MICROBIOLOGICAL ANALYSIS OF PASTEURIZED AND STERILIZED MILK WITH EMPHASIS ON THE IMPACT OF STORAGE TIME AND TEMPERATURE ON THE GROWTH AND DEVELOPMENT OF MICROORGANISMS

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Abstract

Milk is an excellent breeding ground for bacteria and a large number of different species of fungi and other microorganisms. It represents food that easily and commonly gets contaminated with non-pathogenic and pathogenic microorganisms. The aim of this research was to determine the microbiological quality of sterilized and pasteurized milk immediately after opening the product, and to investigate the influence of temperature and storage time on the growth and development of microorganisms in the milk.

Randomly taken 30 samples of commercially available milk were analyzed on presence of *Escherichia coli*, coagulase-positive staphylococci, *Salmonella* sp., sulphite reducing clostridia, and the total number of bacteria. The samples were microbiologically tested immediately after opening the products, afterwards they were left at 25 °C and 4 °C for 72 hours. Microbial analysis was performed according to the standard ISO methods. Statistical method was performed according to Student t-test.

This study concluded out that pasteurized milk is more susceptible to deterioration at room temperature then sterilized milk. Presence of all tested bacterial strains except *E. coli* and sulphite reducing clostridia was recorded. Comparison of the number of microorganisms grown from samples stored at different temperatures showed statistically significant differences at the level of 0.05. Temperature as an important factor for the growth and development of microorganisms affected and the microbiota, stored at temperature of 25 °C.

Study results indicates that there is a difference in the presence of all bacterial species in sterilized and pasteurized milk after 72 hours storage at refrigerator and room temperature.

Key words: Milk, Sterilization, Pasteurization, Total number of bacteria, Time and storage temperature.

1. Introduction

Milk is a complex biological fluid, secreted by mammals for the nourishment of their young. Due to its high nutritional value it represents an important diet of children and some adults. By its nature, milk is a good growth medium for many microorganisms. Its high water activity, mild pH (6.4 - 6.6) and good supply of nutrients makes it an excellent medium for microbial growth, and therefore an agent in the spread of human diseases [1, 7]. Milk is synthesized in specialized cells of the mammary gland and sterile when secreted into the alveoli of the udder [11].

Because of the relatively short shelf life of conventional pasteurized milk, and the undesirable organoleptic changes in milk subjected to more severe heat processes, there has been much interest in alternative methods, both to improve product quality and to extend its shelf life. Some of these processes are now being applied on a commercial scale in North America and Europe. Microfiltration, usually using ceramic membrane filters, can be used in combination with a minimum HTST pasteurization process to remove significant numbers of bacteria from milk, and give a substantial extension to shelf life over conventional pasteurized milk. The fat is separated from the milk before filtration and is heat treated separately before being added back to the milk after processing. Milk produced by this method is on sale in several countries, and is said to have a shelf life of at least 20 days [26, 27]. Unpasteurized milk still represents a public health threat
considering tuberculosis caused by *Mycobacterium bovis* and *Mycobacterium tuberculosis* and brucellosis caused by *Brucella spp.* [2]. *Staphylococcus* and *Streptococcus* species are also commonly isolated bacteria from milk [3]. In late 19th century the dairy industry started pasteurization of milk as protection against spoilage. Nowadays heat treatment as prevention of food borne illness is much more recognizes rather than prevention of spoilage [1]. Among the microbial populations, Gram-negative bacteria usually account for more than 90% in raw milk are psychrotrophic species of: *Pseudomonas*, *Achromobacter*, *Aeromonas*, *Serratia*, *Alcaligenes*, *Chromobacterium*, *Flavobacterium*, and *Enterobacter* [9]. Typically, 65 - 70% of the psychrotrophs isolated from raw milk are *Pseudomonas* species which are characterized by the ability to grow at low temperatures (3 - 7 °C) and to hydrolyze and use large molecules of proteins and lipids for growth [18].

Coliforms are almost always found in raw milk, especially *Escherichia coli*, and their presence is an indication of unsanitary production and handling of either milk [19]. Raw milk is the usual source of spore-forming bacteria in finished dairy products [20]. Their number before pasteurization rarely exceed 5,000/mL [21], but they can also contaminate milk after pasteurization [22]. The most common spore-forming bacteria found in dairy products are: *Bacillus licheniformis*, *B. cereus*, *B. subtilis*, *B. mycoides*, and *B. megaterium*. In one study, *B. cereus* was isolated in more than 80% of raw milks sampled [23].

