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المختبر المايكروبي

التحري عن التلوث البكتيري في الحليب الخام المحلي

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Detection of Bacterial Contamination in local Raw Milk

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الخلاصة

يتعرض الحليب للعديد من الملوثات الفيزيائية والكيميائية و البيولوجية وذلك بسبب طرق الانتاج واسلوب النقل وعمليات التسويق ومن هذه الملوثات درجات الحرارة والبكتيريا وغيرها. صممت الدراسة الحالية لفحص عينات الحليب الخام والتي تم جمعها بشكل عشوائي في الصباح الباكر من مواقع مختلفة مرة واحدة شهرياً خلال فترة الدراسة التي بدأت في أكتوبر 2023 وانتهت في مارس 2024

الاختبارات الميكروبية لعينات الحليب الخام اظهرت نتائج موجبة للنمو البكتيري ،حيث وجدت أنواع مختلفة من البكتيريا مثل

Lactobacilli, Streptococcus spp., Staphylococcus aureus, Escheritia coli, Bacillus cereus, Salmonella typhi, S. epidermidis, Pseudomonas aeruginosa, P. putida, Micrococcus spp, Clostridia sp. and fecal coliforms.

على اية حال، وجدت النتائج ان فقط (1,11%) من المجموع الكلي لعينات الحليب الخام صالحة للاستهلاك البشري و(9,88%) من العينات غير صالحة مما يدل على ان مصدر الاصابة او التلوث في الحليب ربما يعود الى صحة الحيوان، تداول ادوات الانتاج، وعوامل بيئة مثل الاوعية الملوثة والماء الملوث والذباب والغبار الخ....

1.1 Food Contamination

Under the laws administered by [7] a food is adulterated if it contains, a poisonous or otherwise harmful substance that is not an inherent natural constituent of the food itself, in an amount that poses a reasonable possibility of injury to health, or a substance that is an inherent natural constituent of the food itself; is not the result of environmental, agricultural, industrial, or other contamination; and is present in an amount that ordinarily renders the food injurious to health. The includes, for example, a toxin produced by a fungus that has contaminated a food, or a pathogenic bacterium or virus, if the amount present in the food may make risk to health [8].

1.2 Fresh Food Contamination

Contamination of food may take place at all stages during production and processing with possible sources being soil, feces, water, and ice, animals, handling of the products , harvesting and processing equipment and transport. Soil fertilized with farm manure or sewage waste may represent a risk of spreading of different bacterial pathogens. Faecally contaminated water that is used for irrigation and washing of products prior to processing may also be a source of these food-borne pathogens [9].

1.3 Raw Milk Contamination

Cow milk and its products are basic foodstuffs and constitute an important source of nutrients in the daily diet of humans. The content of protein, fat, carbohydrates, vitamins and minerals determine biological and technological properties of milk [10].

The highly nutritious nature of dairy products makes them especially good media for the growth of microorganisms. Milk contains abundant water and nutrients and has a nearly neutral pH. The major sugar, lactose, is not utilized by many types of bacteria, and the proteins and lipids must be broken down by enzymes to allow

sustained microbial growth. In order to understand the source of many of the spoilage microflora of dairy products, it is best to discuss how milk can first become contaminated via the conditions of production and processing [11].

One of the primary sources affecting milk quality at the source is from poor management return from herdsmanship at the herd or at barn. [12] in this study that pathogenic bacteria in milk have been a major factor for public health concern since the early days of the dairy industry. Many diseases are transmissible via milk products. Traditionally raw or unpasteurised milk had been a major vehicle for transmission of pathogens. The health of dairy herd and milking conditions basically determine the milk quality. Another source of contamination by microorganisms is unclean teats. The use of unclean milking and transport equipment also contribute to the poor quality milk [12].

According to Mubarack,H.M. dominant microbial flora associated with raw milk samples in and around Coimbatore District were in the order of *Lactobacillus sp.*, *Staphylococcus aureus*, *Escherichia coli*; *Bacillus sp.*, *Pseudomonas fluorescens*, *Salmonella sp.*, among the isolated pathogens. The presence of those bacteria in milk suggested contamination from various sources, such as animal, human, environment, utensils and others. The high numbers of the isolated microorganisms not only contaminate the milk but also multiply and grow in it. This might be due to the fact that milk is a good nutritive medium for the growth of microorganisms, especially with poor sanitary procedures and lack of the cooling facilities [12].

