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Effects of Freeze Drying on Antioxidants and Immunoglobulins Level of Zebu Bovine Colostrum

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A B S T R A C T

The aim of present study was to investigate the effect of freeze drying on total antioxidant and immunoglobulin levels of bovine colostrum in desi breed (Zebu cattle). A total of 12 colostrum samples were collected from the desi breed (Zebu cattle) reared at LPM section of IVRI, Izatnagar. Collected colostrum was converted aseptically into dry powder form by freeze drying at (-) 40º C with low pressure. Antioxidant content was analysed using free radical scavenging activity (DPPH assay) and total antioxidant capacity (FRAP assay).Qualitatively and quantitative immunoglobulin level was assessed by zinc sulphate turbidity and IgG estimation respectively. Average reduction in DPPH scavenging activity was found to be 35.04 % on freeze drying whereas average reduction of FRAP value was found to be 14.96 % on freeze drying. Immunoglobulin content of bovine colostrum was decreased by 23.86 % of ZST unit after freeze drying whereas with respect to IgG level, the average percentage reduction was 26.05 % after freeze drying of bovine colostrum. Present study concludes that freeze drying reduces antioxidants and immunoglobulin level of bovine colostrum.

Introduction

Bovine colostrum (BC) is the first lacteal secretion after parturition up to 72 hr which is a rich source of immunologically active components and capable of transferring passive immunity to the offspring and also termed as ‘’Immune milk’’ (Nikolic *et al.*, 2017). It has become increasingly popular as a nutritional supplement in humans also for immune support. BC is a rich source of antioxidants both enzymatic and non-enzymatic. Enzymatic antioxidants in colostrum include lactoperoxidase (Shin *et al.*, 2000), catalase (Ito and Akuzawa, 1983), superoxide dismutase (Hill, 1975; Asada, 1976; Korycka-dahl *et al.*, 1979) and glutathione peroxidase (Hojo, 1982). Non-enzymatic antioxidants in colostrum include vitamin E (Goff *et al.*, 2002), Vitamin A (Schweigert and Eisek, 1990; Kume and Toharmat, 2001), vitamin C (Lindmark-Mansson and Akesson, 2000), lactoferrin (Bennett *et al.*, 1986) and selenium (Debski*et*

al., 1987). Persisting copper, zinc and cysteine in BC acts as cofactors of which copper and Zinc are necessary for proper activity of antioxidative enzymes and also itself possess its (Ahmed *et al.,* 2004). Cysteine is a precursor of glutathione (Goldmas *et al.,* 1986). Caseins and whey proteins from colostrum exert their antioxidant activities which can be measured by reducing power, ferrous ion chelating abilities as well as inhibitory effects on lipid peroxidation (Chiang and Chang, 2005).

BC contains several hundred-fold immunoglobulin (Ig) of physiologically bioactive constituents such as growth promoting factors (IGF I and II) as well as a series of antimicrobial and antioxidant peptide including lactoferrin, lactoperoxidase and lysozymes than ordinary bovine milk (Sanchez *et al.,* 1992; Levay and Viljoen, 1995; Lonnerdal and Lyer, 1995; Korhonen *et al*., 1995). The most abundant immunoglobulin class in bovine milk and colostrum is IgG1, while IgA and IgM are present at minimum concentrations. Other important components are various oligosaccharides, acute phase proteins, growth factors, antimicrobial peptides and others (Stelwagen *et al.,* 2009).

Freeze-drying is the most preferred dehydration method for heat-sensitive biological material, as the low processing temperature and rapid local transition of frozen material from hydrated to dehydrated state minimize nutrient losses. Chelack *et al.,* (1993) reported a 10% loss in biological activity of immunoglobulins upon freeze-drying of colostrum, whereas Elfstrand *et al.,* (2002) reported 34% and 25% losses in total immunoglobulins during freeze-drying of colostrum whey and colostrum concentrate prepared from the whey through the application of membrane filtration. Freeze-drying had a significant detrimental effect (i.e., 30% loss) on native TGF-b2 and IGF-1 of a colostrum concentrate, and minor effect on freeze-dried colostrum whey (Elfstrand *et al.,* 2002). Lyophilised colostrum is reported to be stable, easy to handle and suitable for passive immunization (Husu *et al.,* 1993). The present study envisaged to investigate the effect of freeze drying on total antioxidant and immunoglobulin content of bovine colostrum in desi breed (Zebu cattle).