Raw milk on the farm is usually cooled quickly and stored in refrigerated bulk tanks for raw milk on the farm. It is usually cooled quickly and stored in refrigerated bulk tanks at < 7 °C prior to collection. Collection by insulated tanker is often on alternate days, or sometimes less frequently, and therefore some of the milk in the tank could be 48 hours old at the time of collection. Temperature control is thus critical to minimize microbial growth, and tanker drivers are usually permitted to refuse milk stored at too high temperature, or which has an abnormal appearance, or odor. Bacterial numbers in the milk may increase during transport, either as a result of contamination from inadequately cleaned tankers or from the growth of psychrotrophic organisms, particularly *Pseudomonas* spp. Milk temperature and duration of the transport stage are therefore important factors. On arrival at the processing site, the milk is transferred to bulk storage tanks, or silos, prior to processing. The milk may be stored in the silos for 2 - 3 days, and further growth of psychrotrophic bacteria is likely during this period. The degree of growth is dependent on the initial microbial load, and the storage time and temperature. Pseudomonads are the predominant organisms present in stored raw milk, with: *Pseudomonas fluorescens*, *Pseudomonas fragi*, and *Pseudomonas lundensis* being commonly isolated, but *Enterobacteriaceae*, *Flavobacterium*, *Alcaligenes*, and Gram-positive species can also be found. In order to remove different pathogenic organisms different heat treatments are used such as pasteurization, sterilization and ultra-high-temperature (UHT) treatment [4]. Pasteurization aims to reduce the number of viable pathogens so they are unlikely to cause disease, while UHT processing holds the milk at a temperature of 138 °C for a fraction of a second [5]. However pasteurization cannot guarantee the absence of microorganisms, when they are present in large numbers in raw milk or due to post-pasteurization contamination [8]. Microorganisms present in UHT milk are heat treatment resistant strains or originate from post-sterilization contamination. The most common cause of contamination are heat resistant spores [6]. Microbiological status of various types of heat treated milk is gaining a matter of great interest [10].

The objective of this study was to assess the effects that storage conditions (immediately after opening the packaging, milk kept at refrigerator and room temperature for at least 72 h) have on some microbiological properties of pasteurized and UHT milk.

2. Materials and Methods

2.1 Materials

Fifteen different UHT branded milk and fifteen pasteurized milk samples, of different fat content and different manufacturer, were purchased from local markets in Bosnia and Herzegovina. All samples were collected between the periods of March to April 2015 in original package. The samples were analyzed immediately after opening the packaging and 72 h after, kept at refrigerator (4 °C) and room temperature (25 °C).

2.2 Methods

For microbiological analysis the milk samples were examined for: colony count at 30 °C, *E. coli* count, *Salmonella* spp. detection, coagulase-positive staphylococci enumeration, and sulfite-reducing bacteria growing under anaerobic conditions enumeration. Microbial analysis was performed according to the standard ISO methods.

Preparation of the test samples: test samples were mixed thoroughly, so that the microorganisms are distributed evenly, by rapidly inverting the sample container 25 times. Foaming was avoided by allowing the foam to disperse. The interval between mixing and removing the test portion did not exceed 3 min. 1 mL of test sample was removed with a sterile pipette and mixed with 9 mL of diluent - buffered peptone water. The primary dilution was homogenized using vortex mixer for 10 seconds to obtain 10⁻¹ dilution [17].
2.2.1 Aerobic plates count
1 mL of initial test sample was dispensed into an empty Petri dish and mixed with molten Plate Count Agar (PCA) to form a poured plate. The plates were incubated under aerobic conditions at 30 °C for 72 h. The number of microorganisms per milliliter of the test sample was calculated from the number of colonies obtained in the plates containing fewer than 300 colonies [12].

2.2.2 Escherichia coli count
Duplicate plates of tryptone-bile-glucoronic medium (TBX) were inoculated with 1 mL of the initial suspension. The dishes were incubated for 24 h at 44 °C then examined to detect the presence of colonies which, from their characteristics, are considered to be β-glucuronidase-positive Escherichia coli. The number of CFU was calculated per mL [13].

2.2.3 Salmonella spp. detection
Buffered peptone water was inoculated at ambient temperature with the 25 mL of initial portion of milk, and then incubated at 37 °C for 18 h. Rapaport-Vassiliadis Soya Peptone (RVS) broth and Muller-Kauffmann tetraionate-novobiocin broth (MKTTn) broth were inoculated with previously obtained culture. The RVS broth was incubated at 41.5 °C for 24 h, and MKTTn broth at 37 °C for 24 h. Two selective medium Xylose lysine deoxycholate (XLD) and Salmonella-Shigella (SS) agar were inoculated at 37 °C for 24 h. Colonies of presumptive Salmonella are subcultured and their identity was confirmed by biochemical and serological test [14].

2.2.4 Coagulase-positive staphylococci enumeration
0.1 mL of the test sample was inoculated on the surface of solid selective culture medium - Baird-Parker medium, using duplicate plates. Plates were incubated aerobically at 37 °C and examined after both 24 and 48 h. Colonies were confirmed by coagulase test and of the number of coagulase-positive staphylococci per milliliter was calculated [15].