1.4 Environmental Variable

Food is a chemically complex matrix. Predicting whether, or how fast, microorganisms will grow in a food is difficult. Most foods contain sufficient

nutrients to support microbial growth. Several factors encourage, prevent, or limit growth of microorganisms in foods; the most important are pH, and temperature [13].

1.4.1 Hydrogen Ion (pH)

Hydrogen ion concentration, relative acidity or alkalinity. Most bacteria grow best at a neutral (7.0) or slightly higher pH, although this varies with different organisms. Most bacteria will not grow below pH 4.0 or above pH 10.0. The pH of normal milk is around 6.7, which allows the growth of many types of bacteria. Cultured dairy products have lower pH values [14].

1.4.2 Temperature

Temperature: Temperature values for microbial growth, like pH values. The rate of growth at extremes of temperature determines the classification of an organism (e.g., psychrotroph, thermotroph). The optimum growth temperature determines its classification as a thermophile, mesophile, or psychrophile [13].

Muhammad *et al.* (2009) reported that milk is enrichment medium to support growth of contaminating microbes during transportation of milk at ambient temperature; the contaminated microbes may multiply and deteriorate the quality of raw milk [15].

2. Methods

2.1 Samples Collection

Raw milk samples was include cow milk collected from Oct. /2023 –Mar. /2024, from many cows at different locations of Iraq districts and countryside such as (Abu Ghraib, Fal'loga, Azizia, Madain and Ghazaliya) early morning milking directly into sterile screw cap. Cow nipples were also sterilized with cotton diluted with ethanol prior to milking. The samples were then transported to the laboratory immediately in small size ice box in sterile conditions.

2.2 Bacterial isolation and identification

2.3 Morphology and Microscopy

All isolates were identified morphologically with colony characteristics and microscopically, the bacteria appeared under oil immersion lens (100x).

2.4 Isolation and identification of bacterial contamination

One ml of all milk sample was inoculated into 99 ml of peptone broth and Then, about 1 ml directly of inoculated broth were subcultured on Plate Count Agar, Violet Red Bile Agar, Eosin methylene blue EMB, Blood agar, Salmonella Shigella Agar SS Agar [16].

2.5 Microbial quantification

The bacterial load estimation per 1 ml of raw milk was done by adding 25 ml of milk samples into 225 ml of sterilized buffer peptone water and were thoroughly shaken to give one-in-ten initial dilution of the milk sample; the stock solution. Ten-folds of serial dilutions were made from the homogenates up to 10^{-6} with three replicates each. Appropriate spread plates were made with 1 ml aliquots from all serial dilution tubes and incubated at 30 °C for aerobic and coliform bacteria. Bacterial colonies were counted using colony counter to determine colony forming units (cfu)/ml. Dilutions with the total number of colonies on a plate were used for cfu computation according to the following formula.

Cfu/ml= No. of colony counted on plate/ volume plated (ml) * dilution factor

3. Results

3.1. Identification of bacteria microscopically and Morphologically



Fig.1:K.pneumonia colonies grown (Muroid colony) on MacConkey agar incubated at 37°C for 24hr in raw milk (String test).



Fig. 2: *Bacillus cereus* (spore forming) received normal saline. The raw milk sample stained by Gram stain (100x)

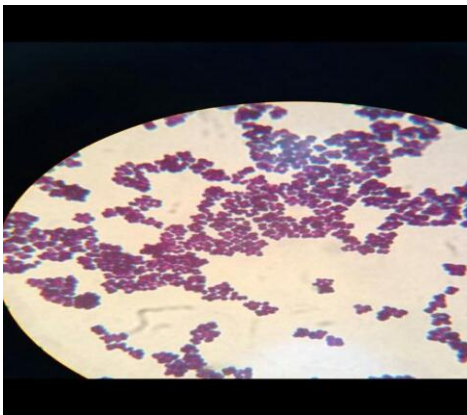


Fig. 3: *Staphylococcus* received normal saline. The raw milk sample stained by Gram stain (100x).

