CHAPTER 2

MATERIALS AND METHODS

One hundred and thirty-five milk samples were obtained over a six-week period from June to August 1998 from five milk-shops in the north-western part of Pretoria. Seventy-nine samples of milk were also purchased from three supermarkets which sold a well-known national distributor's commercial brand of milk, and this milk was used as a reference control milk. The milk-shop milk was either purchased prepackaged or bottled at point of sale by the milk-shop. The national distributor's milk was packaged in sachets.

STUDY DESIGN

Milk samples were purchased from four randomly selected retailers (out of eight available within the specified area) who distributed their milk directly to the consumer from a bulk tank (known as "Milk-Shops"). These shops were all located within a radius of 20 km of Onderstepoort in the north-western part of Pretoria. Two shops were randomly chosen in a poor socio-economic area and two shops in a more middle class area. This was determined by looking at the price of houses in the two areas. These areas were selected because of the good demographic cross-section of the population they represented. Each of the milk-shops looked clean and tidy and did not give an impression of being unhygienic. The owners were all very friendly, giving the shop a personal touch and making the consumer feel happy to return.

Milk samples were also purchased from a selected retailer (a supermarket) who sold both milk from a bulk tank, as well as milk from a commercial national distributor. The milk from the commercial national distributor acted as the control milk. This supermarket was chosen because it sold both bulk tank milk and control milk, and therefore the management of the

shop with respect to the cold chain, should have been the same as both types of milk were kept in the same display cabinet.

A further two samples of "control milk", also from the same commercial national distributor described above, were purchased from two other large supermarkets in the area. An attempt was made to ensure that all the national distributor's milk samples had the same expiry date and were therefore processed on the same day. This was not always possible as sometimes a shop had not bought fresh milk and was still selling milk with an older expiry date. The three supermarkets did however, always sell milk within the "sell-by date".

The farms of origin of the milk were unknown to the researcher as the study attempted to look at the quality of milk available to the consumer, without creating any suspicion on the part of the milk-shop owners. Therefore no direct questions could be asked as to where the milk came from or how many farmers supplied milk to the shop.

Milk was purchased over a six-week period, on alternate days, so that in one week purchases were made on a Tuesday and Thursday, and the following week on a Monday, Wednesday and Friday. From the second week onwards, two milk samples were purchased at each point of sale so as to decrease the variance in the data. There were therefore 8 shops x 2 samples (= 16 samples) purchased for each sampling day over the remaining five-week sampling period. On one of the sampling days, one of the supermarkets selling the national distributor's milk (Supermarket 1), had a power failure and therefore the electricity supply was off and the shop did not open its doors to consumers. Only 25 samples were thus purchased from this particular shop over the six-week sampling period.

All milk samples on sale to the public were kept in their original packaging until they were analysed. The national distributor's milk was packaged in 1 ℓ plastic sachets. Milk-shops usually sell their milk to clients who bring their own containers, thereby keeping down the cost of packaging. All the milk-shops also bottled milk in plastic 500 mℓ or 1 ℓ containers for members of the public who had not brought their own containers. They either pre-packaged them in advance, storing the milk in a refrigerator, or bottled them on demand from the bulk

tank as requested. These pre-packaged milk bottles were purchased at milk-shops so as not to create suspicion by bringing sterile glass bottles to the shop. The temperature of the milk was taken within five minutes of purchase, by decanting approximately 100 mℓ of milk into a separate plastic container and measuring the temperature using an electronic thermometer calibrated to the nearest 0.5 °C. The decanted milk was then discarded. Care was taken not to contaminate the balance of the milk after decanting, and this milk was kept on ice in a cooler box until it was analysed in the laboratory.

The time of purchase was taken as zero hour, and all microbiological analyses were carried out within four hours of the zero hour. The time of sampling and the time of processing in the laboratory were recorded.

At the time of purchase, all the milk samples were labelled and the following data was entered on a separate sheet (see Addendum X):

* sample number
* date and time of purchase
* place of purchase
* purchase price
* temperature of the milk at purchase
* whether the milk was sold as having been pasteurised or not
* whether the milk had been labelled or not
* expiry date.

LABORATORY WORK

Milk samples were kept in a household refrigerator until they were processed for bacterial counts. All milk samples were mixed by inverting them gently in their original containers a minimum of ten times before processing in the laboratory.