2.2.5 Sulfite-reducing bacteria growing under anaerobic conditions count
Two agar plates of iron sulfite medium are inoculated with 1 mL of the test sample. The plates are incubated under anaerobic conditions at 37 °C for 24 and 48 h. Typical black-colored colonies are counted and the number of sulfite-reducing bacteria is calculated per milliliters of milk [16].

3. Results and Discussion
The analysis of sterilized and pasteurized milk immediately after the opening was recorded microbiologically, and no increased colonies tested bacterial species were found. However, in microbiological assay for sterilized and pasteurized milk after storage for 72 hours were observed changes in microbial quantity.

The PCA, TBX, XLD, Baird Parker agar and Iron sulphite agar were sterile after inoculation and incubation of all samples of sterilized milk.

No colonies were formed on agar plates after incubation and inoculation of pasteurized milk which was analyzed immediately after opening. However, analysis of samples standing at a temperature of 25 °C was determined the growth of bacterial colonies on agar plates (Figure 1).

On storage at 25 °C for 72 hours to effect the growth of bacteria in six samples sterilized milk (of 15) we found aerobic mesophilic bacteria, colonies of Salmonella spp., and bacterial colonies of S. aureus and other species. (Table 1).

Pasteurized milk proved to be more susceptible to spoilage, and recorded growth of S. aureus, the total number of aerobic mesophilic counts and species of the genus Salmonella spp., and bacterial colonies of S. aureus and other species. (Table 1).

It should be noted that the samples of milk were different fat content, and that all the samples are treated exactly the same as the analysis required.

Table 1. Detected organisms on agar plate’s microbiological analysis of samples pasteurized milk after 72 hours storage at room temperature

<table>
<thead>
<tr>
<th>ID of sample</th>
<th>Detected organism</th>
<th>Medium</th>
<th>Morphology and color</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 - 15</td>
<td>Aerobic mesophilic bacteria</td>
<td>PCA Incubation during - 72 hours</td>
<td>Yellow color, Mucoid, round colonies</td>
</tr>
<tr>
<td>1 - 15</td>
<td>Staphylococcus aureus and other species</td>
<td>Baird Parker agar Incubation during - 48 hours</td>
<td>Black shiny colonies</td>
</tr>
<tr>
<td>1, 6, 9, 13</td>
<td>Salmonella spp.</td>
<td>XLD and SS agar Incubation during 24 hours</td>
<td>Black colonies with halo, round colonies</td>
</tr>
</tbody>
</table>

Figure 1. Bacterial colonies on different culture media isolated from pasteurized milk left at room temperature for 72 hours
All raw milk and milk products shall be maintained at 7 °C (45 °F) or less until processed. All whey and whey products for condensing and/or drying shall be maintained at a temperature of 7 °C (45 °F) or less; or 57 °C (135 °F) or greater until processed, except that acid-type whey with a titratable acidity of 0.40% or above, or a pH of 4.6 or below, is exempted from these temperature requirements. When milk and milk products are not cooled within a reasonable time, after being received at the milk plant, its bacterial content will be materially increased. The same reasoning applies to cooling the milk and milk products after pasteurization, unless drying is commenced immediately after condensing [24]. We found growth bacterial colonies on Baird Parker agar after incubation 48 hours at 37 °C and mesophilic bacterial colonies on Nutrient agar after 72 hours of incubation at 30 °C (Figure 2).

Figure 2. Growth bacterial colonies from pasteurized milk, after leaving on room temperature for three days, on Baird Parker agar and Nutrient agar after incubation at 37 °C and 30 °C

Anderson et al., [28], found unacceptable levels of Enterobacter spp. and Escherichia coli were in most of samples of milk in a quantitative study used a stratified random sampling technique in the selection of the 20 representative milk samples from six (6) supermarkets. They suggest effective measures to ensure safe milk for human consumption such as the phosphatase test and methylene blue reduction test should be routinely performed on each batch of milk processed by dairy plants [25].

4. Conclusions
- The analysis of sterilized and pasteurized milk immediately after opening the packaging determined microbiological safety of all samples.
- During storage of samples at room temperature for 72 hours were observed changes, and the presence of different species of bacteria.
- The total number of bacteria in pasteurized milk was found in the greatest amount.
- Storage of milk at refrigerator for 72 hours does not change the microbiological properties.
- There was a statistically significant difference in the number of bacterial colonies on the level of 0.05 in the number of bacterial colonies in the samples which were kept at + 4 °C and 25 °C.
- Microbiological analysis found that milk storage temperature significantly affects the quantity of microorganisms, as well as the presence of different types of bacteria in milk.

5. References