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Casein, whey protein and non-protein nitrogen content of milk to identify water, sugar and flour adulterated milk

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ABSTRACT

This study was aimed to assess the potential of using milk's nitrogen distribution pattern, specifically casein, whey protein, and non-protein nitrogen content, as a method for detecting milk adulteration with water, sugar, and flour. Whole milk samples were collected from the Bangladesh Agricultural University Dairy Farm, BAU, Mymensingh, and subjected to adulterations, including 15%, 20%, and 25% water additions and subsequent sugar or flour adjustments to match the fresh milk's specific gravity. The samples were analyzed for specific gravity, fat content, and nitrogen distribution. Results indicated that while specific gravity remained consistent across samples adulterated with sugar or flour, it varied significantly (P<0.05) in those diluted with water. Fat content was significantly (P<0.05) reduced in samples adulterated with water and sugar, particularly at the 25% water addition level. Though total protein, true protein, and casein contents were significantly lower (P<0.05) in the 25% water-added milk compared to fresh milk, they were not significantly different (P>0.05) across the other adulterated samples when compared to fresh milk. Whey protein and non-protein nitrogen levels were statistically consistent (P>0.05) across all the samples. The results indicate that the nitrogen distribution pattern, in its current state, cannot be used to detect milk adulteration effectively. Further research with a larger dataset considering various factors affecting nitrogen distribution in milk is recommended for conclusive results.

Introduction

Milk is a special kind of biological fluid that regarded as one the best nutrient source for over time to support growth and development of human at all ages. It is considered as a best source of dietary fat and protein for its unique fatty acid and amino acid profile (Mapekula *et al.,* 2011; Cabrita *et al.,* 2007). In addition, being a great source of dietary fat and protein, milk promotes the nutritional, immunological, and developmental elements of early life. The main constituents of cow's milk are water (≈87%), macronutrients such as fat (≈3.5%), protein (≈3.2%), lactose (≈4.8%), and micronutrients like salts and minerals (O'Callaghan *et al.,* 2019). Annual need for milk in Bangladesh is 15.85 MMT, while only 14.068 MMT are produced (DLS, 2022-23). So, there is a gap between demand and supply and this production data have already been questioned for exaggeration in different expert opinions. The deficiency of milk encourages some unscrupulous persons to adulterate milk. Adulteration of food, particularly milk, is a global issue in food processing and marketing (Moonajilin *et al.,* 2018). Milk adulteration is the process of

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removing or substituting milk ingredients with inferior one. After olive oil, milk is said to be the food item most prone to be adulterated (Santos *et al.,* 2012). It is well known that water itself is a good adulterant (Xiu and Klein, 2010; Faraz *et al.,* 2013; Mu *et al.,* 2014; Bari *et al.,* 2015; Karmaker *et al.,* 2020). Added water results in reduced viscosity and specific gravity of milk (Shaikh, 2013). It is usually followed by incorporation of cheap and poor quality powdered milk, reconstituted milk, urea, rice flour, sugar, salt, starch, glucose, melamine, and whey powder are in practice to return the viscosity and specific gravity of milk back again to standard range (Motta *et al.,* 2014). Among these sugar and flour are two common adulterants of milk as they are cheap and available. The addition of this kind of stuffs to milk in Bangladesh is more common because the acceptance/rejection of milk depends primarily on its specific gravity. Therefore, it is very important to detect the adulteration of milk in an effective way as it is a solemn concern for food safety. At present several methods have been practiced to detect adulterants in milk such as measurement of freezing point depression, electrical admittance spectroscopy, single-frequency conductance measurements, digital image chromatography, ultraviolet (UV) visible light spectroscopy, enzyme-linked immune-sorbent assay (Santos *et al.,* 2013; Musara and Pote, 2014), RNAase activity in milk (Ju *et al.,* 1991), measurement of the ratio of β-casein to α-lactalbumin by capillary electrophoresis (Chen and Zang, 1993) and the use of NIR spectroscopy (Pedretti *et al*., 1993). But all of these techniques are expensive and require highly specialized equipment. Nitrogen (N) distribution (casein, whey protein and non-protein nitrogen (NPN)) in milk can be measured by Kjeldahl method. This method is more available and less expensive than other methods mentioned. Nitrogen content from external sources affects milk's protein levels (Chen, 2009). In addition, milk is the only natural source of casein protein, therefore, addition of anything to milk may affects the casein concentration in milk. With this connection we are also interested to check the status of other two N-fractions of milk *viz.* whey proteins and NPN in response of milk adulteration. We will also monitor the changes in the fat content as the payment of milk in Bangladesh dairy industry depends on the amount of milk and its fat content. To best of our knowledge, we did not find any literature that report anything about the casein, whey protein and NPN in adulterated milk indicating opportunities to contribute in this domain of milk adulteration. In this context, we designed the present study to monitor the changes in casein, whey protein and NPN concentration of milk adulterated with water and water + flour/sugar.

