USE OF LOCALLY AVAILABLE MATERIALS IN THE DEVELOPMENT OF CULTURE MEDIA FOR THE PRODUCTION OF CHEESE STARTER CULTURES IN ZAMBIA

BY

GREYSON LYSON ELEMSON NKHATA

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CANDIDATE DECLARATION

I declare that this dissertation was written in accordance with the rules and regulations governing the award of Master of Science in Microbiology and that it is my own work which has not been previously submitted for degree purposes to this or any other University. Where others' work has been quoted or commended upon, acknowledgement has been made.

Signature of Candidate: .....

Date: 12th May, 1994

SUPERVISOR DECLARATION

I declare that this dissertation of Greyson Lyson Elemson Nkhata, written according to the rules and regulations of the university of Zambia, is now ready for examination.

Signature of supervisor.....

Date 12-05-95

A P P R O V A L

This dissertation of Greyson Lyson Elemson Nkhata is approved as part fulfilment for the award of a Master of Science degree in Microbiology by the University of Zambia.

Signature of Examiners:	Date:
1. Denys Morgan	30/06/95
2. M.M.Mwamba	30/06/95
3.	30/06/95

D E D I C A T I O N

To my family: My parents Mr and Mrs. E.C.K. Nkhata, my brothers Handsen, Moses, Mayingiso and Genesis, my wife Jean Mervis and my Children Anna, Suzyo and Samson for their untiring mutual support.

ABSTRACT

To determine which farms around Lusaka produce Cheese, a questionnaire was prepared and dispersed to dairy farms affiliated to the Zambia National Farmers Union (ZNFU) around Lusaka.

The Dairy Produce Board of Zambia (DPBZ), Pebble Brook Cheese Factory (PBCF) and Kaposhi Cheese Factory (KCF) were visited and modes of cheese processing studied.

Lyophilized stock cultures obtained from KCF, ZA de Buxiers BP10 of France and a mother culture from DPBZ were used as principal organisms to test the efficacy and sensitivity of all media used in this study.

Eight complex broth media were constructed in the preliminary study. Carbon, nitrogen and growth factors (organic and inorganic) sources varied both quantitatively and qualitatively. Buffering chemicals, distilled water, and sodium chloride were kept constant in amounts of the media dispensed in 250 ml. Pyrex conical flasks cotton wool plugged. They were sterilized by autoclaving ($120^{\circ}C$, 15 min, 0.41Kg/cm) and inoculated with a lyophilized starter culture prior to its activation in pasteurized DPBZ milk.

Based on absorbance readings and total colony counts, the quality of media beginning with the optimal was found to be in the following order: Milk(Mil) --> Soya flour, Orange, Yeast(Soy)--> Whey, Irish potato, Pumpkin (Wip) --> Carrot, Ammonium phosphate (Cap) --> Milk, Glucose (Mig) -->Brown wheat flour, Ammonium phoshate, Sucrose (Bas) --> Meat, Sugarcane, Tomato (Mst) --> Maize bran, Beans, Lettuce (Mbl). Further experiments to estimate the optimal concentrations of ingredients to the growth and propagation of the lactic bacteria were conducted with Mil, Soy, Wip and Cap media.

Lactic bacteria cultivated in Mil, Soy and Za de Buxiers BP10 lyophilized culture were activated in skimmed milk and used to produce six samples of Cream cheese at PBCF. The cheese samples were analysed to compare acidity, moisture and fat content at National Council for Scientific Research (NCSR).

Significant findings in this study are that local raw materials (milk, soya flour, orange juice, yeast, whey, irish potatoes, pumpkin leaves, meat broth, carrot, brown wheat flour, beans and lettuce) can be used to grow and propagate cheese starter cultures. This subculturing technique, when understood and effectively used by the Zambian cheese producers, will help reduce cheese production costs.

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LIST OF ABBREVIATIONS

PBCF	-	Pebble Brook Cheese Factory
ZNFU	-	Zambia National Farmers Union
DPBZ	-	Dairy Produce Board of Zambia
KCF	-	Kaposhi Cheese Factory
DMDT	-	Department of Manpower, Development & Training
SIDO	-	Smallscale Industry Development Organisation
UNZA	-	University of Zambia
NCSR	-	National Council for Scientific Research
FAO-RDDT	-	Food and Agriculture Organisation - Regional and Dairy Training Team.
IDF	-	International Dairy Federation
SPC	-	Standard Plate Count
Mil	-	Milk Medium
soy	-	Soya Flour, Orange, Yeast medium
Wip	-	Whey, Irish potato, Pumpkin medium
Cap	-	Carrot, Ammonium phosphate, Pine apple medium
Mig	-	Milk, Glucose medium
Bas	-	Brown wheat flour, Ammonia phosphate, Sucrose medium
Mst	-	Meat, Sugarcane, Tomato medium
Mbl	-	Maize bran, Beans, Lettuce medium
Min	-	Minutes
Skm	-	Skimmed Milk
Dist.	-	Distilled
Hr	-	Hour
NA	-	Nutrient Agar.
G + C	-	Guanine/Cytosine
g	-	Gramme
Bp	-	Boiling Point
C	-	Degree Celcius

CHAPTER ONE

1.0 INTRODUCTION

1.1 CHEESE

As defined by the FAO-RDTT (1990), cheese is a partially fermented, coagulated milk product with most of the milk components in concentrated form. The biblical cheese of David's time (1 Sm 17:18) was largely made to preserve milk constituents during a season when milk and possibly other sources of protein were scarce (Evans,1973). Today, cheese is an admirable convenience food, affording a wide range of flavours without the need of long and tedious preparation.

Cheese is made mainly in countries that have milk surplus though small amounts are made in developing countries where milk production is below the level required to provide the 0.5 litre per person per day, the lower nutritional limit (Evans, 1973).

Table 1 below shows milk produced and that converted into cheese as compiled by Evans (1973).

TABLE 1 CONVERSION OF MILK INTO CHEESE IN VARIOUS COUNTRIES (1969 VALUES, OBTAINED FROM FAO PRODUCTION YEARBOOK 24, 1970).

COUNTRY	MILK PRODUCTION (10001t.)	MILK CONVERTED INTO CHEESE AS % OF TOTAL MILK PRODUCED
Australia	7,807	10
France	31,010	24
Netherlands	7,922	35
New Zealand	6,479	15
U.S.A.	52,709	23.5
United Kingdom	12,764	10.7

Where lt. = Litre.

In Zambia, there is a paucity of information and interest concerning cheese-making technology, even amongst large scale dairy farmers in Lusaka Province. Commercially, only KCF and the DPBZ make marketable quantities of cheese. Small scale production of soft (Cottage type) cheese is very limited. Mrs Eileen Bender, a farmer's wife, has been the pioneer in the production of a high quality soft cheese, even for export.

One of the main reasons for low cheese production in Zambia, is insufficient milk production (Ng'ambi, 1992). The other reason is that locally produced cheese starter cultures and rennet are not available. The objectives of this research study are:- 1. To establish popularity of cheese starter culture media by excursions to cheese

processing plants in **Lusaka** region. 2. To study the techniques and conducive conditions that ought to prevail in processing a microbial medium meant for propagating and maintenance of cheese starter cultures. 3. To formulate and process cheese starter media. 4. To propose recommendations on production and use of local cheese starter culture media.

1.2 LITERATURE REVIEW

The origin of cheese making is lost in unrecorded history. Methods of making cheese are described by Greek and Roman writers several centuries before the birth of Christ (Foster et al.,1958). This is likely to have been between 9000 B.C and early biblical times (5000 to 3,000 B.C), when man learnt to domesticate cows (Ayres et al, 1980).

The first cheese must have been made from sour milk. When milk goes sour, the increasing acid curdles it into flocculated proteins, fat and fluid (Whey).

The next step is to pour the thickened mass in plaited baskets or perforated vessels like pots. The remaining fluid could thus be removed and a sort of fresh acid curd cheese is achieved.

In Egypt, in the tomb of Horus-aha, second king of first dynasty (3,000 - 2,800 B.C.), pots were found with what chemical analysis has virtually proved to be the remains of cheese while cave paintings in the Libyan Sahara, very much like those in Spain and France, show what appears to be milk processing and dates from the period 5,500 - 2,000 B.C. (Eekhof-Stork, 1976).

By 2,000 B.C. writings from the ancient East reveal that cheese was a cherished food and often used to trade for other items. The Greeks believed cheese to be of divine origin and offered it in sacrifices to their gods while the Romans favoured one type which was made on the island of Cyprus, as a delicacy (Ayres et al., 1980).

Cheese was first introduced into United States of America (U.S.A.) with the early settlers from

England, who brought both cheese and cows on shipboard (Ayres et al., 1980).

How cheese was prepared cannot be established archeologically, neither can the equipment used. The making of cheese is a domestic skill that became an art, an art that became an industry. The successful combination of art and industry has resulted in the manufacture of durable, high quality cheeses capable of being produced on a large scale and transported over long distances without much loss of original character.

Early cheese makers in a given locality made a cheese which when ripened under the conditions available, acquired certain characteristics of its own. Cheese made in other localities and subjected to different manufacturing and ripening conditions acquired other characteristics. Thus specific varieties appeared. Those with desirable qualities became articles of commerce and usually were named after the village or district in which they were manufactured. Today, there are hundreds of cheeses bearing different

names; France boasts of more than 300 different types of cheeses while Italy has at least 50 varieties (Evans, 1973).

In 1876, Louis Pasteur discovered the pasteurization of milk process which has since then found a permanent place in the dairy industry. Following the great advances made in the field of biochemistry, pure starter cultures and standardized rennets were prepared in laboratories. The mysteries of the ripening process and the complex actions of taste and aroma-forming bacteria were gradually unveiled. These discoveries and the many others that were bound to follow, gave the dairy industry the scientific backing that it needed. Production was improved and most important was the uniform quality and excellence guaranteed.

The early factories have now given way to ultramodern mammoth industries, where cheese is made from milk in one continuous process, fully automated and controlled not by skilled cheese makers but by operators.

1.3 CHEESE PROCESSING

1.3.1 MILK TREATMENT

To avoid undesirable fermentations the milk must be from healthy animals and also be of good bacteriological quality. Milk quality tests are routinely used. The methylene blue and resazurin tests are most widely used in cheese factories because of their simplicity. Additionally, many cheese makers perform some kind of fermentation test that supposedly shows the main types of organisms that may grow in the cheese. The test is performed simply by inoculating a sample of the milk with or without added starter culture and rennet and thereafter observing the types of changes that result. The test is most valuable for revealing gas-forming organisms. Since the milk must support active growth of acid forming bacteria, it must be free of inhibitory substances such as residual antibiotics used in treating mastitis.

For many types of cheese, the milk is run through a clarifier to remove extraneous matter. This

treatment probably has little influence on the quality of most cheeses, but for the Swiss, it has a beneficial effect on eye formation. Some Cheddar cheese makers also think clarification improves the quality of their products (Foster et al., 1958).

To reduce fat content, the milk is partly skimmed for many types of cheeses; the creamy layer of the evening milk is ladled off, and the next day the skimmed milk is mixed with the fresh morning milk. In a cheese factory, however, bringing the milk to the right fat content is called standardization and milk is not skimmed by a ladling process, as this would be too slow and costly, but instead a separator is employed. The latter separates milk into skimmed milk and cream. The skimmed milk is then mixed with whole milk till the desired fat content is achieved.

In most modern factories, before the fat content is determined, the milk must be pasteurized. During pasteurizing heat is limited to 75°C

(167 °F) at this temperature the milk is held for 15 seconds. Temperatures above 75 °C are avoided as milk tends to denature and lose some of its natural qualities.

After pasteurization, the milk is cooled to the curdling temperature (29 - 32 °C) and is said to have been "pre-treated".

1.3.2 MILK CURDLING

Cheese making is essentially a process involving concentrating particles of fat and protein in milk until they adhere, at the same time controlling types and activity of microorganisms present so that there is only limited lipolysis and proteolysis. With this process, ten volumes of milk can be reduced to about one volume of (semihard) cheese.

Fresh milk, if left standing long enough, turns sour and thickens by itself, producing a type of cheese that has been made for thousands of years. This traditional cheese processing technique relies on bacteria to present in the milk from

the surrounding environment to produce lactic acid. The bacteria are a mixed culture usually consisting of both harmful and non-pathogenic strains. Nowadays the natural bacteria are destroyed by pasteurization and special strains, non pathogenic but very active at producing lactic acid and sometimes with other characters required for cheese ripening, are purposefully added to cheese milk.

Curdling (coagulating) is the term used to describe the change of milk from a liquid to a solid or gel state by precipitation of casein (Foster et al., 1958). To understand the changes that occur during curd making, it is desirable to consider first the nature of the proteins in milk and the effect of the coagulating agencies on them. Casein is the principle nitrogenous constituent of milk, two and half per cent by weight (Evans, 1973). It is attached to small groups of phosphoproteins which in its natural state exists in combination with calcium (Foster et al, 1958).

In milk, the calcium caseinate complex is dispersed as a colloidal suspension of minute, gelatinous particles in the liquid phase. They have a great capacity for association to form aggregates called submicelles consisting of about thirty-five casein molecules and, in combination with calcium phosphate, fifteen or more sub-micelles associate to form a larger colloidal aggregate called casein micelle about one-tenth micro metre in diameter (Bickerstaff, 1987).

There are normally about one billion micelles in one millilitre of milk (Bickerstaff, 1987) and they are the ones responsible for the characteristic 'milky' appearance.

The structure of the casein particle is still not clearly understood but there are known to be at least three fractions designated as α, β, and ϑ which are surrounded by Kappa (K) casein (Foster et al., 1958, Bickerstaff, 1987).

Besides casein, other proteins in milk are lactalbumin and globulin. These are known as

whey proteins. They are very finely dispersed as a colloidal solution of the hydrosol type. Lactalbumin is a single entity while globulin consists of at least three fractions; α, ß, and ϒ -globulin (Foster et al.,1958). Whereas casein is precipitated by acid and rennin, whey proteins are coagulated by heat.

When the starter culture has acted on lactose to produce lactic acid, the milk pH drops to a range of pH 5.2 - 5.5 (Bickerstaff, 1987). This low pH creates a conducive environment for the rennet enzyme complex activity. Rennet consists principally of an acid protease, rennin (chymosin). It is extracted from calves' stomach. When a calf is older than thirty two days, its rennet enzyme complex consists of rennin and pepsin; thus the younger the calf, the more rennin dominates.

There are two most widely accepted theories on how rennin causes curd formation. One of these states that rennin attacks K casein, which functions as a

protective colloid to keep the other casein fractions in suspension (Ayres et al.,1980); this attack converts K casein from 30,000 Mm molecules to 8,000 Mm molecules and thus sticky patches on the surfaces of the micelles are exposed resulting in micelles aggregating together to produce the coagulum.

The other theory is that which states that rennin first acts on calcium caseinate to form calcium paracaseinate, a compound with greater base-combining power than casein. Next the paracaseinate reacts with free calcium ions and becomes insoluble dicalcium paracaseinate, thus precipitating to form the typical curd (Foster et al., 1958).

Whichever way rennin acts, it has been observed that coagulation is accelerated by heat and acid. Part of the function of the latter being to increase the concentration of calcium ions by dissolving calcium phosphate. Thus calcium chloride may be added in small amounts in milk with less of the substance (Foster et al.,1958).

Lactic acid in the milk causes casein to precipitate by reducing the pH to below the isoelectric point (pH4.7) and eliminating the stabilizing effect of repelling negative charges on the casein molecules (Ayres et al.,1980). At its isoelectric point, pH4.6 to 4.7, at 21°C, casein is in its purest form and at its lowest point of hydration, that is, its minimum solubility (Foster et al.,1958). As acid is formed by the starter organisms, calcium is gradually removed from the calcium caseinate to form calcium lactate.
When the calcium content of the protein is reduced to a low enough point, the casein precipitates (Foster et al.,1958).

Acid precipitation is employed in certain methods of making Cottage cheese and a few other types (Foster et al.,1958).

Acid curd is very fragile and must be handled carefully at first to avoid its dispersion as small particles, with consequent loss of yield of cheese.

Lactalbumin and the globulins are readily precipitated by heat. They comprise the bulk of the protein in the so called whey cheeses that are made by boiling whey (Foster et al., 1958).

Differences between rennin curd and acid curd are that; rennin curd is mainly dicalcium paracaseinate whereas acid curd has less of the calcium. The rennin curd is less fragile than acid curd and rennin curd shrinks more than acid curd during subsequent operations.

Extensive research studies aimed at finding a rennet substitute have and are still going on. The most promising results are those of rennilase, a milk clotting enzyme produced by a selected non-pathogenic strain of the fungus **Mucor miehei**. Rennilase has been found to exert a specific proteolytic effect on Kappa-casein which results in curd formation. It also exerts another slow non-specific proteolytic effect which is of significance to the cheese ripening.

1.3.3 CURD TREATMENT

Curd formation involves formation of a three dimensional web of casein in which fat globules (which form three and half per cent of the milk) become trapped along with the whey (Evans, 1973). Separation of whey from the curd particles is a very delicate part of cheese making process. Nevertheless, for all types of cheese the coagulum formed is caused to shrink by losing whey and becoming more firm. The degree of shrinkage determines the moisture content of the curd thereby affects to a large extent the final consistency of the cheese. It also determines the lactose content and, since most of this disaccharide is rapidly fermented to lactic acid, the acidity of the fresh cheese is related directly to the moisture content of the curd (Foster et al., 1958).

Curd shrinkage is favoured by heat, acid, and rennet while escape of whey is promoted by cutting the curd into small pieces, stirring and subjecting the mass to pressure.

To produce a cheese with low moisture and relatively low acidity, the cheesemaker does one or more of the following:

(a) heats the curd to a fairly high temperature,
(b) cuts it into small pieces,
(c) assures rapid acid development early in the making process or
(d) subjects the curd to high pressure.

Conversely, for a high moisture cheese, the curd is not heated, it is cut little, if at all, and acid develops after most of the whey drainage has ceased.

Methods of handling the curd to adjust its moisture content to the desired level are many. The procedure listed below, according to Foster et al. (1958), represents various possibilities, ranging from the method that results in least moisture loss to those that cause the most.

(a) The curdled milk is ladled directly from the vat into perforated molds or forms that retain the curd but allow whey to escape.

No pressure is applied. The method yields curd with high moisture, high acidity and a very soft consistency.

(b) The curd is cut into cubes, and part of the whey is allowed to separate, usually with stirring. The mixture is dipped into forms as in method (a). Light pressure may or may not be applied.

(c) The curd cut as in method (b) is heated or cooked before dipping into forms where pressure may or may not be applied. High cooking temperature and high pressure on the curd yield cheese with low moisture and relatively low acidity.

(d) The curd cut and cooked as in method (c) is left in the vat, and the whey is drained off. The curd particles may be kept distinct by frequent stirring, or they may be cut into the sizes desired and the pieces may then be placed in molds, or it may be chopped (milled) into small pieces, which are packed into molds and subjected to high pressure.

Methods (c) and (d) are used for cheese in which careful control of the final acidity is important. For most varieties this control is accomplished by reducing the moisture content to such a level that the lactose contained therein will, when completely fermented to acid, yield the desired pH.

For a few varieties it is desirable to have a fairly high moisture content yet a relatively low acidity (e.g. Brick, soaked-curd cheese). This can be accomplished by immersing the curd in water, thus removing part of the lactose without reducing the moisture content. By regulating this treatment, cheese can be made with almost any acidity desired.

1.3.4 SALTING

Sodium chloride is added to practically all varieties of cheese except those eaten shortly after making (Evans, 1973). The most common methods of application are to float the fresh cheese in a strong salt (brine) solution or to rub the surface with dry salt. The amount of

salt taken up by the cheese depends on the concentration of the brine, the time and temperature of exposure, the ratio of surface to volume of the cheese, and its moisture contents. At first the salt is most concentrated near the surface, but in time it diffuses fairly uniformly throughout the cheese.

Among its several functions in cheese, salt contributes to the flavour. In addition, it withdraws whey from the curd and thus helps control moisture and acidity. Of primary importance is the action of salt in controlling growth of undesirable microorganisms. Strongly proteolytic bacteria, for example, are sensitive to sodium chloride in the concentrations found in most cheeses (Foster et al., 1958).

1.3.5 CHEESE RIPENING

Cheese ripening, also known as cheese curing or maturing, is a process of converting the curd (a bland, slightly sour and somewhat salty taste when chewed, fairly tough and, with some

varieties, rubbery) into a palatable delicacy (Foster et al., 1958, Eekhof-stork, 1976,). Enzymes involved during cheese ripening come from three main sources; (a) rennet or other proteases preparation of animal or vegetable origin; (b) microorganisms that grow within the cheese or on its surface; and (c) the milk itself. Foster et al.(1958) observed the following in gross and chemical changes.

