b140 167 46

U.O.V.S. BIBLIOTEEK

University Free State
34300000968358
Universiteit Vrystaat

HIERDIE EKSEMPLAAR MAG ONDER GEEN OMSTANDIGHEDE UIT DIE BIBLIOTEEK VERWYDER WORD NIE

THE OCCURRENCE AND DIVERSITY OF YEASTS IN COMMERCIAL YOGHURT

BRIDGET IKALAFENG

2001

THE OCCURRENCE AND DIVERSITY OF YEASTS IN COMMERCIAL YOGHURT

by

BRIDGET IKALAFENG

Submitted in fulfilment of the requirements for the degree

MAGISTER SCIENTIAE

in the

Department of Food Science,
Faculty of Natural Science and Agriculture
University of the Free State,
Bloemfontein 9300,
South Africa

Supervisor:	Prof B.C.Viljoen
Co-supervisor:	Dr A. Hattingh
	Dr J Myburgh

November 2001

Universiteit van die
Oranje-Vrystaat
BLOEMFONTEIN
26 APR 2002
UOVS SASOL BIBLIOTEEK

Acknowledgements:

Prof. B.C. Viljoen, for his excellent guidance in planning, undertaking as well as constructive criticism of this study;

Dr. A Hattingh, for her advise and invaluable suggestions;

Dr. J Myburg for all the critics and guidance;

To the rest of the colleagues in the lab; for their friendship, interest and co-operation;

To my family, for their love; and

Finally and most importantly, to the Lord, The Almighty, for watching over me and giving me strength to carry on.

CONTENTS

1.1	**Introduction**	3
1.2	**Literature review**	3
1.2.1	Historical background	3-5
1.2.2	Standard yoghurt making	5-6
1.2.3	Microbiology of natural yoghurt	6-9
1.2.4	Yeasts in dairy products	9-17
1.2.5	Microbial Interactions	17-19
1.3	**References**	21-25
	Annex 1	26

CHAPTER 1

1.1 INTRODUCTION

Yeasts have a competitive advantage in yoghurts at elevated temperatures and are major role players in causing spoilage. Spoilage yeasts are responsible for undesirable changes in food or beverages either during processing or there after. However, with yoghurt, this unwanted activity often goes unrecognized and underestimated. Yeasts have been mentioned as suspects for the allergic reactions of consumers to foods. Presently yeasts are considered of limited hygienic significance in the dairy sector. Dairy products present a unique ecological niche, selecting for the growth and occurrence of only a few main species. The growth and predominance of yeasts in dairy products are due its proteolytic and lipolytic activities, growth at low pH, assimilation of lactic acid, assimilation of citric acid, fermentation or assimilation of lactose as well as tolerance to low water activity. These yeasts originated from contaminated ingredients such as fruits, nuts and honey which, in most operations are added to the fermented yoghurt base just before packaging. The yeasts develop on the surfaces of production equipment, such as mixing vessels and filling machines that have been poorly cleaned and sanitized. Consequently, in this study we endeavoured to determine the frequency and diversity of yeasts associated with commercial yoghurts and the influence of storage temperatures on the viability of the contaminating yeasts. In addition the interaction between the yeasts and lactic acid bacteria was also monitored.

1.2 LITERATURE REVIEW

1.2.1 Historical background

The fermented milk produced by the specific acidophilic micro-organisms *Lactobacillus delbrueckii* subsp. *bulgaricus* and *Streptococcus salivarius* subsp. *thermophilus*, known by the traditional name of yoghurt, is usually

manufactured with temperature treated milk of standard composition after inoculation and storage at 45°C (Canganella et al., 1998). It is an extremely popular fermented milk food in Europe, Asia and Africa (Kosikowski, 1966) for decades and is continueing to gain popularity in the United States of America since the seventies (Lundstedt, 1974).

From its early origins in the Balkans and the Middle East, yoghurt has achieved world-wide acclaim and the production of its various forms can now be measured in millions of tons per annum. However, despite of its obvious popularity, no precise definition of yoghurt has been formulated; even the spelling appears to be a matter of personal preference (Robinson and Tamine, 1975). It is spelled several ways namely; yogurt, yoghurt, yohurt, yohourt, (Kosikowski, 1960), yohourt or yaourt (Emmens and Tuckey, 1967) and in some cases the "y" is replaced by the "j" e.g jogurt (Robinson and Tamine, 1975). It is also known by quite different names in different parts of the world; madzoon or matzoon (America), naja (Bulgaria), dahi (India), leben or leben raib (Egypt) (Kosikowski, 1966).

Fermented milks (including yoghurt) are products derived from milk (whole, partially or fully skimmed milk, concentrated milk or milk reconstituted from partially or fully skimmed dried milk) which are homogenised or non homogenised, pasteurised or sterilised and fermented, by means of specific microorganisms (International Dairy Federation, 1969). In the case of yoghurt these organisms are representatives of the genera *Streptococcus thermophillus* and *Lactobacillus bulgaricus*. After incubation and cooling the acidity should be 0.8–0.9 % and a cocci to rods ratio of 1:1 is desirable (Humphreys and Plunkett, 1969).

Yoghurt is currently produced as plain, with added fruits or flavoured, and even has moved to the chest where it appears as a frozen desert or as a

confection on a stick (Christensen, 1970). A study of the literature indicated that European and Scandanavian countries are more inclined to insist upon a strong flavour in yoghurt than other countries. The fruit-flavoured yoghurt is currently the most marketed type, with a ratio of 4:1 compared with the natural type (D'Amicis, 1966, Tealdo et al., 1985).

1.2.2 Standard yoghurt making

The commercial production of yoghurt is a highly technical and well-developed process in which temperature, amount of acid produced, microbial purity and many environmental conditions are regulated in order to produce a sound product. Yoghurt manufacture in many countries is highly mechanized and large volumes of cows milk serve as the predominant substrate. Yoghurt can be made from either "whole" milk or skim milk. "Whole" milk, is milk from cows that contains 3.5 – 3.7% milk fat. Skim milk contains less than 0.1% milk fat derived from whole milk. Following the proper blending of any additional flavouring ingredients and the adjustment to the desired fat and non-fat solids concentrates. The mixture is homogenized and pasteurized. An average of 15 to 20 % of fruit is added but may even range from 10 to 30 %. The fruits are purchased either as frozen or as canned and dried preparations. The increase in yoghurt popularity has been mainly due to the addition of fruits in yoghurt.

The steps involved in the manufacture of yoghurt are illustrated in Fig. 1. The preliminary treatment of milk involves the preparation of the basic mixture by fortification and/or standardisation of milk. Although homogenisation is widely practised by the industry, its effects are not so apparent as those associated with the subsequent heat treatment. The optimum conditions for this treatment can vary from as low as ordinary pasteurisation (72°C for 15sec.) to as high as 133°C for 1s (UHT). The effects of the heat treatment result in denaturation of the whey proteins and an aggregation of the casein molecules which subsequently renders a more viscous coagulum. The heating of the milk is also responsible for a reduction in the microbial load, and hence the

starter cultures have less competition from adventitious organisms; a reduction in the amount of oxygen, and due to limited breakdown products stimulates starter activity.

In order for fermented milks to be labelled as yoghurt, it have to be inoculated and fermented by making use of lactic acid starter cultures. The acidification of milk is a biological process which must be carried out under controlled conditions in special fermentation tanks. As soon as the yoghurt mixture has been cooled to about 45°C, it is inoculated with equal numbers of *Lactobacillus bulgaricus* and *Streptococcus thermophilus* and incubated for 3 to 4 hours at 45°C. During incubation, the starter culture bacteria produce lactic acid and consequently lower the pH (Helferich and Westhoff, 1980). Over-incubation during the growth of bacterial cultures can result in an over production of lactic acid and that will result in a sour product. The bacterial cultures therefore play a major role in the development of flavour, aroma, and taste of the yoghurts. Immediately after the incubation period, the yoghurt is cooled in order to control the level of lactic acid in the product. The normal industrial practise is to cool the yoghurt to 15-20°C before mixing it with flavours/fruit prior to packaging. The final cooling to <5°C takes place in a refrigerated cold store.

1.2.3 Microbiology of natural yoghurt

1.2.3.1 *Normal microflora associated with yoghurt*

The primary microflora of yoghurt consist of the starter cultures, *Streptococcus thermophilus* and *Lactobacillus bulgaricus*, undesired bacteria, yeasts, and milk associated moulds. The latter being present as secondary flora at lesser proportions originating as contaminants. The trend of employing both starter cultures helps to give yoghurt a distinctive character. By promoting the association, reflects in enhanced cell numbers and consequently also enhanced lactic acid production. The existence of a synergistic relationship between the starter cultures encourages *Streptococcus thermophilus* to respond more vigorously in mix culture,

attributed to the proteolytic activity of *Lactobacillus bulgaricus*. The stimulation of *Lactobacillus bulgaricus* is due to a factor originating from the metabolic activity of *Streptococcus thermophilus*. Although the ratio between the two organisms begins as a nominal 1:1 balance, it alters rapidly as *Streptococcus thermophilus* enters its logarithmic phase of growth, and only as lactic acid accumulates in the milk, does *Lactobacillus bulgaricus* become the dominant partner.

There are three distinctive groups of microorganisms other than the starter cultures associated with the manufacture of yoghurt that can be divided based on their contribution: a) Essential microflora, b) non – essential microflora, and c) contaminating microflora. The essential microflora comprise the additional homofermentative lactic acid bacteria that are added as pro-biotic agents. These lactic acid bacteria may be used beneficially for supplementing the yoghurt microflora being capable of influencing intestinal implantation to some extent. The most important pro-biotic species incorporated into yoghurt are *Lactobacillus acidophilus* and *Bifidobacterium bifidum*. The contaminating microflora comprise yeasts, moulds, coliform bacteria and other undesirable organisms. These organisms are undesirable since they substantially decrease the organoleptic and hygienic properties of yoghurt (Rasic and Kurman, 1978), producing excessive amounts of gas and alcohols. Some contaminants, however, like yeasts may contribute to the flavour of the yoghurt product by proteolytic activity.

1.2.3.2 *Microbial spoilage of yoghurts*

The microbial quality of a product is usually concerned with the protection of the consumer being exposed to any health hazard and ensuring that the product does not suffer microbial deterioration during its anticipated shelf life. Both aims are important to the manufacturer accepting the responsibility of the company to secure a safe product and to minimize financial losses that may be initiated by consumer complaints or a public health incident. In general terms, however, yoghurt is regarded as "hygienically safe". The reason stems from the level of acidity present (1% lactic acid), and therefore

potential pathogens like *Salmonella* will be largely inactive. Similarly, coliforms will also be unable to survive the low pH encountered, reinforced by the production of antibiotic substances by the yoghurt organisms. The major risk, however, comes from the possible presence of *Staphylococcus spp.*

Despite the optimism of the lack of pathogens to survive, it does not imply that plant hygiene can be given a low priority, because spoilage organisms are less sensitive to environmental factors than their pathogenic counterparts. Yeasts and moulds are little affected by low pH, and with lactose and sucrose available as energy sources, spoilage can rapidly occur. Yeasts, in particular, are a major concern, and while the lactose utilizing yeasts build up on plant surfaces, the main source of contamination for the popular types of yoghurts is likely to be the fruit. Therefore, not even the pasteurised fruit-purees render an entirely yeast free medium, whereas the sweetened yoghurts provide an ideal medium for growth and metabolism.

Food spoilage by yeasts is well documented. Yeasts are best known for their positive contributions to society, through their activities in the fermentation of bread, alcoholic beverages and other products. Because of the low pH, elevated sugar composition in fruit related yoghurts, and low temperature storage, yoghurt renders a selective environment for the growth of yeasts. The literature contains several references on the spoilage of yoghurts (Davis, 1970; Rasic and Kurmann, 1978; Suriyarachchi and Fleet, 1981) and the beneficial and detrimental effects of lactic acid bacteria (Jin et al., 1996; Link et al., 1995). In contrast with the beneficial contribution of the lactic acid bacteria applied as starter cultures, the presence of heterofermentative lactic acid bacteria, however, reduces the quality of yoghurt by producing carbon dioxide and alcohol, particular if present at high proportions.

Lactic acid bacteria are antagonistic to many bacteria. Consequently, to obtain products with desirable organoleptic properties, and with increased stability, they are incorporated to inhibit the growth and proliferation of undesired microorganisms. Many foods and beverages are therefore fermented with the inclusion of these organisms. These products are

assumed to be free from pathogens, mainly due to environmental stress caused by the lowering of the pH. However, when Ryser and Marth (1987) produced Cheddar, Camembert, and cottage cheeses from milk inoculated with *Listeria monocytogenes,* the bacterial pathogen survived all manufacturing processes, including effects of starter cultures as well as storage temperatures within each type of cheese. It does not mean that all pathogens can survive the manufacturing processes, but accentuates the importance of proper pasteurisation (which with cultured milks commonly involves a heat treatment far greater than conventional pasteurisation) and sanitary practices. *Listeria monocytogenes* should not be present in any cultured milk products (Schaack and Marth, 1988).

Protein hydrolysis by microorganisms in foods may produce a variety of odor and flavour defects. Some of the common psychotrophic spoilage bacteria are strongly proteolytic and cause undesirable changes in dairy products, particularly when high populations are reached after extended refrigerated storage.

1.2.4 Yeasts in dairy products

Yeasts are important in the dairy industry for several reasons. They play an essential role in the preparation of fermented milks and in the ripening of certain cheeses. The significance of them in dairy products is increasingly recognized and a number of reviews focussed on the harmful and beneficial role of yeasts in these products (Fleet, 1990; Marth, 1987). Raw milk, pasteurised milk, fresh milk, cheeses, mould and bacterial ripened cheeses, yoghurt and fermented milk products have been reported to contain significant numbers of yeasts (Fleet, 1990, 1992, 1998; Jakobsen and Narvhus, 1996). Rose and Harrrison (1970) already indicated in the 70's that yeasts are commercially significant in dairy products because they either cause spoilage or conduct desirable fermentations. Their views and results were supported by Reed and Peppler (1973).

Generally, the available information shows that yeasts occur in both raw (Foster et al., 1957; Ingram, 1958; Randolph et al., 1973) and pasteurised

(Jones and Canglois, 1977; Fleet and Mian, 1987; Vadillo et al., 1987) milk at low, insignificant populations, but they may evolve as secondary mycoflora and reach high loads. Milk is an excellent substrate for growth of many microorganisms including yeasts. The varying populations solely depend on hygienic practices used in its handling. Although pasteurisation will kill all the microorganisms except the thermoduric bacteria, the yeasts will develop from secondary contamination. Raw milk held at refrigeration temperatures will support the growth of psychrotrophic strains and yeast counts exceeding 10^4 cfu/ml are frequently reported. Typical yeast genera associated with milk comprise *Cryptococcus, Trichosporon, Debaryomyces, Yarrowia, Kluyveromyces* and *Pichia*.

The high incidence of yeasts associated with cheeses is therefore not unexpected, usually not added as part of the original starter culture, but being present during manufacture and/or maturation of the product originating as environmental contaminants (Fleet, 1990). The main yeast species found during maturation and retailing include *Debaryomyces hansenii, Kluyveromyces marxianus, Yarrowia lipolytica* and various species of *Candida* (Lenoir, 1984; De Boer and Kuik, 1987; Nooitgedaght and Hartog, 1988; Besancon et.al., 1992; Fleet, 1990). They play a very important role in the making of cheese due to their abilities to produce lipolytic and proteolytic enzymes, the fermentation of residual lactose, the utilisation of lactic acid and autolysis, all have an impact on the quality of the final product (a, b; Fleet, 1990).

Nooitgedaght and Hartog (1988) reported yeast counts of $>10^5$ cfu/g in Camembert and Brie cheeses. *Yarrowia lipolytica, Debaryomyces hansenii*, and *Kluyveromyces marxianus* were the most frequently isolated species. Roostita and Fleet (1996) reported yeasts counts up to $10^6 - 10^8$ cfu/g, mainly representatives of the species *Debaryomyces hansenii, Saccharomyces cerevisiae, Candida lipolytica, Candida kefyr* and *Cryptococcos albidus.* According to Reddy and Marth (1995), yeast counts of < 300/g and <100/g for unsalted and salted Cheddar cheeses respectively, were obtained. Prentice and Brown (1983) reported a maximum level of yeasts of 5.0×10^3 cfu /g in

Cheddar cheese. Yeast levels, however, can rise as high as 10^5 cfu/g without any deleterious effect on the quality of the product (Prentice and Brown 1983). Fleet and Mian (1987) found that almost 50% of Australian Cheddar cheese sampled, contain 10^4-10^6 yeast cells/g.

