General hygiene of commercially available milk in the Bloemfontein area

By

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LIST OF ABBREVIATIONS

cfu:	colony forming units
Fig:	Figure
g:	gram
ml:	millilitre
pH:	hydrogen ion concentration
SCC:	somatic cell count

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CHAPTER 1

1. INTRODUCTION

Being a major constituent of the diet, milk can serve as a good medium for the growth of many microorganisms, especially bacterial pathogens. Therefore, its quality control is considered essential to the health and welfare of a community. Being a nutritionally balanced foodstuff that contains a low microbial load (less than 1000 ml^{-1}) when drawn from the udder of a healthy cow, milk gets contaminated at various stages, including the cow itself, the milker (manual as well as automated), extraneous dirt or unclean process water (Lues, et al., 2003). The microbial loads may increase up to 100 fold or more once the milk is stored for some times at ambient temperatures (Richter et al., 1992). However, keeping milk in clean containers at refrigerated temperatures immediately after milking may delay the increase of initial microbial load and prevent the multiplication of microorganisms in milk between milking at the farm and transportation to the processing plant (Adesiyun, 1994; Bonfoh, 2003).

Microorganisms generally associated with milk and milk spoilage are coryneforms, micrococci and lactococci, and include genera such as *Pseudomonas, Brucella, Escherichia, Salmonella, Shigella* as well as B*acillus* and *Clostridium* (Lues, *et al.,* 2002). The threat posed by diseases spread through contaminated milk is well known and the epidemiological impact of such diseases is considerable (Foster, 1990). The presence of these pathogenic microorganisms in milk emerged as a major public health concern, especially for those individuals who still drink raw milk (Ryser, 1998). The growth of microorganisms in milk causes disintegration of fat, protein and lactose and will soon make the product unsuitable for drinking. While elimination of bacterial contamination is an important factor in the production of good flavored, high quality milk, other procedures can be used to protect and maintain good flavor and quality.

In other societies, practices generally used to curb microbial proliferation in milk include refrigeration and pasteurization but there are some concerns about the efficiency of conventional heat pasteurization of milk. For instance, some potential human pathogens, including *Mycobacterium paratuberculosis, Bacillus cereus* and *Prototheca* have been reported to survive conventional heat pasteurization in milk (Stabel *et al.,* 1997; Smith *et al.,* 2002). Food safety has raised public concerns, which may necessitate the actual sterilization of many products in the future, and even though sterile milk is available, the heat required to sterilize it alters its taste and marketability (Smith *et al.,* 2002). It is clear that the only quality acceptable in milk is the best possible and to achieve this goal, certain requirements must be applied.

The most important requirement to be met is that the product must be free of pathogenic bacteria, as well as all forms of antibiotic, insecticide and herbicide compounds. Secondly, it must have a good flavor, which may be characterized as the absence of any objectionable flavor. Thirdly, it should be relatively free from spoilage bacteria and somatic/body cells. Complete absence of such is usually not possible, but all countries do have laws which limit the maximum numbers permitted. The last major factor related to quality is composition, that is, the amount of fat and other solids contained. There are legal restrictions pertaining to milk components, which must be adhered to. The Foodstuffs, Cosmetics and Disinfectants Act (54), of 1972, states that “milk should contain a minimum of 3% fat and 8.5% milk solids not fat and should have nothing added to it or removed from it” (Banwart, 1989). Table 1.1 highlights the National Standards for the acceptable levels of some microorganisms in good quality milk and dairy products. These standards are according to the above-mentioned act.

Therefore, the objectives of this study were to assess the quality of milk sold in the Bloemfontein district thereby investigating their conformity to the National

Standards, as well as evaluating a new effective treatment for keeping quality of milk.

2. LITERATURE REVIEW

2.1. Milk composition

Milk may be defined as the secretion of the mammae of the female mammal used for the feeding of her young, and has been described as close to being nature's perfect food. Fresh milk is neutral or slightly alkaline but on souring becomes acid because of the lactic acid formed by bacterial action on lactose. It has a water content of 88% and 12% of solids which constitute of 4.8% sugars, 3.5% fats, 3.1% protein and 0.6% salts (Stewart, 1978). It has a wide range of positive nutritional benefits and supplies a variety of nutrients including protein for bodybuilding, vitamins, minerals (especially calcium), fat and carbohydrate for energy (Harding, 1995).

2.1.1. Proteins

Proteins are the body's 'building blocks' affecting our growth and immunity. Antibodies, enzymes and hormones all contain proteins, thus the proteins we eat provide the amino acids needed to replace both these and essential body cells. Whilst the body is able to synthesise some amino acids, there are eight essential amino acids it cannot make and have to be supplied in the diet (Harding, 1995).

In the digestion process proteins are broken down, in a process called hydrolyzation, from poly-peptides to smaller oligo-peptides, and then to di-peptides or tri-peptides, which are made up of two or three links of specific amino acids, called free form amino acids, that are finally absorbed into the bloodstream. Proteins in excess of the body's requirements are used for energy.

There are numerous proteins found in milk. The major groups of milk proteins are caseins and whey proteins. Milk provides easily digested protein of a high nutritional value and is a rich source of essential amino acids (Table 1) (Harding, 1995).

Casein

Casein is the principal protein of cow's milk. It forms a curd when milk is left to sour. It is the most commonly used milk protein in the food industry and contains 21 amino acids. Acid casein, a granular milk protein, is available in two types -- edible and technical. Edible acid casein is highly nutritional, low in fat and cholesterol, and flavorful making it ideal for medical and nutritional applications. It is used in coffee whiteners, infant formulas, processed cheese, and for use in pharmaceutical industries. Caseins are found in milk in a form of a micelle (a dense protein granule) and the micelles are composed of alpha-, beta-, and kappa-caseins.

Since casein itself will not dissolve in water, one will more likely see caseinates, which are the salts of casein, on ingredients labels. They are made by dissolving acid casein in a suitable hydroxide and drying it to make a water-soluble product. Calcium caseinate is used as a nutrient supplement. It is used in creamed cottage cheese, powdered diet supplements, nutritional beverages, processed cheese, and frozen desserts because it has a milky appearance and smooth feel in the mouth. Potassium caseinate is used in frozen custard, ice cream, ice milk, and fruit sherbets. Sodium caseinate is highly soluble and is used as an emulsifier in coffee whiteners, cottage cheese, cream liqueurs, yogurt, processed cheeses, and some meat products. It is also used to improve the whipping properties of dessert whips.

Whey casein

Whey protein is one of the proteins found in milk. It accounts for only about 20% of the total protein found in milk, while casein makes up about 80% of milk protein. After the milk fat is removed, spinning the rest of the milk at very high speed will separate out the casein. Once the casein has been removed, then all of the other proteins left in the milk are considered to be whey proteins. The primary whey proteins in cow milk are β-lactoglobulin, which accounts for about 50% and α-lactalbumin for 25%, with two other minor whey proteins making up the final 25%.

Long considered a useless by-product of dairy (cheese) manufacturing, whey protein is enjoying an increased interest as a protein supplement. Whey has a long history of use as a cheap protein source for low-cost protein powders. Recent claims of the high biological activity of whey protein, and the profits to be made by selling something that used to be thrown away, have encouraged dairy processing plants to begin processing and spray-drying in various ways to enhance its benefits in commercial protein powders. Whey proteins are now well known for their high nutritional value and versatile functional properties in food products (de Wit, 1998).

2.1.2. Milk fat

Fats are components of the brain, nerve cells and are essential to many physiological processes. Milk fat being an animal fat, is characterized as being a saturated fat. However about 32% of milk's fatty acids are unsaturated, primarily as mono-unsaturated acids like oleic acid ($C_{18:1}$). Milk supplies the essential fatty acids linoleic acid (2.1%), lanoleic (0.5%) and arachidonic acid (0.14%). These are required by the human body for normal metabolism and growth. Short (C_2 to C_6) and medium chain (C_8 to C_{12}) fatty acids account for about 12% of the fatty acids of milk and being more readily digested. They do not contribute to the elevation of blood lipids nor are they deposited in adipose tissue (Harding, 1995).

2.1.3 Lactose

Lactose with the exception of water is, at about 4.6%, the principal component of milk; however, it is the least important of the solids both nutritionally and commercially. Lactose (milk sugar) is the major carbohydrate in the milk of most mammals; hence mammalian milk is the major source of lactose, one of the most common natural disaccharides. Lactose consists of two molecules, D-glucose and D-galactose and is digested or broken down into these constituents by the enzyme lactase (Harding, 1995).

2.1.4 Minerals

Many trace elements essential for health and growth, are present in milk. Sodium, calcium, potassium and phosphorus account for about 4% by weight of the fat-free human body. Some of the trace minerals are, zinc, cobalt, iodine, iron, etc (Stewart, 1978; Harding, 1995).

2.1.5. Vitamins

Vitamins are complex organic substances that are needed in very small amounts for many of the processes carried out in the body. Usually only a few milligrams (mg) or micrograms (μg) are needed per day, but these amounts are essential for health. Most vitamins cannot be produced within the body, and as a result needs to be provided in the diet, although vitamin D can be obtained by the action of sunlight on the skin, and small amounts of a B vitamin (niacin) can be made from the amino acid (tryptophan). Milk is a source of 12 water-soluble vitamins and four fat-soluble vitamins (Harding, 1995).

Fat soluble vitamins: (i) vitamin A (retinol and beta-carotene) –found in the yellow colouring (carotene), (ii) vitamin D (calciferol)- found in sunlight (iii) vitamin E (tocopherol)- found in nuts, vegetables, oils, etc., (iv) vitamin K-vegetables, cheese, liver, etc. (Bendicho *et al.,* 2002).

Water soluble vitamins: (i) B complex vitamins, e.g. thiamine (B_1), riboflavin (B_2), niacin (B_3), etc.-many common foods, (ii) vitamin C (ascorbic acid)-obtained in small quantities and destroyed by souring, oxidation and heat (Bendicho *et al.,* 2002).

2.2 Factors affecting product quality

The quality of the starting raw milk has a very definite effect on the yield and quality of products made from it. The compositional quality, the hygienic quality, the health of the cow and the level of contaminants present can all have an impact on the yield and quality, and hence financial return from products made from milk (Harding, 1995).

Milk drawn aseptically from the udder of a healthy cow contains only a small number of microorganisms, these being of little importance commercially and presenting no danger to the consumer. While some contamination with bacteria from the milking environment and equipment is inevitable, the total bacterial count of cooled milk, produced under good hygienic conditions, should be lower than 10^3 cfu/ml. If the bacterial count of milk was allowed to increase significantly, e.g. to over 10^3 cfu/ml this could lead to significant degradation of the fat, protein and lactose causing off-flavours and would significantly reduce the flexibility the processor has with respect to storage and use of milk. In order to achieve a high bacteriological quality at farm level, it is important for farmers to be aware of the sources of contamination and to understand how they can be controlled (Harding, 1995).

Milk is synthesized in specialized cells of the mammary gland and is virtually sterile when secreted into the alveoli of the udder. Beyond this stage of milk production, microbial contamination can generally occur from three main sources (Bramley and McKinnon, 1990); from within the udder, from the exterior of the udder and from the surface of milk handling and storage equipment (IDF, 1996). The health and hygiene of the cow, the environment in which the cow is housed

and milked, and the procedures used in cleaning and sanitizing the milking and storage equipment are all important in influencing the level of microbial contamination of raw milk. Equally important are the temperature and length of time of storage, which allow microbial contaminants to multiply and increase in numbers. All these factors will influence the total bacteria count or Standard Plate Count (SPC) and the types of bacteria present in bulk raw milk.

2.2.1 Interior of the udder

Healthy udders

Total counts of milk from individual cows with clinically healthy udders varies considerably from <1 cfu/ml to 690 000 cfu/ml. In healthy cows, the teat cistern, teat canal, and the teat apex may be colonized by a variety of microorganisms, though microbial contamination from within the udder of healthy animals is not considered to contribute significantly to the total numbers of microorganisms in the bulk milk, nor to the potential increase in bacterial numbers during refrigerated storage. Natural flora originated of the cow generally has little influence on total plate counts (Murphy and Boor, 2000).

The microbiological infection of milk taking place inside the udder is called primary infection. The main groups of microorganisms for this infection are the aerobic mesophilic microflora, and they contributed little to the deterioration of good quality raw milk (<5000 cfu/ml) (IDF, 1996).

Infected udders (mastitis)

While the healthy udder should contribute very little to the total bacteria count of bulk milk, a cow with mastitis has the potential to shed large numbers of microorganisms into the milk supply (Bramley and McKinnon, 1990).

Mastitis is defined as the inflammation of the mammary gland. The inflammation is a response of the tissue to injury. The purposes of the inflammatory response are to destroy or neutralize the injurious agent and allow healing and return to normal function. A key component of inflammation is the influx of white blood cells or leukocytes which results in an increase in the somatic (body) cell count (SCC) of milk, thus the SCC is a common measure of mammary gland health and milk quality. Although inflammation can result from a variety of types of injury including infectious agents, physical trauma, or chemical irritants, mastitis in dairy cattle is generally the result of microorganisms which enter the mammary gland, multiply and produce toxins that cause injury to the mammary tissue. Bacteria are the most common causes of mastitis, but other types of organisms such as yeasts, mycoplasmas, and algae may occasionally infect the udder (Harding, 1995).

The causative bacteria of mastitis can be categorized as major or minor pathogens (Bramley and McKinnon, 1990). The most common major pathogens include *Staphylococcus aureus*, the coliforms, streptococci and enterococci of environmental origin and *Streptococcus* spp., most notably *S. agalactiae* and *S. uberis* as organisms which influence most the total bulk milk count (Bramley and McKinnon, 1990; Jeffrey and Wilson, 1987). *Staphylococcus aureus* is not thought to be a frequent contributor to total bulk tank counts though counts as high as 60 000/ml have been documented (Gonzalez *et al.,* 1986). This bacterium is very common on hands and skin of man and cattle (Harding, 1995; IDF, 1996).

Detection of implied pathogens does not necessarily indicate that they originated from cows with mastitis. Potential environmental mastitis pathogens and similar organisms can occur in milk as a result of other contributing factors such as dirty cows, poor equipment cleaning and poor cooling. An increase in somatic cell count (SCC) can sometimes serve as supportive evidence that a mastitis bacterium may have caused an increase in the bulk milk bacteria count. This

seems to hold true more for *Streptococcus* species than for *S. aureus,* which appears to be shed into the milk in lower numbers (Fenlon *et al.,* 1995). *S. agalactiae* and *S. aureus* are not thought to grow significantly on soiled milking equipment or under conditions of marginal or poor cooling. Their presence in bulk tank milks is considered strong evidence that they originated from infected cows (Gonzalez *et al.,* 1986; Bramley and McKinnon, 1990).

Teat skin has been suggested as an important reservoir for intra-mammary infection, while human-to-bovine transmission has also been proposed (Zadoks, *et al.,* 2002). In a small number of mastitis cases, the following bacteria are involved: *Leptospira* spp., *Listeria monocytogenes*, *Bacillus cereus* and *Clostridium perfringens*. The importance of this group however is based on effects on human health and quality of milk products (IDF, 1996). Mastitis caused by the major pathogens results in the greatest compositional changes and increases in somatic cell count in milk and has the most economic impact.

Table 1.3 lists examples of some of the changes in the levels of milk components that accompany mastitis. Lactose and fat are decreased in milk because of reduced synthetic activity of the mammary tissue. Although there may be little change in total protein content, there are dramatic changes in the types of proteins present. The content of casein, the major milk protein of high nutritional quality declines, but there is an increase in whey proteins. Serum albumin, immunoglobulins and transferrin pass into milk because of vascular permeability changes. Lactoferrin, the major antibacterial iron-binding protein in mammary secretions, increases in concentration likely due to increased output by the mammary tissue and a minor contribution from polymorphonuclear nuetrophil. Mastitis also causes marked changes in the ionic environment and increases in the conductivity of milk. Sodium and chloride increase due to passage from blood into milk. Potassium, normally a significant milk mineral, declines because of its passage out of milk between damaged epithelial cells. Since most of the calcium

in milk is associated with casein, the disruption of casein synthesis contributes to lowered calcium levels in milk (PMN) (Harding, 1995).

2.2.2 *Exterior of the udder*

Housing conditions

In temperate regions, cows are housed in winter and pastured in summer. Differences in teat contamination can be found between housing and pasturing (IDF, 1994). Both total plate and aerobic spore counts are lower when cows are at pasture. When cows are housed, bedding material and feedstuffs can be contamination sources. In both cases (housing and pasturing) feaces, or dung is also an important contamination source. Contamination of bedding material can be very high, due to absorption of urine and feaces. For mastitis causing bacteria different bedding materials can be of influence as a vehicle of contamination (IDF, 1996).

Teat contamination

The exterior of the cows' udder and teats can contribute microorganisms that are naturally associated with the skin of the animal as well as microorganisms that are derived from the environment in which the cow is housed and milked.

The groups of microorganisms isolated from teats are mainly micrococci and aerobic sporeformers, but in general most bacteria found are aerobic sporeformers depending on the method of sampling the teats. The aerobic thermoduric organisms on teat surfaces are almost entirely *Bacillus* spores, spore counts ranging from 10^2-10^5 per teat depending on environmental conditions (McKinnon and Pettipher, 1983). The psychrotrophic spore count in summer remains the same as in winter because the proportion within the total

spore population increases, and it is mainly derived from soil contaminating the teat surface (McKinnon and Pettipher, 1983).

Teat surfaces are also a source of clostridial spores in milk. Sources of these spores are feed stuff, silage, bedding and feaces. The numbers decline markedly when cows go out to pasture. Pathogenic bacteria that might contaminate the teats are *Campylobacter jejuni*, *Salmonella typhii, S. dublin* and *Yersinia enterocolytica*. Feacal contamination is very likely to occur (IDF, 1996). Damaged teats can affect milk quality in that any break in the skin can become a reservoir for mastitic bacteria and give rise to a significant increase in bacterial count. Physical injury to teats is usually caused by cows treading on their own teats, usually due to poor housing design, rough concrete, high cubicle steps, and narrow cubicles or overcrowding. This can result in mastitis and in severe cases, blood being drawn into the milk supply during milking (Harding, 1995).

2.2.3 *Milking and storage equipment*

Plant cleaning and disinfection

Having limited the number of bacteria entering milk during milking, it is essential that contamination from equipment situated between the cow and the refrigerated storage unit is kept to a minimum. Bacteria are present in the air, dust and water, especially any water containing traces of milk residues which may have been left in the milking plant overnight, as such residues provide a very good source of food for bacteria, thereby enabling the bacterial counts to increase rapidly. Cleaning regimes are based on removing visible dirt, removing milk residues (fat, protein, milkstones) which harbour bacteria, then sterilization of the cleaned surfaces using heat or chemical sterilants such as sodium hypochlorite (Harding, 1995). Cleaning and sanitizing procedures can influence the degree and type of microbial growth on milk contact surfaces by leaving behind milk residues that support growth, as well as by setting up conditions that might select for specific

microbial groups. More resistant or thermoduric bacteria may endure in low numbers on equipment surfaces that are considered to be efficiently cleaned with hot water (Harding, 1995).

The influence of cleaning and disinfection on the survival of bacteria on milk contact surfaces is not yet fully understood. Attachment of bacteria to different surfaces (Husmark and Ronner, 1990) and possible scaling may cause problems with cleaning and disinfection. In most cases not all bacteria are killed and removed during cleaning and disinfection.

Storage time and temperature

The multiplication of bacteria in milk is dependent on both the temperature and time of storage. After production, milk can be stored in cans and in bulk tanks before collection. The storage temperature influences the types of bacteria which grow and their spoilage characteristics. Spoilage of raw milk is due to streptococci and coliforms, resulting in souring of milk. During storage in bulk tanks and transport, the microflora of the milk changes from micrococci to psychrotrophic gram-negative rods. There are many different microorganisms (mainly bacteria), which can find access to milk, and there are three broad temperature ranges classifying their optimum growth rates. Organisms with an optimum growth rate at low temperatures (0-15˚C) are psychrophiles, at medium temperatures (20-40˚C) are called the mesophiles and at high temperatures (45-55˚C) the thermophiles (Harding, 1995; IDF, 1996).

