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PREVALENCE OF PSYCHROTROPHIC BACTERIA DURING FROZEN STORAGE OF RAW MILK

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Abstract

The study was aimed to enumerate the load of psychrotrophic bacteria in raw milk stored in freezer. Raw milk was collected from 18 different sized dairy farms located in Gazipur and Mymensingh district. From each location equal number (three of each) of small, medium or large farms were randomly selected. The raw milk samples were stored in freezer (-18 to -22 ^{o}C) for 28 days and analyzed for psychrotrophic bacterial load at every 7 days interval. For total viable count, bacteria was grown onto plate count agar at 7^{0}C for 10 days. Bacteria grown under such conditions referred as psychrotrophs. However, to determine the type of psychrotrophs selected colonies were grown further onto pseudomonas agar base and gram staining, oxidase, catalase and methyl red test for each isolate were perfomed. The psychrotrophic bacterial load in all the samples were low until 14 days of storage. However, from the third week of storage the bacterial load was observed to increase which reached to as high as $1.1 \pm 2.3\times10^{7}$ cfu/mL at the end of the storage period (28 days). The milk samples obtained from large farms were lower (1.3×10^{5} to 6.6×10^{6}) than that of medium (1.7×10^{5} to 8.8×10^{6}) and small (2.0×10^{5} to 1.1×10^{7}) type farms. The bacterial load was significantly ($P<0.01$) varied among the samples suggesting the heterogeneous management practices in dairy farms. All the morphological and biochemical tests confirmed the isolates as pseudomonas. Based on the present findings, it could be suggested that raw milk can store in the freezer maximum for two weeks in terms of psychrotrophic bacterial load.

Keywords: Psychrotrophs, herd*, Pseudomonas*, acidification.

Introduction

The term psychrotrophs define, the microorganisms that have the capacity to grow at low temperatures, even in refrigerated and frozen condition but maximum growth temperature is above 10 °C (Moyer and Morita, 2007; Oliveira *et al.,* 2015).

Raw milk provides a favorable physicochemical environment for the growth and multiplication of a broad spectrum of microorganisms, including a range of psychrotrophic bacterial species that contaminate milk during collection, processing and storage (Samarzija *et al.*, 2012; Vithanage *et al.*, 2016).

Psychrotrophic bacteria cause a profound contamination during the storage of milk and milk products in freezing condition

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producing extracellular hydrolytic enzymes where most were heat-resistant. A high level of psychrotrophic bacteria in raw milk is related with a sufficient amount of thermostable lipases and proteinases that may result breakdown of milk fat and protein during storage even after pasteurization (Matta and Punj, 1999; Barbano *et al.,* 2006; Machado *et al.*, 2017).

In Indian-subcontinent as well as Bangladesh, many consumers purchase huge amount of raw milk at a time and kept in freezer (-18 to -22 ^{0}C) for certain periods. Storing long period at freezer enhance to grow psychrotrophs, but consumers think that as they stored in frozen conditions, milk is solely safe. These psychrotrophic bacteria causes severe spoilage of milk in a very limited time without acidification along with sweet curdling which is dangerous for human health (Samarzija *et al.*, 2012).

Therefore, in this study raw milk was collected from unlike sized herds of Gazipur and Mymensingh districts and psychrotrophs load (cfu/mL) were enumerated to establish a safety period for frozen stored milk and were identified to determine the type of psychrotrophs involved in contamination.

Materials and Methods

Collection and storage of samples

Raw milk samples were collected from 18 different dairy farms of Gazipur and Mymensingh districts of Bangladesh. Samples were proportionally collected from small, medium and large dairy farms. After collection, samples of 500 mL were placed in sterile screw-capped jars and transported to the laboratory using an iso-thermal box. Samples were stored at frozen condition (-18 to -22 °C) and assessed after 0, 7, 14, 21 and 28 days of storage period.

Farm classification

Category of dairy farm was strictly maintained to find the individual differences between farms in terms of psychrotrophic bacterial load as well as hygienic quality. Farms were classified based on dairy cows. Dairy cows number 1-5 were considered as small, 6-25 as medium and more than 25 were large farms (Jagadish, 2016).

Enumeration and determination of psychrotrophic bacteria

Samples of 1 mL of milk was serially diluted in 9 mL of 0.1% sterile peptone water and 0.1 mL of each dilution was plated onto plate count agar, and incubated (BJPX-B80II; Biobase) at 7 °C for 10 days (Oliveira and Parmelee 1976). Microorganisms that grown under such conditions were considered primarily as psychrotrophs. Psychrotrophs were further grown onto pseudomonas agar base to determine the type of psychrotrophic bacteria present (Munsch-Alatossava *et al.,* 2006; Moussa *et al.,* 2008; Cosentino *et al.,* 1997).