Yeasts may play a detrimental role in inhibiting the starter cultures in cheeses. In a study by Noda et al. (1980), it was found that osmophillic yeasts, such as *Zygosaccharomyces rouxii spp,* were inhibited by a metabolite produced by *Pediococcus halophillus*. The primary inhibitor seemed to be acetic acid, although lactic acid was also slightly inhibitory. In contrast, yeasts on the other hand may be responsible for inhibiting or eliminating microorganisms (including the starter cultures) which are undesired because they cause quality defects or posses potential pathogenic characters.

Spoilage of cream is observed when the cream becomes foamy in appearance and yeasty in odour. The yeasts are able to ferment residual lactose in the cream, or hydrolyse the fat (Garrison, 1959; Walker and Ayres, 1970; Thomas, 1970). The yeast species that are normally isolated from cream samples comprise strains of *Candida famata*, *Rhodotorula glutinis*, *Candida diffluens*, *Cryptococcus laurentii* and *Rhodotorula rubra* (Fleet, 1990). Lipolytic species of yeasts, and typical air contaminants like *Rhodotorula,* have been reported to grow on the surface of butter (Walker and Ayes, 1970; Thomas, 1971) but the incidence of this problem is very low.

The role of yeasts as spoilage organisms in dairy products is linked with their nutritional requirements, certain enzymatic activities and the ability to grow at low temperatures, low pH values, low water activities and high salt concentrations. Yeasts, however, are presently considered of limited hygienic significance in the dairy sector. Infections arising from the few, known pathogenic yeasts such as *Candida albicans* or *Cryptococcus neoformans* are not transmitted through foods. Consequently, the public health authorities consider the presence of yeasts to be minimal if not negligible. In summaries of statistics on food borne diseases in Canada, Todd (1983) noted cases where yeasts were suspected of causing food poisoning. The allergic

reactions of consumers to foods and their contaminants are of increasing concern to health authorities, and yeasts have been mentioned in this concern.

1.2.4.1 Yeasts associated with fermented milk products

Yeasts are not involved in the fermentation process during the production of yoghurt, but they are a major cause of spoilage in the final product. The main yeasts frequently associated with yoghurt comprise *Candida famata* and *Kluyveromyces marxianus*. *Kluyveromyces marxianus*, a well known dairy associated yeast, is capable of producing β- galactosidase and consequently it can ferment or assimilate lactose which is the main carbohydrate of milk and therefore causing spoilage.

Yeasts are generally described according to their saccharolytic activities. They are capable of attacking a number of substrates, such as gelatin, casein as well as proteins and lipids. *Rhodotorula spp.* are capable of attacking casein in fermented dairy products (Nissen, 1930) whereas *Yarrowia lipolytica* can hydrolyze fats in dairy products such as butter and margarine, even though spoilage of fats and oils by yeasts is rarely found. *Rhodotorula spp.*, *Trichosporon pullulans*, and *Candida scotti* have been described as being very active in the production of lipase (Vorbeck and Cone, 1963; Orla-Jensen, 1931; Miklik, 1953; Eklund et al., 1966). Due to their lipolytic and proteolytic activities, yeasts may become part of the overall enzymatic activity in the development of dairy associated flavours. This also supports the use of yeasts as starter cultures for the production of dairy products. Fermentation of the milk sugar, lactose is the most important modification that is common to most dairy products. Lactose is a dissacharide and must first be cleaved into its monosaccharide, glucose and galactose or their derivatives. The glucose and galactose moieties are then converted through the relevant energy–yielding pathways (Lund, 1958).

The second most important transformation of a milk component into an essential flavour compound in a cultured dairy product involves citrate metabolism. Milk contains an average of 0.2% citrate. Citrate is converted into diacetyl by "flavour bacteria", included in starter mixtures for cultured dairy products. Diacetyl is the single most important and essential flavour compound that imparts the characteristic "buttery", nut-meat like aroma and flavour of dairy products (Lund, 1958). Although pH is not necessarily a reliable indicator of the effectiveness of an acid for controlling growth at low pH and the presence of bacteria, yoghurts are a selective environment for the growth of acid tolerant yeasts and moulds. Optimum growth of yeast species normally occurs in the pH range of 4.5 – 6.5. Literature contains general references on the spoilage of yoghurts (Davis, 1970, Rasic and Kurman 1978, Suriyarachchi and Fleet 1981) and the beneficial effects of lactic acid bacteria (Jim et al., 1996; Link et al., 1995). Yeasts do not play a major role in the spoilage of frozen or refrigerated dairy products, however, they are resistant to low temperatures and can survive frozen storage (Lund, 1958).

Despite the presence of yeasts at insignificant numbers in both raw (Foster et al., 1957; Ingram, 1958; Randolph et al., 1973) and pasteurized (Jones and Langlois, 1977; Fleet and Mian, 1987; Vadillo et al., 1987) milks, they develop in yoghurts as secondary flora, after bacterial growth and during spoilage. According to Yong and Wood (1976), the lactic acid fermentation occurs before yeast fermentation. The lactic acid bacterial numbers grow up rapidly, lowering the pH and permitting yeast growth to take place and to compete actively. Walker and Ayres (1970) reported the frequent occurrence of pigmented yeasts of the genus *Rhodotorula*. Similar results were obtained by Fleet and Mian (1987) when they recovered *Rhodotorula glutinis* from milk, cream, yoghurt, butter and cheese. Fleet and Mian (1987) isolated *Candida famata, Kluyveromyces marxianus, Cryptococcus flavus*, *Candida diffluens* as well as *Saccharomyces cerevisiae*. Fleet (1990) reported that these species could grow to 10^8 –10^9 cells/ml when they were inoculated and incubated in UHT – treated milk. The frequent occurrence of yeasts in pasteurized milks suggests that they have some degree of tolerance to the pasteurization

process (Fleet and Mian, 1987; Vadillo et al., 1987) but this possibility requires investigation (Garrison, 1959).

1.2.4.2 Characteristics of yeasts associated with yoghurt

Yeasts, like other living organisms require sources of carbon, nitrogen, phosphorus, trace elements and growth factors (Phaff et al., 1979). Yeasts cannot grow anaerobically, and therefore require oxygen. All wild-type yeasts utilize glucose, mannose, and fructose. Different species may also utilize nitrates, others only ammonium salts. They can utilize a wide range of organic nitrogen compounds, including both D- and L- amino acids (La Rue and Spencer, 1966). All yeasts require trace elements. Some species can synthesize the growth factors they require, and many cannot, and must be supplied.

Nitrogen: An extracellular supply of nitrogenous material is essential for the continued production of new protoplasm and yeasts generally derive this element from such relatively simple substances as ammonium salts, nitrates, amino acids and amides although there is evidence that dipeptides or even higher peptides may also be assimilated (Cook, 1958). Diammonium phosphate is utilized most efficiently and ammonium chloride least. Until recently it has been a common belief that yeasts are unable to fix atmospheric nitrogen, but evidence is now available that certain strains of *Rhodotorula* and at least one strain of *Saccharomyces* have this property. All yeasts but *Cyniclomyces gluttalatus* can utilize ammonium salts as sole source of nitrogen. This species has absolute requirements for numerous amino acids (Rose, 1987). Although some yeasts may not utilize nitrate, nitrogen, many can use not only L- amino acids such as L- lysine, but also many D – amino acids and purines, pyrimidines, and other organic nitrogen compounds (La Rue and Spencer, 1967). Brewer's and baker's yeasts, *Saccharomyces cerevisiae*, excrete amino acids, oligopeptides and amides which may be reabsorbed, depending on the energy source present. In addition, nucleotides may be excreted from yeast cells when they are

suspended in water or glucose solutions and unlike the amino acids, are not reabsorbed.

Not all yeasts are able to utilize other sources of inorganic nitrogen and in fact the ability to use nitrates is regarded as a diagnostic feature. Thus in general most of the species comprising the genera *Saccharomyces, Pichia, Hanseniaspora* and *Debaryomyces* fail to assimilate nitrite in a nitrate medium (Lodder and Kreger- van Rij, 1952).

Phosphates: All yeasts as far as known, utilize inorganic phosphates for growth. It is taken up as a monovalent anion, HPO_4, and more is taken up of the monobasic potassium salt than the dibasic sodium form.

Sulphates: Uptake of sulfate by yeasts requires energy, therefore the medium H_2PO_4 contains both glucose (or other metabolizable compounds) and available nitrogen. The cell can take up sulfate under either aerobic or microaerophilic conditions.

Mineral requirements: The role played by mineral salts in the growth of yeasts is extremely difficult to ascertain because of the technical problems encountered in ensuring that the basal media are entirely free of the elements under examination. Consequently it is not surprising that little precise information is available concerning this fundamentally important and interesting topic. Most of the synthetic media contain relatively few elements and even supposing that the requirements for nitrogen, carbon and vitamins are satisfied, it is almost certain that the media are unbalanced so far as trace metals are concerned, particularly if salts of a high degree of purity are used. The synthetic media defined generally contain almost exclusively the following salts: potassium dihydrogen phosphate, dipotassium hydrogen phosphate, disodium hydrogen phosphate, calcium salts as the chloride, carbonate and/or nitrate, magnesium sulfate, and the chlorides of sodium and potassium.

Calcium: Calcium does not appear to be essential for growth of yeast cells, although it has been established that the amount of yeast ash is reduced by approximately one-half when the medium in which the cells have been grown is devoid of calcium (Cook, 1958).

Potassium: Potassium is required for yeast growth and fermentation, and can be partially replaced by sodium and ammonium ions. Yeasts containing abnormal amounts of potassium or having the potassium ions replaced by Na (or ammonium ferment more slowly than normal yeasts. Potassium can also be replaced by Rb, Li, and Cs ions with similar effects (Spencer and Spencer, 1977).

There can be no doubt that magnesium is essential for the production of high yields of yeasts and it is known that it is associated with metabolism both of carbohydrates through the activation of enzymes concerned with the transfer of phosphates and of nitrogenous compounds (Cook, 1958). Deficiency of magnesium in synthetic media reduces the response of certain strains of *Saccharomyces cerevisiae* to "bios" factors, a fact that has been usefully employed by Lesk et al. (1958) to differentiate strains of this species.

Growth factors: Biotin is required by *Saccharomyces cerevisiae* but not by some yeasts including species of *Pichia, Brettanomyces, Candida utilis* or *Hansenula anomala*. Yeasts may require pantothenic acid, inositol, thiamin, nicotinic acid, pirydoxin, riboflavin, p-aminobenzoic acid and folic acid is a component of coenzyme A and participates in transfer of acyl groups, especially in fatty acid metabolism.

1.2.4.3 Sources of yeast contamination

The major spoilage problem in fermented milks like yoghurt is experienced when the product is supplemented or decorated with fruits, honey, sugar, nuts and flavouring agents being sources of infections and providing nutrients for yeast growth and fermentation. This fact makes yoghurt a less selective growth environment, as these products are likely to support the growth of a

number of a yeast species (Suriyarachchi and Fleet, 1981; Deak, 1991; Canganella et al., 1992).

The yeasts originate not only from the ingredients but also from processing equipment, the surface of production equipment, such as mixing vessels and filling machines that has not been properly cleaned and sanitized. When they are produced under conditions of good manufacturing practice, yoghurt should contain less than 10 yeast cells/g (but preferably less than 1 cell/g) and if refrigerated at 5°C or less they should not undergo spoilage by yeasts (Davis, 1970). Starter cultures including the lactic bacteria used to ferment the yoghurt is another potential source of yeast contamination.

1.2.5 Microbial Interactions

Yeasts and lactic acid bacteria participate in the fermentation of kefir, koumiss, kvass, and the production of various cheeses. Microbial interactions are indicated by a number of studies in blue cheese (Kaminarides and Anifantakis, 1989), white mould cheeses (Seiler and Busse, 1990), bacterial ripened cheeses (Lenoir, 1924) and fermented milk products like kefir (Robinson and Tamine, 1990). These fermentations are mostly anaerobic and produce alcohol and CO_2. This inhibits the growth of spoilage organisms, including filamentous fungi and toxin-producing bacteria. These fermentations are carried out to obtain a product of the desired flavour and texture, and with beverages for the ethanol content rather than for preservation.

In cheeses, however, the interaction between lactic acid bacteria and yeasts like the lactose-fermenting yeast species, *Kluyveromyces marxianus*, contribute to blue type cheeses due to the production of CO_2 causing openings in the curd that helps *Penicillium roquefortii* to grow in the internal fissures. This attributes to the characteristic blue vein appearance of the cheese. Yeasts also contribute to the ripening of Camembert cheese due to the fermentation of lactose adding to aroma and taste (Lenior, 1984). *Kluyveromyces marxianus, Kluyveromyces lactis, Candida versatilis,*

Debaryomyces hansenii and *Saccharomyces cerevisiae* are frequently isolated from the inner and outer part of Camembert cheese. The yeast species present in the soft cheeses furthermore inhibit the growth of Mucor; *Penicillium roquefortii* and *Penicillium camemberti* responsible for slow development of the mould cultures. Most of the yeasts isolated from Camembert cheese are able to assimilate lactose and lactic acid, and exhibit lipolytic and proteolytic activity. All these characteristics contribute to the development of cheese aroma. The use of yeast species as part of starters for the manufacturing of Camembert cheese, however, is still the exception.

Yeasts also play an important spoilage role in the production of feta cheese, being present in the brine of the cheese. *Saccharomyces cerevisiae* and *Candida famata* were the dominant yeasts isolated by Kaminarides and Laskos (1992) responsible for flavour development. In addition, yeasts added to the formation of aroma components, or precursors of aroma (amino acids, fatty acids, esters, etc.) due to their proteolytic, lipolytic and esterifying activities (Lenoir 1984). Furthermore, yeasts excrete vitamins resulting in growth stimulation of other microorganisms, which include the starter cultures (Purko et.al., 1951).

The positive interaction between yeasts and starter cultures and the abilities of yeasts to assist the starter cultures during cheese processing based on proteolytic and lipolytic activities and the production of amines, are well documented for surface ripening cheeses (Besancon et.al., 1992; Kalle et.al., 1976; Kaminarides and Anifantakis, 1989; Lenoir, 1984). It has been mentioned that yeasts improve the quality of numerous cheeses, mainly by their lipolytic activity. The lipolytic enzymes excreted by *Yarrowia lipolytica* have been added to cheese milk to improve the taste of Cheddar cheese and blue-veined cheeses (Parmelee and Nelson, 1949a; Parmelee and Nelson, 1949b).

Yeasts, lactic acid bacteria and other microorganisms are often found together in natural ecosystems (Narendrnath et al., 1997; Fleet, 1998). These microorganisms interact differently in their habitats. Jakobsen and Rossi

(1994) reported that yeasts in dairy products may interact with other microorganisms in three different ways: i) They may inhibit or eliminate undesirable microorganisms because they cause quality defects or possess potential pathogenic characters; ii) they may inhibit the starter culture or iii) they may contribute positively to the fermentation or maturation process by supporting the function of the starter culture. These results were confirmed by (Jakobsen and Narvhus, 1996; Fleet, 1998; Fleet, 1999) indicating that yeasts in dairy products may inhibit or eliminate other microorganisms by the production of acetic acid, ethanol, antibiotics and killer toxins.

1.2.5 References

Ahmed, A.H., Moustafa, M.K. and Bassiony, T.A., 1986. Growth and survival of *Y. enterocotica* in yoghurt. J. Food Protect. 49, 983 – 985.

Besancon, X., Smet, C., Chabalier, Rivemmele, M., Reverbel, J.P., Ratomahenina, R. and Galzy, R., 1992. Study of yeast flora of roquefort cheese. Int. J. Food Microbiol. 17, 9-18.

Budu – Amoako, E., Toora, S., Ablett, R.F. and Smith, J., 1993. Competitive growth of *Listeria monocytogenes* and *Yersinia enterocolitica* in milk. J. Food Protect. 56, 528 – 532.