2.3. Bacteriological quality of milk

2.3.1 *Non-pathogenic microorganisms*

Milk is an ideal balanced food for man; however, the composition of milk makes it not only an excellent food for man but also an ideal medium for the growth of bacteria and other microorganisms (Hayes, 1981).

The vast majority of bacteria in the following groups of organisms are non-pathogenic, however, these organisms are of particular concern to the dairy industry because they affect product quality. They may have contaminated the milk from external sources such as the animal's coat, faeces or urine, dust and dirt of the dairy, hay and feed, manure, milking operatives and utensils but are destroyed by pasteurization, although some thermoduric organisms are capable of surviving the prescribed heat treatment conditions. Cleaning and sterilizing equipment, together with bulk tank collection and refrigeration are believed to reduce the bacterial content of raw milk. However, they also create conditions favourable for the growth of psychrotrophic rather than mesophilic organisms (Hayes, 1981).

Thermoduric organisms

Spore-forming organisms

Spore-forming bacteria are divided into two main genera. The first, the genus *Bacillus*, comprises aerobic and facultatively anaerobic species, whilst the second, the genus *Clostridium,* contains mainly obligately anaerobic species (Hayes, 1981)

Bacillus species included in this group are frequently present in raw milk and are the most common cause of sweet curdling, bitter flavour and bitty cream in

pasteurized milk. These defects occur because the spores of these organisms survive pasteurization and in pasteurized products held at ambient temperatures the spores can germinate and grow to produce vegetative cells in large numbers. These organisms gain entrance to milk from unsterile utensils, and the dust of hay, straw and grains (Chalmers, 1955).

The primary source of contamination by *Clostridium* species e.g. *Clostridium butyricum* and *Clostridium sporogenes*, is soil, but they may also be normal inhabitants of silage, feeds and manure. Clostridia often produce butyric acid under conditions which inhibit the formation of lactic acid, whilst certain clostridia produce the gases hydrogen and carbon dioxide as well as acid (Hayes, 1981).

Non-spore forming organisms

The presence of high levels of thermoduric organisms indicates that milk was produced or processed in unclean surroundings or poor sanitary conditions. The most important thermoduric organisms include *Streptococcus thermophilus, Lactobacillus bulgaricus, Micrococcus luteus, Micrococcus varians, Microbacterium* species, and coryneforms. Some species of *Streptococcus* and *Lactobacillus* are thermophilic as well as thermoduric (Hayes, 1981).

Coliform organisms

Coliforms are a broad class of bacteria found in our environment, including the feaces of man and other warm-blooded animals. The group includes species from several genera. They are facultatively anaerobic, gram-negative, short rods that cannot form spores but do produce acid and gas from lactose within 48 hours at 35°C thus giving milk and its produts a very undesirable flavour. Organisms belonging to this group include *Eschericia coli*, the closely related *Enterobacter aerogenes* (a non-faecal coliform), *Klebsiella pneumoniae*, *Citrobacter* species, and others. These organisms have "sanitary significance," in

that their presence at detectable levels in finished drinking water or at higher than minimal levels in pasteurized milk is considered cause for alarm. Most are not capable of causing disease in humans (Cliver, 1999) except *E. coli* enterobacteriaceae (ETEC) strains (IDF, 1994).

Fungi

Fungi is a collective term for yeasts and moulds.

Moulds: are under the division of Mycota and they include true slime moulds (Myxomycetes) and cellular slime moulds (Acrasiomycetes). Moulds are filamentous, multi-celled fungi with an average size larger than both bacteria and yeasts (10 X 40 µm). Each filament is referred to as a hypha. The mass of hyphae that can quickly spread over a food substrate is called the mycelium. Moulds may reproduce either asexually or sexually, sometimes both within the same species. Moulds are undesirable in most dairy products because they produce a musty odour, affect flavour and, when growing on the surface of a product, appear unsightly. Mould growth may be an indication of poor storage or unhygienic production of dairy products (Hayes, 1981).

Yeasts: belong to the protoascomycetes. They may be defined as fungi whose growth form is normally unicellular. However, some yeasts do have a pseudomycelium. The shape of the yeasts varies from spherical to ovoid, lemon-shaped and occasionally almost cylindrical. Their sizes vary greatly but are generally larger than bacterial cells. Yeasts may be divided into two groups according to their method of reproduction. One group reproduces by both budding and spore formation, the other only by budding. Yeasts may be beneficial or harmful in foods. Generally, those associated with the production of dairy products by fermentation are true yeasts whereas false yeasts are usually associated with spoilage (Hayes, 1981).

Psychrotrophs

When considering the quality of refrigerated milk, the concern is almost exclusively with microorganisms that grow at storage temperatures. These bacteria are referred to as psychrotrophs or psychrotrophic bacteria, however nowadays they are called psychrotolerant. They are defined based on their capability of appreciable growth at commercial refrigeration temperatures of 2-7°C, irrespective of their optimum growth temperature (IDF, 1976).

Psychrotolerants are ubiquitous in nature and are common contaminants of milk. They are mostly gram- negative, predominantly strains of the genus *Pseudomonas,* other genera include *Flavobacterium* and *Alcaligenes*. Psychrotolerants are very rare amongst gram-positive bacteria but *Bacillus* and *Clostridium* species have been isolated. These organisms are introduced into milk when these sources become established on milk contact surfaces, equipment, flooring, and drains in the milking parlor and processing plant. Microorganisms can adhere to stainless steel surfaces, grow there and release a large number of cells into the milk (Bouman *et al*., 1982; Driessen *et al*., 1984; Langeveld *et al*., 1995). Environmental factors that may contribute to contamination are water, soil, vegetation, bedding material and to a lesser extent the air (Suhren, 1989). Milk produced or processed under sanitary conditions usually contains less than 10 percent of the total microflora as psychrotolerants. But milk produced or processed under unsanitary conditions can contain more than 75 percent psychrotolerant bacteria.

From a standpoint of quality control of milk, these bacteria, particularly of the gram – negative, are the most important microorganisms. Their importance has increased as storage and holding times on the farm have lengthened with changes in technology and marketing conditions. They can cause a variety of flavour defects and colour changes, all depending on their biochemical activity (Jay, 2000; Frank, 1997; Ray, 1996).

2.3.2. *Pathogenic bacteria*

Salmonella

Food-borne disease outbreaks associated with *Salmonella* have been known for a long time and are still a continuing problem in both developed and third-world countries (Bean, *et. al.,* 1990). *Salmonella* is a well known contaminant of poultry and poultry products (eggs), meat and meat products, milk and milk products. There have been several outbreaks of salmonellosis for which milk and milk products were responsible. Raw milk may be contaminated with salmonellae derived from either an infected animal or from a human carrier. Since it is generally agreed that salmonellae are killed during HTST pasteurization, incidents associated with pasteurized milk must be attributed to post-pasteurization contamination of the milk (IDF, 1994).

Salmonellosis is caused by the ingestion of living bacteria of the *Salmonella* group. The primary habitat of *Salmonella* species is the intestinal tract of animals such as birds, domestic and farm animals, reptiles and occasionally insects. As intestinal organisms, these bacteria are excreted in feaces from which they may be transmitted to a large number of places. Consumption of contaminated milk may lead to a number of gastrointestinal diseases. Gastroenteritis has been attributed to species of *Salmonella* especially *Salmonella typhimurium* and *Salmonella enteridis* and symptoms occur 7-72 hours following ingestion of contaminated food. Typhoid and paratyphoid fevers are caused by organisms such as *Salmonella typhi* and occur less frequently than outbreaks of gastroenteritis (IDF, 1994).

Campylobacter jejuni

Amongst the organisms known to be the cause of enteritis in man and animals, *C. jejuni* was not recognized until the beginning of the 1980's. In the mean time this species has been established as one of the most common food-borne bacterial pathogens, comparable to Salmonellae. *Campylobacter jejuni* is a common inhabitant of the alimentary tract of milking cows, but it is not clear how the milk becomes contaminated with this organism. Pasteurization if carried out effectively will eliminate *Campylobacter* from milk. The global prevalence of *C. jejuni* in humans and in domestic, wild and laboratory animals and birds is a reason for very complicated epidemiological relations. The incidence of this organism worldwide corresponds to those caused by Shigellae and salmonellae (IDF, 1994).

Brucella abortus

Brucella is the causative agent of brucellosis, a zoonosis of worldwide importance also called 'Malta fever', 'Mediterranean fever', or 'Undulent fever'. Brucellosis affects man and animals mainly cattle, sheep, goats and swine. Principal manifestations of animal brucellosis are reproductive failure, that is, abortion and birth of unthrifty offspring in the female and orchitis and epididymitis in the male. Persistent infection with shedding of *Brucella* in reproductive and mammary secretions is common. Brucellosis in man is usually characterized by an intermittent influenza-like clinical pattern (IDF, 1994).

Humans are accidental and almost always dead-end hosts of *Brucella* infections. The disease is primarily an occupational risk and occurs mainly in exposed professions, that is, veterinarians, farmers, laboratory technicians, abattoir workers and others who work with animals and their products. The primary source is an animal, and infection is contracted either through direct or indirect contact through skin or mucous membranes or ingestion of contaminated

products, dairy fresh products in particular. Because of the frequency and sometimes the severity of human cases directly or indirectly acquired from animals, brucellosis is regarded as a major anthropozoonosis. Brucellosis causes economic losses to livestock industry due to abortions, infertility, losses in milk production and trade restrictions. Although progress in the control and local eradication of brucellosis in several parts of the world has been achieved, brucellosis remains a major public health hazard (IDF, 1994).

Mycobacterium tuberculosis

This organism is responsible for tuberculosis in man and animals. It has almost certainly inflicted more suffering and death than any other bacterial infection. This species of bacterium is considered pathogenic not only to humans but also to several other species of animals, normally in association with humans. Dairy animals were the first to catch the attention in this regard. However, it was only after 16 years that Theobald Smith in 1898 demonstrated a distinct variation in the organism causing tuberculosis in dairy animals and now known as *Mycobacterium bovis* (IDF, 1994).

Tuberculosis is a disease that spreads either due to the infectious agent that comes primarily from the producing animal through milk secretion or the infectious agent gaining access to milk at the post-secretory stage through infected humans or animals (IDF, 1994). The extent of infection of milk with *Mycobacterium tuberculosis* varies greatly, but probably 5-7 percent of samples of raw ungraded milk, taken in urban areas from individual herd supplies, contain the organism. Pasteurization when carefully carried out is lethal to the organism but it has been found in practice that not all samples of commercially pasteurized milk are free from the pathogen (Chalmers, 1955). Tuberculosis is observed in all age groups of humans. It is difficult to indicate how far milk and milk yielding animals contribute to the morbidity and mortality of humans, but tuberculosis still

ranks as the principal cause of death among infectious and parasitic diseases (IDF, 1994).

The other pathogenic microorganisms in raw milk are: *Staphylococcus aureus, Streptococcus agalactiae, Yersinia enterocolitica, Escherichia coli, Listeria monocytogenes* and *Coxiella burnetti* (IDF, 1994, 1996). Table 1.4 summarizes the diseases transmissible to man through milk.

Changes in food production and distribution practices may affect the microbial ecology in food processing systems and the transmission of food borne diseases. Similarly, changes in food consumption patterns, as well as in the human population (such as an increase in the number of elderly an immunocompromised people) can alter patterns of food borne infections. Separately, or together, changing factors that affect our food system may furthermore contribute to the emergence or reemergence of newly recognized food borne pathogens. Improved diagnostic methods also may lead to the discovery of pathogens that had been previously unrecognized as causes of food borne infections. Continuous efforts to better understand and control transmission of food borne pathogens in the dairy food system are thus key to assure safe dairy foods for the future.

2.4. Methods of treatment of milk

There is no such thing as absolute safety in milk, but experience has shown that adoption of certain practices has produced a satisfactory level of safety. There are three officially recognized methods by which milk sold to the consumer may be treated by heat, namely pasteurization, sterilization and ultra heat treatment (UHT). Lowering the number of microorganisms initially within raw milk will undoubtedly also enhance the level of safety, quality and shelf-life.

2.4.1. Sterilisation

Milk sterilization (130˚C, at least 1 second) is a well-established method for prolonged milk storage. The heat treatment is severe enough to kill all microorganisms present, both spoilage and food borne pathogens, to an acceptable level. There is a statistical chance of an organism surviving the process, but this is acceptable in the normal sense of safe food production (Forsythe, 2000). However, milk processed by this method suffers some reduction in nutritional value. The biological value of the proteins is slightly reduced and about one third of the thiamin and half of the vitamin C, folic acid, and vitamin B_{12} are destroyed (Hayes, 1981).

2.4.2. Ultra heat treatment

Although ultra heat treatment (UHT) was developed in the 1940's, it was not accepted as a legal designation for milk until 1965. The UHT procedures are based on the discovery that higher processing temperatures with much shorter holding times produced a product in which all vegetative bacterial cells had been killed but in which there was much less change in milk colour flavour and nutritional value than with sterilization procedures.

UHT milk involves sterilization of homogenized milk by a continuous process employing either direct or indirect methods of heating. With the indirect heating process, a temperature of at least 138˚C is achieved by passing the milk through a heat exchanger using steam under pressure for the final stage of heating. The milk is then aseptically cooled prior to packing. Milk heated by the direct method reaches a temperature of at least 145˚C either by injecting steam directly into the milk or by forcing the milk through a nozzle into a tank filled with steam. The UHT process has the effect of killing all bacteria although some spores are capable of surviving this heat treatment (Hayes, 1981).

2.4.3. Pasteurization

Pasteurization is defined by the International Dairy Federation (IDF) as “a process applied to a milk product with the object of minimizing possible health hazards arising from pathogenic microorganisms associated with milk, by heat treatment which is consistent with minimal chemical, physical and organoleptic change of the product”.

The object of pasteurization primarily is to render milk safe by inactivating microorganisms and enzymes, followed by cooling and holding at low temperature. There are numerous combinations of time and temperature having the desired effect of destroying bacteria, but in practice there are limits for both parameters, which, if exceeded produce undesirable effects such as the destruction of the cream line or caramelization of the lactose in milk (Hayes, 1981). Milk is often pasteurized using two methods, the high temperature short time (HTST) and the holder method. The holder method involves holding fixed batches of milk for at least 30 minutes at not less than 62.8˚C and not more than 65.6˚C. The HTST method is a 72˚C, 15 seconds process. The whole of the heating, holding and cooling of the milk is done as the milk flows in a continuous stream through a single unit composed of a series of plates arranged in parallel (Chalmers, 1955). This is designed to kill all pathogenic bacteria such as *Salmonella* and *Brucella* species at levels expected in fresh milk. However, there are microorganisms that survive pasteurization such as *Streptococcus thermophilus* and *Micrococcus luteus* to name a few, and they are called thermoduric organisms (Forsythe, 2000).

2.4.4. ***Ultra violet radiation***

Ultra-violet (UV) light is invisible radiation within a range of the solar spectrum. It is similar to the wavelengths that are produced by visible light, but much shorter. UV light is found between X-rays and visible rays on the electromagnetic

spectrum but its wavelengths are longer than those of x-rays and have a range of 14-400 nm. UV rays of wavelength 300-400 nm have a mildly biocidal effect. These are the typical wavelengths occurring in sunlight penetrating the atmosphere. Within the UV radiation spectrum, there are three main groups or sub-bands. These sub-bands are UV-A, having the longest wavelengths, UV-B and UV-C having the shortest wavelengths.

The radiation of the UV-C and the lower end of the UV-B sub-bands show the highest absorption rates by the nucleic acids contained in microorganisms. The maximum absorption of UV light by these nucleic acids occurs between 260-265 nm, and it is because of this characteristic that the UV-C band is known to be germicidal.

Ultra-violet radiation of 260 nm must hit the microorganism to inactivate it, and each microorganism must absorb a specific amount of energy to be destroyed. Proteins and nucleic acids which store the entire microorganism's genetic data absorb UV radiation. The absorbed energy from the emitted UV light then breaks down the links between the bases in the nucleic acids and rearranges their genetic information. This destroys or inactivates the DNA, thus preventing the microorganisms from reproducing.

UV lamps, however, are designed to emit wavelengths in the most lethal range of around 260 nm being an effective microbicide and are used for killing microorganisms in air or in liquids (such as water). In experimental conditions, the characteristics of death are similar to those of ionizing radiations. The UV rays are absorbed by the intracellular RNA and DNA resulting in cell death, or if the cells survive, resulting in an increased frequency of mutation. But because UV rays are not very penetrating, unlike ionizing radiations, they are most effectively used either for sterilization of surfaces or of thin liquid films. Generally it cannot be used with any certain effect for opaque or turbid liquids since the

rays must actually strike the microorganisms and not be absorbed by particles in the liquid.

The South African patent, however, overcame this difficulty by making use of an elongate sterilizer arranged tangentially with respect to the housing creating a swirling effect of the liquid. Consequently, the swirling effect of the turbid liquid (milk in this case) enhances the contact time/striking of the UV rays with the microorganisms leading to an increase in cell death. However, there are concerns regarding the effects of UV rays on the composition of the milk.

OBJECTIVES

Our objectives of the current study were to determine the number of undesired microorganisms including pathogens in raw and pasteurized milk sold in the Bloemfontein area. These microorganisms will be characterized and their contribution assessed. A possible means, using UV radiation, to reduce these numbers on the dairy farm level will be investigated.

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Table 1.1 National standards applicable to milk

Analysis	Raw milk before further processing	Raw milk directly to the public	Pasteurized milk
Total count/ml	<200 000 cfu/ml	<50 000 cfu/ml	<50 000cfu/ml
Coliforms/ml	<20 cfu/ml	<20 cfu/ml	<10 cfu/ml
E. coli/ml	0	0	0
Pathogens	0	0	0

Foodstuffs, Cosmetics and Disinfectants Act (54), 1972

Table 1.2 Essential amino acids

Amino acid	Daily requirement (g)	g/100g Milk protein
Phenylalanine	1.1	5.5
Methionine	1.1	2.8
Leucine	1.1	12.1
Valine	0.8	7.1
Lysine	0.8	7.4
Isoleucine	0.7	6.7
Threonine	0.5	4.6
Tryptophan	0.3	1.4
Histidine	80	2.2

Table 1.3 Compositional changes in milk constituents associated with elevated somatic cell counts (SCC) [a]

Constituent	Normal milk (%)	Milk with high SCC (%)	Percentage of normal
SNF	8.9	8.8	99
Fat	3.5	3.2	91
Lactose	4.9	4.4	90
Total protein	3.61	3.56	99
Total casein	2.8	2.3	82
Whey protein	0.8	1.3	162
Serum albumin	0.02	0.07	350
Lactoferrin	0.02	0.10	500
Immunoglobulins	0.10	0.60	600
Sodium	0.057	0.105	184
Chloride	0.091	0.147	161
Potassium	0.173	0.157	91
Calcium	0.12	0.04	33

Table 1.4 Diseases transmissible to man through milk.