1.3.5.1 GROSS CHANGES

Changes in the elasticity, firmness, cohesiveness, and plasticity of the body are associated largely with enzymatic hydrolysis of the protein, that is, making the casein soluble. As a result, the cheese loses its toughness and elasticity becoming softer and, in low moisture cheese, more crumbly. If large amount of acid are developed during manufacture, the fresh curd tends to be crumbly and brittle and the body is described as ``short'' meaning that it will not stretch without breaking. Shortness is encouraged or discouraged depending on the variety of cheese, e.g.

the careful control of acid development in making Cheddar, Swiss, Brick and similar varieties is intended partly to avoid shortness of body and to retain the elasticity characteristic of these types of cheese.

Since scent and flavour are due to chemical substances, then the characteristic cheese flavour and taste must be a result of a balanced mixture of compounds. Cheese being mainly a mixture of fat, protein and water, the flavourful compounds can account for far less than one per cent of the cheese (Evans, 1973). Because flavour and taste compounds are present in minute quantities, knowledge of the specific compounds is difficult to obtain. While lactic acid and sodium chloride serve as background flavour giving compounds, more distinctive flavours are caused by products of decomposition of lactic acid, lactates, citrates, proteins and fats (Foster et al., 1958). For example, volatile acids such as acetic, propionic, butyric, caprylic, and capric, including their esters or

ketones, appear in varying amounts during ripening of many cheeses.

Texture, the amount of openness or gas space within the cheese, can be a quality determining factor; for instance cheese with no gas is said to have a close texture; that with considerable space has an open texture.

Openness may result from failure of curd particles to fuse during pressing or from subsequent gas production. With many types of cheese, a close texture is desired and any considerable amount of openness is considered to be a defect (e.g. Cheddar). With others (e.g. Roquefort) an open texture is necessary to permit vigorous growth of molds throughout the cheese. Moderate openness caused by gas formation is acceptable in some varieties (e.g. Brick and Limburger) and necessary in others (e.g. Swiss and Gruyere), but excessive or non-characteristic gas production is undesirable in all cheeses.

1.3.5.2 CHEMICAL CHANGES

The bulk of lactose disappears within the first few days after curd formation in most cheeses but may take relatively longer in very soft and high-moisture cheeses. Lactose is hydrolysed to glucose and galactose. The glucose is then readily fermented as opposed to galactose. Most of the sugar is fermented to lactic acid, but some of it is changed to volatile acids, alcohol, and small amounts of other products. Part of the lactic acid reacts with basic radicals in the cheese to form salts. In a few varieties a portion of the lactic acid is converted to propionic and acetic acids, and other compounds.

As ripening progresses, part or all of the protein is hydrolyzed enzymatically to simpler compounds that are soluble in water. The amino acids may be deaminated to yield ammonia and fatty acids or, less commonly, may be decarboxylated to liberate carbohydrate and amines. The extent of proteolytic action and the specific compounds

resulting therefrom help to determine the characteristics of the final cheese. In some of the softer cheeses such as Camembert and Limburger, practically all of the protein is converted to water - soluble compounds, including appreciable amounts of simple peptides, amino acids and ammonia.

In contrast, hard cheeses undergo relatively much less protein proteolysis. In Cheddar and Swiss, for instance, rarely more than 25 to 35 per cent of the protein is made soluble even on extended ripening.

Fat, though not as extensive as protein, also undergoes hydrolysis. Volatile lower fatty acids, including butyric, caproic, caprylic, and capric hydrolytic products are probably of greatest significance.

1.3.5.3 METHODS OF RIPENING

Cheeses are either ripened by holding the curd under conditions that discourage growth on the surface and limit acitivity to microbial as well

as enzymes activity inside the mass; this procedure is applied on the hard grating cheeses and with practically all of the hard varieties, or the cheese is held under conditions that favour growth of organisms on the surface, the so-called "smear" or "slime" development. With the latter, enzymes produced diffuse into the cheese and contribute to the ripening changes.

The surface smear technique is applied to soft and semi-soft cheeses; such cheeses are small in size to maximise the optimum surface area in relation to mass. The cheese ripened by this method is held at a high relative humidity to permit the surface growth. Surface ripening according to Foster et al.(1958), is usually faster than normal internal ripening. While all soft cheeses are ripened by the surface smear method, semi soft cheeses are ripened by a combination of both surface smear and internal methods.

Hard cheeses take long to ripen partly because of their large sizes (eg. Cheddar and Swiss).

During ripening of the hard cheeses, the relative humidity of the air in the curing room is kept fairly low to discourage surface growth but high enough to prevent excessive evaporation (Foster et al.., 1958). For some varieties of hard cheeses, loss of moisture is minimised by coating the surface with paraffin or a plastic film (Smulders, 1992). In long curing cheeses, a wide range of cheeses different in degree of ripenness can be produced within any single variety. In Blue cheese, a mold introduced in the interior of the cheese, spreads and causes blue-veining and often sharp, piquant taste, as in Roquefort.

The basic flavour of cheese is modified by differences in milk and in processing, by addition of herbs, seeds, spices, and vegetables during processing, by differences in wrapping and in maturation, by smoking (eg. Italian provolone and Indian Dacca) by salting and by selection of strains of bacteria and/or of moulds that exert their influence during making and maturation (Evans, 1973).

1.4 CHEESE CLASSIFICATION

The world of cheese, with its overwhelming variety of types, is almost impossible to classify as many countries apply their own system; for instance there are over 300 types of cheese in France and over 50 types in Italy (Evans, 1973).

In this study the approach of classifying cheeses adopted, is that used by Eekhof-Stork (1976), who recognized eight groups of cheese.

(A) The milk used is a very obvious criterion; and thus one can talk of cow, goat, sheep, buffalo, reindeer, or camel, cheese (Bickerstaff, 1978).

(B) The International Dairy Federation (IDF) places emphasis on consistency as a way of classifying cheese. Consistency is determined by the proportion of dry matter to moisture, the less moisture the cheese contains, the harder it is. The difficulty is that this ratio is never constant as it changes as the cheese gets older. Thus a mature semihard

cheese would be called "hard" when old and "soft" when young. According to the consistency, four groups of cheeses can be identified which include;

i) Fresh cheeses; these are not/or hardly ripened. They are eaten almost immediately. Examples are; Fromage frais, Speisequark and Cream cheese.

ii) Soft cheeses; these stick to the knife and are spreadable. They include Camembert, Limburger and Brinza.

iii) Semihard cheeses, these are firm but smooth and easy to cut. They include Gouda, Stilton and Fontina.

iv) Hard cheeses; these range from being hard to very hard, sometimes are difficult to cut but ideal to grate. They include Emmental, Sbrinz, and Parmesan.

(C) Another criterion of classifying cheeses is by considering the exterior - the rind; according to this approach, five groups of cheeses exist;

1. Those which have no rind or hardly any rind. This is the case with most fresh cheeses.

2. Those with a dry rind; a natural crust develops during ripening under normal conditions.

3. Those with a rind consisting of a white mould on the surface.

4. Those with a rind consisting of the orange-red smear of Corynebacteria on the surface ("washed cheese"), and

5. Those called Blue-veined cheeses due to a blue or greenish-blue mould spread throughout the interior of the cheese.

(D) Another, method of classifying cheeses is by grouping them according to fat content. This is calculated according to the percentage of fat in the dry matter of the cheese. For example, if the fat content is 50 per cent and the total dry matter 40 per cent, it

means that 100 parts of cheese contain 20 parts of fat, 40 parts of dry matter, and 60 parts of water. The content is expressed as a percentage of the dry matter because a cheese, as it gets older it loses moisture while the dry matter remains constant.

Some countries indicate fat content with the symbol '+' (e.g. 48 Positive) because the percentage is a minimum requirement and the actual fat content may be higher.

The categories mentioned above under A,B,C, and D are the easiest and most useful ways to differentiate the many cheeses of the world. However, cheeses can also be classified by the following ways:-

(E) By the way cheese is prepared; there are as countless recipes as there are variations on these recipes. Most popular cheeses are made according to one of the following methods;

i) Emmental production - This represents hard cheese with heated ("Cooked") and pressed curd.

ii) Cheddar production - This represents hard cheese with cheddared and pressed.

iii) Gouda production - This represents semihard, pressed cheese.

iv) Camembert production - This represents unpressed cheese with or without rind flora, and

v) Cottage production - This represents fresh cheese.

(F) Another way of classifying cheese is by the curdling method used; a division is made between curdling with the aid of rennet (most cheeses) and curdling without rennet.

(G) Yet still, cheeses can be classified by the mode of ripening; long or short-ripened, under moist or dry conditions, resulting in large, medium, small or no holes, etc. and finally,

(H) Cheese can be classified by observing differences in shape and weight of the cheese.

1.5 JUDGING CHEESE

A Cheese gourmet does or looks for the following in determining the quality of cheese as observed by Eekhof-Stork (1976): a good look to note the shape and smoothness; the cheese should be well shaped, neither bulging, too flat nor too thick, and a smooth, well sealed rind without cracks or cloth creases, shows it has been carefully produced. If a cheese has rind flora, it should be evenly distributed and free of any colouring that is not expected. The cheese paste itself should have no mould unless it is Blue cheese. It should have fresh colour - varying from white to light cream or ochre-yellow according to variety.

The cream surface should be unbroken; cracks indicate faulty production or poor storage.

When hard or semihard cheese is held next to an ear by one hand, taps with the other hand by a fist or a special cheese hammer, gives a characteristic hollow or dead sound; the dead

sound indicates a 'blind' body with hardly any 'eyes' or 'holes' thus by listening to the sound one can judge whether the holes are properly distributed over the body and whether they are too large or too small.

An instrument called a cheese trier or cheese iron is usually pushed into cheese and the boring tested. Questions answered by this test include; Is the trier well filled with cheese? Does the cheese look springy? Are there any holes? When the cheese trier is removed from the cheese or the cheese is cut, an aroma is released. Six basic aromas can be smelt; fruity, flowering, resinous, spicy, foul and burned. Experts, however, find cheese aroma either good, fresh, sweet or sour (Eekhof-Stork, 1976). The aroma of the particular milk with regard to the source is always there.

Sometimes, cheese may be mouldy, gassy or tainted with ammonia, none of which means necessarily that the cheese is of below standard. This could be a characteristic of a variety. An important fact

to bear in mind is that the full aroma is not released until the cheese has been brought to room temperature.

Taste invariably follows scent, there are four basic flavours; sweet, salt, sour and bitter. When the cheese is finely divided in the mouth, coming in contact with entire surface of the tongue, the aroma is fully released to the senses. Then, memory stores the experience, ready for future acceptance or rejection, it then becomes a personal matter outside of the practical judgement of experts.

Soft varieties of cheeses are often judged in the first place by touch; the resilience of the body is checked by pinching. This way, one can check whether the cheese is firm or pliant and soft, that is to say, unripe or well ripened respectively. If the body of a soft cheese can be pressed out of the rind, it is of excellent quality.

To make sure that the consumer gets what he expects, most countries have grading and

supervision systems. Cheeses are graded and classified and given a marking that tells the original fat content, type and the quality of the cheese.

1.6 CHEESES MADE IN AND AROUND LUSAKA

DPBZ, KCF and PBCF were visited while Galaunia Ngwerere cheese plant was not visited because cheese production was suspended. Other farmers with dairy cattle who are affiliated to the Zambia National Farmers Union (ZNFU) around Lusaka were asked to respond to a few questions on their knowledge as well as their interest in cheese technology.

1.6.1 DAIRY PRODUCE BOARD OF ZAMBIA

At DPBZ Lusaka Plant, only soft cheese (Feta) is processed; the manufacturing process takes about 12 hours. The process begins with milk fat standardization using a separator. Standardization is followed by pasteurization; when milk has cooled, cheese starter culture, calcium chloride, potassium chloride and rennet

are added. The milk is then allowed to stand for about 45 minutes. At the end of this time the curd formed is transferred into a cheese tray called `hoop´. The whey left in the milk cane is mixed with sodium chloride and stirred vigorously by steam. The steam has a duo function; dissolving the salt and sterilizing the solution. The milk can is then tightly closed and left to stay over night.

In the meantime, the curd is wrapped tightly in a cheese cloth and a heavy sterile plank is placed on top of the curd; this helps in pressing the curd and hence forcing the whey out of the former. The curd is left to stay in this position for the night.

The following day the curd is unwrapped and placed on a sterilized table where it is carefully cut into small cubes which are transferred into the salted whey. The Feta cheese is then ready for marketing.

The Lusaka DPBZ cheese plant cheese processor, Mr. V. Sakala, commonly known as a cheese man, mentioned that the customers of his cheese are mainly foreigners who buy it to serve as an ingredient in recipes like those of cakes, porridge or gravy soup. And the factory production manager, Mr. R.M. Simphanya, mentioned that where as the workers tend to scrumble for Feta cheese at the end of each year during the party the board throws, cheese is too expensive for the average Zambian.

1.6.2 KAPOSHI CHEESE FACTORY

At KCF, the cheerful and very kind production manager, is Ms. K. Smulders from Holland.
The dairy farm has about 300 Friesian dairy cattle with milk production capacity of 300,000 - 400,000 litres per day.

Of this milk produced each day 300,000 litres of the milk is processed into cheese while 100,000 litres is stored and every third day of storing, 300,000 litres is supplied to DPBZ Lusaka Plant.

The first stage of cheese processing is pasteurization; the pasteurizing unit, though still functioning well, is old and would have to be replaced in the near future. After pasteurization, the milk is divided into three fractions and each going into its own cheese vat. Addition of necessary ingredients such as cheese starter cultures and rennet, according to the type of cheese (whether Cheddar, Cheshire or Gouda) is then done. The process of making Cheddar and Cheshire cheeses takes about five and half hours while that of Gouda cheese takes three hours, prior to maturation.

While the curd is forming, the whey draining off from cheese vats goes into conduits which lead it to calves or pigs.

When the curd has been moulded into the desired shape and has been smoothed, it is taken for salting. Salting for Cheshire and Cheddar cheeses is done directly, that is, sprinkling sodium chloride directly onto the curd while that

for Gouda cheese is done by submerging the curd into the brine solution where it is left for one and half days.

After salting, the curd is taken for waxing. This process is carried out in order to protect the cheese from fungal and bacterial attack. During waxing carotenoides and other similar pigments can be added so as to make the cheese colourful.

After waxing, the cheese is taken to cold rooms for ripening. Cheddar and Cheshire cheeses take about six months to ripen while Gouda cheese takes about three months.

At KCF only imported commercial starter cultures are used as there is no provison for subculturing. Subculturing is discouraged because of the high risk of contamination by undesirable and sometimes pathogenic microorganisms.

1.6.3 PEBBLE BROOK CHEESE FACTORY

PBCF, situated some twenty kilometres South-East of Lusaka, is a small scale factory. The production manager, Mrs. E. Bender buys 300 litres of milk every day from a dairy farm along Chalimbana road.

At the factory milk is pasteurized by heating to 72°C for 30 seconds then cooled to 18 - 22°C. The starter culture and rennet (both imported from Denmark) are then added. The milk stays in the cheese vat till enough acid has been made for the curd to cut. The curd is then transferred into a presser where it stays while being pressed by a weighty object for about eight hours. Thereafter it is tranferred into small pieces of cloth in which the small pieces of curd are left undisturbed for 30 to 60 minutes to allow more moisture loss till the curd is firm enough. Before the small cheese lumps are made, the cheese curd in a container is salted by addition of ten per cent Sodium chloride directly into the curd where it is mixed mechanically.

The curd is then wrapped in plastic after being weighed (800 g) and is moulded into a smooth oval or sphere shape.

PBCF operates on a small scale. The subculturing technique of the lactic acid bacteria is brilliantly done. Of the lyophilised lactic acid bacteria, 2.5 ml is added to 50 ml of skimmed milk. This then becomes an inoculum for one to two weeks. Only 10 ml of the inoculum is used to inoculate 250 ml of milk. The later, when incubated at 37 °C for about 24 hours is used to inoculate one cheese vat (about 30 litres capacity). The factory uses one ampoule of the lyophilised commercial culture every 8-12 months.

1.6.4 QUESTIONNAIRE

The questionnaire was sent out to farmers affiliated to the ZNFU around Lusaka. Farmers were asked to give a statement about their involvement in cheese production. Only Galaunia dairy farm responded positively in the sense that

they used to make cheese while others have not as yet made any cheese.

At the Galaunia Ngwerere Cheese Plant the following types of cheese used to be made prior to 1992; Gouda, Cheddar, Mozzarella, Pecorino, Parmesan and Asiago. At the time of replying to the questionnaire, the respondent stated that they were only manufacturing Mozzarella cheese but when their head office was contacted to confirm, it was discovered that they have completely suspended cheese production.

Reasons advanced for not making cheese on other dairy farms are that some farms are not electrified, others do not know cheese technology. Similarly, there is a general lack of the necessary equipment available in Zambia.

1.7 MICROORGANISMS USED IN THE DAIRY INDUSTRY

Microorganisms, most of which are either homofermentative-fermenting lactose to chiefly

lactic acid or heterofermentative-producing other substances besides lactic acid, produce lactic. The lactic acid which provides an optimal environment for coagulum formation besides inhibiting the presence of unwanted non-lactic fermenting. Whereas the traditional small scale cheese producer relied on naturally occurring microorganisms found in fresh milk, modern cheese factories eliminate such organisms by pasteurization and purposely add the carefully isolated strains which have been proved as suitable for starter cultures.

Microorganisms also contribute to cheese ripening (maturing) by secreting enzymes or merely their presence in or on the cheese. With the help of microorganisms biochemical processes such as lipolysis, proteolysis and degradation of the products of these, the reactions are made possible.

For any given cheese, at least more than one strain of microorganism is required for the

processing, that is, a form of synergism is part of cheese technology. For example, in Swiss cheese manufacturing, **Streptococcus thermophilus** contributes to lactic acid production, **Lactobacillus cremoris** is essential for diacetyl production, while **Propionibacterium freudenreichii** is required for 'eye' formation.

1.8. STREPTOCOCCACEAE

These bacteria consist morphologically of spherical or ovoid cells arranged in pairs or chains. Cells are usually non-motile and do not form endospores.

All species of Streptococcus are Gram-positive and ferment carbohydrates with the production of lactic acid but never form gas. The catalase test is variable and the benzidine test is negative. They are facultatively anaerobic, with G + C content of DNA ranging from 33-44 moles per cent (Foster et al., 1958, Buchanan et al., 1974, Wilson et al., 1975).

Genera of the **Streptococcaceae** needed in cheese starter cultures are; **Streptococcus** and **Leuconostoc**.

1.8.1. STREPTOCOCCUS

The term '**Streptococcus**' was first applied by Billroth and Ehrlich in 1877 to a chain forming coccus that they saw in infected wounds (Wilson et al., 1975).

Most **Streptococci** produce a group specific polysaccharide (C substance) which enables one to classify them serologically by a precipitin reaction (Lancefield typing) into Lancefield groups with capital letter designations (A,B,C,D, etc) (Foster et al., 1958, Frazier et al., 1978)

âStreptococciÇ found in milk and milk products are known as lactics and serologically belong to group N. They grow at 10 °C and not at 45 °C. **Streptococcus** decolourise litmus milk before clotting and usually survive 60 °C for 30 minutes, (Wilson et al., 1975).

Of the **Streptococcus** genus, only the lactic **streptococci** have been found to have desirable character in the cheese industry. The three most commonly applied spp are: **S. lactis**, **S. cremoris**, and **S. thermophilus** (Frobisher et al., 1974, Schlegel, 1986).

S. lactis is always present in market milk even of the best quality! (Frobisher et al., 1974). It occurs in cow dung, dust, soil, on plants and utensils. Its persistence in such environments shows that it is a relatively hardy organism (Foster et al., 1958). It occurs as elliptical or oval cocci in pairs or short chains and rarely in long chains. The individual cells range from 0.5 to 1.0 micro metre in diameter. It is Gram-positive, non-motile, asporogenous, and stains easily with common bacteriological dyes. S. lactis belongs to group N antigen and grows at 10°C but not at 45°C (Foster et al., 1958, Frobisher et al., 1974, Wilson et al., 1975). S. lactis does not survive 63°C for 30 minutes and hence is a post-pasteurization contaminant if found in pasteurised milk (Foster et al., 1958).

S. lactis, usually mixed with **S. cremoris**, is used as an acid producer-except the subspecies **S. lactis** subsp. diacetylactis which when present in the curd, contributes to cheese flavour imparting substances (Ayres et al., 1980).

S. cremoris, like **S. lactis** serologically belongs to group N antigen. It grows at 10 °C but not at 45 °C and reduces litmus milk prior to coagulating it. Apart from the tendency of occurring in long chains, it has many properties resembling those of **S. lactis**, for example, both in starter cultures are chosen as acid producers having weak proteolytic and lipolytic activity. They have approximately the same vitamin and amino acid requirements, but can be differentiated in sofar that **S. lactis** does not produce ammonia from arginine (Foster et al., 1958, Buchanan et al., 1974, Wilson et al., 1975).