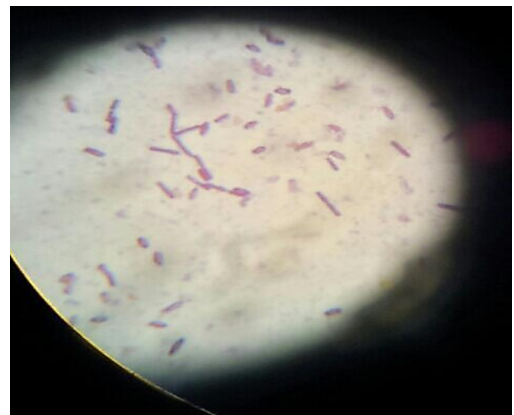


Fig. 4: *Lactobacillus* received normal saline. The raw milk sample stained by Gram stain (100x).



Fig. 5: *Escherichia coli* colonies grown on EMB agar (Green metallic sheen) incubated at 37°C for 24hr in raw milk



Fig. 6: *Escherichia coli* (Green metallic sheen) and *K pneumoniae* (pink colony) colonies grown on EMB agar incubated at 37°C for 24hr in raw milk.

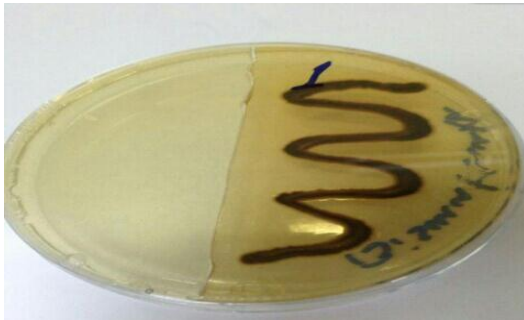


Fig. 7: *S. typhi* colonies grown (dark colony) on Bismuth agar incubated at 37°C for 24hr in raw milk.

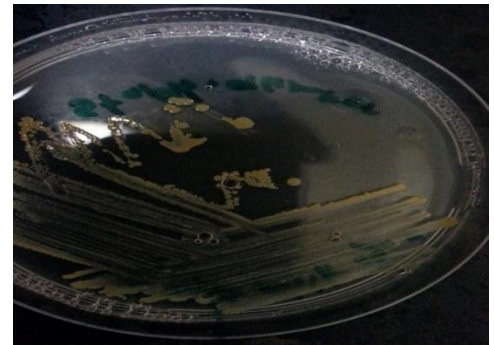


Fig. 8: *Staphylococcus aureus* colonies (golden) grown on Milk agar incubated at 37°C for 24hr in raw milk



Fig. 9: Compare between *S.aureus* β -hemolysis and *S. epidermidis* Non-hemolysis growth on blood agar incubated at 37°C for 24hr in raw milk.



Fig. 10: *S. epidermidis* Non-hemolysis growth on blood agar incubated at 37°C for 24hr in raw milk.

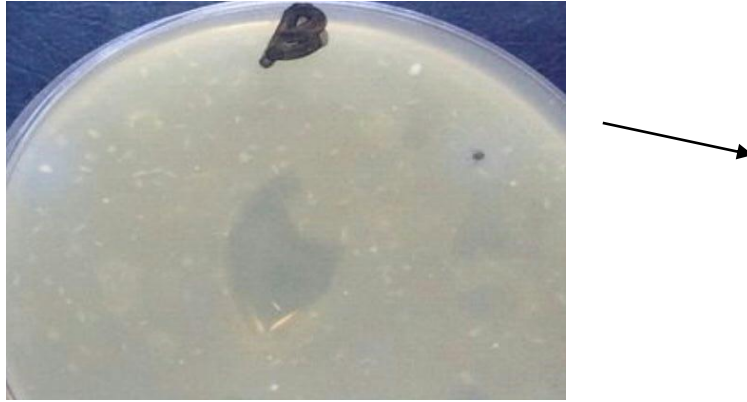


Fig. 11: *Clostridia sp.* colonies (dark colony) grown on iron sulfate agar incubated at 37°C for 72hr in raw milk.

3.2. Bacterial Biochemical Identification

Thirty six samples of raw milk from different sites during study period were cultured using different biochemical tests and the bacteria identified morphologically with colony characteristics. The result of bacteria genera and species isolated from raw milk revealed the gram negative isolates including species *Salmonella typhi*, *E. coli*, *K. pneumonia*, and *Pseudomonas aeruginosa*, as well as gram positive bacteria including *Bacillus cereus*, *Enterococci*, *Clostridium species*, *Staphylococcal aureus*, *Staphylococcal epidermidis*, *Lactobacillus* and *Micrococcus spp.* Table (3-1).