DISEASE		PRINCIPAL SOURCES OF INFECTION		
		Man	**Milk animal**	**Environment**
Bacterial	Anthrax *		X	X
	Botulism (toxin)			x
	Brucellosis		X	
	Cholera	X		
	Coli infections (pathogenic strains of *E. coli*)	X	x	
	Clostridium perfringes (welchii) infection			X
	Diphtheria	X		X
	Enteritis * (non-specified, from large numbers of killed or living coli, proteus, pseudomonas, welchii, etc.)			
	Leptospirosis		X	
	Listeriosis		X	
	Paratyphoid fever		X	
	Rat-bite fever	X	X	
	Salmonellosis (other than typhoid and paratyphoid fevers)	X	x	
	Shigellossis	X		
	Staphylococcal enterotoxic gastroenteritis	X	X	
	Streptococcal infections	X	X	
	Tuberculosis	X	x	
	Typhoid fever	X		
	Infections with adenoviruses	X		
Viral	Infections with enteroviruses (including polioviruses and the Coxsacchie groups)	X		
	Foot and mouth disease		x	
	Infectious hepatitis	X		
	Tick-borne encephalitis		x	
Rickettsial	Q-fever		x	
Protozoal	Amoebiasis	X		
	Balantidiasis	X		x
	Giardiasis	X		
	Toxoplasmosis		X	

* not conclusively incriminated as milk-borne, but epidemiolocally probable or suspect (IDF, 1994; IDF, 1996)

CHAPTER 2

THE HYGIENIC QUALITY OF COMMERCIALLY PRODUCED FRESH MILK IN BLOEMFONTEIN, SOUTH AFRICA

Abstract

The objective of this study was to assess the general hygiene of fresh milk in the Mangaung area of Bloemfontein, South Africa. A total of 52 milk samples (45 pasteurized and 7 raw milk samples) were collected at different milk selling points in the Bloemfontein area and examined for the food-borne pathogens *Listeria monocytogenes, Salmonella* spp.*, Clostridium botulinum, Staphylococcus aureus, Bacillus cereus* and *Escherichia coli*. Proteolytic and lipolytic organisms, coliforms and total bacterial counts were also determined. Milk was directly plated on selective agars for direct bacterial enumeration and was enriched in selective broths to increase detection sensitivity. None of the pathogens was detected in either the pasteurized or raw milk samples. However, strong proteolytic activity was detected in 16 of the pasteurized milk samples, whereas none tested positive for lipolytic activity. Coliforms were detected in 69% of the pasteurized milk samples and the counts exceeded the milk standards of South Africa for pasteurized milk of more than 1.0 log cfu/ml. All (100%) of the raw milk samples tested positive for coliform counts which exceeded the maximum limits according to South African standards for raw milk intended for human consumption (1.3 log cfu/ml). Most (83%) of the pasteurized milk samples had total bacterial counts which exceeded the maximum limits according to South African standards for pasteurized milk quality (4.7 log cfu/ml), and 100% raw milk samples had counts exceeding 4.7 log cfu/ml.

2.1 INTRODUCTION

The biological value of milk is second to eggs regarding the availability of essential amino acids, energy, calcium and vitamins. In many parts of the world it contributes significantly to the wholesomeness of human diets, especially during childhood. The increasing demand for milk and its products also makes it one of the prime commodities for marketing and trade. Milk is considered an attractive source of energy, proteins and calcium for infants and young children who have few alternative sources for these nutrients. Besides its beneficial effects on nutrition, milk is ideally suited for growth of microorganisms.

Throughout the world, food safety and quality is a topic of public concern. Food-borne diseases have a major public health impact and their well-publicized and widespread outbreaks have created an awareness of their potential threats to human health. The epidemiology of foodborne diseases is rapidly changing as newly recognized pathogens emerge and well-recognized pathogens increase in prevalence or become associated with new food vehicles (Alterkruse *et al.,* 1997).

Milk can act as a vehicle for the transmission of diseases of bacterial (brucellosis, tuberculosis, salmonellosis, listeriosis), viral (hepatitis, foot-and-mouth-disease), ricketsial (Q-fever) or parasitological (toxoplasmosis, giardiasis) origin. Milk-borne illnesses have been recognized since early days in the dairy industry (Ryser, 1998). The diseases transmissible to humans through the consumption of milk like salmonellosis, listeriosis, *E. coli* infections and many others were described extensively by Kaplan *et al*. (1962). Listeriosis and salmonellosis can have serious health implications in calves and cattle, but asymptomatic shedding in feces also occurs (Van Kessel *et al.,* 2003). Listeriosis can also cause miscarriages or result in meningitis in patients with chronic disease, whilst salmonellosis can be a result of invasive disease or reactive arthritis (Alterkruse *et al.,* 1997). Most *E. coli* strains are commensal intestinal organisms that do not

cause disease, but a small percentage of *E. coli* is enteropathogenic. Infection with enteropathogenic *E. coli* usually results in mild illness, however, some serotypes are enterohemorrhagic *E. coli* and can lead to hemolyticuremic syndrome. *Eschericia coli* O157:H7 is the most common entero-hemorrhagic strain (Van Kessel *et al.,* 2004), which resulted in acute kidney failure in children in the United States (Alterkruse *et al.,* 1997). Pathogenic microorganisms in milk are derived from the cow itself, from human handlers and from the environment. Because *Listeria, Salmonella,* and *E. coli* O157:H7 are shed in the animal's feces, there is a risk of these pathogens entering the milk (Van Kessel *et al.,* 2004).

Cows suffering from mastitis, discharge large numbers of pathogens into the milk, especially *Staphylococcus aureus*, *E. coli* and *Clostridium perfringens*. *S. aureus* is a leading cause of gastroenteritis resulting from the consumption of contaminated food including milk. Staphylococcal food poisoning is due to the absorption of enterotoxins preformed in the food (Loir *et al*, 2003). The organisms multiply in infected lesions or colonized teat canals and can readily enter the udder. Infected heifers at calving may represent the most important reservoir to uninfected herd mates. How heifers become infected before calving is unknown at this time. Mastitis control programs need to address the presence of this disease in heifers (Jones *et al*., 1998). *Clostridium botulinum* produces a potent neurotoxin (Brown, 2000) and the spores are heat resistant and can survive in foods that are incorrectly or minimally processed. Food-borne botulism is a severe type of food poisoning caused by the ingestion of foods containing the potent neurotoxin formed during growth of the organism. The toxin is heat labile and can be destroyed if heated at 80°C for 10 min or longer. Botulinum toxin causes paralysis by blocking motor nerve terminals at the myoneural junction. The resulting asphyxia causes death. The incidence of the disease is low, but the disease is of considerable concern because of its high mortality rate if not treated immediately and properly.

Another type of food poisoning organism associated with milk and milk products is *Bacillus cereus.* It causes two distinct types of illnesses. The diarrheal type illness is caused by a large molecular weight heat-labile protein and causes a watery diarrhea, abdominal cramps, and pain. The vomiting (emetic) type of illness causes nausea and vomiting and is caused by a low molecular weight, heat-stable peptide.

Pasteurization is very effective against bacterial organisms such as *Salmonella, Listeria* and *Escherichia coli*, and as a result foodborne outbreaks associated with these organisms in pasteurized milk or milk products are rare, and when they do occur, are typically the result of improper pasteurization or post-pasteurization contamination.

Milk is not only an excellent culture and protective medium for pathogens, but also spoilage microorganisms. Spoilage is characterized by any change in a food product that renders it unacceptable to the consumer from a sensory point of view. This may be physical damage, chemical changes (oxidation, colour changes) or appearance or off-flavours and off-odours resulting from microbial growth and metabolism in the product. Microbial spoilage is by far the most common cause of spoilage and may manifest itself as visible growth (slime colonies), as textural changes (degradation of polymers) or as off-odours and off-flavours (Gram *et al.,* 2002).

Since the microbial spoilage of milk is generally associated with the growth of bacteria, very little consideration has been given to the ability of yeasts to grow in milk (Fleet, 1990). However, a range of observations indicates an ability of yeasts to metabolise milk constituents. These observations include the occurrence and growth of yeasts in many cheeses (Roostita and Fleet, 1996), the spoilage of condensed milk and yoghurts by yeasts (Fleet, 1990), incidences of yeast spoilage of pasteurized milks (Fleet and Mian, 1987). Generally, the available information shows that yeasts that occur in both raw and pasteurized milks, are

mostly reported at low insignificant populations of less than 10^3 cfu/ml, but occasionally counts as high as 10^4 cfu/ml can occur. Such yeasts rarely grow in milk during refrigerated storage and are quickly overgrown by psychrotrophic bacteria (Fleet, 1990). Psychrotrophs are spoilage microorganisms that are able to grow in milk held at refrigerated storage temperatures. Psychrotrophic counts may reach 10^3-10^6 cfu/ml before processing of milk and proteases, which are also of concern to the dairy industry, have been detected at these populations (Cousin, 1982). Proteolytic enzymes are of concern because they may be detrimental to milk or dairy products (Cousin, 1982; Fairbairn and Law, 1986). Numerous problems in dairy products have been associated with the actions of proteases, which include off-odours and flavours, poor or non-existent curd formation in cheesemaking, and gelation of UHT milk (Cousin, 1982; Mitchell and Ewings, 1985). Proteolytic enzymes from microorganisms may be located within the cell (intracellular), cell wall associated (periplasmic), or excreted into the media (extracellular). Most information exists for extracellular proteases from psychrotrophic microorganisms (Cousin, 1982).

Where milk is produced under poor hygienic conditions and is not cooled, the main contaminants are usually lactic acid producers, which cause rapid souring. Lactic acid has an inhibitory effect on pathogenic bacteria but since souring of the milk is undesired, this cannot be depended upon to provide a safe milk product (Heeschen, 1994). Pasteurization or more severe heat-treatments applied to raw milk is the only way to ensure that pathogens present are killed and that the milk is safe. It also improves the shelf life of milk by reducing the number of non-pathogenic microorganisms that would otherwise cause spoilage (Burton, 1986), therefore, potential threats to human health related to the dairy industry include errors during pasteurization, consumption of raw milk products, contamination of milk products by heat-resistant pathogens, chemical adulteration of milk, and food-borne disease transmission by the feces of cows. The potential for additional human or animal pathogens to survive current food

processing methods such as pasteurization is an area of ongoing research (Stabel, 2001).

The objective of this study was to assess the hygienic quality of milk sold around the Bloemfontein area.

2.2 MATERIALS AND METHODS

2.2.1 Sample collection

A total of 52 different milk samples including seven from tank milk and 45 from packaged milk were randomly collected at different milk selling points in the Bloemfontein area over a period of two weeks. Care was being taken not to take samples older than one day after packaging and all refrigeration procedures in the outlays were in good working order. This was repeated on four occasions over a time span of four months.

Pasteurized and raw milk (500ml) from cooler tanks were collected in 1L sterile Schott bottles whereas all other pasteurized milk samples were purchased as being sold in either plastic sachets or plastic bottles. The samples were transported in cooler boxes with ice to the laboratory and analyzed within 1h after collection. Plastic sachets were cleaned with 70% ethanol and opened with sterile scissors whereas plastic bottles were aseptically sampled after swirling the bottles. On each sampling, at least two samples were collected from each container and individually enumerated on the different selective media.

All samples were microbiologically examined for the standard coliform, total aerobic bacteria, yeasts, psychrotolerant counts, and the presence of the milk-borne pathogens *Listeria monocytogenes, Salmonella, Escherichia coli, Clostridium botulinum, Staphylococcus aureus* and *Bacillus cereus.* The

presence of proteolytic and lipolytic activity in all microorganisms was also detected.

2.2.2 Enumeration of microbial loads and the detection of pathogens

Food-borne pathogens*:*

L. monocytogenes. Milk (1 ml) was added to a *Listeria* enrichment broth (1/2 Fraser, Biolab South Afric) and the tubes were incubated at 35˚C for 24h. The broth was plated (0.1 ml) directly onto *Listeria* selective medium (ALOA, AES Laboratoire). Plates were incubated at 37˚C for 24h and determined for presumptive *Listeria* colonies (blue to green-blue colonies with an opaque halo).

Salmonella. Milk samples were pre-enriched by adding 1ml into 9ml buffered peptone water (Biolab, South Africa) and incubated at 37˚C for 24h. After the 24h incubation, 0.1 ml of the pre-enrichment was transferred into 5 ml Rappaport-Vassiliadis Soya Peptone (Oxoid, Basingstoke) broth for enrichment and incubated at 42˚C for 24h. After incubation, the enrichment broth was streaked onto XLD (Biolab, Merck, Darmtadt) agar and further incubated at 37˚C for 24h for the detection of presumptive *Salmonella* species (dark pink to red colonies).

E. coli and **coliforms.** For each sample decimal dilutions were carried out as required for microbial assays in 9 ml sterile peptone water and plated in duplicate by the spread plate technique onto VRB-Mug (Biolab). These were incubated at 37˚C for 24h after which all plates containing coliforms (pink to dark red colonies) (Hall *et al.,* 1967) between 25 and 250 colony forming units (cfu) on the highest dilution were counted and mean values determined from duplicate plates. For the determination of *E. coli* colonies, all plates were evaluated under UV light and those, which fluoresced, were considered positive for *E. coli*. Random colonies from the plates were then transferred to Sorbitol Mac Conkey agar for the enumeration of pathogenic *E. coli* (*E. coli* 0157: H7). Lack of sorbitol fermentation

within 24hrs was considered a stable phenotypic character of *E. coli* 0157: H7 (March and Ratman, 1986).

Clostridium botulinum. Milk samples (0.1 ml) were plated directly onto CBI agar (Biolab), a medium selective for *Clostridium botulinum.* The plates were incubated in anaerobic flasks with Anaerocult A (Merck) to confirm an anaerobic atmosphere at 35˚C for 48h. After incubation, plates with a good luxuriant and lecithinase positive were possible suspects for the pathogen.

Staphylococcus aureus. *S. aureus* was isolated from milk by directly plating 0.1ml of milk samples onto Baird-Parker agar (Biolab) according to Baird-Parker (1962) and incubated at 37˚C for 24h. After incubation, typical isolates of *S. aureus* (typical black, shiny convex colonies surrounded by clear zones) were confirmed by a coagulase test.

Bacillus cereus. Milk (0.1 ml) was plated directly onto M.Y.P agar, a medium proposed by Mossel *et al.,* (1967). The plates were incubated at 30˚C for 18-40h. Presumptive colonies for *B. cereus* (rough dry colonies with pink to purple base surrounded by a ring of dense precipitate) were confirmed by their positive nitrate reduction and glucose fermentation tests.

Non-pathogenic microorganisms

Proteolytic organisms: Milk samples (0.1 ml) were plated onto plate count agar (PCA; Biolab) and mixed with sterile skim milk. Plates were incubated at 32˚C for 48-72 h. After incubation, plates were flooded with a solution of 10% acetic acid for 1 min. Colonies surrounded by clear zones after flooding were presumptive of proteolytic activity.

Lipolytic organisms: The evaluation was done by plating the milk samples (0.1 ml) on Tributyrin agar and incubating them at 30˚C for 72 h. Colonies presumptive for lipolytic activity were surrounded by clear zones.

Total plate count: Total aerobic populations were determined by carrying out decimal dilutions of each sample in buffered peptone water (9 ml) and plating them by the spread plate technique onto PCA agar plates (Biolab). These were then incubated at 30˚C for 48 h, after which all plates containing colonies between 25 and 250 colonies were counted.

2.2.3 Phosphatase test

The phosphatase test was performed to check for proper pasteurization of the milk samples. The milk samples (1ml) were diluted with a buffer substrate (5ml) containing disodium p-nitrophenyl phosphate and incubated at 37˚C for 30 min. Liberation of p-nitrophenol, which was detected by its yellow colour, was conformation of unpasteurised samples whereas no colour change indicative of proper pasteurization.

2.3 RESULTS

None of the selected food-borne pathogens could be detected in any of the pasteurized or raw milk samples. Also, there was no lipolytic activity detected for all the samples. However, 45% of the milk samples (all pasteurized) tested positive for proteolytic activity (Table 2.1).

From the phosphatase test, all the samples were found to be properly pasteurized.

The coliforms could be isolated from both the raw milk and pasteurized milk samples (Figures 2.1A and 2.1B). The numbers ranged from 0-10^7 cfu/ml. Twenty percent of pasteurized milk samples contained between10^5 -10^7 cfu/ml, while 43% of the raw milk samples had about 10^5 –10^6 cfu/ml. About 69% of the pasteurized milk samples and 100% of raw milk samples exceeded 10^1 cfu/ml, which is the maximum limit according to the Foodstuffs, Cosmetics and

Disinfectants Act (54), 1952. About 23.8% of all milk samples (pasteurized and raw) contained *E. coli* in the range of 10^4 –10^6. Therefore 76.2% of the samples conformed to the national standard for *E. coli,* which is 0 per 1ml.

Viable total bacterial numbers ranged between 1 to 8 log units (cfu/ml) for pasteurized milk and 4 to 8 log units for raw milk (Figs. 2.2A and 2.2B). Bacterial numbers exceeding the national standard for viable plate count (10^4cfu/ml) were observed in 83% of the pasteurized milk samples, whereas all the raw milk samples (100%) exceeded the standard. Psychrotolerant numbers were depicted in Figs. 2.3A and 2.2B representative of pasteurized and raw milk samples respectively. High counts were detected in the range of 10^5 –10^8 cfu/ml for pasteurized milk whereas raw milk showed counts ranging between 10^5 –10^6 cfu/ml. However, there is no national standard set at present concerning the limit of psychrotolerant species present in milk.

Results of the distribution of yeast counts in pasteurized and raw milk samples are shown by Figs. 2.4A and B respectively. Only 6% of the pasteurized milk samples contained detectable counts of yeasts between 10 and 10^3 cfu/ml. In the raw milk, 57% of the milk samples contained counts at 10^3 cfu/ml.

2.4 DISCUSSION

Pathogenic microorganisms have long been a concern in the dairy due to their potential impact on animal health, milk production, and economics. In recent years, increasing concerns over zoonotic pathogens in the dairy environment and in milk and meat products have added a new dimension to the challenge of biosecurity and biocontainment. Local programs have been developed to help dairy producers implement best management practices (BMPs) that minimize the incidence of animal disease, improve profitability, assure product safety, and address issues of biosecurity (Van Kessel, 2004). Consumers are now more aware of the potential for food-borne pathogens and demand that their food is

clean and wholesome. Public perception of food quality is critical in the marketing of any product. Therefore, even though pasteurization is an effective control method for bacterial pathogens, it is important to maintain high preprocessing standards (Van Kessel, 2004).

Based on the microbial results obtained, it appears that the milk sold at the different selling points around the Mangaung area, despite highly contaminated in some cases, were in all cases pathogen-free as none of the pathogens tested for were detected. These findings conform to the National Legislation concerning the presence of pathogens in raw and pasteurized milk. The standard enforces complete absence of pathogenic organisms in milk intended for consumers. Absence of pathogens in the samples may be explained by lack of competence of these organisms with other microorganisms present, and also no outbreak related with food poisoning caused by any of these organisms was reported in the study area, Bloemfontein.

However, a high percentage of these milk samples showed excessive high numbers of coliforms and viable total bacterial counts exceeding standards proposed by the National Standard. An unacceptable 74% of all milk samples had counts which exceeded the accepted limit of coliforms by the National Standards of log 1.0 cfu/ml for pasteurized milk and 1.4 cfu/ml for raw milk sold to consumers. These results are similar to the findings by Lues *et al.,* (2003) who conducted a study at Botshabelo, a township nearby the present study area (Bloemfontein). These authors obtained 100% of milk samples exceeding the coliform National Standard. Van Kessel *et al*., (2004) also obtained the same results in a study conducted in 2004, whereby only 7% of the samples tested were identified as not being contaminated with fecal coliforms. Fecal coliforms are often used as an indicator of fecal contamination and the potential risk of zoonotic pathogens. Fecal coliforms such as nonpathogenic *E. coli* are prevalent in the digestive tracts of cattle (Van Kessel, 2002). *E. coli* is*,* furthermore, a

known causative agent of diarrhoea and other food-borne related illnesses through the ingestion of contaminated foodstuffs (Lues, *et al.,* 2003).

A total of 23.8% of all the milk samples tested positive for nonpathogenic *E. coli* thereby leaving 72.2% conforming to the National Standard of 0 *E. coli* per ml. The same results were observed by Lues, *et al.,* (2003) who obtained a 23.3% incidence of *E. coli* whereas Van Kessel *et al.,* (2004) obtained an extremely high positive percentage of 93% regarding the incidence of this microorganism.

