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Original Article

Effect of refrigeration on prevalence and enumeration of psychrotrophic bacteria in raw milk

ShaguftaAndleeb*, Alveena Khalid and Urooj Fatima

Division of Science and Technology, University of Education, College Road Township Lahore (SA, UF); Department of Zoology, University of the Punjab, New Campus Lahore (AK), Pakistan.

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Abstract
Fifty milk samples were assessed for prevalence and enumeration of coliform bacteria under refrigeration. Refrigeration exerted profound effect conferring diminished growth of *Citrobacter*, *Enterobacte*r and *Serratia*, however, itfavoured S*almonella*, *E.coli* and *Klebsiell*a on other hand. Among them, *Salmonella* appeared with highest load both in pre-refrigerated (23%) as well as post-refrigerated (46.3%) samples. A blend of responses toward erythromycin and polymyxin B was observed by various coliform isolates, however,polymyxin B was found more effective comparatively. Predominantly, these isolates exhibited Gamma (γ) hemolysis, while only *Serratia* and *Klebsiella* arose as possible pathogenic being β-hemolytic.
Key words: Psyhrotrophs, Raw milk,*E.coli, Shigella, Enterobacter, Klebsiella, Salmonella, Citrobacter, Serratia.*

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INTRODUCTION

Being a natural nutritious drink milk becomes heavenly ideal for growth of microorganisms when it gets contaminated by soil, water or skin and hairs of the animals or utensils(Murphy and Boor, 2000) or from the milk handlers (Kohlmann *et al.*, 1991) with*Lactobacillus*, *Streptococcus*, *Escherichia*, *Bacillus*, *Salmonella*, *Pseudomonas*,*Staphylococcus* and *Micrococcus sp.*(Mubarack *et al.*, 2010; Quigley *et al.*, 2013). As it leaves the udder raw milk encounters high total bacterial count in summer (Elmoslemany *et al.*, 2009) with possible sources of contaminations of infected mammary glands or environment (Rysanek *et al.*, 2007). Air, milking equipment, feed, soil, faeces and grass are rich in microbial contaminations including pathogens (Oliver *et al.*, 2005; Torkar and Teger, 2008). Among them, many pathogens in milk become inactive and stop manipulating until favourable conditionsare met (Sangoyomi *et al.*, 2010).Refrigeration selects psychrotrophic microorganisms which affect milk adversely by releasing proteolytic enzymes(Perko, 2011).Psychrotrophic bacteria and some members of *Enterobacteriaceae* have significant proteolyticand lipolytic activities in refrigerated milk (Nornberg *et al.*, 2010) except *E.coli* which isneither proteolytic nor lipolytic (Prakash *et al.*, 2007). Poor cooling conditions allow bacteria other than psychrotrophs to grow rapidly in raw milk (Perko, 2011), hence increasing the acidity and causing deterioration (Jay, 1992). Moreover, under such conditions, proteinases and lipases released by psychrotrophicbacteria cause spoilage (Braun and Fehlhaber, 2002)like degradation of casein (Vyletelova *et al.*, 2000), off-flavouring and even putrification(Canigova and Benczova, 2001).Present study was aimed to investigate the intensity and diversity of psychrotrophic bacteria to estimate their pathogenicity and resistance /sensitivity towards various antibiotics to emphasize health risks associated with their contamination.

MATERIALS AND METHODS

Fifty milk samples collected from different dairy shops were processed to prepare three serial dilutions by mixing initially 0.1ml of original sample in 9.9 ml of sterile water. From each of the original sample and its dilutions, 0.1 ml was spread evenly over the surface of Eosin Methylene Blue EMB agar plates with

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*Corresponding author: s_andleeb@yahoo.com

subsequent incubation at 37 °C for 24 hours. The prepared milk samples after refrigeration for 168 hours were also processed in the same way. Serial dilutions' spread plate technique and their subsequent incubation were used to calculate their growth rate as well as different types of coliforms. The plates having 30-300 colonies were selected for study. Size, shape elevation, margins, surface texture, consistency, pigmentation and optical nature of well separated representative bacterial colonies were noted. Various physiobiochemical tests like Gram's/endospore staining, motility, catalase and oxidase tests were performed to characterize the isolates.

