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CORRELATION OF SPOILAGE MICROFLORA IN CHILLER STORED BUFFALO MEAT WITH CHEMICAL INDICATORS

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ABSTRACT

The aim of the study was to analyze the total microflora in aerobically packed buffalo meat stored under chiller (4±1°C) condition using chemical indicators of spoilage. Buffalo meat was kept in chiller (4±1°C) and analyzed on alternate days from 1st day to 7th day. Various chemical indicators analyzed were pH, TVBN, free amino acids (FAA), FDA hydrolysis and D-glucose concentration. Standard plate count (SPC) values of meat sample were also evaluated on each experimental day. Except D-glucose concentration, all other parameters including SPC increased significantly (P<0.05) as storage advanced. The SPC values of buffalo meat increased from 3.44 log cfu/g on day zero to 6.96 log cfu/g on day 7. The SPC showed a correlation coefficient (r) of 0.943 with both pH and TVBN, 0.953 with FAA, 0.978 with FDA hydrolysis and -0.919 with D-glucose concentration. Further, regressions equations were generated to predict SPC using correlated chemical indicators. Based on correlation coefficient and standard error of estimate, the suitability of regression equation to predict SPC using various chemical parameters as independent variable was found to be, FDA>FAA>pH=TVBN>D-glucose.

Key words: Buffalo meat, chiller storage, spoilage microflora, chemical indicators, correlation.

INTRODUCTION

Meat is considered as a highly perishable food and is invariably spoiled by microorganisms because of its higher moisture content and ability to supply abundant and free nutrients (Ercoloini *et al*., 2006). The inadvertent sensorial change that may develop upon storage of meat, like discolouration, off-odours and surface slime formation depend on microbial

growth contaminating the meat (Fung 2010). Moreover, growth and proliferation of the contaminating microflora depends on conditions under which meat is stored (Doulgeraki *et al*., 2010, 2011; Pennacchia *et al*., 2011 and Casaburi *et al*., 2011), especially storage temperature and packaging conditions (Nychas *et al*., 2008). For fresh aerobically stored meat, *Pseudomonas* spp. has been identified as the specific spoilage organism (Raab *et al*., 2008). A pseudomonades population of 10^7 – 10^8cfu/g causes meat surface slime formation and bad smell. Both these changes appear, however, when pseudomonades exhaust the glucose and lactic acid in the meat and begin to metabolise amino acids (Nychas *et al*., 2008). Therefore, to establish a microbiological acceptance criterion for fresh and frozen meat, ICMSF (2002) has put forward a standard plate count (SPC) value of $\log_{10}$ 7 cfu/g as the upper microbiological limit.

Determination of microbial load in meat samples using conventional plate count methods are time consuming and laborious (Venkitanarayanan *et al*., 1997). Therefore, meat industry and various inspection services are in continuous search for rapid and cheap alternative methods to establish the microbial load meat products. Moreover, such methods can also be used for monitoring HACCP and sanitary standard operating proceduresin meat plant. Therefore, the possibility of using chemical indicators to establish bacterial spoilage of fresh meat is an area of interest for meat researchers. In the present study, five chemical indicators of meat spoilage namely pH, total volatile basic nitrogen (TVBN), free amino acids (FAA), FDA hydrolysis and D-glucose concentration were evaluated in buffalo meat stored under chiller (4±1°C) condition, along with measurement of standard plate count (SPC). Correlation analysis was carried out between SPC and each chemical indicator to develop linear regression models to predict SPC using chemical indicators, so as to establish the bacteriological quality of chilled buffalo meat.

MATERIALS AND METHODS

Sample preparation

The meat samples were collected from buffalo slaughter house, Bareilly district, Uttar Pradesh State, India. The round muscles, without excessive fat and connective tissue, were collected from nine different buffaloes which were slaughtered according to traditional halal method and brought to the laboratory under refrigerated conditions within 4 h of slaughter. Meat collected from nine different carcasses was packed separately in LDPE bags and kept in a chiller room maintained at 7-10°C for 24 h for rigor mortis to complete so as to

avoid cold shortening and excessive drip loss.