Materials and Methods

Collection and preparation of freeze dried bovine colostrum (FDBC)

Excess colostrum at the time of first milking was collected from Indian zebu cattle reared at LPM section (Cattle & Buffalo farm), ICAR - IVRI, Izatnagar under strict hygiene. A total of 12 samples were collected from the desi breed (Zebu cattle). The collected colostrum was transported to laboratory in cold condition and kept at -20º C till processing. The collected colostrum was thawed and subjected to freeze drying at - 40º C with low pressure to make it as dry powder and kept under cold condition for further use (Klobasa *et al*, 1998).

Antioxidant potential of FDBC

Antioxidant activity of FDBC was assessed by following methods. All the samples were analysed in triplicate and average values were noted.

Free radical scavenging activity (DPPH method)

The free radical scavenging activity of FDBC was measured by DPPH (1, 1 diphenyl 2, picrylhydrazyl) assay with slight modification (Brand-Williams *et al.,* 1995). It measures the free radical scavenging activity in terms of hydrogen donating ability or radical

scavenging property of any biological fluids using the stable free radical DPPH solution. Colostrum sample (100 µl) was mixed with 2 ml of DPPH solution (0.2 mM) prepared in methanol. The mixture was allowed to incubate at room temperature for 30 min. After completion of incubation period, 1 ml of chloroform was added and centrifuged at 3000 x g for 5 min. The absorbance of clear solution was measured at 517 nm. A 100 mM of DPPH solution prepared in methanol was used as a control. The percentage inhibition of DPPH free radical (scavenged %) was calculated based on reading of control solution by employing the following equation:

Scavenging activity (%) = [(absorbance of the control – absorbance of the sample)/ absorbance of the control] ×100

FRAP assay to determine total antioxidant activity

To determine the total antioxidant capacity of colostrum, a modified FRAP assay was used with little modification (Benzie and Strain, 1996). FRAP reagent was freshly prepared by mixing 300 mmol/L acetate buffer (3.1 g of CH_3COONa and 16 ml of CH3 OOH), pH 3.6, 10 mmol/L TPTZ (2, 4, 6-tripyridyl-s-triazine) in 40 mmol/L HCl and 20 mmol/L $FeCl_3$ in 10:1:1 ratio. Colostrum sample (50 µl) was mixed with 1.5 ml of FRAP reagent and kept at dark for 10 minutes. The resulting intense blue colouration (Ferrous tripyridyltriazine complex) was subsequently measured at 593 nm. Aqueous solutions of $FeSO_4 \cdot 7H_2O$ (100–1000 µM) was used as standard curve. The data was expressed as FRAP values (µM/mL Fe (II)).

Immunoglobulin assessment

Zinc sulphate turbidity test (ZST)

Zinc sulphate turbidity test was done to determine the immunoglobulins present in FDBC. Zinc sulphate turbidity reaction (ZST) was measured using McEvan's method with little modifications (McEvan *et al.,* 1970; Hogan *et al.,* 2016). Colostrum serum was collected by using 10 % acetic which precipitated the casein protein at 37ºC. The fat was separated by adding diethyl ether and ethanol. 50 µl of the tested colostrum serum was mixed with 3.4 mL of zinc sulphate (350 mg/l) solution which was immediately prepared in boiling water bath in a screw capped tube. The mixture was shaken and left to stand at room temperature for 60 minutes. Light absorption due to turbidity was measured photometrically at 680 nm. The immunoglobulins contents of the tested sample were derived from a calibration curve plotted on a basis of turbidity values corresponding to different dilutions of the standard barium sulphate solution. Six ml of 11.5 g/l $BaCl_2$ solution was made up to 200 ml in a volumetric flask with 0.2 N H_2SO_4. The absorbance of this barium sulphate standard was measured spectrophometrically and the resultant absorbance value was assigned a value of 12.5 ZST units. ZST unit of the tested samples were calculated from standard curve.