Morphological and biochemical tests

Several morphological and biochemical tests were done to determine the type of psychrotrophs present in stored frozen raw milk samples.

Gram staining

Clean and grease free slide was taken. Air dried and heat-fixed smear of cells was prepared. Crystal violet was poured and kept for about 30 seconds and rinsed with water. Gram's iodine was flooded for 60 seconds and washed with water. Then washed with 95% alcohol for about 10-20 seconds and rinsed with water. Safranin was incorporated for about 60 seconds and washed with water. After air drying slides was observed under microscope (Holt *et al.,* 1994). Thin layer of peptidoglycan-10% of cell wall, high lipid content and red/pink stains indicate gram negative and purple stains specify gram positive. (Diene *et al.,* 2014). Gram stained bacterial image were captured by Compound Biological Digital Microscope with Camera (LB-1270, Thomas Scientific).

Oxidase test

Oxidase test was done by wet filter paper method. A strip of filter paper was soaked with a little freshly made 1% solution of tetramethyl-p-phenylenediamine dihydrochloride. A speck of culture was rubbed. A positive reaction was described by an intense deep-purple hue, and absence of coloration indicated negative reaction (Holt *et al.,* 1994).

Catalase test

This was done by sliding method. A loop was use to transfer a small amount of colony to a glass slide. A drop of 3% H_2O_2 then placed on glass slide. Observed for the evolution of oxygen bubbles (MacFaddin, 2000). Production of copious bubbles and active bubbling indicate positive catalase test and no or very few bubbles designate negative catalase test (Scaccabarozzi *et al.,* 2015).

Methyl red (MR) test

Methyl Red Voges Proskauer (MRVP) broth and 0.02% methyl red solution were prepared to perform the test. 7.0 g buffered peptone, 5.0 g glucose and 5.0 g dipotassium phosphate was properly mixed with 1 litre deionized water to produce MRVP broth (pH 6.9). 0.1 g of methyl red were dissolved in 300 mL of 95% ethyl alcohol. Sufficient distilled water were added to make 500 mL of 0.02% methyl red solution. Prior to inoculation, medium was allowed to equilibrate at room temperature. Using organisms taken from an 18-24 h pure culture, medium was lightly inoculated. Then, incubated aerobically at 37 °C for 24 h. After 24 h of incubation, 1 mL aliquot of the broth was transfer to a clean test tube. Remaining broth was re-incubated for an additional 24 h. Two to three drops of methyl red indicator were added to aliquot and then observed for color. A distinct red color in reaction describe positive MR reaction and yellow color indicate negative MR test (MacFaddin, 2000).

Statistical analysis

Statistical analysis was performed using SPSS (IBM@version25) statistical software. All results were represented as the means ± S. E. For comparison, one-way analysis of variance (ANOVA) was carried out. Differences were considered to be statistically significant when the p value was less the 0.01 ($P<0.01$).

Results and Discussion

Enumeration of psychrotrophic bacteria

Each three samples were collected from small, medium and large dairy farms of Gazipur and Mymensingh districts and analyzed after 0, 7, 14, 21 and 28 days of storage period. Just after collection, the psychrotrophs load/mL for Gazipur and Mymensingh districts samples varied from $1.3 \pm 0.08 \times 10^5$ to $2.77 \pm 0.09 \times 10^5$ and $1.4 \pm 0.05 \times 10^5$ to $2.67 \pm 0.09 \times 10^5$ respectively. These mean counts were within the standard range. In each districts, large herds psychrotrophic bacterial load/mL were lower than medium herds. GM1 and GM2 samples count were little bit same but GM3 were slightly higher (Table 1). Though, both samples were collected from medium sized farms but colony count/mL altered. These

Table 1. Psychrotrophs count in stored frozen milk collected from different areas of Gazipur district