After the initial chilling period, the total meat was divided into 3 replicates and each replicate represents a mixed sample comprising meat from three randomly chosen animals. All the three replicates were packed in polyethylene bags with zip lock and stored under chiller (4±1°C) condition. Composite samples were collected from all three replicates shortly before chilling and various analytical parameters like SPC, pH, TVBN, FAA, FDA hydrolysis and D-glucose concentration were evaluated in duplicate. Same parameters were analyzed further on days 3, 5 and 7.

Standard plate count (SPC)

Test portion of meat sample for SPC were collected separately from three different laboratory samples representing three replicates, following ISO standard (ISO/TS 17728: 2015). Preparation, initial suspension and decimal dilutions of test samples were done following ISO/DIS 6887-2: 2013. Duplicate plates of plate count agar (Hi-Media Laboratories Pvt Ltd., Mumbai, M091) were prepared for each dilution and for all test samples. All the plates were inoculated by pour plate method and incubated at 30ºC following ISO 4833-1: 2013. The standard counts were expressed as colony forming units (cfu) per gram, after multiplying average number of colonies with reciprocal of dilution.

Measurement of chemical parameters of meat spoilage

pH

The meat homogenate was prepared by blending 10g test sample with 90 ml distilled water in an Ultra Turrax tissue homogenizer for one min and the pH was recorded by immersing combined glass electrode of digital pH meter into the meat homogenate.

Total volatile basic nitrogen (TVBN)

Micro-diffusion technique (Pearson 1968) was adopted to determine the TVBN content of buffalo meat samples. Meat homogenate (10 g of meat in 90 ml distilled water) was filtered and 5 ml of filtrate was mixed with 5 ml of 10% trichloro acetic acid in a test tube. The covered tubes were kept for 30 min at room temperature and filtered through a Whatman filter paper No. 1. In a Conway micro-diffusion unit, 2 ml of boric acid reagent was added in its centre compartment and 1 ml of filtrate was pipetted onto the outer compartment. Saturated potassium carbonate (1ml) was added to the filtrate in outer compartment and cover lid applied immediately. After proper mixing, the dishes were incubated

at 37°C for 3-4 h with intermittent rotations. After incubation, boric acid solution (turned to green from reddish) was titrated with 0.02 N sulphuric acid till end point (reddish colour reappeared). The TVBN content was calculated by using the following equation:

TVBN (mg/100 g of meat) = Reading of burette (volume of 0.02N sulphuric acid consumed) × Normality of acid used for titration ×14 × 100

FAA content

A modified colorimetric ninhydrin analysis (Rosen, 1957) was followed to estimate the α-amino acids present in buffalo meat as nihydrin reactive substances. Meat homogenate was prepared from 10 g meat and 100 ml distilled water and kept overnight in refrigerator. Homogenate was transferred into a polycarbonate centrifuge tube and centrifuged at 3000 rpm for 15 min. After the first centrifugation, 10 ml of supernatant was mixed with 10 ml of 10% trichloro acetic acid and centrifuged at 3000 rpm for 15 min. To 1 ml of aliquot in duplicate, 1 ml of both 80% phenol in ethanol and 10% ninhydrin reagent in acetone was added. Ten micro liter of pyridine solution was added to the reaction mixture to improve the colour sensitivity. In blank, 1 ml of distilled water was added instead of aliquot. This mixture was stirred and kept in boiling water bath for 10 min, cooled in running tap water and 5 ml of ethanol was added. The optical density was determined in a spectrophotometer at 570 nm and converted to mg free amino acid per ml using leucine standard curve and expressed as mg per 100 g of meat.

FDA hydrolysis

The FDA hydrolysis of meat samples were measured following the procedure described by Venkitanarayanan *et al*., (1997). One gram of meat was collected observing necessary aseptic precautions and transferred into a tube containing 10 ml sterile peptone water (0.1%). After proper shaking, the tubes were centrifuged at 100×*g* for 30 s to sediment meat particles. The supernatant was transferred into another tube and centrifuged at 3000×*g* for 30 min to pellet the bacterial cells. The supernatant was decanted and the bacterial pellet was washed and resuspended in 5 ml sterile sodium phosphate buffer (pH 7.6). The resultant 5 ml solution was sonicated in a bath sonicator in 4 episodes of 15 sec each. To 3 ml of the resulting clear solution, 100µL FDA reagent [500µg FDA/ml acetone] was added. The mixture was incubated at 25°C for 3 h and the absorbance of the solution at 490 nm was recorded using a spectrophotometer. A tube containing 3 ml sterile phosphate buffer and 100 µl FDA reagent designated