S. thermophilus is commonly used in dairy products. It is not truly thermophilic but

thermodiuric surviving temperatures as high as 63 °C (Pasteurization) and thus may appear in large numbers in pasteurized milk. It's 'pin point' colonies on agar plates are used to determine the number of bacteria in milk. It grows well at 45 °C with a growth range of 40 - 45 °C. **S. thermophilus** resists heating at 60 °C for 30 minutes and it can not grow at 10 °C. It belongs to viridans group which is serological group S. **S. thermophilus** coagulates and reduces litmus milk and hydrolyses arginine (Foster **et al.**, 1958,, Frazier **et al.**, 1974, Wilson **et al.**, 1974, Ayres **et al.**,1980).

S. thermophilus is generally used in the manufacture of cheese whose curd is cooked at high temperatures e.g. as in Swiss cheese.

1.8.2. **LEUCONOSTOC**

This genus was known earlier as **Betacoccus** by Orla-Jensen (Foster et al.,1958,Frazier et al.,1974) and the present generic name is derived from the Greek words `Leucus' or `Leukos' meaning colourless or white and `nostoc' referring to a genus of encapsulated cyanobacteria.

Leuconostoc, the bacterium resembles **Nostoc** in forming spherical cells in tangled chains and in general forming thick outer coatings of slime or gum in sucrose media but **Leuconostoc** bacterial cells are small (1.0 to 1.5 micro metres long) (Foster et al.,1958, Frobisher et al.,1974).

Leuconostoc is pleomorphic and often resembles **Lactobacillus**. It is distinguished among the lactic acid bacteria in being heterofermentative - fermenting sugar to lactic acid plus considerable amounts of acetic acid, ethyl alcohol and carbon dioxide (Frazier et al.,1974, Frobisher et al., 1974).

Leuconostoc is found on the leaves of green vegetables, also in butter, sour milk, cream and fresh milk. Some of the characteristics of Leuconostoc species that make them useful in the production of dairy foods are:

1. Production of diacetyl and other flavouring products.
2. Tolerance of salt concentrations, e.g. in sauerkraut and dill-pickle fermentation.
3. Ability to initiate fermentation in vegetable products more rapidly than other lactic bacteria or other competing forms and to produce enough acid to inhibit nonlactic bacteria.
4. Tolerance to high sugar concentrations (up to 55 to 60 per cent), permitting the organism to grow in syrups, liquid cake and ice-cream mixers etc.
5. Production of considerable amounts of carbon dioxide gas from sugars, for cheese 'eye formation' in some cheeses, undesirable spoilage of foods high in sugars and to leavening in some breads (Frazier et al.,1974).

The two common **Leuconostoc** spp. in the cheese industry are **L. cremoris** and **L. dextranicum**. The old name of **L.cremoris** is **L. citrovorum** as cited in Foster et al.(1958).

Differences between **L.cremoris** and **L. dextranicum** are minor; both are Gram positive cocci, 0.6 to 1.0 micro metre in diameter and cell division occurs in two planes resulting in pairs and tetrads. They are both aerobic or facultatively anaerobic and require supplements of yeast or beef infusion for optimal growth at 8°C but not at 45°C and they do not survive pasteurization (Foster et at, 1958, Buchanan et al.,1974).

The ability of the two spp; **L. cremoris** and **L. dextranicum,** to ferment citric acid of milk and produce diacetyl, the more reduced acetoin and 2,3-butanediol and to stimulate lactic streptococci has led to their inclusion as cheese 'starters' in the cheese industry (Frazier et al.,1974).

1.9 LACTOBACILLACEAE

The term 'Lactobacillus' is derived from the latin word 'lactis' for 'milk' and 'bacillum' for 'staff' or 'stick', hence a rod shaped organism (Foster et al.,1958, Frobisher et al., 1974). The first member of the Lactobacillus genus was isolated by Kern (1881, in Wilson et al., 1975) from the fermented milk of the Caucasus known as Kefir. Kern called it Dispora kausisca which today is called L. caucasicus.

Lactobacilli comprise of straight or curved rods usually occurring singly or in chains. They are usually non-motile and vary from long and slender to short coccobacilli. Chain formation amongst Lactobacilli is common particularly in later logarithmic phase of growth. Lactobacilli do form spores but give a positive Gram test becoming Gram-negative with increasing age and acidity. They ferment sugars to chiefly lactic acid if homofermentative or to lactic acid, acetic acid, carbon dioxide, and trace products if heterofermentative. Lactobacilli rarely reduce

nitrate and do not liquefy gelatin. Neither do they digest casein nor produce indole and hydrogen sulphide.

Lactobacilli surface growth on solid media is often enhanced by anaerobiosis and 5 - 10 per cent carbon dioxide. The growth temperature range is 5 - 53 °C with an optimal range of 30 - 40 °C. **Lactobacilli** are usually killed by heat of about 60-65 °C for 30 minutes. They are aciduric and grow well in an optimum pH 5.5-5.8. **Lactobacilli** are present in dairy products, effluents, grain and meat products, water, sewage, fruits, pickled vegetables, and intestinal tract. Their G + C content of the DNA ranges from 34.7 - 53.4 per cent moles. They are non pathogenic to man and laboratory animals (Foster et al., 1958, Frazier et al.,1974, Buchanan et al.,1974, Wilson et al., 1975,).

Lactobacilli involved as cheese starters include : **L. helveticus**, **L. bulgaricus** and **L. casei** (Frobisher et al., 1974, Schlegel,1986).

L. helveticus and L. bulgaricus are *homofermentative*. They produce lactic acid as the major product from glucose (generally 85 per cent or more). They do not produce gas from glucose or gluconate fermentation. Ribose is not fermented. Their G+C ratio is 34.7 - 50.8 per cent. They generally grow at 45°C or higher and not at 15°C. Their colonies are normally rough becoming smooth and compact in the presence of Tween or sodium oleate (Rogosa et al., 1950 in Buchanan et al., 1974).

L. casei, though homofermentative like L. helveticus and L. bulgaricus differs from the latter in belonging to group B (Buchanan et al., 1974). L. casei does not produce gas from glucose, gluconate, or ribulose. L. casei does need thiamine for its growth. Its optimal growth occurs at 15°C and has a G+C ratio of 45-46.4%.

L. bulgaricus was isolated by Grigoroff (1905 in Wilson et al., 1975) from the fermented milk of Bulgaria. It is a large rod ranging from 2-20 m long and about 1 μm broad with parallel sides and slightly rounded ends Its cells are arranged singly or in short chains. It is non-motile but gives a positive Gram test (Wilson et al, 1975).

Two morphological types **L. bulgaricus** were described by White and Avery (1910, in Wilson et al., 1975): type A consists of chains of short bacilli with oval or reniform nodules extruding from the cell substance which stain uniformly while type B comprise long bacilli arranged singly, having spherical bodies attached to the cell wall but not stemmed bodies as in A. Cells in B show intense granular staining with Loefflers' methylene blue or Neissers' stain and grow best at 45 °C (Gibbs et al, 1966).

L. bulgaricus, in serological group E (Foster et al, 1958), is amongst the strains in the starter cultures used for Swiss cheese production (Ayres et al, 1980).

L. helveticus was isolated by Orla-Jensen (1919 in Wilson et al., 1974) from sour milk and Emmental cheese. It is a large bacillus which forms long rods and filaments. It produces a high degree of acidity in milk, and forms inactive lactic acid. **L. helveticus** ferments glucose, lactose, maltose, and usually trehalose and dextrin but does not hydrolyze arginine or aesculin. It grows best at 45°C but not at 15°C and requires riboflavin and pyridoxal for its growth (Gibbs et al., 1966 and Wilson et al., 1975).
L. helveticus is amongst cultures used as starter cultures in manufacturing of Emmental cheese (Ayre et al., 1980).

L. casei is a pleomorphic bacillus. Sometimes it is coccoid. It occurs characteristically in short or long chains, some of which may be long and tangled closely resembling **streptococci**.
For its growth, riboflavin, pyridoxal and folic acid are essential. On nutrient agar medium, deep colonies are smooth, compact and shaped like a disc or a biconvex lens. It grows best at 30°C

but not at 15°C (Biggs et al., 1966, Wilson et al., 1975). In the cheese industry, L. casei is added to cheese milk as a starter culture for the maturation of hard cheeses (Schlegel, 1986).

1.10 PROPIONIBACTERIACEAE

Members of this family are like **Lactobacillus.** They are non-motile, nonsporeforming but give a positive gram test (Frobisher et al., 1974). They are generally short rods with rounded ends but may assume very pleomorphic, club-shaped, and branched forms. Unlike **Lactobacillaceae**, most **Propionibacteriaceae** produce catalase. They are nutritionally fastidious, facultative anaerobic and heterofermentative (Frobisher et al., 1974, Schlegel, 1986).

Propionibacterium does not produce lactic acid from carbohydrate fermentation but ferments it to produce carbon dioxide, acetic acid, and propionic acid (Frobisher et al., 1974). **Propionibacterium freudenreichii** subspecies

shermanii and **P.acidi-propionici** (formerly called **P. pentosaceum**) are the best known (Schlegel, 1986). **Propionibacterium** requires supplements of yeast extract and fermentable carbon compounds for optimal growth; with a growth temperature range 15 - 45 °C, growing best at 30 °C. A salt concentration of 10 per cent prevents their growth (Foster et al.,1958). **Priopionibacteria** are commonly found in hard cheeses where during ripening they contribute flavour and formation of eyes in the cheese (Schlegel,1986, Frobisher et al., 1974).

1.11 PENICILLIUM spp

Penicillia consist of conidiophores which are basically hyphae that branch at the tip into finger-like clusters of sterigmata. Spores extend in chains from the ends of sterigmata. This arrangement gives the whole conidiophore, with its chains of conidiospores, a form suggestive of a tiny paint brush from which the generic name is derived (**L. penicillus** = paint brush or pencil) (Frobisher et al., 1974).

Penicillium roqueforti is used in the ripening of Blue cheeses. A common practice in processing Blue cheese, is to separate cream from raw milk, bleach the cream with benzyl peroxide, then homogenize it and recombine it with the skim milk to provide a fat content of about 3.8 per cent, the bleaching provides a white cheese against which the blue mould, **P. roqueforti**, distinctly and attractively appears (Ayres et al., 1980).

Another **Penicillium**, **P. camemberti**, is involved in the ripening of Camembert cheese, manufactured in France since 1791. The greyish conidia, of **P. camemberti** produced are useful in the ripening of the cheese. The mould is sprayed onto the cheese curd on the second day and thereafter the curd is cured at 12 °C (Ayres et al., 1980).

1.12 CULTURE MEDIA

Cultivation of microbial culture involves provision of optimal growth conditions; nutrients, growth factors (organic and inorganic), pH, temperature, salt concentration, water, and aeration usually in vitro (L. vitro = glass) but can also be in vivo (i.e. in living

cells or tissues) resulting into growth - an increase in number or size of the organisms or both.

Joseph Lister (1827-1912), physist, physician and pioneer in aseptic surgery, described a method of isolating bacteria from aqueous suspensions by successive dilutions of the suspensions in sterile fluid to the point that one tube among the highest dilutions would contain only one of the desired organisms, a point extremely difficult to determine. He succeeded but rarely, and perhaps only three or four of his contemporaries were ever able to succeed with the dilution method (Frobisher et al.,1974).

In 1872, a Microbiologist named Schroeter had observed the growth of different sorts of bacteria in isolated masses (colonies) of various colours on slices of decaying potato. With his microscope he saw that all the microorganisms in any one colony were always exactly the same. It was obvious that by cultivating microorganisms on solid nutrient surfaces, it was possible to obtain isolated colonies of any single kind- each a pure culture.

Extending this principle, Koch (1880) used a five to ten per cent gelatin solution to prepare a transparent solid jelly with a moist, sticky nutrient surface on flat pieces of glass. However, gelatin melts at body temperature and, being a protein, it is often digested and liquified by the metabolic processes of the microorganisms. Besides that, if particles of dust settle on it, results are obscured and hence confused (Frobisher et al, 1974).

The wife of W. Hesse (a microbiologist) in 1881 suggested use of agar-agar (commonly called agar) as a substitute for gelatin. This gum is a polysaccharide derived from a seaweed (**Rhodophyceae** and a sp. of **Gelidium**) (Blair et al., 1970, Frobisher et al., 1974). It melts at 80°C and gels at 39°C. Agar, non-nutritive, indigestible to most bacteria and humans, and transparent with no colour, has since then to-date proved very useful as a solidifying agent for culture media.

However, chemolithotrophic microorganisms of the soil are 'poisoned' or inhibited by the presence of an organic substance such as commercial agar and besides these, a number of species of the heterotrophic microorganisms of the sea and soil readily digest and liquefy agar. For these, silica (Silicon oxide) was found to be an acceptable substitute for agar when made to assume a jelly like state (Silica gel) (Blair et al., 1970).

Polyacrylic gels, agarose along with agarpectin which are agar dissociation products, are used as solidifying agents in immunoelectrophoretic diffusion tests where clarity is especially important (Blair et al., 1970).

Historically, culture media were natural materials, or infusions thereof, used for human nutrition, milk, eggs, potatoes, meat, etc. These materials are still in use today, but most modern media formulations frequently contain more defined components, such as the peptones.

Media, chemically undefined are known as complex or empirical media while those whose chemical composition is known are called defined or synthetic media (Frobisher et al., 1974).

1.13 NUTRIENTS IN CULTURE MEDIA

In preparing a medium for any microorganism, the primary goal is to provide a balanced mixture of the required nutrients at concentrations conducive for optimal growth.

In industrial fermentations, ingredient composition of the formulations, are carefully controlled to a specification often regarded as confidential `know-how' (Rhodes et al, 1969).

The type of medium used depends upon several interrelated variables of which cost, availability and standardization of the individual raw materials are of primary concern.

For instance, a cheap raw material is of little use if the world supply is severely limited, and an abundant waste product is equally undesirable if it cannot meet a standard specification. It is also important to relate the medium ingredients to the type of product required e.g. vitamin B12(B compound) as an end product of microbial metabolism then a cobalt salt must be amongst the ingredients required.

Nutrient media form the environment in which microorganisms grow and from which they draw substances necessary to ensure the synthesis of cellular components and to produce the energy needed for biochemical processes. As such, the medium has to contain individual substrates in a form best accessible to microorganisms which usually means in solution. Main substrate ingredients include; water, carbon, nitrogen sources and growth factors (vitamins and mineral elements).

Water is required for metabolic activities. It is also needed as a solvent for various ingredients.

lower-quality water chemical composition usually involves removal of iron and free chlorine. Hard water is treated by removing calcium ions. Microorganisms are either removed by filtration or killed by sterilization. Finally, colloids are removed by reverse osmosis (Sikyta, 1983).

If lower-quality water shows traces of ammonia, salts of nitrous or nitric acids, it is polluted. Such water is not suitable for microbial media preparation as it unfavourably affects the physiological state of microorganisms (Sikyta, 1983).

Quantitatively, carbon sources represent the main component of nutrient media. They serve not only for the biosynthesis of cell matter, but also as the main source of energy. The nature of the microbial process requirements for product purity

and the metabolism of the production strain may dictate the use of chemically defined carbon sources such as glucose, sucrose, lactose, or starch, wood hydrolysates, cellulose, potatoes, maize, wheat, barley, whey and vegetable oils. Some branches of the microbial industry have recently begun using entirely new substrates such as synthetic alcohols and n-alkanes (Sikyta, 1983).

Next to carbon sources in quantitative composition are nitrogen sources. Potato liquors, yeast extracts, corn-steep liquor, soy bean and peanut flour are among important natural sources of nitrogen.

Growth factors, vitamins and mineral elements, are usually present in natural sources of carbon or nitrogen compounds when these natural substrates are used as the only sources. Table 2 shows content of vitamins in some industrially important raw materials.

TABLE 2: CONTENT OF VITAMINS IN SOME INDUSTRIALLY IMPORTANT RAW MATERIALS: after Sikyta (1983).

VITAMIN (µg/g)	MAIZE FLOUR	BARLEY	S/BEAN FLOUR	BEET MOLAS.	CANE MOLAS.	YEAST EXTRACT	STILLAGE
Thiamine	4.5	6.5	13.5	0.8	0.8	10	3.5
Riboflavine	0.9	1.2	3.5	-	-	20	11.9
Nicotinic-acid	23.0	115.0	25.2	35.0	15.0	400	75.8
Pantoth - enic acid	4.6	4.4	26.1	50.0	20.0	50	10.8
Pyridoxine	6.9	11.5	8.5	-	-	25	1.0
Biotin	0.1	-	0.7	0.1	1.5	1	0.3
Inositol	-	-	3850.	5000.	2000.0	1500	7170.0
Chlorine	-	-	1100.	2880.	-	-	3080.0

It is sometimes necessary to add additional growth factors in some industrially important raw materials. Mineral elements required for microorganisms structural or physiological functions include; hydrogen, oxygen, phosphorus, sulphur, potassium, calcium, magnesium, sodium and sometimes chlorine and the following are required in trace (minute) quantities: manganese, copper, zinc, molybdenum, cobalt and boron (Rhodes et al, 1966).

1.14 HYDROGEN ION CONCENTRATION

The attainment of the optimal initial pH (degree of acidity i.e. H+ ion concentration or alkalinity ie. OH- ion concentration) and its maintenance during cultivation of microorganisms is of great importance particularly for acid producers which are not acid tolerant. Most organisms grow best when H+ and OH- ions are present in approximately equal concentrations (pH7). However, while many prefer alkaline medium, only a few are acid tolerant (Schlegel, 1986).

To avoid self-poisoning of long term cultures by acid production, the media must be buffered or be free from fermentable substances. The phosphate buffers consisting of monohydrogen phosphate mixtures, are the most useful ones. KH_2PO_4 (potssium dihydrogen phosphate) is a weakly acidic salt and the other K_2HPO_4 (potassium monohydrogen phosphate) is slightly basic so that an equimolar solution of the two is very neutral, having a pH of 6.8. If a limited amount of strong acid is added to such a solution, part of the basic salt is converted to the weakly acidic one:

$$K_2HPO_4 + HCl \longrightarrow KH_2PO_4 + KCl$$

If however, a strong base is added, the opposite conversion occurs.

$$KH_2PO_4 + KOH \longrightarrow K_2HPO_4 + H_2O$$

But when a great deal of acid is produced by a culture, the limited amounts of phosphate buffer that may be used become insufficient for the maintenance of a suitable pH. In the case of more pronounced acid production, addition of calcium carbonate to the medium or sodium bicarbonate if insoluble components are undesirable, is recommended.

In the presence of hydrogen ions, carbonate is converted further to carbonic acid which decomposes spontaneously to carbon dioxide and water.

$$CO_3^{2-} \underset{-H^+}{\overset{+H^+}{\rightleftharpoons}} HCO \underset{-H^+}{\overset{+H^+}{\rightleftharpoons}} H_2CO_3 \rightleftharpoons CO_2 + H_2O$$

The damage produced by an unsuitable pH is not actually due to the hydrogen and/or hydroxyl ions,

these only increase the undissociated proportion of weak acids or bases which penetrate more rapidly into the cell than their dissociation products.

For example, cells take up the dibasic succinic acid and tribasic citric acid more rapidly the lower the pH of the medium is (Schlegel, 1986).

The cost of buffers (such as phosphates) to control pH is negligible for laboratory work but would be near prohibitive industrially. Cheaper materials e.g. hydrochloric acid, sodium hydroxide or ammonia are usually used to adjust the pH, being added according to the results of successive titrations or in response to a signal from a pH electrode immersed in the medium (Riviere, 1977).

As a rule, increases in temperature leads to an increased dissociation of acids. Thus, a solution which is neutral or slightly alkaline, and therefore favourable to growth of most

microorganisms at room temperature (about 22°C), may become definitely acid and lethal if incubated at a commonly used incubator temperature (37°C). Similarly, if a nutrient solution is prepared at a definite pH while near the boiling point, it will be more alkaline when cool (Frobisher et al.,1974).

1.15 OSMOTIC PRESSURE

If living cells are immersed in fluids with an abnormally high osmotic pressure, exomosis of water will occur, being drawn out of the cells until they rupture (plasmoptysis). Ordinary osmotic potentials within the cell are sufficient to keep it slightly distended (turgid). Extremely hypertonic solutions like pickling brines and concentrated sugar syrups have a "preservative" value because they withdraw water from cells and hence they have a microbiostatic effect (Frobisher et al., 1974).

Probably because of their minute volume, relatively strong cell wall, and thin cytoplasmic

membrane which permits rapid adjustment of osmotic equilibria, most bacteria are not highly sensitive to variations in salt concentrations between half and three per cent. Concentrations much above this may adversely affect some of more sensitive strains (Frobisher et al., 1974).