The viable total bacterial counts of pasteurized milk samples generally ranged between 10^2 to 10^6 cfu/ml with the exception of one sample that had counts of up to 10^8 cfu/ml. Only 17% of these samples were within the acceptable range (50 000 cfu/ml) in terms of the National Standard, which means 83% of these samples exceeded the limit. All the raw milk samples exceeded the National Standard (50 000 cfu/ml) for viable total bacterial counts in raw milk sold directly to the public. Counts for these samples ranged between 10^5- 10^8 cfu/ml. The majority of the samples (71%), however, had counts higher than 10^6 cfu/ml. The total bacterial count is used in the dairy industry as an indication of milk quality, and therefore the 79% of the total milk samples not conforming to the National Standard were a clear reflection of the poor quality of milk in the study area.

Despite indications of yeasts associated with dairy products, especially in yoghurts and cheeses, and milk being the raw material of these products, surprisingly few studies have been conducted on the specific occurrence of yeasts in either raw or pasteurized milk (Fleet, 1990). Although counts of yeasts were found during this study, their growth in milk is considered rather uncommon, mainly because of the neutral pH of milk which favors for the predomination of bacteria (Frazier and Westhoff, 1988; Pitt and Hocking, 1997). However, literature frequently refers to yeasts being present in both raw (Foster *et al.,* 1957; Ingram, 1958; Engel 1986) and pasteurized milk (Jones and Langlois, 1977; Fleet and Mian, 1987) at low insignificant populations of less than 10^3

cfu/ml. These reports correspond to the results of yeast counts obtained in the present study. In the pasteurized milk, yeast counts between 10^1-10^3 cfu/ml were obtained for 6% of the samples whereas a higher incidence of 57% was obtained in the raw milk at loads of 10^3 cfu/ml. These low populations may be a result of yeast growth restriction by faster growing bacteria (Fleet and Roostita, 1996) and being overgrown by psychrotolerant bacteria at refrigerated storage (Cousin, 1982). Although there are many possible sources of yeast contamination associated with raw milk, this is not so for pasteurized milk. According to Fleet and Mian (1987), the occurrence of yeasts in pasteurized milk suggests that they have some degree of tolerance to the pasteurization process. However, the results of the phosphatase test performed on all the milk samples in the present study showed that all the pasteurized milk samples were fully pasteurized. Therefore, yeast contamination may have occurred after pasteurization.

Psychrotolerants were detected in 85.7% of the pasteurized milk. The highest counts were obtained between 10^5 and 10^8 cfu/ml for 56% of these samples, whereas the remaining 44% showed counts ranging from 10^4 cfu/ml and below. All the raw milk samples were positive for the presence of psychrotolerant species with counts as high as 10^4 to 10^6 cfu/ml. These counts were generally lower than obtained in the pasteurized milk samples. The psychrotolerant are capable of growth at refrigerated storages, thereby making them common contaminants of milk, which can cause a variety of defects leading to spoilage. Nevertheless, there is currently no standard for the acceptable limit of these microorganisms in milk and dairy products. But the high counts of psychrotolerants in this study suggested that standardization based on the number of psychrotolerants may be just as important.

Based on the extremely high counts of microorganisms found in raw and pasteurized milk marketed in the Bloemfontein area, it is obvious that we need better and improved health care in the food market. Although no pathogens were found, the alarming high numbers and the presence of *E. coli* in the milk sold to

consumers may pose serious health risks in the study area. Non-conformance to the National Standard is, however, not necessarily indicative of the presence of pathogens but rather a measure of the general hygiene.

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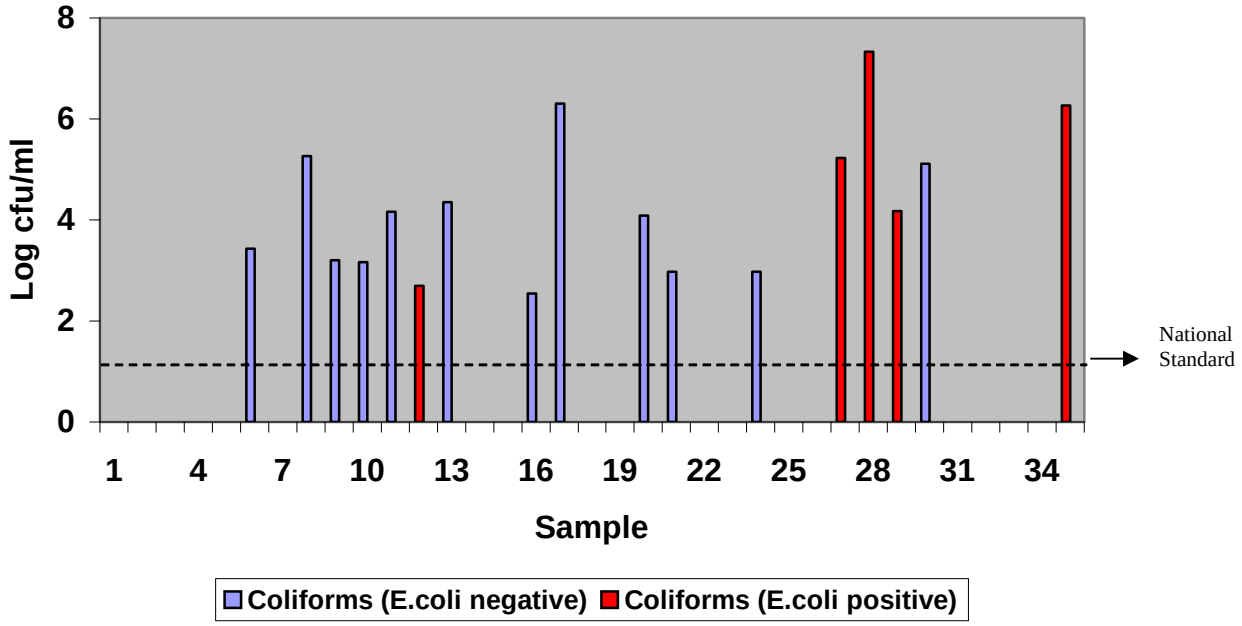
Table 2.1 Results of the proteolytic activity detected in the milk samples.

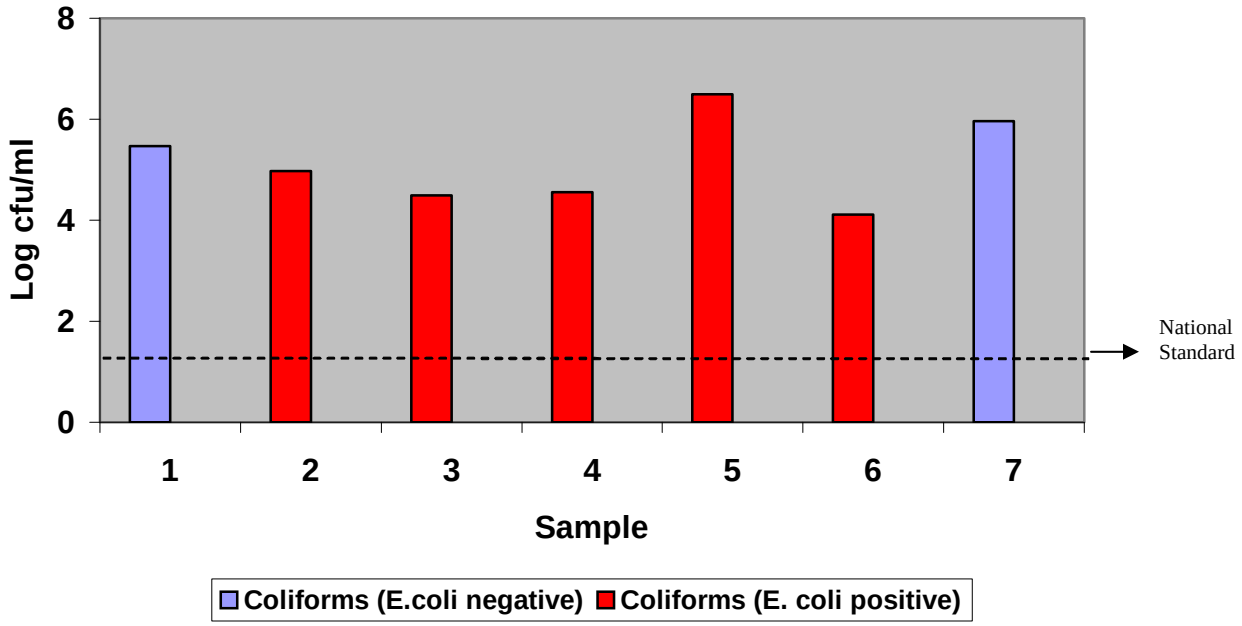
Sample	% Negative	% Positive
Raw milk	0	0
Pasteurized milk	55	45

This table represents the percentage proteolytic activity detected for the milk samples

2.1A Coliform counts in pasteurized milk

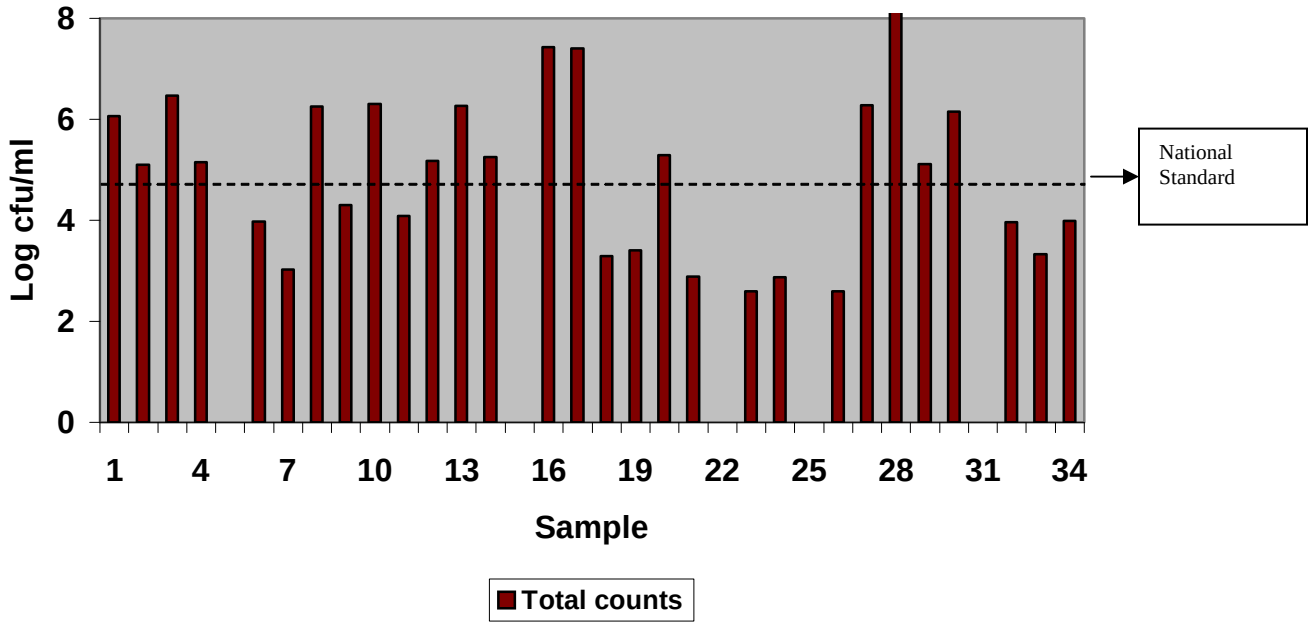


2.1B Coliform counts in raw milk

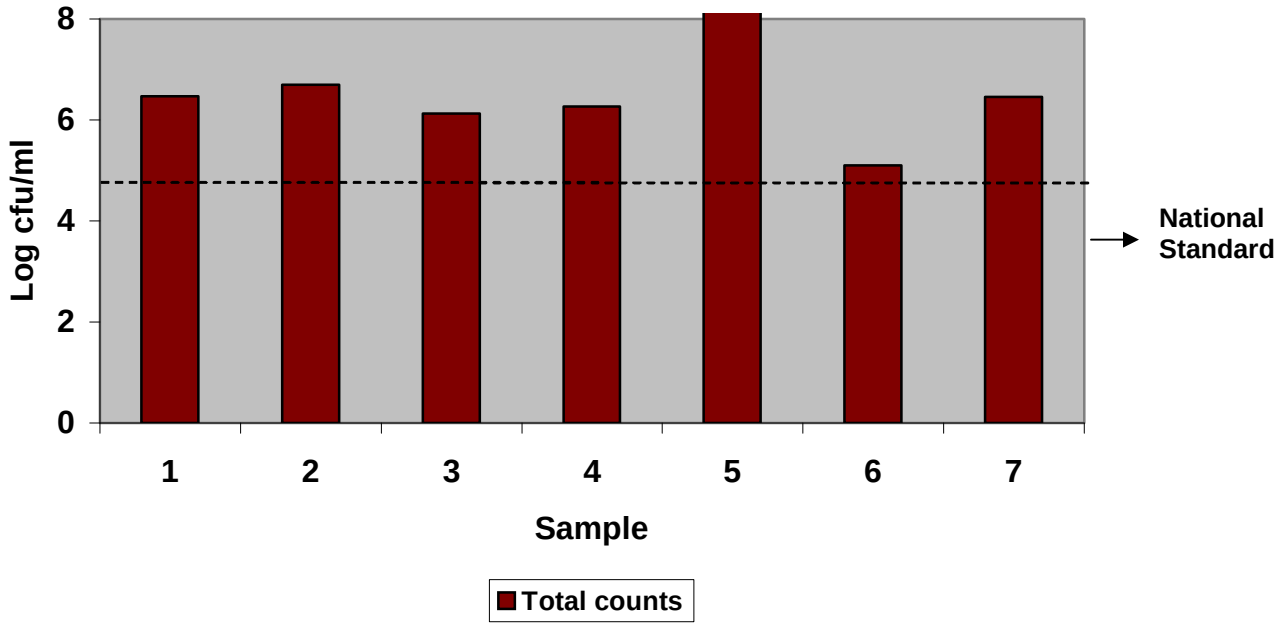


Figures 2.1A and 2.1B. Frequency distribution of fecal coliforms for a set of pasteurized, replicates and raw milk samples respectively.

2.2A. Total aerobic counts in pasteurized milk

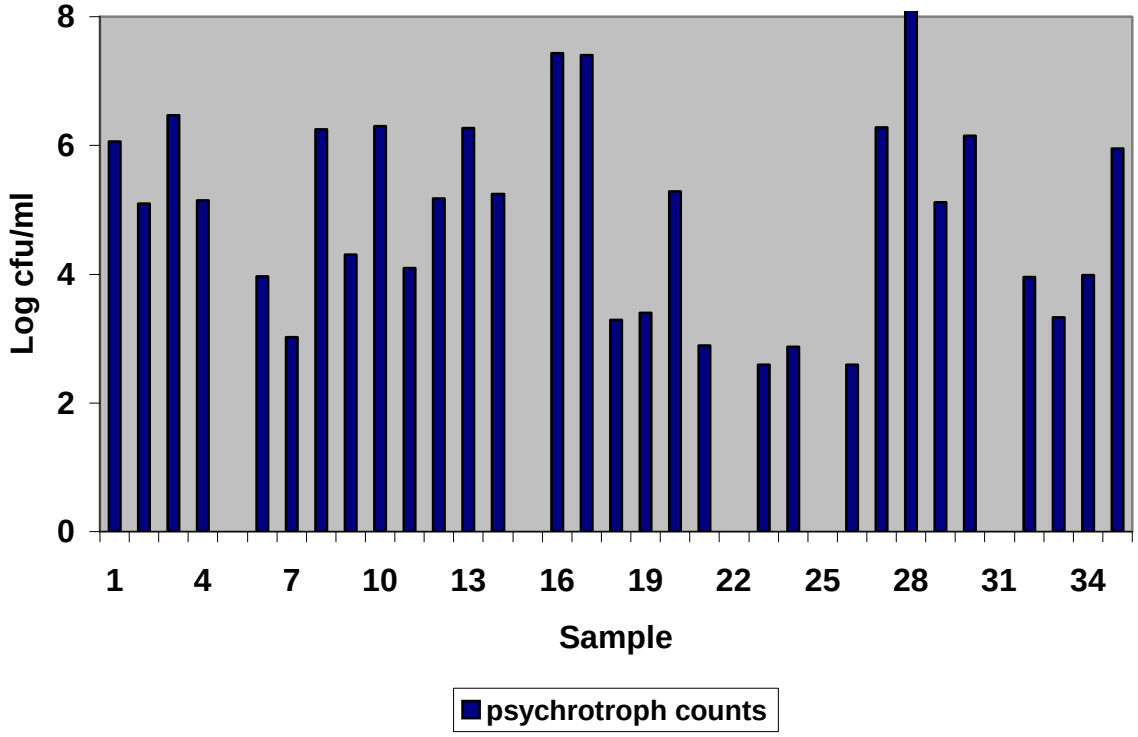


2.2B. Total aerobic counts in raw milk

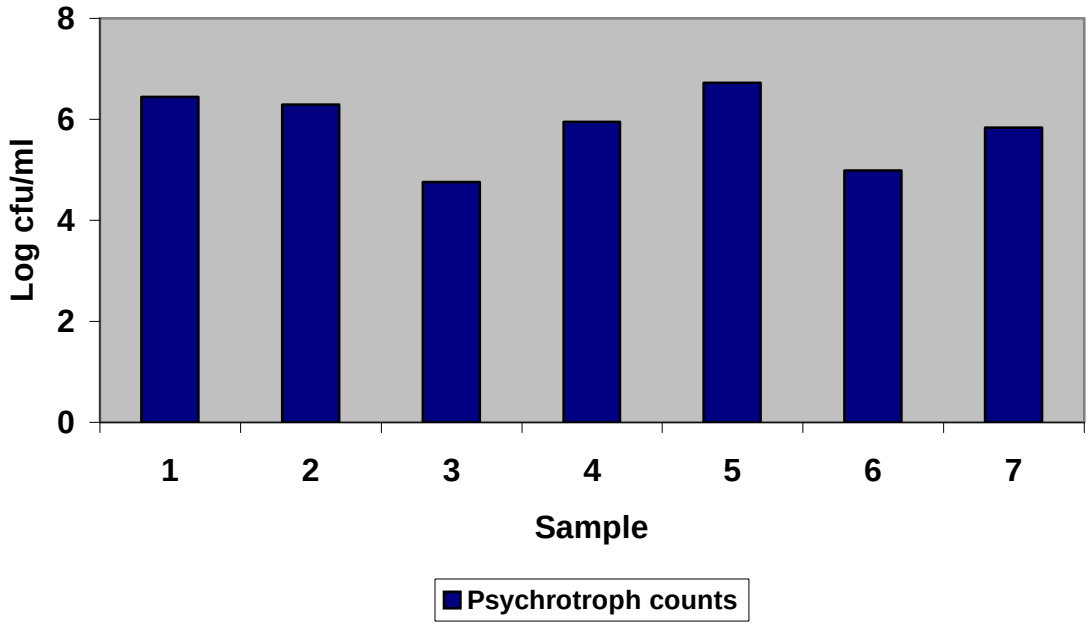


Figures 2.2A and 2.2B. Frequency distribution of total aerobic counts for pasteurized, replicates and raw milk samples respectively.