Table 1. Standard plate count (SPC) and chemical indicators of spoilage in buffalo meat stored under chiller (4 ± 1°C) condition (Mean±SE)

Parameters	Storage period (days)			
	0	3	5	7
SPC (log cfu/g)	3.44±0.07^{d}	3.95±0.26^{c}	5.00±0.16^{b}	6.96±0.14^{a}
pH	5.27±0.03^{d}	5.39±0.02^{c}	5.55±0.07^{b}	5.71±0.05^{a}
TVBN (mg/100g)	8.13±0.09^{d}	11.48±0.13^{c}	13.70±0.07^{b}	16.55±0.14^{a}
FAA (mg/100g)	4.80±0.17^{d}	19.72±0.10^{c}	27.28±1.07^{b}	42.18±1.32^{a}
FDA Hydrolysis	0.24±0.005^{d}	0.30±0.004^{b}	0.35±0.005^{c}	0.46±0.005^{a}
D-Glucose (mg/100g)	142.00±1.03^{d}	122.50±1.26^{c}	53.23±0.72^{b}	33.77±0.73^{a}

Table 2. Linear regression equations to predict standard plate count (SPC) of buffalo meat stored under chiller (4 ± 1°C) condition using chemical indicators of spoilage as independent variables.

Standard plate count from	Regression equation
pH	$Y = 7.498X_1 - 36.246$ (Y=SPC, X1= pH)
TVBN	$Y = 0.415X_2 - 0.333$ (Y=SPC , X2= TVBN)
FAA	$Y = 0.096X_3 + 2.584$ (Y=SPC , X3= FAA)
FDA	$Y = 16.519X_4 - 0.716$ (Y=SPC , X4= FDA)
D-glucose	$Y = -0.027X_5 + 7.254$ (Y=SPC , X5= D-glucose)

Standard error of estimate for the 'estimated SPC value for chiller stored buffalo meat' using different independent variables at $P<0.05$ level of significance are 0.482 for pH, 0.474 for TVBN, 0.430 for FAA, 0.296 for FDA and 0.558 for D-glucose.

Table 3. Summary of regression analysis used to predict the SPC values of buffalo meat stored under chiller (4 ± 1°C) condition using different independent variables

Independent variables (X)	Correlation Coefficient (r)	Standard error of estimate	Regression constants (β)
pH	0.943	0.482	-36.246
TVBN	0.943	0.474	-0.333
FAA	0.953	0.430	2.584
FDA	0.978	0.296	-0.716
D-glucose	-0.919	0.558	7.254

as ''blank'' was incubated simultaneously. The FDA hydrolysis was expressed as mean absorbance at 490 nm.

D-glucose concentration

D-glucose concentration in buffalo meat was determined using glucose oxidase/peroxidase assay kit (Sigma-Aldrich, USA). Meat homogenate was prepared from 1 g meat sample and 10 ml distilled water. It was then filtered through a Whatman filter paper No. 1 using glass funnels. One ml aliquot in duplicates was drawn from the clear supernatant. Distilled water (1 ml) was taken in one test tube marked as blank. To all the tubes, 2 ml of assay reagent was added and kept at 37° C for 30 min. The reaction was stopped after 30 min by addition of 2 ml of 12 N sulphuric acid. The optical density was determined with a spectrophotometer at 540 nm and converted to mg D-glucose concentration per ml of aliquot using glucose standard curve. The D-glucose content of meat was expressed as mg per 100 g of meat.

Statistical analysis

A randomized block design with three completely random replicates was followed and the data generated were compiled and analyzed using SPSS (version 20.0). The smallest difference ($D_{5\%}$) for two means was reported as significantly different (P<0.05). Pearson coefficient of correlation (r) between SPC and various chemical parameters was calculated. Regression equations were developed to predict SPC using chemical parameters. A linear regression model using SPC as dependent variable and chemical parameters as independent variable was followed.