Table (3-1) Bacterial Identification Morphologically and Microscopically.

| Bacterial species | Identified Morphologically and Microscopically |
|--|---|
| <i>Salmonella typhi</i> | motile, non-sporeforming, Gram-negative, rod-shaped bacterium in the family Enterobacteriaceae. |
| <i>Escherichia coli</i> | Highly motile, Gram-negative, rod-shaped bacteria. |
| <i>Pseudomonas aeruginosa</i> | Gram-negative, motile, aerobic rods. |
| <i>K. pneumonia</i> | Gram-negative, nonmotile, aerobic, mucoid large colony explained in (string test) (Fig.1). |
| <i>Bacillus cereus</i> | Gram-positive, facultative anaerobic, endospore forming, large rod, <i>B. cereus</i> strains are motile (Fig. 2). |
| <i>Enterococci</i> | Gram-positive, facultative anaerobes that normally are spherical and ovoid, are less than 2 µm in diameter, and occur in chains or pairs or singly. |
| <i>Clostridium</i> species | An anaerobic, Gram-positive, spore-forming rod. |
| <i>Staphylococcal aureus</i> and <i>S. epidermidis</i> | Gram-positive, nonmotile, catalase-positive, small, spherical bacteria (cocci), which, on microscopic examination, appear in pairs, short chains, or bunched in grape-like clusters. The genus <i>Streptococcus</i> is comprised of Gram-positive, microaerophilic cocci that are nonmotile and occur in chains or pairs, and in long chains in broth culture. Cells are normally spherical, ovoid, and less than 2 µm in diameter (Fig. 10). |
| <i>Lactobacillus</i> | Gram-positive, non-spore-forming, motile rod or coccobacilli, catalase- negative (Fig. 4). |
| <i>Micrococcus spp.</i> | Gram-positive and catalase-positive, motile cocci appeared diplococci or tetrads (Fig. 5). |

Current results agree with Rogelj [17] who found *E. coli*, *Enterobacter Spp*, *Pseudomonas Spp*, *Klebsiella Spp*, *Staphylococcus aureus* , *Staph. epidermidis*, *Proteus spp*, *Yersinia enterocolitica* and *Salmonella* in raw cow milk.

The major group of bacteria in milk is the group of lactic acid bacteria. These are able to use the lactose in the milk and to convert it into lactic acid. The most important family in this group is the *Streptococcus lactis*. They multiply and grow very fast when the milk is kept at ambient temperatures after milking. They produced lactic acid causes the natural souring of milk. The primary source of these bacteria is the environment: air, dust, dirty equipment and operators, etc. The milk turns sour depends on the degree of contamination and on the temperature of the milk [18].

The presence of *Staphylococcus aureus* in raw milk generally comes from cows with mastitis, from handlers or from deficient hygiene. When found in milk, high levels of contamination can be reached quickly under favorable conditions. Its presence in milk can be a risk to human health, causing a public health problem, as these bacteria produces toxins that can cause toxic food infections [19]. Various conditions favor the growth of *Staphylococcus aureus* and the production of enterotoxins such as the temperature, activity of water, concentrations of salts and pH, and even the competitiveness of the micro-flora [20].