Samples (cfu/mL)	0 days	7 days	14 days	21 days	28 days
GS1	$2.77 \pm 0.09\times10^5$	$3.73 \pm 0.14\times10^5$	$4.57 \pm 0.09\times10^5$	$5.08 \pm 2.89\times10^6$	$8.75 \pm 2.14\times10^6$
GS2	$2.5 \pm 0.12\times10^5$	$3.67 \pm 0.11\times10^5$	$4.8 \pm 0.06\times10^5$	$6.24 \pm 5.76\times10^6$	$9.62 \pm 3.15\times10^6$
GS3	$2.0 \pm 0.07\times10^5$	$3.43 \pm 0.12\times10^5$	$4.75 \pm 0.1\times10^5$	$4.59 \pm 1.77\times10^6$	$1.06 \pm 3.34\times10^7$
GM1	$1.72 \pm 0.13\times10^5$	$2.72 \pm 0.11\times10^5$	$3.52 \pm 0.09\times10^5$	$3.07 \pm 1.93\times10^6$	$7.25 \pm 1.44\times10^6$
GM2	$1.77 \pm 0.12\times10^5$	$2.94 \pm 0.29\times10^5$	$3.77 \pm 0.26\times10^5$	$4.04 \pm 1.5\times10^6$	$8.34 \pm 2.52\times10^6$
GM3	$2.35 \pm 0.05\times10^5$	$3.33 \pm 0.12\times10^5$	$4.23 \pm 0.14\times10^5$	$5.37 \pm 2.53\times10^6$	$8.84 \pm 0.94\times10^6$
GL1	$1.33 \pm 0.09\times10^5$	$1.99 \pm 0.12\times10^5$	$3.31 \pm 0.12\times10^5$	$2.48 \pm 0.84\times10^6$	$6.4 \pm 0.53\times10^6$
GL2	$1.3 \pm 0.08\times10^5$	$2.43 \pm 0.04\times10^5$	$3.68 \pm 0.09\times10^5$	$2.24 \pm 2.2\times10^6$	$5.36 \pm 2.1\times10^6$
GL3	$1.6 \pm 0.15\times10^5$	$2.7 \pm 0.08\times10^5$	$3.72 \pm 0.15\times10^5$	$1.63 \pm 0.9\times10^6$	$4.33 \pm 2.6\times10^6$
LSD	0.219	0.294	0.286	5.580	4.773
LS	**	**	**	**	**

** = Significant at 1% level, * = Significant at 5% level, LS= Level of significance, LSD = Least Significant Difference, GS1-GS3= Samples collected from small farms of Gazipur district, GM1-GM3= Samples collected from medium farms of Gazipur district, GL1-GL3= Samples collected from large farms of Gazipur district

may be due to variation in farm biosecurity or post collection contamination. Mymensingh areas moderate size herds psychrotrophs load was marginally higher than Gazipur. In both areas large herds colony load/mL were around 1.5 lacs. Small herds psychrotrophic bacterial load showed worst result. The highest load/mL were found in GS1 sample, which is 2.77×10^5 (Table 1). In Mymensingh areas, small farms highest load/mL was found in MS2 sample, and this is alike to GS1 sample. All samples of Gazipur and Mymensingh areas varied significantly. Psychrotrophic bacteria secrete high amounts of extracellular hydrolytic enzymes, and the extent of recontamination of stored milk with these bacteria is a major causal agent of their shelf life and within three days of storing their number varies 1.5×10^5 cfu/mL to 1.5×10^5 cfu/mL (Ledenbach *et al.,* 2009) and this is agreed with 0 days colony counts. The average of culturable psychrotrophic bacteria of frozen stored milk is 6.4 to 14.7% of total mesophilic population and their count is 1.8×10^5 to 2.3×10^5 cfu per mL on SPC agar (Hantsis-Zacharov *et al.,* 2007).

After 7 days of frozen conditions, notable amount of psychrotrophic bacterial number was increased. GL3 sample contained highest amount of psychrotrophic bacteria among large farms. ML3 contained the maximum population among Mymensingh areas large farms. In case of medium farms, the lowest mean count/mL were found in MM1 and the highest were in MM1 (Table 2). Medium type farms of Gazipur areas psychrotrophic bacterial population were within the range of Mymensingh areas. Comparing small farms of both areas bacterial load/mL were higher in MS3 and lower in MS1. The mean bacterial load/mL for small farms varied from $3.35 \pm 0.04\times10^5$/mL to $4.5 \pm 0.10\times10^5$/mL.