Petrifilm plates (dry rehydrateable films)

Petrifilm (3M) plates were used for aerobic, psychrotrophic, coliform and *E. coli* counts. Petrifilm plates are a commercially available, time and labour-saving method for enumerating bacteria by eliminating the need to prepare media. This sample-ready system consists of nutrients and/or selective and differential agents coated onto two dry rehydratable films along with a cold-soluble gelling agent and tetrazolium indicator dye which facilitates colony enumeration (Bishop & Juan 1988, Blackburn *et al.* 1996). Colonies growing on Petrifilm appear pink owing to the reduction of the dye. Coliform colonies, including *E. coli*, which grow on the Petrifilm produce gas by the fermentation of lactose. Petrifilm plates are ready to receive 1 mℓ of milk with no sterilization or plate pouring required. There is therefore no between-lot medium variation and less incubator space is needed. They are particularly useful in quality assurance laboratories which have limited facilities.

The Petrifilm aerobic count and the Petrifilm *E. coli* and coliform counts were found to be suitable and practical alternatives to standard methods used in enumerating bacteria in raw milk (Ginn *et al.* 1984, Betts *et al.* 1994, Blackburn *et al.* 1996). The different authors found that there was a good correlation between the Petrifilm aerobic count and the standard aerobic colony count method; the Petrifilm coliform colony count and the Standard Violet Red Bile Agar plating method, and the Petrifilm *E. coli* count and modified Eijkman test. Blackburn *et al.* (1996) found that the aerobic count Petrifilm and the coliform count Petrifilm both showed better repeatability than the standard methods. In South Africa an investigation into using the Petrifilm as an alternative for the evaluation of aerobic micro-organisms, coliforms and *E. coli* in fresh and pasteurised milk was conducted by Vermaak and Petzer (1996), who also concluded that the Petrifilm technique compared well with, and was even more sensitive than present conventional counting methods.

All milk samples were subjected to the following tests:

pH of the milk

The pH of the milk on the day of purchase and after incubating for 18 hours at 21 °C was determined by means of the Orion SA 250 pH meter autocalibrated with buffers at 7.00, 4.01 and 10.01 in accordance with the manufacturer's specifications.

Standard aerobic plate count / Total aerobic count (Petrifilm 3M)

The standard plate count is used to estimate viable bacterial populations in the milk and reflects the hygienic practices used in the production and handling of the milk (Houghtby *et al.* 1994).

Standard procedures for the use of the dry rehydrated film method for standard colony count were used (see Addendum I). The method used was according to the Foodstuffs, Cosmetics and Disinfectants Act, No 54 of 1972: Regulations relating to milk and milk products, No R.1555. *Government Gazette* No 18439, 21 November 1997, 4-29.

Serial milk dilutions of 10^{-2}, 10^{-3}, 10^{-4} and 10^{-5} were made for the standard plate count. Plates were incubated at 32 °C for 48 hours. A few standard plate count values are missing for some of the milk-shops during the first few days as insufficient dilutions were made and the bacterial numbers present were too numerous to count. Thereafter dilutions as described above, even though far above what is acceptable, were made so that exact counts could be made. Single-use disposable pipettes were used for each dilution.

Modified psychrotrophic bacterial count (Petrifilm 3M)

Milk samples were plated onto an aerobic Petrifilm plate as described in Addendum I, except that approximately 80 mℓ of milk was first pre-incubated at 21 °C for 18 hours, after which 1 mℓ of the serial dilutions were placed onto Petrifilms and incubated at 21 °C for 48 hours (Bishop & Juan 1988, Byrne *et al.* 1989, Phillips & Griffiths 1990). This method has shown good correlations with the psychrotrophic bacterial count done at 7 °C for 10 days.

Phillips & Griffiths (1990) described a similar method whereby milk was pre-incubated at 15 °C for 25 hours and then plated onto Petrifilms for 48 hours at 21 °C. This method showed a high correlation with the European Economic Community (EEC), now the European Union (EU), directive which requires incubation at 6 °C for 5 days, and specifies that the bacterial count of pasteurised milk shall not be greater than 100 000 CFU per mℓ after incubation (Council Directive of 5 August 1985 on health and animal health problems affecting intra-community trade in heat-treated milk (85/397/EEC). *Journal of the European Communities* No. L226, 13, cited by Phillips & Griffiths 1990 and Suhren & Heeschen 1990).

