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

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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*, *Enterobacter* and *Serratia*, however, it favoured *Salmonella*, *E.coli* and *Klebsiella* 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: Psychrotrophs, Raw milk, *E.coli*, *Shigella*, *Enterobacter*, *Klebsiella*, *Salmonella*, *Citrobacter*, *Serratia*.

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 conditions are 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 proteolytic and lipolytic activities in refrigerated milk (Nornberg et al., 2010) except *E.coli* which is neither 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 psychrotrophic bacteria cause spoilage (Braun and Fehlhaber, 2002) like degradation of casein (Vyvletelova et al., 2000), off-flavouring and even putrification (Canigova and Benzova, 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 0.1 ml 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
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, motilility, catalase and oxidase tests were performed to characterize the isolates.

Some more tests like Indole, citrate utilization, methyl red, Voges-Proskauer 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. Antimicrobial 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

**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 $80 \times 10^6$ CFUs /ml. In refrigerated group only 3 different types of colonies appeared with significant decreased growth ($P<0.05$) harvesting only up to $32 \times 10^6$ 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 Voges-Proskauer 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* by adversely 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.](image-url)

<table>
<thead>
<tr>
<th>Isolate</th>
<th>Size (mm) / Color</th>
<th>Configuration / Elevation</th>
<th>Consistency / Opacity</th>
<th>Indole test</th>
<th>Gram’s &amp; Endospore staining</th>
<th>Catalase / Oxidase test</th>
<th>MR / Citrate test</th>
<th>VP-I &amp; II</th>
<th>Hemolysis</th>
<th>Antibiotic sensitivity test (mm)</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>E.coli</em></td>
<td>3/Metallic Sheen</td>
<td>Round/Convex</td>
<td>Creamy/Opaque</td>
<td>-ve</td>
<td>-ve</td>
<td>+ve/-ve</td>
<td>-ve/-ve</td>
<td>γ</td>
<td>R</td>
<td>15</td>
</tr>
<tr>
<td><em>Salmonella</em></td>
<td>3/Maroon</td>
<td>Round/Raised</td>
<td>Creamy/Opaque</td>
<td>-ve</td>
<td>-ve</td>
<td>+ve/-ve</td>
<td>-ve/-ve</td>
<td>γ</td>
<td>S</td>
<td>18</td>
</tr>
<tr>
<td><em>Enterobacter</em></td>
<td>4/Pink</td>
<td>Round/Droplike</td>
<td>Rubber/Opaque</td>
<td>-ve</td>
<td>-ve</td>
<td>+ve/-ve</td>
<td>-ve/-ve</td>
<td>γ</td>
<td>R</td>
<td>13</td>
</tr>
<tr>
<td><em>Citrobacter</em></td>
<td>1.5/Pinkish purple</td>
<td>Round/Raised</td>
<td>Creamy/Opake</td>
<td>-ve</td>
<td>-ve</td>
<td>+ve/+ve</td>
<td>-ve/+ve</td>
<td>γ</td>
<td>I</td>
<td>17</td>
</tr>
<tr>
<td><em>Serratia</em></td>
<td>2/Metallic sheen</td>
<td>Round/Convex</td>
<td>Creamy/Opake</td>
<td>-ve</td>
<td>-ve</td>
<td>+ve/-ve</td>
<td>-ve/-ve</td>
<td>B</td>
<td>I</td>
<td>15</td>
</tr>
<tr>
<td><em>Klebsiella</em></td>
<td>4/Purple</td>
<td>Round/Convex</td>
<td>Creamy/Opake</td>
<td>-ve</td>
<td>-ve</td>
<td>+ve/-ve</td>
<td>-ve/-ve</td>
<td>B</td>
<td>R</td>
<td>9</td>
</tr>
</tbody>
</table>

E(15)= erythromycin, PB(300) = polymyxin B
PSYCHROTROPHIC BACTERIA IN RAW MILK

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 Psychrophils 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 $5 \times 10^6$, $5 \times 10^3$ to $5 \times 10^5$ and $>5 \times 10^7$ 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 (Norberget 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). Enterobacteriaceae 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* (Metcchick, 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 diseases and 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 and their pathogenicity as well as resistance toward antibiotics poses more threats to the consumers. It is obvious that pasteurization can lessen these threats to a 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.

**REFERENCES**


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