. In unboiled milk sample coagulum formed after gastric digestion was firm and closely knitted. Fat molecules were spread across as one single huge clot. After every 1-hour interval for the unboiled sample, it appeared that all of the caseins and fat globules were incorporated into the clot. In boiled milk sample coagulum formed after pepsin digestion at low pH was less integrated. The clot formed was loose and fragmented at the end of pepsin digestion. Hence heating of the milk will alter the coagulum structure as well as protein and fat distribution. In homogenized milk fat globule formed were small in both digested unboiled and

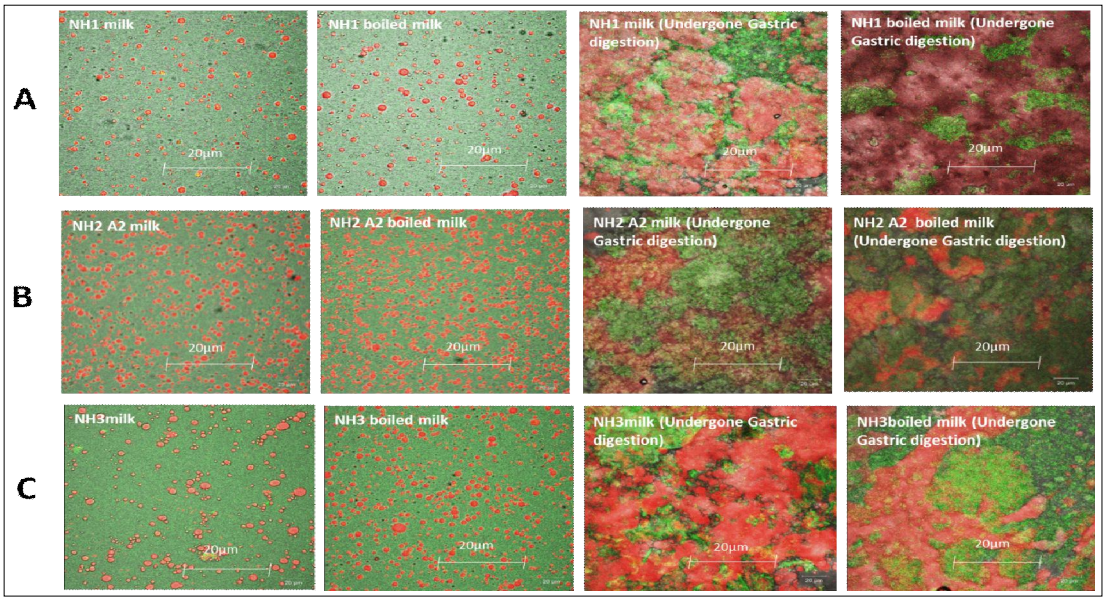

Fig 1: Confocal images of non-homogenized milk samples in unboiled and boiled form, with and without undergoing gastric digestion. A-NH1, B- NH2 and C- NH3 are images of non-homogenized milk samples. Fat is stained as red (Nile red) and Protein is stained as green (Fast green).