Materials and Methods

Collection of milk sample

Milk samples were collected from the Bangladesh Agricultural University Dairy Farm, Department of Dairy Science, BAU, Mymensingh. Pooled cow milk was collected in the morning and was transferred to the Dairy Chemistry and Technology Laboratory, department of Dairy Science, BAU for analyses. Milk was diluted by adding 15, 20 and 25% water. Sugar or flour was added to the diluted milk in an amount to get the specific gravity back approximately as it was in the original milk. Hence, the total types of samples were Fresh milk, Milk + 15% water, Milk + 15% water + sugar, Milk + 15% water + flour, Milk + 20% water, Milk + 20% water + sugar, Milk + 20% water + flour, Milk + 25% water and Milk + 25% water + sugar. Because of the solubility issue, we were unable to prepare Milk + 25% water + flour adulterated sample. The adulterated milk preparation is summarized in Table 1.

Table 1. Preparation of adulterated milk by using water, sugar and flour

Ingredients	Fresh Milk	M+15 % W	M+15% W+S	M+15% W+F	M+20 %W	M+20% W+S	M+20%W +F	M+25 %W	M+25% W+S
Milk (mL)	300	255	255	255	240	240	240	225	225
Water (mL)	0	45	45	45	60	60	60	75	75
Flour (g)	0	0	0	7	0	0	9.25	0	0
Sugar (g)	0	0	3.5	0	0	5.21	0	0	6.2

M, Milk; W, Water; S, Sugar; F, Flour. N.B. We observe solubility issue of flour and, therefore, cannot correct the specific gravity of 25% added water milk by using flour.

Determination of Specific Gravity and fat of milk

A Quevenne lactometer was used to estimate the specific gravity of the milk. The fat content was determined by the Gerber method. Ten milliliters of milk was digested by 11 mL of H_2SO_4 added with 1 mL amyl-alcohol in a butyrometer. This was followed by centrifugation for 5 min in a Gerber centrifuge. Then the reading was recorded.

Analysis of nitrogen distribution in the samples

Total nitrogen (TN), non-casein nitrogen (NCN)/whey protein, true protein nitrogen and non-protein nitrogen (NPN) were estimated by the Kjeldahl method following the method used by Islam *et al.* (2014) with some modifications. Before estimating the nitrogen content of the samples, the milk samples were skimmed through centrifugation (3500 rpm for 5 minutes at 4 – 5 ℃).

Total Nitrogen estimation

To estimate the total nitrogen, 5 mL of skimmed milk from each type of sample was digested (with added H_2SO_4 and mixed catalyst) for 3 hrs at 420°C. Then the content was cooled down to room temperature. These digested samples were mixed with 40% sodium hydroxide (60 mL) and the mixture was then underwent the distillation. Distillates were collected in 20 mL of 4% boric acid in a conical flask. The content was then titrated against the 0.1 N HCl using a mixed indicator. A blank test was made by applying the described method using 5 ml of distilled water instead of sample. The following formula was used to calculate the N% -

% Nitrogen = $(1.4 \times (V_1\text{-}V_0) \times N) / P$; where, V_1, HCl consumption on sample titration; V_0, HCl consumption on blank titration; N, Normality of HCl; P, Sample weight.