GS1 contain the highest amount and GS3 the lowest amount and GS2 between GS1 and GS3 among Gazipur areas (Table 1). MS2 samples mean psychrotrophs count/mL lower than GS1 and GS2. Storing raw milk samples up to 7 days in frozen conditions clearly indicated that, there is a proportional increasing of psychrotrophic bacterial load among small, medium and large farms. Large farms showed the best result. Statistical analysis showed significant variation among the samples. Deep frozen storage of raw milk has favored the growth of psychrotrophic bacteria and six days storage of frozen raw milk shows psychrotrophic bacteria number 3.7×10^5 cfu/mL, additionally, this milk does not show off flavor (Perko, 2011). Torkar *et al.* (2008) enumerated total bacteria, number of coliforms, psychrotrophic bacteria, yeasts and moulds of one week stored frozen milk and found psychrotrophic micro-organisms number 3.9×10^5 cfu/mL.

Storing of milk 14 days afterwards, consistent amount of psychrotrophs were increased. In large farms Mymensingh areas showed highest and lowest bacterial load (Table 2). ML1 showed highest and ML1 showed lowest, whereas both three samples of Gazipur areas large farms within the range. Comparing within the samples of large farms of Gazipur areas GL1 showed lowest and GL3 showed the highest psychrotrophic bacterial load/mL (Table 1). GL2 samples load higher than ML1 and ML2. In case of medium farms, GM1 contained lowest bacterial load but MM3 showed highest result. Medium farms psychrotrophic bacterial load fluctuated from $3.52 \pm 0.09\times10^5$/mL to $4.3 \pm 0.11\times10^5$/mL. Likewise, all other samples count of these areas were within the range. On the other hand, small farms psychrotrophs load/mL was remarkably higher than medium and large farms. Higher values were found in GS2 which was $4.8 \pm 0.06\times10^5$ /mL and lowest for MS1 which was $4.27 \pm 0.09\times10^5$/mL. GS1, GS3, MS2 and MS3 samples load/mL were within the range of MS1 to GS2. Samples were varied within significantly ($P<0.01$).

Prakash *et al.* (2007) collected raw milk from different collection centres of kanchipuram district, India (south) and stored in frozen conditions for 15 days to enumerate and identify proteolytic and lipolytic activity of psychrotrophs. They found psychrotrophic bacterial load 4.9×10^5 cfu/mL and no significant proteolysis and lipolysis were occurred that can create any off flavor or health disorder on human health. They referred the psychrotrophic bacteria more than 5.0×10^5 cfu/mL of milk is detrimental for human health. In frozen condition (-18 to -22 °C) up to 14 days psychrotrophs counts were within the limit as maximum load/mL for this period was found in GS2 sample, which was $4.8 \pm 0.06\times10^5$ cfu/mL. Almeida *et al.* (2017) stockpiled raw milk samples from cooling tanks of five dairy farms and corresponding bulk tank after arriving industries and stored for frozen conditions for 2 weeks. They quantified psychrotrophic populations and find 4.8×10^5 cfu/mL in number. They do not find any organoleptic delusion of this samples. These findings were agreed with enumerated load/mL of two weeks stored frozen raw milk. Fagnani *et al.* (2017) enumerated and evaluated the effect of the extended (2 weeks) cold storage (-18^0C) of raw milk on the kinetics of fermentation on milk product production, additionally on the product's microbiological and physicochemical properties. They mentioned psychrotrophic population 4.95×10^5 cfu/mL and no off flavor and putrefaction is found for this milk and milk products.

Following 21 days of storage period, massive psychrotrophic bacterial load were found. Between the gap from 14 to 21 days, their number increased exponentially. During 14 days storage period highest count were found $4.8 \pm 0.06\times10^5$ cfu/mL, but after 21 days storage period highest count were found $6.24 \pm 5.76\times10^6$ cfu/mL (Table 1). So, in this stage psychrotrophs per mL of milk increased 13 times than of last stage. Among large farms GL3 showed lowest count (Table 1) but ML1 showed highest number/mL (Table 2). Their count varied from $1.63 \pm 0.9\times10^6$ cfu/mL to $2.64 \pm 0.3\times10^6$ cfu/mL. Likely, medium farms load also increase aggressively. In medium farms the highest load/mL were found in MM3 ($5.58 \pm 2.40\times10^6$ cfu/mL) but the lowest were found in GM1 ($3.07 \pm 1.93\times10^6$ cfu/mL). GM2 and MM2 contained around same amount which was 4 million/mL. MM3 load slightly higher than GM3 count. However, maximum bacterial load on this stage were found in small farms. GS2 represented the highest number and MS1 the lowest count. Small farms bacterial loads varied from $4.54 \pm 2.01\times10^6$ cfu/mL to $6.24 \pm 5.76\times10^6$ cfu/mL. Every samples of 21 days storage period contained more than 5.0×10^5 psychrotrophs per mL, so, consumption of milk at this stage may causes severe health disorders (Prakash *et al.,* 2007; O'brien and Guinee, 2011). Minimum amount of load were found in GL3 sample, 1.63 million/mL, which was more than 3 times higher than standard count. Storing of milk more than two weeks (18 days) at frozen condition causes surge on psychrotrophic microorganisms curve and their estimated number is around 7.0×10^6 million (Alrabadi, 2015; Mcphee and Griffiths,

