MICROBIOLOGICAL STUDIES ON "NONO"
A NIGERIAN FERMENTED MILK PRODUCT

A Dissertation

BY

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Major Subject - Food Fermentation Microbiology

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DECLARATION

I hereby declare that the content of this thesis is purely a record of my research findings. No part of this work has been submitted or accepted in any previous application elsewhere. All quotations and literature cited from sources are duly acknowledged.

M. O. Bankole

February, 1991

C E R T I F I C A T I O N

This project thesis entitled "Microbiological studies on "Nono", a Nigerian, fermented milk product' by M. O. Bankole meets the regulation governing the award of the degree of Ph.D Microbiology of Ahmadu Bello University, and is approved for its contribution to knowledge and literary presentation.

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DEDICATION

To my husband

DURO

for patiently giving
all he could
towards the conpletion
of this work.

ACKNOWLEDGEMENT

I am extremely indebted particularly to my main supervisor Dr. R. N. Okagbue and Dr. N. E. Gomwalk a co-supervisor without whose help, continuous push and encouragement in varying degrees, this work would never have been completed.

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ABSTRACT

The microorganisms involved in the traditionally produced fermented milk were many but the most predominant ones were Streptococcus lactis, Streptococcus cremoris, Streptococcus diacetilactis, Lactobacillus cellobiosus, Lactobacillus brevis, Lactobacillus plantarum, Bacillus spp., Candida pseudotropicalis, Endomyces spp., Trichosporon beigelii and Saccharomyces cerevisiae.

The pH of the fermenting medium dropped from around 6.8 ± 0.1 to 4.3 ± 0.1 while the total titratable acidity increased from 0.2 gram % to 1.04 gram % lactic acid. The proximate and mineral contents of "nono" sold in the markets varied slightly.

The optimum temperature for growth of the Lactobacillus species was 40°C while that of Streptococcus species was 35°C.

The pH level optimal for growth were 4 for L. cellobiosus, 5 for L. plantarum, L. brevis, S. diacetilacitis, S. lactis, C. pseudotropicalis, S. cerevisiae and 6. for S. cremoris.

S. diacetilactis, S. lactis, S. cremoris and S. cerevisiae produced (4, 2, 1.4, 0.4) µg/ml amount of diacetyl respectively at 24 hours of fermentation.

The most acceptable starter culture for the processing of "nono" was the combination of S. diacetilactis, L. brevis and S. cerevisiae.

Agitated fermentation yielded a product with less viscosity, more diacetyl and more total titratable acidity than stationary fermentation while closed system fermentation yielded a product with gas, stronger flavour and less sour than the product of open system fermentation.

"Nono" produced at temperatures of 25°C and 30° C were best appreciated. "Nono " stored at 4° C was more preferred and more acceptable than "nono" stored at 25°C.

Lactic acid bacteria present in "nono" in single and mixture had inhibitory effects on the growth of some spoilage organisms such as Staphylococcus aureus and Escherichia coli. These lactic acid bacteria had no inhibitory effect on one another

No bacteriophage was observed in the samples of "nono" examined.

The findings were discussed with special reference to the possible development of the technology for large scale production of "nono".

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Chapter 1.

INTRODUCTION

"Nono", a product of milk fermentation, is a common food-drink in the northern part of Nigeria. It had been traditionally produced by the Fulanis, a nomadic cattle-rearing tribe, for a long time, and is consumed either alone or as a homogenate with "Fura", a sorghum-millet meal. The local name of the homogenate is "Fura-da-nono".

"Nono" production has remained a traditional art. Cattle is milked by hand by the nomads and the milk contained in a calabash is covered with a mat and allowed to undergo a natural fermentation for approximately 24 hours. The traditional method of production has limited production scale, quality and acceptability of "nono".