Canganella, F., Ovidi, M., Peganini, S., Vettraino, A.M., Bevilacqua, L. and Trovatelli L.D., 1998. Survival of undesirable micro–organisms in fruit yoghurts during storage at different temperatures. Food Microbiol. 15, 71 –77.

Christensen, V.W., 1970. Yoghurt – the bright star among dairy products. Food Product Development 4, 18-19.

Cook, A.H., 1958. The chemistry and biology of yeasts. Academic Press Inc. New York.

D'Amici, A., 1996. Yoghurt and other fermented milk. Trend of marketing (translated). Abstracts of the meeting on probiotic foods, I.N.N. Roma, Italy.

Davis, J.G., 1971. Standards for yoghurt. Dairy Indust. 36, 456 –462

Davis, J.G., 1970. Laboratory control of yoghurt. In: Ca, J.G., Cutting, C.V., Whitting, G.C., (Eds.) Lactic acid bacteria in beverages and food. Academic Press, London, pp. 245-266.

Deak, T., 1991. Foodborne yeasts. Adv. Appl. Microbiol. 36,

De Boer, E.M. and Kuik, D., 1987. A Survey of the microbiol quality of blue-veined cheeses. Netherlands Milk Dairy. J. 41, 227-237.

Deeth, H.L. and Tamine, A.V., 1981. Yoghurt: nutritive and therapeutic aspects. J. Food Protect. 44, 78 –86.

Engel, G., Einhoff, K., Prokopek, D. and Teuber, M., 1987. Relationship between yeast species, numbers of yeasts and sensoric yeast –

depending defects in skim milk quarg after adding defined yeasts. Milchwissenschaft 42, 428 – 421.

Eklund, M.W., Spinelli, J., Miyauchi D. and Dassow, J., 1966. Development of yeast on irradiated pacific crab meat. J. of Food Sci. 31, 424 -431

Emmens, D.B. and Tuckey, S.L., 1967. Cottage cheese and other cultured milk products. New York, Chas. Pfizer.

Fleet, G.H. and Mian, M.A., 1987. The occurrence and growth of yeasts in dairy products. Int. J. of Food Microbiol. 4, 145 – 155.

Fleet, G.H., 1990. Yeasts in dairy products. J. Appl. Bacteriol. 68, 199 – 211.

Fleet, G.H., 1992. Spoilage yeasts. Critical Reviews in Biotechnol. 12, 1 – 44.

Fleet, G.H., 1998. Yeasts – What reactions and interactions really occur in natural habitats. Food Technol. and Biotechnol. 36(4), 285 – 289.

Fleet, GH., 1999. Micro-organisms in food ecosystems. Int. J. Food Microbiol. 50, 101-117.

Foster, E.M., Nelson, F.E., Speck, R.N., Doetsch, R.N. and Olson, J.L., 1957. Dairy Microbiol. New Jersey: Prentice Hall.

Garrison, E.R., 1959. Lipolytic yeasts in cream from dairy farms. J. of Dairy Sci. 42, 905 – 910.

Griffiths, M.W., 1989. *Listeria monocytogenes*: its importance in the dairy industry. J. of Sci. Food Agric. 47.

Helferich, W. and Westhoff, D., 1980. All about yoghurt. Prentice-Hall, Inc., London.

Humphreys, C.L. and Plunkett, M., 1969. Yoghurt: a review of its manufacture. Dairy Science Abstracts 31, 607 – 622.

Ingram, M., 1958. Yeasts in food spoilage. In The Chemistry and Biology of yeasts ed. Cook, A.H. 603 –633. New York: Academic Press.

International Dairy Federation, 1969. Compositional standard for fermented milks. Int. Stand. FIL-IDF 47: 1962

Jakobsen, M. and Narvhus, J. 1996. Yeasts and their possible beneficial and negative effects on the quality of dairy products. Int. Dairy J. 6, 755–768.

Jin, L.Z., HO, Y.W., Abdullah, N., Ali, M.A. and Jalaludin, S., 1996. Antagonistic effects of intestinal Lactobacillus isolates on pathogens of chicken. Lett. Appl. Microbiol. 23, 67 -71.

Jones, F.T. and Langlois, B.E., 1977. Microflora of retail fluid milk products. J. of Food Protect. 40, 693 – 697.

Kaminarides, S.E. and Anifantakis, N.S., 1989. Evolution of the microflora of Kopanisisti cheese during ripening. Study of the yeast flora. Le Lait 69, 537 – 546.

Kaminarides, S.E. and Laskos, N.S., 1992. Yeasts in factory of brine of Feta cheese. Aust. J. Dairy Technol. 47, 68-71.

Kalle, G.P., Deshpande, S.Y. and Laskaris, B.Z., 1976. Fermentative production of cheese-like flavour concentrate by Candida lipolytica. J. Food Sci. and Technol. 13, 124-128.

Kosikowski, F., 1966. Cheese and fermented milk foods. 3rd ed. Ann Arbor: Edwards Brothers, Inc., Michigan, 47-50.

La-Rue, T.A. and Spencer, J.F.T., 1968. Utilisation of organic nitrogen compounds by yeasts of the genus Saccharomyces. Antonie van Leeuwenhoek 34: 153-158.

Leinor, J. 1984. The surface flora and its role in the ripening of cheese. International Dairy Federation Bulletin. 171, 3 – 20.

Link, H., Rochat, F., Saudan, K.Y. and Schiffrin, E., 1995. Immunomodulation of the gnotobiotic mouse through colonization with lactic acid bacteria. In Advances in mucosal Immunology (Eds J. Mestecky et al.) New York; Plenum Press.

Lodder, J. and Kreger–van Rij N.J.W., 1952. In: The Yeasts , A taxonomic study. North – Holland, Amsterdam

Lund, A., 1958. Ecology of yeasts. In "Chemistry and biology of yeasts." ed. A.H. Cook

Lundstedt, E., 1974. Improved methods for manufacturing yoghurt. American Dairy Review. 38: A-D.

Massa, S., Trovatelli, L.D. and Canganella, F., 1991. Survival of *Listeria monocytogenes* in refrigerated cultured milks and yoghurt. J. Food Protect. 51.

Marshall, V.M., 1993. Starter cultures for milk fermentation and their characteristics. Journal of the Society of Dairy Technology 46 49 –56.

Marth, E.H., 1978. Dairy Products. In : Beuchat CR (ed) Food and everage mycology. AVI, Connecticut, 145-172.

Miklik , E., 1953. Uber das Eiweissabbaw – und Fettspaltungsvermogen einer *Rhodotorula* aus butter. Milchwissenschaft 8.

Miller, M.W. 1979. Yeasts in food spoilage. An update. Food technol.

Narendrnath, N.V., Hynes, S.H., Thomas, K.C. and Inledew, W.M., 1997. Effects of lactobacilli on yeast – catalysed ethanol fermentations. Applied and Environmental Microbiology 63(11), 4158 –4163.

Nissen, 1930. Untersuchen uber das Vorkommen von Hefen in der Erde, mit bessonderer Berucksichtiging der fur die Milchwinschaft wechtigen Arten Milchwirtsch Forsch.

Nooitgedaght, A.J. and Hartog, B.J., 1988. A Survey of the microbiological quality of blue-veindd cheeses Netherlands. Milk and Dairy J. 41, 227-237.

Noda, F., Hayashi, K. and Mizunuma, T., 1980. Antagonism between osmophilic lactic acid bacteria and yeasts in brine fermentation of soy sauce. Appl. Environ. Microbiol. 40.

Orla–Jensen, S., 1931. " Dairy Bacteriology", 2nd English ed. Blakiston Div., McGraw – Hill Book Co., New York.

Parmelee, C.E. and Nelson, F.E., 1949a. The use of Candida lipolytica on the manufacturing of blue cheese made from pasteurized homogenized milk. J. Dairy Sci. 32, 993.

Parmelee, C.E. and Nelson, F.E., 1949b. The relation of chemical analysis to the scores of blue cheese made from pasteurized homogenized milk. Dairy Sci., 32, 1007.

Phaff, H.J., Miller, M.W. and Mrak ,E.M., 1966. "The life of yeasts. Their nature, activity, ecology and relation to mankind. "Harvard University Press, Cambridge, Massa.

Purko, M., Nelson, W.O. and Wood, W.A., 1951. The associative action between certain yeasts and bacterium linens. J. Dairy Sci. 34, 699-701.

Randolph, H.E., Chakraborty, B.K., Hampton, O. and Bogart, O.L. 1973. Microbial counts of individual producer and commingled grade A raw milk. J. Food Technol. 36, 146 –151.

Rasic, J.Lj. and Kurman, J.A., 1978. Yoghurt Scientific Grounds, Technology, manufacture and preparations. Technology Dairy Publishing House, Copenhagen.

Reddy, K.A. and Marth, E.H., 1995. Microflora of Cheddar Cheese made with Sodium Chloride. J. Food Prot. 58, No. 1, pp. 45-61.

Reed, G. and Peppler, H.J., 1973. Yeast Technology Westport Connecticut: AVI Publishing.

Rose, A.H. and Harrison, J.S. (eds), 1970. The Yeasts, 3, Yeasts Technology. London: Academic Press.

Rosi, I., Vinella, M., and Domizio, P., 1994. Characterization of β-glucosidae activity in yeasts of oenological origin. J. Appl. Bacteriol. 77, 519-527.

Robinson, R.K. and Tamine, A.Y., 1990. Microbiology of Fermented Milks: In Robinson, , R.K. (ed). Dairy Microbiology, 2. The Microbiology of Milk Products, 2nd Ed. Elsevier London, 291 –343.

Robinson, R.K. and Tamine, A.Y.,1975. Yoghurt – a review of the product and it`s manufacture. J. Soc. Dairy Technol. 28, 149 –163.

Rose, A.H., 1987. Fermented foods. 2nd ed., 1. Elsevier Applied Science Publishers.

Ryser, E.J. and Marth, E.H., 1987. Behaviour of *Listeria monocytogenes* during the manufacturing and ripening of camembert cheese. J. Food Protect. 50, 372-378.

Schaack, M.M. and Marth, E.H., 1988. Survival of *Listeria monocytogenes* in refrigerated cultured milks and yoghurt. Journal of Food Protection. 51, 848 - 852

Schiemann, D.A., 1987. *Yersinia enterocolotica* in milk and dairy products. Journal of Dairy Science. 70.

Seiler, H. and Busse, M., 1990. The yeasts of cheese brines. Int. J. of Food Microbiol. 11, 289 – 304.

Spencer, J.F.T. and Spencer, D.M., 1977. Hybridization of non-sporulating strains of brewer's and distiller's yeast. J. Inst. Brew. 83, 287-289.

Suriyarachchi, V.R. and Fleet G.H., 1981. Occurrence and growth of yeasts in yoghurts, Appl. Environ. Microbiol. 42, 574 - 579.

Tealdo, E., Tutta, C., Barcarolo, R., Eastioni, R., Faccin, M., Fellin A., Castioni, R., Faccin, M., Fellin, A., Gaburro, L. and Tapparoro, M. Caratterizzazione chimico Fisica Dello Yoghurt. INT. Meeting of Technol. Sperim. Inst. of Dairy and Milk, 36016 Thiene.

Thomas, S.B., 1970. Psychrotrophic microorganims in market cream – a review Part 1. Dairy Industry 35, 79 – 84.

Thomas, S.B., 1971. Psychrotrophic microorganisms in butter: Part II. Dairy Industry 36, 145 – 150.

Todd, E.C., 1983. Foodborne disease in Canada – a 5 year summary. J. of Food Protect. 46, 34 - 40.

Vadillo, S., Paya, J., Cutuli, M.T. and Suarez, G., 1987. Mycoflora of milk after several types of pasteurization. Le Lait. 67, 265-273.

Vorbeck, M.L. and Cone, J.F., 1963. Characterization of an intracellular proteinase system of a *Trichosporon* species isolated from trappist-type cheese. Appl. Microbiol. 11, 23-27.

Walker, H.W. and Ayres, J.L., 1970. Yeasts as spoilage organisms. In The Yeasts, Vol. 3, Yeast Technology. Ed Rose, A.H. and Harrison J.S. 464 –527. London Academic Press.

Wood, B.J.B., 1985. Microbiology of fermented foods. 1, Academic Press; New York.

Yong, F.M. and Wood B.J.B., 1976. Microbial succession in experimental soy sauce fermentation. J. Food Technol.

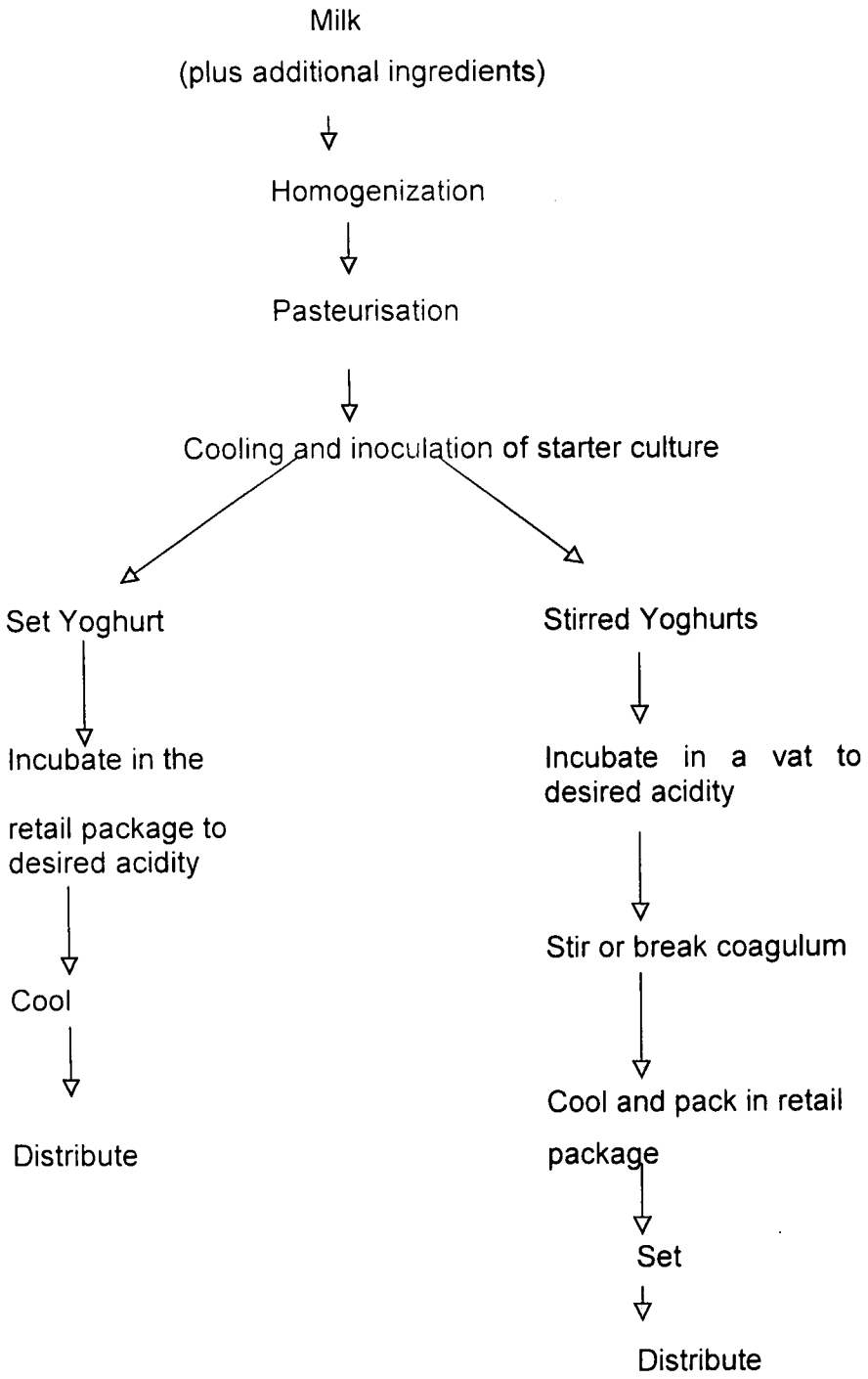

Fig. 1 Steps involved in the manufacture of yoghurt

CONTENTS

2.	**Abstract**	28
2.1	**Introduction**	29
2.2	**Materials and methods**	30
2.2.1	Yoghurt samples	30
2.2.2	Enumeration of microorganisms	31
2.2.3	Yeast identification	31
2.2.4	Nitrite tolerance	32
2.2.5	Lipolytic activity	32
2.2.6	pH measurement	32
2.3	**Results and discussion**	33
2.3.1	Incidence of yeasts	33
2.3.2	Key properties encouraging yeast growth	35
2.3.3	Enumeration of yeasts	36
Table 1.1	Key properties of yeast strains isolated from yoghurt	37
2.4	**References**	38

CHAPTER 2

THE OCCURRENCE AND DIVERSITY OF YEASTS IN YOGHURT

Abstract

Yeasts have a competitive advantage in yoghurt due to its ability to grow at low pH values and temperature, and therefore are major role players in causing spoilage. In this study yoghurt samples were purchased from various supermarket outlets to determine the incidence and diversity of yeast species associated with commercial yoghurt. Yeast species were isolated and identified according to conventional identification techniques. *Saccharomyces exiguus* (25%), *Yarrowia lipolytica* (17,9%), *Saccharomyces cerevisiae* (14,3%) and *Kluyveromyces marxianus* (10.7%) were most frequently encountered. *Rhodotorula* spp. occurred at a very low incidence. Populations not less than 10^3 cfu/ml were observed, and counts as high as 10^6 cfu/ml occurred.

