CHAPTER 3

MATERIALS AND METHODS

3. 1 Sample preparation

3. 1. 1 Cereal and skim milk samples

Traditionally processed finger millet (*Eleusine coracana*) meal was purchased from rural farmers in Manicaland, a province in the Eastern Highlands in Zimbabwe. The traditional process of making finger millet meal involved roasting of the grain to loosen the pericarp and to develop flavour. The pericarp was removed by pounding with the traditional mortar and pestle. This was followed by winnowing to separate the loose pericarp from the clean grain. The clean grain was then milled using hammer milling. The finger millet meal was stored in sealed plastic bags at 10°C until required. Skim-milk powder was sourced in South Africa and stored under cool dry conditions until required.

Table 1 shows the proximate composition of the finger millet meal and the skim-milk powder.

Table 1. Proximate composition of finger millet meal and skim-milk powder*

Component (g/ 100 g DM)	Finger millet	Skim-milk Powder
Protein	8.9	32
Crude fat	3.3	1
Ash	3.1	8
Starch	66.2	nd
Lactose	nd	48
Crude fibre	6.1	nd

*based on analysis

nd - not detected

3. 1. 2 Preparation of cereal-dairy composite gruels

Skim-milk powder was reconstituted, according to the manufacturer's instructions. One part skim-milk powder was added to nine parts water and stirred until completely dispersed. The finger millet gruel was prepared by cooking five parts of finger millet meal in 95 parts of water at low heat for 10 min while stirring continuously. Proportions of finger millet greater than 5% gave a gruel that solidified on cooling. The reconstituted skim milk was added to the gruel to give composites containing 100%, 90%, 80%, 70%, 60%, 50% and 0% (skim milk only) finger millet gruel by volume. The unfermented gruels were sterilised by autoclaving for 15 min at 121°C.

3. 2 Starter cultures

Three starter cultures were used for fermentation. YC380 (Chr. Hansen, Denmark) and Joghurt V2 (Wiesby, Denmark) were commercial yoghurt type starter cultures that were supplied by Darleon CC. in South Africa. They are often used in the dairy

industry in South Africa for the manufacture of yoghurt. According to the suppliers, they contain strains of the bacteria *Lactobacillus delbrueckii* subsp. *bulgaricus* and *Streptococcus thermophilus* subsp. *salivarius*. Both cultures are mixed cultures with Gram positive cocci and rods. The third starter culture, JC, was a mixed strain culture developed in our laboratory over a period of time to ferment raw cereal slurries at 25°C. Table 2 summaries the characteristics of the three starter cultures

Table 2. Some characteristics of starter cultures YC380, V2 and JC

	YC380	V2	JC
Description	A mixture of rods and cocci.	A mixture of rods and cocci.	Predominantly rods with some in chain formation, some cocci, ocurring in clusters.
Growth on agar	Grew on acidified MRS agar as round, whitish colonies	Grew on Rogosa agar as pin-head round, whitish colonies	Grew on acidified MRS agar as round, whitish colonies
Gram reaction	+ ve	+ ve	+ ve
Catalase reaction	- ve	- ve	- ve

3. 3 Starter culture propagation

Bacterial starter cultures YC380 and V2 were each added to sterilised, reconstituted skim milk at a rate of 1 g of starter culture per 99 ml of skim milk. This was done under aseptic conditions. The mixture was thoroughly mixed and incubated at 45°C for 3 h. The fermented products were cooled to 5°C. These preliminary yoghurt cultures were subcultured into sterilised skim milk at a rate of 1 ml of culture per 99 ml of skim milk. After thorough mixing they were incubated at 45°C for 3 h and then

cooled to 5°C. Subculturing was repeated twice leading to batches of yoghurt culture, hereafter referred to as Starter Cultures. These were stored in the refrigerator.

The starter culture JC was prepared by mixing 50 g dehulled sorghum meal and 64 ml of water at 30°C into a slurry which was then fermented at 25°C until the pH dropped to 3.6 . The culture was maintained by inoculating a mixture of 50 g of dehulled sorghum meal and 64 ml of water with 5 ml of the fermented slurry.

