Sudan University of Science and Technology
College of Graduate Studies

Evaluation of Bacteria in Row Milk in Khartoum State

تقيم البكتريا في اللبن الخام في ولاية الخرطوم

A thesis Submitted in a Partial Fulfillment for the Requirement of the Master Degree in Preventive Medicine

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الآيـــــة

بسم الله الرحمن الرحيم

قال تعالى:

﴿ وَإِنَّ لَكُمْ فِي الْأَنْعَامِ لَعِبْرَةً نُّسْقِيكُم مِّمَّا فِي بُطُونِهِ مِن بَيْنِ فَرْثٍ وَدَمٍ لَّبَنًا خَالِصًا سَائِغًا لِلشَّارِبِينَ ﴾

صدق الله العظيم

سورة النحل الآية (66)

Dedication

Every challenging work needs self-efforts as well as guidance of

elders especially those whom were very close to our heart.

To those of the fingers to give us a life of happiness.

My humble effort I dedicate to my sweet and lovely mother.

To reap the thorns out of my way for me to pave the way science

To heart the great my father

I ask Allah to mercy my dear father and forgive you and shed your soul paradise

Along with all hard working and respected teachers

To my husband, my sons brothers, sisters and my friends

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Many thanks due to Allah who owned me with health and courage to accomplish this work.

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Abstract

The study was conducted to evaluate the bacteria in row milk during 2018 in Khartoum state. In this investigation total of 30 samples were collected from different marketing and supermarket of Khartoum State (Omdurman , Khartoum and Bari localities). For isolation and identification of bacteria in these samples cultural methods and biochemical tests were used. The results revealed that 3 types of bacteria were identified and these were *Staphylococcus spp* (40%) –*Salmonella spp* (26, 7%) and *E.coli* (33.3%). Gram negative bacteria represented the higher percentage (60 %) compared to gram positive (40%). In conclusions the contamination of row milk by microorganisms indication of poor sanitary measures.

المستخلص

هذه الدراسة أجريت لتقيم البكتريا في اللبن الخام اثناء عام 2018 م بولاية الخرطوم. وفي هذا التقصي لعدد 30عينه جمعت من مختلف الأسواق والسوبر ماركات في ولاية الخرطوم (محليات أم درمان، الخرطوم وبحري). وذلك للعزل والتعرف على البكتريا في تلك العينات باستعمال الزراعة والاختبارات الكيميائية. والنتائج كشفت عن وجود ثلاثة أنواع من البكتريا تم التعرف عليها وهي المكورات العنقودية (40 %) وسالمونيلا (26,7%) والأي كولاي (33,3%). البكتريا السالبة تمثل اعلي نسبة (60%) مقارنة بالبكتريا الموجبة (40%).وفي الختام التلوث في اللبن الخام بالجراثيم يوضح ضعف الإجراءات الصحية.

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Introduction

Milk is a natural liquid food, is one of our most nutritionally complete foods, adding high-quality protein, fat, milk sugar, essential minerals, and vitamins to our diet. However, milk contains bacteria that–when improperly handled–may create conditions where bacteria can multiply. Most of the bacteria in fresh milk from a healthy animal are either harmless or beneficial. But, rapid changes in the health of an animal, or the milk handler, or contaminants from polluted water, dirt, manure, vermin, air, cuts, and wounds can make raw milk potentially dangerous.

Raw milk is milk from cows or other animals that has not been pasteurized,

Raw milk may contain harmful bacteria or germs such as Salmonella, E. coli and Listeria, These bacteria can lead to very serious illnesses including fever, vomiting, diarrhea, life-threatening kidney failure, miscarriage and death.

Pasteurization is the process of heating milk to a high enough temperature for a long enough time to kill bacteria or germs in the milk that can cause illness. Pasteurization is exactly the same type of process as cooking poultry or meat. It does not affect the quality or the amount of calcium, protein, riboflavin and vitamin A in milk. Pasteurization of raw milk has prevented thousands of illnesses and deaths. According to the Food and Drug Regulations, milk must be pasteurized before it can be sold in Canada.

Objectives

- To isolate Gram –negative and positive bacteria from row milk in Khartoum state .
- To identify the bacteria isolated from row milk using biochemical tests.

Chapter One

Literature review

1.1.Definition of milk

Milk is a highly nutritious food, ideal for microbial growth and the fresh milk easily deteriorates to become unsuitable for processing and human consumption (FAO 2001). High bacterial counts are indicator of poor production hygiene or ineffective pasteurization of milk (Harding 1999). Milk and milk products derived from dairy cows milk can harbor a variety of microorganisms and can be important sources of food borne pathogens (Oliver *et al.,* 2005; Yagoub *et al.* 2005). The presence of food-borne pathogens in milk is due to direct contact with contaminated sources in the dairy farm environment and to excretion from the udder of an infected animal (El Zubeir *et al.* 2006). The raw milk distributed for consumption in Sudan does not find the real quality control measures needed to be of good quality food (Mohamed and El Zubeir 2007). However, some new private dairy plants started the processing of fluid milk and some dairy products. These are faced with many problems of which the quality control measures constitute an important concern. Hence, the present study was designed to assess the chemical, physical and microbial properties of raw milk supplied to the Blue Nile Dairy Company plant (CAPO) and to compare it with the produced pasteurized milk. The hygienic quality problems of milk may arise from raw milk of diseased animals (Murphy and Boor,2000). Kang *et al.* (2005) reported that the presence of antimicrobial substances in raw milk could have serious toxicological and technical consequences. Raw milk may contain over 2,000,000 cfu/ml before processing of liquid milk or cheese making (Kameni *et al.*, 2002). Milk is an indispensable food for human being from infancy to

old age. It contains all the nutrients necessary for health in almost ideal proportion. So, it is considered as natural single most complete food (O'Mahony, 1988). Milk contains all nutrients but during heating, some of the enzymes are destroyed which are essential for the absorption of calcium and vitamin D. Pasteurization is a heating process to prevent the spoilage of milk. Pasteurization has been used since the early 1900s (heating raw milk to 161◦F for 15 minutes) is expected to remove microorganisms from milk (Imele *et al*., 2002). Milk is considered as nature's single most complete food (O'Mahony, 1988) and is definitely one of the most valuable and regularly consumed foods. Similarly it is a good growth medium for spoilage and pathogenic micro-organisms (Speer, 1998). Milk should have normal composition, not adulterated and produced under hygienic condition (Chamberlian, 1990). Contamination of milk and milk products with pathogenic bacteria is largely due to handling, processing and unhygienic conditions (Maity *et al*., 2010). Milk may be contaminated by the microbial agents during processing, transportation, storage and preparation of milk products for consumption. Microbes have access to milk to reproduce at a rapid rate. Pasteurization is done for the improvement of keeping quality of milk. The purpose of chemical and microbial quality control carried out by the government inspection service is to provide good and safe food for human consumption. Due to the non-enforcement of Milk Inspection Act, consumers are deprived of getting quality milk. Due to widespread consumption by infants, children and adults, milk has been marked as the most important food for which chemical and microbial quality control should be attained. Staphylococci may come into milk and milk product from food handlers (Pelczar *et al*., 1965). Animal source foods have found guilty for the majority of food-borne diseases (De Buyser *et al*., 2001) and incidences increase with increasing access to such foods especially

without adequate hygiene, inspection for safety or satisfactory heating for killing pathogens (McCrindle, 2008). Being highly perishable commodity and highly nutritious food, milk serves as an ideal medium for the growth and multiplication of various microorganisms (Parekh and Subhash, 2008). According to Bertu *et al*. (2010) humans may be infected with milk-borne pathogens through consumption of infected raw or unpasteurized milk and milk products. Although milk and milk products are minor constituents in most diets but contaminated milk are responsible for up to 90% of all dairy related diseases of humans (De Buyser *et al*., 2001). Shirima *et al*. (2003) reported several pathogens resulting to milk-borne zoonotic diseases including brucellosis, enterotoxaemia and tuberculosis.

1.2.Common pathogens in milk

Illnesses from contaminated milk and milk products have occurred worldwide since cows have been milked. In the 1900s it was discovered that milk can transmit tuberculosis, brucellosis, diphtheria, scarlet fever, and Q-fever (a mild disease characterized by high fever, chills, and muscular pains) to humans. Fortunately, the threat of these diseases and the incidence of outbreaks involving milk and milk products has been greatly reduced over the decades due to improved sanitary milk production practices and pasteurization.