2.1 Introduction

Yoghurt is a fermented milk product produced by specific lactic acid bacteria, namely *Lactobacillus bulgaricus* and *Streptococcus thermophilus*, is highly viscous, firm and cohesive. Its typical body characteristics are greatly influenced by the careful regulation of production conditions. Top quality yoghurt is smooth, without grittiness or granules and without effervescence. It is a highly acidic product, with a low pH of 4 – 4.5. Because of the low pH and storage at low temperatures, yoghurts render a selective environment for the encouragement of yeast growth (Wood, 1985). Consequently, yeasts may cause spoilage in yoghurts. Literature contains several references on the spoilage of yoghurts (Davis, 1970; Rasic and Kurmann, 1987; Suriyarachchi and Fleet, 1981) associated with yeast growth.

A spoilage yeast is defined as being responsible for undesirable changes in foods or beverages either during processing or thereafter. Generally, spoilage is recognized by the development of yeasty and bitter off-flavours, gassy and frothy texture, swelling and blowing of the product container (Fleet, 1990). Manufacturing practices to prevent the occurrence of yeast growth in yoghurt have been discussed extensively (Fleet and Arora et al., 1991). These control measures all embrace the general principal of good manufacturing practices. Critical points to monitor are the usage of accepted hygienic practices before, during, and after manufacturing. Processing areas and machinery should be checked regularly for freedom from contamination. Machinery mould (*Geotrichum candidum*) is often used to assess the sanitation of processing operations (Arora et al., 1991). Proper mixing and heating of ingredients before fermentation are also imperative. Doyle and Marth (1975) found that fungal spores are readily inactivated by heat processes, a procedure common to the dairy industry, and hence it is especially important for heated products to be handled in a sanitary manner. Absence of fungi in the starter culture of lactic acid bacteria is a necessity since many mould spores and yeasts are transmitted by air in the

dairy-processing facility. Furthermore, the total absence of yeasts (not detectable) in 1.0g fruit and other ingredients added to the fermented yoghurt base is required. Packaging to the standard that will essentially eliminate oxygen in the surrounding atmosphere is effective in eliminating mould growth whereas rapid cooling of the product to 5°C, and maintenance of this temperature throughout retailing (Davis, 1975; Suriyarachchi & Fleet, 1981) all assure a good quality product.

The introduction of sugar and fruit into yoghurt makes yoghurt a less selective environment, and such yoghurts are likely to support the growth of a wider diversity of microorganisms. Consequently, competition between the yeasts and other bacteria, including the lactic acid bacteria may be present. *Saccharomyces cerevisiae, Kluyveromyces marxianus* and *Yarrowia lipolytica* are typical yeast species frequently found in yoghurt (Suriyarachchi and Fleet, 1981). Other yeasts encountered comprised *Debaryomyces hansenii, Candida versatilis, Pichia toletana* and *Issatchenkia orientalis* (Deak and Beuchat, 1996). The dominance of these species in yoghurts is related to their ability to ferment or utilize sucrose and lactose, utilize casein and organic acids, and grow at refrigeration temperatures.

The aim of this study was to determine the occurrence and diversity of yeasts associated with commercial yoghurts at retail which may contribute to spoilage.

2.2 Materials and methods

2.2.1 Yoghurt Samples

Fifty yoghurt samples in 175 ml plastic containers were purchased from various supermarkets irrespective of the type, brand or expiry date. The varieties of yoghurts sampled comprised plain yoghurts, flavoured and fruit yoghurts

ran
ic box to th laboratory, and analyz d microbiologically within 12 hours.

2.2.2 Enumeration of microorganisms

Th yoghurt carton was vigorously shak n, wip d with 90% alcohol and10ml sampl s as ptically transf rr d into 90ml of p pton wat r (Biolab C134, M rck, Darmstadt). Portions (0.1 ml) of th appropriat dilutions as r quir d, w r spr ad plat d in duplicat on s l ctiv m dia for th num ration of y asts and lactic acid bact ria.

Lactic acid bact ria w r num rat d using D Man Rogosa agar (MRS) (Biolab, C86) and th plat s incubat d at 25°C for 48h. For th num ration of y asts, Y ast Extract Glucos Chloramph nicol Agar (YGC) (Biolab, C98) was us d and th plat s incubat d at 25 °C for 7 days. All visual diff r ntiating y asts bas d on colour, shap and siz w r isolat d from th high st dilution and purifi d on Y ast – Malt Extract Agar (YM) by thr succ ssiv str akings and v rifi d by microscopy. Th pur strainsw r maintain d on YM (Y ast xtract Malt xtract) agar slants at 5°C until id ntification.

2.2.3 Yeast identification

Strains w r id ntifi d to th sp ci s l v l according to th conv ntional id ntification m thods of Kr g r-van Rij (1984), Barn tt t al. (1990) and Kurtzman and F ll (1998).

Each isolat was inoculat d into 6 f rm ntation m dia, 35 carbon sourc assimilation m dia, vitamin fr m dium, 0.01% and 0.1% cycloh ximid . Assimilation of nitrog n compounds was p rform d by m ans of th auxanographic m thod (Lodd r and Kr g r-van Rij 1952). Additional t sts p rform d includ d growth at 37°C, in 50% D-glucos m dium, ur a hydrolysis,

starch formation, acetic acid formation and staining of 4-week-old cultures with Diazonium blue B salt reagent (Van der Walt and Hopsu-Havu, 1976).

Ascospore formation was examined on McClary's acetate agar, potato glucose agar, Gorodkowa agar, corn meal agar and malt extract agar (Kreger-van Rij, 1984). The inoculated media were incubated at 18°C for 4 weeks and examined at 4-day intervals. Cell morphology and mode of reproduction were examined on malt extract (Difco) and on Dalmau plates (Kreger-van Rij, 1984). The formation of pseudomycelium and true mycelium was examined on corn meal agar according to the Dalmau plate technique (Wickerham, 1951).

2.2.4 Nitrite Tolerance

Yeast isolates (Table 1.1) were streaked on YM Agar containing 80ppm and 240ppm nitrite and incubated at 24°C for 5 days (Table 1.1).

2.2.5 Lipolytic activity

Isolated yeasts were streaked on sterile plates with olive oil and Rhodamine agar (Kouker and Jaeger, 1987) and Tributyrin (Glycerol tributyrate) (Fryer et al., 1966) agar and the plates were incubated at 30°C for 6 days.

2.2.7 pH measurement

The pH of each sample was determined by a Cybersan pH meter, model 500. The mean initial pH for the fruit yoghurt samples was 3.80 and for the plain yoghurt 3.89.

2.3 RESULTS AND DISCUSSION

2.3.1 Incidence of yeasts

The restricted diversity of yeasts isolated from yoghurt samples is reported in Table 1.1, revealing only six different species. The most prevalent spp. were *Saccharomyces exiguus* (25%), *Yarrowia lipolytica* (17%), *Saccharomyces cerevisiae* (14.3%) and *Kluyveromyces marxianus* (10.7%). *Rhodotorula spp* were isolated but occurred at a very low incidence. The frequent occurrences of *Saccharomyces cerevisiae* and *Kluyveromyces marxianus* were also reported by Tilbury et al. (1974) and Suriyarachchi and Fleet (1981) from yoghurts containing added sucrose and fruits. *Yarrowia lipolytica*, a typical lipase and protease producer, occurs frequently in milk products and refrigerated foods. Other species encountered included, *Debaryomyces hansenii*, which occurs in dairy products being a common contaminant of equipment surfaces and air.

Dairy products in general, present a unique ecological niche, selecting for the growth and occurrence of only a few dominant yeast species that will survive the immediate stresses of the environment (Fleet, 1990). *Yarrowia lipolytica* occurs frequently in milk products and refrigerated foods. Due to its enzymatic activities, it is regarded as a good candidate as ripening agent in milk products. The resistance of these yeasts against the environmental stresses is attributed to specific key properties. *Saccharomyces* is a typical fermentative species, usually associated with sugar originating from the added sugar and fruits. The role of all these yeasts as spoilage organisms in yoghurts is linked with their nutritional requirements, certain enzymatic activities and the ability to grow at low temperatures, low pH values and water activities. All the yeasts isolated in this study were reported to have these properties. Lactose fermenting strains of *Candida pseudotropicalis* (Van Uden and Carmo Sousa, 1957) and *Kluyveromyces marxianus* (Dubois et al., 1980) have been implicated in the spoilage of plain and fruit yoghurts, whilst our studies indicated *Kluyveromyces*

only in the spoilage of plain yoghurt. Fleet (1990) also reported on the presence of *Kluyveromyces marxianus* and *Saccharomyces cerevisiae* as the most prevalent yeasts in dairy products. *Rhodotorula* isolates were reported to be recovered from walls and equipment surfaces within dairy plants according to Connel and Skinner (1953), confirmed by Viljoen and Welthagen (1998). This strain occurred at very low numbers (3.6%), mainly attributed to the equipment (Fleet, 1990). The pink spots that usually develop on the top of sour milk and cream are reported to be colonies of *Rodotorula glutinis* probably arising through contamination from air (Ingram, 1958). This species did not grow at 5°C or 10°C in our study although it is reported in literature to grow well at 4°C.

There are two main mechanisms by which yoghurts become contaminated with yeasts. Although the packaging of yoghurt protects the product against dirt, microorganisms within the environment, gases (oxygen), and light (Robinson, 1981), the handling of the equipment with hands are responsible for various contaminating yeasts. These yeasts may also originate from contaminated ingredients which in most operations are added to the fermented yoghurt base just before packaging (Fleet, 1990). Although the packaged yoghurt material must be non – toxic and no chemical reaction should take place between the material and the yoghurt, this may not be true in all cases (Ministry of Agriculture, Fisheries and Food (MAFF), 1983). During the packaging of yoghurt, the handling of the processed fruit in the dairy is dependant on the degree of the automation and/or the type of the container used to package the fruit. For example, if metal cans are used, the normal approach is to open the can (i.e. hand operated, semi – automatic equipment) and then meter the fruit directly to the fruit/yoghurt blending equipment, such as mixing vessels and filling machines (Davis, 1975; Suriyarachchi and Fleet, 1981).

2.3.2 Key properties encouraging yeast growth

The key properties of the isolated yeasts are listed in Table 1.1 indicating the ability of these species to ferment or utilize sucrose, lactose, galactose, and lactic acid, growth at 5°C and 10°C, lipase and protease activity and nitrite tolerance. *Yarrowia lipolytica* and *Saccharomyces exiguus* exhibited the ability to grow at 80 to 240 ppm nitrite. *Kluyveromyces marxianus, Saccharomyces cerevisiae* and *Rhodotorula glutinis* despite being unable to tolerate nitrite, were previously recovered from milk, cream, yoghurt, butter and cheese (Fleet and Mian, 1987). Suriyarachchi and Fleet (1981) and Fleet and Mian (1987) isolated *Kluyveromyces marxianus*, *Saccharomyces cerevisiae* from 169 samples of yoghurts retailed in Sydney. Although *Saccharomyces* lacks the ability to utilize lactose and citric acid, or to produce lipase and protease, and also weakly utilize lactic acid (Fleet and Mian, 1987), its growth is attributed to trace amounts of glucose and galactose in plain yoghurt derived from the breakdown of lactose by the lactic acid bacteria. *Debaryomyces hansenii* (Barnett and Punkhurt, 1974) was able to utilize lactic acid but lacked the ability to ferment lactose. This species, however, possesses lipase and protease activity and therefore it could contribute to the significant growth of the species. The presence of *Debaryomyces hansenii* in yoghurts is consistent with reports in literature indicating that the species are prominent in dairy products (Cook, 1958). The frequent occurrences of this species in dairy products were also reported by Deak and Beuchat (1996). Seiler (1991) and Viljoen and Greyling (1995) also indicated on the dominance of *Debaryomyces* strains in cheese brines, cheese and other dairy products (Roostita and Fleet, 1996).

All the yeasts species isolated during this study were able to grow at 10°C and some showed growth at 5°C. Since yoghurts are stored at refrigeration temperatures, this is probably the main reason for the abundance of yeasts in yoghurts. *Yarrowia lipolytica, Saccharomyces exiguus* as well as *Saccharomyces cerevisiae* were unable to ferment lactose (Table 1.1), whereas *Kluyveromyces*

marxianus strains showed positive lactose fermenting abilities. Davis (1975) indicated that glucose and fructose may occur in yoghurts due to the usage of invert sugar by some manufacturers and that small amounts of galactose may arise from lactose. These three sugars, therefore, could also act as fermentable substrates for yeast growth in yoghurts of species such as *Debaryomyces hansenii* and *Rhodotorula glutinis*.

Most dairy yeasts possess the ability to utilize all the other available sugars other than the normally present lactose, lactic acid and other organic acids, and to produce protease and lipase enzymes which enable them to hydrolyze milk casein and fat (Roostita and Fleet, 1996).

2.3.3 Enumeration of yeasts

The yoghurts examined in this study were all examined the same day of purchasing. Only 25% of the yoghurt samples contained less than 10 yeast cells/g whereas 50% of the samples showed yeast counts in excess of 10 cells/g which suggested an unsatisfactory degree of contamination during production. Counts exceeding 10 cells/g are most often encountered in yoghurts that were not adequately refrigerated after packaging during marketing (Cook, 1958).

The presence of yeasts could be clearly seen on the third day after incubation, and counts as high as $10^3 - 10^4$ cfu/g were found. Inadequate refrigeration leads to rapid growth of yeasts in yoghurts. Yoghurt spoilage, however, was observed only when yeast counts reached 10^5 cfu/g which frequently occurred at storage temperatures above 5°C and especially at temperatures above 10°C. These yeasts where not added as part of the starter culture, and hence originated from equipment surfaces as contaminants.

Table 1.1 Key properties of yeast strains isolated from yoghurt.

Isolates	NitriteTolerance 80 160 240	Growth at 5°C 10°C	Fermentation of Sucrose lactose galactose	Casein digestion	Utilization of lactic acid	Tributyrine Rhodamine
Yarrowia lipolytica	+ + +	− +	− − −	+	+	+ +
Yarrowia lipolytica	+ + +	+ +	+ − +	+	+	+ +
Saccharomyces exiguus	+ + +	− +	+ − +	−	−	+ +
Saccharomyces exiguus	+ + +	− +	+ − +	−	−	+ +
Saccharomyces exiguus	+ + +	− +	+ − +	−	−	+ +
Saccharomyces cerevisiae	− − −	− +	+ − +	−	−	+ +
Kluyveromyces marxianus	− − −	− +	+ + +	−	−	− +
Rhodotorula glutinis	+ + +	+ +	− − −	+	+	− +
Debaryomyces hansenii	+ + +	+ +	+ − +	+	+	− −

2.7 REFERENCES

Arora, D.K., Mukerj, K.G. and Marth, E.H., 1991. Handbook of Applied Mycology. Foods and Feeds, 3 U.S.A, pp. 400.

Barnett, J.A., and Pankhurt, R.J., 1974. A new key to the yeasts, a key for identifying yeasts based on physiological tests only. North – Holland Publishing Co., Amsterdam.