Since "nono" is a fermented milk product, it probably belongs to the same family as yoghurt of more developed countries. Consequently, several nutritive and therapeutic values which have been described for yoghurt (Keogh 1978, Jay 1978) may be applicable to "nono". Indeed experience shows that many tribes other than the Fulani are developing interest in "nono" especially since imported milk and yoghurt have become very expensive. Consequently, there is a need to optimize production scale and quality of "nono" in order to make it more attractive to prospective consumers. Indeed current national

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interest for self-reliance necessitate an intensive effort towards developing "nono" as a national product.

Very little scientific information is available on the fermentation of milk for "nono" production. These include studies of the changes in the acidity and lactic acid content of milk during Nono production (Atanda and Ikenebomeh 1988), the microbiological quality of prepared "nono" (Eka and Ohaba 1977) and the effect of "kuka" (cream of tartar) on the bacterial load of "nono" (Makinde, 1979). There is need to carry out further studies into the fermentation of milk for "nono" production. Such investigations include the identification and the activities of the microorganisms involved in the fermentation and the conditions favouring the activities of the microorganisms. The physico-chemical conditions that lead to the selection of the natural fermenting flora will need to be determined to develop suitable pure culture inocula for the fermentation. Pure starter culture which have been developed for other processes have been found to improve fermentation process as well as make for good product quality and stability (Nout 1985). The presence and activities of phages cannot also be ruled out. All this ivestigations will be needed to optimize the fermentation processs and then popularise "nono" among the populace.

This work was therefore embarked upon with the following objectives:

(1) Isolation and identification of the micro-organisms involved in the fermentation of milk for "nono" production;

(2) Determination of the physico-chemical changes during the fermentation process;

(3) Investigation of the effects of varying processing conditions on the fermentation;

(4) Investigation of the effect of some starter culture on the fermentation, on one another and on other organisms;

(5) Studying the effect of some storage environments on "nono" stability;

(6) Investigation of the presence of phages in "nono",

Chapter 2

LITERATURE REVIEW

2.1. Food and Beverage Fermentation

Fermentation and drying are the oldest methods of food preservation known to mankind (Pederson, 1979). Prehistoric man had little or no difficulty in finding sufficient food which he consumed in the raw state as he roamed about. Such food however was perishable due to degradation and spoilage by its natural enzymes or by the enzymes elaborated by microoganisms. As man developed, he learned to store some of his food for future use by trial and error, using the natural processes of drying and fermentation (Pederson, 1979). Seeds from wild grasses were sun-dried and stored for months and even years without spoiling as evidenced from the Biblical account of the storage of grains for the seven lean years by the Egyptians upon Joseph' recommendation (Pederson, 1979). Some of these dried seeds were put into powder and used in various forms. Sausages, one of the oldest form of processed food, are prepared from fermentation of meat of many animals and also fermentation of fish (Pederson, 1979). Individual and mixed vegetables are fermented and preserved as acid products while many types of alcoholic beverages from cereals, potatoes and other starchy foods either alone or in combinations have been known in practically every civilisation. These processes of preservation introduced microbiological, physical, chemical and organo-

leptic changes to the food, which are important to mankind (Jay, 1978).

Fermented food range from fermented legumes (Wood 1979, Hedger and Basuki 1979), fermented fish (Beddows 1979), fermented alcoholic drinks (Bassir 1968), fermented carbohydrate rich foods (Hedger and Basuki 1979, Gashe 1987 and Odunfa 1988) to fermented dairy products (Pederson 1979, Cox 1979). Fermentation though primarily a method of preserving food (Hesseltine and Wang 1967, Pederson 1979), imparts a new and better aroma flavour on the product (Steinkraus 1973). It has also been found to have other benefits such as improvement of digestibility of food materials (Padhye and Sarunkhe 1979), reduction in volume of materials to be transported, destruction of undesirable factors, reduction of energy required for cooking, increasing the nutritional value of the food materials and making the fermented food safer (Hesseltine and Wang, 1979).