IgG estimation

IgG was estimated in bovine colostrum as well as freeze dried bovine colostrum by Quantia IgG kit with the modification of human IgG was replaced with bovine specific IgG. Colostrum serum was prepared following the method casein precipitation with 10 % acetic acid method. A calibration curve was plotted against concentration and absorbance to find the linear equation.

Results and Discussion

For uniqueness of result, all the tests performed both with BC and FDBC. Freeze drying of bovine colostrum was done to make

it dry powder at (-) 40°C and low pressure using standard protocol. Freeze drying efficiency was calculated for every representative sample. Average percentage recovery of freeze dried bovine colostrum was 21.67 %. Collected BC was pale yellow in colour which after freeze drying converted into light pale crystalline powder.

Antioxidant potential of FDBC

Antioxidant activity of FDBC was assessed by free radical scavenging activity (DPPH methods) and Total antioxidant activity (FRAP assay) methods.

Free radical scavenging activity (DPPH methods)

Free radical scavenging activity of biological samples was determined by DPPH assay which is based on the electron donation or hydrogen atom acceptance. In the present study, 12 first day bovine colostrum (BC) and their corresponding FDBC samples were analysed by DPPH methods. All the samples were analysed in triplicates. Scavenging activity of bovine colostrum and corresponding FDBC has been depicted in the Table 2. The average scavenging activity of BC was 50.46 % whereas the average scavenging activity of FDBC was found to be 32.75% which revealed a reduction in the DPPH scavenging activity after freeze drying. Average reduction in DPPH scavenging of BC was found to be 35.04 % after freeze drying.

Total antioxidant activity (FRAP assay)

Total antioxidant activity of colostrum samples and FDBC was determined by FRAP assay. In the present study, 12 first day bovine colostrum and their corresponding FDBC samples were evaluated by FRAP methods. All the samples were analysed in triplicates. A standard regression equation ($R^2 = 0.9917$) was plotted using freshly prepared aqueous solution of ferrous sulphate solution (100-1000 µM) and absorbance. Concentration of FRAP value of BC and FDBC were derived using the linear equation and expressed as µM/mL Fe (II). The average FRAP value for BC and FDBC were 876.83 µM/mL Fe (II) and 745.87 µM/mL Fe (II) respectively. Average reduction of FRAP value on freeze drying was found to be 14.96 %. The data has been presented in Figure 2 and table 3.

Table.1 Yield percentage of freeze dried bovine colostrum

Sl. No.	Weight of Colostrum (gm)	Weight of FDBC (gm)	% Yield
BC-1	16.25	2.93	**18.03**
BC-2	15.40	2.78	**18.05**
BC-3	15.48	2.88	**18.60**
BC-4	15.20	2.72	**17.89**
BC-5	16.15	3.06	**18.94**
BC-6	15.24	2.98	**19.55**
BC-7	25.29	6.15	**24.31**
BC-8	24.28	5.92	**24.38**
BC-9	24.35	6.03	**24.76**
BC-10	24.20	6.32	**26.11**
BC-11	25.14	6.04	**24.03**
BC-12	24.80	6.29	**25.36**
Average yield of FDBC from all samples			**21.67 %**

Table.2 Scavenging activity of BC and FDBC with % reduction of Scavenging activity upon freeze drying

Sl. no	% Scavenging activity of BC	% Scavenging activity of FDBC	% Reduction on freeze drying
BC 1	50.53	33.31	34.06
BC 2	45.95	29.32	36.19
BC 3	53.92	33.75	37.42
BC 4	52.60	34.81	33.82
BC 5	51.93	31.01	40.30
BC 6	53.30	30.18	43.37
BC 7	45.88	31.19	32.01
BC 8	51.90	34.99	32.57
BC 9	48.49	31.58	34.88
BC 10	53.01	36.80	30.57
BC 11	47.79	30.62	35.93
BC 12	50.20	35.43	29.41
Average	**50.46**	**32.75**	**35.04**

Table.3 FRAP assay of BC and FDBC with % reduction of FRAP value upon Freeze drying