1.2.1.*Salmonella*

Salmonellosis is the most common disease transmitted in raw milk. This organism is shed in the feces of cattle and picked up on the animals' hair or teats. Many strains of *Salmonella* can cause foodborne illness in humans, and all strains exhibit the same symptoms such as gastroenteritis (vomiting and diarrhea). Pasteurization destroys the *Salmonella* organism, and although pasteurized milk, powdered milk, and cheese have been implicated

in salmonellosis outbreaks, in these cases, the pasteurized milk was contaminated during further processing.

1.2.2Staphylococcus aureus

is a common cause of mastitis in dairy cattle and can enter the milk supply from sores on the teats of cows or from the hands and nasal discharges of dairy farmers and workers. The *Staphylococcus* organism produces an enterotoxin (toxins causing vomiting and diarrhea) in raw milk when it is held at temperatures above 50 degrees Fahrenheit. Sufficient amounts of enterotoxin in foods can cause illness. The incidence of staphylococcal intoxication has been greatly reduced by pasteurization.

1.2.3.*Escherichia coli 0157: H7*.

Recent studies show that young dairy cattle are host to *E-coli* and fecal contamination is a likely source of *E-coli* in row milk. It can cause hemorrhagic colitis and hemolytic uremic syndrome in humans. Milk should be stored at temperatures below 40 degrees Fahrenheit to inhibit the growth of *Escherichia coli 0157: H7*. Temperature abuse during holding and shipping can cause significant growth of the organism. Pasteurization destroys this organism.

1.2.4.Listeria monocytogenes.

This widespread organism is found principally in soil. Listeriosis in humans may cause serious illness, and is especially dangerous to pregnant women, causing stillbirths or infant death soon after birth. Pasteurization inactivates *Listeria monocytogenes*.

1.2.5.*Yersinia enterocolitica*.

This common organism has been found in many foods of animal origin including milk, cheese, and red. Yersinia, found in streams, lakes, and wells, spreads from the water to warm-blooded animals. The most common

symptom of yersinosis is gastroenteritis and mimics the symptoms of appendicitis. *Yersinia enterocolitica* is destroyed by pasteurization.

1.2.6.*Campylobacter jejuni.*

This organism, isolated in raw milk and meat, can cause mastitis in dairy cattle. It has also been isolated in the feces of many species including dogs, cats, rodents, cattle, sheep, swine, and poultry. Symptoms include vomiting, cramps, bloody diarrhea, mild enteritis, or severe enterocolitis. Individuals who have recovered from the disease may suffer a relapse. *Campylobacterjejuni* is destroyed by pasteurization.

1.2.Microorganisms enter milk from a variety of sources

These are beneficial or harmful, for example *Lactococcus*, *Lactobacillus* , *Streptococcus* , *Propionibacterium* and fungal populations facilitate dairy fermentations, *lactobacilli* and *bifidobacteria* promote health while *Pseudomonas* , *Clostridium* , *Bacillus* and other spore-forming or thermoduric microorganisms cause spoilage and *Listeria monocytogenes* , *Salmonella* , Shiga toxin producing *Escherichia coli* (STEC), *Campylobacter* and mycotoxin-producing fungi cause disease (Quigley *et.al.*,2013).

1.3.How do microorganisms enter the milk supply?

the environment contains an abundance of microorganisms that find their way to the hair, udder, and teats of dairy cows and can move up the teat canal. Some of these germs cause an inflammatory disease of the udder known as mastitis while others enter the milk without causing any disease symptoms in the animal. In addition, organisms can enter the milk supply during the milking process when equipment used in milking, transporting, and storing the raw milk is not properly cleaned and sanitized. All milk and milk products have the potential to transmit pathogenic (disease- causing) organisms to

humans. The nutritional components that make milk and milk products an important part of the human diet also support the growth of the organisms. Drinking raw milk causes foodborne illness, and dairy producers selling or giving raw milk to friends and relatives are putting them at risk.

1.4.Milk Value Chain

According to Kaplinsky and Morris (2000), the value chain describes the complete range of activities required to bring a product from conception to the delivery to final consumers, and the final disposal after use. It includes activities like design, production, marketing, distribution and support to the final consumer (Ruijter de Wildt *et al*., 2006). Milk value chains have several outlets through which milk products flow from the producer to the consumer, which impacts the quality of milk and transaction costs as well as potential risk of contamination with pathogens. Hence, an understanding of functional market chains is an important first step towards understanding and dealing with milk safety risks (Kilango, 2011).

1.5.Milk Composition and Milk Quality

On average 87.4 % of the cow's liquid milk is water, 3.7% is milk fat (milk lipids or butter fat), 8.9% is solids-not-fat (SNF), 3.4% is protein (2.8% casein, 0.6% whey protein), 4.8% is lactose, 0.7% includes minerals (micronutrients such as Zinc, Iron and Copper as well as macronutrients such as Calcium, Phosphate, Magnesium, Sodium, Potassium, Citrate and Chlorine). This group also includes sulphate, bicarbonate, acids (citrate, formate, acetate, lactate and oxalate), enzymes (peroxidase, catalase, phosphatase and lipase), gases (oxygen and nitrogen) and vitamins A, C, D, Thiamine and Riboflavin(Nangwala, 1996; Tesha, 2010). In addition, milk is a good source of many other vitamins such as B6, B12, K, E, niacin, biotin, folates, and pantothenic acid (Goff and Hill, 1993). In general, milk has a high

nutritional value and it is a good diet for the children (FAO, 2005). It provides nourishment and immunological protection (Bauman, 2004). However, if not handled properly, milk can be easily destroyed through contaminations and bacterial growth and becomes unfit for human consumption. Some of the microbial contaminants are responsible for milk spoilage while others are pathogenic with potential health effects to cause milk borne diseases (Kivaria *et al.,* 2006). Bacterial count in milk is influenced by the temperature at which milk is stored and the time that elapses since milking. Once the milk is cooled to 4ºC within 2 – 3 hours after milking, it preserves its original quality and remains safe for processing and consumption (Omore *et al.*, 2005). East African countries (EAC) have harmonized standards for some products including milk. Standards are reference points and tools for ensuring quality and safety. East African Standard (EAS67) Prescribes quality requirements for raw, normal cow's milk. It covers bacteriological quality. It is important that all players in the milk value chain implement standard at their level of operation to protect the consumer (EAS, 2006).

1.6.Sources of Microbial Hazards in the Milk Value Chain

Microbial contamination of milk in the value chain can originate from a diseased cow, unhygienic milking practice, poor personal hygiene, unsanitary utensils and/or milking equipment and water supplied in sanitary activities (Parekh and Subhash, 2008; Kilango, 2011; Lubote *et al.*, 2014). Bacterial contamination of raw milk can originate from different sources: air, milking equipment, feed, soil, faeces and grass (Coorevits *et al*., 2008). The number and types of micro-organisms in milk immediately after milking are affected by factors such as animal and equipment cleanliness, season, feed and animal health (Rogelj, 2003). It is hypothesized that differences in feeding and housing strategies of cows may influence the microbial quality of milk

(Coorevits *et al.*, 2008). Rinsing water for milking machine and milking equipment washing also involve some of the reasons for the presence of a higher number of micro-organisms including pathogens in raw milk (Bramley, 1990). A cow with an infectious disease can shed pathogens from its blood into the milk. Findings by Streeter *et al*. (1995) indicate that infected cows with clinical disease and subclinical infections shed *Mycobacterium avium* subspecie *Paratuberculosis* in both milk and faeces. Detectable levels of the organism were observed in milk from both clinically infected and asymptomatic carrier animals. Also, infected mammary quarters or cows and the environment, in which animals are kept, are known to be chief sources of bacteria that cause udder infections in a herd. Transmission of infectious bacteria to teats of uninfected mammary quarters or cows occurs mostly at milking (Kilango, 2011). Appropriate milking hygiene practices reduces the rate of new infections during milking (Robert, 1996). The use of pre- and post-milking teat disinfectants is an effective measure in reducing the risk of new infections. Pre-dipping reduces the resident teat skin bacterial population, which is the main source of infection for the mammary gland. It can reduce new environmental streptococcal infections and *E. coli* by 50%. Post-dipping prevents the transmission of contagious bacteria such as *S. aureus* (NADIS, 2013).