Non-Casein Nitrogen (NCN) or Whey Protein Nitrogen estimation

Five milliliters of milk sample was tempered to 35°C and subsequently cooled to room temperature in order to perform NCN or whey protein nitrogen separation. After adding 8 mL of acetate buffer (0.53 mL of 10% V/V acetic acid, 0.53 mL of 1N sodium acetate, and 6.94 mL of distilled water), the mixture was centrifuged for 25 minutes at room temperature at 3500 rpm. Then, 3 mL of supernatant containing NCN or whey protein nitrogen was applied to the Kjeldahl method to assay the nitrogen content.

NPN estimation

The NPN fraction was separated using the same protocol as the NCN fraction, with the exception of using 20 mL of 10% (W/V) tri-chloro acetic acid in place of 8 mL acetate buffer and using 6 mL of supernatant to estimate the nitrogen content of the non-protein fractions using the Kjeldahl method.

True protein nitrogen estimation

True protein nitrogen was estimated by deducting NPN from total nitrogen. Then the value was multiplied with the nitrogen conversion factor 6.38 to get the true protein.

Casein nitrogen estimation

Non-protein nitrogen and NCN were deducted from the total nitrogen (TN) to obtain the casein nitrogen (CN), which was then multiplied by 6.38 to achieve the casein protein.

CN= (TN - (NCN+NPN))

Casein = CN x 6.38

Statistical analysis

In order to determine the pattern of nitrogen distribution in samples (fresh milk and adulterated milk), data were collected, visualized and analyzed by the statistical program Minitab version 17. A one-way ANOVA was used to compare the means, and the Tukey's HSD test was used to separate the means, if the differences were significant.

Results and Discussion

Specific Gravity and fat content of milk samples

Table 2 represents the specific gravity, fat percentage and nitrogen distribution of fresh milk and milk samples adulterated by adding water, flour and sugar. There was a significant difference (p<0.01) in specific gravity among fresh milk, water added milk and milk with added water and sugar/flour. Specific gravity was found similar (1.028-1.029) in fresh milk and milk samples adulterated with sugar or flour which is supposed to be so as mentioned in the methodology earlier. Sugar and flour have specific gravity of 1.59 and 1.438, respectively, which are higher than that of water (The Engineering ToolBox 2017; Wichser, 1947). As these two components were added to fix the specific gravity of milk samples adulterated

with water, the specific gravity of the samples containing sugar or flour was similar to fresh milk. It also reveals that the specific gravity of 15% water added sample was significantly different from the specific gravity of 25% water added sample, however, 20% water added milk differed non-significantly (p>0.05) from both of them. The specific gravity of milk samples gradually decreased with the increased amount of water added with milk. This may be due to the addition of water whose specific gravity (1.00) is lower than that of milk (1.028-1.032) (Burke *et al.*, 2018). Barham *et al.* (2020) also found similar result that water adulterated milk had lower specific gravity. Fat percentage differed significantly (p<0.05) among the samples. Fat percentage of samples M+20%W+S and M+25%W+S were found significantly lower (p<0.05) than that of fresh milk (Table 2). However, the rest of the samples with fat percentages ranging from 3.40 to 4.00% differed non-significantly (p>0.05) between them and from other samples. Granulated sugar has 0% fat in proximate analysis (Obiegbuna *et al*., 2023). On the other hand, flour has around 2% fat (Kashlan *et al.,* 1991). These may be the reason of milk above 20% water plus sugar addition had a lower fat percentage than that of milk adulterated with water and flour. However, Memon *et al*. (2018) found no significant deviation of milk fat from fresh one after adding 1-2% cane sugar with fresh milk.