Sl. no	FRAP value of BC [µM/mL Fe (II)]	FRAP Value of FDBC [µM/mL Fe (II)]	% Reduction in FRAP value upon freeze drying
BC 1	819.70	690.37	15.74
BC 2	932.03	826.20	11.35
BC 3	930.87	769.70	17.31
BC 4	844.20	733.20	13.15
BC 5	970.70	865.37	10.85
BC 6	803.03	671.37	16.40
BC 7	914.70	741.87	18.89
BC 8	941.37	800.20	15.00
BC 9	892.03	773.37	13.31
BC 10	847.37	724.53	14.49
BC 11	745.53	638.20	14.39
BC 12	880.37	716.03	18.65
Average	**876.83**	**745.87**	**14.96**

Table.4 Zinc sulphate turbidity test of BC, FDBC and % reduction upon freeze drying

Sl.no	BC (ZST unit)	FDBC (ZST unit)	% Reduction on freeze drying
BC 1	65.92	50.55	23.32
BC 2	65.69	48.65	25.93
BC 3	59.57	49.31	17.23
BC 4	67.47	52.63	21.99
BC 5	61.12	48.06	21.36
BC 6	73.22	53.34	27.15
BC 7	61.53	46.69	24.11
BC 8	75.24	53.82	28.47
BC 9	70.26	52.04	25.93
BC 10	72.21	54.53	24.49
BC 11	75.30	58.50	22.30
BC 12	71.03	55.12	22.39
Average	**68.21**	**51.94**	**23.86**

Table.5 Concentration of IgG in BC and FDBC with % reduction in IgG upon Freeze drying

Sl. no	IgG Conc. of BC (mg/dl)	IgG Conc. of FDBC (mg/dl)	% reduction in IgG
BC 1	781.0	591.0	24.33
BC 2	773.5	573.5	25.86
BC 3	713.5	586.0	17.87
BC 4	728.5	598.5	17.84
BC 5	803.5	603.5	24.89
BC 6	778.5	583.5	25.05
BC 7	771.0	556.0	27.89
BC 8	803.5	553.5	31.11
BC 9	828.5	573.5	30.78
BC 10	771.0	571.0	25.94
BC 11	803.5	566.0	29.56
BC 12	838.5	591.0	29.52
Average	**782.8**	**578.9**	**26.05**

Fig.1 Fresh bovine colostrum (BC) after collection, (B) Freeze dried bovine colostrum (FDBC)