1.7.Health and Economic Impact of Unsafe Milk

Food safety is an area of great concern in terms of public health management especially from an economic point of view (Mangwayana *et al*., 2000). Food-borne diseases due to microbial pathogens in milk are a serious threat to the health of millions of people (FAO, 2006). Raw milk continues to be a staple in the epidemiological literature linked to campylobacteriosis, salmonellosis, tuberculosis, brucellosis, hemorrhagic colitis, Brainerd diarrhoea, Q fever,

listeriosis, yersiniosis, and toxoplasmosis to name a few (Plotter, 2002). These impose a substantial burden on health care systems and reduce economic productivity (FAO, 2006). Seventy percent of deaths among children fewer than five years are linked to biologically contaminated food and water (Unnevehr and Hirschhorn, 2000). Also, unsafe milk and milk borne illnesses cause producers, vendors and wholesalers to earn a poor reputation, which may take time to overcome and consequential loss of income. These important players may also become prey of milk borne illness thus perpetuating the cycle of poverty (Nhachi and Kasilo, 1996; FAO, 2006).

1.8.Antibiotic Residues in Milk

Although milk is such an important source of nutrition to people in different age categories, it sometimes contains an unacceptable high level of antibiotic residues so causes problems to consumers of such milk and its products (Muhammad, 2014). Antibiotic residues in milk originate from various sources namely residue of herbicides on feedstuffs, drugs given to cow orally, by injection or as an intramammary infusion for the treatment of mastitis (Jahed, 2007). This problem has led to the development of various techniques to check the level of antibiotic residues in milk to name few DelvoTest and CharmEZ methods. Drug residues in milk apart from other hazardous effects it also affects negatively the health of the consumer of milk with high level of antibiotic residues. These effects include allergic reactions and bacterial resistance in the body of humans (Muhammad, 2014). The HACCP approach has an important role in preventing and controlling of chemical contamination in milk and dairy products especially antibiotics in raw milk transported from the producer (Jahed, 2007). The HACCP concept is dealing with hazard and risk identification, process decomposition, designation of critical control

points, documentation and verification of the programme, is an alternative to the ISO system (Noordhuizen, 2005)

1.9.The Microbiological Quality of Milk

Microbial quality of milk refers to the cleanness of milk. This is defined by a number of bacteria present in milk. The high bacterial count as well as the presence of pathogenic bacteria in milk not only degrades the milk quality and shelf-life of milk or milk related products but also poses a serious health threat to consumers (Yuen *et al*, 2012). Milk being a wholesome food with high nutritive value is often prone to early contamination and spoilage if not handled properly (Minj and Behera, 2012). The fewer the number of micro-organisms in milk the higher the quality of milk. The microorganism may originate from the cow or the environment. Examples of these are lactic acid bacteria and mastitis causing bacteria. The lactic acid bacteria convert milk sugar i.e lactose into lactic acid. The common spoilage bacteria in milk multiply at the temperature range of 20℃ - 25℃. The rate of multiplication for microorganisms can be slowed down through cooling the milk and keeping it at the temperature of 4℃ and below. The microbiological quality of milk is affected by storage temperatures and time taken in milk transporting. The longer the time taken to transport the milk, the more likely the milk is going to spoil. The rate of cooling and milk handling procedures during and after milking are also important in determining the quality of milk. Tanzania uses the standards as described by the Tanzania Bureau of Standards and by the East African Standards and COMESA (EAC, 2006). These state that quality of raw milk is poor if it has a coliform count of more than 50 000 CFU/ml and a total bacterial count of more than 2 million CFUs/ml. Many types of bacteria live harmlessly in the digestive systems of people and animals. For example,

E.coli is one of them, but some strains of *E.coli* such as *E.coli* O157:H7, produce toxins that can cause serious illness (Pennington, 2005).

1.10.*Enterobacteriaceae* in milk

The *Enterobacteriaceae* is a large family of gram-negative, rod shaped bacteria, which includes more familiar pathogens, such as *E. coli, Salmonella* ***spp.,*** *Klebsiella spp., Shigella spp., Yersinia pestis* and other disease causing bacteria such as *Proteus spp., Serratia spp., Enterobacter spp.* and *Citrobacter spp.* (Brenner *et al*., 2005). As several of these organisms are potential pathogens, consumption of raw milk is considered highly risk (Anand and Griffiths, 2011). In recent years, there has been emergence of new milk borne bacterial pathogens with serious and even life-threatening complications such as enteric *E. coli* serotypes (Sivapalasingams *et al*., 2004). Verocytotoxigenic *E. coli* sero-groups may infect humans through consumption of infected raw unpasteurized milk and milk products, which have significant contribution to the reported cases of Shiga toxin producing *E. coli* (STEC) in humans (Baylis, 2009). Also, *Salmonella* infections of food animals play an important role in public health and particularly in food safety, as food products of animal origin are considered to be the major source of human *Salmonella* infections (OIE, 2008). It has adapted to survive and recognize temperature and pH extremes, oxygen limitations, presence of bile salts, digestive enzymes, and competing micro flora. The hostile environment within the gastrointestinal tract is tolerated and serves as a signal to induce transcription of genesrequired for host cell attachment and invasion (Ahlstrom, 2011). In contrast with other pathogens of the family, the reservoirs of *Salmonella* cover a greater variety of warm and cold blooded animal. *Salmonella* may be found in milk, and has been associated in milk borne disease. *Enterobacteriaceae* infections are among the most killing diseases of children in developing countries (Frey and

Sherk, 2006). Moreover, gastrointestinal infections due to pathogenic *Enterobacteriaceae* in particular ***Escherichia*** and *Salmonella spp.* are significant causes of morbidity and mortality worldwide (Bisi-Johnson et al., **2011).** Then in addition *Staphylococcus aureus* in milk *Staphylococcus aureus* is a facultative anaerobic**,** Gram-positive coccus, which appears as grape-like clusters when viewed through a microscope and has large, round, golden yellow colonies, often with haemolysis, when grown on blood agar plates (Ryan and Ray, 2004). It is a versatile pathogen of humans and animals and causes a wide variety of diseases ranging in severity from slight skin infection to more severe diseases such as pneumonia and septicemia. It is an important food-borne pathogen, which ranks as one of the most prevalent causes of gastroenteritis worldwide (Dinges *et al.*, 2000). It survives in as much as 15% NaCl and can grow at pH = 4.2 – 9.3 and in temperatures ranging from 7 to 48.5ºC. These characteristics enable *S. aureus* to grow in a wide variety of foods. The bacterium may occur in the milk of cows with clinical or sub-clinical mastitis or as the result of contamination by handlers. When toxigenic strains of this organism replicate to numbers exceeding 105 CFU/ml, they may produce staphylococcal enterotoxins that cause staphylococcal food poisoning (Hudson, 2010). The intoxication is characterized byenteric responses such as diarrhea, abdominal cramps and vomiting within 1 – 6 hours of consumption of contaminated food. The bacterium is heat labile and does not compete well with other microorganisms. Contamination usually occurs when there is little competition from other microorganisms. Although *Staphylococci* are also commonly found in other materials including animal skins, water and soil, bacteria from food handlers and other human sources are considered as the most important contributing factors to intoxications associated with food (Kilango, 2011).

1.11.*Escherichia Coli*

This bacterium is classified under the family *Enterobacteriaceae*. They are normal flora in the guts of human, cattle and sheep where it produces vitamin K and protects the host animal from digestive infection by suppressing and preventing growth and colonisation of the gut by pathogenic bacteria. *E.coli* is able to live with or without oxygen and can use different sources for food requirements (Theodor, 1982). Contamination of the milk and other food is done through faecal-oral route. A study was done in Indonesia byStreet and Bogor (2013) and in Khartoum by Yuen *et al*. (2012) shows a high load of contamination of milk and milk products by *E. coli*. This imposes high health risk to the consumers of milk since there is an evidence of the presence of pathogenic *E coli* strain. Another study (Kumar and Prasad, 2010). *E.coli O:157* strain is pathogenic strain was identified in 1975 and 7 years later i.e. in 1982 was found to cause bloody diarrhoea infection in human (Theodor, 1982). The disease caused by this strain has the incubation period ranging from 1-16 days and is common in young, elderly and immune-compromised people (University, 2009). The disease is associated with the consumption of contaminated and unpasteurised milk. In Washington it was found that 44% of identified cases were laboratory confirmed to be associated with *E. coli* 0157:H7 infection due to consumption of raw milk the study done in Pakistan (Hussaina *et al,* 2014) showed that the *E. coli* counts of Dahi (yoghurt) were amazingly higher counted up to $212.16\pm17.54\times10^3$ and $189.35\pm3.42\times10^3$ for the poor and better sanitation areas, respectively. This creates the need for the further studies in Tanzania to establish the status of milk contamination levels as milk is commonly consumed by young and people who are immune-compromised.

Chapter Two
Materials and Methods

2.1. Collection of milk samples:

Thirty row (unpasteurized) milk from different markets and super markets from different localities of Khartoum state these localities were Khartoum , Omdurman and Bahri . theses samples were taken in sterile bottles and they keep in ice box and transported to Sudan university micro biology laboratory and transferred to deep freezer until used (table 1) .