2.3.A Psychrotolerant counts in pasteurized milk

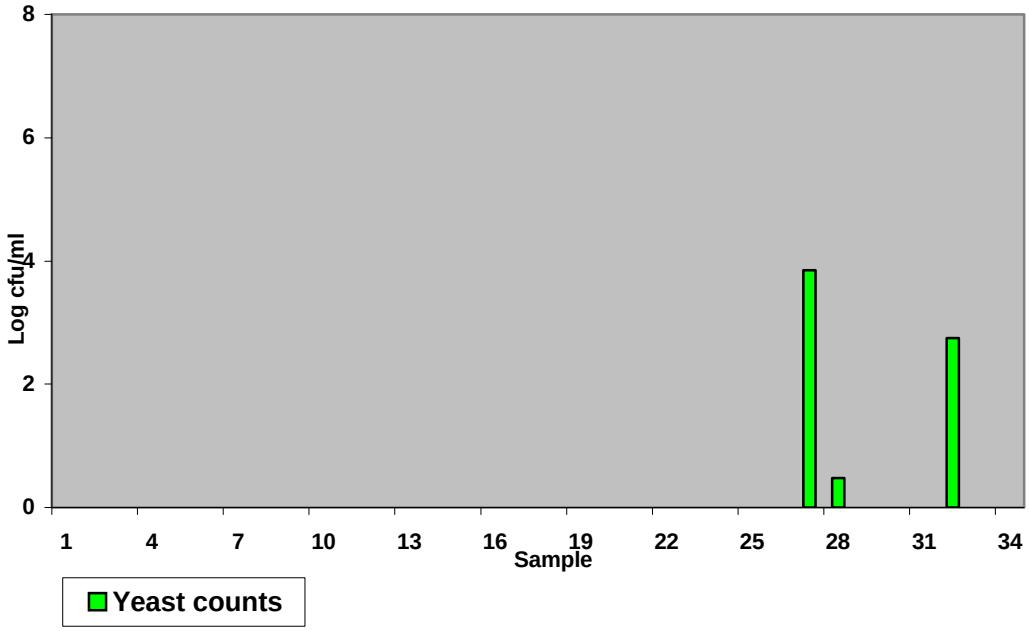


2.3B Psychrotolerant counts in raw milk

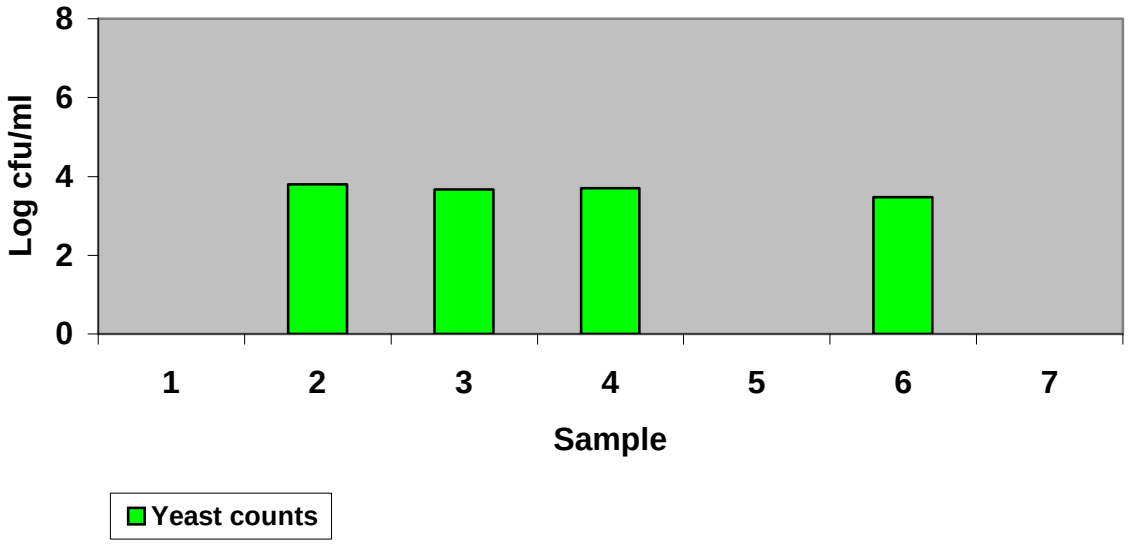


Figures 2.3A and 2.3B. Frequency distribution of psychrotolerant counts for pasteurized, replicates and raw milk samples respectively.

2.4A Yeast counts in pasteurized milk



2.4B. Yeast counts in raw milk



Figures 2.4A and 2.4B. Frequency distribution of yeast counts in pasteurized and raw milk samples respectively.

CHAPTER 3

DIVERSITY OF YEAST SPECIES IN RAW AND PASTEURIZED MILK

Abstract

Yeasts were isolated and characterized from 42 different raw milk samples and 23 pasteurized milk samples collected in the vicinity of Bloemfontein. These were isolated and identified to the species level according to conventional identification techniques and molecular techniques (D1/D2 homology). A total of 81 yeast strains representative of 6 genera and 14 species, were isolated from the raw and pasteurized milk. The predominant species found were *Kluyveromyces marxianus, Debaryomyces hansenii*, *Clavispora lusitaniae, Cryptococcus flavus, Cryptococcus curvatus* and *Candida albicans*.

3.1 INTRODUCTION

Yeasts are important in the dairy industry for several reasons. They play an essential role in the preparation of fermented milks and in the ripening of certain cheeses. Their occurrence in dairy products is also significant because they can cause spoilage, effect desirable biochemical changes and may adversely affect public health (Fleet and Mian, 1987). However, the public health significance of yeasts in foods is considered by most health authorities to be very minimal if not negligible, because infections arising from the few, known pathogenic yeasts such as *Candida albicans* and *Cryptococcus neoformans* are not transmitted through foods (Fleet, 1992).

A variety of dairy products offer a special ecological niche that selects for the activity and occurrence of specific yeast species. Investigations of the occurrence of yeasts in a particular food that has been exposed to certain environmental conditions can give an overall insight as to which species might be encountered in spoilage (Deak and Beuchat, 1996).

Although milk is an excellent substrate for the growth of many microorganisms including yeasts, the microbial spoilage of milk is generally associated with the growth of bacteria (Cousin, 1982; Bishop and White, 1986) and very little consideration has been given to the ability of yeasts to grow in milk (Fleet, 1990). Generally, the available information shows that yeasts occur in both raw (Foster *et al.,* 1957; Ingram, 1958; Randolph *et al.,* 1973) and pasteurized (Jones and Langlois, 1977; Fleet and Mian, 1987) milk at low insignificant populations, but they may evolve as secondary flora and reach high loads after bacterial growth and spoilage. The varying populations depend solely on hygienic practices used in milk handling. Pasteurization will kill most of the microorganisms present in raw milk except thermoduric bacteria, and yeasts will come from secondary contamination. Raw milk held at refrigeration temperatures will support the

growth of psychrotrophic strains which quickly overgrow the yeasts (Cousin, 1982; Bishop and White, 1986).

Many factors affect the growth and biochemical activities of yeasts in foods. Their role as spoilage organisms in dairy products is linked to their nutritional requirements, certain enzymatic activities and the ability to grow at low temperatures, low pH values, low water activities and high salt concentrations (Jakobsen and Narvhus, 1996). Despite frequent references to the presence of yeasts in dairy products, little is known of the diversity of yeasts being present in milk. Therefore, the main objective of the current study was to determine all possible yeasts present in raw and pasteurized milk to give as an insight of possible spoilage yeasts that may contribute to the final dairy products. With a better understanding of the yeasts associated with milk, it may be possible to construct better quality measurements to avoid yeast contamination.

3.2 MATERIALS AND METHODS

3.2.1 Milk samples

Forty two raw and twenty three pasteurized milk samples were purchased from supermarkets and small-scale producers around the Bloemfontein area. After collection, the milk samples were kept on ice and microbiologically analyzed within 1 h.

3.2.2 Isolation of yeasts

Yeast enumerations were performed using the spread plate technique. Serial dilutions were prepared by aseptically transferring a portion of the milk (1 ml) into peptone water (9 ml) (Merck, Darmstadt, Germany). Portions (0.1 ml) of the dilutions were spread plated in duplicates on Yeast- Extract Malt- Extract (YM) (Wickerham, 1951) agar plates. Yeast colonies from the highest dilutions were

isolated and purified by subculturing on YM agar (Wickerham, 1951) plates and incubating for 72 h at 25˚C. The pure cultures were kept on YM slants at 4˚C during the period of investigation.

3.2.3 Identification of the yeasts isolates

Identification of the yeast isolates to species level was performed according to the conventional identification methods as proposed by Kreger-van Rij (1984), Barnett *et al.* (1990) and Kurtzman and Fell (1998). These conventional methods used to determine the biochemical activities of the isolates included fermentation of sugars, assimilation of carbon compounds, and growth on vitamin free medium (Van der Walt and Yarrow, 1984). Additional tests performed included growth at 37˚C, in 50% D-Glucose medium, urea hydrolysis and 0.01% and 0.1% cycloheximide. Assimilation of nitrogen compounds was determined by means of the auxonographic method of Lodder and Kreger-van Rij (1952).

Ascospore formation was examined on McClary's acetate agar, potato glucose agar (Oxoid), Gorodkowa agar (Oxoid), corn meal agar and malt extract agar (Oxoid) (Kreger-van Rij, 1984). The inoculated media were incubated at 18˚C for 4 weeks and examined at 4 d intervals. Cell morphology, type of budding and mode of reproduction were examined on malt extract agar (Difco) and on Dalmau plates (Kreger-van Rij, 1984). The formation of pseudomycelium and true mycelium were examined on corn meal agar according to the Dalmau plate technique.

Further confirmation of the identity of the species was performed through sequence analysis of the D1/D2 domain using primer pairs NL-1(5'-GCATATCAATAAGCGGAGGAAAAG) and NL-4 (5'-GGTCCGTGTTTCAAGACGG (Kurtzman and Robnett, 1998). Sequencing reactions were performed with the ABI Prism™ Big Dye terminator™ v3.1 cycle sequencing ready reaction kit and data collected on an ABI Prism 377

DNA sequencer (Applied biosystems). Data was analyzed using sequencing analysis V3.3 and sequences assembled using Auto-assembler V1.4.0.

3.2.4 pH determination

The pH of each sample (raw and pasteurized milk) was determined using a digital pH meter (Cyberscan 500, Eutech Instruments, Germany) at 24˚C. The pH meter was calibrated using commercial buffers (Merck) of pH 4 and 7.

3.3 RESULTS

3.3.1 Incidence of yeasts

The diversity of yeasts isolates from raw and pasteurized milk samples is shown in Table 3.1, revealing 14 different species. Kluyveromyces marxianus, Debaryomyces hansenii, Clavispora lusitaniae, Cryptococcus flavus, Cryptococcus curvatus, Candida famata and Candida albicans were the most frequently isolated species.

Kluyveromyces marxianus, Debaryomyces hansenii and *Candida famata* were the predominant species both in pasteurized and raw milk samples. The frequent occurrence of K. marxianus and C. famata was also reported by Fleet and Mian (1987) in pasteurized milk. Fleet (1990) also reported on the presence of K. marxianus as one of the most prevalent yeasts in dairy products. D. hansenii was obtained at higher percentages in both raw and pasteurized milk samples which corresponds with results obtained by Fleet and Mian (1987) who obtained high numbers of this species in raw milk. Other yeast species that were isolated but at lower percentages included C. blankii, C. catenulata, C. diffluens, C. emobi, C. parapsilosis, C.rugosa and Saccharomyces cerevisiae.

3.3.2 Physiological and biochemical properties

The mean initial pH values of all the raw and pasteurized milk samples were 6.71 and 6.76 respectively (data not shown). The technologically important physiological and biochemical properties of the yeasts isolated from raw and pasteurized milk samples are shown in Table 3.2. These included growth at 5˚C, ability to ferment glucose, lactose and galactose, and the ability to assimilate lactose, lactate and citrate. Of the 14 different yeast species isolated, only *K. marxianus* and its imperfect form *C. famata* were capable of fermenting lactose, which is the major carbohydrate fraction of milk. Variable results regarding the fermentation of other sugars were detected, although most of the species assimilated lactate and other available organic acids.

3.4 DISCUSSION

Although milk is the raw material of dairy products, surprisingly only a few studies have been conducted on the specific occurrence of yeasts in either raw or pasteurized milks. Mean counts of 10^3 cfu/ml were obtained in this study for the raw milk samples, while for pasteurized milk samples the counts ranged from counts of 10-10^3cfu/ml. Populations less than 10^3 cfu/ml are frequently reported (Fleet and Mian, 1987), although counts as high as 10^4 cfu/ml can occasionally occur (Fleet, 1990). According to literature, yeasts rarely grow in milk during refrigerated storage, as they are quickly overgrown by psychrotolerant bacteria (Cousin, 1982; Bishop and White, 1986). However, yeast proliferation might occur in milk where bacterial growth has been inhibited by residual antibiotics, at lower pH values and when bacterial growth is restricted.

All of the yeast species isolated from the milk samples had the ability to grow at low temperature of 5˚C, and this result is consistent with their occurrence in dairy products which are stored under refrigeration during most times. The most frequent occurrence and growth of *Kluyveromyces marxianus* and *Candida*

famata in milk (Table 3.3) should be seen in the light of their ability to ferment and assimilate lactose (Devoyod, 1990), which is the major sugar of milk, and to assimilate lactic acid and citric acid which are the main organic acids of dairy products (Fleet and Mian, 1987). *Debaromyces hansenii,* lacks the ability to ferment lactose (Barnett *et al.,* 1990), that characteristic was also observed in this study but being capable to assimilate lactose, lactate and strong utilization of citrate for survival. Furthermore, the species exhibited lipolytic and proteolytic activity which may further guaranteed its proliferation. The presence of this species in milk in the present study is consistent with literature reports indicating that this species is prominent in dairy products (Cook, 1958; Deak and Beuchat, 1996; Rohm *et al.,* 1992; Roostita and Fleet, 1996; Seiler, 1991; Welthagen and Viljoen, 1998). Viljoen and Greyling (1995) also reported the dominance of this strain in cheese. The predominance of this species in dairy products is certainly related to its biochemical properties, as all the strains isolated in this study showed an ability to grow at low temperatures and to assimilate lactose, lactate and citrate.

In general, the presence of all the yeasts in the milk can be explained by their various abilities to assimilate milk sugars, available organic acids and/or proteolytic and lipolytic activities (Fleet and Mian, 1987) as consistently indicated in this study. While it is possible to understand the growth of these species in milk, it is difficult to explain the survival of *S. cerevisiae* which lacks these properties. Presumably, other milk components are used and these could include the small amounts of free amino acids and fatty acids. Due to these uncertainties, further research is necessary to understand the biochemical basis of *S. cerevisiae* growth in milk and know the nature of the metabolic end-products generated.

There are no reports so far of high levels of *C. albicans* from milk as obtained in this study. However, since yeast mastitis has been known to occur in 2-3% of all cases of mastitis in dairy cows (Stanojevic and Krnjajic, 2003) and the

main yeasts associated with it are *C. albicans* and *Cr. neoformans*, it is possible, therefore, that the high prevalence of *C. albicans* could be attributed to milk samples derived from cows with mastitic udders. Although some yeasts like *Candida albicans* and *Cryptococcus neoformans* have long been known to be pathogenic to humans, it is previously believed that they cannot be transmitted through foods (Fleet, 1990). However, according to Kockova-Kratochvilova (1990), *C. albicans* and the related species such as *C. tropicalis*, *C. rugosa*, and *C. parapsilosis* occur in humans as commensals or saprophytes only as long as the host is not providing them suitable conditions. Reports also showed an increase in the incidence of an invasive disease caused by *Candida lusitaniae* (the anamorphic stage of *Clavispora lusitaniae*) (Hadfield *et al.*, 1987; Merz, 1984). In addition to the pathogenic *Candida,* reports also indicated some species of *Pichia* and *Cryptococcus* encountered as rare and emerging causative agents of opportunistic diseases that develop in immunocompromised patients (Araissie *et al*., 1989; Chakarabarti *et al*., 2001; Ikeda *et al.,* 2002). *Cr. curvatus* and *Cr. laurentii* have been isolated from AIDS or cancer patients suffering from myeloradiculitis and fungemia (Dromer, *et al*., 1995; Johnson *et al*., 1998; Kunova and Krcmery, 1999). Consequently, all the above species, being opportunistic pathogens, can turn out to cause serious disease under certain conditions of the host. Therefore their high percentage of occurrence in milk samples, especially that of *Cr. curvatus* and *C. albicans* may pose a serious health threat in view of the very high prevalence of immuno-compromised people amongst the African population.

3.5 CONCLUSIONS

Raw and processed dairy products become contaminated by microorganisms from the environment (Deak, 1991, Deak and Beuchat, 1996), and since yeasts are generally heat sensitive, their presence in pasteurized milk can be assumed to be post-pasteurization contamination.

Most dairy associated yeasts have the ability of utilizing all the other available sugars other than the naturally present lactose, lactic acid and other organic acids. They are also able to produce protease and lipase enzymes which enable them to hydrolyze milk casein and fat (Roostita and Fleet, 1996). The overall results of the key properties encouraging yeast growth clearly indicated that all species isolated from the milk samples had the ability to utilize one or more of the milk constituents.

In this study we clearly demonstrated the frequent occurrence and diversity of yeasts in milk. *Kluyveromyces marxianus, Candida famata* and *Debaryomyces hansenii* predominated and this could be attributed to their ability to grow at a lower temperature of 5˚C. Other properties include the ability of protease and lipase production (Fleet and Mian, 1987) and these could be more important than lactose fermentation in determining the occurrence of yeasts in dairy products.

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Table 3.1 The incidence of yeasts isolated from raw and pasteurized milk in the vicinity of Bloemfontein, South Africa

Yeast species	Raw milk	Pasteurized milk
C. albicans	+	-
C. blankii	-	+
C. catenulate	-	+
C. diffluens	+	+
C. emobi	+	-
C. famata	+	+
C. parapsilosis	+	-
C. rugosa	+	-
Cl. lusitaniae	+	-
Cr. Curvatus	+	-
Cr. Flavus	-	+
D. hansenii	+	+
K. marxianus	+	+
S. cerevisiae	+	-

Table 3.2 Characteristic physiological and biochemical properties of dominant yeasts isolated from raw and pasteurized milk that determine their growth.

Yeast isolates	Fermentation of			Assimilation of			Growth at 5˚C
	Glucose	Lactose	Galactose	Lactose	L. acid	C. acid	
C. albicans	+	-	+	-	-	+	+
C. famata	+	+	+	+	+	+	+
Cl. lusitaniae	+	-	+	-	+	-	+
Cr. curvatus	-	-	-	+	+	+	+
Cr. flavus	-	-	-	+	+	+	+
D. hansenii	-	-	-	+	+	+	+
K. marxianus	+	+	+	+	+	+	+

L. acid= lactic acid

C. acid= citric acid

Table 3.3 Representation of the yeast species from raw and pasteurized milk samples.

Species	**Raw milk (% isolates)**	**Pasteurized milk (% isolates)**
C. albicans	11	0
C. blankii	0	8
C. catenulata	0	8
C. diffluens	4	8
C. emobi	4	0
C. famata	15	15
C. parapsilosis	4	0
C. rugosa	4	0
Cl. lusitaniae	7	0
Cr. curvatus	7	0
Cr. flavus	0	8
D. hansenii	18	23
K. marxianus	22	30
S. cerevisiae	4	0
Total	**100**	**100**

CHAPTER 4

THE EFFECT OF ULTRA VIOLET RADIATION TREATMENT OF MILK FOR IMPROVED SAFETY AND QUALITY ON THE DAIRY FARMS

Abstract

Milk is part of the daily diet of all people; therefore achieving top quality and safety are essential. The severe consequences of food poisoning caused by the consumption of contaminated milk are not acceptable. Due to concerns that some potentially dangerous and high numbers of undesired microorganisms may derive from the dairy farm, the ability to efficiently control these microorganisms at the farm level become more desirable. In this study, we investigated the effect of ultraviolet irradiation on the microbial loads and chemical composition of raw milk. Dairy bulk tank milk was treated with UV radiation emitted from a UV unit (260 nm) installed. Milk samples were analyzed over a 14 day period twice every day for possible alterations in chemical compounds and for surviving bacteria using the standards as proposed by the International Dairy federation (IDF). The results showed a 90% reduction on the microbial populations while the chemical analysis indicated no significant alterations in the milk composition. Based on the results obtained, it was suggested that the usage of UV radiation on the milk resulted in an enhanced shelf-life and better microbial quality.