Fig.2 Ferrous sulphate calibration curve

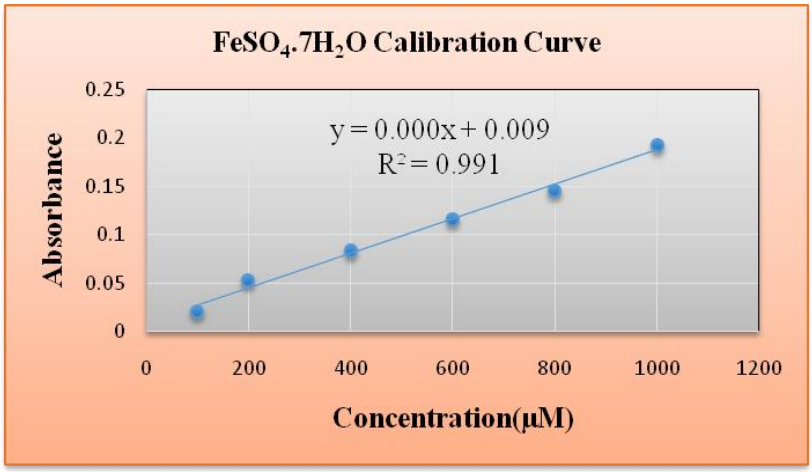


Fig.3 Barium sulphate calibration curve

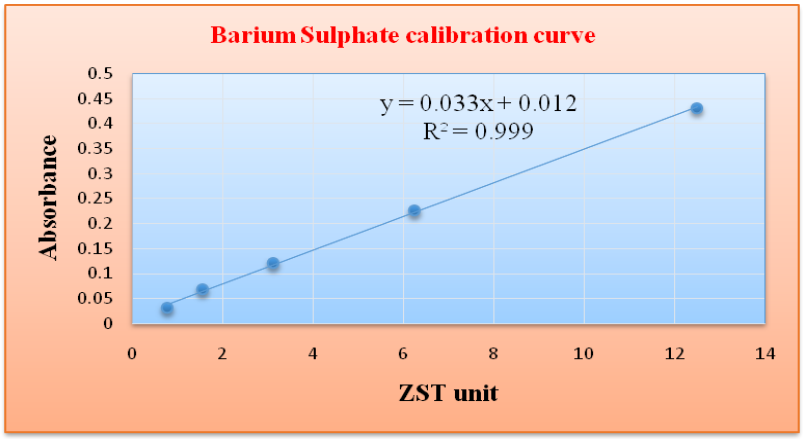
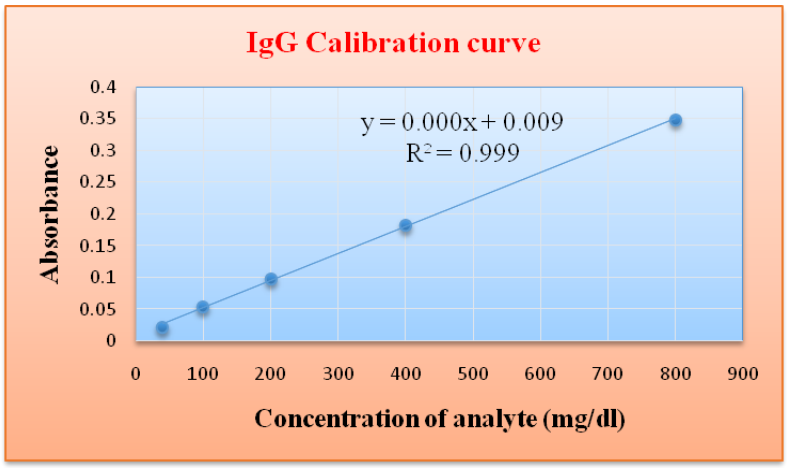


Fig.4 IgG calibration curve



Immunomodulatory potential of FDBC

Immunomodulatory potential of FDBC was assessed by zinc sulphate turbidity test (ZST) and IgG concentration estimation in colostrum serum.

Zinc sulphate turbidity test (ZST)

Zinc sulphate turbidity test was done to estimate the immunoglobulins content of BC and FDBC serum which is qualitative test. A stand regression equation was plotted using serial dilution of barium sulphate (R^2=0.9991). Immunoglobulin content was estimated using absorbance by linear equation plotted and represented in ZST unit/ dl. The average content of immunoglobulin in BC and FDBC was found to be 68.21 ZST unit/dl and 58.94 ZST unit/dl. Data analysis of BC and FDBC revealed reduction of 23.86 % ZST unit after freeze drying. The data has been presented in Figure 3 and table 4.

Immunoglobulin G estimation

IgG was estimated in bovine colostrum as well as freeze dried bovine colostrum by Quantia IgG kit with the modification of human IgG was replaced with bovine specific IgG. Colostrum serum was prepared following the method casein precipitation with 10 % acetic acid method. The average concentration of IgG in BC and FDBC was 782.8 mg/dl and 578.9 mg/ dl respectively. The average percentage reduction of IgG was calculated to be 26.05 %. The data has been presented in Figure 4 and table 5.

Bovine colostrum of zebu cattle was processed by lyophilisation and dried powdered colostrum was recovered. The recovery percentage was 21.67 % which is approximately equivalent to the total solid (22.0 %) depicting the removal of water by freeze drying process. During freeze drying process, water is sublimated at low temperature (- 40 °C) with low pressure to preserve the thermolabile component of biological samples (Nireesha *et al.,* 2013) to prolong the self-life and storage quality. Basically, colostrum as such cannot be stored for longer period due to possible microbial attack. So, freeze drying process removes the watery component and decrease water activity of the biological sample thereby increases self-life of colostrum with minimum loss of the active components. Pasteurization and other heat treatment methods have been reported to produce detrimental effect on protein by denaturing the original structure of protein components (Moreti *et al.,* 2012).