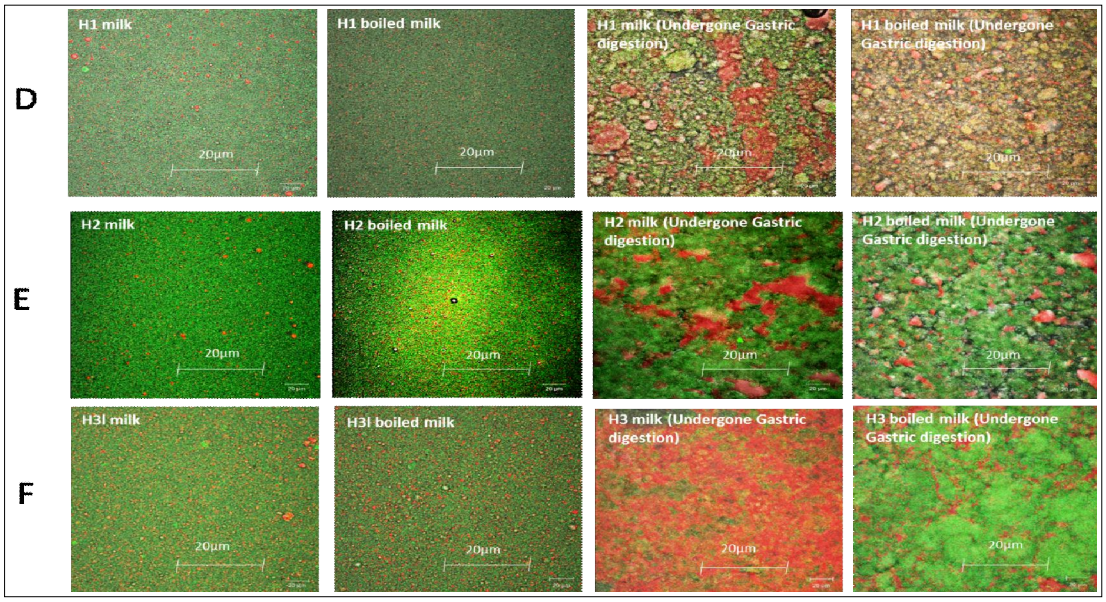

Fig 2: Confocal images of homogenized milk samples in unboiled and boiled form, with and without undergoing gastric digestion. D- H1, E- H2 and F- H3 are images of homogenized milk samples. Fat is stained as red (Nile red) and Protein is stained as green (Fast green).

boiled milk as compared to non-homogenized milk. In boiled homogenized milk, fat globules are smaller with respect to rest of the samples, it may alter protein hydrolysis and fat globule release from clot. These results are similar to the work carried out by, Ye *et al.* 2016. As explained by Michalski 2006, 2009, Homogenization and intensive heat process leads to alteration in fat globule structure and milk fat globule membrane (MFGM). Homogenization process leads to rupture of fat globule membrane, hence MFGM cannot cover fat globules entirely. This leads to adsorption of other surface active component like casein micelles forming a new membrane around fat droplets. Liang *et al.* 2017 have also discussed that fat globule in non-homogenized milk is coated by membrane rich in polar lipids, cholesterol, proteins and glycoproteins. Also as explained by Argova *et al.* (2008), textural characteristics of milk and milk products are contributed by milk lipid fraction. It also influences thermal and physiological properties of milk.

Clot study

When milk is treated with low pH and pepsin enzyme, it

leads to formation of clot. Clot weight of milk samples after pepsin enzyme treatment reduced at 180 minutes (3 hrs) as compared to 0-hour weight indicating that sample is getting digested. In all samples clot weight was less at the end of gastric digestion. In Fig 3A and B, non-homogenized milk sample NH2 is showing higher clot weight. This result can be correlated with confocal image Fig 1A, B and C, wherein confocal image of NH2 milk sample in both unboiled and boiled sample are showing more fat content as compared to NH1 and NH3. As explained by Ye *et al.* 2016, during gastric digestion pepsin hydrolyses κ-casein. Casein micelles are stabilized by κ-casein, hence destruction of κ-casein leads to coagulation. Continuous proteolysis of protein is initiated by pepsin enzyme once clots are formed. Larger weight of coagulum is observed in case of boiled milk sample for both non-homogenized and homogenized milk except for NH3 milk. Homogenization leads to formation of large number of new fat globule surface. Physical and chemical properties are modified once new fat globules are formed (Argova *et al.* 2008).

pH study

Before initiation of gastric digestion, all milk samples showed pH in the range of 6.6-6.8. In unboiled milk throughout the gastric digestion process pH is maintained at lower units as compared to boiled milk, refer Fig 4. As discussed in confocal study, in case of unboiled milk protein is trapped in coagulum hence cannot buffer the mixture. In general, the pH of all samples decreased with increasing digestion time, but there was a difference in the pH profiles of the milks with and without heat treatment in case of non-homogenized milk. The pH of the unboiled whole milk treated with pepsin decreased and then changed only slightly during further digestion. The boiled milk showed significantly less decrease in pH with digestion time than the unboiled milk. Similar trend is not seen in homogenized milk, as it has undergone heating process at the time of homogenization. This difference in pH profile may be because of structures of the clots. In unboiled milk, clot firmly holds protein, hence diffusion through clot is restricted and as a consequence buffering action of protein present in coagulum is not possible. Whereas in case of boiled milk sample, clot formed is loose and open, allowing diffusion through clot. Hence greater buffering action by protein and slowing down of decrease in pH. As discussed in research article by Marie-Caroline Michalski, (2006, 2009) in case of heated milk coagulum formed is loose and protein is not embedded within clot leading to buffering of mixture.