2.2 Bacteriology

2.2.1 Media:

2.2.1.1 Solid Media:

2.2.1.2 Blood Agar (Oxoid, CM17):

Consisted of:

1-Proteose peptone	15g
2- Liver digests	2.5g
3- Yeast extracts	5g
4- Sodium chloride	5g
5- Agar No3	12g
6- pH 7.3	(approx)

Twenty eight grams of blood agar base were suspended in one liter of distilled water. This was brought to the boiling temperature to dissolve

completely in a steamer, mixed and sterilized by autoclaving at 121ºC under 15 lbs/in^2 for 15 minutes, then cooled to 45–50ºC in a water bath before addition of 10% defibrinated ovine blood . It was mixed gently and dispensed into sterile Petri dishes in 15ml portion each.

2.2.4. Nutrient Agar:

Consisted of:

1. Protease Peptone 15g
2. Liver digest 2.5g
3. Yeast Extract 5g
4. Sodium Chloride 5g
5. Agar No3 12g
6. pH 7.3 (approx)

Twenty eight grams of the powder of nutrient agar were suspended in one liter of distilled water, brought to the boiling temperature to dissolve completely in the steamer, mixed and sterilized by autoclaving at 121ºC under 15 lbs/in^2 for 15 minutes, then cooled to 45–50Cº in water bath . These were dispensed into sterile Petri dishes in 15ml portion each.

2.2.5. Mac Conkey's Agar (Oxoid CM7):

The medium was prepared by dissolving 52 grams in one liter of distilled water. It was boiled to dissolve and then autoclaved at 121ºC for 15 minutes. After cooling, the mixture was poured aseptically in sterile Petri dishes.

2.2.6. Semi-Solid Media

2.2.6.1. Hugh and leifson′s (O.F) medium (OXOID):

The medium was prepared by dissolving 10.3 grams of solid in 1 liter of distilled water by heating, and pH was adjusted to 7.1 filtered bromothymol blue 0.2% aqueous solution was added and then the medium was sterilized at

115˚C for 20 minutes. Sterile solution of glucose was aseptically added to give a find concentration of 1%. The medium was mixed and distributed aseptically as 7ml volume in sterile test tubes.

2.2.6.2. Motility medium (OXOID):

Thirteen grams of nutrient broth was add to 4 grams of agar and dissolved in 1 liter of distilled water and the pH was adjusted to 7.2. The medium was distributed as 5ml volumes in test tubes containing carigie- tubes and sterilized by autoclaving at 115˚C for 15 minutes.

2.3. Liquid media:

2..3.1. Nutrient broth (Oxoid CM1):

Consisted of:

1. Peptone
2. Beef extract
3. Sodium chloride

Thirteen grams of the powder were dissolved in one liter of distilled water and sterilized by autoclaving at 121^{o}C under15 lbs/in^{2} for 15 minutes. The pH was adjusted to 7.2–7.4 before sterilization.

2.3.2. Peptone water:

Fifty grams of peptone water powder (Oxoid, CM9- CM10) were added to 1 litre of distilled water, mixed well, distributed in 3 ml amount into clean test tubes and sterilized by autoclaving at 121°C for 15 minutes.

2.4. Solutions and Reagents:

2.4.1. Oxidase test reagents:

Tetraethyl-P-Phenylene diamine hydrochloride reagent was prepared as 1% solution as described by Barrow and Felltham (1993).

2. 4.2. Kovac's regent:

This regent is composed of 5g of Para- dimethyl- amino Benz aldehyde; 75 ml amyl alcohol and 25ml concentrated hydrochloric acid. The alcohol and the acid were added carefully. The regent was stored at 4ºC for later use in indole test.

2.4.3. Methyl red solution:

This solution was prepared by dissolving 0.04 g of methyl red powder in 40 ml ethanol and the volume was made up to 100 ml with distilled water.

2.4.4. Alpha- naphtol solution:

It was prepared as 1% aqueous solution and also used for V.P test.

2.4.5. Bromothymol blue solution:

This indicator was prepared by dissolving 0.2 gram of bromthymol blue powder in 100 ml distilled water. It was used for oxidation fermentation test.

2. 4.6. Normal saline:

Physiological or isotonic saline was prepared by dissolving 8.5 grams of sodium chloride in 1 liter of distilled water to obtain 0.85% concentration.

2.5. Sterilization:

2.5.1. Flaming:

Flaming was used to sterilize slides, cover slips and glass rods.

2.5.2 Hot air oven:

Glassware like Petri dishes, pipettes, tubes, flasks and glass rods were sterilized in hot air oven at 160C for one hour.

2.5.3. Autoclaving (121ºC 15lbs/in2) Moist heat:

Screw capped bottles, rubber caps, tips of micropipette, media solution etc were sterilized in autoclave at 121C for 15 minutes and 110C for 10 minutes for sugar media. Sugar media were sterilized at 110C for 10 minutes.

2.5.4. Steaming at 100 Cº:

Repeated steaming (tyndallization) was used for sterilization of sugars and media that could not be autoclaved without detriment effect to their constituents.

2.5.5 Disinfection of bench:

Alcohol (70%) and phenolic solution were used for disinfecting working place in the media preparation room and in the floors and bench in laboratory.

2.5.6. Irradiation:

Ultraviolet irradiation for 10 min was used to sterilize media pouring room and safety cabinet.

2.6. Cultural methods:

2.6.1. Primary isolation:

Three loopfull from milk sample were streaked on Blood agar, McConkey'sagar, and Nutrient agar and then the streaking over the plate was completed using the wire loop.

2.6.2. Incubation of culture:

All inoculated solid and liquid media were incubated aerobically at 37C^{o} for 18-24 hours.

2.6.3. Examination of cultures:

Cultures on semi-solid media were examined grossly for colonial morphology and haemolysis whereas, broth media were checked for turbidity, change in colour, accumulation of gases in Carbohydrates media and for sediment formation.

2.6.4. Preparation and staining of smears:

From one colony on each plate one half was taken with a sterile loop, emulsified in a drop of normal saline on a clean microscopic slide. The smear was allowed to dry and then fixed by passing the slide over a flame. The slides were placed on the rack and flooded with crystal violet stain for one minute

and rinsed with water. They were then covered by iodine a minute and rinsed with water. Alchohol was poured and immediately the slides were rinsed with water. The slides were counter stained with nuteral red for two minute and rinsed with water again and allowed to dry by blotting with filter paper. A drop of immersion oil was added to each slide and examined under microscope. Colonies which showed Gram–positive cocci, Gram positive bacilli and Gram-negative bacilli were subcultured on nutrient agar.

2.6.5 Subculturing and purification:

Purification was based on the characteristics of colonial morphology and smear. Discrete colonies were picked, smeared, fixed, and Gram–stained. Then the same colonies were subcultured on nutrient agar.

2.6.6. Biological and biochemical identification:

All the biochemical tests were performed according to Smith *et al* (1986) and Barrow and Filtham (1993).

2.7. Primary tests:

2.7.1 Catalase test:

A drop of 3% aqueous solution of hydrogen peroxide was placed on a clean glass slide. A drop of the culture under test grown on nutrient agar was added and mixed with the hydrogen peroxide. Production of gas bubbles indicates positive results for the catalase test.

2.7.2. Oxidase test:

The tested bacteria were grown on nutrient agar. A disk of tetramethyl - p–phenylene diamine dihydrochloride was put on a clean slide. The tested

organism was picked with sterile bent glass rod and rubbed on the disk. A violet color that developed within 5-10 seconds indicates a positive result.

2.7.2. Oxidation fermentation test (O.F):

This test was used to determine the way by which the bacterium attacks a carbohydrate (by fermentation or by oxidation). The test performed by growing the bacterium in tow tubes of Hugh and Leifson's medium. One of them was covered with a layer of sterile paraffin oil. All tubes were incubated at 37ºC and examined daily for 14 days. Fermentative organisms produced a yellow color on both tubes while oxidative organisms produced a yellow color only on open tube. When the two tubes were green, the result was negative.

2.7.3. Motility test:

Motility medium was inoculated by stabbing the examined bacterium with straight sterile wire. The medium was then incubated for up to 3 days together with uninoculated medium as control. The growth of non-motile organism was confined to the stab, while the motile one was distributed out the stab.