Some more tests likeIndole, citrate utilization, methyl red,VogesProskauer I and II tests were also performed. The cultural response on EMB agar was also noted to identify different types of isolates (Holt *et al.*, 1994). All the isolates were examined for their degree of pathogenicity by growing over the blood agar medium. Antimicrobic zones of inhibition against erythromycin (15 µg) and polymyxin B (300 µg) were evaluated for each isolate by using Kirby-Bauer disk-diffusion method (Pelczar *et al.*, 1986; Benson, 2001). Plates were examined after 24 hrs. A zone of inhibition (a clear area) around the disk indicated that the organism was inhibited by the drug which diffused into the agar from the disk.

Statistical analysis

Data were analyzed by One-way Analysis of variance (ANOVA) using Microsoft Excel 2010.

RESULTS

Based on differing morphologies 6 different colonies were recognized on the surface of EMB agar prior to refrigeration (Table I). The pre-refrigerated milk samples yielded 80×10^6 CFUs /ml. In refrigerated group only 3different types of colonies appeared with significant decreased growth ($P<0.05$) harvesting only upto32×10^6 CFUs /ml of original sample (Fig. 1).

Colonies of variable color, elevation, consistency, and size, ranging from 2-4mm in diameter were observed. Most of them were round, opaque, of convexed configuration and creamy consistency. All of the isolates were found motile, non-spore former, Gram's –ve, catalase +ve, indole and oxidase –ve. Except *Enterobacter*, all the Isolates were found methyl red +ve. Only *E.coli* appeared as non-citrate utilizer. *Enterobacter* and *Klebsiella* were found +ve for VogesProskauer I and II,whereas others were found –ve for both of these tests. *Serratia* and *Klebsiella* were found β hemolytic, while others were γ hemolytic (Table I).Interestingly, refrigeration affected *Citrobacter, Serratia,* and*Enterobacter*byadversely diminishing them entirely. *Salmonella* was found dominant over all the isolates both in pre and post refrigerated samples (Fig. 2).

Table I: Colonial and biochemical characteristics of bacteria isolated from the milk samples.

Isolate	Size(mm)/ (Color)	Configuration (Elevation)	Consistency/ Opacity	Indole test	Gram's &Endospore staining	Catalase/ Oxidase test	MR/ Citrate test	VP-I & II	Hemolysis	Antibiotic sensitivity test (mm)	
										E(15)	PB(300)
E.coli	3/Metallic Sheen	Round/ Convex	Creamy/ Opaque	-ve	-ve	+ve/-ve	+ve/-ve	-ve/-ve	γ	R 13	I 11
Salmonella	3/Maroon	Round/ Raised	Creamy/ Opaque	-ve	-ve	+ve/-ve	+ve/+ve	-ve/-ve	γ	S 18	I 10
Enterobacter	4/Pink	Round/ Droplike	Rubbery/ Opaque	-ve	-ve	+ve/-ve	-ve/+ve	+ve/+ve	γ	R 13	I 11
Citrobacter	1.5/Pinkish purple	Round/ Raised	Creamy/ Opaque	-ve	-ve	+ve/-ve	+ve/+ve	-ve/-ve	γ	I 17	S 13
Serratia	2/Metallic sheen	Round/ Convex	Creamy/ Opaque	-ve	-ve	+ve/-ve	+ve/+ve	-ve/-ve	B	I 15	I10
Klebsiella	4/Purple	Round/ Convex	Creamy/ Opaque	-ve	-ve	+ve/-ve	+ve/+ve	+ve/+ve	B	R 9	I 11