Table 2. Psychrotrophs count in stored frozen milk collected from different areas of Mymensingh district

Sample (cfu/mL)	0 days	7 days	14 days	21 days	28 days
MS1	$2.15 \pm 0.13\times10^5$	$4.5 \pm 0.10\times10^5$	$4.27 \pm 0.09\times10^5$	$4.54 \pm 2.01\times10^6$	$9.29 \pm 3.95\times10^6$
MS2	$2.67 \pm 0.09\times10^5$	$3.59 \pm 0.10\times10^5$	$4.67 \pm 0.11\times10^5$	$6.20 \pm 5.44\times10^6$	$9.80 \pm 4.47\times10^6$
MS3	$2.5 \pm 0.23\times10^5$	$3.35 \pm 0.04\times10^5$	$4.68 \pm 0.13\times10^5$	$4.79 \pm 0.9\times10^6$	$1.06 \pm 2.31\times10^7$
MM1	$2.32 \pm 0.04\times10^5$	$2.71 \pm 0.08\times10^5$	$3.87 \pm 0.09\times10^5$	$3.18 \pm 3.56\times10^6$	$7.84 \pm 3.46\times10^6$
MM2	$1.81 \pm 0.08\times10^5$	$2.76 \pm 0.13\times10^5$	$3.80 \pm 0.25\times10^5$	$4.13 \pm 1.20\times10^6$	$8.28 \pm 2.14\times10^6$
MM3	$2.42 \pm 0.06\times10^5$	$3.34 \pm 0.08\times10^5$	$4.3 \pm 0.11\times10^5$	$5.58 \pm 2.40\times10^6$	$8.78 \pm 1.09\times10^6$
ML1	$1.4 \pm 0.05\times10^5$	$1.86 \pm 0.16\times10^5$	$3.28 \pm 0.04\times10^5$	$2.64 \pm 0.3\times10^6$	$6.64 \pm 1.10\times10^6$
ML2	$1.44 \pm 0.16\times10^5$	$2.36 \pm 0.08\times10^5$	$3.58 \pm 0.09\times10^5$	$2.27 \pm 2.32\times10^6$	$5.87 \pm 0.35\times10^6$
ML3	$1.63 \pm 0.10\times10^5$	$2.49 \pm 0.15\times10^5$	$3.82 \pm 0.07\times10^5$	$1.79 \pm 1.62\times10^6$	$4.39 \pm 2.63\times10^6$
LSD	0.252	0.232	0.256	5.533	5.730
LS	**	**	**	**	**

** = Significant at 1% level, * = Significant at 5% level, LS= Level of Significance, LSD = Least Significant Difference, MS1-MS3= Samples collected from small farms of Mymensingh district, MM1-MM3= Samples collected from medium farms of Mymensingh district, ML1-ML3= Samples collected from large farms of Mymensingh district

2011). Tribst *et al.,* 2019 assessed the effects of freezing and refrigeration over long periods (20 days) on the microbiological quality of sheep milk specially psychrotrophs and find psychrotrophic bacterial number 7.3×10^6 cfu/mL. They found serious putrefaction and off-flavor from these frozen milk samples.