2.8. Secondary tests:

2.8.1 Indole production test:

Indole production test was carried out as described by Barrow and Feltham (1993). The organism was inoculated into peptone water and incubated at 37oC for 48 hours. One milliliter of Kovac's reagent was run down along side of the test tube. Appearance of pink colour in the reagent layer within a minute indicated positive reaction. Yellowish color in the tube covered with paraffin oil was an indication of fermentation reaction and in opened tube an indication of oxidation reaction.

2.8.2. Methyl red (MR) test:

Methyl red test was carried out as described by Barrow and Feltham (1993). The test organism was inoculated into glucose phosphate medium (MR-VP medium), then incubated at 37oC for 48 hours. Two drops of methyl red reagent were added, shaken well and examined. Appearance of red colour indicated positive reaction, whereas orange or yellow colour indicated negative reaction.

2.8.3. Voges-Proskauer (VP) test:

The test was performed as described by Barrow and Feltham (1993). The test culture was inoculated into glucose phosphate medium (MR-VP medium) and incubated at 37oC for 48 hours. Three milliliter of 5% alpha-naphthol solution and one milliliter of 40% potassium hydroxide were added. When bright pink colour developed within 30 minutes, the reaction was regarded as positive.

2.8.4 Citrate utilization test

Simmon´s citrate medium was inoculated with test organism and incubated at 37°C for up to 7 days and was examined daily for growth and color change. Blue color and streak of growth indicated positive citrate utilization.

2.8.5. Coagulase test:

2.8.5.1. Slide coagulase test:

This was performed on a clean, grease–free slide, by suspending a discrete well isolated colony from an overnight culture in normal saline to give a milky suspension. A drop of undiluted human plasma was then placed and the slide was rocked till visible clumping of cocci occurred within 10 seconds. The slide test was confirmed by the tube test, both the positive and the negative results.

2.8.5.2. Tube coagulase test:

Human plasma was diluted (1:10) by normal saline, and then 0.5ml of it was placed in each tube. A volume of 0.5ml of 24 hours old broth culture was added and the tubes were incubated at 37^{o}C, coagulation was observed after an hour, three hours of and finally after 24 hours of incubation.

2.8.6. Urease test:

A slope of urea agar medium was inoculated with the test organism and incubated at 37°C. Change in color to red indicated positive reaction.

Chapter Three

Results

In this investigation a total of 30 bacterial isolates were obtained from 30 row milk samples collected from different localities of Khartoum State. the results Showed in(table 1) the identified bacteria were : 12 *Staphylococcus spp* (40%), 8 *Salmonella spp* (26.7%) , 10 *E.coli* (33.3%) Shown in table 1.

As shown in table 2 the cultural characteristics, bacterial morphology and biochemical tests of bacteria isolated from row milk (30samples).In table3 Gram negative Bacteria represented the higher percentage (60%) compared with Gram positive bacteria (40%).The result revealed that *Staphylococcus spp* was the predominant organism (40%), isolated from row milk samples while *E. coli* (33.3%) and *salmonella* spp *(*26.*7*%) were lowered (Table 3).

In Khartoum locality *Staphylococcus* spp high percentage (44.4%) compared with *E.coli* (22.2%) and *salmonella* spp. But the total of Gram negative (Table 4) was higher(55.5%) than Gram positive (44.4%) .

Also as shown in Table 4 the organism *Staphylococcus spp* was high percentage (60%) than *E.coli* (30%) and *salmonella* spp (10%), but the percentage of Gram negative was lowered(40%) than Gram positive in Omdurman locality.

Table 5 showed the organism re percentage higher percentage (60%) compared with *Staphylococcus spp(33.3%)* and *salmonella* spp (16.7%) while the total of Gram negative was higher (76.7%) than Gram positive(33.3%).

Table (1)

Bacteria isolated from raw milk samples(n=30) collected from different localities of Khartoum State.

Bacterial isolated	**Khartoum locality**	**Omdurman locality**	**Bahri locality**	**Total%**
Staphylococcus spp	4	6	2	12(40)
E. coli	2	5	3	10(33.3)
Salmonella spp	3	4	1	8(26.7)
Total%	9(30)	15(50)	6(20)	30(100)

Table (2)

Cultural characteristics, bacterial morphology and biochemical tests of the isolated bacteria from row milk samples (n=30) in Khartoum State

Test	***Staphylococcus spp***	*E. coli*	*Salmonella spp*
Aerobic growth	+	+	+
Colonies on MacConkey	Bright pink	Bright pink	Pink pale
Haemolysis on blood agar	+	+	-
Gram-reaction	+	–	–
Shape	Cocci	Rods	Rods
spore	-	-	-
Motility	-	+	+
Catalase	+	+	+
Oxidase	-	-	-
Indole	-	+	-
Methyl red	+	+	+
VP	-	-	+
Citrate	-	-	-
H2S	-	-	
O/F	+	+	+
Glucose	+	+	+
Lactose	+	+	-

Key :(+) = positive (-) = negative

Table (3)

Total number and percentage of bacteria isolated from milk samples collected from Khartoum localities.

Bacterial spp.	**Number**	**Percentage**
Staphylococcus spp	12	40%
E.coli spp	10	33.3%
Salmonellas spp	8	26.7%
Total	30	100%

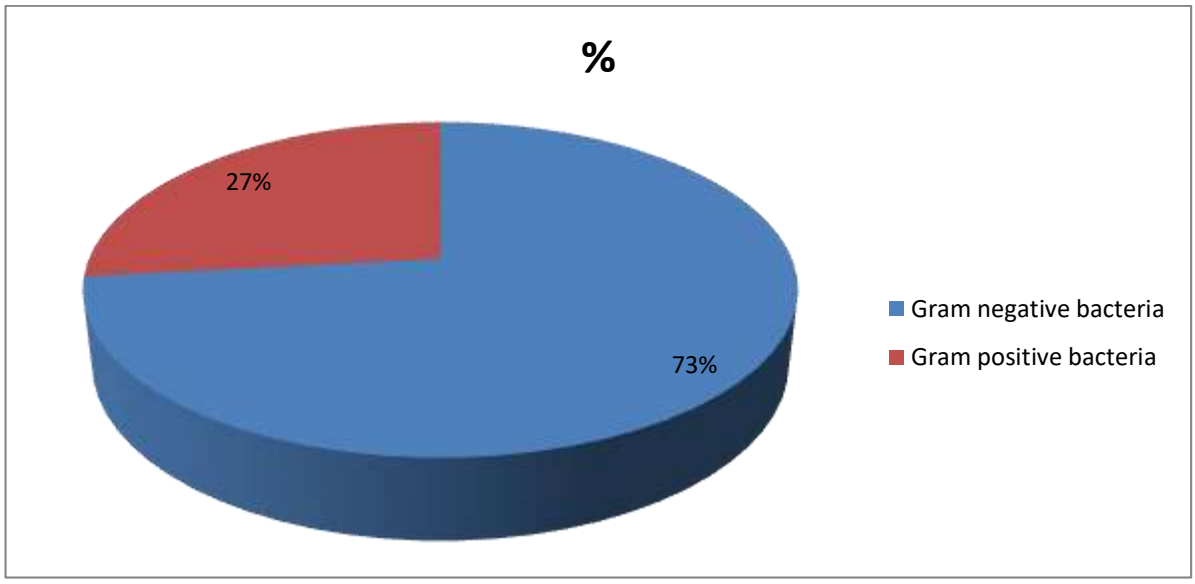

Fig (1): Gram negative and Gram positive bacteria isolated from milk sample samples collected from Khartoum state.