Connel, G.H., and Skinner, C.E., 1953. The chemistry and biology of yeasts. Academic Press Inc. New York.

Cook, A.H., 1958. The chemistry and biology of yeasts. Academic Press Inc. New York.

Davis, J.G., 1975, The microbiology of yoghurt. In:Lactic acid bacteria in beverages and food Ed. Carr, J.G., Cutting, C.V. and Whitting, G.C. London: Academic Press. pp 245 – 266.

Davis, J.G., 1970. Laboratory control of yoghurt. Dairy Indus. 39, 149-177.

Deak, T., and Beuchat., L.R., 1996. Handbook of food spoilage. CRC Press, New York.

Dubois, G., Desaullniers – Therrien, F., Charbonneau, R., 1980, Role d'une levure dans un accident de fabrication de yoghourt brasse. Le Lart 60, 393-396.

Doyle, M.P. and Marth, E.H., 1975. Thermal inactivation of conidia from Aspergillus and *Aspergilillus parasiticus*. 1. Effects of moist heat age of conidia and sporulation medium. J. Milk Food Technol. 38, 678 – 682.

Fleet, G.H., Yeasts in dairy products.1990. J. of Appl. Bacteriol. 68, 199-211.

Fleet, G.H. and Mian, M.A., 1987. The occurrence and growth of yeasts in dairy Products. Int. J. Food Microbiol. 4, 145 – 155.

Fleet, G.H., 1990. Food spoilage yeasts. Yeast technology by Spencer J.F.T., Spencer D.M.

Fryer, T.F., Reiter, B. and Lawrence, R.C., 1967. Lipolytic activity of lactic acid bacteria. J. Dairy Sci., 50, 388-589.

Ingram, M., 1958. Yeasts in food spoilage. In the chemistry and bioloy of yeasts ed. Cook, A.H. pp 603 –633. New York; Academic Press.

Kreger–van Rij, N.J.W., 1984. In: The Yeasts, A Taxonomic Study. 3rd ed Amsterdam, Elsevier.

Kouker, G., Jaeger, K.E., 1987. Specific and sensitive plate assay for bacterial lipases. Appl. Environ. Microbiol. 53, 211-213.

Kurtzman, C.P., and Fell, J.W., 1998. The Yeasts. A Taxonomic Study, 4th Edn. Elsevier Science Publishers, Amsterdam, pp. 1055.

Lodder, J., Kreger-van Rij, N.J.W., 1952. The Yeast, A Taxonomic Study. North-Holland Publishing Co., Amsterdam.

Marth, E.H., 1991. Handbook of Applied Mycology. 3, Marcel Dekker, Inc. New York.

Miller, M.W., 1979. Yeasts in food spoilage, Institute of Food Technology, 33, 76 – 80, California.

Robinson, R.K., 1981. Dairy Microbiology 2 London and New Jersey: Applied Publishers.

Rasic, J.L.J. and Kurman, J.A., 1987. Yoghurt Scientific Grounds, Technology, Manufacture and Preparations. Technology Dairy Publishing House, Copenhagen.

Roostita, R. and Fleet, G.H., 1996. The occurrence and growth of yeasts in blue veined cheeses, Int. Food Microbiol. 28, 393 – 404.

Seiler, H., 1991. Some additional physiological characteristics for the identification of foodborne yeasts. Netherlands. Milk Dairy J. 45, 253 – 258.

Soulides, D.A., 1956. Lactose fermenting yeasts in yoghurt and their effect upon the product and the bacterial flora. Appl. Microbiol. 274 –318.

Spencer, J.F.T. and Spencer, D.M., 1990. Yeast technology. Germany, pp 125.

Suriyarachchi, V.R. and Fleet, G.H., 1981. Occurrence and growth of yeasts in yoghurts. Appl. Environ. Microbiol., 42, 574 – 579.

Tilbury, R.H., Davis, J.G., French, E.K., Imrie, K., Campbell – lent, K. and Orbell, C., 1974. Taxonomy of yeasts in yoghurts and other dairy products. In fourth International Symposium on yeasts ed. Klaushofer, H. and Sleyter, V.B. pp 265 – 266.. Vienna: Hochschuler shaft an der Hoschle for Bodenkultur.

Van der Walt, J.P. and Hopsu-Havu, V., 1976. A colour reaction for the differentiation of ascomycetous and hemibasidiomycetous yeasts. Antonie van Leeuwenhoek 42, 157-163.

Van Uden and Carmo Sousa, 1957. Presumptive tests with liquid media for coliform organisms in yoghurt in the presence of lactose fermenting yeasts. Dairy Indus. 22, 574 – 579.

Viljoen, B.C. and Welthagen, J., 1998. Yeast profile in gouda cheese during processing and ripening. Int. J. of Food Microbiol. 41, 185 – 194.

Viljoen, B.C. and Greyling, T., 1995. Yeasts associated with Cheddar and Gouda making. Int. J. Food Microbiol. 28, 79-88.

Wickerrham, L.J., 1951. Taxonomy of Yeasts. Tech. Bull. No. 1029. U.S. Dept. Agric., Washington, D.C.

Wood, B.J.B., 1985. Microbiology of fermented foods. 1. Elsevier, London and New York.

CONTENTS

3.	**Abstract**	43
3.1	**Introduction**	44
3.2	**Materials and methods**	45
3.2.1	Sampling and enumeration of contaminating yeasts	45
3.2.2	Characterization and identification of yeast isolates	46
3.2.3	Chemical analysis	47
3.3	**Results and discussion**	47
3.3.1	Microbial enumeration	47
3.3.2	Yeast identification	49
3.4	**References**	52
Annexure		55

CHAPTER 3

TEMPERATURE ABUSE INITIATING YEAST GROWTH IN YOGHURT

Bridget Ikalafeng, Analie Lourens-Hattingh, **Gabor Peter and Bennie C. Viljoen*

**Department of Microbiology and Biochemistry, University of the Orange Free State, P.O. Box 339, Bloemfontein, 9300, South Africa*

***National Collection of Agricultural and Industrial Microorganisms, H-1118, Budapest Somloi ut 14-16, Hungary*

*Corresponding author : Phone: +27-51-4012621

Fax: +27-51-4443219

E-mail: ViljoenBC@micro.nw.uovs.ac.za

Abstract

Allergic reactions of consumers to food and its contaminants are of increasing concern to health authorities. The involvement of yeasts has been mentioned in this respect. The fermentative and spoilage activities of yeasts at elevated temperatures are well known in many food and beverage commodities, while little attention has been given to the specific occurrence and significance of yeasts in dairy products at these temperatures. Since yeasts play a substantial role in the spoilage of commercial fruit yoghurts, especially when cold storage practices are neglected, the deterioration of yoghurt samples obtained from the manufactures were evaluated at different temperatures for a period of 30 days during this study. Based on the results obtained, the interaction between the yeasts and lactic acid bacteria resulted in a decline in pH values and the stabilization of viable lactic acid bacterial loads.

The highest number of yeast populations, up to 10^4 and 10^6 cfu/g, was found when yoghurts were exposed to elevated temperatures in the range of 25°C, while lower yeast counts were obtained from samples kept refrigerated at a temperature of 5°C. The most prevalent yeast species isolated, included strains of *Kluyveromyces marxianus, Saccharomyces cerevisiae, Saccharomyces exiguus* and *Yarrowia lipolytica*.

Key words: yeasts, spoilage, food, yoghurt

3.1 Introduction

Yoghurt is a fermented end product produced by a symbiotic culture of *Streptococcus thermophilus* and *Lactobacillus bulgaricus* growing at temperatures in the range of 40-45°C (Wood, 1985). Yeasts are not involved in the fermentation process during yoghurt production, but they are a major cause of spoilage of the final product (Fleet, 1990). Yeasts counts as high as $10^6 - 10^7$ cells/g have been recorded in this respect (van Uden and Carmo Sousa, 1957; Green and Ibe, 1986; Fleet and Mian, 1987). Counts exceeding 10^4 cells/g were encountered by surveys undertaken in Portugal (van Uden and Carmo Sousa, 1957) and Australia (Suriyarachchi and Fleet, 1981; Fleet and Mian, 1987). Green and Ibe (1986) examined 100 yoghurt samples from which species of *Kluyveromyces* and *Saccharomyces* were frequently isolated. *Saccharomyces cerevisiae* was also frequently isolated by Giudici et al. (1995), whilst Canganella et al. (1998) reported on the survival of *Kluyveromyces marxianus* in yoghurt. These yeasts may originate from contaminated ingredients such as fruits, nuts and honey which, in most operations, are added to the fermented yoghurt base just before packaging (Fleet, 1990). The yeasts may also develop on the surfaces of production equipment, such as mixing vessels and filling machines, that have been poorly cleaned and sanitized (Davis, 1970; Davis, 1975; Suriyarachchi and Fleet, 1981; Walker, 1977).

Due to the inherent low pH of the yoghurt, the product acts as a selective environment for the growth of yeasts (Suriyarachchi and Fleet, 1981). The yeasts therefore have a competitive advantage compared to the psychrotrophic bacteria. The greater size of the yeast cell results in greater metabolic activity per cell (Ingram, 1958). Yeasts grow relatively slow, having generation times of approximately an hour, in contrast to the much faster growth of bacteria under optimal conditions (Ingram, 1958). The inhibitory effects of alcohol on vegetative bacteria also contribute to the competitive advantage of yeasts over bacteria. The addition of sugar and fruits, acting as fermentable growth substrates, further

enhances the growing capabilities of yeasts, making the yoghurt prone to yeast spoilage. The competitive growth of yeasts is also encouraged by low storage temperatures and its resistance against preservatives which might be added in some instances (Green and Ibe, 1986). When good manufacturing practices are employed during yoghurt production, the product should contain less than 1 yeast cell/g and, if refrigerated at 5°C or less, it should not undergo spoilage by yeasts (Davis, 1975). Under normal storage conditions at low temperatures, yoghurt has an expected shelf life of 30 days (Davis, 1970). However, when storage temperatures are abused, the rapid growth of yeasts results in excessive gas formation, off flavours and discolouration. Spoilage becomes evident when the yeast population reaches $10^5 - 10^6$ cells/g, and is first seen as a swelling of the yoghurt package due to gas production by yeast fermentation (Fleet, 1990). Eventually, the package ruptures and the yoghurt acquires a yeasty, fermentative flavour and odour, and gassy appearance (Suriyarachchi and Fleet, 1981; Green and Ibe, 1986). Occasionally, yeast colonies are seen on the bottom of the package.

In this study we endeavored to determine the influence of storage temperatures on the viability of the contaminating yeasts. In addition, the interaction between the yeasts and the lactic acid bacteria was also monitored. Lactic acid fermentation occurs before yeast fermentation (Yong and Wood, 1976). Certain yeasts are inhibited by these metabolites excreted during the fermentation. The primary inhibitor is acetic acid with lactic acid being only slightly inhibitory to yeast growth.

3.2 Materials and methods

3.2.1 Sampling and enumeration of contaminating yeasts

Yoghurt samples (in total 32) from the same batch, prepared on the same day and packed in 175 ml plastic containers, were obtained from a local dairy plant

directly after production on two separate occasions. The yoghurts were prepared according to standard methods as described by Tamime and Robinson (1985). In both yoghurts, *Streptococcus thermophilus* and *Lactobacillus bulgaricus* were applied as starter cultures. Plain yoghurt (16 containers) and fruit yoghurt (16 containers) varieties were transported to the laboratory within 1 hr after production in an ice box without shaking and immediately analysed for microbial purposes. Four plain yoghurt containers, and four fruit yoghurt containers were incubated at each temperature interval of 5°C, 10°C, 15°C and 25°C, respectively, and sampled in duplicate at five-day intervals, over a period of 30 days. Prior to sampling, the yoghurt carton was cleaned with 70% alcohol. Sampling was performed by aseptically transferring 1ml samples into 9.0 ml of buffered peptone water, (Biolab C134, Merck, Darmstadt) and mixed thoroughly. Serial dilutions as required, were made and aliquots (0.1 ml) of the appropriate dilutions were spread-plate inoculated onto the surface of chloramphenicol agar (CA, Biolab) for the isolation of yeasts and De Man, Rogosa and Sharpe Agar (MRS, Biolab) for the isolation of lactic acid bacteria. Inoculated plates for yeast enumeration were incubated at 25°C for 96 hr and those for the lactic acid bacteria at 25°C for 48hr after which the colonies were counted and individual counts calculated for each type.

3.2.2 Characterization and identification of yeast isolates

All visually differentiating yeast colonies obtained from the highest dilutions were isolated and purified by streaking on Yeast Extract Malt Extract (YM) agar plates (Wickerham, 1951). Pure cultures were maintained on YM slants at 5°C. Identification to species level of the individual yeast isolates was performed according to the conventional identification methods as proposed by Kreger – van Rij (1984), Barnett et al. (1990) and Kurtzman and Fell (1998). Each of the isolates was inoculated into 6 fermentation media, 35 carbon source assimilation media, vitamin free medium, 0.01 % and 0.1 % cycloheximide. Assimilation of nitrogen compounds was performed by means of the auxanographic method

(Lodder and Kreger-van Rij, 1952). Additional tests performed included growth at 37°C, in 50 % D- glucose medium, urea hyrolysis, starch formation, acetic acid formation and staining of 4-weeks-old cultures with Diazonium blue B salt reagent (Van der Walt and Hopsu-Havu, 1976).

Ascospore formation was examined on McClary`s acetate agar (Oxoid, Basingstoke), potato glucose agar (Oxoid), Gorodkowa agar (Oxoid), corn meal agar and malt extract agar (Oxoid) (Kreger-van Rij, 1984). The inoculated media were incubated at 18°C for 4 weeks and examined at 4-day intervals. Cell morphology and mode of reproduction were examined on malt extract (Difco) and on Dalmau plates (Kreger-van Rij, 1984). The formation of pseudomycelium and true mycelium were examined on corn meal agar according to the Dalmau plate technique (Wickerham,1951).

3.2.3 Chemical analysis

The pH of each sample was measured on a regular basis during each sampling occasion as indicated in Figs. 1-4. The pH of the samples was measured at 25°C with a HI 9321 Microprocessor pH meter (Hanna Instruments, Germany).

3.3. Results and discussion

3.3.1 Microbial enumeration

Figures 1 and 2 represent the microbial growth as log cfu/ml of yoghurt samples, encountered during incubation at 25°C, 15°C, 10°C and 5°C, respectively, over a period of 30 days during the present study. Fig. 1 is representative of the microbial growth at higher temperatures (25 and 15°C) whereas Fig. 2 represents the data obtained when the yoghurts were stored at lower temperatures (5 and 10°C). According to Cousin (1982), yeasts rarely grow in refrigerated dairy

products due to the competitive growth of the rapidly growing psychrotrophic bacteria. Our results partially corresponded with the absence of yeasts grown at low temperatures, since no yeast growth was observed initially in yoghurt samples incubated at 5°C and 10°C (Fig. 2 a and b) during the first ten day period of incubation directly after being fresh. The yeast counts, however, increased significantly after 15 days of incubation reaching counts as high as 5 log cfu/ml. At the same time, a decline in lactic acid bacterial numbers was observed. The decline in lactic acid bacterial numbers at the lower storage temperatures was more significant compared to the decline at higher storage temperatures, despite the presence of an increased number of yeasts (> 6 log cfu/ml) at the higher storage temperatures. According to Canganella et al. (1998), the loss of viability for lactic acid bacteria is increased when associated with the growth of yeasts. In contrast, our results indicated that the number of lactic acid bacteria remained higher in the presence of an increased number of yeasts (Fig. 2). This may be attributed to a synergistic interaction between the yeasts and the lactic acid bacteria, as the yeasts produce stimulants encouraging the growth of the lactic acid bacteria (Fleet, 1990).

Yeasts grew well in the fruit yoghurt incubated at the higher temperatures, reaching viable numbers of 7.0 log cfu/ml, whereas the yeast numbers increased to a slightly lower maximum of 6.3 log cfu/ml when grown in plain yoghurt. The higher yeast counts observed in fruit yoghurt (Fig. 1a) compared to yeasts present in plain yoghurt (Fig.1b), may be an indication of contamination evolved from the addition of fruits, and more available utilizable carbohydrates derived from the fruit. However, no significant differences in viable yeast counts were observed in the fruit and plain yoghurts when incubated at lower temperatures. A maximum yeast count of log 5.2 cfu/ml was reached in fruit yoghurt whereas only a slightly lower count of log 5.0 cfu/ml was observed in plain yoghurt.