Four weeks stored samples showed proportionately increasing of psychrotrophic bacterial number/mL than of three weeks. Large farm samples count varied from $4.33 \pm 2.6 \times 10^6$ cfu/mL (Table 1) to $6.64 \pm 1.10 \times 10^6$ cfu/mL (Table 2). GL3 and ML3 showed around same result and their number is $4.3 \pm 2.6 \times 10^6$ cfu/mL. Both GL1 and ML1 crossed the level of 6.0×10^6 cfu/mL. Moreover, medium farms load varied from $7.25 \pm 1.44 \times 10^6$ cfu/mL to $8.84 \pm 0.94 \times 10^6$ cfu/mL and Gazipur areas samples contained these number (Table 1). Highest load were found in GM3 and lowest were GM1. Both GM3 and MM3 number closed to 9.0×10^6 cfu/mL. GM2 and MM2 samples crossed over the level of 8.0×10^6 cfu/mL. Correspondingly, small farms bacterial number crossed the load of both large and medium farms. Maximum psychrotrophic bacterial count/mL were found in MS3, which was $1.06 \pm 2.31 \times 10^7$ cfu/mL (Table 2) and lowest in GS1, that was $8.75 \pm 2.14 \times 10^6$ cfu/mL (Table 1). Except GS1, all samples of small farms in both areas exceeded the level of 9.0×10^6 cfu/mL. GS2 and MS2 samples count within the range of 9.6×10^6 to 9.8×10^6 cfu/mL. Significant differences were found among the samples. Frozen storing of milk for extended period of time (one month) showed psychrotrophic bacterial count 1.1×10^7 cfu/mL and profound spoilage of milk due to proteolysis and lipolysis, so, consumption of such milk causes certain diseases (Porcellato *et al.,* 2018). Similar agreements also referred by Li *et al.* (2016), Gschwendtner *et al.* (2016) and Pinto *et al.* (2014). Wei *et al.* (2019) cited psychrotrophic microorganisms in one month frozen stored milk reproduce rapidly and produce high thermal stable proteases and lipases that completely spoilage milk and intake of this milk causes several food-borne-diseases. In this stage psychrotrophic microorganisms number is around 1.20×10^7 cfu/mL. Same results were also reported by Pal *et al.* (2016) and Yamazi *et al.* (2013). All of these agreements and references were similar with four weeks stored frozen milk psychrotrophs load/mL.

Determination of psychrotrophic bacteria gram staining

Selected colonies of all samples showed gram negative rods (Fig. 1). In milk most of the psychrotropic bacteria were pseudomonas (Gschwendtner *et al.,* 2016). Pseudomonas are also gram negative rods.

Oxidase test

Colonies of all samples showed positive oxidase test (Fig. 2). Pseudomonas shows positive oxidase test (Caldera *et al.,* 2016).

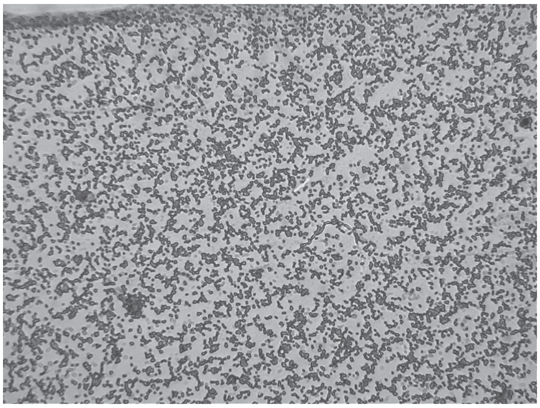
Catalase test

Pseudomonas showed positive catalase test (Fig. 3)

Methyl red (MR) test

Picked colonies showed yellow color in reaction, indicated negative MR test (Fig. 4). McAuley *et al.* (2016) worked on psychrotrophs of raw and pasteurized milk and find negative methyl red reaction for pseudomonas.

Almeida *et al.* (2017) described most of the psychrotrophs in milk is Pseudomonas



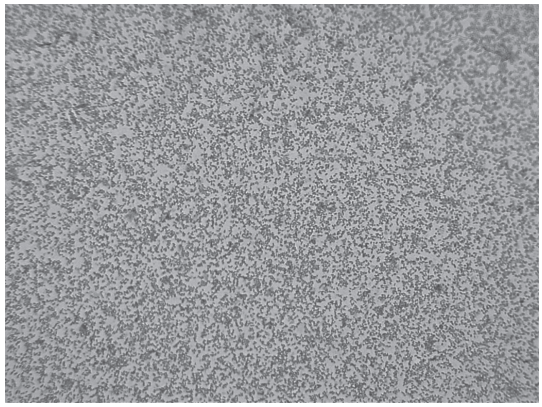
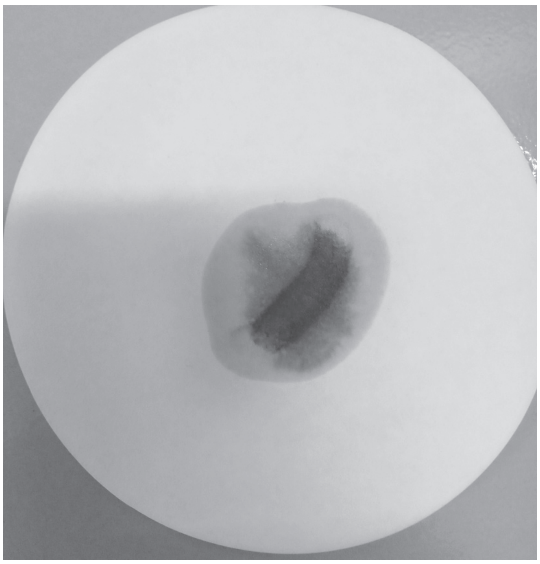


Fig. 1. Gram negative rods after gram staining of selected colonies. Image were captured by Compound Biological Digital Microscope with Camera.