Even though the favourable osmotic pressure of a given culture medium may continue unchanged, there often appears in it, as a result of aging and metabolic activities, substances that alter the permeability of the cell membrane so that excessive water diffuses inward and the cells become swollen and distorted. Such alternative substances may be various waste products (e.g., acid or alchols) of cells that have grown in the culture and the distorted forms of bacteria are often called involution forms. Cell lysis finally results (Frobisher et al, 1974).

1.16.1 **AERATION**

When aerobic bacteria grow on the surface of agar plates or in thin layers of liquid, in contact with air, the oxygen supply is usually

sufficient. However, in liquid media of great depth, they can grow only in the surface layers because oxygen is continuously consumed and the deeper layers become anaerobic. Continuous oxygen supply by aeration thus becomes necessary if aerobic bacteria have to grow throughout a deep liquid medium. Fortunately, most microorganisms are adapted to very low concentrations of dissolved oxygen but a certain minimal value, the 'critical oxygen concentration' must be maintained for adequate respiration of the cells.

The rate of solution of oxygen in the liquid can be increased by providing a large area of gas-liquid interphase and by increasing the partial pressure of oxygen in the gas phase. Liquid cultures are usually aerated by air, or by gas mixtures of oxygen, nitrogen and carbon dioxide. Ways of trying to obtain large surface areas include: thin layer cultures, agitation of the liquid by shaking, rotation of horizontal held flasks around their longitudinal axis,

forced aeration of a liquid column with air under pressure through a gas distributor, percolation through columns of granular material, and mechanical stirring.

However, even in a well-aerated fermentor, or in natural waters, the distribution of oxygen is not always even. Clumping of bacteria, for example, can produce local microenvironments with lower partial pressure of oxygen. Such semi-anaerobic microenvironments can also be provoked by suspended material in natural waters (Schlegel, 1986).

1.16.2 **TEMPERATURE**

Bacteria have different growth requirements such as temperature; most soil and acquatic bacteria are mesophilic, i.e. they have their maximal growth rates at temperatures in the range of 20-42 $^{\circ}$C while thermophilic bacteria grow at maximal rates above 40 $^{\circ}$C and their temperature limit is in the region of 70 $^{\circ}$C (Schlegel, 1986).

Temperature may be measured by mercury thermometers, thermocouples, thermistors and resistance thermometers. Mercury thermometers are however, the most commonly used (Sikyta, 1983). Disadvantages with the latter are: in most cases they are placed in well pockets with contact liquid such as a mineral oil which causes a considerable delay between the thermometer reading and the actual attainment of a given temperature in the culture.

In order to maintain viability, homogeneous stock cultures must be periodically sub-cultured on appropriate media and at optimum temperatures, usually 25°C for fungi, 28°C for actinomycetes, 37°C for bacteria, and 60°C for thermophilic microorganisms (Rhodes et al., 1966).

1.17 MAINTENANCE AND PRESERVATION OF PURE CULTURE

Because of the tendency towards instability in sub-cultures, microorganisms are most conveniently stored over long periods in a metabolically inhibited state and preferably as

replicated cultures to insure against accidental loss. Since strain variability is highly undesirable, a variety of methods have therefore been developed for culture preservation (maintenance of stability of the production culture in its initial form) (Salle, 1973).

Bacterial strains can be preserved in several ways:

(a) Agar slant cultures: For general use, bacteria are generally preserved on nutrient agar slants contained in screw-cap tubes to prevent drying. Some exceptions to the use of nutrient agar slants are; certain pathogenic bacteria which require the addition of serum, blood, or other body fluids to the medium; anaerobic organisms which must be provided with a reducing environment; disease - producing streptococci which prefer semi-solid media; and some autotrophic species which grow only in mineral salt media. The toxic metabolic waste products secreted by bacteria diffuse into the agar slant and away from the

growth. Because of this property, bacteria are able to survive longer on agar than in liquid media. After preparation, cultures are incubated for 24 hours or more, stored in a cool dark place or refrigerator, and used as needed. They are referred to as stock cultures.

(b) Agar slant cultures covered with oil: Agar slants in screw-cap tubes are inoculated and incubated until good growth appears, then covered with sterile mineral oil to a depth of one centimetre above the agar slant. Transfers are made by removing a loopful of the growth, touching the loop to the glass surface to drain off excess oil, and transferring the inoculum to fresh medium. This method has the following advantages; practically all species tested live longer under oil than in the control tubes without oil; subcultures may be prepared when needed without affecting the preservation of the stock cultures; the method is especially advantageous in working with unstable

variants where occasional transfers to fresh media or growth in mass cultures results in changes in the developmental stages of the strains; and special equipment such as a centrifuge, desiccator or vacuum pump is not required.

(c) Saline suspension: Sodium chloride in high concentration is frequently used as an inhibitor of bacterial growth. Chance (1963 in Salle, 1973) suspended various bacteria in one per cent salt solution (sublethal concentration) in screw-cap tubes to prevent evaporation. The tubes were stored at room temperature. After almost four years, transfers were made to agar slants and incubated. In every case vigorous growth occurred in less than 24 hours. The procedure offers an easy method of storing bacterial cultures for two or three years, possibly longer. It appears to be particularly valuable in keeping stock cultures for class use in laboratories with limited equipment.

(d) Freezing: The preservation of organisms by freezing has been practised for many years. Gibson et al., (1965 in Salle, 1973) froze 16 cultures of lactic streptococci, followed by storage at -77.8 to - 23.3°C. The numbers of surviving bacteria decreased as storage time was increased. For most species, the survival rate was greater at the lower temperature.

(e) Storage in liquid nitrogen: Hwanga (1966 in Salle, 1973) stored species and strains of fastidious molds in liquid nitrogen and reported excellent results for long-term preservation of stock cultures. The cultures were sealed in ampoules and cooled to -35°C at the rate of approximately one degree Celcius per minute. Further cooling to the storage temperature of -165 to -196°C was uncontrolled and took place at an accelerated rate. Viable cultures developed from specimens after a storage period of at least five years.

(f) **Drying in vacuum:** Miller and Simons (1962 in Salle, 1973) dried 202 cultures, representing 67 species of bacteria, over calcium chloride in a vacuum, then stored the organisms in the refrigerator at 10 °C. After a period of ten years, they found that only 13 cultures failed to grow.

However, duplicates of nine of the 13 cultures did show growth. The Gram-positive species survived better than the Gram-negative bacteria. This appears to be a simple and satisfactory procedure for the preservation of bacteria for long periods.

(g) **Freeze drying:** Freeze-dry or lyophilization is based on freezing vegetative cells or spores to temperatures far below the freezing point and drying them under high vacuum. The suspension obtained by washing the culture from the surface of the medium which contains agar is transferred to test tubes containing either a protective colloid such as broth, blood serum, whey, or a

mixture of soil and sand. The samples are frozen in solid carbon dioxide in ethanol and then dried in a vacuum of about 7 - 70 Pa. The test tubes are then sealed under vacuum. The resulting stock cultures can be stored for several years without any change in the culture properties. The shortcoming of the technique is the limited amount of the culture so present (about one gram) which can serve to inoculate only a small number of subcultures (Sikyta, 1983).

Shackell (1909 in Salle, 1973) recommended freezing as a preliminary step to rapid desiccation. The cultures may be frozen in a mixture of dry ice (carbon dioxide) and alcohol or acetone, then dried in a vacuum over sulphuric acid as the desiccant. This method is universally employed for the preservation of bacteria, viruses, sera, toxins, enzymes, and other biological materials.

To reactivate the lyophilised microorganisms it is only necessary to transfer them to a suitable liquid medium and then incubate.

Survival of microorganisms under these conditions has both practical and philosophical implications.

Practically, once preserved, a large number of cultures may be stored in a small volume, and many cultures will remain viable for decades. When cultures are maintained in this state, contamination and mutation are avoided, and both time and money are saved as compared with frequent transfer of active cultures. While the percentage of viable cells slowly decreases in these preserved cultures, the "half-life" is frequently measured in years. Cultures of various pathogenic species of streptococci kept for reference and research, for example, have survived for 25 years, while diphtheria bacilli have survived for 17 years (Frobisher et al.,1974).

However, though many species of bacteria tolerate freeze drying, this is not true of all. Philosophically, in a cell, in a state of virtually complete desiccation and in a high vacuum, there must be an unimaginably small vital activity; metabolic processes must stop almost completely as they depend on osmosis, diffusion, ionization and the colloidal state, all of which are dependent on hydration, how then, can microorganisms exist desiccated in a vacuum? How can they live in the entire absence of vital activity?

CHAPTER TWO

2.0 MATERIALS AND METHODS

2.1 ACTIVATION OF LYOPHILIZED CHEESE STARTER CULTURE

Twenty millilitres of pasteurised DPBZ milk was autoclaved (120 °C, 15min, 0.41Kg/cm. sq.) in four sterile 12 x 1.5 cm cubic test tubes. When it was cool enough to be handled, each test tube was aseptically inoculated with a loop of lyophilized cheese starter culture. The cotton-plugs used to close the mouths of the test-tubes were themselves wrapped in an aluminium foil to guarantee the required anaerobic environment. The four test tubes, standing on the base of a beaker with their sides resting against the beakers' rim, were left in an incubator set at 37 °C. The starter culture was allowed to grow for 48 hours before it could be used as a source of inoculum for the eight formulated media.

2.2 PREPARATION OF EMPIRICAL MEDIA

2.2.1 PRELIMINARY EXPERIMENTAL MEDIA

For the preliminary experiments, eight media were designed. The carbon, nitrogen and growth factors

sources varied from one medium to another while the buffering chemicals, sodium chloride, and distilled water including other ambient conditions were maintained uniformly as far as was possible. Raw materials were bought from Soweto and Kamwala markets in **Lusaka**. Below are formulations of the designed media and a brief account of how they were prepared. Medium A, designated `Mig´ consisted of the following components:

SUBSTANCE	QUANTITY
Glucose.....	2.5 g
Milk.....	1.25ml
K_2HPO_4	0.5 g
KH_2PO_4	2.21g
NaCl	0.25g
Distilled water.....	250ml

The medium was prepared in a 500 ml Erlenmeyer conical flask. The solid substances were weighed on an analytical balance and 1.25 ml pasteurised DPBZ milk was then poured into the flask followed by 250 ml of distilled water. The mixture was then vigorously stirred to dissolve all

substances. The conical flask mouth was securely closed with a cotton wool plug which was itself wrapped in an aluminium foil.

Medium B, designated `Soy´ comprised the following:

SUBSTANCE	QUANTITY
Orange juice	1.0 ml
Soya flour	0.63 g
Yeast extract	0.63 g
K_2HPO_4	0.5 g
KH_2PO_4	2.21 g
NaCl	0.25 g
Distilled water	250ml

Medium C, designated `Mst´ was made up of the following;

SUBSTANCE	QUANTITY
Meat extract	2.5 ml
Sugar cane juice	2.5 ml
Tomato juice	75 ml
K_2HPO_4	0.5 g
KH_2PO_4	2.21 g
NaCl	0.25 g
Distilled water	250ml

To extract the sugar cane juice, the hard outer coverings of a 15 cm long sugar cane was peeled off and its whitish fleshy mass was cut into small bits which were placed into a mortar, crushed into even smaller bits by pounding using a pestle. The juicy mash mixture that resulted was wrapped in a clean mutton cloth and squeezed to force the juice out of the cloth. The cane juice was collected by a measuring cylinder placed directly below the mixture being squeezed till 2.5 ml was collected.

The meat infusion was prepared in the same way soup is made in Kitchens; a small piece of beef (steak) was placed into a pot. Water was then added and the pot was then placed onto a hot plate. Cooking continued till a broth was obtained and used for the experiment.

To get tomato juice, the outer coverings of two ripe tomato fruits, which collectively weighed 116 g, were peeled off and the remaining fleshy mass cut into small bits to produce a juicy mass which was boiled in a pyrex beaker for about

10 minutes, then 75 ml of the broth that resulted was measured and used for the experiment. The rest of the ingredients were prepared as in medium 'A' above.

Medium D, designated 'Cap' had the following constituents:

SUBSTANCE	QUANTITY
Pine apple juice	2.5 ml
Ammonium sulphate	1.25 g
Carrot juice	0.63ml
K_2HPO_4	0.5 g
KH_2PO_4	2.21 g
NaCl	0.25 g
Distilled water	250ml

Carrot juice was prepared from three carrots which collectively weighed 56.03 g. Outer coverings of the carrots were peeled off. The remaining orange mass was cut into small bits which were placed into a 500 ml pyrex beaker. Thereafter, 200 ml of distilled water was added to the beaker and the mixture boiled using a hot plate till an orange broth resulted. Of the broth, 0.63 ml was

measured and added to other ingredients in the flask. The rest of the ingredients were prepared just as those in medium `A' above.

The composition of complex medium E, designated `Wip' was as follows:

SUBSTANCE	QUANTITY
Irish potato extract.....	1.25ml
Whey	2.5 ml
Pumpkin leaves juice	2.5 ml
K_2HPO_4	0.5 g
KH_2PO_4	2.21 g
NaCl	0.25 g
Distilled water.....	250ml

The Irish potato infusion was prepared by peeling off outer coverings of two irish potato. The tubers were cut into small bits which were placed into a 500 ml pyrex beaker. When 300 ml of water had been added, the beaker was placed on a hot plate and the mixture boiled till a white potato infusion needed for the experiment resulted.

Whey was prepared by boiling previously pasteurized DPBZ milk bought the previous day and kept in a refrigerator. After 10 minutes of boiling, a yellow soft curd formed and floated on top of the liquid whey. The mixture was poured onto a piece of clean cloth spread over a glass funnel. Of the filtrate, 2.5 ml was measured and used for the experiment.

An attempt to get an extract of the pumpkin leaves by crushing the leaves using a pestle failed. The alternative was to get a pumpkin leaves infusion. This was done; the infusion was prepared by boiling 67.7 g pumpkin leaves with 400 ml of distilled water in a pyrex beaker. After 15 minutes boiling, a greenish broth was obtained and 2.5 ml of it was measured and used for the experiment.

The rest of the substances were prepared as those in medium A above.

Medium F, designated 'Mb1' consisted of the following:

SUBSTANCE	QUANTITY
Maize bran infusion.....	5.0 ml
Beans infusion.....	2.5 ml
Lettuce juice	2.5 ml
K_2HPO_4	0.5 g
KH_2PO_4	2.21 g
NaCl	0.25 g
Distilled water.....	250ml

To make medium F, the following procedures were carried out:

1. Maize bran infusion was prepared by boiling 70.0 g of maize bran in 1.5 litres of distilled water.
2. A bran infusion was made by boiling 60.23 g of dry brown beans in 1.5 litres of distilled water.
3. A lettuce juice was prepared by boiling 78.8 g of lettuce leaves for ten minutes in 400 ml of distilled water.

Each preparation was allowed to cool to room temperature then filtered. The filtrates were then used in making up Medium F. The medium was finally autoclaved. The rest of the ingredients were prepared as in medium A above.

Medium G, designated `Mil' was made up of the following ingredients:-

SUBSTANCE	QUANTITY
Milk	5.0 ml
K_2HPO_4	0.5 g
KH_2PO_4	2.21 g
NaCl	0.25 g
Distilled water	250ml

Medium H, designated `Bas' was made as follows:-

SUBSTANCE	QUANTITY
Sucrose	2.5 g
Ammonium phosphate	1.0 g
Brown wheat flour	1.0 g
K_2HPO_4	0.5 g
KH_2PO_4	2.21g
NaCl	0.25g
Distilled water	250ml

2.2.2 FINAL EXPERIMENTAL MEDIA

The final experimental four media: Soy, Mil, Cap and Wip were chosen from the preliminary experiments after showing that they supported cultivation and propagation of lactic bacteria more satisfactorily. They were prepared just as before except for a few minor variations. This time each medium was prepared in triplicate and each replicate was duplicated. Their formulations are written below.

2.2.2.1 THE SOY MEDIUM

SUBSTANCE	SOY1	SOY2	SOY3
Orange juice	1.0 ml	0.5 ml	2 ml
Soya flour	0.63 g	0.5 g	1 g
Yeast extract	0.63 g	0.5 g	1 g
K_2HPO_4	0.5 g	0.5 g	0.5 g
KH_2PO_4	2.21 g	2.21 g	2.21g
NaCl	0.25 g	0.25 g	0.25g
Distilled water	250 ml	250 ml	250ml

2.2.2.2 THE CAP MEDIUM

SUBSTANCE	SOY1	SOY2	SOY3
Pine apple juice	2.5 ml	1.0 ml	3.0 ml
Ammonium sulphate	1.25 g	0.5 g	2.0 g
Carrot juice	0.63 ml	1.0 ml	2.0 ml
K_2HPO_4	0.5 g	0.5 g	0.5 g
KH_2PO_4	2.21 g	2.21 g	2.21g
NaCl	0.25 g	0.25 g	0.25g
Distilled water	250 ml	250 ml	250ml

2.2.2.3 THE WIP MEDIUM

SUBSTANCE	SOY1	SOY2	SOY3
Irish potato extract	1.25 ml	1.0 ml	2.0 ml
Whey	2.5 ml	1.0 ml	3.0 ml
Pumpkin leaves juice	2.5 ml	1.0 ml	3.0 ml
K_2HPO_4	0.5 g	0.5 g	0.5 g
KH_2PO_4	2.21 g	2.21 g	2.21g
NaCl	0.25 g	0.25 g	0.25g
Distilled water	250 ml	250 ml	250ml

2.2.2.4 THE MIL MEDIUM

SUBSTANCE	SOY1	SOY2	SOY3
Lactose (Milk).....	2.5 ml	1.0 ml	3.0 ml
Protein (Milk).....	2.5 ml	1.0 ml	3.0 ml
K_2HPO_4	0.5 g	0.5 g	0.5 g
KH_2PO_4	2.21 g	2.21 g	2.21g
NaCl	0.25 g	0.25 g	0.25g
Distilled water.....	250 ml	250 ml	250ml

2.3 pH ADJUSTMENT

Lactic bacteria are aciduric and the recommended initial pH for appropriate media is 6.5.

To adjust media pH to pH6.5, the pH meter electrode, rinsed with distilled water and dried up with blotting paper, was immersed into each medium one at a time. Either 0.1 M NaOH(aq) or 0.1M HCl (aq) was added until the pH meter read 6.5.

2.4 MEDIA STERILISATION

To sterilize the devised media, an autoclave was used. The media were sterilized at 120 °C for 15 minutes at 0.41Kg/cm sq.

2.5 CHARACTERISATION

To show that the microorganisms cultivated were lactic bacteria used as cheese starters, the following tests, as recommended by researchers (Foster et al, 1958, Wilson et al., 1975) were conducted: Gram-stain, benzidine, catalase, and fermentation (glucose and lactose) tests.

2.5.1 GRAM-STAIN TECHNIQUE

The Gram stain technique was carried out as follows: by aseptic technique a loop of each of the medium was placed onto a dry sterile unused microscope slide.

A smear from each culture was made and the slides were heat fixed. They were then placed on a slide rack suspended over a sink. The smears were

flooded with crystal violet (Gram-stain) solution for 30 seconds and thereafter rinsed with tap water. They were again flooded for thirty seconds but this time with Grams' iodine solution. After that, the smears were rinsed with tap water before decolourizing them with 95 per cent alcohol for ten seconds. The smears were again rinsed with tap water before flooding them with safranin (two per cent safranin in 95 per cent alcohol) for ten seconds. They were then rinsed with tap water and air dried.

The slides, one at a time, were placed onto the stage of a light microscope where a drop of an immersion oil was applied onto the smear. The smear was then observed using an oil immersion lens of a light microscope. The picture of a smear shown in figure two was taken using a camera mounted on the eye-piece of a light microscope.

2.5.2 BENZIDINE TEST

The benzidine test determines the presence or

absence of iron-porphyrin substances if these substances are present in the culture, a blue colour develops in the microbial growth, which otherwise remains colourless (Gibbs et al.,1966).

This test clearly differentiates lactic acid bacteria (including pseudocatalase- producing strains) which do not posses a cytochrome system from morphologically related organisms which do.

In the present study, nutrient agar (NA) media, with the following formulation, was prepared:-

SUBSTANCE	QUANTITY
Lab-lemco powder	1.0 g
Yeast extract	2.0 g
Peptone	5.0 g
Sodium chloride	5.0 g
Agar	15.0 g
Dist. Water	1000 ml

The medium was sterilized in a steam autoclave as in 2.4. While the medium was still in a molten state, it was poured into nine Petri dishes.

The Petri dishes were aseptically inoculated (except the control). The inoculum was from the cultures grown in the devised media in 2.2.1. Petri dishes were then incubated at 37 °C for 24 hours.

After 24 hours of incubation, scattered, small (0.1 mm to 0.5 mm in diameter), silverly and smooth colonies were observed except in the control experiment.