Due to the higher number of lactic acid bacteria observed at the higher temperatures, the decline in pH levels of these yoghurts (Fig. 1) was significantly

higher compared to the samples incubated at lower temperatures (Fig. 2). During yoghurt fermentation, lactose, the main disaccharide in milk, is metabolized into its monomers, *i.e* glucose and galactose (Van Denmark and Batzing, 1987). The lactic acid bacteria and yeasts metabolize these sugar monomer(s) through the relevant energy-yielding pathway(s), to produce end products like lactic acid (Van Denmark and Batzing, 1987) which resulted in a decrease in the pH. Interesting to note is that the decrease in pH values corresponded with an increase in yeast numbers, possibly initiated by the stimulation of lactic acid bacteria incurred by the yeasts (Figs 1 and 2). The pH values of the yoghurts incubated at elevated temperatures (Figs. 1a and b) continued to decrease directly after processing correlating with rapidly increasing yeast numbers, whereas the pH values of the yoghurts incubated at lower temperatures (Figs, 2a and b), initially remained stable. Similarly, the presence of viable yeasts in the fresh yoghurts stored at low temperatures remained undetected during this period. After 10 days of incubation at lower temperatures, the pH within the fruit yoghurt decreased slightly from 3.8 to 3.7 (Fig. 2a) and from 3.9 to 3.8 within the plain yoghurt sample (Fig. 2b). Enhanced trends of decreasing pH values (pH 3.8 to 3.1 in fruit yoghurt; pH 3.9 to 3.0 in plain yoghurt), correlating with increasing yeast numbers were observed in the yoghurts incubated at higher temperatures, and hence creating a competitive environment for yeast growth. The enhanced decline in pH may be attributed to the enhanced number of lactic acid bacterial loads which remained viable as a result of increased yeast populations which stimulated the growth of these bacteria.

3.3.2 Yeast identification

A total of 6 different yeast species representative of five genera were isolated from plain and fruit yoghurt samples incubated at 5°C, 10°C, 15°C and 25°C. The predominant species isolated in order were *Saccharomyces cerevisiae, Debaryomyces hansenii, Saccharomyces exiguus, Kluyveromyces marxianus, Yarrowia lipolytica*, and *Rhodotorula glutinis*. The frequency of presence of each

of the species isolated during six consecutive intervals of five days from fresh until 30 days old are shown in Table 1. Our results corresponded with data of Giudici et al. (1996), indicating that galactose/ sucrose fermenting yeasts predominated in plain and fruit yoghurts. Moreover, the only lactose fermenting yeast species observed during this study, *Kluyveromyces marxianus,* was more prominent in fruit yoghurts than in plain yoghurts.

Despite indications in literature (Canganella et al., 1998) that low temperature storage plays a minimal role on the growth of yeasts in yoghurts, our results indicated that the species growth was retarded at the lower temperatures (Table 1, Fig. 2). At 5°C, only species of *S. exiguus, D.* hansenii and *R. glutinis* were recovered and only at a low frequency. Although all these species lack the ability to assimilate or ferment lactose, the main carbohydrate in yoghurt, they are capable to utilize lactic and citric acids, and galactose that accumulates in yoghurts due to the breakdown of lactose by the lactic acid starter cultures (Giudici et al., 1996). These species predominantly evolved as contaminants in yoghurts originating from the equipment, improper sanitation, and fruits and sugar added as part of the ingredients (Fleischer et al., 1984; Fleet, 1990). *Rhodotorula* strains, may originate from the air as they are typical air contaminants (Kurtzman and Fell, 1998) and therefore only isolated sporadically. At 10°C, *Kluyveromyces marxianus* species were the most prominent being present on several occasions from fresh to 30 day old yoghurts, whereas high incidences of *Saccharomyces cerevisiae* were also observed. The frequent appearances of these species are in agreement with results obtained by Tilbury et al., (1974), Suriyarachchi and Fleet (1981), Green and Ibe (1987), Fleet and Mian (1987), and Fleet (1990). When the yoghurts were incubated at higher temperatures, 15 and 25°C, increased yeast populations were evident and a wider diversity of yeast species was observed. The yoghurts at these temperatures showed clear indications of swelling and deterioration much earlier during their shelf-life. The frequency of appearances of *S. cerevisiae, S. exiguus, D. hansenii* and *Y.lipolytica* strains isolated from the yoghurts incubated at

higher temperatures significantly increased, while the presence of *R. glutinis* was undetected. The presence of these species might be expected, since they are typical dairy associated yeasts frequently associated with yoghurt spoilage (Salji et al., 1987; McKay, 1992; Jordano et al., 1991; Rohm et al., 1990). *Y. lipolytica* and *D. hansenii* strains possess proteolytic and lipolytic activities (Alford and Pierce, 1961), although the characteristic is strain specific, and may therefore contribute to spoilage (Deak and Beuchat, 1996).

It has been observed that dairy products in general present a unique ecological niche, selecting for the growth and occurrence of only a few main yeast species (Fleet, 1990). The presence of the number of isolated yeasts in the current study, enhanced by elevated temperatures, clearly indicated on the importance of rapid cooling of the final product. When these low temperatures are neglected, the growth of yeasts is exaggerated and may lead to spoilage of the product. In contrast to literature, we clearly showed that the storage temperature played a significant role on the proliferation of yeasts. At higher temperatures, not only a wider diversity of yeasts developed, but the yeast loads developed much earlier during the shelf life of the yoghurts. Furthermore, the enhanced yeast numbers clearly stimulated the growth of the lactic acid bacteria resulting in an enhanced decline in pH levels. As a result, the lower pH levels present in the yoghurts, encouraged the growth of yeasts and may inhibit the growth of undesired bacterial species. This association between the yeasts and the lactic acid bacteria within the yoghurts, indicates on a typical synergistic interaction where both microorganisms will benefit from the other.

i15698063

U.O.V.S. BIBLIOTEEK

3.4 References

Alford, J.A., Pierce, D.A., 1961. Lipolytic activity of microorganisms at low and intermediate temperatures. Activity of microbial lipases at temperatures below 0°C. J. Food Sci. 26, 518-524.

Barnett, J.A., Payne, R.W., Yarrow, D., 1990. Yeasts: Characteristics and Identification, 2nd Edn., Cambridge University Press, Cambridge, UK, pp. 15-21.

Canganella, F., Ovidi, M., Paganini, S., Vettraino, A.,M., Bevilacqua, L., Trovatelli, L.D., 1998. Survival of undesirable micro-organisms in fruit yoghurts during storage at different temperatures. J. Food Microbiol. 15, 71-77.

Cousin, M.A., 1982. Presence and activity of psychrotrophic micro-organisms in milk and dairy products. A review, J. Food Protect. 45, 172 – 207.

Davis, J. G., 1970. Laboratory control of yoghurt. Dairy Indust. 39, 149 – 177.

Davis, J.G., 1975. The microbiology of yoghurt. In: Carr, J. G., Cutting, C.V., Whitting, G.C. (Eds.) Lactic acid bacteria in beverages and food. Academic Press, London, pp. 245-266.

Deak, T., Beuchat, L.R., 1996. Handbook of Food Spoilage. CRC Press, New York, pp. 210.

Fleet, G.H., 1990. Yeasts in dairy products. A review. J. Appl. Bacteriol. 68, 199-211.

Fleet, G., Mian, M.A., 1987. The occurrence and growth of yeasts in dairy products. Int. J. Food Microbiol. 4, 145-155.

Fleischer, M., Shapton, N., Cooper, P.J. 1984. Estimation of yeast numbers in fruit mix for yoghurt. J. Soc. Dairy Technol. 37, 63-65.

Giudici, P., Masini, G., Laggia, C., 1995. The role of galactose fermenting yeast in plain yoghurt spoilage. Annali di Microbiol. ed Enzimol. 46, 11-19.

Green, M.D., Ibe, S.N., 1986. Yeasts as primary contaminants in yoghurts produced commercially in Lagos, Nigeria. J. Food Protect. 50, 193-198.

Ingram, M., 1958. Yeasts in food spoilage. In: Cook, A.H (Ed.), The Chemistry and Biology of Yeast's. Academic Press, New York. pp. 603-633.

Jordano, R., Medina, L.M., Salmeson, J., 1991. Contaminating microflora in fermented milk. J. Food Prot. 54, 131-132.

Kreger – van Rij, N.J.W., 1984. The Yeasts, A Taxonomic Study. 3rd Edn, Elsevier Science Publishers, Amsterdam. pp. 1082.

Kurtzman, C.P., Fell, J.W., 1998. The Yeasts. A Taxonomic Study, 4th Edn. Elsevier Science Publishers, Amsterdam. pp. 1055

Lodder, J., Kreger-van Rij, N.J.W., 1952. The Yeasts, A Taxonomic Study. North-Holland Publishing Co., Amsterdam.

McKay, A.M., 1992. Growth of fermentative and non-fermentative yeasts in natural yoghurt stored in polystyrene cartons. Int. J. Food Microbiol. 15, 383-388.

Rohm, H., Lechner, F., Lehner, M., 1990. Microflora of Austrian natural-setyoghurt. J. Food Prot. 53, 478-480.

Salji, J.P., Saadi, S.R., Mashhadi, A., 1987. Shelf life of plain liquid yoghurt manufactured in Saudi Arabia. J. Food Prot. 50, 123-126.

Suriyarachchi, V.R., Fleet, G.H., 1981. Occurrence and growth of yeasts in yoghurts. Appl. Environ. Microbiol. 42, 574 –579.

Tamime, A.Y., Robinson, R.K., 1985. Yogurt: Science and Technology. Pergamon Press, Oxford.

Tilbury, R.H., Davis, J.G., French, E.K., Imrie K., Campbell-Lent, K., Orbell, C., 1974. Taxonomy of yeasts in yoghurts and other dairy products. In: Klaushofer, H., Sleytr, V.B., (Eds.), Fourth International Symposium on Yeasts, Vienna, Hoschschule fur Bodenkultur. pp. 265-266.

Van Denmark, P.J., Batzing, B.L., 1987. The microbes: an introduction to their nature and importance. Benjamin/Cummings Publishing Company, Inc., Menlo Park, Carlifornia.

Van der Walt, J.P., Hopsu-Havu, V K., 1976. A colour reaction for the differentiation of ascomycetous and hemibasidimycetous yeasts. Antonie van Leeuwenhoek. 42, 157-163.

Van Uden, N., Carmo Sousa, L.D., 1957. Presumptive tests with liquid media for coliform organisms in yoghurt in the presence of lactose fermenting yeasts. Dairy Indust. 22, 1028-1029.

Walker, H.W., 1977. Spoilage of food by yeasts. Food Technol. 31, 57-61.

Wood, B.J.B., 1985. Microbiology of fermented foods. Vol 1. Elsevier Science Publishers, London.

Wickerham, L.J., 1951. Taxonomy of yeasts. Tech. Bull. No. 1029. U.S. Dept. Agric, Washington, D.C.

Yong, F.M., Wood, B.J.B., 1976. Microbial succession in experimental soy sauce fermentation. J. Food Technol. 11, 525-528.

Table 1. Frequency of occurrence of yeasts in fruit and plain yoghurt, sampled consecutively at 5 day intervals from fresh until 30 days old, incubated at various temperatures.

ISOLATES	5°C		10°C		15°C	
	PLAIN	FRUIT	PLAIN	FRUIT	PLAIN	FRUIT
K. marxianus	0/6	0/6	4/6	4/6	1/6	2/6
S. cerevisiae	0/6	0/6	3/6	2/6	1/6	6/6
S. exiguus	2/6	0/6	0/6	0/6	5/6	4/6
R. glutinis	0/6	2/6	1/6	2/6	0/6	0/6
Y. lipolytica	0/6	0/6	0/6	0/6	6/6	1/6
D. hansenii	2/6	2/6	1/6	2/6	1/6	3/6

CONTENTS

Abstract		57
4.1	**Introduction**	58
4.2	**Materials and methods**	59
4.2.1	Yoghurt sampling	59
4.2.2	Enumeration of Microorganisms	60
4.2.3	Yeast identification	60
4.3	**Results and discussion**	61
4.3.1	Yeast enumeration	61
4.3.2	Yeast identification	62
4.3.3	Enumeration of Lactic acid bacteria	63
4.4	**References**	65
Appendix		68

CHAPTER 4

A COMPARISON OF THE ECOLOGICAL DIVERSITY OF YEASTS BETWEEN SOUTH AFRICAN AND HUNGARIAN YOGHURTS

Abstract

The yeasts in 60 samples of Hungarian yoghurt were enumerated and identified according to conventional methods. A similar survey was conducted in South Africa and the results compared. The yeast counts ranged from 10^3 – 10^5 cfu/g in both countries whereas the lactic acid bacterial loads showed similar declining values with their maximums ranging from 10^5 – 10^6 cfu/g. Despite similar manufacturing procedures, types of yoghurt, and starter cultures, Hungarian yoghurts showed a less diverse population of yeasts. Only *Saccharomyces cerevisiae* was isolated from yoghurts from both countries. High proportions of *Candida parapsilosis, Pichia cactophila* and *Torulaspora delbrueckii* found in Hungarian yoghurts were absent in South African yoghurts.

4.1 Introduction

The consumption of yoghurt in Europe in the late 1930s was negligible and seen only in certain ethnic groups. The market for yoghurt remained low until the 1950s, when the addition of artificial fruit juices was introduced and responsible for a slight but steady increase in the consumption for the next decade. The real boom in consumption of yoghurt began in the middle 1960s with the addition of real fruits and flavourants. The sweet taste of the fruit and added sugar were a perfect complement for the taste of the yoghurt. Although fruit and flavoured yoghurts are now very popular, plain yoghurts, that contain no non-milk ingredients, are still prepared. Details of the production, composition and microbiology of the yoghurts were discussed in Chapter 1.

Yeasts are not involved in the fermentation during the production of yoghurt, but they are a major cause of spoilage of the final product. When yoghurts are produced under conditions of good manufacturing practice, they should contain less than 10 yeast cells/g (preferably less than 1 cell/g) and, if refrigerated at 5°C or less, they should not undergo spoilage by yeasts. In these cases, a shelf life of four weeks is expected and is limited by factors other than yeasts. Yoghurts, contaminated with an initial load of 100 or more yeast cells/g will probably spoil, as the yeasts multiply and progress (Fleet, 1990). Spoilage becomes evident when the yeast population reaches 10^5 – 10^6 cells/g, and is first seen as a swelling of the yoghurt package due to gas production by yeast fermentation. Eventually the package ruptures, and the yoghurt acquires a yeasty, fermentative flavour and odour, and gassy appearance (Chapter 3).

It is not uncommon to find yeast populations exceeding 10^3 cfu/g in retail samples of either plain or fruit yoghurts. Examination of yoghuts randomly purchased at retail outlets in the United Kingdom and Canada has shown that 25 to 30% of the samples contain greater than 10 yeast cells per g (Suriyarachchi and Fleet, 1981). Akyuz and Coskun (1990) reported that 40% of the commercially yoghurt sold in Turkey, showed coliform organisms at an average of 3.55×10^3 cfu/g, while the rest of the samples contained yeasts

and moulds with an average of 2.84 x 10^5 cfu/ g. Similar studies in Australia (Suriyarachchi and Fleet, 1981), and Nigeria (Green and Ibe,. 1987) revealed a much higher incidence of contamination, with 60% of samples having yeast counts exceeding 10^4 cells/g. In many cases, counts of 10^6 – 10^7 cfu/g have been recorded (Van Uden and Carmo, 1957; Green and Ibe, 1987; Fleet and Mian, 1987).

Yeasts are the primary spoilage microorganisms of yogurt, especially fruit yoghurt, due to growth in or contamination of the mix before blending. Consequently, the yeast count is used as an index of proper plant sanitation and high quality products. Various species of yeast may induce several defects and spoilage of dairy products (Foster et al., 1958; Walker, 1977). The levels of yeasts are therefore constantly checked in the mix and final product (Fleischer et al., 1984). Almost any kind of food will permit yeasts to grow if it has not been adequately heat-treated. High concentrations of sugar, salt, organic acids, the exclusion of air, refrigeration and application of other storage conditions will not safeguard a food from the action of yeasts provided storage is sufficiently long. The higher the initial contamination, the sooner spoilage symptoms become apparent. For this reason, observance of sanitation in food- processing plants offers the best protection against losses caused by microbial spoilage (Phaff et al., 1978).