RESULTS AND DISCUSSION

Standard plate count and chemical indicators

Standard plate count, pH, TVBN, FAA, FDA hydrolysis and D-glucose concentration of buffalo meat stored under chiller (4 ± 1°C) condition and evaluated on alternate days up to 7th day are presented in Table 1. Except D-glucose concentration, all other indicators of buffalo meat spoilage increased significantly (P<0.05) as storage period advanced. The SPC values of buffalo meat increased from 3.44 log cfu/g on day zero to 6.96log cfu/g on day 7. The association between SPC values and chemical indicators was established through analyzing the Pearson coefficient (r) of correlation. SPC showed a correlation coefficient of 0.943 with pH values (Fig. 1), 0.943 with TVBN (Fig. 2), 0.953 with FAA (Fig. 3), 0.978 with FDA hydrolysis (Fig. 4) and -0.919 with D-glucose concentration (Fig. 5). All the correlation coefficients were found to be highly significant at (P<0.01).

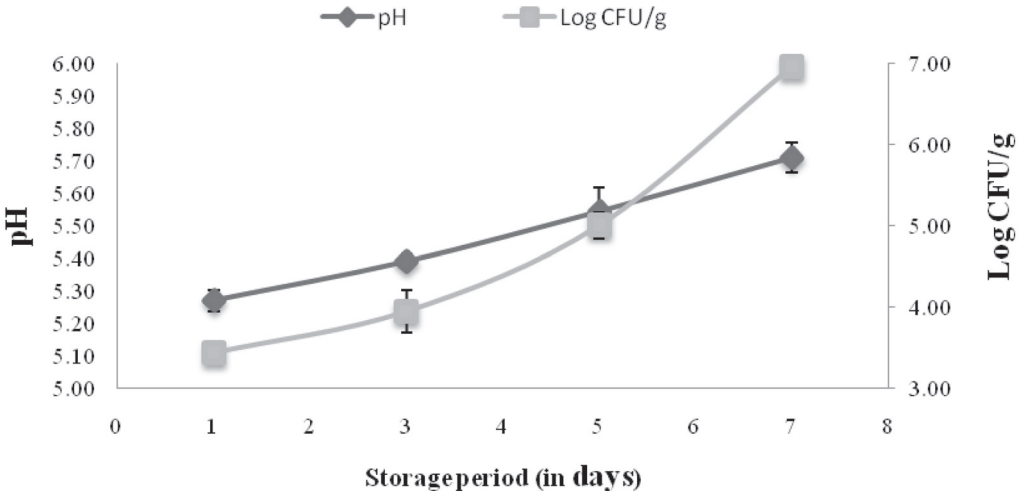

Fig. 1. Standard plate counts (SPC) and pH values of buffalo meat stored under chiller (4 ± 1°C) condition.