3. 4 Gruel fermentation

Initially, the inoculated samples were incubated at 10°C, 25°C, 30°C, 37°C and 45°C for 10 h. All analyses were carried out in triplicate. In the second phase of the experiment the optimum time of incubation was determined. This was measured as the time that it took for the pH of inoculated samples to fall to 4.6 . Sterile samples were inoculated with bacterial starter cultures YC380 and V2 at an inoculation rate of 2% (v/v) under aseptic conditions. The samples were incubated at 37°C and 45°C. At 1 h intervals the pH of the samples was determined. The control samples were not inoculated.

3. 5 Gruel inoculation

Inoculation of gruel samples was carried out aseptically at room temperature and each mixed by shaking the bottle. Gruel samples that had been measured to give a volume of 98 ml were inoculated with 2 ml of starter culture to give an inoculation rate of 2%. The samples were thoroughly mixed to ensure even distribution of the inoculum.

3. 6 Freeze-drying

Gruel samples that were used for the determination of crude fat, crude fibre, amino acids and energy were freeze-dried (Specht Scientific, Model no. SJ-FD-5/PC, Asslar, Germany). The freeze-dried samples were milled using a Waring blender and stored in sample bottles at 10°C away from light until required.

3. 7 Physicochemical analyses

3. 7. 1 pH and titratable acidity

Acidity was determined by the potentiometric procedure (International Dairy Federation, 1991) by weighing 10 g of the sample into small beakers, adding 10 ml of distilled water and titrating with 0.1M sodium hydroxide to a pH of 8.3. Acidity was expressed as % lactic acid. The pH was determined using a combined glass electrode connected to a Mettler DL-25 pH meter (Mettler-Toledo AG, Greifensee, Switzerland) by immersing the electrode directly into the gruel sample.

3. 7. 2 Syneresis

The % syneresis (v/m) was determined by a modification of the centrifugation procedure described by Harwarkar & Kalab (1983). Samples of the fermented gruel, which had been incubated at 30°C, 37°C and 45°C, were chilled to 4°C for 24 h and then centrifuged at 1000 x *g* for 20 min. The lower temperatures of incubation (10°C and 25°C) that were used for pH determination were left out because the gruels fermented at these temperatures did not attain the desired pH of 4.5 or lower. The volume of serum (cm^3) was expressed as a percentage of the mass (g) of the sample centrifuged.

3. 7. 3 Consistency

The consistency of the fermented gruel incubated at 37°C and 45°C was determined as the distance flowed, in centimetres, within a time period of 30 s using a Bostwick consistometer (Gould, 1974). This instrument is used widely by the dairy industry in South Africa for quality control.

3. 7. 4 Gruel firmness

Gruel firmness was measured as the maximum force required to penetrate undisturbed gruel in glass containers using a texture analyser (TA-XT2; Stable Microsystems, Surrey, England) and a cylindrical probe which had a base diameter of

20 mm (Hess, Roberts & Ziegler, 1997). The containers were half filled and had a maximum capacity of 55 cm^3, a base diameter of 35 mm and a height of 70 mm.

3. 8 Proximate analyses

3. 8. 1 Moisture

A commonly used procedure for determining the moisture content of a food product is based on the separation of water from the solids and its measurement as the resulting loss in weight or by measurement of the amount of water lost (Joslyn, 1970). The accurate determination of moisture is difficult because of the problem of completely separating all the water from the food product without completely causing its decomposition with concomitant production of water which would be included in the determination (Joslyn, 1970). The loss of volatile constituents from the food is another factor. A modification of the oven drying method was used (AOAC, 1980a).

A sample of known mass was dried in an oven at 100°C for 3 h. This was followed by cooling in a desiccator to room temperature and the weighing of the cooled sample. The moisture content was determined as loss in moisture using the following formula:

$$\% \text{ Moisture} = \frac{A \times 100}{B}$$

Where A was moisture loss in grams and B was the original weight of the sample.