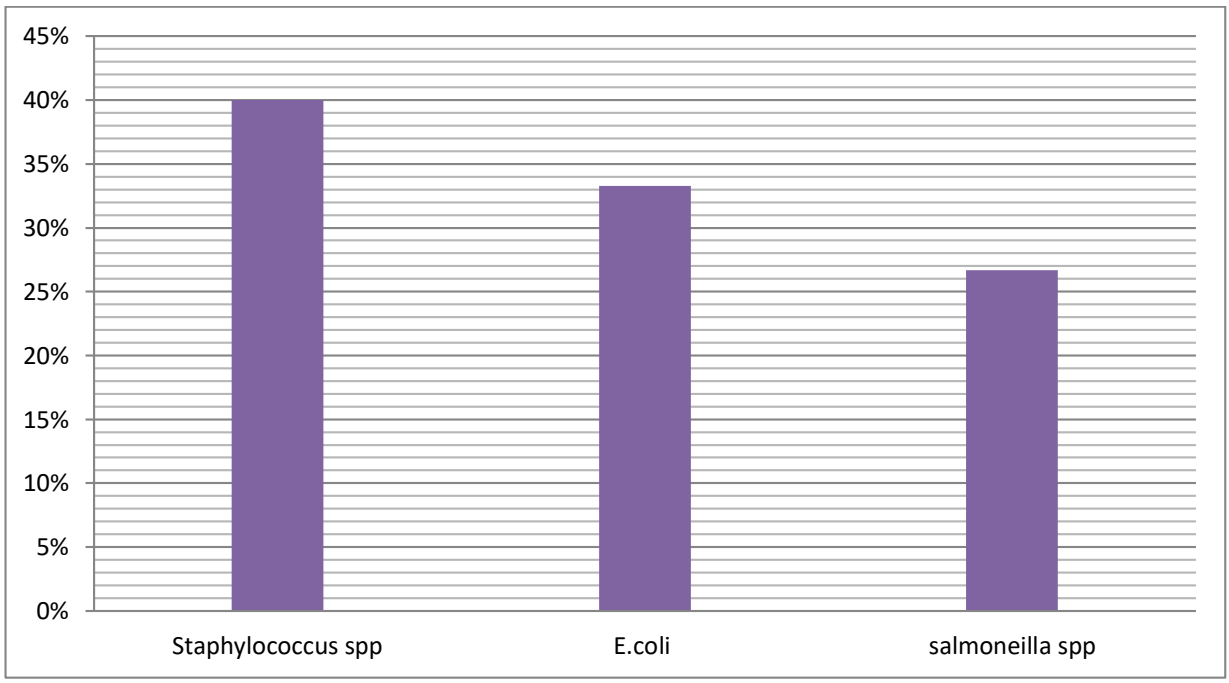

Fig (2) Bacteria isolated from milk samples collected from Khartoum state.(Appendix1)

Bacteria isolated from milk samples collected from Omdurman locality:

Table (4)

Total number and percentage of bacteria isolated from milk samples collected from Omdurman locality:

Bacterial spp.	**Number**	**Percentage**
Staphylococcus spp	6	60%
E.coli	3	30%
Salmonella spp	1	10%
Total	10	100%

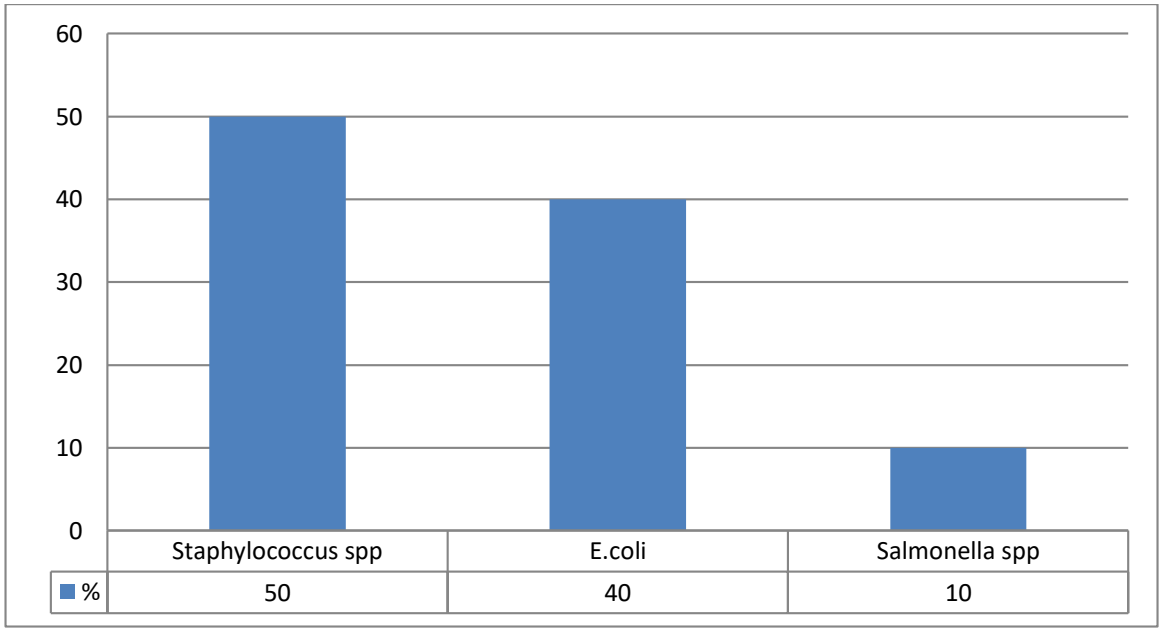

Fig (3): Bacteria isolated from milk samples collected from Omdurman locality:

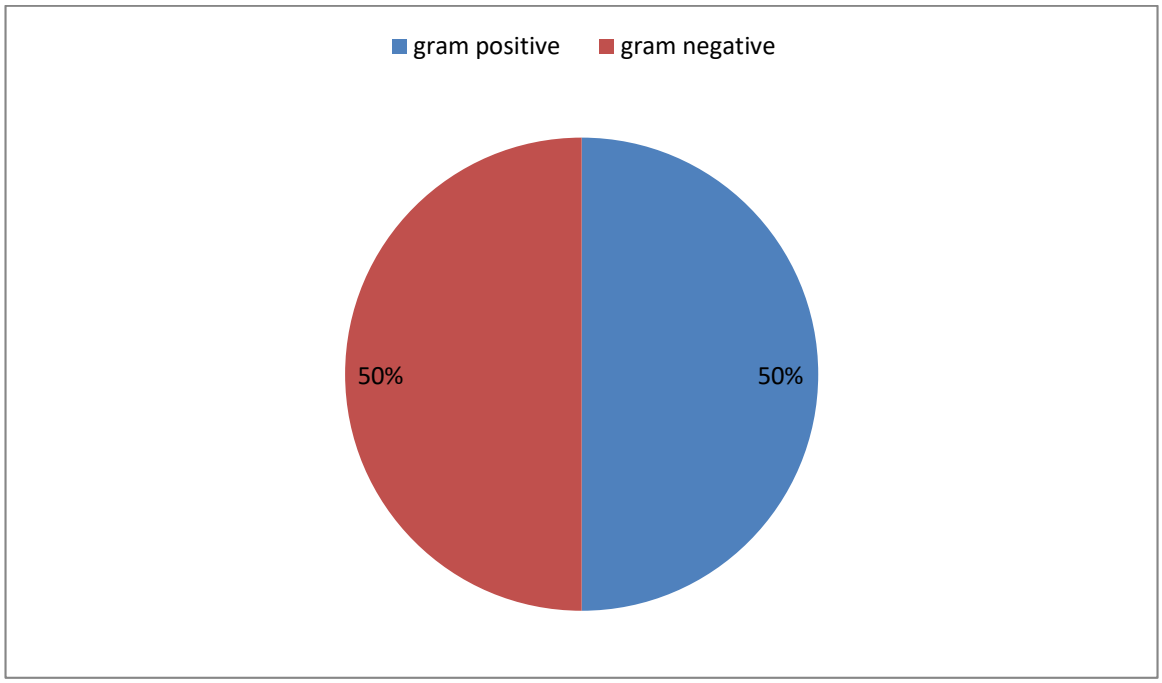

Fig (4): Gram negative and gram positive bacteria isolated from milk samples collected from Omdurman locality. (Appendix2)

Table (5)

Total number and percentage of bacteria isolated from milk samples collected from Omdurman locality:

Bacterial spp.	**Number**	**Percentage**
Staphylococcus spp	2	33.3%
E.coli	3	60%
Salmonella spp	1	16.7%
Total	6	100%