Table 2. Specific gravity, fat content and nitrogen distribution of fresh milk and milk adulterated with water, sugar and flour

Parameters	Treatment groups									
	Fresh Milk	M+15% W	M+15% W+S	M+15% W+F	M+20% W	M+20% W+S	M+20% W+F	M+25% W	M+25 %W+S	P-value
Specific Gravity and Fat percentage										
Sp. gr.	1.028^{a}±0.0	1.023^{b}±0.0	1.028^{a}±0.0	1.029^{a}±0.0	1.022^{bc}±0.0	1.028^{a}±0.0	1.029^{a}±0.0	1.021^{c}±0.0	1.029^{a}±0.0	0.000
Fat (%)	4.20^{a}±0.6	4.00^{ab}±0.3	3.77^{ab}±0.5	3.75^{ab}±0.2	3.60^{ab}±0.2	3.23^{b}±0.1	3.40^{ab}±0.3	3.43^{ab}±0.2	3.20^{b}±0.2	0.027
Nitrogen distribution in the samples										
Total protein (%)	3. 20^{a}±0.6	3.06^{ab}±0.0	2.74^{ab}±0.3	2.38^{ab}±0.3	2.63^{ab}±0.4	2.57^{ab}±0.5	2.38^{ab}±0.1	2.06^{b}±0.2	2.92^{ab}±0.2	0.028
True protein (%)	3.15^{a}±0.6	3.00^{ab}±0.0	2.69^{ab}±0.3	2.32^{ab}±0.3	2.52^{ab}±0.4	2.53^{ab}±0.5	2.32^{ab}±0.2	2.02^{b}±0.2	2.86^{ab}±0.2	0.034
Whey protein (%)	0.31±0.1	0.30±0.1	0.30±0.1	0.18±0.1	0.20±0.1	0.27±0.1	0.17±0.0	0.25±0.0	0.20±0.1	0.285
Casein (%)	2.78^{a}±0.6	2.62^{ab}±0.1	2.33^{ab}±0.4	2.07^{ab}±0.3	2.21^{ab}±0.3	2.23^{ab}±0.4	2.10^{ab}±0.2	1.72^{b}±0.2	2.60^{ab}±0.2	0.044
NPN (%)	0.06±0.0	0.07±0.0	0.05±0.0	0.06±0.1	0.10±0.1	0.03±0.0	0.06±0.0	0.04±0.0	0.06±0.0	0.345

M, Milk; W, Water; S, Sugar; F, Flour; NPN, Non-Protein Nitrogen; Mean with different letter differ significantly. The data has been represented as (mean±SD)

Nitrogen distribution in the samples

Table 2 shows that the 25% water added sample had a significant (p<0.05) reduction in the total protein content of the milk compared to the fresh one. The highest total protein was found in fresh milk (3.20%) and the lowest protein found was 2.06% in milk with 25% added water. Protein content in all other adulterated milk samples ranged from 2.38 to 3.06% and they were found statistically similar (p>0.05). The normal range of total protein is 3.0-3.6% in cow milk (Lin *et al.*,2021; Guetouache *et al.,* 2014; Ceballos, *et al*., 2009; Heck *et al.,* 2009; Bijl *et al.,* 2013). In our study the total protein of fresh milk was within this range. Addition of 15% water did not take the protein out of the lower limit, and interestingly protein content in milk with 25% added water and sugar is marginally behind the lower limit. Sugar has no protein content in it, which may be the reason for lowering the value below the standard level **(**Obiegbuna *et al.*, 2023). In spite of having higher protein content in flour (≈10%), still it couldn't overcome the dilution effect of milk which may be due to the addition in small amount (David *et al.,* 2015). Present findings are in