E(15)= erythromycin, PB(300) = polymyxin B

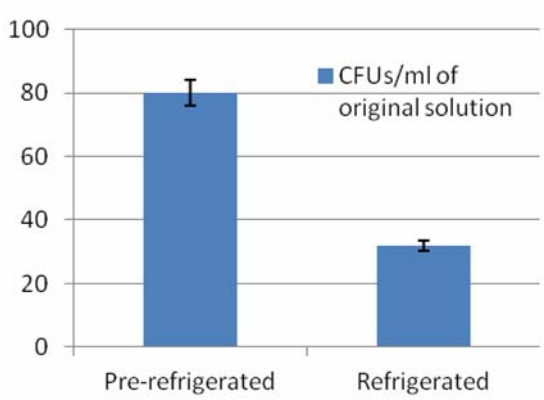

Figure 1 Pre and Post-Refrigerated number of CFUs ×10^6/ml of original milk samples

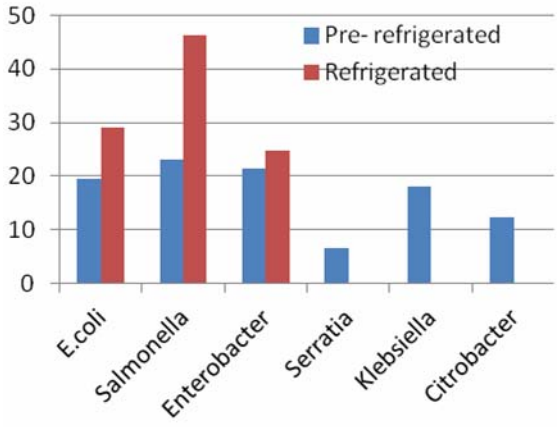

Figure 2 Pre and Post-refrigerated Number of CFUs×10^6 of various isolates /ml of original milk samples

DISCUSSION

This study was designed to analyze the prevalence of pathogenic content in milk with special emphasis on effect of refrigeration of milk.There was a significant difference between the CFUs/ml of refrigerated and non-refrigerated samples which means that refrigeration had affected the growth of Psychrotrophs negatively. Coliform bacteria had previously been isolated as the dominant psychrophilic types from milk samples of dairy products after storage at 4° C for 96 hrs.(de Garnica *et al.*, 2011).*Salmonella* was found dominant over all isolates both in pre as well as post-refrigerated samples. On the other hand, *Serratia* appeared only in pre-refrigerated with least %age. Increase in microbial content of *E.coli*, *Salmonella* and *Klebsiella* (Fig 2) reflects the compatibility of these isolates with their environment (Sangoyomi *et al.*, 2010).*Lactococcus lactis* and *Enterococcus faecium* are known as antagonistic strains in milk and have been found to show inhibitory action against *Salmonella* (Nero *et al.*, 2008) but they not inhibit *Salmonella* spp. at refrigeration temperatures (Brashears and Durre, 1999). *Salmonella, Shigella, E.coli* have been isolated from different food items like dairy and meat (Ahmed and Shimamoto, 2014).

Food containing <10^4 CFU/g, 10^4 to 5×10^6, 5×10^6 to 5×10^7 and >5×10^7 CFU/g (aerobic plate count) are rated as good, average, poor and spoiled food, respectively. In this study it is seen that milk samples represented the poor category of food before and after refrigeration.

Regarding the nature of bacteria isolated from milk samples collected from different shops, comparable microbial diversity had been isolated from dairy cattle (Botrel *et al.*, 2010)and from various locations within dairy farm environments such as water, feed, manure, and bird droppings (Kirk *et al.*, 2002). *E. coli* has been documented at highest percentage in raw milk by (Singh *et al.*, 2011). For *E. coli*,10^6 to > 10^{10} CFUs/g have been labeled as the estimated illness dose (DuPont *et al.*, 1971).