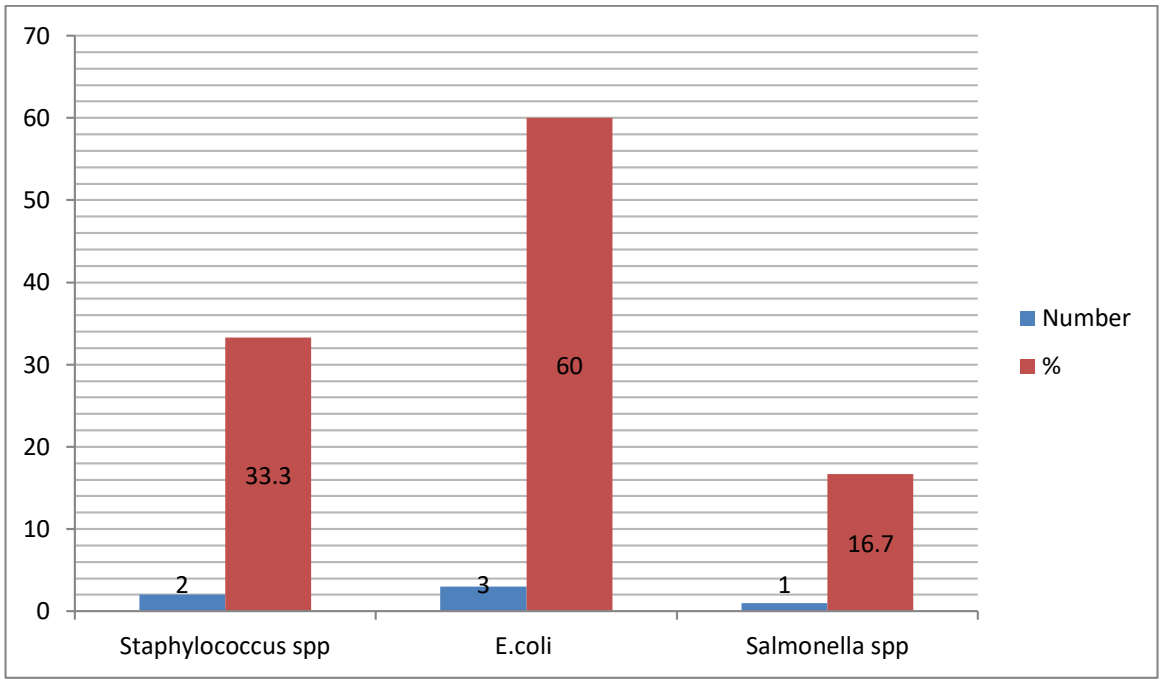

Fig (5): Bacteria isolated from milk samples collected from Bahri locality:(Appendix3)

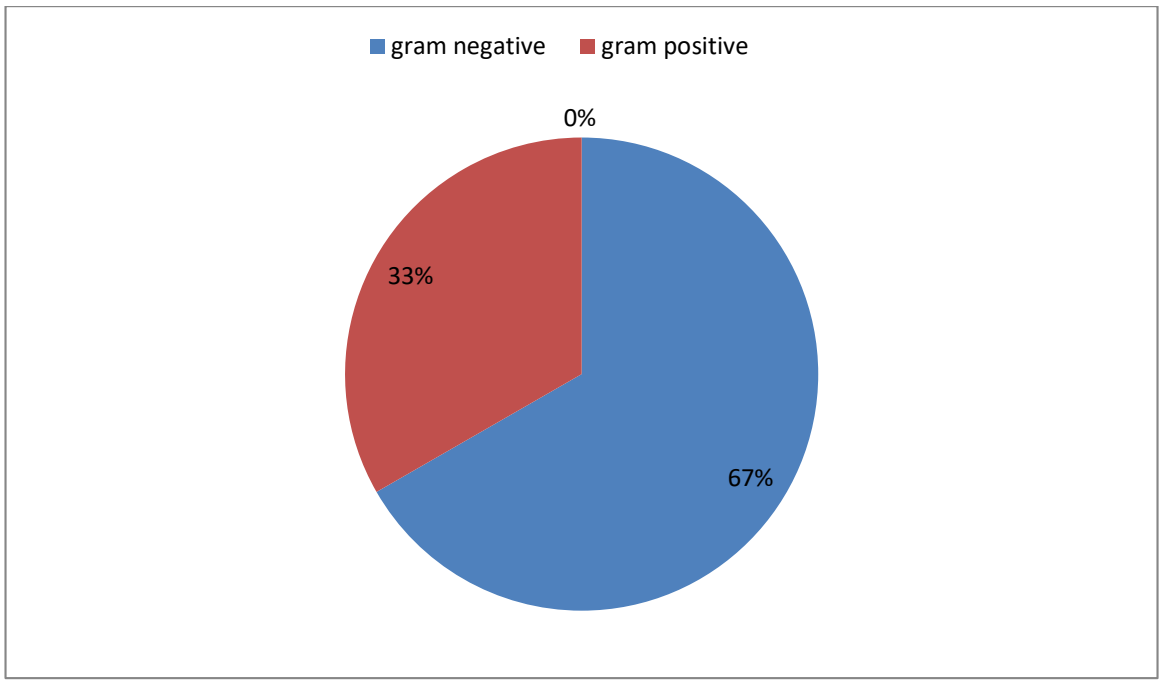

Fig (6): Gram negative and gram positive bacteria isolated from milk samples collected from Bahri locality.

Chapter Four

4.1 Discussion

In the past two decades, urban dairy production constituted an important sub-contributing immensely towards filling in the large demand-supply gap for milk and milk products in urban centers, where consumption of milk and milk products is remarkably high (Azage and Alemu, 1998). In this study a total of 30 bacterial isolates were obtained from milk sample collected from different localities of Khartoum State. The identified bacteria were: *Staphylococcus spp* 12. (40%), *E.coli* 10. (33.3%), *Salmonella spp* 8. (26.7%).*Staphylococcus spp* represented 12. (40%) of total bacteria isolated from The State and different localities (4 (13%) Khartoum, Omdurman 6(20%) and Bahri 2(7%). Karmen *et al. (2008)* reported that coliforms and coagulase-positive staphylococci were present of raw milk samples in 2.1 log10 cfu/ml and 1.97 log10 cfu/ml respectively. The literature reviewed in the present study shown evidence that *Escherichia coli* is frequently occurring organism in milk. The methods of production, transportation, handling and sale of milk are entirely unhygienic the raw milk poses a great hazard to public health without adapting hygienic measures because of possibilities of contamination with *E. coli.* The result of milk sample showed that all samples were contaminated with *E. coli.* In this study the *E.coli* represented 2 (7%) of total bacteria isolated from the Khartoum localitiy. (Bhat *et al., 1948;* Marrieer, *1973;* Triq Masud *et al., 1988);* it is reported *the* unclean hands of workers, poor quality of milk, unhygienic conditions of manufacturing units, inferior quality of material used and water supplied for washing the utensils could be the source of accelerating the bacterial contamination of milk product and the post manufacturing

contamination this study were in line with (Hahn, 1996). It is mentioned that *E. coli* frequently occurring organiss in milk and its products, the incidence of this species of *E. coli* itself in milk and milk product as a possible for food born disease is insignificant because *E. coli* normally is ubiquitous organisms. *Salmonella spp* represented *8. (26.7%)* of total bacteria isolated from the this study State and different localities (3,10%) Khartoum, Omdurman (4,13.4%) and Bahri (1,3.3%).Abdoul-latif., *et.al*. (2017) reported that *Salmonella spp and Staphylococcus spp* presence of food-borne diseases.

4.2. Conclusions:

High bacterial count obtained in this work is an indication of poor sanitary condition. Moreover, the milking bucket, milk maids and milking environment is an important source of milk contamination. Coli forms came in first place no.18 (60%) in Khartoum State followed by *Staphylococci* spp 12(40%).The contamination by microorganism especially *salmonella* is represent grater to hazard for consumer.

4.3. Recommendation

1. Further studies should include a survey of more animals in different farms and an extensive study to cover the significance of other bacteria in raw milk should be conducted.
2. From results of the present study, it is imperative to provide adequate extension inputs and training to dairy farmers to increase their awareness on milk hygiene, cattle and milk-borne zoonosis and their control methods in order to reduce public health risks.
3. Hygienic measures should be practiced before and after milking.
4. Daily cleaning and disinfection of equipment and workers hand should be applied.