A pre-incubation temperature of 21 °C was chosen for this study as one incubator could then be used for both the pre-incubation of the milk and the incubation of the Petrifilm plates. A water bath was not used.

Serial milk dilutions of 10^{-2}, 10^{-3}, 10^{-4}, 10^{-5} and 10^{-6} were used for the modified psychrotrophic bacterial count. A new single-use disposable pipette was used for each dilution so that there would be no carry-over of milk from one dilution to another. Psychrotrophic values are missing for some of the milk-shops during the first few days as insufficient dilutions were made and the bacterial numbers present were too numerous to count. Thereafter dilutions as described above, even though far above what is acceptable, were made so that exact counts could be made.

I 14542900
B 14270456

E. coli and coliform count (Petrifilm 3M)

Coliforms in milk are suggestive of unsanitary conditions or practices during production, processing or storage. Coliforms are destroyed by pasteurisation, and therefore their presence after correct pasteurisation are indicative of bacterial recontamination post-pasteurisation (Christen *et al.* 1992).

Escherichia coli is a faecal indicator organism, whose recovery from milk suggests that other organisms of faecal origin, including pathogens, may also be present (Christen *et al.* 1992).

Standard procedures for the use of the dry rehydrated film method for coliform and *E. coli* count were used (see Addendum II). The method used was according to the Foodstuffs, Cosmetics and Disinfectants Act, No 54 of 1972: Regulations relating to milk and milk products, No R.1555. *Government Gazette* No 18439, 21 November 1997, 4-29. This Act prescribes an incubation temperature of 32°C and not 35°C as prescribed by the Petrifilm manufacturers. Coliform and *E. coli* counts were tested in 1 mℓ of milk using the combined coliform and *E. coli* petrifilm plates.

Serial dilutions of 10^{-1}, 10^{-2} and 10^{-3} were also made and the milk was plated onto petrifilm rapid coliform count plates. These plates only give a coliform count. Serial dilutions were made because some of the milk-shops had counts that were too numerous to count in 1 mℓ of milk. The petrifilm rapid coliform count plates, and not the combined coliform and *E. coli* petrifilm plates, were used for the serial dilutions as they were donated by the manufacturer 3M.

Aschaffenburg and Mullen alkaline phosphatase test

Pasteurisation is universally used to kill pathogens, and the alkaline phosphatase test is used to determine the effectiveness of pasteurisation (Murthy *et al.* 1992). The presence of alkaline phosphatase may also indicate the possible addition of raw milk to pasteurised milk. The

thermal resistance of alkaline phosphatase is greater than that of non-spore-forming pathogenic micro-organisms, and therefore milk heated to 62.8 °C for 30 minutes or to 71.7 °C for 15 seconds, will inactivate alkaline phosphatase, and will also kill all non-spore-forming pathogenic micro-organisms (Murthy *et al.* 1992).

The Aschaffenburg and Mullen alkaline phosphatase test was performed, using the method specified in the Foodstuffs, Cosmetics and Disinfectants Act, No 54 of 1972: Regulations relating to milk and milk products, No R.1555. *Government Gazette* No 18439, 21 November 1997, 4-29 (see Addendum III).

Somatic cell count

The Regulations (Foodstuffs, Cosmetics and Disinfectants Act, No 54 of 1972) state that milk should not contain any inflammatory product which may render the milk unfit for human consumption. Cows in very early or very late lactation, or cows with a low-grade or latent udder infection, are likely to produce milk containing an excessive number of somatic cells, consisting mainly of leucocytes and some epithelial cells (Hinz *et al.* 1992).

The somatic cell count was determined using the Fossomatic apparatus. Standard operating procedures for counting somatic cells in milk by means of the Fossomatic were used (see Addendum IV).