The aim of this study was to isolate, enumerate and identify the yeasts in Hungarian yoghurts and to compare the results with commercial South African yoghurts.

4.2 Materials and methods

4.2.1 Yoghurt sampling

Sixty yoghurt samples in 200 ml plastic containers were purchased from various supermarkets irrespective of the type, brand or expiry date. The varieties of yoghurts sampled comprised plain yoghurts, flavoured and fruit

yoghurts randomly selected from different outlets. The samples were transported in an icebox to the laboratory, and analyzed microbiologically within 12 hours.

4.2.2 Enumeration of microorganisms

The yoghurt carton was vigorously shaken, wiped with 90% alcohol and 10ml samples aseptically transferred into 90ml of peptone water (Biolab C134, Merck, Darmstadt). Portions (0.1 ml) of the appropriate dilutions as required, were spread plated in duplicate on selective media for the enumeration of yeasts and lactic acid bacteria.

Lactic acid bacteria were enumerated using De Man Rogosa agar (MRS) (Biolab, C86) and the plates incubated at 25°C for 48h. For the enumeration of yeasts, Yeast Extract Glucose Chloramphenicol Agar (YGC) (Biolab, C98) was used and the plates incubated at 25 °C for 7 days. All visual differentiating yeasts based on colour, shape and size were isolated from the highest dilution and purified on Yeast – Malt Extract Agar (YM) by three successive streakings and verified by microscopy. The pure strains were maintained on YM (Yeast extract Malt extract) agar slants at 5°C until identification.

4.2.3 Yeast identification

Strains were identified to the species level according to the conventional identification methods of Kreger-van Rij (1984), Barnett et al. (1990) and Kurtzman and Fell (1998).

Each isolate was inoculated into 6 fermentation media, 35 carbon source assimilation media, vitamin free medium, 0.01% and 0.1% cycloheximide. Assimilation of nitrogen compounds was performed by means of the auxanographic method (Lodder and Kreger-van Rij 1952). Additional tests performed included growth at 37°C, in 50% D-glucose medium, urea hydrolysis, starch formation, acetic acid formation and staining of 4-week-old

cultures with Diazonium blue B salt reagent (Van der Walt and Hopsu-Havu, 1976).

Ascospore formation was examined on McClary's acetate agar, potato glucose agar, Gorodkowa agar, corn meal agar and malt extract agar (Kreger-van Rij, 1984). The inoculated media were incubated at 18°C for 4 weeks and examined at 4-day intervals. Cell morphology and mode of reproduction were examined on malt extract (Difco) and on Dalmau plates (Kreger-van Rij, 1984). The formation of pseudomycelium and true mycelium was examined on corn meal agar according to the Dalmau plate technique (Wickerham, 1951).

4.3 Results and discussion

4.3.1 Yeast enumeration

The yeast counts obtained from different yoghurt samples purchased and analysed in Hungary ranged from 10^1 to 10^5 cfu/g (Table 4.1). Natural yoghurt and yoghurt with added forest fruits had the highest viable yeast counts of 10^5 cfu/g. Similar yeast counts were observed from yoghurt samples purchased in South Africa (Chapter 2). Again the number of yeasts associated with the yoghurts is related to the date of production, expiry date and temperature control whereas the type of yoghurt played a minimal role.

The viable yeast counts encountered from Hungarian and South African yoghurts are in correspondence with results obtained world-wide. It is not uncommon to find yeast populations of 10^3 cfu/g or more in retail samples of either plain or fruit yoghurts. In some extreme cases, counts as high as 10^6 to 10^7 cfu/g were detected in Portugal and Nigeria (Van Uden and Carmo Sousa, 1957; Green and Ibe, 1987). Surveys of retail yoghurts in the UK (Davis, 1974) and Canada (Arnott et al., 1974) found that more than 30% of samples had yeast counts exceeding 10^3 cfu/g. Similar surveys in Portugal, Australia (Fleet, 1990) and Nigeria revealed a much higher incidence of 60% of samples having counts exceeding 10^4 cfu/g. Lesser degrees of

contamination were reported for retail yoghurts purchased in the USA and Netherlands. Most yoghurts sold in the European countries are flavoured and sweetened, and unless severe heat treatment is applied, yeast and mould contamination can occur. Duru and Ozgunes (1981) revealed that 35% of commercially sold yoghurt in Ankara, Turkey, contained more than 10^5 cfu/g of viable yeasts. Tamine et al. (1993) indicated that any yoghurt sample containing more than 100 cfu/g yeast cells is considered unacceptable.

These results showed that yeasts constitute a significant part of the microflora of yoghurts. At such high levels, the yeast metabolism should impact on the overall quality and acceptability of the product. In our study, samples taken from the same source showed varying yeast counts. The variance in counts may be attributed to the quality of the milk, which could be indicative of an inconsistent microflora, and yeasts could be involved. The high and variable yeast counts could also be attributed to contamination from air, the fruits and flavourants, or improper sanitation practices during yoghurt production (Con et al., 1995).

4.3.2 Yeast identification

A total of 60 strains were isolated from yoghurt samples examined in Hungary (Table 4.2). The isolated yeast species from nine different types purchased from various retail outlets are shown in Table 4.1. Only one species was similarly encountered in both South Africa and Hungary, namely *Saccharomyces cerevisiae* (Table 4.3). Yeasts have been mentioned as contaminating microorganisms in yoghurts, but in the production, only partial interpretations of the complex microflora interactions have been discussed.

Candida parapsilosis (29.4%) and *Pichia cactophila* (35.3%) were the most predominant species frequently isolated from Hungarian yoghurt. According to Rossi (1978), *Candida parapsilosis* selectively attribute with a tendency towards increased viability and cell survival of *Lactobacillus bulgaricus*. Several strains of *Pichia cactophila* were isolated from Hungarian yoghurt, despite being a yeast species not frequently reported to be associated with

foods. Phaff et al. (1966) reported that this yeast species is found in decaying stems of various cacti in the Sonoran desert of Northern Mexico, Southern Arizona, and Baja California. Since the cactus necrosis is believed to be initiated by bacteria, the yeasts may be introduced by cactophilic *Drosophila* that feeds and breeds on the rotting tissue. The presence of this species at high incidences in Hungarian yoghurts is unexplainable. Similar data, however, regarding the identification of this species, were also observed by the Hungarian group.

Saccharomyces cerevisiae, a melibiose-negative brewer's yeast is commonly found in dairy products and has been observed at frequent intervals (17.6%). This species does not only occur in spoiled foods, but also has a positive role in fermentation of specific foods e.g. baking of bread and brewing beer. King and Torok (1991) isolated this species most frequently from fruits. Their results are in correspondence with our data indicating that the species was mainly found in fruit yoghurts in Hungary and South Africa.

Although *Torulaspora delbrueckii* had a low incidence in the samples, it is known to be important in dairy products and its presence could be important (Fleet, 1990). The species was the predominant species found in South African traditional fermented milk (Loretan et al., 1998), and Cheddar cheese (Fleet, 1990; Deak and Beuchat, 1996; Welthagen and Viljoen, 1998). *Pseudozyma* was found in lesser numbers in Hungarian yoghurt, whereas none was found in South African yoghurt.

4.3.3 Enumeration of Lactic acid bacteria

The viability of the lactic acid bacteria, which plays a fundamental function in the production of yoghurt, was also determined in this study. Results are presented in Table 4.1. Of all the samples analyzed, pineapple yoghurt had the lowest lactic acid bacterial count (1.2×10^5 cfu/g), whereas the highest count was observed in the yoghurt with added fruit pieces (3.6×10^6 cfu/g). Duration of storage, however, played an important role in the growth and survival of lactic acid bacteria in the samples analyzed. The decline in the

number of viable lactic acid bacterial cells may be due to the accumulation of ambient lactic acid. Similar findings were reported by Con et al. (1995). Hamann and Marth (1984) also reported on the decline of lactic acid bacterial counts from 10^9 cfu/g present in fresh yoghurt to 10^6 cfu/g when the yoghurt was stored at 5°C for 60 days or longer.

Similar viable counts of lactic acid bacteria were found in Hungarian and South African yoghurts ranging between $10^5 - 10^6$ cfu/g. In both cases, initial viable counts exceeded 10^8 cfu/g (results not shown).

4.4 References

Arnott, D.R., Duitschaever, C.I. and Bullock D.H. 1974. Microbiological evaluation of yoghurt produced commercially in Ontario. J. Milk Food Technol. 37, 11-13.

Akyuz, N., and H. Coskun, 1990. A study on the chemical, hygienic and microbiological properties of yoghurt commercially sold in Van, Turkey (in Turkish). Yuzuncu Yil Univ., College of Agriculture Publication 1 (1): pp 71-79.

Barnett, J.A., Payne, R.W. and Yarrow, D., 1990. Yeasts: Characteristics and Identification. 2nd Edn., Cambridge University Press, Cambridge, UK, pp. 14-21.

Con, A.H., Cakmakci, S., Cagcar, A., and Gokalp, H. Y., 1995. Effects of different fruits and storage periods on microbiological qualities of fruit – flavoured yoghurt produced in Turkey. J. of Food Protect. Vol 59, no. 4 pp 402 – 406.

Deak, T. and Beuchat, L.R., 1996. Handbook of Spoilage. CRC Press. New York.

Duru, S. and Ozgunes, H., 1981. A study of the hygienic qualities of yoghurt commercially sold in Ankara, Turkey (in Turkish). GIDA 4:19 –23.

Fleet, G.H., and Mian, M.A., 1987. The occurrence and growth of yeasts in dairy products. New York, Chas. Pfizer.

Fleet, G., 1990. Yeasts in dairy products. A review. J. Appl. Bacteriol. 68, 199-211.

Fleischer, M., Shapton, N. and Cooper, P.J., 1984. Estimation of yeast numbers in fruit mix for yoghurt. J. of the Society of Dairy Technol. 37 no. 2 .

Foster, E., Nelson, E., Spech, M., Doetsch, R. and Olson, J., 1958. Dairy Microbiol. McMillan and Co. Ltd., London

Green, M.D. and Ibe, S. N.,1987. Yeasts as primary contaminants in yoghurt produced commercially in Lagos, Nigeria. J. Food Protect. 50, 195-198.

Hamann, W.A.T. and Marth, E.H., 1984. Survival of *Streptococcus thermophilus* and *Lactobacillus bulgaricus* in commercial and experimental yoghurts. J. Food Prot. 47, 781 – 786.

Helferich, W. and Westhoff, D., 1980. All about yoghurt. Prentice – Hall, Inc., London.

King, A.D. JR. and Torok, T., 1991. Comparative study on the identification of food – borne yeasts. Appl. Environ. Microbiol. pp 1207 – 1212.

Kurtzman, C.P. and Fell, J.W., 1998. The Yeasts. A Taxonomic Study. North-Holland Publishing Co., Amsterdam.

Kreger-van Rij, N.J.W., 1984. The Yeasts, A Taxonomic Study. 3rd Edn., Elsevier Science Publishers, Amsterdam. pp 1082.

Lodder, J. and Kreger-van Rij, N.J.W., 1952. The Yeasts, A Taxonomic Study. North-Holland Publishing Co., Amsterdam.

Loretan, T., 1999. The diversity and technological properties of yeasts from traditional South African Fermented milks. M.Sc. thesis. University Orange Free State, Bloemfontein, South Africa.

Phaff, H. J., Miller, M.W., and Mrak, E.M., 1966. The life of yeasts. Their nature, activity, ecology and relation to mankind. Harvard University Press, Cambridge, Massa. pp 237.

Rossi, J., 1978. The kefir microorganisms. Yeasts Sci. Technol. Cat. Cas. 29, 59-67.

Suriyarachchi, V.R., and Fleet, G.H., 1981. Occurrence and growth of yeasts in yoghurts. Appl. Environ. Microbiol. 42, 574 – 579.

Torok, T. and King, A.D., 1991. Comparative study on the identification of foodborne yeasts. Appl. Environ. Microbiol. 57, 1207-1212.

Van der Walt, J.P. and Hopsu-Havu, V.K., 1976. A Colour reaction for the differentiation of ascomycetous and hemibasidiomycetous yeasts. Antonie van Leeuwenhoek. 42, 157-163.

Van Uden, N. and Carmo Sousa, 1957. Presumptive tests with liquid media for coliform organisms in yoghurt in the presence of lactose fermenting yeasts. Dairy Ind. 22, 574 –579.

Walker, H.W., 1977. Spoilage of food by yeasts. Food Technol. 31: pp 57 – 65.

Welthagen, J. and Viljoen, B.C., 1998. Yeast profile in gouda cheese during processing and ripening. Int. J. Food Microbiol. 41, 185-194.

Wickerham, L.J. 1951. Taxonomy of yeasts. Tech. Bull. No. 1029, US Dept. Agric. Washington, D.C.

Table 4.1 Microbiological counts of yoghurts obtained in Hungary

Yoghurt sample	Yeast count (cfu/g)	Bacterial count (cfu/g)
F1-fruit pieces	1.3×10^3	3.64×10^6
F2- layer yoghurt with apricot	3.0×10^2	1.02×10^6
F3-apricot	1.1×10^2	$7. \times 10^5$
F4-layer yoghurt with forest fruits	1.6×10^5	5.6×10^5
F5-strawberry	1.2×10^3	1.7×10^6
F6-cherry and muesly	1.9×10^2	9.2×10^5
F7-pineapple	4.0×10^4	1.2×10^5
F8-strawberry pieces	3.3×10^3	2.8×10^5
F9-natural	1.2×10^5	4.8×10^5

Table 4.2 Proportional incidence (%) of yeasts isolated from yoghurt in Hungary.

Isolate	Proportional incidence (%)
Pichia cactopila	35.3
Candida parapsilosis	29.4
Saccaromyces cerevisiae	17.6
Torulaspora delbrueckii	11.8
Pseudozyma sp.	5.9

Table 4.3 Proportional incidence (%) of yeasts isolated from South African yoghurt

Isolate	Proportional incidence (%)
Debaryomyces hansenii	27.1
Kluyveromyces marxianus	10.7
Saccharomyces cerevisiae	14.3
Rhodotorula glutinis	3.6
Saccharomyces exiguus	25.0
Yarrowia lipolytica	17.0

CONTENTS

General Discussion and Conclusions 72

Chapter 2 – The occurrence and diversity of yeasts in yoghurt 73

Chapter 3 – Temperature abuse initiating yeast growth in yoghurt 73

Chapter 4 – A comparison of the ecological diversity of yeasts 75

References 76

CHAPTER 5

GENERAL DISCUSSION AND CONCLUSIONS

Currently there is a major unawareness of the presence of yeasts in commercial yoghurt. The dairy industry generally belief that only lactic acid bacteria are present due to its involvement in the fermentation process as starter cultures, and normal bacterial populations causing spoilage. The contribution of yeasts to spoilage is considered of little importance since bacteria normally out-competed yeast growth and none of the yeasts is of any health concern.

In this study 11 different yeast species were isolated and identified, although only four species proofed to be predominant. The high numbers of yeasts, up to 8.08 log cfu g^{-1}, however, suggested that the yeasts are able to grow and multiply in the yoghurt and therefore may cause spoilage despite a conserved diversity.

The occurrence of yeasts is due to the standard of hygiene in the production of the fermented milk. Therefore yeasts evolved from environmental contamination, including the air, water, as well as from equipment used in the processing of yoghurt. Although some of the yeast strains, such as *Saccharomyces cerevisiae* are known to be ubiquitous in nature, their presence cannot be attributed to the contamination factor as they predominantly originated from fruits added to the yoghurts.

Chapter 2 - The occurrence and diversity of yeasts in yoghurt

The restricted diversity of yeasts isolated from yoghurt samples revealed only six different species. The most prevalent species were *Debaryomyces hansenii* (32%), *Saccharomyces exiguus* (25%), *Yarrowia lipolytica* (17%), *Saccharomyces cerevisiae* (14.3%) and *Kluyveromyces marxianus* (10.7%). *Rhodotorula spp* were isolated but occurred at a very low incidence. The presence of these species in yoghurts is consistent with reports in literature indicating that the species are prominent in dairy products (Fleet, 1990).