2.5.3. CATALASE TEST

The medium for this test was prepared as in 2.5.2. except that instead of using Petri plates, McConkey bottles were used for the formation of NA slopes. The agar slopes were then aseptically inoculated using cultures from the devised media in 4.2 and thereafter incubated for 24 hours at 37 °C.

After 24 hours of incubation, smooth, raised, silverly colonies ranging from 0.1 to 0.5 mm in width were observed except in the control agar slope. Three drops of five per cent $H_2O_2(aq)$ was applied onto the colonies.

2.5.4 FERMENTATION TEST

The micoorganisms incubated in the formulated media for 48 hours were then tested against the fermentation test.

Fermentation of glucose and lactose was investigated. The composition of glucose fermentation medium was as follows:

SUBSTANCE	QUANTITY
Glucose	5 g
Peptone	10 g
NaCl	0.5 g
Dist. Water	1000 ml

The glucose medium was prepared in a 500 ml Erlenmeyer conical flask. Using a 10 ml pipette, 10 ml of the medium was pipetted into each of 9, 12 x 1.5 ml test tubes. One Durham fermentation tube, was inverted and put into each test tube. The mouths of the test tubes were then plugged and loaded into an autoclave.

The lactose medium was also prepared in the same way as the glucose fermentation medium except for its composition which was as written below:-

SUBSTANCE	QUANTITY
Beef extract	3.0 g
Peptone	5.0 g
Lactose	5.0 g
Dist. water	1000 ml

Autoclaving of the fermentation medium was done as in 2.4 above. After sterilization of the medium, and when the test tubes had cooled enough to be handled, they were transferred into test tube racks; those for glucose fermentation on a separate rack from that of the lactose fermentation test tubes. Two to three drops of methyl red indicator, using a sterile pipette, were put into each test tube. They all became yellow. Each test tube, except two which served as controls, one for each medium, were then aseptically inoculated with activated cheese starter cultures grown in the devised media in 2.2.1.

2.6 MEDIA SENSITIVITY TESTS

To study media suitability in supporting growth and/or propagation of cheese starter cultures, two techniques were employed namely turbidimetry and standard plate count (SPC).

2.6.1 TURBIDIMETRY

To study rate of growth, a spectrophotometer, an instrument that can be used for measuring cell

density, was employed. The units are in Absorbance (A)-where A is defined as the logarithm of the ratio of intensity of light striking the suspension (Io) to that transmitted by the suspension (I) as summarized in the equation below:

$$A = \log \frac{Io}{I}$$

To measure A, 5.0 ml of each medium devised as described in 2.2.1 and 2.2.2 was used. The medium was aseptically pipetted into a 10 ml measuring cylinder after every twenty-forth hours of incubation. This was done over a period of nine days. Each sample was diluted with 5 ml of distilled water as the original samples were too concentrated to be measured on the A scale. When each of the diluted samples had been carefully shaken to homogenise, they were transferred into clean sample cuvettes.

Before A could be read, the following were done to the spectrophotometer; it was connected to electricity (240 V.AC) and left to warm up for 15 minutes (this was done to allow stability of the

needle on the measuring dial): then it was set to minimum scale (A= infinite) after which the wavelength control knob was adjusted to select a band of 450 nm; this was followed by inserting a blank (the blank was filled with distilled water being the solvent used in preparation of the devised media in 2.2.1 and 2.2.2.) into the sample chamber whose mouth was then securely closed with the lid; the needle was thereafter set to full scale (A=0) and the blank cuvette was then removed from the sample chamber.

Fingerprints on the sample cuvette were carefully wiped off using dry cotton wool before the cuvette was inserted into the sample chamber. Absorbance was read and recorded. The sample cuvette was then removed and its contents discarded.

The sample cuvette was thoroughly rinsed with distilled water before it was filled with the next sample and the whole process repeated. This was done for all the media devised in 2.2.1. and 2.2.2.

2.6.2 STANDARD PLATE COUNT (SPC)

To estimate the numbers of bacteria in each medium, the SPC technique was used. Since this technique involves pouring of molten nutrient agar and a sample culture concomitantly into a Petri plate, it is also known as the pour plate technique. The technique involves successive dilutions of the inoculum which are placed into sterile Petri plates and mixed with the molten but cool nutrient agar (NA) medium and then allowed to solidify. Colonies subsequently develop, embedded in the NA.

In the present study, the NA medium used for the experiment consisted of the following:

SUBSTANCE	QUANTITY
Lab-lemco powder	1.0 g
Yeast extract	2.0 g
Peptone	5.0 g
NaCl	5.0 g
Agar	15.0 g
Milk	10.0 ml
Dist. Water	1000 ml

The SPC medium, in a five litres Erlenmeyer conical flask, was sterilized by autoclaving as in 2.4 above.

After sterilizing, the medium was apportioned into 54 sterile McConkey bottles (pouring the media into McConkey bottles was done after flaming to minimise contamination).

Each McConkey bottle was filled with about 15.0 ml of the molten NA. While the SPC medium was being autoclaved, 54 (12x1.5 ml) Pyrex test tubes, cotton wool plugged, 54 Petri dishes and 54 one millilitre pipettes were simultaneously sterilized in an air drier oven set at 160 °C. They were left undisturbed for one hour. After sterilization, each test tube was aseptically filled with 9 ml of sterile distilled water. Six test tubes were assigned to each inoculum (inoculated media in 2.2.1 above). One millilitre of the inoculum was aseptically transferred into the first test tube. The test tube was shaken to ensure even distribution of

the inoculum before one millilitre of its mixture was aseptically transferred into the next tube. This process continued till the fifth test tube had its share of the inoculum. The sixth test tube served as a control.

Six sterile Petri plates, each with a label indicating the type of inoculum and dilution, were brought next to the McConkey bottles containing molten NA. The latter were near the inoculated test tubes.

Pouring of the molten NA and the inoculum into each of the Petri plates was done simultaneously. As soon as the NA had been poured into the Petri plate, 1 ml of the appropriate dilution was pipetted into the Petri plate which was then rotated gently to allow even distribution of the inoculum into the NA. This was done for each of the six Petri plates as illustrated in figure 1 below.

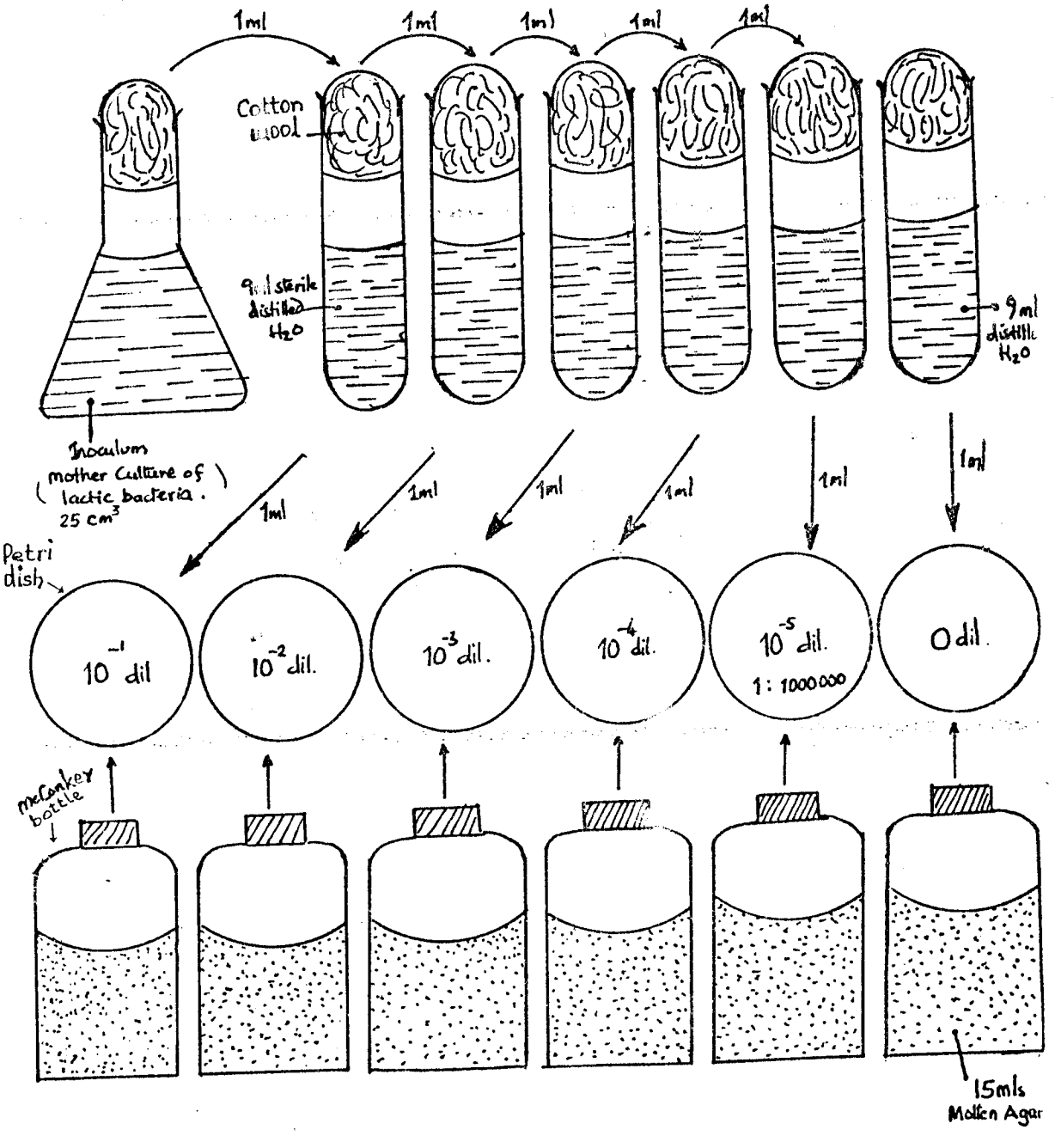


Figure 1 shows how SPC experiment was prepared.

For the media devised in 2.2.2 the SPC technique was done similar to those devised in 2.2.1. There was only 1/1 dilution inoculum used due to limited space in the autoclave. Colonies were counted after 18 hours.

For preliminary media, colonies were counted after 48 hours of incubation. Since the concentration of colonies increased with reduced inoculum dilution, only colonies with 1:1000 000 inoculum dilution were counted. Colony counting was done by placing a Petri dish upside down in a counting chamber of the Quebec Colony Counter (QCC).

2.7 EXPERIMENTAL CHEESE

After final experimental media sensitivity tests, **lactic bacteria** cultivated in Soy and Mil media plus the lyophilized culture from France were used to produce Cream cheese at PBCF. For this experiment, thirty litres of milk was used. At the factory, the milk was pasteurized. The milk was cooled to 20 °C and 10 litres apportioned for each medium culture sample.

The lyophilized culture was activated by aseptically inoculating half a teaspoonful into 50 ml of previously pasteurized skimmed milk and thereafter incubating at 37 °C for 24 houre. From each of the three media (Soy, Mil and skm milk), 50 ml were then used to inoculate the three 10 litres samples of pasteurized milk and Cream cheese was made as described in 1.6.3 above. Table 3 below summarizes what was obtained for each medium:

TABLE 3: AMOUNT OF MILK, WEIGHT AND SOME CHARACTERISTICS OF CHEESE PRODUCED USING LACTIC BACTERIA CULTIVATED IN SOY, MIL AND SKM.

MEDIUM	AMOUT OF MILK	AMOUNT OF CHEESE	CHEESE APPEARANCE AND TEXTURE	CHEESE TASTE
Soy	10 lt	1.485 Kg	Normal	Excellent
Mil	10 lt	0.875 Kg	Normal	Not that good
Skm milk	10 lt	1.060 Kg.	Normal	Not that good

2.7.1. CHEESE ANALYSIS

The cheese samples produced as in 2.7 above were subjected to moisture, acidity and fat analytical tests carried out at NCSR in the food technology unit.

2.7.1.1. WATER CONTENT

For each of the six cheese samples, six aluminium cylindrical containers each with a diameter of 4 cm, a height of 3 cm, and an air tight fitting lid, were weighed. Then 3 g of cheese samples were placed into each container. Each container was weighed again. The lids were taken into an oven set at 130 ^{o}C while the containers were steam heated over a water bath set at 100 ^{o}C. Before steam heating could start, 3 ml of distilled water was added into each container to help the cheese sample spread. Steam heating continued till nearly all the water had been driven off the cheese. Using tongs, containers were transferred into an oven set at 130 ^{o}C where they stayed for another one hour. Container mouths were then closed with their corresponding lids using tongs.

The containers were then transferred to a desiccator where the hygroscopic substance used was $CaCl_2$ (calcium chloride). When the containers had cooled enough, after thirty minutes, they were weighed and differences in weights noted as water quantity for each cheese sample as seen in the table below.

TABLE 4: TYPE OF CULTURE MEDIA USED AND AMOUNT OF WATER IN CHEESE.

CHEESE SAMPLE	ALUMINIUM +3 g CHEESE BEFORE HEATING	ALUMINIUM CONT. +3 g CHEESE AFTER HEATING	WATER QUANTITY IN CHEESE SAMPLE (g)
Soy a	20.4785	18.7662	1.7123
Soy b	20.3503	18.6514	1.6989
Mil a	20.7089	19.2172	1.4917
Mil b	20.0173	18.5087	1.5086
Skm a	21.2547	19.5654	1.6893
Skm b	20.8667	19.2504	1.6163

2.7.1.2 ACID CONTENT

To determine acid content in the cheese samples, 10 g of each cheese sample was weighed in a 100 ml flat bottomed flask. Fifty millilitres of distilled water were added into each flask.
The samples were steam heated for five minutes and then vigorously shaken to homogenize the mixture.

The mixture was thereafter filtered. Two to three drops of phenolphthalein indicator were added into each filtrate. From each sample, 25 ml of the filtrate was titrated with 0.1M NaOH. The amount of lactic acid in the cheese was calculated from the formula one millilitre NaOH = 0.0090 g lactic acid (Pearson, 1970).

Table 5 shows the volume of NaOH used and the calculated amount of lactic acid for each sample:

TABLE 5: THE VOLUME OF NaOH USED AND THE CALCULATED AMOUNT OF LACTIC ACID FOR EACH SAMPLE.

	Soy a	Soy b	Mil a	Mil b	Skm a	Skm b
Vol.NaOH (ml)	2.6	3.1	3.3	2.7	2.6	2.9
Lactic acid (g)	0.0234	0.0279	0.0297	0.0243	0.0234	0.0261

2.7.1.3. **FAT CONTENT**

For fat content determination, a fat extraction machine connected to a steam generating apparatus fitted with a thermometer (the Soxhlet method),

was used. Fat extraction beakers were labelled and weighed. Three grams of cheese samples were placed into six dry fat extraction tubes which were inserted into thimbles. The beakers were then two thirds filled with petroleum spirit (Bp 70-80 °C) and placed directly below the thimbles. The steam generating tank was three forth filled with distilled water and the machine was then set at 82.5 °C.

The machine was left to run for about 16.00 hours when petroleum spirit was driven off the fat extraction beakers into a solvent storing tank by raising the temperature to 95.5 °C. The fat extraction beakers were taken into an oven set at 110 °C where they were left undisturbed for one hour. They were then transferred into a desiccator to cool for about 30 minutes and thereafter weighed again.

CHAPTER THREEE

3.0 RESULTS

The table below summerises the fat analysis procedure

TABLE 6: AMOUNT OF CHEESE FAT

CHEESEC SAMPLES	FAT EXTRAC-TION BEAKER	THIMBLE	THIMBLE AND CHEESE	WEIGHT OF CHEESE	FAT EXT-RACTION BEAKER + FAT	WEIGHT OF FAT
Soy a	101.8739	3.2542	6.2649	3.0107	102.8732	0.99
Soy b	105.4956	4.2312	7.7247	3.4935	106.7267	1.22
Mil a	98.3923	3.6504	8.5167	4.8663	99.6141	1.21
Mil b	99.1062	4.2571	9.0010	4.7439	100.5611	1.45
Skm a	105.6035	3.9974	8.4656	4.4682	106.8682	1.25
Skm b	103.4553	3.9850	8.6893	4.7063	105.9766	2.51

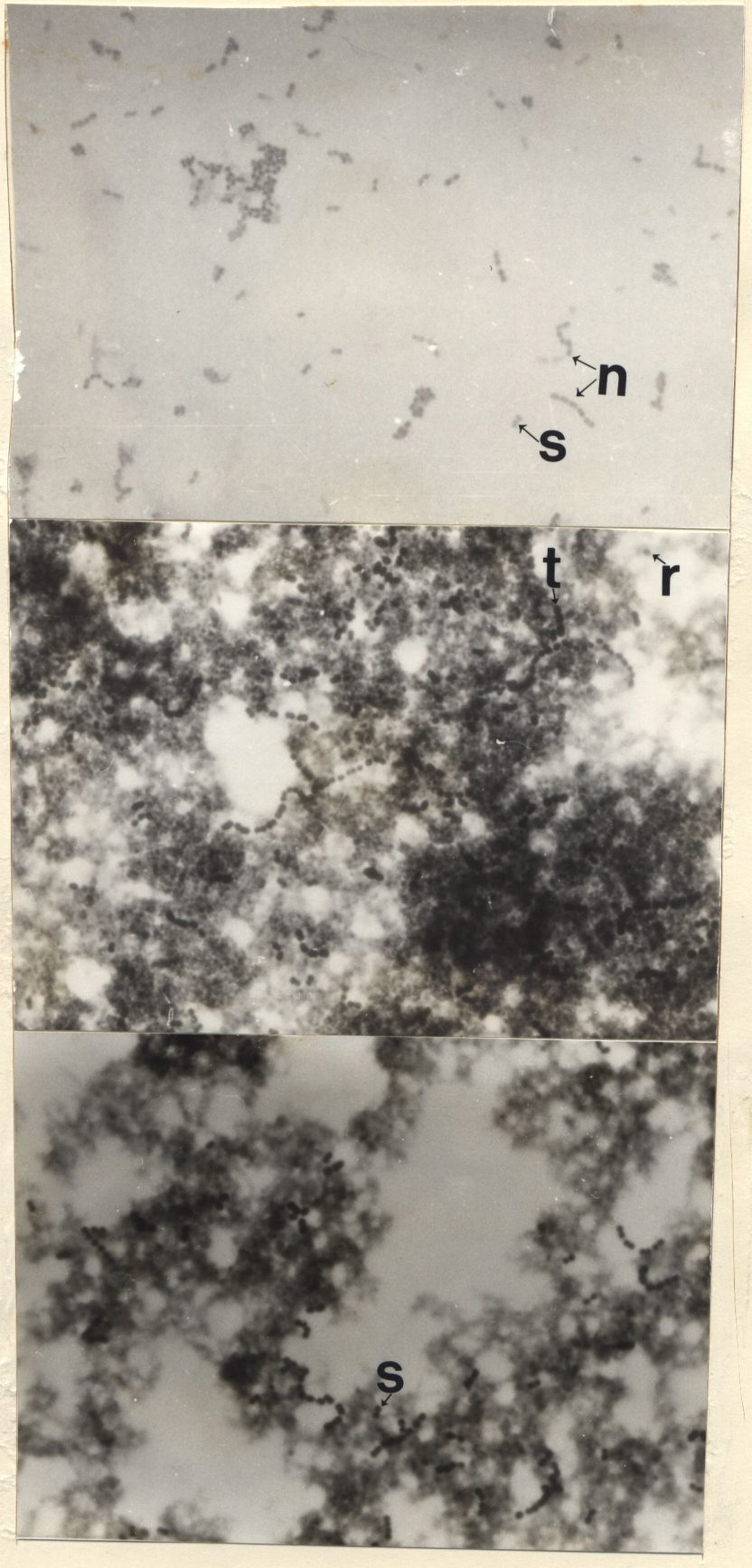
3.1 GRAM-STAIN TEST

After 24 hours of incubation at 37 °C smears of the cultures from the media devised in 2.2.1 above gave a positive Gram-stain test.
Figure 2 below shows a plate of lactic bacteria.

3.2 BENZIDINE TEST

When two to three drops of benzidine chloride solution, followed by five per cent $H_2O_2(aq)$ were dropped onto the silverly colonies scattered all over the NA surface after 24 hours of incubation at 37 °C, the colonies remained silverly. This is a negative benzidine test reaction as is expected of lactic bacteria (Gibbs et al.,Wilson et al.,1975).

Figure 2: Shows a plate of Gram-stained mixed culture of lactic bacteria where illustrative letters point to the following: r = Coccus, t = Lactobacillus, s = dicocci, n = streptococci


n
s
t
r
S

3.3 CATALASE TEST

When the 24 hours old, silverly, smooth, small (0.1 to 0.5 mm wide) raised colonies were in contact with the introduced five per cent H_2O_2 (aq), effervescence took place, oxygen was evolved as the colonies diminished in size until all that remained on the NA surface was a colourless liquid. This is a characteristic positive catalase reaction.