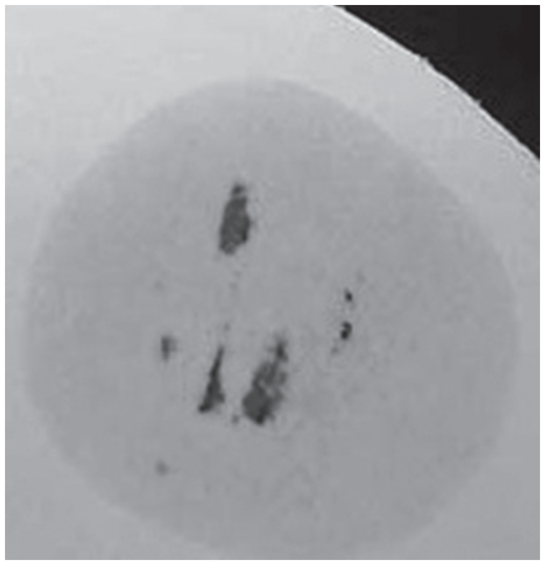


Fig. 2. Positive oxidase result of selected colonies.

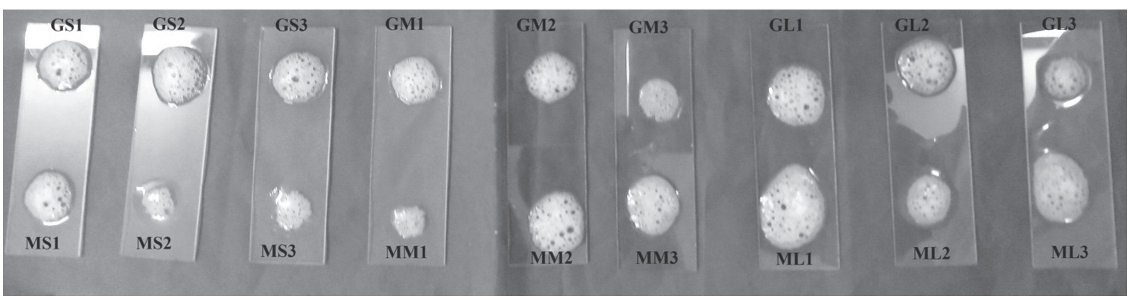


Fig. 3. Positive catalase test of selected colonies.