3. 8. 2 Ash

Ash was determined as the residue that remained after all the moisture had been removed and the organic material (including fats, proteins, carbohydrates, vitamins and organic acids) had been combusted at a temperature not less than 550°C to carbon dioxide and oxides of nitrogen (James, 1995). Ash content was calculated as follows:

$$\% \text{ Ash} = \frac{(\text{weight of crucible + ash}) - \text{weight of empty crucible} \times 100}{\text{weight of sample (g)}}$$

3. 8. 3 Fat

Fat in skim milk was determined using a modification of the Babcock test (Davis, 1959). Milk fat exists in milk as an emulsion of the oil-in-water type. In the modified Babcock procedure, sulphuric acid and alcohol are added to coagulate the protein and to remove the phospholipid layer around the fat globule. This breaks the emulsion and separates the fat allowing it to coalesce (Joslyn, 1970). Hot water is added to raise the liberated fat layer above the water layer.

To 17.6 ml of milk, 4 ml iso-butyl alcohol was added. This was followed by thorough mixing and addition of 17.5 ml of sulphuric acid (specific gravity 1.82-1.83). The mixed sample was centrifuged for 6 min in a Babcock centrifuge to separate the fat from the water component of the emulsion. The flask was filled with hot water and centrifuged for a further 14 min. The contents of the flask were rapidly mixed and centrifuged for 10 min after which a reading was taken.

3. 8. 4 Crude fat

Fats, oils and fatty acids are characterised by their extreme insolubility in water, very slight solubility in alcohol and by the readiness with which they are dissolved by ethyl ether, petroleum ether, carbon disulphide and carbon tetrachloride (Joslyn, 1970). The Soxhlet apparatus is constructed to permit the passage of the vapours of the solvents into the condenser by a separate tube and return of the condensed solvent after having stood in contact with the sample, to the evaporating flask by an external siphon. The advantage of the process lies in freeing the sample entirely from the rise in temperature resulting from contact with the hot vapours of the solvent (Joslyn, 1970).

Crude fat was determined on freeze-dried gruels using a modification of the AOAC procedure (AOAC, 1980b). Samples were milled using a Waring blender.

Approximately 4 g of thoroughly mixed sample was weighed onto filter paper. The filter paper was folded and inserted into an extraction thimble. Fat-free cotton wool was used to plug the extraction thimble. Extraction was carried out for 4 h on a Soxhlet extraction unit using petroleum ether (boiling point 40-60°C). The extract was dried at 100°C for 30 min, cooled and weighed. Crude fat was then calculated as follows:

$$\% \text{ Crude fat} = \frac{(\text{weight of flask + fat}) - \text{weight of flask}) \times 100}{\text{weight of sample (g)}}$$

3. 8. 5 Crude protein

The Kjeldahl procedure for the determination of nitrogen in biological materials is characterised by the use of boiling, concentrated sulphuric acid to effect the oxidative destruction of the organic matter of the sample. The acid also reduces organic nitrogen to ammonia. The process is facilitated by the use of a catalyst. The ammonia is retained in the acid digest as ammonium bisulphate (Chang, 1994). The digest is made alkaline using sodium hydroxide and the ammonia is distilled off. The ammonia is measured by titration (Lillevik, 1970).

The traditional method of estimating the protein content of a food is to multiply its content of nitrogen by a suitable conversion factor. This factor will vary according to the nitrogen content of the particular proteins and can vary from 12 to 30%. The factor of 6.25 is generally used and it would apply to a protein containing 16% nitrogen (Lillevik, 1970) For dairy products, a factor of 6.38 is used (Pomeranz & Meloan, 1994).