3.4 FERMENTATION TEST

After 48 hours of incubation, all test tubes (except the controls) became reddish brown even though the intensity of colour was more intense in some test tubes than in others.

The controls, however, remained yellowish.

In all cases, there was no gas collected in the Durham tubes. This is a characteristic positive reaction of lactic bacteria (Gibbs et al.,1966, Buchanan et al.,1974, Wilson et al.,1975). According to the colour intensity exhibited by each medium, the following table illustrates what was observed.

TABLE 7: RESULTS OF THE PRELIMINARY MEDIA FERMENTATION REACTIONS.

MEDIUM	Mig	Soy	Mst	Cap	Wip	Blm	Mil	Bas	Control
COLOUR INTENSITY	+	++	+	++	++	++	++	+	-
GAS EVOLUTION	-	-	-	-	-	-	-	-	-

NB ++ designates more intense than + and - means a negative response.

3.5 SENSITIVITY TESTS RESULTS

3.5.1 PRELIMINARY MEDIA TURBIDIMETRY RESULTS

TABLE 8: ABSORBANCE READINGS FOR PRELIMINARY MEDIA.

	MEDIA							
DURATION	Mig	Soy	Mst	Cap	Wip	Blm	Mil	Bas
DAY 1	0.13	0.49	0.07	0.04	0.12	0.07	0.39	0.05
DAY 2	0.19	0.56	0.09	0.05	0.17	0.09	0.48	0.29
DAY 3	0.20	0.61	0.09	0.06	0.19	0.11	0.81	0.31
DAY 4	0.37	0.66	0.15	0.07	0.23	0.15	1.00	0.31
DAY 5	0.39	0.69	0.16	0.08	0.25	0.15	1.14	0.35
DAY 6	0.41	0.73	0.20	0.08	0.27	0.17	1.50	0.45
DAY 7	0.41	1.30	0.20	0.09	0.28	0.22	1.60	0.47
DAY 8	0.64	1.50	0.20	0.11	0.29	0.26	1.80	0.70
DAY 9	0.75	1.80	0.32	1.35	0.48	0.40	1.80	0.73
TOTALS	3.49	8.34	1.48	1.93	2.28	2.62	10.52	3.66
MEANS	0.39	0.93	0.16	0.21	0.25	0.29	1.17	0.41

3.5.2 FINAL MEDIA TURBIDIMETRY RESULTS

TABLE 9: FINAL MEDIA TURBIDIMETRY RESULTS

	DURATION								
MEDIA	DAY1	DAY2	DAY3	DAY4	DAY5	DAY6	DAY7	DAY8	DAY9
Wip1A	0.089	0.1	0.091	.085	0.15	.09	.099	.085	.079
Wip1B	0.11	0.085	0.15	.14	0.085	.08	.182	.141	.181
Wip2A	0.16	0.11	0.165	.21	0.21	.143	.179	.163	.189
Wip2B	0.14	0.11	0.17	.11	0.09	.171	.2	.161	.26
Wip3A	0.199	0.189	0.15	.085	0.16	.179	.16	.19	.19
Wip3B	0.21	0.21	0.2	.15	0.25	.185	.27	.24	.22
Mil1A	0.43	0.36	0.265	.42	0.27	.245	.293	.13	.22
Mil1B	0.58	0.56	0.281	.29	0.41	.31	.26	.28	.32
Mil2A	1.0	0.89	0.42	.45	0.82	.47	.36	.5	.4
Mil2B	0.94	0.95	0.251	.228	0.57	.56	.499	.56	.59
Mil3A	1.1	1.10	0.51	.155	1.45	.71	.581	.46	.64
Mil3B	1.2	1.15	1.21	.211	0.163	.28	.182	.16	.322
Soy1A	0.055	0.37	0.251	.43	0.51	.53	.72	.5	.62
Soy1B	0.208	0.45	0.48	.51	0.57	.58	.6	.6	.6
Soy2A	0.175	0.15	0.175	.215	0.365	.65	.681	.62	.65
Soy2B	0.079	0.03	0.24	.17	0.152	.165	.74	.519	.79
Soy3A	0.08	0.04	0.143	.13	0.145	.171	.37	.44	.55
Soy3B	0.032	0.02	0.071	.07	0.162	.128	.182	.14	.18
Cap1A	0.038	0.019	0.036	.068	0.14	.14	.14	.13	.141
Cap1B	0.05	0.03	0.049	.09	0.079	.07	.085	.08	.11
Cap2A	0.035	0.011	0.131	.11	0.151	.135	.13	.125	.14
Cap2B	0.021	0.1	0.041	.04	0.033	.036	.09	.05	.13
Cap3A	0.037	0.049	0.11	.08	0.07	.054	.085	.051	.06
Cap3B	0.241	0.16	0.11	.092	0.33	.27	.23	.19	.22

3.5.3 PRELIMINARY MEDIA COLONY COUNT RESULTS

TABLE 10: COLONY COUNT RESULTS FOR PRELIMINARY MEDIA 1:1,000,000 DILUTION AFTER 48 HOURS.

MEDIUM	COLONIES COUNTED	BACTERIA IN MOTHER CULTURE
Mig	800	2.0×10^{10}
Soy	1398	3.495×10^{10}
Mst	472	1.18×10^{10}
Cap	1028	2.57×10^{10}
Wip	1078	2.695×10^{10}
Blm	336	0.84×10^{10}
Mil	1530	3.825×10^{10}
Bas	544	1.36×10^{10}

3.5.4 FINAL MEDIA COLONY COUNT RESULTS

TABLE 11: COLONY COUNT RESULTS FOR FINAL MEDIA 1:10 DILUTION AFTER 18 HOURS.

MEDIUM	1st READING	2nd READING	1st + 2nd/2	BACTERIA IN MOTHER CULTURE
Wip 1A	254	214	234.0	5.85×10^5
Wip 1B	213	304	258.5	6.46×10^5
Wip 2A	245	192	218.5	5.46×10^5
Wip 2B	255	441	348.0	8.70×10^5
Wip 3A	502	436	469.0	1.17×10^6
Wip 3B	742	636	689.0	17.22×10^5
Mil 1A	518	696	607.0	15.17×10^5
Mil 1B	808	604	706.0	17.65×10^5
Mil 2A	940	614	777.0	19.45×10^5
Mil 2B	786	868	827.0	20.67×10^5
Mil 3A	969	1144	1056.5	26.41×10^5
Mil 3B	874	704	789.0	19.72×10^5
Soy 1A	1079	854	966.5	24.16×10^5
Soy 1B	1067	746	906.5	22.66×10^5
Soy 2A	510	398	454.0	11.35×10^5
Soy 2B	304	123	213.5	5.34×10^5
Soy 3A	206	230	218.0	5.45×10^5
Soy 3B	-	-	-	-
Cap 1A	24	50	37.0	0.93×10^5
Cap 1B	13	46	29.5	0.74×10^5
Cap 2A	47	40	43.5	1.09×10^5
Cap 2B	99	100	99.5	2.49×10^5
Cap 3A	370	312	341.0	8.52×10^5
Cap 3B	102	99	100.5	2.51×10^5

3.5.5 CHEESE ANALYSIS RESULTS

TABLE 12: CHEESE FAT, WATER AND LACTIC ACID ANALYSIS RESULTS

CHEESE SAMPLES	FAT (g)	WATER (g)	LACTIC ACID (g)
Soy a	0.99	1.7123	0.0234
Soy b	1.22	1.6989	0.0279
Mil a	1.21	1.4917	0.0279
Mil b	1.45	1.5086	0.0243
Skm a	1.25	1.6893	0.0234
Skm b	2.51	1.6163	0.0261

3.6.1 ANALYSIS OF DIAGNOSTIC TEST RESULTS

TABLE 13: COLLECTIVE RESULTS OF THE DIAGNOSTIC TESTS.

				FERMENTATION			
	GRAM			GLUCOSE		LACTOSE	
MEDIUM	STAIN	BENZIDINE	CATALASE	ACID	GAS	ACID	GAS
Mig	+	-	+	+	-	+	-
Soy	+	-	+	++	-	++	-
Mst	+	-	+	+	-	+	-
Cap	+	-	+	++	-	++	-
Wip	+	-	+	++	-	++	-
Blm	+	-	+	++	-	++	-
Mil	+	-	+	++	-	++	-
Bas	+	-	+	+	-	+	-

Nb:+ = positive result; ++ = more colour intensity than + for fermentation reactions, and - = negative result.

3.6.2 ANALYSIS OF PRELIMINARY MEDIA TURBIDIMETRY RESULTS

Preliminary turbidimetry results were tested at five per cent significant level by Duncan's multiple range test (see appendix A). The order of means, when arranged in a descending order from the largest to the smallest was as follows;

Mil	**Soy**	**Bas**	**Mig**	**Blm**	**Wip**	**Cap**	**Mst**
1.17	0.93	0.11	0.39	0.29	0.25	0.21	0.16

Media Mil and Soy, at five per cent significant level by the Duncan's multiple range test were found to be none significantly different from each other but the two were significantly different from the rest of the media as summarised below;

Mil	Soy	Bas	Mig	Blm	Wip	Cap	Mst
1.17	0.93	0.11	0.39	0.29	0.25	0.21	0.16

The differences between the observed preliminary media turbidimetry results, were statistically proved to be significantly different at five per cent significant level by the analysis of variance (ANOVA) test (see appendix A).

To compare growth rate of bacteria between the devised preliminary media, turbidimetry results were converted to logarithm and thereafter plots of logarithm versus time were made. The table below shows turbidimetry results and corresponding logarithm values.

TABLE 14: TURBIDIMETRY RESULTS (TR) AND THEIR CORRESPONDING LOGARITHM VALUES (LV) OF PRELIMINARY MEDIA

MEDIA	DAY1	DAY2	DAY3	DAY4	DAY5	DAY6	DAY7	DAY8	DAY9
MigTR	0.13	0.19	0.20	0.37	0.39	0.41	0.41	0.64	0.75
LV	-0.88	-0.72	-0.70	-0.43	-0.41	-0.39	-0.39	-0.19	-0.12
SoyTR	0.49	0.56	0.61	0.66	0.69	0.73	1.30	1.50	2.80
LV	-0.31	-0.25	-0.21	-0.18	-0.16	-0.14	0.11	0.18	0.25
MstTR	0.07	0.09	0.09	0.15	0.16	0.20	0.20	0.20	0.32
LV	-1.15	-1.04	-1.04	-0.82	-0.80	-0.70	-0.70	-0.70	-0.49
CapTR	0.04	0.05	0.06	0.07	0.08	0.08	0.09	0.11	1.35
LV	-1.40	-1.30	-1.22	-1.15	-1.10	-1.10	-1.04	-0.96	0.13
NipTR	0.12	0.17	0.19	0.23	0.25	0.27	0.28	0.29	0.48
LV	-0.92	-0.77	-0.72	-0.64	-0.60	-0.57	-0.55	-0.54	-0.32
BlaTR	0.07	0.09	0.11	0.15	0.15	0.17	0.22	0.26	0.40
LV	-1.15	-1.04	-0.96	-0.82	-0.82	-0.78	-0.66	-0.58	-0.40
MilTR	0.35	0.48	0.81	1.00	1.14	1.50	1.60	1.80	1.80
LV	-0.41	-0.32	-0.09	0.00	0.06	0.18	0.20	0.25	0.25
BasTR	0.05	0.29	0.31	0.31	0.35	0.45	0.47	0.70	0.73
LV	-1.30	-0.54	-0.51	-0.51	-0.46	-0.35	-0.33	-0.15	-0.14

FIGURE 3

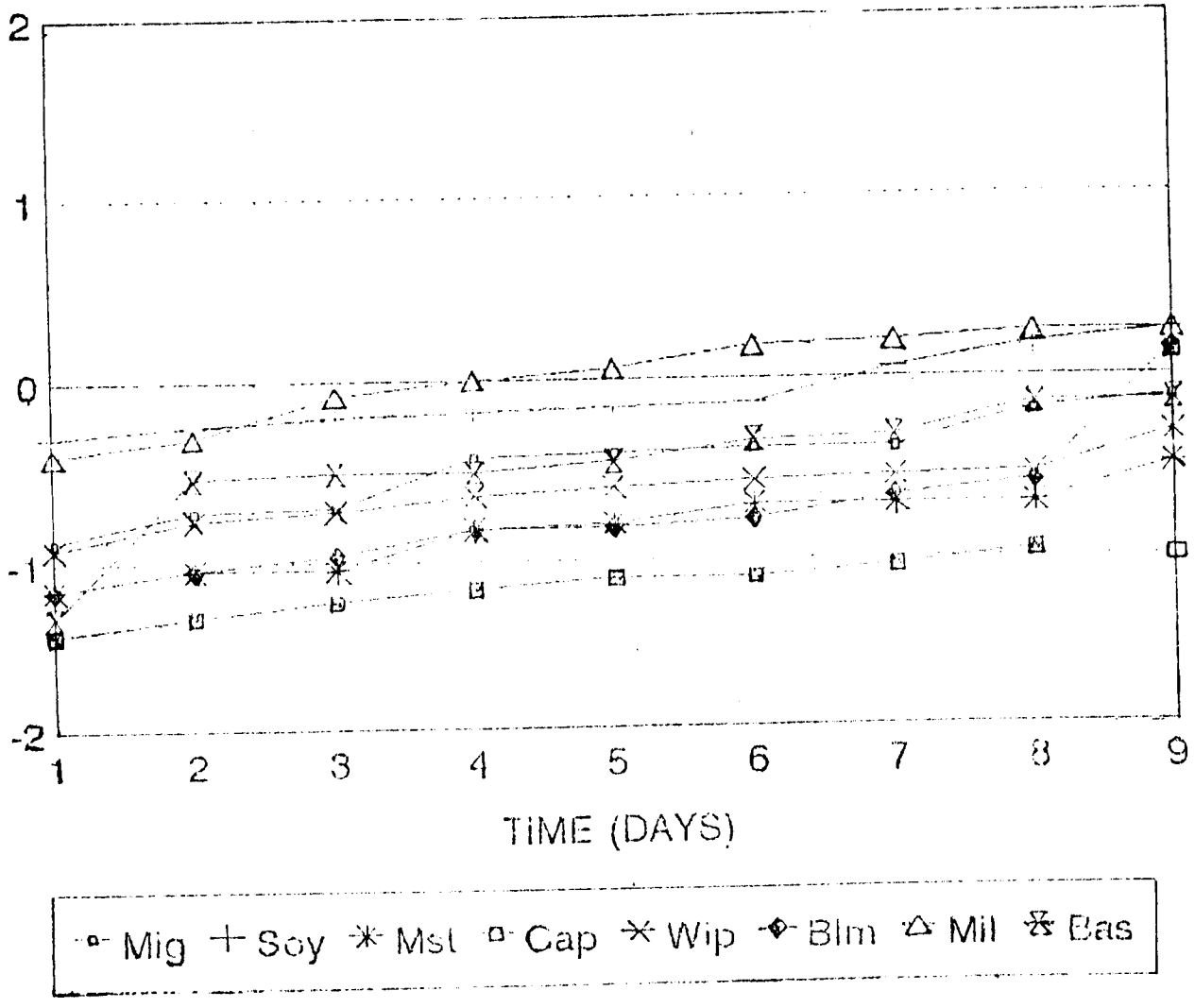


FIGURE 3: Shows plots of logarithm values against time for preliminary media turbidimetry sensitivity test.

3.6.3 ANALYSIS OF FINAL MEDIA TURBIDIMETRY RESULTS

By the Bailey (1981) significance test at five per cent significant level, based on normal distribution, duplicates of final media turbidimetry results were proved to be insignificantly different from each other (see appendix A).

The Duncan's multiple range test, at five per cent significant level, showed that final media results of Mil3 and Mil2 were not significantly different from each other but that the two were significantly different to the rest of the media (see appendix A).

When the means were arranged in a descending order, from the largest to the smallest, the following order was obtained:-

Mil3	**Mil2**	**Soy1**	**Mil1**	**Soy2**	**Wip3**	**Soy3**	**Wip2**
0.68	0.58	0.47	0.36	0.36	0.19	0.17	0.16
Cap3	**Wip1**	**Cap2**	**Cap1**				
0.13	0.11	0.09	0.08				

The ANOVA test, at five per cent significant level, showed that observed means of final media turbidimetry reults, were not significantly different from each other (see appendix A).

To compare growth rate of bacteria between the devised final media, turbidimetry results were converted to logarithm and plots of logarithm against time were made thereafter. Table 15 below shows turbidimetry results (TR) and their corresponding logarithm values (LV).

3.6.4 ANALYSIS OF PRELIMINARY AND FINAL MEDIA COLONY COUNT RESULTS

The null hypothesis ``that given the same environmental and incubation conditions, nutrients of various media will not affect the rate of culture growth and therefore, at any given time there should be the same number of organisms where each medium is inoculated with the same quantity and strains of a culture,'' was rejected at five per cent significant level by the chi-square (X sq.) as a goodness-of-fit test on both the preliminary and final media colony results (see appendix B).

TABLE 15: TURBIDIMETRY RESULTS (TR) AND THEIR CORRESPONDING LOGARITHM VALUES (LV) FOR FINAL MEDIA SENSITIVITY TEST.

MEDIA	DAY1	DAY2	DAY3	DAY4	DAY5	DAY6	DAY7	DAY8	DAY9
Wip1TR	0.10	0.09	0.12	0.11	0.12	0.09	0.14	0.11	0.13
LV	-1.00	-1.04	-0.92	-0.96	-0.92	-1.04	-0.85	-0.96	-0.89
Wip2TR	0.15	0.11	0.17	0.16	0.15	0.16	0.19	0.16	0.22
LV	-0.82	-0.96	-0.77	-0.80	-0.82	-0.80	-0.72	-0.80	-0.66
Wip3TR	0.20	0.20	0.17	0.12	0.20	0.18	0.21	0.21	0.20
LV	-1.70	-1.70	-0.77	-0.92	-0.70	-0.74	-0.68	-0.68	-0.70
Mil1TR	0.50	0.45	0.27	0.56	0.47	0.27	0.28	0.20	0.27
LV	-0.30	-0.34	-0.57	-0.25	-0.33	-0.57	-0.55	-0.67	-0.57
Mil2TR	0.97	0.92	0.33	0.34	0.69	0.51	0.43	0.53	0.49
LV	-0.01	-0.04	-0.48	-0.47	-0.16	-0.29	-0.37	-0.28	-0.32
Mil3TR	1.15	1.12	0.86	0.18	0.81	0.49	0.38	0.31	0.48
LV	0.06	0.05	-0.06	-0.74	-0.09	-0.31	-0.42	-0.51	-0.32
Soy1TR	0.13	0.41	0.36	0.47	0.54	0.55	0.66	0.55	0.61
LV	-0.89	-0.39	-0.44	-0.33	-0.27	-0.23	-0.18	-0.23	-0.21
Soy2TR	0.13	0.09	0.21	0.19	0.26	0.41	0.71	0.57	0.72
LV	-0.89	-1.05	-0.68	-0.72	-0.58	-0.39	-0.15	-0.24	-0.14
Soy3TR	0.06	0.03	0.11	0.10	0.15	0.15	0.28	0.29	0.36
LV	-1.22	-0.52	-0.96	-1.00	-0.82	-0.82	-0.55	-0.53	-0.44
Cap1TR	0.04	0.02	0.04	0.08	0.11	0.10	0.11	0.10	0.12
LV	-1.40	-1.70	-1.40	-1.10	-0.96	-1.00	-0.96	-1.00	-0.92
Cap2TR	0.03	0.11	0.09	0.07	0.09	0.08	0.11	0.09	0.13
LV	-1.52	-0.96	-1.05	-1.15	-1.05	-1.10	-0.96	-1.05	-0.89
Cap3TR	0.14	0.10	0.11	0.09	0.20	0.16	0.16	0.12	0.13
LV	-0.85	-1.00	-0.96	-1.05	-0.70	-0.80	-0.80	-0.92	-0.89

See figure 4 below showing plots of logarithm values against time for final media turbidimetry sensitivity test.