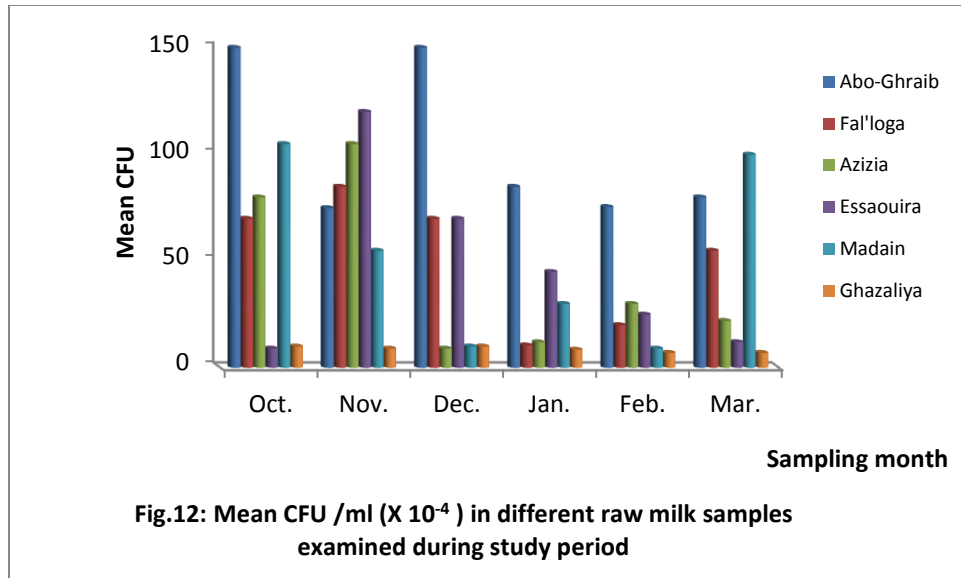
E. coli is a most common member of the normal flora, i.e. the natural habitat of it is the intestinal tract of warm-blooded organisms. It is therefore considered an indicator organism for fecal contamination of water and foods. Within a few hours or days after birth *E. coli* will colonize in the human bowel. *E. coli* becomes pathogenic only when they reach tissue outside of their normal location [21].

Salmonella also are a leading cause of foodborne disease in humans, and consumption of both meat and milk has been implicated in salmonellosis outbreaks in

people. In addition, strains of *Salmonella* resistant to multiple antibiotics have been isolated from dairy cows during salmonellosis outbreaks on dairy operations. These same strains have been isolated from ill people [22].

Pandey and Voskuil, [18] revealed the physical and chemical changes occurring after milking either due to microbial reactions or environmental factors, mitigation action to prevent further milk quality spoilage has to be observed. The raw milk is one of the most suitable media for the growth of a wide variety of bacteria. Especially immediately after milking when it is almost at body temperature. However, milk contains a natural inhibitory system which prevents a significant rise in the bacterial count during the first 2-3 hours. If milk is cooled within this period to 4°C, it maintains nearly its original quality. Timely cooling ensures that the quality of the milk remains good for processing and consumption. Ayub [23] mentioned that the bacterial load in fresh raw milk should be less than 5×10^4 cfu/ml when it reaches the collection point or processing plant. To prevent high multiplication of bacteria, the milk has to be produced and should be cooled or heated at the earliest.

The results of Viable count bacteria test 10^{-4} are given for $\times 10^{-4}$ test, again highest (150 x 104 CFU/ml) and lowest (7 CFU/ml) contamination were recorded in milk samples of Abo-Ghraib during Desember and Ghazaliya during March respectively (Fig. 12) and the study found that LSD value ($P \leq 0.05$) between these data was 27.18 showing significant differences some of these data.



This study was carried out to evaluate the quality of raw milk measured by Total Bacterial Count (TBC). Results showed a wide variation of TBC during study period. The TBC higher than 100.000 cfu/ml considered second grade (acceptable) was found in 11 samples (30.5%) of total number of samples which was recorded area indicating the most inconvenient condition of milk handling and management and TBC 100.000 cfu/ml considered first grade (acceptable) was found in 21 samples (58.3%) and TBC 50.000 cfu/ml considered first grade (good) was found in 4 samples (11.1%) area showing the suitable condition of milk production according to Iraqi Standards (IQS) (2006). Similar observation, the high variability of TBC was found in the present study was supported by the finding with [24,25] .

However, the growth of bacteria accompanied by lacking of cooling environment through long distance of milk transportation. [24] also reported similar problems concerning hygienic quality of raw milk received in Morocco. Similarly, [1] observed similar results due to lapses in milk sanitation. [26, 27] documented difficulties to obtain high quality milk during summer season. They reported that the increase in air temperatures favour the increase of bacterial growth, especially on the

surfaces of not well cleaned milking equipment which was the potential source of infection.

3.3 Trend of monthly recordings of cfu/ml

During October, November and December at high mean ambient temperature recorded 31, 27, 23°C respectively, than the rest of months. Lower microclimatic temperature during winter was recorded 13, 11, 29°C in January, February and March respectively, also reduced bacterial count ($<10 \times 10^6$). The present observation of reduced number of cfu/ml, as winter advanced, was in agreement with earlier finding of [24] who also observed the highest cfu/ml of raw milk during the hottest months of October followed by November and December and the lowest cfu/ml during the coolest or driest month of January and February [24].