Samples were analysed for crude protein using a Kjeldahl method. Approximately 0.5 g sample was weighed accurately into a digestion flask. One Kjeltab (Thompson & Capper, Cheshire, England), consisting of 100 parts K_2SO_4, 6 parts $CuSO_4.5H_2O$ and 2 parts selenium was added. To this, 20 ml of concentrated H_2SO_4 was added. Samples were digested for approximately 2 h using a Büchi 430 Digestor (Büchi,

Flavil, Switzerland). Distillation of ammonia, reaction with boric acid and titration with standard HCl (0.1*M*) were done with a Büchi 322 Distillation Unit (Büchi, Flavil, Switzerland). The crude protein content was calculated as follows:

$$\% \text{ Protein} = \frac{(\text{ml std NaOH} \times \text{M of NaOH}) \times \text{factor} \times 1.4007}{\text{weight of sample (g)}}$$

One of the products that was analysed was a composite containing cereal and milk hence the factor that was used was 6.38 .

3. 8. 6 Crude fibre

The hot sulphuric acid and sodium hydroxide used in the determination of crude fibre precipitates protein, hydrolyse the protein into smaller molecules that are removed through washing. The reagents also hydrolyse starch and pectin into smaller molecules that are soluble and can be removed by washing and remove mineral matter that is not bound in the cell wall (Woodman, 1941). The acetone wash helps to remove water that is not bound to the cellulose prior to drying.

Fibre was determined using a Fibertec System M (1020 Hot extractor, Tecator AB, Hoganas, Sweden). A ground sample (2 g) was weighed into a crucible that had been left in a muffle furnace at 500°C for 1 h and then cooled. The first extraction was carried out for 30 min using 150 ml of pre-heated sulphuric acid (0.128*M*). The sample was rinsed three times with hot water and then filtered. The second extraction was carried out for 30 min using 150 ml of hot potassium hydroxide (0.128*M*). The sample was washed with hot water and filtered, washed three times with acetone and dried at 100°C overnight. The dried sample was ashed in a muffle furnace at 500°C for 3 h and then cooled in a desiccator followed by weighing.

3. 8. 7 Energy

The energy (measured as heat of combustion) content was measured on 0.5 g freeze-dried sample. A waterless bomb calorimeter was used (dds, model no. CP 500,

Midrand, South Africa). The sample was completely combusted in the presence of oxygen at a pressure of 3000 kPa.

3. 8. 8 Lactose

A sample (1 g) was weighed into a 100 ml volumetric flask, 60 ml of water was added followed by 5 ml of Carrez I solution (potassium hexacyanoferrate (II) 85 mol/l $K_4[Fe(CN)_6].3H_2O$) and 5 ml of Carrez II solution (zinc sulphate, 250 mmol/l $ZnSO_4.7H_2O$). The contents of the flask were thoroughly shaken and the pH was adjusted to 7.5-8.5 with 0.1 mol/l NaOH. The contents of the flask were again mixed and the volumetric flask filled up to the mark with distilled water. The solution was filtered and the clear filtrate was stored at 7°C.

A lactose/ D-galactose enzymatic kit (Boehringer, Mannheim, Germany) was used for the determination of lactose. Lactose was hydrolysed to D-glucose and D-galactose at pH 6.6 by the enzyme β-galactosidase in the presence of water. D-galactose was converted by nicotinamide adenine dinucleotide (NAD) to D-galactonic acid in the presence of the enzyme β-galactose dehydrogenase. The absorbance of the sample was then read at 340 nm against a reagent blank. The concentrations of lactose and D-galactose in the sample were calculated using the following formula:

$$c = \frac{V \times MW}{\varepsilon \times d \times v \times 1000} \times \Delta A \text{ [g/l]}$$

Where:

c = Lactose calculated as lactose monohydrate / D-galactose

ΔA = Absorbance of the sample, less the absorbance of the blank

V = final volume (ml)

v = sample volume (ml)

MW = molecular weight of lactose monohydrate / D-galactose

d = light path (cm)

ε = extinction coefficient of NADH at 340 nm (= 6.3)

The amount of lactose was determined as the difference between the concentrations of lactose and D-galactose.