Chapter Five

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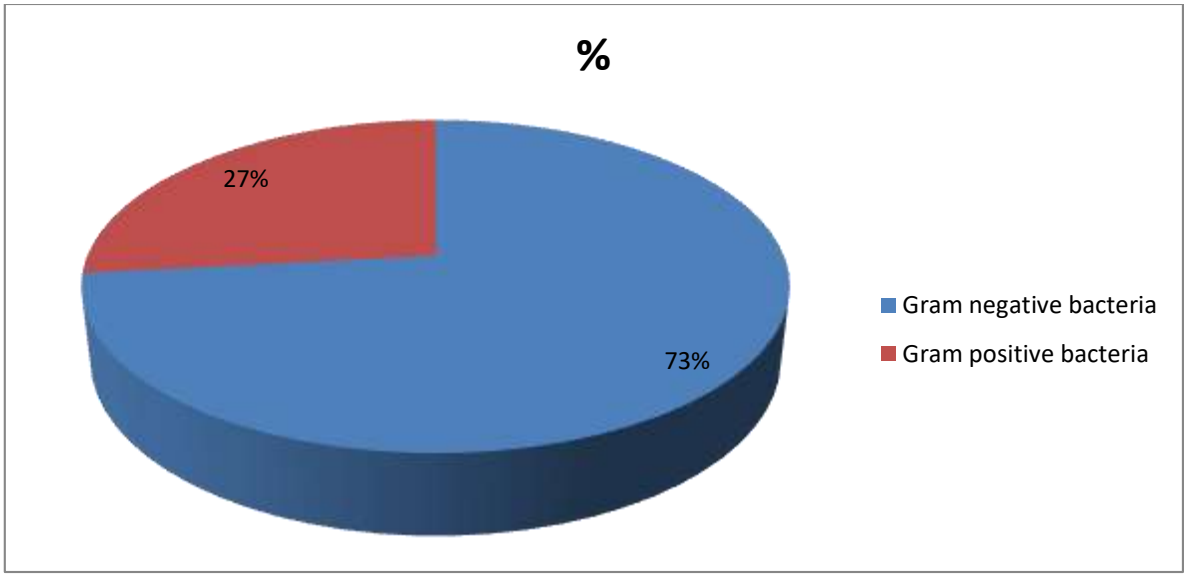
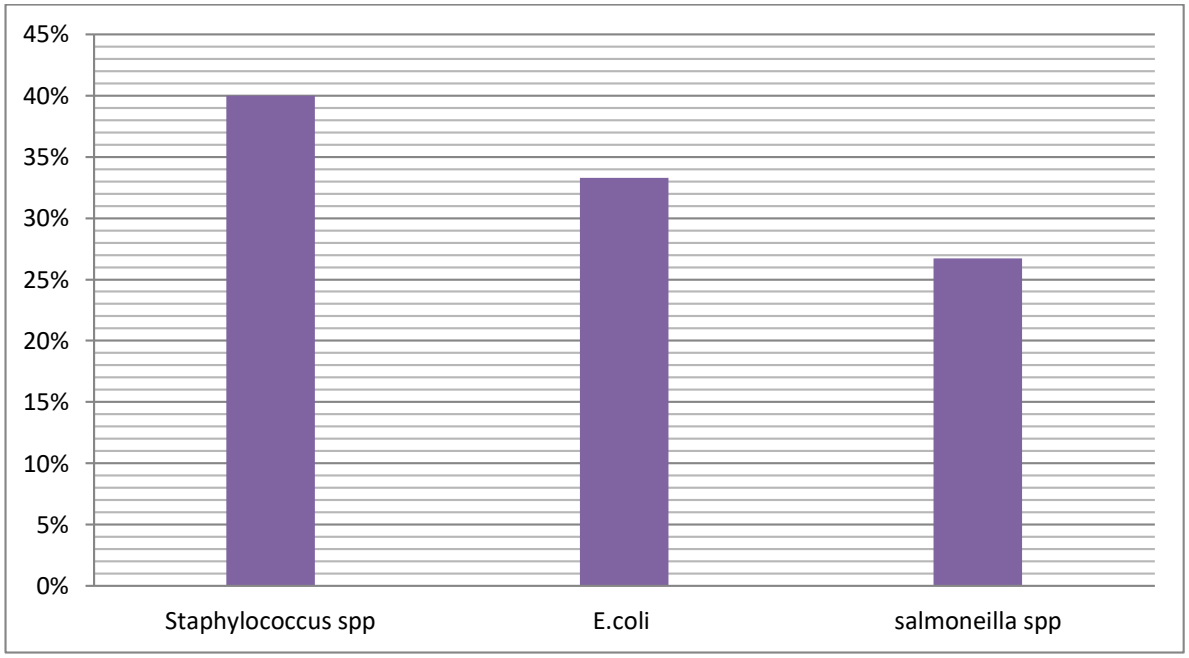
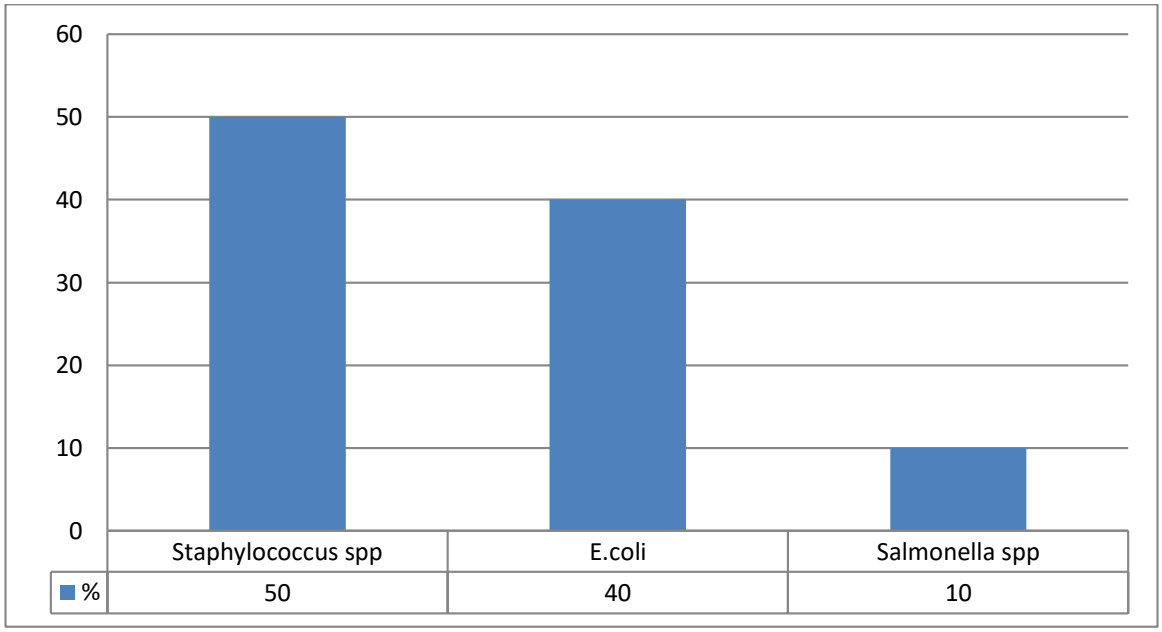
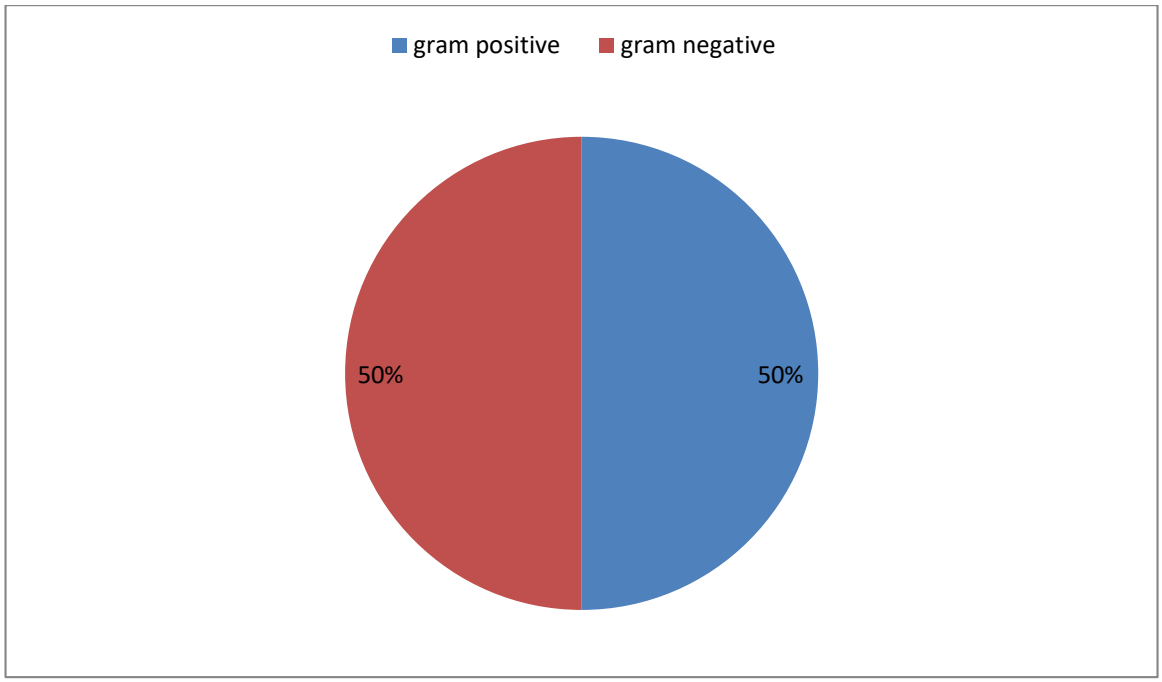
Appendices 1





Appendices 2





Appendices 3