FIGURE 4

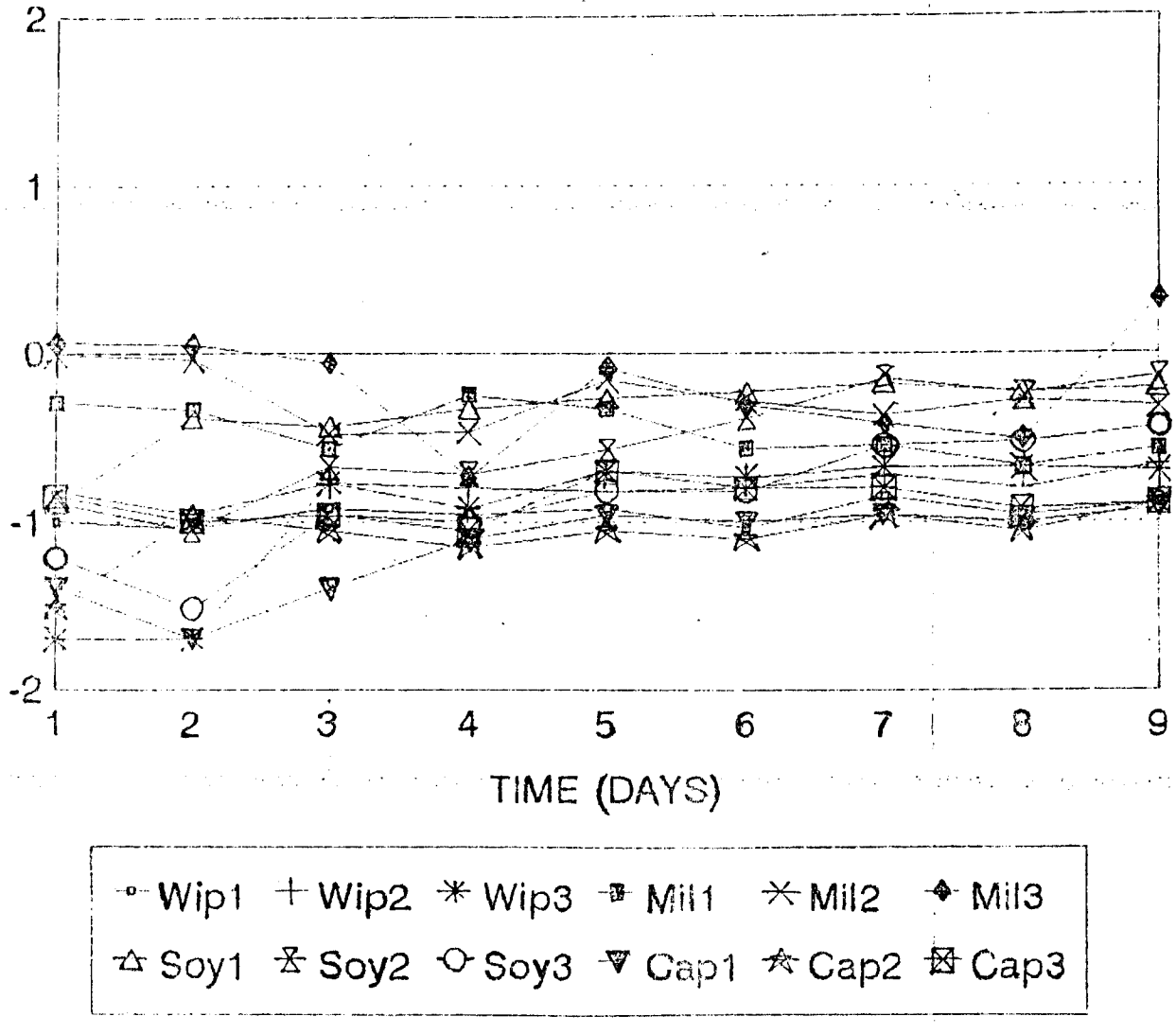


Figure 4: Shows plots of logarithm values against time for final media turbidimetry sensitivity test

Figure 5 below shows histograms that illustrate quantities of the lactic bacteria in the preliminary and final media respectively.

3.6.5 ANALYSIS OF CHEESE RESULTS

TABLE 16: WEIGHT AND % OF FAT, WATER AND ACID IN CHEESE SAMPLES.

CHEESES SAMPLES	FAT CONTENT		WATER CONTENT		ACID CONTENT	
	Fat (g)	Fat %	Water (g)	Water %	Acid (g)	Acid %
Soy a	0.99	32.88	1.7123	57.08	0.0234	0.117
Soy b	1.22	34.92	1.6989	56.63	0.0279	0.139
Mil a	1.21	24.86	1.4917	49.72	0.0297	0.148
Mil b	1.45	30.57	1.5086	50.28	0.0243	0.121
Skm a	1.25	27.97	1.6893	56.31	0.0234	0.117
Skm b	2.51	53.33	1.6163	53.88	0.0261	0.130

FIGURE 5a

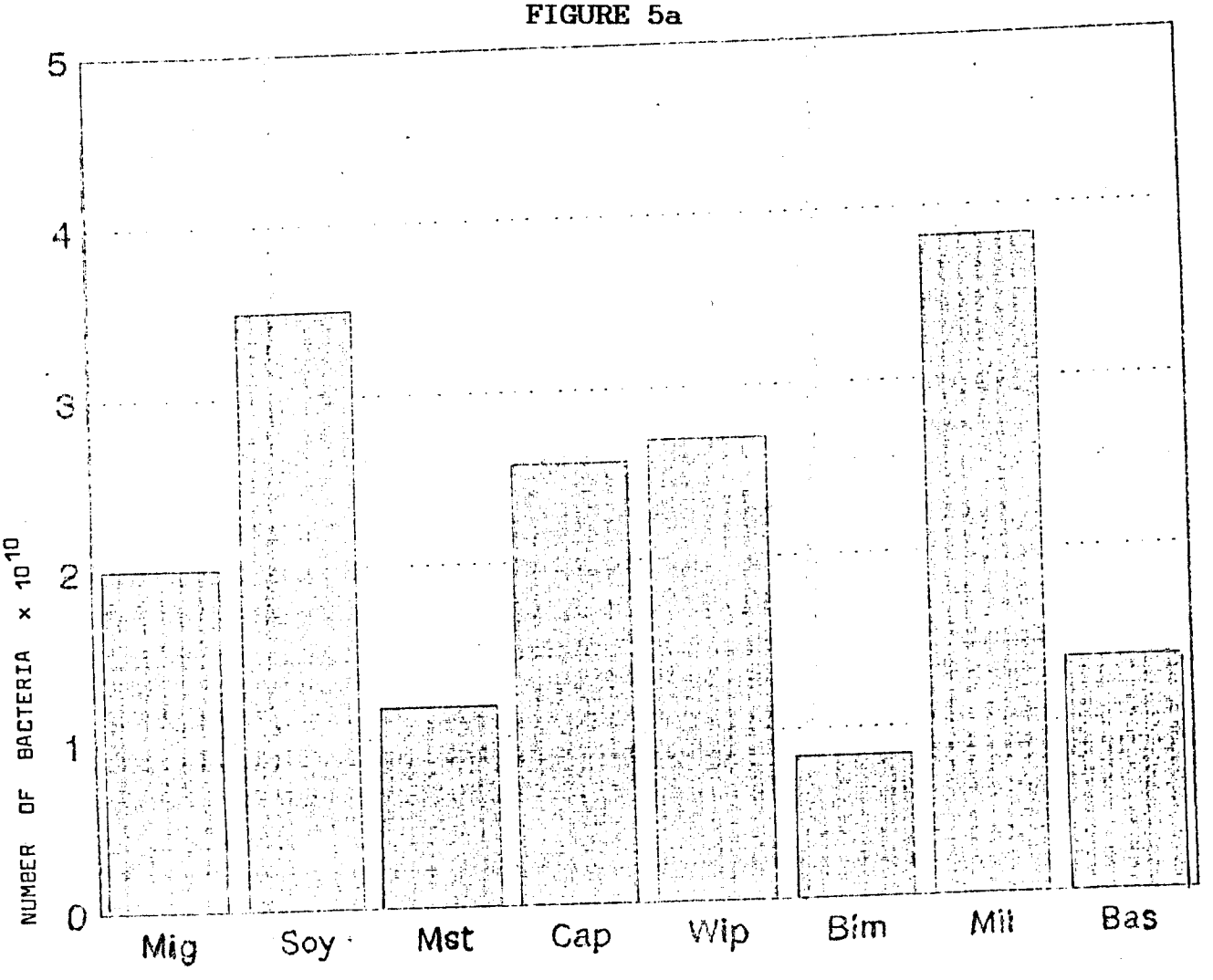


Figure 5a: Shows plots of logarithm values against time for preliminary media turbidimetry sensitivity test

FIGURE 5b

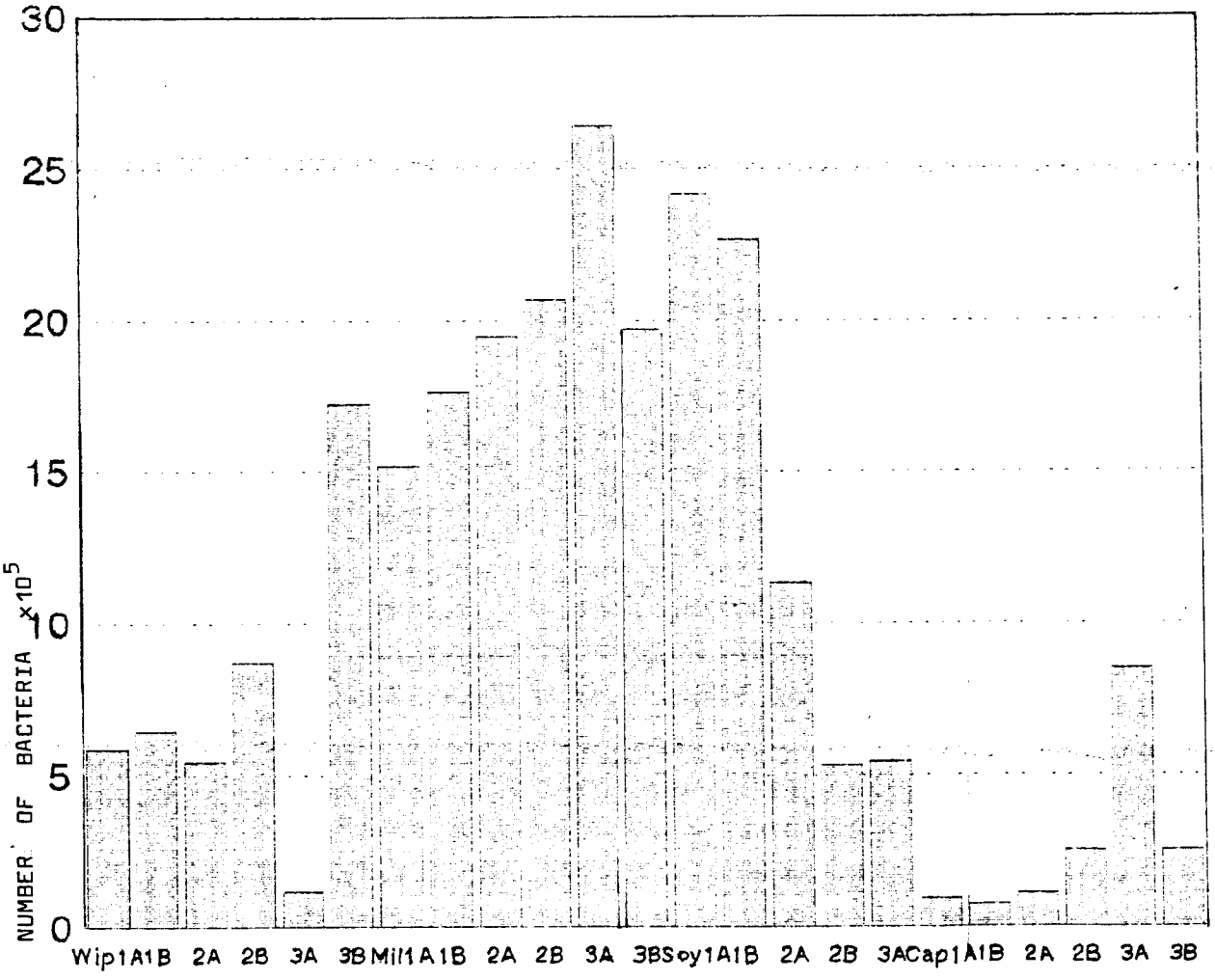


Figure 5b: shows plots of logarithm values against time for final media turbidimetry sensitivity test.

CHAPTER FOUR

4.0 DISCUSSION, CONCLUSION, RECOMMENDATIONS

4.1 DISCUSSION

Excursions to cheese processing plants in the **Lusaka** region revealed that no local institution nor individual dairy farmers have committed themselves to isolating commercial dairy starter cultures. This is unfortunate because by prolonging the viability as well as multiplying dairy starter cultures, isolation and subculturing techniques greatly reduce cost of dairy produce manufacturing. On the other hand, it is only due to lack of knowledge that makes some commercial cheese producers, such as KCF, hesitate to apply subculturing techniques in their operations.

Bacteria used in this study were **Lactobacillus, Streptococcus,** and **Leuconostoc** species in mixed culture. The relative numbers of these bacteria was determined by the turbidimetry and colony count techniques. All of these gave positive Gram stain reaction, negative benzidine reaction, and positive glucose and lactose fermentation reations. The positve catalase

reaction gave a rather unconsitent **Lactic Bacteria** character, but going by the latest edition (8th) of Bergey's Manual of determinative bacteriology, the catalase test is inconsitent for **Lactic bacteria** and thus the presnt observed result is still suggestive of **Lactic bacteria** trait.

Going by the ANOVA five per cent significant analysis result on preliminary media by which it was clearly shown that the media were significantly different from each other, an observation which was further supported by the varying quantities of organisms from medium to medium in the standard plate count results, the fact that sources of carbon, nitrogen and essential nutrients varied from medium is availed.

The Duncan's muiltiple range test on preliminary media turbidimetry results reveales that Soy and Mil media at five per cent significant level are not significantly different to each other but are significant to the rest of the media. these media, though

with different constituents, are both very rich in growth factors.

But how can one reconcile with the observed significant difference between Mil and Mig? This is a tricky situation as one would expect Mig to be a better medium than Mil since Mil only has milk while Mig has glucose besides milk. A tentative explanation to this, is that, since the lyophilized starter culture were cultivated in milk NA, the cells' enzyme complex was better adapted to growth in milk and glucose. The reason for this is because microorganisms generally prefer glucose to lactose as a source of carbon. Thus cells which are already adapted to lactose have glucose metabolism enzymes repressed.

Due to for going, if the purpose for cultivating lactic bacteria is to rapidly grow the organisms, then Mil would be recommended but if it does not matter if it took long to cultivate them, then Mig would be better.

Overlooking the fact that at the beginning

of the experiment, clarity of media in the turbidimetry experiment was not uniform due to differences in opacity of raw materials used. Figure 3 shows the following general trend of growth rate beginning with the most optimal: Mil-> Soy-> Bas-> Mig-> Wip->Blm-> Mst->Cap.

Inerestingly, this descending order of growth rate only resembles the order of quantity of bacteria in the SPC experiment with the first two media. On the other hand, plots of the growth rates exhibited by figure 4 are clustered, espacially from day 3 to day 9. The close relationship of growth rate plots highlights the fact that the four media were each replicated.

On both, the preliminary and final media colony count results, the chi-square (X sq.), as a goodnesss-of-fit test, at five per cent significant level, gave a strong evidence against the null hypothesis. Therefore, the hypothesis that "given the same environmental and hence, at any given

time, there should be different numbers of organisms where each medium is inoculated with an equal quantity and strain(s) of a culture," is accepted.

though cheese is classified differently from country to country (Eekhof-stork, 1976), Pearson (1970) gives the following general composition of fat acidity and moisture in cheese

TABLE 17: ACID AND MOITURE IN CREAM CHEESE ACCORDING TO PEARSON (1970)

	%W/W
ACIDITY	0.3 - 1.9
WATER	48 - 60
FAT	- -

Thus going by Pearson's data, the cheese that was made according to data in 7.2 had normal moisture composition but the acid was lower by 0.15 per cent to 0.18 per cent. Factors that are likely to have contributed are short culture

incubation time or short time of **Lactic bacteria** to act on curd in the vat or both.

In this study, due to limited resources and time, it was not possible to carry out more SPC tests on final media which would have provided more useful information about the the potentiality of the final devised media.

Further studies on the devised media with specific species of the **Lactic bacteria** should provide interesting and useful information for the dairy industry in the country.

4.2 CONCLUSION

The high cost of commercial dairy products, partly due to the high cost of importing commercial dairy starter cultures, can be offset by establishing local commercial dairy starter culture isolating centres and effective use of the subculturing technique.

The fact that various cheese starter culture media, processed from raw materials bought from Kamwala and Soweto markets in **Lusaka,** were successfully used to propagate and grow cheese starter cultures should ignite interest to dairy farmers. With specialisation and commitment, better cheese starter culture media could be locally processed and contribute greatly to the growth of the dairy industry in the country.

4.3 RECOMMENDATIONS

At the DPBZ **Lusaka** plant, it would be a good idea to recycle the whey to by selling the commodity to dairy farmers who sell milk to the plant instead of trearing as waste product.

A microbiology laboratory at KCF for isolation and subculturing of commercial starter cultures would be a positive development.

It is now a high time that Zambian as a a nation should set up a commercial starter cultures and rennet processing centre.

APPENDICES

APPENDIX A

SIGNIFICANCE, DUNCAN'S MULTIPLE RANGE, AND ANALYSIS OF VARIANCE (ANOVA) TESTS

Preliminary turbidimetry results were tested at five per cent significant level by Duncan's multiple range test. The totals and means required for the test were obtained as follows.

TABLE: PRELIMINARY TURBIDIMETRY RESULTS WITH SUMS AND MEANS FOR EACH MEDIUM.

	M E D I A							
DURATION	Mig	Soy	Mst	Cap	Wip	Blm	Mil	Bas
DAY 1	0.13	0.49	0.07	0.04	0.12	0.07	0.39	0.05
DAY 2	0.19	0.56	0.09	0.05	0.17	0.09	0.48	0.29
DAY 3	0.20	0.61	0.09	0.06	0.19	0.11	0.81	0.31
DAY 4	0.37	0.66	0.15	0.07	0.23	0.15	1.00	0.31
DAY 5	0.39	0.69	0.16	0.08	0.25	0.15	1.14	0.35
DAY 6	0.41	0.73	0.20	0.08	0.27	0.17	1.50	0.45
DAY 7	0.41	1.30	0.20	0.09	0.28	0.22	1.60	0.47
DAY 8	0.64	1.50	0.20	0.11	0.29	0.26	1.80	0.70
DAY 9	0.75	1.80	0.32	1.35	0.48	0.40	1.80	0.73
TOTALS	3.49	8.34	1.48	1.93	2.28	2.62	10.52	3.66
MEANS	0.39	0.93	0.16	0.21	0.25	0.29	1.17	0.41

The order of means, when arranged in a descending order from the largest to the smallest is as follows:

Mil	**Soy**	**Bas**	**Mig**	**Blm**	**Wip**	**Cap**	**Mst**
1.17	0.93	0.41	0.39	0.29	0.25	0.21	0.16

The table below shows differences between corresponding largest and smallest means:

TABLE: SHOWING MEAN DIFFERENCES

A DESCENDING ORDER OF MEANS MINUS THE SMALLEST							AN ASCENDING ORDER OF MEANS MINUS THE LARGEST	
Mil	Soy	Bas	Mig	Blm	Wip	Cap		
1.01	0.77	0.25	0.23	0.13	0.09	0.05	Mst	0.16
0.96	0.72	0.20	0.18	0.08	0.04		Cap	0.21
0.92	0.68	0.16	0.14	0.04			Wip	0.25
0.88	0.64	0.12	0.10				Blm	0.29
0.78	0.54	0.02					Mig	0.39
0.76	0.52						Bas	0.41
0.24							Soy	0.93

The required standard error (S.E), whose formula is;

$$S.E = \sqrt{\frac{S^2}{N}}$$, where, S^2 = Standard deviation
N = Number of varieties,

Therefore $$S.E = \sqrt{\frac{(0.3456493)^2}{8}}$$

$$= 0.12$$

Degrees of freedom (n-1) = 8 - 1 = 7. The value obtained for degrees of freedom = 7 from the standard table for Duncan's multiple range test

at five per cent significant level was multiplied by the S.E to obtain D - Values as follows:

$D_2 = 3.35 \times 0.12 = 0.43$

$D_3 = 3.47 \times 0.12 = 0.45$

$D_4 = 3.54 \times 0.12 = 0.46$

$D_5 = 3.58 \times 0.12 = 0.47$

$D_6 = 3.60 \times 0.12 = 0.47$

$D_7 = 3.61 \times 0.12 = 0.47$

$D_8 = 3.61 \times 0.12 = 0.47$

See the completed Duncans' multiple range table below.