4.1 INTRODUCTION

Due to the heightened public awareness over food-poisoning, it is important that all companies in the food chain maintain high hygienic standards and assure the public of the safety of the produce (Forsythe, 2000). Numerous outbreaks of foodborne illnesses have been attributed to the consumption of unpasteurized food products. Several strains of *Salmonella (S. enteritidis, S. pullorum, S. typhimurium*) are responsible for the overwhelming majority of cases of food borne disease. In the United States alone five million cases are reported annually and as many as two thousand of them with lethal outcome. Symptoms include headaches, chills, fever, vomiting and severe dehydration.

Milk is no exception in being an excellent carrier of undesired microorganisms. Despite being a nutritious food for humans, it also serves as a good medium for the growth of many microorganisms, especially bacterial pathogens. Pathogens that have been involved in foodborne outbreaks associated with the consumption of milk include *Listeria monocytogenes*, *Salmonella, Campylobacter*, *Staphylococcus aureus*, *Bacillus cereus* and *Clostridium botulinum*. (Fook Yee Chye, *et al.,* 2004). There are some concerns about the efficiency of conventional heat pasteurization of milk because some potential human pathogens have been reported to survive standard pasteurization (Smith, *et al.,* 2002). Potential human pathogens like *Mycobacterium paratuberculosis, Bacilius cereus* and *Prototheca* have been reported to survive conventional heat pasteurization in milk (Stabel *et al.,* 1997; Smith *et al.,* 2002). Furthermore, the consumption of raw milk, as still in practice, will remain a major health risk if not properly looked after. Under normal milking situations as experienced on these farms, improper hygienic milk procedures are still a major concern enhanced by limited cooling systems and may lead to undesired high microbial counts. Therefore, because of these concerns it is essential to continue searching for

effective means of bacterial destruction, Especially if these microbial loads can be minimized on the farm directly after milking.

Ultra-Violet radiation has been successfully applied as a possible means of pasteurization for certain foods (Arrage *et al.,* 1993; Dizer *et al.,* 1993; Hanis *et al.,* 1989). The antimicrobial effects of ultraviolet radiation on a variety of foodborne bacterial pathogens and other bacterial loads (on surfaces, sterile foods and liquids) have been studied using a broad spectrum light with varying amounts of UV content. The FDA has given market approval to use UV radiation for the treatment of water and juices under specific conditions and mentioned that milk is no exception, as exposure of milk to UV light may adequately control bacterial loads.

Ultraviolet light has been extensively used for more than 40 years as an effective treatment for the elimination of various microorganisms in water. Wavelengths of UV light in the range of 200 to 280 nm have been demonstrated to effectively inactivate bacteria and viruses due to DNA mutations induced by the absorption of UV light by DNA molecules. There is wide variety of damages that UV can cause to hereditary apparatus, proteins and membranes of the target cells. Each of those damages (for example thymine dimerisation or breakage of DNA sugar-phosphate backbone) may either disable or kill target cells. Ultraviolet radiation is very valuable not only because of its effectiveness, but also because UV does not leave any hazardous residue behind and thus is not a cause for the consumer concern (Caudle and Shneider, 1995). However, it could not be used to disinfect turbid fluids like milk because of the potential for physical and chemical components present to absorb the UV energy thereby making the liquid less impenetrable leading to inefficient disinfection. Excessive UV treatment on the other hand may lead to oxidation and sensory defects and as a consequence, the flow turbulence of the milk becomes essential.

A recent patent registered in South Africa however, overcame this difficulty by making use of an elongate sterilizer (Fig. 4.1.) containing two manifolds, the inlet

and outlet, whereby the milk gets in and out of the sterilizer. Inside the sterilizer there is a flourescent tube and a sheath which creates a swirling effect of the liquid. Consequently, the swirling effect of the turbid liquid (milk in this case) enhances the contact time/striking of the UV rays with the microorganisms leading to an increase in possible cell death.

Concerns' regarding the effectiveness of UV radiation on the composition of the milk, however are understandable as it's not generally applied in improving the quality and safety of milk. Therefore the objective of this study was to evaluate the effectiveness of UV treatment for reducing microbial loads and the influence on some critical chemical compounds.

4.2 MATERIALS AND METHODS

4.2.1 Milk sample collection

Milk samples were aseptically collected during milking from a farm in the Free State equipped with a fitted UV-unit. The samples were taken from the same main stream milk pipe directly before going through the installed UV-unit and at the end of the line after running through the UV-unit. The milk was kept under controlled conditions (<5^{o}C) and transported to the laboratory in a cooler box lined with ice and sampled within 1 h. This procedure was repeated over 14 consecutive days, taking samples during the morning and afternoon milking.

4.2.2 Standard microbial counts in milk

Duplicate milk samples were prepared for microbiological analysis on each sampling occasion. For each sample decimal dilutions were carried out in sterile peptone water (9 ml) and plated in duplicate by the spread plate technique onto selective media as proposed by the International Dairy Federation and incubated at the relevant temperatures.

Yeasts were enumerated by spread plating on Rose Bengal Chloramphenicol Agar (RBCA) (Oxoid, Bassingstoke, UK). The plates were incubated at 25^{o}C for 72 hrs.

Total aerobic mesophilic bacteria, psyhrotolerant bacteria and coliform bacteria were enumerated by spread plating on Plate Count Agar (PCA) (Oxoid) and Violet Red Bile Agar (VRBA) (Oxoid), respectively. The PCA plates were incubated at 30^{o}C for 48 hrs, whereas for the detection of psychrotolerant bacteria the plates were incubated at 7^{o}C for at least 7 days, while the VRBA plates were incubated at 37^{o}C for 24 hrs. In all cases, a portion of the appropriately diluted fermented milk samples (0.1ml) was used for spreading on to the surfaces of the respective duplicate plates. All plates containing between 25 and 150 colony forming units (CFU) on the highest dilution (or the highest number if below 25) were counted and the mean values determined from duplicate plates.

4.2.3 Microbial detection of presumptive pathogens

E. coli: For each sample decimal dilutions were carried out as required for microbial assays in 9 ml sterile peptone water and plated in duplicate by the spread plate technique onto VRB-Mug (Biolab). These were incubated at 37˚C for 24h after which all plates containing coliforms (pink to dark red colonies) (Hall *et al.,* 1967) between 25 and 250 colony forming units (cfu) on the highest dilution were counted and mean values determined from duplicate plates. Typical isolates of *E. coli* were confirmed based on their IMViC pattern.

Salmonella: Milk samples were pre-enriched by adding 1ml into 9ml buffered peptone water (Biolab, South Africa) and incubated at 37˚C for 24h. After the 24h incubation, 0.1 ml of the pre-enrichment was transferred into 5 ml Rappaport-Vassiliadis Soya Peptone (Oxoid, Basingstoke) broth for enrichment and incubated at 42˚C for 24h. After incubation, the enrichment broth was streaked

onto XLD (Biolab, Merck, Darmtadt) agar and further incubated at 37˚C for 24h for the detection of presumptive *Salmonella* species (dark pink to red colonies).

L. monocytogenes: Milk (1 ml) was added to a *Listeria* enrichment broth (1/2 Fraser), (Biolab) and the tubes incubated at 35˚C for 24h. The broth was plated (0.1 ml) directly onto *Listeria* selective medium (ALOA, AES Laboratoire). Plates were incubated at 37˚C for 24h and determined for presumptive *Listeria* colonies (blue to green-blue colonies with an opaque halo).

Clostridium botulinum: Milk samples (0.1 ml) were plated directly onto CBI agar (Biolab), a medium selective for *Clostridium botulinum.* The plates were incubated in anaerobic flasks with Anaerocult A (Merck) to confirm an anaerobic atmosphere at 35˚C for 48h. After incubation, plates with a good luxuriant and lecithinase positive were possible suspects for the pathogen.

Staphylococcus aureus: Baird–Parker agar (Oxoid, Basingstoke, UK) was used to quantitatively detect *S. aureus* according to Baird-Parker (1962) and incubated at 37˚C for 24h. After incubation, typical isolates of *S. aureus* (typical black, shiny convex colonies surrounded by clear zones) were confirmed by a coagulase test.

Mycobacterium tuberculosis: TB medium base according to Löwenstein-Jensen (Merck) was used for the detection of *M. tuberculosis.*

4.2.4 Chemical and physical analysis

On every sampling occasion the lactose content, % fat content, % protein content, total solids, acidity, amino acids, vitamins, moisture, and minerals were measured. All of these tests (Table 4.1) were determined by the methods proposed by the International Dairy Standard (IDF) and the Association of Official Analytical Chemist (AOAC) (1990).

4.2.4.1 *Evaluation of casein breakdown in milk*

The purity and the relative molecular mass (M_r) of the casein and/or the specific breakdown products were assessed using sodium dodecyl sulphate polyacrylamide gel elecrophoresis (SDS-PAGE). The relative molecular mass of the products were estimated by comparing its electrophoretic mobility with those of standard proteins of known molecular masses. The major casein components may be distinguished by electrophoresis and are designated as α-, β-, γ- and K-caseins, in order of decreasing mobility at pH 7.0. The β-, casein has a molecular weight of 23600 Da.

A modification of the method of Laemmli (1970) was used.

SDS-PAGE was performed using the "Mighty Small" miniature slab gel electrophoresis unit, SE 200, from Hoefer Scientific Instruments. Electrophoresis was performed on approximately 30 μg protein. The protocol used was that described by Sharpiro and Maizel (1969). When the tracking dye almost reached the bottom of the gel, the power supply was turned off and the flow of the coolant was stopped.

The gels were stained with Coomassie blue for 1 hr as described in the Hoefer instruction manual. Thereafter, it was destained with destaining solution 1 (7.5 % ethanol, 5 % acetic acid) and then with destaining solution 2 (40 % ethanol, 10 % acetic acid). To detect bands not visible after Coomassie blue staining, the gel was exposed to silver staining according to Switzer *et al*., (1979). The gels were stored in 20 % ethanol and then sealed in a small plastic bag.

The protein standards used were: α_2-Macroglobulin (subunit M_r = 170 000), β-galactosidase (M_r = 116 400), fructose-6-phosphate kinase (M_r = 85 200), glutamate dehydrogenase (M_r = 55 600), aldolase (M_r = 39 200), triose

phosphate isomerase (M_r = 26 600), trypsin inhibitor (M_r = 20 100) and lysozyme (M_r = 14 300).

4.2.4.2 *Evaluation of fat hydrolysis and fat oxidation*

Lipid extraction and fatty acid analysis

Lipids were extracted from the milk using chloroform:methanol (2:1 v/v) (Kendrick and Ratledge, 1992) and washed twice with distilled water (Folch et al., 1957). The organic solvents were evaporated under vacuum. The lipids were dissolved in a minimal volume of diethyl ether and transferred to vials after which they were dried to constant weight in a vacuum oven over P_2O_5 at 50°C. The samples were dissolved in chloroform and methylated with trimethyl sulphonium hydroxide (TMSOH) (Butte, 1983). The fatty acid methyl esters were analysed using a Varian 3300 gas chromatograph equipped with a polar Supelcowax 10 glass capillary column (0.75 mm x 30 m) with N_2 (5 $ml.min^{-1}$) as carrier gas. Lauric acid (C:12) (3 mg) were included as a standard while peaks were identified by reference to authentic standards.

Free fatty acid extraction and analysis

Free fatty acids were extracted from the milk using ethyl acetate, which was subsequently evaporated under vacuum. The lipids were dissolved in a minimal volume of diethyl ether and transferred to vials after which they were dried to constant weight in a vacuum oven over P_2O_5 at 50°C. The samples were dissolved in methanol and methylated with diazomethane. The fatty acid methyl esters were analysed as described above.

4.3 RESULTS AND DISCUSSION

4.3.1 Effectiveness of UV radiation on the microbial populations

There is no such thing as absolute safety in milk, but experience has shown that adoption of certain practices can produce a satisfactory level of safety. Without a doubt, pasteurization is the most important practice followed as a means of assuring the safety of milk. When properly done, and if accompanied by the use of sterile containers and effective protection from recontamination, pasteurization does result in milk free of pathogenic organisms (Prescott *et al.,* 1996). Lowering the number of microorganisms initially within raw milk will, however undoubtedly also enhance the level of safety, quality and shelf-life.

When considering the quality of raw or refrigerated milk, the concern is almost exclusively with microorganisms that grow at storage temperatures as they mainly contribute to the spoilage of the product. These bacteria are referred to as psychrotolerant bacteria. They are defined based on their capability of appreciable growth at commercial refrigeration temperatures of 2-7°C, irrespective of their optimum growth temperature (IDF, 1976).

Psychrotolerants are ubiquitous in nature and are common contaminants of milk. They are introduced into milk when these sources become established on milk contact surfaces, equipment, flooring, and drains in the milking parlor and processing plant. Microorganisms can adhere to stainless steel surfaces, grow there and release a large number of cells into the milk (Bouman *et al*., 1982; Driessen *et al*., 1984; Langeveld *et al*., 1995). Environmental factors that may contribute to contamination are water, soil, vegetation, bedding material and to a lesser extent the air (Suhren, 1989). Milk produced or processed under sanitary conditions usually contains less than 10 percent of the total microflora as psychrotrotolerants. But milk produced or processed under unsanitary conditions can contain more than 75 percent psychrotolerant bacteria.

From a standpoint of quality control of milk, these bacteria, particularly of the gram – negative, are the most important microorganisms; their importance has increased as storage and holding times on the farm have lengthened with changes in technology and marketing conditions. They can cause a variety of flavour defects, ropiness and colour changes, all depending on their biochemical activity (Jay, 2000; Frank, 1997; Ray, 1996). Psychrotolerant counts of 3.3 log units were obtained from the untreated raw milk being reduced to 2.3 log units after UV radiation, resulting in a 90% reduction. Aerobic counts were also reduced from 4.8 log units to 3.8 log units, again a reduction of 90% in bacterial cells. The highest reduction in microbial loads was obtained with the coliforms, being reduced from 2.1 log units to 0.7 log units, a reduction of 93% (Fig. 4.2). There have been reported differences in UV radiation bactericidal efficiency levels for different bacterial species as well as for different strains of the same species (MacGregor *et al.,* 1998; Sommer *et al.,* 2000).

Similar to the reduction in bacterial numbers, the mould count was also reduced by 92% from 1.7 to 0.5 log units. Yeast numbers on the other hand proved to be slightly more resistant showing a reduction of only 71% from 2.9 to 2.1 log units (Fig. 4.2). According to Schlegel (1993) fungal spores are less sensitive to UV and are killed much more slowly than bacteria.

Due to the specific circumstances, sine the experiment was conducted on a dairy farm during normal milking, it was impossible to inoculate the raw milk with pathogenic strains. Determination of all the relevant pathogens was however conducted during every sampling occasion. Low numbers of *Escherichia coli* (3) was found only on a few occasions before radiation, *Staphylococcus aureus* (7) and *Salmonella* (1). On all occasions, none was found after radiation (results not shown). *Listeria, Clostridium* and *Mycobacterium* were not detected before or after radiation of milk.

Based on the results obtained, therefore the effectiveness of UV radiation applied on raw milk seemed to show positive results regarding the reduction of microbial loads. Previous studies on the bactericidal effect of UV light have used different sources of energy including cold quartz generators or mercury lamps of relatively low irradiance power (Sommer *et al.,* 2000; Wright *et al.,* 2000), or have used higher intensity pulsed broad-spectrum light with variable amounts of UV content (MacGregor *et al.,* 1998; Rowan *et al.,* 1999). In this study, however allowing the milk to swirl for improved penetration and contact by the UV, resulted in very promising reductions.

4.3.2 The influence of UV radiation on the chemical compounds

A discussion of the requirements for high quality milk must consider the composition of the product. Composition refers to the milk's content of major nutrients like fat, protein, lactose, and minerals. When offered for sale, to be legal, all milk must meet specific requirements as to the composition. These also may not be altered, before selling.

The major constituent of cow's milk is water; the remainder consists largely of proteins, lipids and carbohydrate materials synthesized in the mammary gland. Also present, in smaller quantities, are mineral components and other water-soluble and lipid-soluble materials, specific blood proteins and traces of enzymes. The physical form of the lipid and the caseins affects profoundly the characteristics of whole milk, and has important consequences for milk processing.

In order to evaluate the effect of the UV unit on milk quality, it is therefore imperative to also determine whether differences in milk composition occurred. Based on the results obtained for the chemical analysis, the differences obtained after UV radiation, none of the compounds tested, i.e. minerals, vitamins, proteins, fats, solids, lactose and amino acids, was significantly altered (Figs. 4.3

– 4.9; Table 4.2). Comparative experimental results on the influence of UV radiation on milk constituents are limited, however electrophoretic studies carried out to establish the effect of ultraviolet treatment on cow's milk at various rates of irradiation by Filipov (1976) showed no substantial changes in the level of the total protein. These results corresponded with the findings in this study.

4.3.2.1 *Casein breakdown*

Casein is the principal protein of cow's milk. It is the most commonly used milk protein in the food industry. It is used in coffee whiteners, infant formulas, processed cheese, and for use in pharmaceutical industries. It is also used in creamed cottage cheese, powdered diet supplements, nutritional beverages, processed cheese, and frozen desserts to name but a few.

Fig. 4.9 represents the samples obtained over a period of 14 days (Lane M = Markers and lanes marked according to samples received). The protein patterns are all similar with no significant products appearing in latter stages of samples obtained. Initially a 10 and 12 % gel was used for evaluation, but increased to 15 % to evaluate products formed smaller than the 14 kDa. The data obtained showed no significant difference in concentration or fragmentation of the caseins that could indicate that without smaller peptides present no significant breakdown occurs. The protein patterns are also similar, indicating a good consistence between the samples.

4.3.2.2 *Vitamins present in the milk*

Vitamins are complex organic substances that are needed in very small amounts for many of the processes carried out in the body. Usually only a few milligrams (mg) or micrograms (µg) are needed per day, but these amounts are essential for health. Vitamins have a variety of functions in the body: some are *co-factors* in enzyme activity, some are *antioxidants* (prevent oxygen from doing damage in

the body) and one (vitamin D) is a *pro-hormone*. If insufficient amounts of vitamins are available to the body because of a poor diet or some medical condition (e.g. malabsorption), certain symptoms will appear and can develop into a deficiency disease. Vitamins have been traditionally grouped into two categories; the *fat soluble vitamins*, and the *water soluble vitamins*. Based on the results obtained, minimal changes in the vitamin content occurred due to UV radiation (Fig. 4.4 and 4.5).

4.3.2.3 *Amino acids in the milk*

Amino acids are organic molecules that form the basic constituents of protein. Proteins are simply collections of large particles of accumulated links of peptides (or poly-peptides). In the digestion process proteins are broken down, in a process called hydrolyzation, from poly-peptides to smaller oligo-peptides, then to di-peptides or tri-peptides, which are made up of two or three links of specific amino acids, called free form amino acids, that are finally absorbed into the bloodstream. Therefore, we can see that amino acids are, quite simply, the most basic building blocks of proteins.

Typically, discussions of amino acids revolve around about 20 or so amino acids that are involved in body function. Of these, 8 to 10 are deemed to be essential due to the fact that; 1) the body can not make them so that they must be taken in from an external source, and 2) the body can not survive with a deficiency of any one of them. The essential amino acids are; Leucine, Isoleucine, Valine, Methionine, Lysine, Threonine, Phenylalanine, and Tryptophan. All of these amino acids are present in milk. Based on the results obtained, minimal changes in content occurred (Fig. 4.6).