***In vitro* protein digestibility**

Protein digestibility of boiled milk is superior to unboiled milk in all the tested milk, especially in case of non-homogenized milk samples difference is evident, refer Fig 5. H2 milk sample which is mixed milk has shown 84% protein digestibility and is higher as compared to other milk samples. In our study significant difference (p value <0.05, Anova two factor) is observed between non-homogenized and homogenized milk samples in unboiled condition. Homogenized unboiled milk showed superior protein digestibility as compared to non-homogenized, unboiled milk samples. However, no significant difference was observed between non-homogenized and homogenized milk samples in boiled condition. As explained by Kim *et al.* (2002), emulsion based products like homogenized milk, once homogenized are capable of withstanding thermal processing like heating, cooking, sterilization, pasteurization *etc*. May be because of this no significant difference is observed between unboiled and boiled homogenized milk

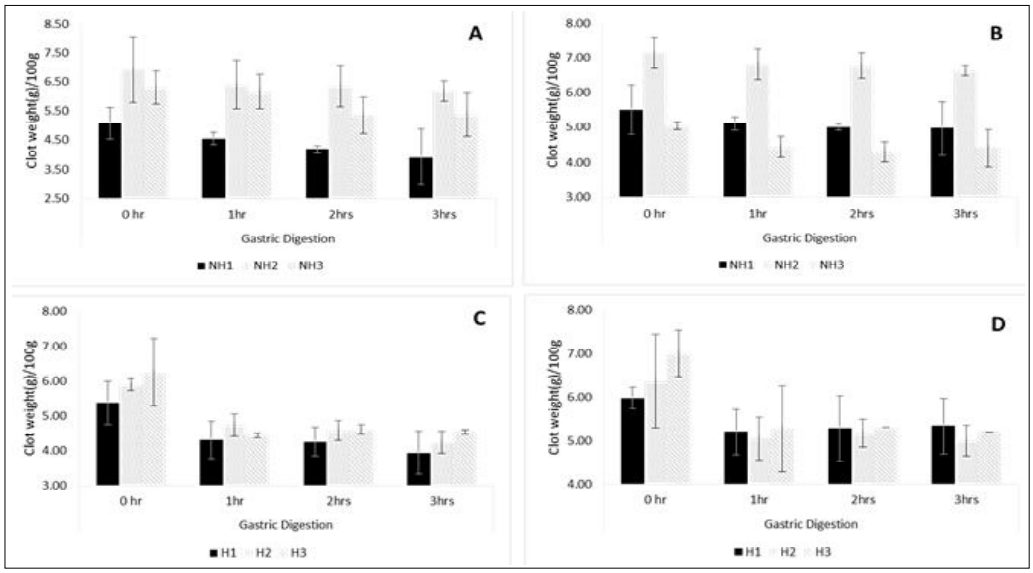

Fig 3: Changes in fat content of non-homogenized and homogenized milk samples in unboiled and boiled condition were represented as dried clot weigh/100 g of milk. Graph A and B represents results of non-homogenized milk (NH1, NH2 and NH3) in unboiled and boiled conditions respectively, Graph C and D represents results of homogenized milk (H1, H2 and H3) in unboiled and boiled conditions respectively. Data represents values of three independent experiments (Mean±SD).

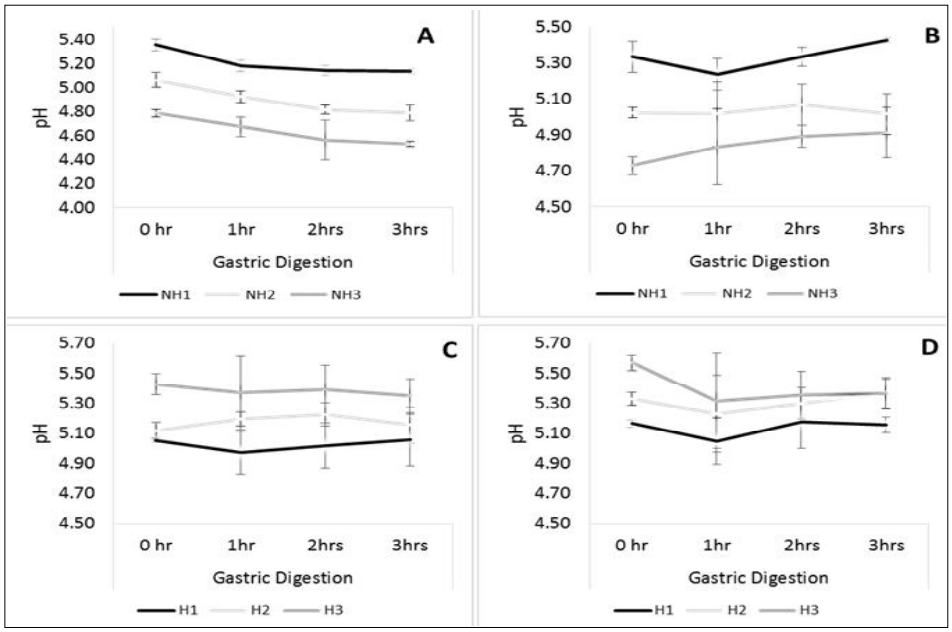

Fig 4: These graphs depict variation in pH of non-homogenized (NH1, NH2 and NH3) and homogenized (H1, H2 and H3) milk samples in unboiled and boiled condition. Graph A and B represents results of non-homogenized milk in unboiled and boiled conditions respectively, Graph C and D represents results of homogenized milk in unboiled and boiled conditions respectively. Data represents values of three independent experiments (Mean±SD).