agreement with that of Memon *et al*. (2018), who reported the lower total protein content of adulterated milk with water, cane sugar and starch. In addition, it may be attributed to the impurities in the sugar and/or flour used their interference in the Kjeldahl procedure or unintentional error from the sampling and/or Kjeldahl analysis. True protein and casein also followed a similar trend as it was in the case of total protein (Table 2). Fresh milk contained the highest true protein (3.15%) which differed non-significantly (p>0.05) from the other samples, except milk adulterated with 25% water that had 2.02% true protein. The highest and lowest amount of casein was found in fresh milk and milk added with 25% water which was 2.78% and 1.72%, respectively. Milk protein comprises two fractions, namely, casein and whey protein. Casein is the chief protein in milk which comprises about 75-85% of the total protein (Brunner, 1981; Bijl *et al.,* 2013; Bhat *et al.,* 2016). On the other hand, flour produced from wheat is mainly composed of two types of protein. These are water-soluble non-gluten protein (15%) and water-insoluble gluten (85%). Casein protein is not present in flour (Khalid *et al*., 2023). In our study, though the casein percentage in most of the adulterated milk differed non-significantly (p>0.05) with the fresh milk sample, the adulterations still caused 6 – 26% reduction in the casein content. This reduction may have some implications for the dairy industry. However, need a large set of national data for that purpose. Because Islam *et al.* (2014) reported 2.7% casein in buffalo milk and 1.8 – 2.9% casein in milk from different genotypes of the dairy cow, indicating other factors to be involved in the casein content variation in milk. The whey protein in all the milk samples was found statistically similar (0.17 to 0.31%). Islam *et al*. (2014) found 0.8 to 0.9% whey proteins in buffalo and cow milk. The result of the present study appears well below of that reported value (≈1/3). The non-protein nitrogen ranged from 0.03 to 0.10% in different milk samples (fresh and adulterated) and was found statistically similar to each other (p>0.05). In cow milk, NPN accounts for about 3–5 % of the total protein (Rushka and Jonkus, 2014). Non-protein nitrogen from animal blood enters into the milk after metabolism. Milk urea forms one of the major (~50%) and most stable elements of NPN. These NPNs also include free amino acids, creatine, uric acid, peptides, organic acids, and phospholipids in addition to milk urea (DePeters and Ferguson, 1992; DePeters and Cant, 1992). Flour contains some non-protein nitrogen fraction, ≈0.025% (Bell, 1963). Islam *et al.* (2014) reported 0.035% NPN in buffalo and cow milk, which is much lower than we found in this study.

Conclusions

The findings from our study indicate that while adulteration of milk with water, flour, or sugar alters its nitrogen content, the variations are not significant enough to reliably identify adulteration using nitrogen distribution patterns alone. Consequently, this method cannot be recommended as a routine assay for detecting milk adulterated with these substances in the market.

However, this study uncovers a valuable alternative application for the nitrogen pattern analysis. By compiling a comprehensive national dataset on the nitrogen distribution in milk, accounting for variables such as species, breed, season, and feeding practices, we can provide a resource of immense value to the dairy industry. This is particularly pertinent considering the critical role of casein content in milk processing and cheese production. Thus, while the technique may fall short as a direct tool for identifying milk adulteration, it holds significant potential for enhancing industry standards and quality control through informed, data-driven insights.

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Authors Contribution

MA Islam conceived and designed the study. S Islam and MMH Khandakar executed the experiment and analyzed the milk samples. MMH Khandakar, M Abunaser and S Islam analyzed the data. M Abunaser and M Mannan prepared the draft manuscript. MH Rashid, MMH Khandakar and MA Islam critically revised the manuscript for important intellectual contents and approved the final version.

Data Availability

Data can be available in reasonable request.

Conflict of interest

The authors declare that there is no conflict of interest.

Consent to Participate

The authors provide full consent to participate.

Consent for Publication

All authors are fully agreed to publish this article in the Bangladesh Journal of Animal Science.

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