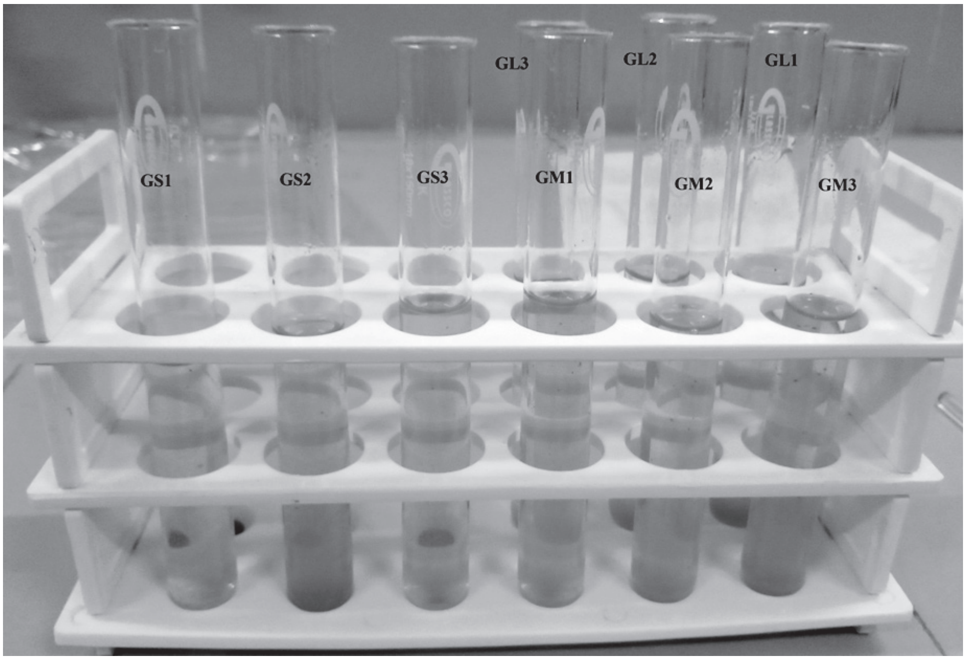


Fig. 4a. Negative MR reaction of selected colonies of Gazipur district.

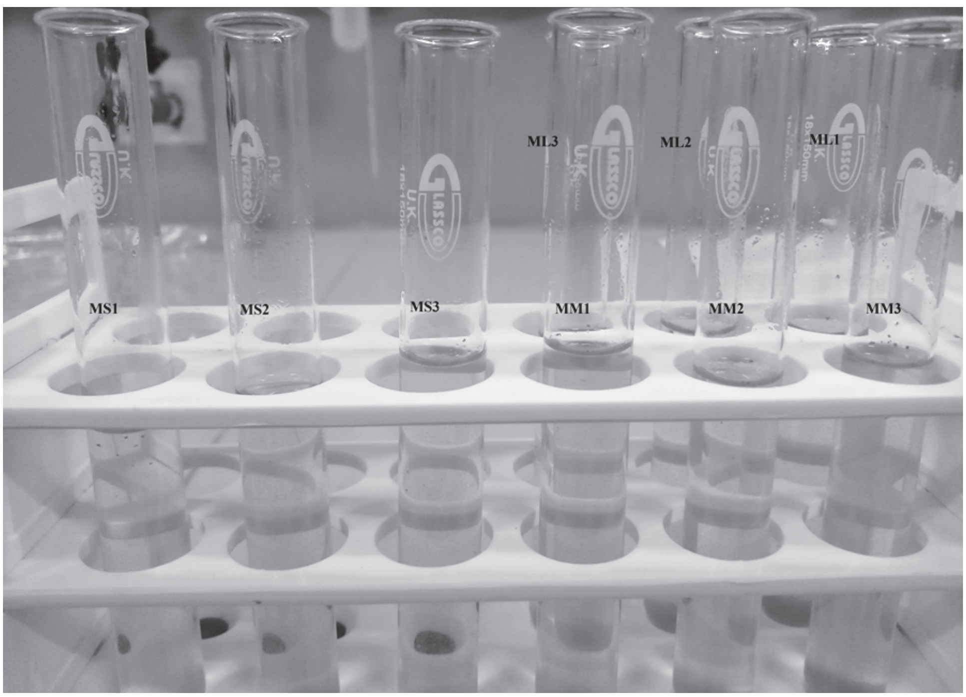


Fig. 4b. Negative MR reaction of selected colonies of Mymensingh district.

group. Gram Staining, oxidase, catalase and methyl red (MR) tests were on the favoured of Pseudomonas group. Munsch-Alatossava *et al.,* 2006 cited that, members of the genus *Pseudomonas* have long been recognised as the predominant group of psychrotrophic bacteria, recovered from spoiled refrigerated milk. Among the pseudomonads, *P. fluorescens* is generally considered to be the principal spoilage agent of stored milk. Beales (2004), Nörnberg *et al.* (2010) and Pinto *et al.* (2006) referred that, the present and subsequent replication of populations of psychrotrophs may lead to the spoilage of cold milk.

Wiedmann *et al.* (2000) quoted that *Pseudomonas* spp., with predominance of *P. fluorescens,* are the most commonly isolated spoilage organisms in frozen raw milk and majority of these bacteria (58-91%) have the ability to show distinct enzymatic extracellular proteolytic, lipolytic and phospholipolytic activity.

Conclusions

Though psychrotrophs growth rate was slow for first two weeks but, after two weeks their number increased exponentially. Following 14 days of storing, psychrotrophic bacterial number exceeded their standard range. Three weeks stored milk showed off flavor and four weeks stored milk results serious putrefaction, where psychrotrophic bacterial load exceeded more than 1.0×10^7 cfu/mL. Gram Staining, oxidase, catalase and methyl red tests were favoured on pseudomonas. Comparing types of farm, large farms showed lowest number of psychrotrophic bacterial load, both in Gazipur and Mymensingh areas.

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