Brilliant Black Reduction Test for the detection of antibiotic and other antimicrobial residues

The Regulations (Foodstuffs, Cosmetics and Disinfectants Act, No 54 of 1972) do not specify any specific tests to determine whether inhibitory substances are present in the milk. The Brilliant Black Reduction Test was chosen as it is highly sensitive to a number of antibiotics (see Addendum V). It is also the test of choice used by some of the European Union countries

(Suhren & Heeschen 1996). This test is easy to use, cheap and detects a broad spectrum of antibiotics for screening purposes. The test principle is based on the detection of growth inhibition, noticed visually by interpreting the colour of a pH-indicator in the test medium. *Bacillus stearothermophilus*, a thermophilic organism which is very sensitive to penicillin, is used as the test organism.

To avoid the influence of the micro flora of the sample on the test micro-organisms, a high incubation temperature of 64°C is used, because only minor parts of the milk flora can multiply and metabolize at this temperature (Suhren 1995, Suhren & Heeschen 1996).

Brucella milk ring test

Brucella abortus is a zoonosis which causes undulant fever in man. Brucellosis in animals has not yet been eradicated in South Africa. Commercial pasteurisation effectively kills *B. abortus* with a large margin of safety (Flowers *et al.* 1992).

Standard procedures for the brucella milk ring test were used to identify *B. abortus* antibodies in the milk (see Addendum VI).

Isolation of *Staphylococcus aureus* on Baird-Parker Agar Base and confirmation by means of the Staphylase test

Staphylococcus aureus isolation was done on Baird Parker Agar Base containing Potassium Tellurite solution and Egg Yolk Emulsion (see Addendum VII). One mℓ of milk was used, and a positive colony was confirmed as being *S. aureus* by means of the Staphylase test (see Addendum VIII). No counts were performed.

Detection of Staphylococcal enterotoxins

Several strains of *S. aureus* can produce heat stable enterotoxins which survive the pasteurisation process and cause food poisoning in man (Flowers *et al.* 1992). Individual *S. aureus* cultures were inoculated in Tryptone Soya Broth and incubated overnight at 37 °C, and subsequently tested for the presence of Staphylococcal enterotoxins A, B, C and D by means of reversed passive latex agglutination, using the SET-RPLA Staphylococcal enterotoxin test kit (Oxoid) (see Addendum IX). This kit was used as the sensitivity of the assay was found to be 0.25 ng/mℓ in pasteurised milk, which was more sensitive than that claimed by the manufacturer (2 ng/mℓ) (Rose *et al.* 1989, Park & Szabo 1986).

The Staphylococcal enterotoxin test was done on all positive *S. aureus* cultures. Fifteen milk samples from the national distributor were also tested, one from each day of sampling. These samples were centrifuged for 15 minutes at 3 400 rpm and the sediment was discarded. Enterotoxin detection was carried out on the supernatant.

Detection of *Salmonella* spp.

One milk sample from every pair of milk samples purchased which was positive for *E. coli* was cultured for *Salmonella* spp. Samples were inoculated for pre-enrichment onto a non-selective medium and incubated at 37 °C for 24 hours. They were then inoculated onto Rappaport Vassiliadis Soya Broth and incubated at 42 °C for 24 hours, after which they were inoculated into a selective solid agar medium (Brilliant green, Bismuth sulphite and XLD) at 37 °C for 24 hours for presumptive identification of *Salmonella* spp.

DATA ANALYSIS

A data sheet was completed for each day of sampling (see Addendum X), and all the raw data, including all the results of the different dilutions was completed on this sheet.

The Department of Information Technology and the Department of Statistics of the University of Pretoria analysed the data using the statistical computer package SAS (SAS Institute Inc., CarY, NC).

Comparisons were made between milk bought from "Milk-shops" and milk bought from the commercial national distributor. All count data were transformed to $\log_{10}$ values because of their non-normal distribution. Frequency distributions were made and correlation analyses were performed to provide association between different variables.

Analysis of variance (ANOVA) was done to determine whether the week number, day of the week, place of purchase, temperature at purchase, presence or absence of alkaline phosphatase, presence or absence of inhibitory substances, and the pH of the milk (independent variables) from the five milk-shops and the milk from the commercial national distributor differed with regard to aerobic standards plate count, psychrotrophic bacterial count, coliform count and somatic cell count (dependent variables). Probability (p) values were reported, and any value less than or equal to ($\leq$) 0.05 was reported as significant.

The computer spreadsheet packages of Sigma Plot (Jandel Scientific, San Rafael, CA) and Microsoft Excel 97 (1985-1997 Microsoft Corporation) were used to make the graphs.