New bacterial population has also been observed to evolve following storage at refrigeration (Lafarge *et al.*, 2004).*Citrobacter* and *Serratia* have been isolated from raw cow milk (Ercolini *et al.*, 2009).*Klebsiella* has been found highly proteolytic in refrigerated milk (Nornberg*et al.*, 2010). A gene apr has been identified as responsible for proteolytic activity in psychrotrophs from refrigerated raw milk (Martins *et al.*, 2005). *Enterobacter* has not only been isolated from refrigerated milk but it showed capability to grow at refrigeration temperature (Iversen *et al.*, 2004), contrary to the present findings.

E.coli and *Enterobacter*, as in present study,have been found resistant to erythromycin over a period of time (Makovec and Ruegg, 2003; Khan *et al.*, 2011). *Enterobacteriacea* and *E.coli* have also been found resistant against polymyxin B (Castanheira *et al.*, 2008; Urban *et al.*, 2011) unlike the present results.Polymyxin B is used against multiple drug resistant pathogens including many Gram's –ve bacteria like *Klebsiella*(Bratu *et al.*, 2005; Zavascki *et al.*, 2007).Supporting the result of present study. *Klebsiella* has been involved in outbreaks in

infants(Stillwell *et al.*, 2014). Several genes have been found conferring resistance to many drugs in *Klebsiella*(Yong *et al.*, 2009). Erythromycin has been found effective against *Klebsiella* isolated from evaporated milk (Oladipo and Omo-Adua, 2011) contrary to our findings.

Unlike present study, erythromycin has not been found quite potent against *Salmonella*(Metchock, 1990; Singh *et al.*, 2012).*Serratia*has been isolated from raw milk contaminated by bovine feces (Kagkli *et al.*, 2007)and it exhibits intermediate response for polymyxin B (Lin *et al.*, 2014). *Serratia* has been found to show intermediate response toward erythromycin as it was found resistant by Chen *et al.*(2003) for many other drugs too. *Citrobacter* showed intermediate response unlike another study where it was found resistant against Erythromycin (Fass, 1993),and sensitive to polymyxin B as also found by (Gales *et al.*, 2006)Mastitis is one of the most frequent infectious diseases in dairy cattle and is a reason for antimicrobial drug usage in dairy cows(Pol and Ruegg, 2007). Use of antibiotics in adult dairy cows and other food-producing animals does contribute to increased antimicrobial resistance (Oliver *et al.*, 2011).

Among the isolates from milk samples, *E.coli*, *Salmonella*, *Enterobacter* and *Citrobacter* showed gamma hemolysis while *Serratia* and *Klebsiella* showed beta hemolysis. Non hemolytic nature may not be allied to non-pathogenic attribute because such strains may also cause diseases, like *E. coli* may cause diarrhea or other enteric diseasesand even kidney failure(Plews *et al.*, 1985).It is found that the bacteria that survive pasteurization and other which grow under refrigeration are found on the surface of teats (Bramley and McKinnon, 1990).

High coliforms bacterial content of the milk samples in the present study indicates that they contain pathogens andtheir pathogenicity as well as resistance toward antibiotics poses more threats to the consumers. It is obvious that pasteurization can lessen these threats to certain extent but that post pasteurization contamination can negate this practice (Juven *et al.*, 1981).The present and the earlier studies do not recommend the use of refrigerated raw milk, as prolonged storage at low temperature entertains psychrotrophic bacteria which are real culprits of milk spoilage (Barbano *et al.*, 2006). Mahgoub *et al.*(2011) suggested the use of certain proteins and their methylated esters capable of inhibiting pathogens in raw milk. However, the use of certain chemical preservatives to enhance the shelf life of raw milk has been reported to increase resistance against antibiotics like penicillin, ampicillin and gentamycin in *Citrobacter*, *Klebsiella* and *E.coli*(El-Zubeir and El-Owni, 2009). Finally it may be concluded that proper hygienic conditions must be managed and maintained during handling of raw milk to minimize the chances of contamination. Moreover, prolonged refrigerated storage should be avoided for possible outbreaks resulting from ingestion of raw milk.

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