4.3.2.4 *Fat oxidation and hydrolysis*

Milk fat contains 98% of glyceride neutral lipids characterised by a wide variety of fatty acids (FA) of which they are composed (Choisy *et al.*, 1984). The hydrolysis of milk fat is normally induced by lipases derived from the microbial flora associated with the milk (Chilliard *et al*., 1984), natural lipases present, and may possible also be induced by UV radiation. Lipolysis of triglycerides in milk results in free fatty acids (FFA), influencing the flavour directly by imparting specific flavour notes or indirectly as precursors for other flavour compounds (Engels *et al*., 1997).

The free fatty acids in milk are derived from either the breakdown of milk fat, by lipolysis or the metabolism of carbohydrates and amino acids by bacteria. Free fatty acids derived from milk fat hydrolysis are believed to contribute to the flavour (Badings *et al*., 1980) and aroma. However, an appropriate concentration of individual FFA's is required to obtain the desirable flavour and to avoid undesirable rancid off-flavours. Total free fatty acids (TFFA) were the sum of the content of butyric ($C_{4:0}$), caproic ($C_{6:0}$), caprylic ($C_{8:0}$), capric ($C_{10:0}$), lauric ($C_{12:0}$), myristic ($C_{14:0}$), palmitic ($C_{16:0}$), stearic ($C_{18:0}$), oleic ($C_{18:1}$) and linoleic ($C_{18:2}$) acids (Kheadr *et al.*, 2002) whereas FFA's are grouped into short ($C_{4:0}$ to $C_{8:0}$), medium ($C_{10:0}$ to $C_{14:0}$), or long chain ($C_{16:0}$ to $C_{18:3}$) fatty acids. Based on the results obtained, minimal changes in content occurred (Table 4.2).

4.4. CONCLUSIONS

The growth of microorganisms in milk causes disintegration of fat, protein, and/or lactose and will soon make the product unsuitable for drinking. While elimination of bacterial contamination is an important factor in the production of good flavored, high quality milk, other procedures can be used to protect and maintain good flavor and quality. The first of these is prompt cooling to near or below 5^{o} C with maintenance of that low temperature for the usable life of the milk (unless

heat processing is involved in the manufacture of a product from the milk). The second is pasteurization to inactivate microorganisms and enzymes, followed by cooling and holding at low temperature. Such heat treatments may vary from basic pasteurization to ultra high temperature processing.

However, by lowering the microbial loads directly after milking and keeping it at low temperatures to inhibit the microorganisms growth will benefit the farmer financially by achieving a higher grading for their milk, sustaining better quality and safety. The milk processors will, however also benefit by better quality milk especially when certain cheeses need to be manufactured. By evaluating the effectiveness of UV radiation on the milk based on the microbial populations it was clear from the results obtained that a reduction in all microbial loads was possible. Generally, a reduction in microbial numbers exceeding 90% was achieved. As a result of this reduction in microbial loads, a definite increase in the shelf-life will be achieved. Despite a reduction in microbial loads exceeding 90%, it must be remembered, a reduction of 90% in the microbial numbers does not necessarily make the milk safe. It depends on the initial number of microbial organisms, and therefore the usage of the UV unit must not be compared to pasteurization. Since the application of UV radiation also has no significant influence on the chemical compounds tested, it may be conclude that the installation of a UV unit on the dairy farm may lead to enhanced safety and quality of the milk.

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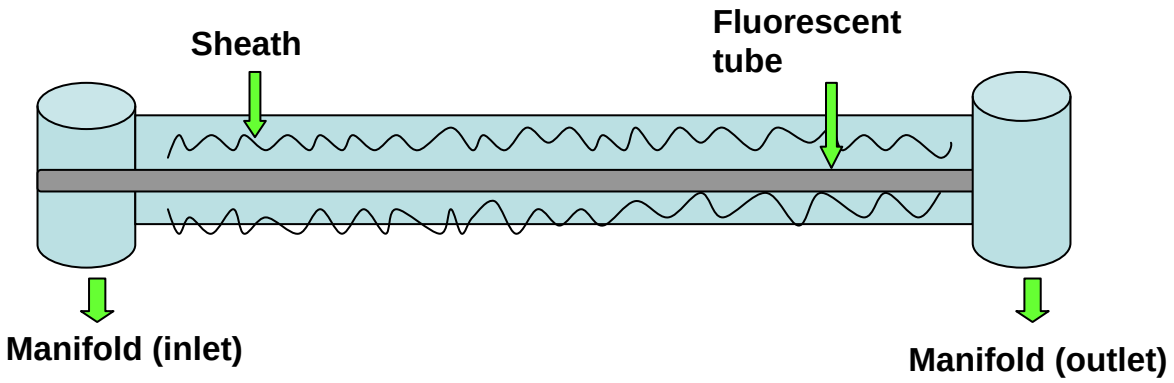

Fig.4.1 A diagram of the UV unit for ultraviolet radiation.

Table 4.1 The analyzed biochemical methods and the correspondent analytical techniques.

Parameter	Analytical techniques/reference
Acidity	NP 470/1983 and AOAC 947.05 - 33.2.06
Fat	Method AOAC 905.02, 33.2.25 or NP 468/1990 Extraction followed by gravimetry or Roese-Gottlieb method
Total solids	AOAC 925.23, NP 580/1970 and NP 475/1983, Gravimetry
Protein	Kjeldhal method, NP 1986/1991, conversion factor = 6.38
Amino acids	AOAC 991.21 - 33.2.12, Kjeldhal method
Casein	AOAC 927.03 – 33.2.18 and AOAC 991.20 -33.2.11, Kjeldhal method, conversion factor = 6.38, separation by IDF 29:1964 method
Vitamins	Handbook of Food Analysis, Chapter 17, vol.1 Ed. Leo M. L. Nollet High, AOAC 981.17- 45.1.21 RP-HPLC method, AOAC 991.25-33.7.08, UV/vis spectrophotometry method
Minerals	Spectrophotometry by Atomic Absorption (AA)

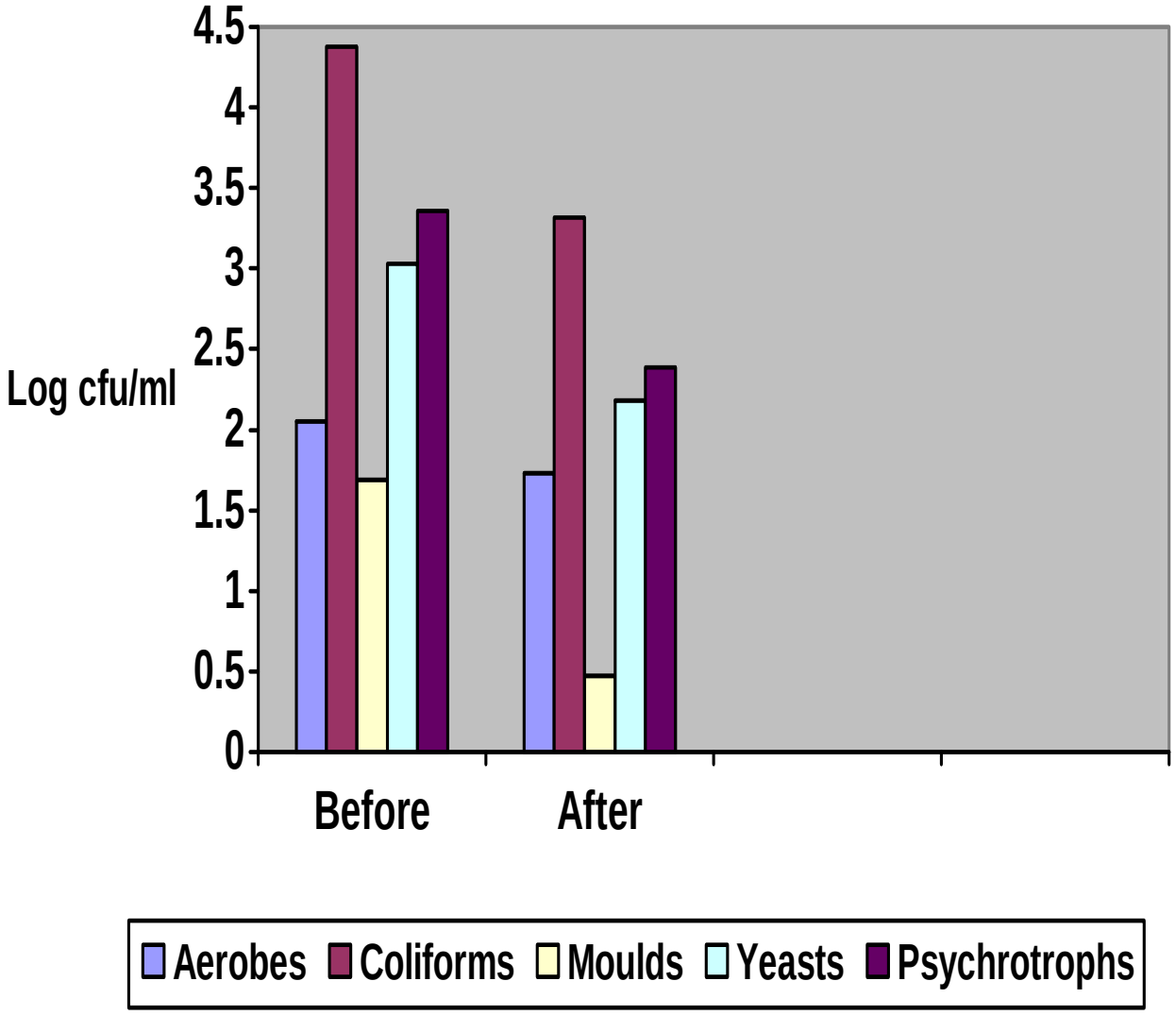

Fig.4.2 The reduction of aerobic bacteria, coliforms, moulds, yeasts and psychrotolerants before and after UV radiation. Results are the means of samples taken over a 14 day period, twice per day and enumerated in duplicate.

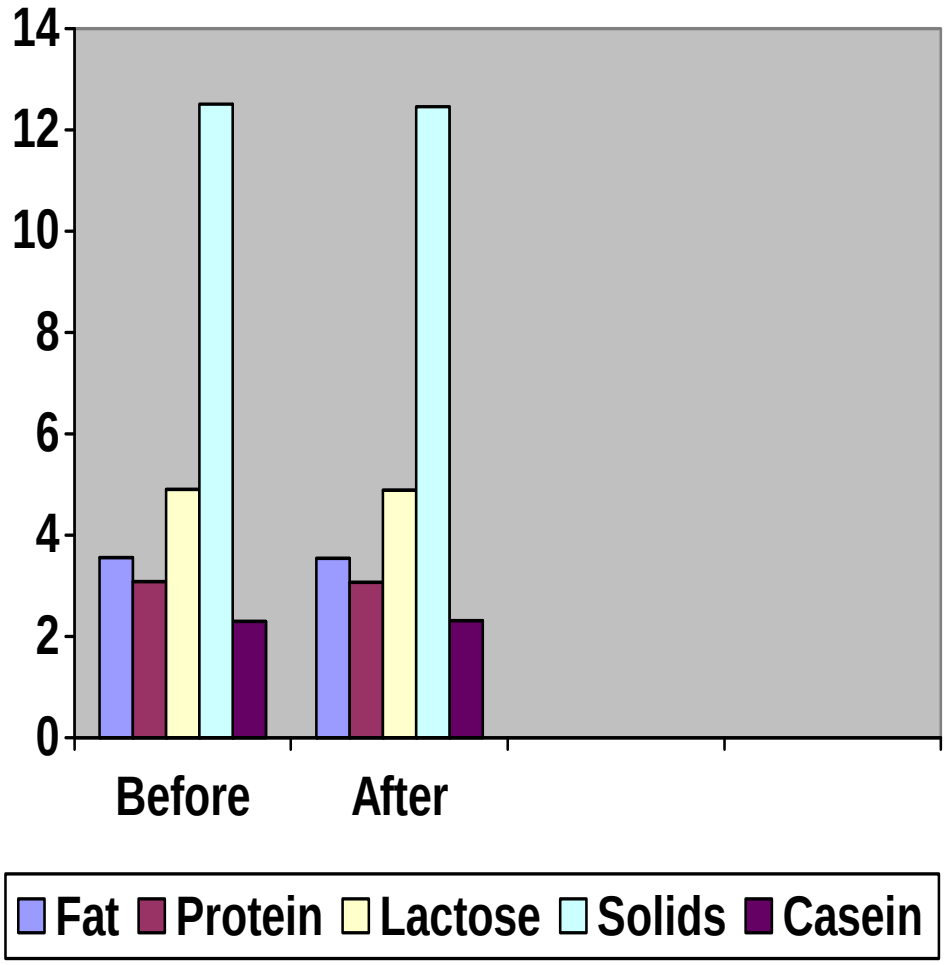

Fig.4.3 Differences in the composition (%) of chemical compounds present in raw milk both before and after UV radiation. Results are the means of samples taken over a 14 day period, twice per day.

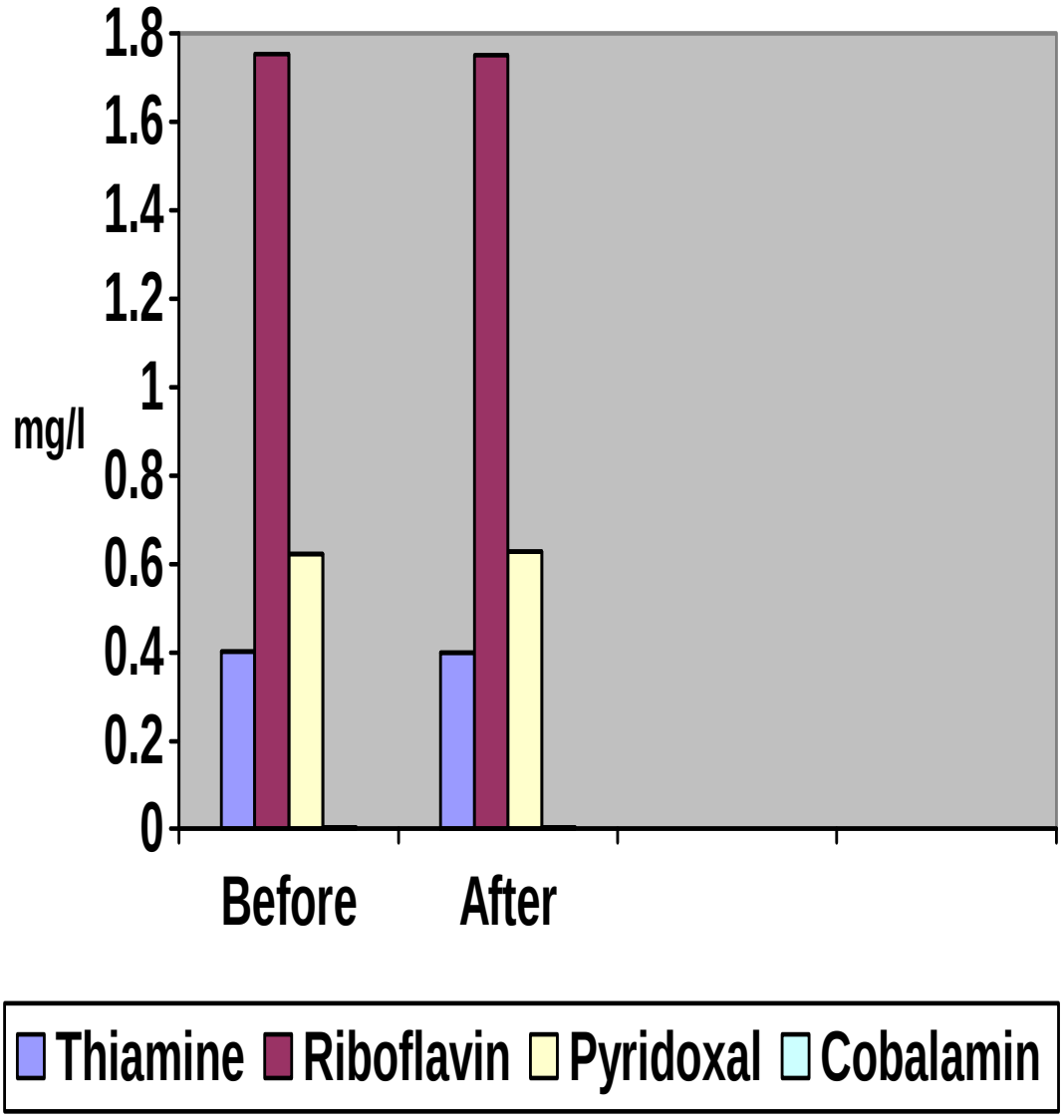

Fig.4.4 Differences in water-soluble vitamins present in raw milk before and after UV radiation. Results are the means of samples taken over a 14 day period, twice per day.

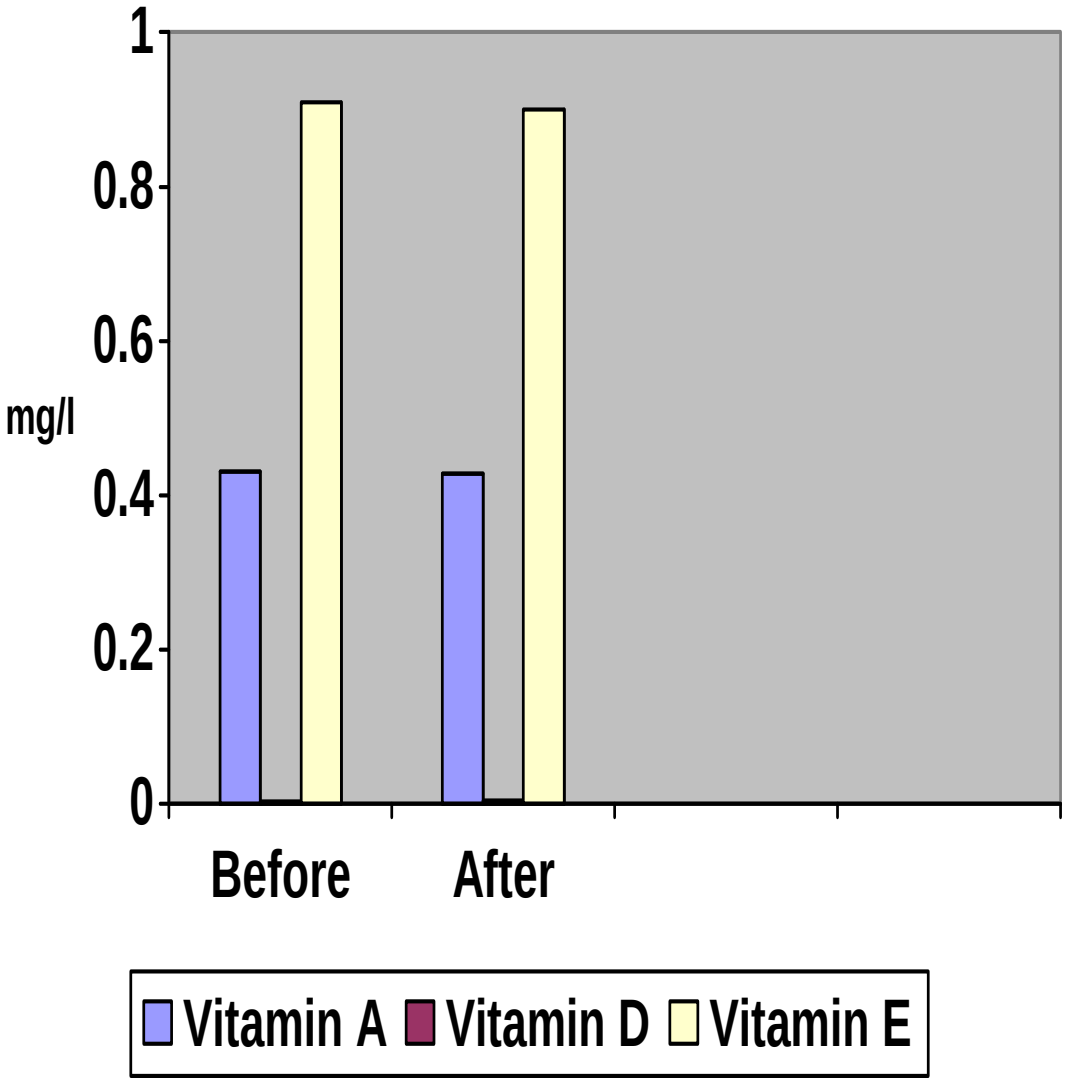

Fig.4.5 Differences in fat-soluble vitamins present in raw milk before and after UV radiation. Results are the means of samples taken over a 14 day period, twice per day.