The key properties of the isolated yeasts verified their presence, indicating the ability of these species to ferment or utilize sucrose, lactose, galactose, and lactic acid, growth at 5°C and 10°C, lipase and protease activity and nitrite tolerance.

Only 25% of the yoghurt samples contained less than 10 yeast cells/g whereas 50% of the samples showed yeast counts in excess of 10 cells/g which suggested an unsatisfactory degree of contamination during production. Counts exceeding 10 cells/g are most often encountered in yoghurts that were not adequately refrigerated after packaging during marketing (Cook, 1958). Yoghurt spoilage, however, was observed only when yeast counts reached 10^5 cfu/g which frequently occurred at storage temperatures above 5°C and especially at temperatures above 10°C. These yeasts where not added as part of the starter culture, and hence originated from equipment surfaces as contaminants.

Chapter 3 - Temperature abuse initiating yeast growth in yoghurt

According to Cousin (1982), yeasts rarely grow in refrigerated dairy products due to the competitive growth of the rapidly growing psychrotrophic bacteria. Our results partially corresponded with the absence of yeasts grown at low temperatures, since no yeast growth was observed initially in yoghurt samples incubated at 5°C and 10°C. The yeast counts, however, increased significantly after 15 days of incubation reaching counts as high as 5 log cfu/ml. A decline in lactic acid bacterial numbers corresponded with an increased number of yeasts at low temperatures. The decline in lactic acid bacterial numbers at the lower storage

temperatures was more significant compared to the decline at higher storage temperatures, despite the presence of an increased number of yeasts (> 6 log cfu/ml) at the higher storage temperatures. This phenomenon is attributed to a synergistic interaction between the yeasts and the lactic acid bacteria, as the yeasts produce stimulants encouraging the growth of the lactic acid bacteria (Fleet, 1990).

Enhanced trends of decreasing pH values correlating with increasing yeast numbers were observed in the yoghurts incubated at higher temperatures, and hence creating a competitive environment for yeast growth. The enhanced decline in pH was attributed to the enhanced number of lactic acid bacterial loads which remained viable as a result of increased yeast populations which stimulated the growth of these bacteria.

The frequency of presence of each of the yeast species isolated during six consecutive intervals of five days from fresh until 30 days revealed only six dominant yeast species. Our results corresponded with data of Giudici et al. (1995), indicating that galactose/ sucrose fermenting yeasts predominated in plain and fruit yoghurts. Despite indications in literature (Canganella et al., 1998) that low temperature storage plays a minimal role on the growth of yeasts in yoghurts, our results indicated that the species growth was retarded at the lower temperatures. At 5°C, only species of *Saccharomyces exiguus, Debaryomyces* hansenii and *Rhodotorula glutinis* were recovered and only at a low frequency.

When the yoghurts were incubated at higher temperatures, 15 and 25°C, increased yeast populations were evident and a wider diversity of yeast species was observed. The yoghurts at these temperatures showed clear indications of swelling and deterioration much earlier during their shelf life. At these temperatures, not only a wider diversity of yeasts developed, but the yeast loads developed much earlier during the shelf life of the yoghurts. Furthermore, the enhanced yeast numbers clearly stimulated the growth of the lactic acid bacteria resulting in an enhanced decline in pH levels. As a result, the lower pH levels present in the yoghurts, encouraged the growth of yeasts and may inhibit the growth of undesired bacterial species. This association between the yeasts and the lactic acid bacteria within the yoghurts, indicates on a typical synergistic interaction where both microorganisms will benefit from the other.

Chapter 4 - A comparison of the ecological diversity of yeasts between South African and Hungarian yoghurts.

The viable yeast counts encountered from Hungarian and South African yoghurts are in correspondence with results obtained world-wide. The yeast counts ranged from 10^3 – 10^5 cfu/g in both countries whereas the lactic acid bacterial loads showed similar declining values with their maximums ranging from 10^5 – 10^6 cfu/g. Despite similar manufacturing procedures, types of yoghurt, and starter cultures, Hungarian yoghurts showed a less diverse population of yeasts. *Candida parapsilosis* (29.4%) and *Pichia cactophila* (35.3%) were the most predominant species frequently isolated from Hungarian yoghurt although none was found in yoghurts sampled in South Africa. Several strains of *Pichia cactophila* were also isolated from Hungarian yoghurt, despite being a yeast species not frequently reported to be associated with foods. Only *Saccharomyces cerevisiae* was commonly found in both countries.

Similar viable counts of lactic acid bacteria were found in Hungarian and South African yoghurts ranging between 10^5 – 10^6 cfu/g. In both cases, initial viable counts exceeded 10^8 cfu/g.

REFERENCES

Canganella, F., Ovidi, M., Paganini, S., Vettraino, A.M., Bevilacqua, L. and Trovatelli, L.D., 1998. Survival of undesirable micro-organisms in fruit yoghurts during storage at different temperatures. J. Food Microbiol. 15, 71-77.

Cook, A.H., 1958. The chemistry and biology of yeasts. Academic Press Inc. New York.

Cousin, M.A., 1982. Presence and activity of psychrotrophic micro-organisms in milk and dairy products. A review. J. Food Protect. 45, 172-207.

Fleet, G.H., 1990. Yeasts in dairy products. J. Appl. Bacteriol. 68, 199-211.

Giudici, P., Masini, G., Laggia, C., 1995. The role of galactose fermenting yeast in plain yoghurt spoilage. Annali di Microbiol. ed Enzimol. 46, 11-19.

CONTENTS

SUMMARY 78

Appendix 80

CHAPTER 6

SUMMARY

Yeasts have a competitive advantage in yoghurt due to its ability to grow at low pH values and temperatures and therefore are major role players in causing spoilage. The yeasts occurring as natural microflora in commercial yoghurt were isolated and identified according to conventional identification and enumeration techniques. Characteristics of the naturally contaminating yeasts of commercial yoghurt revealed a limited diversity of yeast species. The yeasts most frequently isolated were *Kluyveromyces marxianus, Debaryomyces hansenii, Saccharomyces cerevisiae*, *Saccharomyces exiguus* and *Yarrowia lipolytica*. *Rhodotorula* spp. occurred at a very low incidence.

Since these yeasts play a substantial role in the spoilage of commercial fruit yoghurts, especially when cold storage practices are neglected, the deterioration of yoghurt samples obtained from the manufactures were evaluated at different temperatures for a period of 30 days during this study. Based on the results obtained, the interaction between the yeasts and lactic acid bacteria resulted in a decline in pH values and the stabilization of viable lactic acid bacterial loads.

The highest number of yeast populations, up to 10^4 and 10^6 cfu/g, was found when yoghurts were exposed to elevated temperatures in the range of 25°C, while lower yeast counts were obtained from samples kept refrigerated at a temperature of 5°C. Populations not less than 10^3 cfu/ml were generally observed in commercial yoghurt samples. All the isolated yeasts were examined based on relevant key properties that governed their growth and survival in yoghurt. All the yeasts were tolerant to 4-8% NaCl, except for *Saccharomyces cerevisiae,* and 40 – 80 ppm nitrite. The lipolytic and proteolytic activity appeared variable.

The yeasts in 60 samples of Hungarian yoghurt were enumerated and identified according to conventional methods and compared with a similar survey conducted in South Africa. The yeast counts ranged from 10^3 – 10^5 cfu/g in both countries whereas the lactic acid bacterial

loads showed similar declining values with their maximums ranging from 10^5 – 10^6 cfu/g. Despite similar manufacturing procedures, types of yoghurt, and starter cultures, Hungarian yoghurts showed a less diverse population of yeasts. Only *Saccharomyces cerevisiae* was isolated from yoghurts from both countries. High proportions of *Candida parapsilosis, Pichia cactophila* and *Torulaspora delbrueckii* found in Hungarian yoghurts were absent in South African yoghurts.

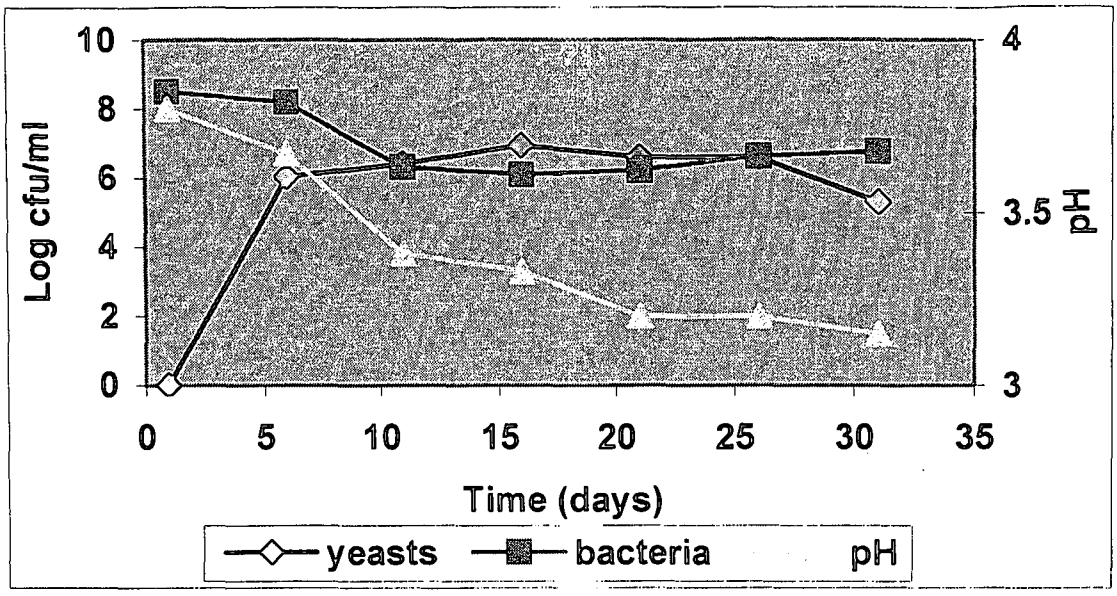

Fig. 1(a) Growth of yeasts and lactic acid bacteria in fruit yoghurt at higher temperatures.

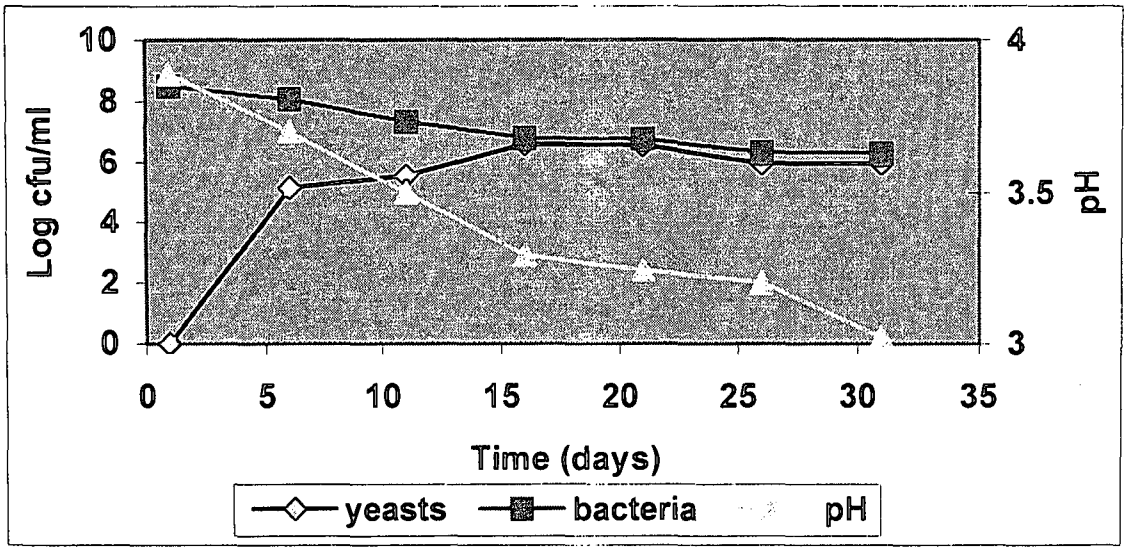

Fig.1(b) Growth of yeasts and lactic acid bacteria in plain yoghurt at higher temperatures.

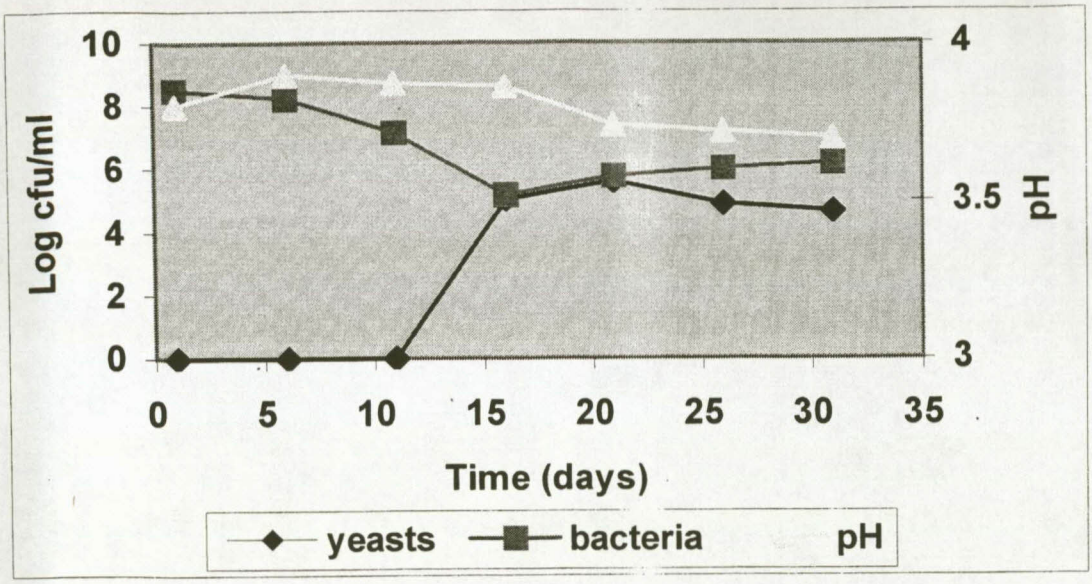

Fig. 2 (a) Growth of yeasts and lactic acid bacteria in fruit yoghurt at lower temperatures.

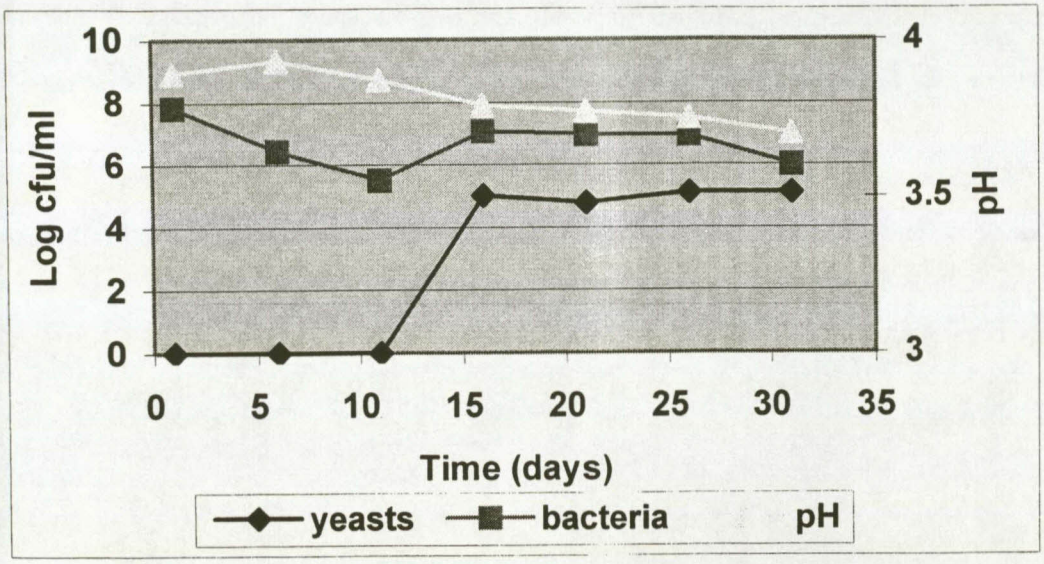

Fig.2 (b). Growth of yeasts and lactic acid bacteria in plain yoghurt at lower temperatures.

U.D.V.S. BIBLIOTEEK