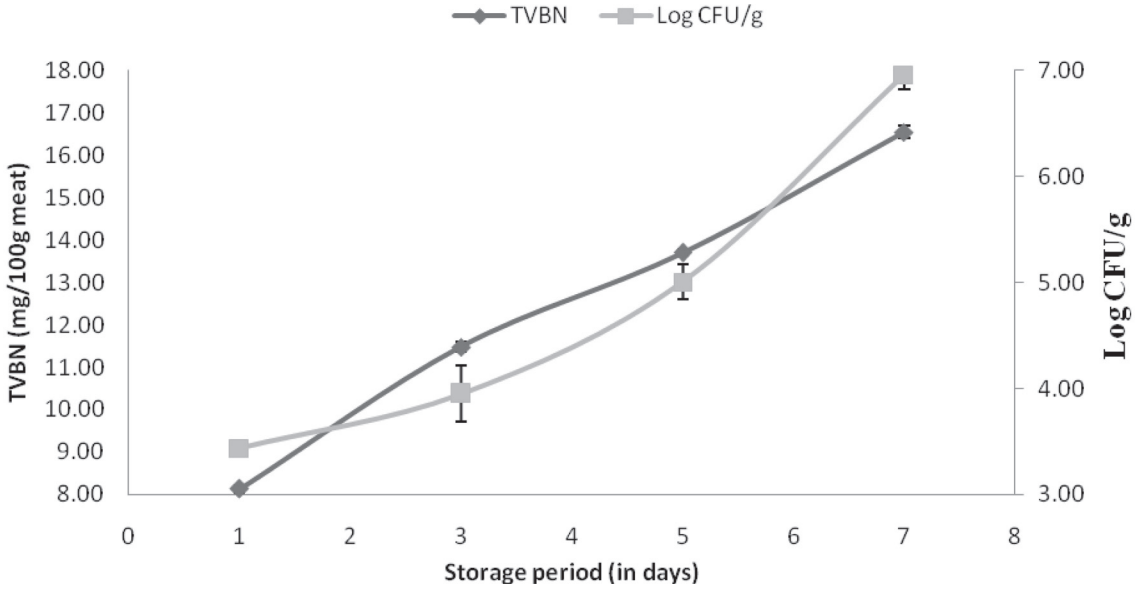

Fig. 2. Standard plate counts (SPC) and TVBN values of buffalo meat stored under chiller (4 ± 1°C) condition.

In accordance with the three attribute sampling system for fresh meat (ICMSF, 2002), the upper limit for microbiological acceptance of fresh chilled meat is 7 log cfu/g. In the present investigation, SPC value of ca. 7 log cfu/g was observed on 7th day of storage and this is attributed to the initial higher level of contamination. Studies conducted by Lavieri and Williams (2014) emphasized the importance of packing on microbial spoilage, since in their study the ground beef stored either with MAP or VP under 4°C has showed only an SPC of 6.2 log cfu/g even on 25th

day of storage. Similarly, beef stored under 4°C with initial microbial count of log 2.06 cfu/cm^2 has shown the final count of log 7.31 cfu/cm^2 after 12 days (Byun et al. 2003).

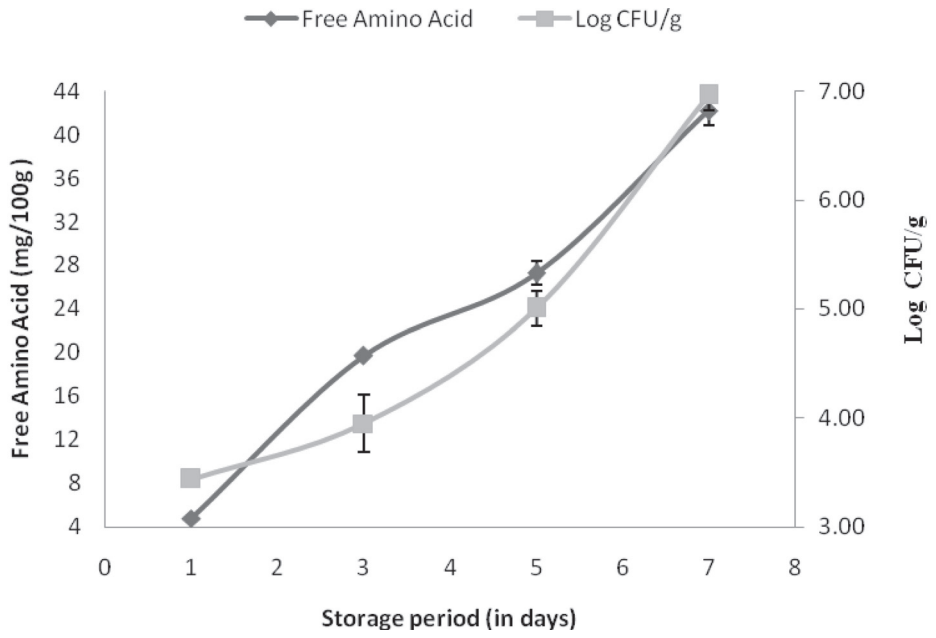

Fig. 3. Standard plate counts (SPC) and FAA values of buffalo meat stored under chiller (4 ± 1°C) condition.