Bovine colostrum is a combined source of enzymatic and non-enzymatic antioxidants (Pandey *et al.,* 2011). These antioxidants have potential to protect the body from excessive production of free radicals or ROS, a process commonly linked to oxidative tissue injury and may be useful as a therapeutics of certain diseases like cancers, diabetes mellitus *etc* (Jackson *et al.,* 2002).

The free radical scavenging activity of biological samples was determined by DPPH assay which is based on the electron donation or hydrogen atom acceptance. In present study, the average scavenging activity of BC was 50.46 % whereas the average scavenging activity of FDBC was found to be 32.75%. Mann *et al.,* (2016) reported free radical scavenging activity of 55.42±0.50 % in Sahiwal cattle (Indian Zebu cattle). Present findings reported that decreased DDPH scavenging activity after freeze drying might be due to loss of some antioxidant component during the process. Similar findings were reported on losses of active components of colostrum and colostrum whey during freeze drying process (Chelack *et al.,* 1993: Elfstrand *et al.,* 2002).

The FRAP assay evaluates the capacity to reduce ferric ions of any biological sample. The present study revealed total antioxidant activity (FRAP Value) of BC and FDBC were 876.83 µM/ml Fe (II) and 745.87 µM/ml Fe (II) respectively. Similar finding was reported by Mann *et al.,* (2016) who stated that FRAP values for Sahiwal cow colostrum was found to be 627.38 µM/ml Fe (II) and also reported that FRAP value decreases with the lactation progress. The present studies revealed average reduction of FRAP value after freeze drying and was (14.96 %) which could be explained as loss of some component during the process. Similar findings were also reported on losses of active components of colostrum and colostrum whey during freeze drying process (Chelack *et al.,* 1993: Elfstrand *et al.,* 2002).

Bovine colostrum is a condensed source of immunoglobulins such as IgG, IgM, IgA, IgD, and IgE. IgG and IgM play important role to protection from invading bacteria, virus and fungi and parasites whereas IgA protects the intestinal surface and facilitates the removal of microorganisms thus inhibiting the first step of infection. When given orally, immunoglobulins in colostrum protect rabbits from *E.coli* infection due to improvement of cell mediated or humoral immunity (Nagaraja, 2010; Pandey *et al.,* 2011).

Total immunoglobulin was estimated qualitatively using zinc sulphate turbidity test in BC serum and FDBC serum in ZST unit/ dl. The average content of immunoglobulin in BC and FDBC was found to be 68.21 ZST unit/dl and 58.94 ZST unit/dl respectively. The data analysis of BC and FDBC revealed a reduction of 23.86 % ZST unit upon freeze drying. Zinc sulphate test is a qualitative method used to determine the immune status of neonatal animals. This estimation gives a total globulin status rather than specific globulin described by McEvan *et al.,* (1970). According to the Hogan *et al.,* (2016), 1 ZST unit is equivalent to the 10 mg/ml of immunoglobulins. Present study revealed the 23.86 % of reduction of immunoglobulins after freeze drying of BC which is in accordance with the Elfstrand *et al.,* (2002) who reported 25% losses in total immunoglobulins during freeze drying of colostrum whey.

IgG was estimated in bovine colostrum as well as freeze dried bovine colostrum by Quantia IgG kit with the replacement of Human IgG with Bovine specific IgG. The average concentration of IgG was 782.8 mg/dl and 578.9 mg/ dl in BC and FDBC respectively. The average percentage reduction of IgG was 26.05 %. Chelack *et al.,* (1993) reported a 10% loss in biological activity of Immunoglobulin G upon freeze-drying of colostrum, whereas Elfstrand *et al.,* (2002) reported 34% and 25% losses in total Ig during freeze-drying of colostrum whey and colostrum concentrate prepared from the whey through application of membrane filtration.

In conclusion the Study revealed reduction in total antioxidant capacity, DPPH % scavenging activity as well as low reduction in immunoglobulin level after freeze drying of zebu cattle colostrum. However decrease in water activity of fresh bovine colostrum increases the self-life for antioxidant and immunomodulatory property of freeze dried bovine colostrum. Hence freeze dried bovine colostrum supervened fresh bovine colostrum and may be a potent source of antioxidant and immunoglobulin supplementation in ailing as well as ill thrift conditions of animals.

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