3. 8. 9 Total Starch

A total starch assay kit (α-amylase/amyloglucosidase method, AA/AMG 9/97, Megazyme International Ireland Limited, Wicklow, Ireland) was used to determine total starch. The analysis included solubilisation of the starch with dimethyl sulphoxide, hydrolysis with thermostable α-amylase and hydrolysis with amyloglucosidase. The glucose formed was then determined using a glucose oxidase/peroxidase reagent (GOPOD). The absorbance was read at 510 nm. The total starch was calculated using the following formula:

$$\% \text{ Starch} = \frac{\Delta E \times F \times 90}{W}$$

Where ΔE was the absorbance read against a reagent blank, F was the conversion from absorbance to μg glucose, 90 the adjustment from free glucose to anhydro glucose (as occurs in starch) and W was the weight of the sample.

3. 8. 10 Amino acids

High performance liquid chromatography uses high pressure to force a solution containing the compounds to be separated rapidly through resin held in a strong metal tube (McDonald, Edwards, Greenhalgh & Morgan, 1995). For the separation of the amino acids, the first stage is the hydrolysis of the proteins using a strong mineral acid such as hydrochloric acid (White, Handler & Smith, 1964). The products of hydrolysis are ammonia and free amino acids. Phenol is added to the acid during hydrolysis as an oxygen scavenger to minimise the destruction of labile amino acids such as cysteine and arginine. Reaction with phenylisothiocyanate produces amino acid derivatives which can be detected in the ultraviolet region below 250 nm (Chiou, 1988). The sample is then dissolved in a solvent to obtain all the amino acids present in the food sample in a liquid phase (Macrae, 1985).

Amino acid analysis was done using the Pico.tag® method (Bidlingmeyer, Cohen and Tarvin, 1984). Approximately 10-20 mg of defatted freeze-dried sample was put into a hydrolysis flask into which 1 ml 6*M* HCl and 1% phenol were added. The flask was evacuated and flushed with nitrogen to remove oxygen and then sealed under vacuum. The sample was left at 110°C for 24 h. After cooling the sample was made up to 5 ml and 25 µl were taken to dry.

Ten microlitres of a mixture containing methanol, water and triethylamine in the ratio 2:2:1 was added to the sample. The sample was mixed, left to dry and 20 µl of a mixture containing methanol, water, triethylamine and phenylisothiocyanate was added to the dried sample. The flask was left at room temperature for 20 min and the sample dried under vacuum to remove excess reagent. The dried sample was dissolved in 200 µl of a solution consisting of 710 mg Na_2HPO_4 made up to 1 litre with water and the pH adjusted to 7.40 using 10% H_3PO_4 and 5% acetonitrile.

Separation of amino acids was done using a reverse phase column (Pico.tag column for hydrolysate amino acid analysis, 3.9 mm x 15 cm). Two pumps were used to create a gradient for optimum separation. The monitor was set at 254 nm.

3. 9 Microbiological tests

3. 9. 1 The Gram stain

A heat-fixed smear was prepared from 18-24 h cultures of YC380, V2 and JC. The smear was stained with crystal violet for two minutes. The crystal violet solution was washed off the smear using Gram's iodine solution and the iodine was allowed to react for one minute. The smear was blotted dry and washed with 95% ethanol until no dye could be detected in the ethanol. The slide was rinsed under running water and counter-stained with dilute carbol fuchsin for ten seconds. The slide was washed with water, blotted and examined using light microscopy (Harrigan, 1998). The crystal violet, Gram's iodine and carbol fuchsin were prepared according to the procedures described by Harrigan (1998).

3.9.2 Catalase reaction

The catalase test is used to identify strains of bacteria that possess the enzyme catalase and can break down hydrogen peroxide to water and oxygen (Harrigan, 1998). The catalase test was carried out on 24 h old cultures of YC380, V2 and JC that had been enriched in nutrient broth. The nutrient broth was prepared according to the manufacturer's instructions (Biolab Merck, Midrand, South Africa). Five millilitres of starter culture were added to 1 ml of freshly prepared 3% hydrogen peroxide in a clean test-tube. The mixture was observed for the formation of bubbles (Harrigan, 1998).