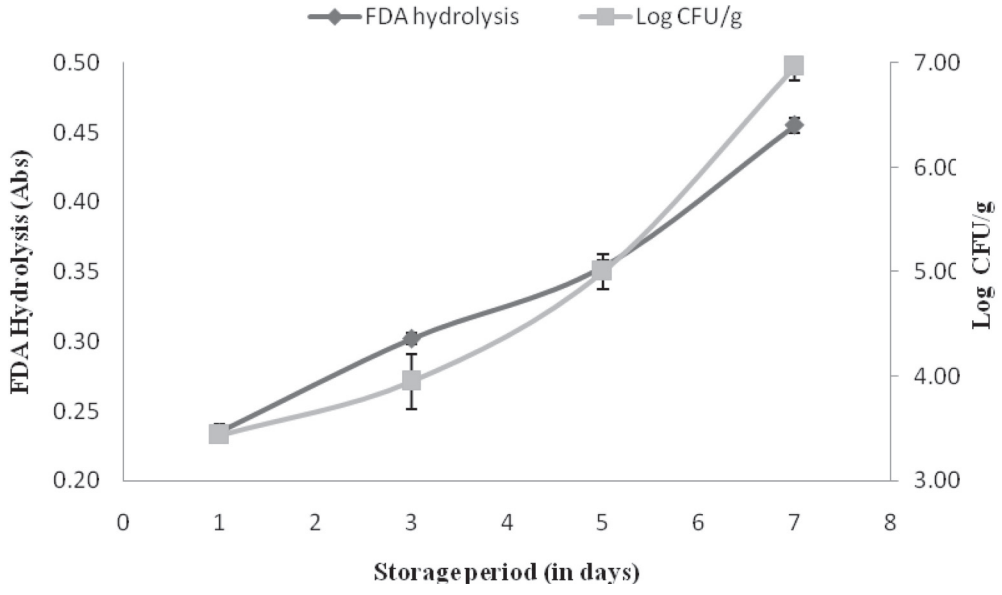

Fig. 4. Standard plate counts (SPC) and FDA hydrolysis values of buffalo meat stored under chiller (4 ± 1°C) condition.

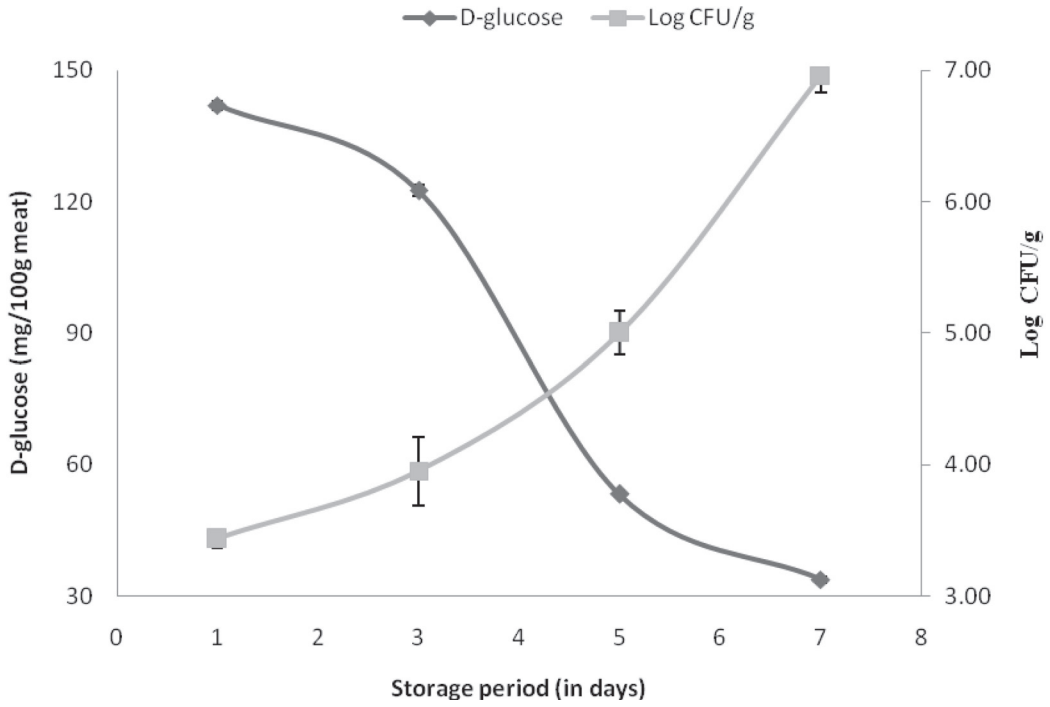

Fig. 5. Standard plate counts (SPC) and D-glucose concentration of buffalo meat stored under chiller (4 ± 1°C) condition.