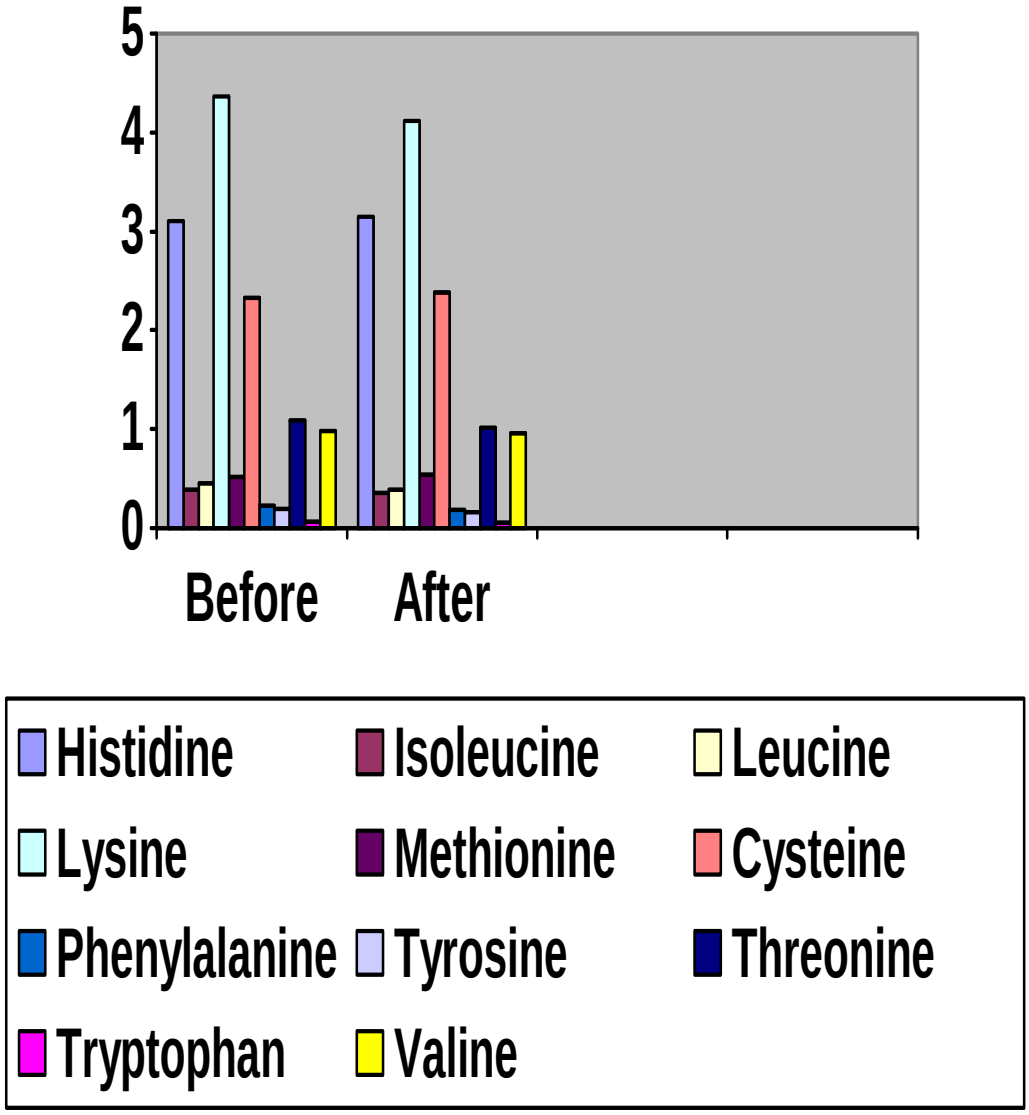

Fig.4.6 Differences in amino acids present in milk before and after UV radiation. Results are the means of samples taken over a 14 day period, twice per day.

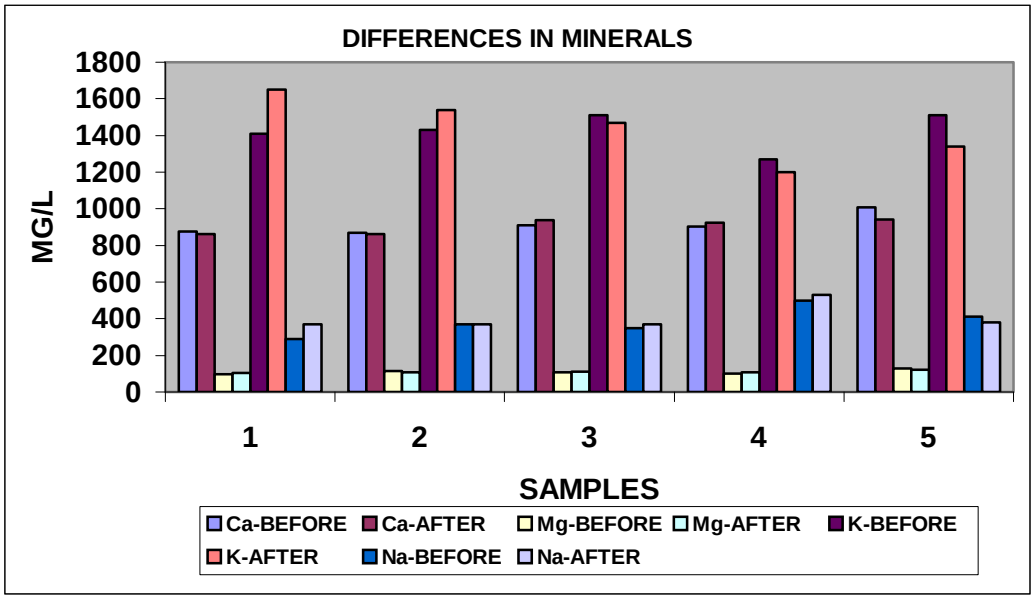

Fig.4.7 Differences in minerals present in milk before and after UV radiation. Results are the means of five pool samples taken over a 14 day period, twice per day.

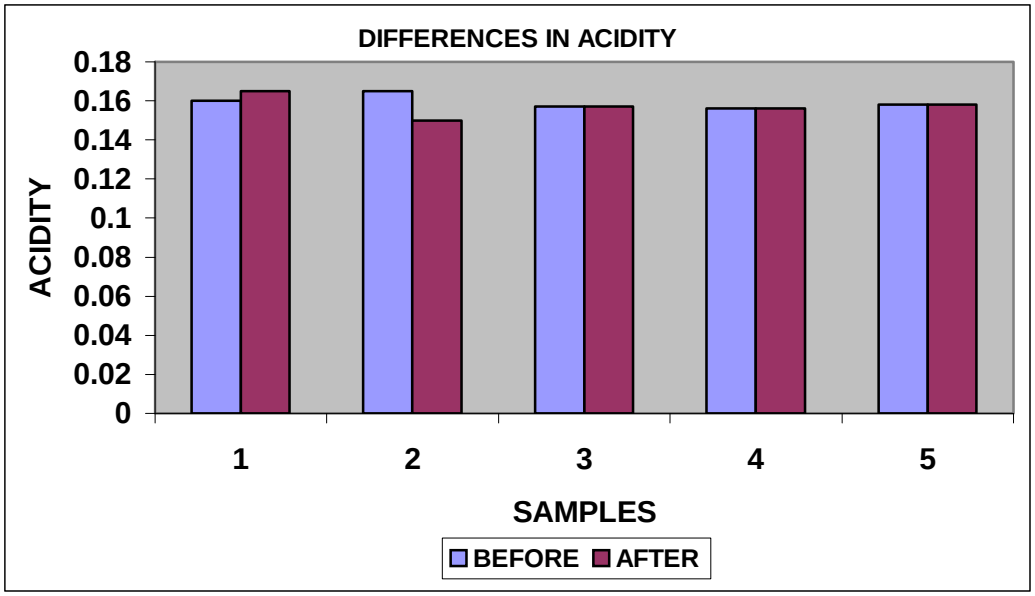

Fig. 4.8 Differences in acidity present in milk before and after UV radiation. Results are the means of five pool samples taken over a 14 day period, twice per day.

Table 4.2 The influence of UV radiation of milk on the fat hydrolysis and oxidation. Results are the means of five pool samples taken over a 14 day period, twice per day.

Relative percentage short chain fatty acids

Sample	**8:0**	**10:0**	**12:0**	**14:0**
A	1.86	4.83	5.76	15.84
B	2.08	5.75	7.03	16.49
2A	1.77	4.49	5.04	15.26
2B	1.81	3.87	4.52	14.38
3A	2.03	4.91	5.50	16.56
3B	2.28	4.92	5.56	16.42
4A	1.56	3.51	3.90	13.51
4B	1.74	3.62	4.01	13.48
5A	2.05	4.18	4.60	15.13
5B	1.58	3.38	3.81	13.24

Relative percentage long chain fatty acids

Sample	**16:0**	**16:1**	**18:0**	**18:1**	**18:2**
A	32.50	2.02	10.53	23.26	3.40
B	33.95	1.87	9.65	20.36	2.81
2A	32.35	2.11	11.67	24.40	2.91
2B	31.62	1.98	12.22	25.96	3.64
3A	34.42	1.84	11.19	20.96	2.60
3B	34.70	1.87	11.20	20.51	2.54
4A	31.16	2.01	13.54	26.95	3.86
4B	30.94	2.00	13.45	26.83	3.92
5A	31.94	1.76	12.26	24.63	3.46
5B	30.50	1.68	14.09	27.46	4.26

Relative percentage free short chain fatty acids

Sample	**8:0**	**10:0**	**12:0**	**14:0**
A	1.47	3.69	4.77	7.90
B	4.62	2.55	6.27	9.40
2A	2.77	5.54	5.38	10.20
2B	3.15	5.74	5.14	9.45
3A	2.94	5.91	5.34	8.62
3B	3.51	7.57	6.21	12.86
4A	3.72	7.09	5.50	11.29
4B	2.82	9.08	6.78	10.00
5A	3.94	7.54	6.53	14.70
5B	5.85	11.79	6.49	10.98

Relative percentage of free long chain fatty acids

Sample	**16:0**	**18:0**	**18:1**	**18:2**	**20:1**
A	22.49	7.96	23.48	12.63	15.61
B	24.33	6.20	18.15	6.79	21.68
2A	23.90	7.14	20.80	4.48	19.80
2B	22.67	6.86	20.30	4.02	19.90
3A	21.41	6.10	16.77	3.89	29.02
3B	27.15	7.76	18.72	2.95	13.27
4A	23.69	8.69	24.14	4.36	11.51
4B	21.34	6.19	20.66	3.99	19.15
5A	26.74	9.23	20.28	3.62	7.42
5B	22.91	5.98	17.09	0.00	18.90

Oxidation: reduction of double bindings = not significant

Hydrolysis: increase in free fatty acids = not significant

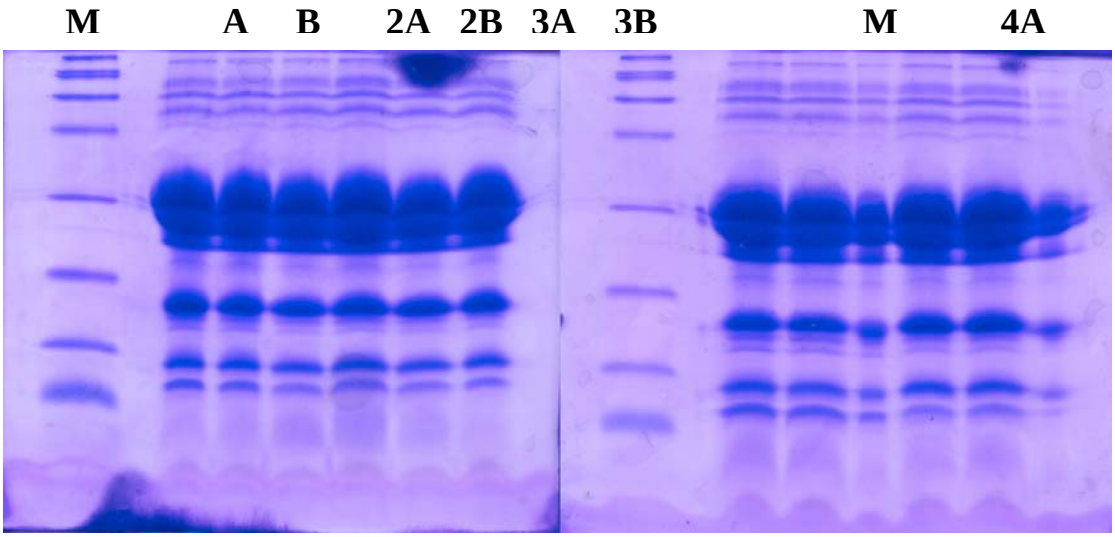

Fig. 4.9 Possible protein breakdown in milk samples obtained before and after UV radiation. Results are the means of five pool samples taken over a 14 day period, twice per day. (15%.gel).

CHAPTER 5

GENERAL DISCUSSION AND CONCLUSION

It is well established that food borne diseases cause significant economic and social losses. Potential threats to human health related to the dairy industry include consumption of raw milk products, pasteurization errors, contamination of milk products by heat-resistant pathogens and chemical adulteration of milk. Non-typhoidal S*almonella* spp., are considered important threats to food safety because of the enormous number of illnesses they cause. On the other hand, *Listeria monocytogenes,* and *Escherichia coli* 0157: H7 are priority pathogens because of the severity of symptoms associated with infection and because of the number of deaths that occur in infected people. All of these pathogens are shed in cattle feces and can contaminate dairy farm premises including unpasteurized bulk tank milk. When regulatory standards for bacterial counts in milk are met, pasteurization of milk is highly effective in destroying all of these organisms.

5.1. The hygienic quality of commercially produced fresh milk in Bloemfontein, South Africa

The initial step of this investigation was to collect raw and pasteurized milk from small scale dairy producers as well as in major milk retailers in the vicinity of the study area. After collection, all the milk samples were analyzed for selected pathogens including *E. coli, L. monocytogenes, B. cereus, C. botulinum, Salmonella* and *Staphylococcus aureus.* In addition, proteolytic and lipolytic activities were determined and all the standard microbial enumeration determinations including total aerobic counts, LAB, yeasts, moulds and coliforms.

The analyses were done by plating the milk samples on media selective for each of the test organisms followed by identification. None of the pathogens tested were positive on all the milk samples. These findings conform to the National Legislation concerning the presence of pathogens in raw and pasteurized milk. However, high counts of total aerobic bacterial counts and coliforms were observed for 74% of the milk samples which were unacceptable as they did not confirm to the National Standards with regards to microorganisms in both pasteurized and raw milk. Similar findings by Lues *et al,* (2003) who conducted a study at Botshabelo, a township nearby the present study area (Bloemfontein) were obtained with regards to coiliforms. These authors obtained 100% of milk samples exceeding the coliform National Standard. Van Kessel *et al*. (2004) also obtained the same results in a study conducted in 2004. Only 17% of all the milk samples were within an acceptable range of total bacterial counts.

About 23% of the milk samples tested positive for non-pathogenic *E. coli.* Similar results were observed by Lues *et al.* (2003) who obtained a 23.3% incidence of *E. coli* whereas Van Kessel *et al.* (2004) obtained an extremely high positive percentage of 93% regarding the incidence of this microorganism.

Also, high counts of psychrotolerants were observed in the milk samples, however, there is currently no standard for the acceptable limit of these microorganisms in milk and dairy products. Only a small percentage of the samples had yeasts present at low counts, and these results corresponded with the literature indicating that low insignificant counts of yeasts are obtained in raw ((Foster *et al.,* 1957; Ingram, 1958; Engel 1986) and pasteurized milk (Jones and Langlois, 1977; Fleet and Mian, 1987).

2. Diversity of yeast species in raw and pasteurized milk

Yeasts were isolated and characterized from 42 different raw milk samples and 23 pasteurized milk samples collected in the Bloemfontein vicinity by serially diluting and spread plating on selective media.

These isolates were identified to species level according to the conventional identification methods as proposed by Kreger-van Rij (1984), Barnett *et al.* (1990) and Kurtzman and Fell (1998). Further confirmation of the identity of the species was performed through sequence analysis of the D1/D2 domain using primer pairs NL-1(5'-GCATATCAATAAGCGGAGGAAAAG) and NL-4 (5'-GGTCCGTGTTTCAAGACGG (Kurtzman and Robnett, 1998).

The results revealed 14 different species, whereby**,** *Kluyveromyces marxianus, Debaryomyces hansenii*, *Clavispora lusitaniae, Cryptococcus flavus, Cryptococcus curvatus, Candida famata* and *Candida albicans* were the most frequently isolated species. The yeasts occurred in a higher percentage in raw milk and since they are heat sensitive, their occurrence in pasteurized milk can be assumed to be post-pasteurization contamination.

All of the yeast species isolated from the milk samples had the ability to grow at low temperature of 5˚C, and this result is consistent with their occurrence in dairy products which are stored under refrigeration during most times. The ability of yeasts to assimilate milk sugars, available organic acids and proteolytic and lipolytic activities explains their presence in milk (Fleet and Mian, 1987).

3. The effect of ultra violet radiation for raw milk treatment to reduce microbial loads

Due to concerns that some potentially dangerous and high numbers of undesired microorganisms may derive from the dairy farm, the ability to efficiently control these microorganisms at the farm level become more desirable. In this study, we investigated the effect of ultraviolet irradiation on the microbial loads and chemical composition of raw milk.

The results showed a 90% reduction on the microbial populations, except yeast numbers showing more resistance being reduced by 73%. Chemical analysis compared from results performed before and after UV radiation showed no significant alterations in the milk composition. Based on the results obtained, it was suggested that the usage of UV radiation on the milk resulted in an enhanced shelf-life and better microbial quality.

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CHAPTER 6

SUMMARY

From the extensive literature review given in Chapter 1, it is evident that milk is an important part of the diet and can also serve as a good medium for growth of microorganisms. These microorganisms can be either pathogenic or being undesired causing spoilage. The pathogenicity and incidence of undesired microorganisms were reviewed and as a result one of the aims of this study was to assess the hygienic quality of milk sold in and around Bloemfontein. The results obtained during this study proved interesting as it showed an alarming high percentage of these milk samples were of a poor microbial quality as they did not confirm to the National Legislation regarding milk sold to consumers.

The importance of yeasts in the dairy industry has been highlighted on a number of occasions by various authors. Despite indications of yeasts associated with dairy products, especially in yoghurts and cheeses, and milk being the raw material of these products, surprisingly few studies have been conducted on the specific occurrence of yeasts in either raw or pasteurized milk. The results obtained showed an ability of these yeasts to survive and proliferate in both raw and pasteurized milk. However, the number of yeast cells was low and insignificant to cause major problems. A wide diversity, including 14 different species was isolated and characterized. The alarming effect remains that predominant species like *Candida albicans* was found, a severe human pathogen.

Due to concerns that some potentially dangerous and high numbers of undesired microorganisms may derive from the dairy farm, the ability to efficiently control these populations at the farm level seemed desirable. Consequently, the effect of ultraviolet irradiation on the microbial loads and chemical composition of raw milk was investigated.

The results showed a 90% reduction on the microbial populations, except yeast numbers showing more resistance being reduced by 73%. Chemical analysis compared from results performed before and after UV radiation showed no significant alterations in the milk composition. Based on the results obtained, it was suggested that the usage of UV radiation on the milk resulted in an enhanced shelf-life and better microbial quality.