A higher number of cfu/ml was observed in October, November and December as compared to January, February and March which might be due to high ambient temperature. The trend of bacterial load showed a positive relation with prevailing ambient temperature. Data obtained from pervious study also indicated higher microbial counts during summer. A similar observation was recorded by [28, 29] in different agro-climatic region in goat milk.

Coorevits [30] reported that the bacterial contamination of raw milk can originate from different sources: air, milking equipment, feed, soil, faeces and grass. The number and types of micro-organisms in milk immediately after milking are affected by factors such as animal health, season, food and animal health [17].

The present study results showed that the pH of raw milk ranged from (6.62-6.80) through study sampling from Oct. /2023 –Mar. /2024, No differences were observed in pH. It was slightly acidic duo to the lactic acid present in it.

The milk has a pH of around 6.5 to 6.7, which makes it slightly acidic. Milk contains lactic acid [31].

Conclusions

1- Bacterial contamination was found in raw milk samples which were collected from different location of Iraq. Microbial counts as quality indicator of raw milk and the possible impact of specific influence factors are of central importance, and such specific influencing factors are therefore of great concern in hygienic milk production.

2- In this study, the present are TBC in milk varied from one region to another and varied among seasons, due to environmental pollution and poor cleaning of the milking system. Milk can have bacteria in it, either from the cow (if it is ill) or from the handling of the milk before it is packaged and delivered to the store in region with highest TBC, while region with lowest TBC was showing healthy environment suitable for cattle breeding and the better condition of milk production and sample collected milk from clean, healthy cows.

Recommendation

1- Examine other biological content such as virus and other dangerous bacterial strains, again in raw milk in Iraq.

2-The assessment of other environmental variables of breeding and manufactured dairy products.

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Study framework

The current study was designed to examine raw milk samples which were collected randomly at early morning from different location such as (Abu Ghraib, Fal'loga, Azizia, Madain and Ghazaliya) at a rate of once each month during study period from Oct./ 2023 and ended in Mar./ 2024 testing in bacteriological laboratory.

Aim of the study

- 1- Determine level of bacterial contamination in milk samples.
- 2- Study some affecting environmental factor on milk quality as pH and Temperature.

Summary

Milk is exposed to various physical, chemical and biological pollutants due to producing, transporting and marketing processes such as certain temperature, bacteria and others. This study was designed to examine raw milk bacterial loads and micro-organisms associated with milk handling practices which collected randomly at early morning from different location once monthly during study period which commenced in Oct. 2023 and ended in Mar 2024. The microorganisms were isolated and identified.

Microbial tests of raw milk samples were positive for bacterial growth, as different bacterial species were observed such as *Lactobacilli*, *Streptococcus spp.*, *Staphylococcus aureus*, *Escheritia coli*, *Bacillus cereus*, *Salmonella typhi*, *Staphylococcus epidermidis*, *Pseudomonas aeruginosa*, *P. putida*, *Micrococcus spp*, *Clostridia sp.* and *feacal coliforms*.

That, only (11.1%) of the total collected samples of raw milk were suitable for human consumption and (88.9%) unsuitable, this indicating the source of infection or contamination of milk may be: the animal health, human handler and the environmental factors, e.g. ,contaminated vessels, polluted water, flies and dust, etc...

Introduction

Milk is considered as a nearly complete food since it is a good source for protein, fat and major minerals. Also, milk and milk products are main constituents of the daily diet, especially for vulnerable groups such as infants, toddler and elderly [1], and it is a major constituent of the diet, its quality assurance is considered essential to the welfare of a community. In the last few years, the contamination of milk is considered as one of the main dangerous aspects [2].

A wide variety of chemicals from man-made sources may be found in or on foods. Contaminants in foods may come from application of pesticides to crops, from transport of industrial chemicals in the environment, or from chemicals used in food packaging products [3, 4]. Bacteria are one of the biggest competitors for our food supply. We are constantly struggling to prevent food from spoiling. Spoilage is the result of the action of bacteria and other microorganisms [5]. Milk server as a good medium for the growth of many microorganisms, especially *Lactobacillus*, *Streptococcus*, *Staphylococcus* and *Micrococcus sp.* Bacterial contamination of raw milk can originate from different sources from animals such as air, milking equipment, feed, soil, feces and grass [6].