pH value has been suggested as an important chemical indicators of bacterial spoilage in meat and meat products (Korkeala *et al*., 1987; De Pablo *et al*., 1989; Yano *et al*., 1995) and it is evident from the significantly (P<0.01) higher correlation coefficient (r = 0.943) observed between SPC and pH in the present study. Many microbes, including pseudomonads, can produce ammonia during amino acid metabolism, which is the major cause of increase in pH. Incipient spoilage is accompanied by a rise in pH and an increase in bacterial numbers along with other changes (Shelef and Jay, 1970). Higher meat pH can be considered as an intrinsic stimulus of multiplication for bacteria that colonizes meat (Nychas *et al*., 2007), and this is more evident from the higher SPC value of meat with higher pH.

Utilization of free amino acids by bacteria leads to increased levels of ammonia and other nitrogenous compounds in meat on prolonged storage (Dainty *et al*., 1988). Therefore, the amount of TVBN present in meat can be used as an indicator to detect the quality of meat. Studies conducted by Byun *et al*., (2003) on beef and Shukla *et al*., (2015) on carabeef stored under 4° C showed an increasing trend in TVBN content as storage days proceeded. Higher TVBN content is associated with sensorial rejection of meat and the upper

limit of sensorial rejection of buffalo meat was observed at TVBN concentration of 16.50 mg/100g (Vishnuraj *et al*., 2014a). The correlation coefficient (r = 0.943) observed between SPC and TVBN for buffalo meat in the present study agreed with the findings of Byun *et al*. (2003), who observed a corresponding value of 0.91 to 0.96.

Glucose concentration of buffalo meat stored under refrigeration decreased from 142 to 33.7 mg/100g from zero day to 7th day and the correlation coefficient between SPC and D-glucose concentration was found to be -0.919. Depending on its initial concentration, glucose may become depleted and only at this point do other substrates begin to be metabolized (Gill, 1983). Therefore, glucose and lactate along with their oxidative products have been proposed to serve as spoilage indicators. The correlation coefficient observed between SPC and FAA was 0.953 in the present study. Under aerobic storage condition, spoilage is most frequently associated with post- glucose utilization of amino acids by *Pseudomonas* and it has been shown that sum of free amino acids along with water soluble protein content increased during storage and this corresponded well with colony counts (Nychas *et al.,* 2008). Among all the chemical parameters evaluated, the highest degree of association was shown between SPC and FDA hydrolysis (r = 0.978). The use of FDA hydrolysis to monitor the microbial spoilage of higher temperature abused buffalo meat was already established (Vishnuraj *et al*., 2014b).

Regression analysis to predict SPC using chemical indicators

Linear regression equations to predict standard plate count (SPC) of aerobically packed buffalo meat stored under chiller condition, using chemical indicators of spoilage as independent variables are presented in Table 2. The standard error of estimate of SPC, which determines the deviation of expected value of SPC from the observed value, was also calculated and presented in Table 3. Since this parameter depends on coefficient of correlation (r), independent variable FDA showed the lowest value for standard error of estimate and D-glucose showed the highest value. Therefore, the suitability of regression equation to predict SPC using various chemical parameters as independent variable is in the order, FDA hydrolysis > FAA > pH =TVBN > D-glucose concentration.

SUMMARY

Results of the present study proposes that during aerobic storage of buffalo meat at chiller condition (4±1°C),

pH, TVBN, FAA, FDA hydrolysis and SPC increased significantly ($P<0.05$) whereas, D-glucose concentration decreased. Coefficient of correlation between chemical indicators and SPC value of buffalo meat was also established. Through various regression models, this study offers a comprehensive approach to predict a time-consuming parameter *i.e.* SPC of buffalo meat using some of the easily quantifiable physico-chemical parameters.

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