3.9.3 Enumeration of lactic acid bacteria

Sterile peptone water at a concentration of 0.1% was used as the diluent of choice (Houghtby, Maturin, Koenig & Messer, 1992). Using sterile pipettes and following the procedure outlined in Figure 8, serial dilutions of the samples were prepared and plated up to a dilution of 10^{-7}.

MRS (de Man, Rogosa, Sharpe) agar (Merck, Darmstadt, Germany) and acidified Rogosa agar (Merck, Darmstadt, Germany) were used for the enumeration of lactic acid bacteria in the fermented product. The agar media were prepared according to the manufacturers' instructions.

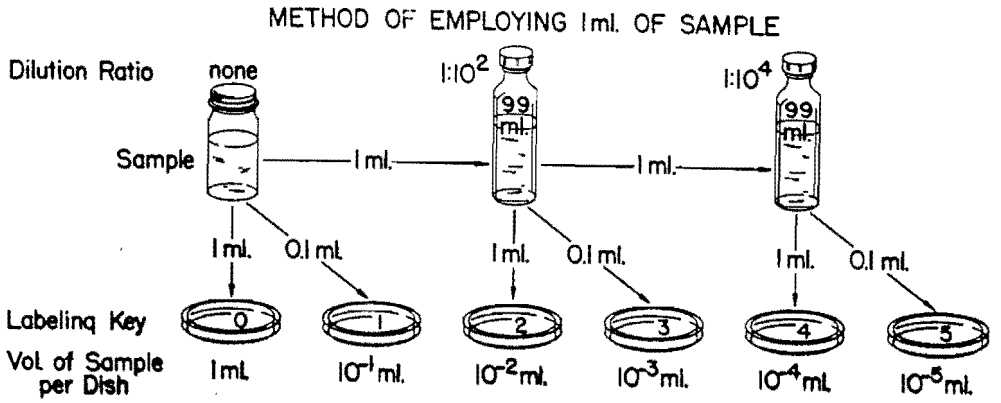

Figure 8. Examples for preparing dilutions (Houghtby, Maturin, Koenig & Messer, 1992)

Molten agar at approximately 45°C was added to petri dishes in which the diluted samples had been added. The agar in the petri dish was thoroughly mixed with the sample and left to cool and solidify before incubation.

The petri dishes were incubated at 37°C for 3 d in an inverted position in sealed anaerobic jars. The anaerobic conditions were created by using Anaerocult A® (Merck, Darmstadt, Germany). The strips of anaerocult were activated according to the manufacturer's instructions.

3. 10 Statistical analysis

The experimental design was a completely random design with the following variables: bacterial starter culture, temperature of incubation, temperature of storage, proportion of finger millet gruel and period of incubation. SAS (1982) was used to analyse the data based on the following model:

$$Y_{ijklm} = \mu + a_i + b_j + c_k + d_m + (ab)_{ij} + (bc)_{jk} + (ad)_{im} + (bd)_{jm} + (cd)km + (abc)_{ijk} + (abcd)_{ijkm} + \varepsilon_{ijklm}$$

Where:

Y_{ijklm} = the response variable

μ = the overall mean

a_i = change from the mean value due to the bacterial starter culture (i=1 to 3)

b_j = change from the mean value due to temperature (j=1 to 5)

c_k = change from the mean value due to proportion of finger millet gruel to skim milk (k= 1 to 7)

d_m = change from the mean value due to the effect of storage temperature (m= 1 to 2)

$(ab)_{ij}$, $(bc)_{jk}$, $(ad)_{im}$, $(bd)_{jm}$, (cd)km, $(abc)_{ijk}$ and $(abcd)_{ijkm}$ = the effects of interaction and

ε_{ijklm} = the random residual term.

The least squares analysis was used to obtain the least squares estimates of differences between sub units for comparison purposes. A probability level of $p = 0.05$ was used to test the significance of the results.