	A DESCENDING ORDER OF MEANS MINUS THE SMALLEST							AN ASCENDING ORDER OF
D-VALUES	Nil	Soy	Bas	Nig	Blm	Wip	Cap	MEANS MINUS THE LARGEST
	1.17	0.93	0.41	0.39	0.29	0.25	0.21	
D8=0.47	1.01	0.77	0.25	0.23	0.13	0.09	0.05	Mst 0.16
D7=0.47	0.96	0.72	0.20	0.18	0.08	0.04		Cap 0.21
D6=0.47	0.92	0.68	0.16	0.14	0.04			Wip 0.25
D5=0.46	0.88	0.64	0.12	0.10				Blm 0.29
D4=0.46	0.78	0.54	0.02					Nig 0.39
D3=0.45	0.76	0.52						Bas 0.41
D2=0.43	0.24							Soy 0.93

From the Duncan's multiple range table, at five per cent significant level, media Mil and Soy are not significantly different from each other but the two are significantly different from the rest as summarised below:-

Mil	**Soy**	**Bas**	**Mig**	**Blm**	**Wip**	**Cap**	**Mst**
1.17	0.93	0.41	0.39	0.29	0.25	0.21	0.16

To determine whether the observed differences in turbidimetry results are significant or not, they were tested by the analysis of variance (ANOVA) test at five per cent significant level.
Data in the table below shows how N(sum of n observations), $\leq x$ (sum of absorbance readings for each medium), $(\leq x)$sq./n (sum of absorbance readings squared for each medium divided by number of observations), $(\leq x)^2/n$ (sum of absorbance readings squared for each medium divided by number of observations), $\leq x$ squared/n (sum of squares of absorbance readings for each medium) were calculated.

TABLE: DATA FOR ANOVA ON PRELIMINARY MEDIA TURBIDIMETRY RESULTS.

MEDIUM	Mig	Soy	Mst	Cap	Wip	Blm	Mil	Bas
n	9	9	9	9	9	9	9	9
$\leq x$	3.49	8.34	1.48	1.93	2.28	2.62	10.52	3.66
$(\leq x)^2/n$	1.35	7.73	0.24	0.41	0.58	0.76	12.30	1.49
$\leq x^2$	1.69	9.55	0.29	1.87	0.65	2.17	14.63	1.85

From the table; $N = \leq n = 81$

$G = \leq(\leq x) = 34.32$

$\leq[(\leq x)^2/n] = 24.45$

$\leq(\leq x^2) = 30.70$

c (correction factor) $= G^2/N = \frac{(34.32)}{81} = 14.54$

SS(sum of squares) $= \leq(\leq x^2) - C = 30.70 - 14.54 = 16.16$

SST(sum of squares total)$= \leq[(\leq x)^2/n] - C = 24.45 - 14.54$

$= 9.91$

SSE(Variance error) = SS - SST = 16.16 - 9.91

$= 6.25$

Df(Degree of freedom) = K(number of varieties)(n-1)

$= 8(9-1) = 64$

$$F(\text{variance ratio}) = \frac{SST}{SSE} \times \frac{Df}{N} = \frac{9.91}{6.25} \times \frac{64}{9}$$

$$= 11.27$$

ANOVA standard table value for DF = 64 at five per cent significant level is 2.22. Therefore, as the calculated F (11.27) is greater than the table value (2.22), differences between the observed preliminary media turbidimetry results are statistically significant.

To satistically determine that there were no significant differences between duplicate absorbance readings of each medium, turbidimetry results were tested against a significance test based on normal distribution according to the following formula as outlined by Bailey (1987);

$$d = \frac{\bar{x}_1 - \bar{x}_2}{\sqrt{\frac{S_1^2}{n_1} + \frac{S_2^2}{n_2}}}$$

where; d= normal variable with zero mean.

$\bar{x}_1$ = mean 1

$\bar{x}_2$ = mean 2

S_1^2 = Standard deviation 1

S_2^2 = Standard deviation 2

n_2 = No. of observations 1

n_2 = " " " 2

d values were calculated as outlined in the following tables.

TABLE : Showing Preliminary Wip Media d computations.

	Wip1A	Wip1B	Wip2A	Wip2B	Wip3A	Wip3B
DAY1	0.089	0.11	0.16	0.14	0.199	0.22
DAY2	0.10	0.085	0.11	0.11	0.189	0.21
DAY3	0.091	0.15	0.165	0.17	0.15	0.20
DAY4	0.085	0.14	0.21	0.11	0.085	0.18
DAY5	0.15	0.085	0.21	0.09	0.16	0.28
DAY6	0.09	0.08	0.143	0.171	0.179	0.185
DAY7	0.099	0.182	0.179	0.20	0.16	0.27
DAY8	0.085	0.141	0.163	0.161	0.19	0.24
DAY9	0.079	0.181	0.189	0.26	0.19	0.22
Σx	0.868	0.154	1.529	2.941	1.502	1.965
$\bar{x}$	0.09644	0.12822	0.16988	0.163388	0.1668888	0.218333
$\bar{x}_1-\bar{x}_2$		0.0317778		0.0065		0.0514445
S^2/n	0.002216	0.004198	0.0033425	0.0046066	0.0036703	0.0031426
$\sqrt{\frac{S_1^2}{n} + \frac{S_2^2}{n}}$		0.00088		0.0891577		0.0825408
d		0.396786		0.0729045		0.6232614

Table: Showing preliminary Mil media d of computations.

	Mil1A	Mil1B	Mil2A	Mil2B	Mil3A	Mil3B
DAY1	0.43	0.58	1.0	0.94	1.10	1.20
DAY2	0.36	0.56	0.89	0.95	1.10	1.15
DAY3	0.265	0.281	0.42	0.251	0.51	1.21
DAY4	0.42	0.29	0.45	0.228	0.155	0.211
DAY5	0.27	0.41	0.82	0.57	1.45	0.163
DAY6	0.245	0.31	0.47	0.56	0.71	0.28
DAY7	0.283	0.26	0.36	0.499	0.581	0.182
DAY8	0.13	0.28	0.50	0.56	0.46	0.16
DAY9	0.22	0.36	0.40	0.59	0.64	0.322
Σx	2.623	3.331	5.31	5.148	6.706	4.878
$\bar{x}$	0.291444	0.3701111	0.59	0.572	0.7451111	0.542
$\bar{x}_1 - \bar{x}_2$		0.0786667		0.018		0.2031111
S/n	0.010141	0.0128311	0.0254183	0.0263361	0.0417841	0.0509797
$\sqrt{\frac{S_1^2}{n} + \frac{S_2^2}{n}}$		0.1515675		0.2274967		0.3045715
d		0.5190209		0.0791222		0.6668749

Table: Showing preliminary Soy media d of computations

	Soy1A	Soy1B	Soy2A	Soy2B	Soy3A	Soy3B
DAY1	0.055	0.208	0.175	0.079	0.08	0.032
DAY2	0.37	0.45	0.15	0.03	0.04	0.02
DAY3	0.251	0.48	0.175	0.24	0.143	0.071
DAY4	0.43	0.51	0.215	0.17	0.13	0.07
DAY5	0.51	0.57	0.365	0.152	0.143	0.162
DAY6	0.53	0.58	0.65	0.165	0.171	0.128
DAY7	0.72	0.60	0.681	0.74	0.37	0.182
DAY8	0.50	0.60	0.62	0.519	0.44	0.14
DAY9	0.62	0.60	0.65	0.79	0.55	0.18
Σx	3.986	4.598	3.681	2.885	2.067	0.985
$\bar{x}$	0.442888	0.5108888	0.409	0.3205555	0.2296666	0.1094
$\bar{x}_1 - \bar{x}_2$		0.068		0.0884444		0.1202221
S/n	0.020828	0.0132764	0.0248681	0.0300942	0.018641	0.006554
$\sqrt{\frac{S_1^2}{n} + \frac{S_2^2}{n}}$		0.1846759		0.2344404		0.1587293
d		0.3682126		0.3772575		0.7574033

Table: Showing preliminary Cap media d of computations

	Cap1A	Cap1B	Cap2A	Cap2B	Cap3A	Cap3B
DAY1	0.038	0.05	0.035	0.022	0.37	0.241
DAY2	0.019	0.03	0.011	0.10	0.049	0.16
DAY3	0.036	0.049	0.131	0.041	0.11	0.11
DAY4	0.068	0.09	0.11	0.04	0.08	0.092
DAY5	0.14	0.079	0.151	0.033	0.07	0.33
DAY6	0.14	0.07	0.135	0.036	0.054	0.27
DAY7	0.14	0.085	0.13	0.09	0.085	0.23
DAY8	0.13	0.08	0.125	0.05	0.051	0.19
DAY9	0.141	0.11	0.14	0.13	0.06	0.22
Σx	0.852	4.643	0.968	0.542	0.929	1.843
$\bar{x}$	0.094666	0.0714444	0.1075555	0.0602222	0.1032222	0.204777
$\bar{x}_1 - \bar{x}_2$		0.0232221		0.0473332		0.1015555
S/n	0.005574	0.0025735	0.005191	0.0038861	0.0106798	0.0079469
$\sqrt{\frac{S_1^2}{n} + \frac{S_2^2}{n}}$		0.0902636		0.0952741		0.1364799
d		0.2572698		0.4968107		0.7441059

Since d values, range from -0.623264 to 0.7574033, they are all less than 1.96 and thus differences between means of absorbance readings for final media turbidimetry results are insignificant at five per cent level of significance.

To determine which media were statistically similar, the duplicate means were tested at five per cent signicant level by the Duncan's multiple range test.

The totals and means required for the test were obtained as illustrated in the table below:

Table: Showing duplicate means, mean totals and subsequent means

MEDIA	DAY1	DAY2	DAY3	DAY4	DAY5	DAY6	DAY7	DAY8	DAY9	$\leq x$	$\bar{x}$
Wip1	0.10	0.09	0.12	0.11	0.12	0.09	0.14	0.11	0.13	1.01	0.11
Wip2	0.15	0.11	0.17	0.16	0.15	0.16	0.19	0.16	0.22	1.47	0.16
Wip3	0.20	0.20	0.17	0.12	0.20	0.18	0.21	0.21	0.20	1.69	0.19
Mil1	0.50	0.46	0.27	0.56	0.47	0.27	0.28	0.20	0.27	3.28	0.36
Mil2	0.97	0.92	0.33	0.34	0.69	0.51	0.43	0.53	0.49	5.21	0.58
Mil3	1.15	1.12	0.86	0.18	0.81	0.49	0.38	0.31	0.48	5.78	0.64
Soy1	0.13	0.41	0.36	0.47	0.54	0.55	0.66	0.55	0.61	4.28	0.47
Soy2	0.13	0.09	0.21	0.19	0.26	0.41	0.71	0.57	0.72	3.29	0.36
Soy3	0.06	0.03	0.11	0.10	0.15	0.15	0.28	0.29	0.36	1.53	0.17
Cap1	0.04	0.02	0.04	0.08	0.11	0.10	0.11	0.10	0.12	0.72	0.08
Cap2	0.03	0.11	0.09	0.07	0.09	0.08	0.11	0.09	0.13	0.80	0.09
Cap3	0.14	0.10	0.11	0.09	0.20	0.16	0.16	0.12	0.13	1.21	0.13

Means, when arranged in a descending order are as follows;

Mil3	Mil2	Soy1	Mil1	Soy2	Wip3	Soy3	Wip3
0.64	0.58	0.47	0.36	0.36	0.19	0.17	0.16

Cap3	Wip1	Cap2	Cap1
0.13	0.11	0.09	0.08

$$S.E = \sqrt{\frac{S^2}{N}}$$

Where, S.E = Standard error

S^2 = Standard deviation

N = Number of varieties

$$S.E = \sqrt{\frac{0.1894216}{12}}$$

$$= 0.1256389$$

The Duncan's multiple range table is as outlined below.

DUNCAN'S MULTIPLE RANGE TABLE FOR FINAL MEDIA

A DESCENDING ORDER OF MEANS MINUS THE SMALLEST											AN ASCENDING ORDER
Mil3	Mil2	Soy1	Mil1	Soy2	Wip3	Soy3	Wip2	Cap3	Wip1	Cap2	OF MEANS MINUS THE
0.64	0.58	0.47	0.37	0.36	0.19	0.17	0.16	0.13	0.11	0.09	LARGEST.
0.56	0.50	0.39	0.28	0.29	0.11	0.09	0.08	0.05	0.03	0.01	Cap1 0.08
0.55	0.49	0.38	0.27	0.27	0.10	0.08	0.07	0.04	0.02		Cap2 0.09
0.53	0.47	0.36	0.25	0.25	0.08	0.06	0.05	0.02			Wip2 0.11
0.51	0.45	0.34	0.23	0.23	0.06	0.04	0.03				Cap3 0.13
0.48	0.42	0.31	0.20	0.20	0.03	0.01					Wip2 0.16
0.47	0.41	0.30	0.19	0.19	0.02						Soy3 0.17
0.45	0.38	0.28	0.17	0.17							Wip3 0.19
0.28	0.22	0.11									Soy2 0.36
0.28	0.22	0.11									Mil1 0.36
0.17	0.11										Soy1 0.47
0.06											Mil2 0.58

Degrees of freedom (n-1) = 12 - 1 = 11. The value obtained for degrees of freedom = 11 from the standard table for Duncans' multiple range at five per cent significant level was multiplied by the S.E to obtain D-values as follows.

$D_2 = 3.115 \times 0.12 = 0.37$

$D_3 = 3.26 \times 0.12 = 0.39$

$D_4 = 3.35 \times 0.12 = 0.40$

$D_5 = 3.39 \times 0.12 = 0.41$

$D_6 = 3.43 \times 0.12 = 0.41$

$D_7 = 3.44 \times 0.12 = 0.41$

$D_8 = 3.45 \times 0.12 = 0.41$

$D_9 = 3.455 \times 0.12 = 0.41$

$D_{10} = 3.46 \times 0.12 = 0.41$

$D_{11} = 3.47 \times 0.12 = 0.42$

$D_{12} = 3.48 \times 0.12 = 0.42$

See completed Duncan's multiple range table outlined.

TABLE: DUNCAN'S MULTIPLE RANGE COMPLETED TABLE FOR FINAL MEDIA

D-VALUES	A DESCENDING ORDER OF MEANS MINUS THE SMALLEST											AN ASCENDING ORDER OF MEANS MINUS THE LARGEST.
	Mil3	Mil2	Soy1	Mil1	Soy2	Wip3	Soy3	Wip2	Cap3	Wip1	Cap2	
	0.63	0.58	0.47	0.36	0.36	0.19	0.17	0.16	0.13	0.11	0.09	
D12 = 0.42	0.56	0.50	0.39	0.28	0.29	0.11	0.09	0.08	0.05	0.03	0.01	Cap1 0.08
D11 = 0.42	0.55	0.49	0.38	0.27	0.27	0.10	0.08	0.07	0.04	0.02		Cap2 0.09
D10 = 0.41	0.53	0.47	0.36	0.25	0.25	0.08	0.06	0.05	0.02			Wip2 0.11
D9 = 0.41	0.51	0.45	0.34	0.23	0.23	0.06	0.04	0.03				Cap3 0.13
D8 = 0.41	0.48	0.42	0.31	0.20	0.20	0.03	0.01					Wip2 0.16
D7 = 0.41	0.47	0.41	0.30	0.19	0.19	0.02						Soy3 0.17
D6 = 0.41	0.45	0.39	0.28	0.17	0.17							Wip3 0.19
D5 = 0.41	0.28	0.22	0.11									Soy2 0.36
D4 = 0.40	0.28	0.22	0.11									Mil1 0.36
D3 = 0.39	0.17	0.11										Soy1 0.47
D2 = 0.37	0.06											Mil2 0.58

To determine whether observed absorbance readings are significantly different or not from each other turbidimetry results were tested by the analysis of variance test as in 4.8.6.3. See table on data for ANOVA on final media turbidimetry results.

TABLE : SHOWS DATA FOR ANOVA ON FINAL MEDIA TURBIDIMETRY RESULTS.

MEDIUM	n	$\leq x$	$(\leq x^2)/n$	$\leq x^2$
Wip1	9	1.01	0.11	0.12
Wip2	9	1.47	0.24	0.25
Wip3	9	1.69	0.32	0.32
Mil1	9	3.28	1.20	1.33
Mil2	9	5.21	3.02	3.45
Mil3	9	5.78	3.71	4.72
Soy1	9	4.28	2.03	2.24
Soy2	9	3.29	1.20	1.69
Soy3	9	1.53	0.26	0.36
Cap1	9	0.72	0.06	0.07
Cap2	9	0.80	0.07	0.08
Cap3	9	1.21	0.16	1.17

From the table; $N = 81$

$G = 30.27$

$\leq[(\leq x)^2/n] = 12.38$

$$\Sigma(\Sigma x^2) = 15.80$$

$$C = G^2/N = 11.31$$

$$SS = \Sigma(\Sigma x^2) - C = 4.49$$

$$SST = \Sigma[(\Sigma x)^2/n] - C = 1.07$$

$$SSE = SS - SST = 3.42$$

$$Df = K(n-1) = 12(9-1) = 96$$

$$F = \frac{SST}{SSE} \times \frac{Df}{N} = 0.37$$

ANOVA standard table value for Df = 96 at five per cent significant level is 2.25. Therefore, as the calculated F(0.37) is less than the table value (2.25), differences between the observed means of the final media turbidimetry results are statistically insignificant.

APPENDIX B

CHI-SQUARE AS A GOODNESS-OF-FIT TEST

To determine whether preliminary media colony count results are due to merely a chance happening or there were significant differences between them, they were tested against chi-square (X Sq.), as a goodness-of-fit test. This test, tests results on the basis of whether they are good enough to fit the null hypothesis or not. The null hypothesis in the present study is that 'given the same environmental and incubation conditions, nutrients of various media will not affect the rate of culture growth and therefore, at any given time there should be the same number of organisms where each medium is inoculated with the same quantity and strain(s) of a culture'.

The table below shows how X^2 was calculated.

Table : Showing how X sq. for preliminary media colony count results was calculated.

	(O) COLONY COUNT	(E) EXPECTED	(0 - E)	$(0 - E)^2$	$(0 - E)^2/E$
Mig	800	898.25	- 98.25	9653.06	10.75
Soy	1398	898.25	499.75	249750.06	278.04
Met	472	898.25	- 426.25	181689.06	202.27
Cap	1028	898.25	129.75	16835.06	18.74
Wip	1078	898.25	179.75	32310.06	35.97
Blm	336	898.25	- 562.25	316125.06	351.93
Mil	1530	898.25	631.75	399108.06	444.32
Bas	544	898.25	- 354.25	125493.06	139.71
Total	7186				
Mean	898.25				$X^2 = 1481.73$

Nb. 'O' denotes number of colonies observed and 'E' the expected number of colonies which would be counted should there be no nutrient differences in media.

Df (degrees of freedom) = n-1 = 8 - 1 = 7. The value of X^2 obtained at 5 per cent confidential level for Df = 7 is 14.07. Since 14.07 is much less than the calculated X. sq. (1481.73), there is thus a strong evidence against the null hypothesis which can thus be discarded.

4.8.6.3.4 ANALYSIS OF FINAL MEDIA COLONY COUNT RESULTS

To determine whether final media colony count results are due to merely a chance happening or

there were significant differences between them, they were tested against X Sq. as a goodness-of-fit test as in 4.8.6.3.3. The table below shows how X sq. was calculated.

TABLE : SHOWING HOW X SQ. FOR FINAL MEDIA COLONY COUNT RESULTS WAS CALCULATED.

	(O) COLONY COUNT	(E) EXPECTED	(O - E)	$(O - E)^2$	$(O - E)^2/E$
Wip1A	234.0	451.7	- 217.7	47393.29	104.92
Wip1B	258.5	451.7	- 193.2	37326.24	82.63
Wip2A	218.5	451.7	- 233.2	54382.24	120.39
Wip2B	348.0	451.7	- 103.7	10753.69	23.81
Wip3A	469.0	451.7	17.3	299.29	0.66
Wip3B	689.0	451.7	237.3	56311.29	124.66
Mil1A	607.0	451.7	155.3	24118.09	53.39
Mil1B	706.0	451.7	254.3	64668.49	143.17
Mil2A	777.0	451.7	325.3	105820.09	234.27
Mil2B	827.0	451.7	375.3	140850.09	311.82
Mil3A	1056.5	451.7	604.8	365783.04	809.79
Mil3B	789.0	451.7	337.3	113771.29	251.87
Soy1A	966.5	451.7	514.8	265019.04	586.71
Soy1B	906.5	451.7	454.8	206843.04	457.92
Soy2A	454.0	451.7	2.3	5.29	0.01
Soy2B	213.5	451.7	- 238.2	56739.24	125.61
Soy3A	218.0	451.7	- 233.7	54615.69	120.91
Soy3B					
Cap1A	37.0	451.7	- 414.7	171976.09	380.73
Cap1B	29.5	451.7	- 422.2	178252.84	394.63
Cap2A	43.5	451.7	- 408.2	166627.24	368.89
Cap2B	99.5	451.7	- 352.2	124044.84	274.62
Cap3A	341.0	451.7	- 110.7	12254.49	27.13
Cap3B	100.5	451.7	- 351.2	123341.44	273.06
Total	10389.0				
Mean	451.8				$X^2 = 5271.6$

Df = n - 1 = 23 - 1 = 22. The value of X^2 obtained at five per cent confidential level for Df = 22 is 35.17. Since 35.17 is much less than the calculated X sq. (5271.6), there is thus a strong statistical evidence against the null hypothesis which can thus be discarded.

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