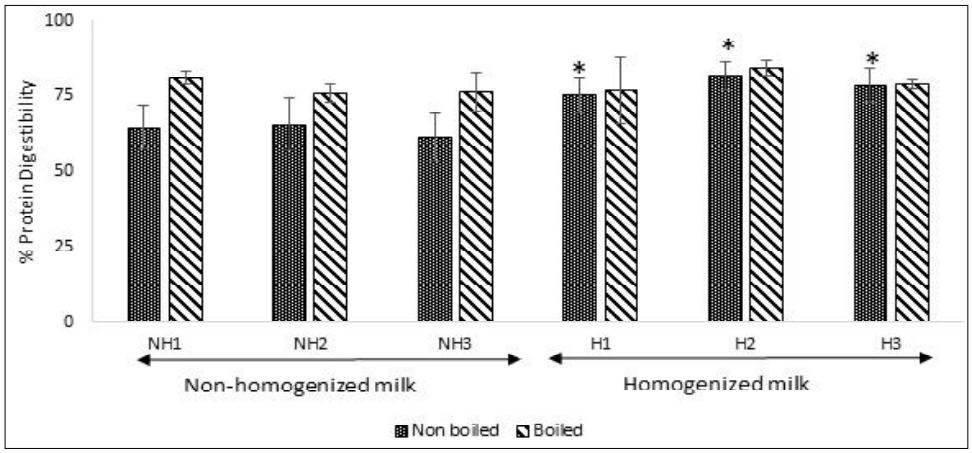

Fig 5: Digestibility of milk protein in non-homogenized and homogenized milk samples in unboiled and boiled condition are expressed in% protein digestibility. NH1, NH2 and NH3 represents results of non-homogenized milk samples and H1, H2 and H3 represents results of homogenized milk samples in both unboiled and boiled condition. Data represents values of three independent experiments (Mean±SD).* p value <0.05, Anova two factor, homogenized unboiled milk showed significantly high protein digestibility as compared non-homogenized unboiled milk.

protein digestibility. As discussed by Lieshout *et al.* (2019) and Wada *et al.* (2014), milk heated above 75°C leads to denaturation of β-lactoglobulin and this in turn resulting in improved digestion by enzymes by *In vitro* protein digestion method. As heating above 75°C leads to unfolding of protein and also increases accessibility of cleavage site of protein. This result is in confirmation with Rat study where in intact β-lactoglobulin is found in rat stomach for unboiled milk and heated milk did not show intact β-lactoglobulin. Digestion process is likely to be dependent on structure of milk Michalski (2007). During digestion of homogenized milk, coagulation of casein and lipid droplets occurs simultaneously in stomach. In case of homogenized milk, fine structure of coagulated matter is formed leading to fast digestion as compared to non-homogenized milk. It is observed that subjects suffering from intestinal disease could digest homogenized milk easily as compared to untreated milk. In mini pig model, raw and pasteurized milk showed slower gastric emptying rate as compared to pasteurized and homogenized milk Heat processing leads to change in state of protein and this impacts protein digestibility. Heat processing changes structure of protein

and results in chemical modification. However, while evaluating digestion of heated milk, it is very important to understand different protein modification (Lieshout *et al.* 2019).

CONCLUSION

In conclusion, homogenized milk has shown satisfactory results in all tested parameters with respect to non-homogenized milk. It has been observed that heating has improved protein digestibility in case of non-homogenized milk as compared to unboiled milk sample. Homogenized milk sample has already undergone heat processing and high pressure treatment, hence no significant difference is found between unboiled and boiled milk samples. Common population considers bovine milk as superior milk and easily digestible. In our study H2 milk sample is homogenized, mixed milk and has 84% protein digestibility as per Pepsin-Pancreatin *in vitro* protein digestion method. In addition, unboiled homogenized milk showed significantly higher protein digestibility as compared unboiled non-homogenized milk. This data can be further validated using different approaches like illeal digestibility, protein absorption and human study.

Conflict of interest: None.

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