2.2. Food Fermentation in Nigeria and Africa

Fermented foods are important in the diet of the people of Africa, Asia and the Orient (Steinkraus 1973). Odunfa (1988), categorized the fermented foods in West Africa to include starchy gruels and solid foods, alcoholic beverage, vegetable proteins and animal proteins. The fermented starchy gruels include "ogi", "ogi-baba", "soy-ogi" and "kenkey". "Ogi" and "ogi-baba", are produced from maize and guinea-corn respectively,

while "soy-ogi" is a protein - enriched, soya - incorporated "ogi". The fermented solid starchy foods are those produced from cassava and they include "gari", "fufu" and "lafun". Studies have been carried out on the microorganisms involved in the production of these carbohydrate products. Akinrele (1970), reported that the microorganisms isolated from fermenting maize during "ogi" production include Cephalosporium spp.; Fusarium spp.; Aspergillus spp.; Penicillium spp.; Candida mycoderma, Saccharomyces cerevisiae, Rhodotorula spp.; Corynebacterium spp., Aerobacter spp. and Lactobacillus plantarum. Okafor (1977), reported the involvement of Corynebacterium manihot, Geotrichum candidum, Leuconostoc, Lactobacillus and some yeasts in the production of "gari" from cassava. Similar spectrum of microorganisms have been found to be involved in the fermentation of cassava for "fufu" (Okafor et. al. 1984) and "lafun" (Oyewole and Odunfa, 1988) productions.

Alcoholic beverages vary considerably from society to society. Generally those produced from cereals are referred to as beers while those from fruits are classified as wine. Some other beverages could be produced from blends of fruits, cereals, milk, saps, honey, molasses and or other foods containing fermentable carbohydrates (Pederson, 1979). The fermented alcoholic beverages common in West Africa include palm-wine (Faparusi and Bassir, 1972), cocoa-wine (Bassir 1969, Ojeh, 1981);

"pito" (Ekundayo, 1969); "busa" (Nout 1980); "agadagidi" (Sanni, 1988); kunun - tsamiya (Omakwu 1980), "burukutu" and "sekete" among others. Reports are available on the microbial spectrum involved in the production of most of these alcoholic beverages. A wide spectrum of micro-organisms have been isolated from ripe palm-wine. These include Saccharomyces cerevisiae, Pichia spp., Micrococcus spp., Lactobacillus spp., Sarcinia spp., Aerobacter spp., Corynebacterium spp., Streptoccus spp., Bacillus spp., Leuconostoc spp., Acetobacter spp. and Serratia spp. (Bassir, 1962; 1968; Okafor, 1975). However, Faparusi and Bassir, (1972) implicated Saccharomyces cerevisiae, Candida spp. and Schizo-saccharomyces pombe as being responsible for alcohol production in palm wine. Ekundayo (1969), implicated Geotrichum candidum, Candida spp, and Lactobacillus spp in the conversion of Sorghum into "pito" wine while Bassir (1968), reported that only Saccharomyces cerevisiae is involved in cocoa wine production. In the fermentation of banana to produce a native wine called "agadagidi", Sanni (1988), reported the involvement of Saccharomyces spp., Leuconostoc spp., Streptococcus spp., Bacillus spp. and Micrococcus spp.. Nout (1980) studied the production of a Kenyan alcoholic beverage called "busa" and reported the involvement of Lactobacillus brevis and Saccharomyces cerevisiae in the conversion of soured roasted maize to this drink.

Fermented plant protein foods are common in West Africa. These are usually added to foods as condiments. The common ones include daddawa, made from either the Africa locust bean (Odunfa 1981), or soya beans (Ogbadu and Okagbue 1988); "ogiri" which is produced from melon (Citrulus vulgaris) seeds, (Odunfa 1983) and Ugba, which is made from Pentachlethra macrophylla (Obeta 1982). An important observation on these fermented plant protein foods is the involvemet of Bacillus subtilis strains in the fermentation process (Odunfa and Oyewole, 1986). Other organisms which have been reported include Staphylococcus spp. and Lactobacillus spp.. These fermented legumes are important in the diet of Africans as they are sometimes used as "Meat substitutes" in poor homes (Odunfa 1988).

Unlike other fermented food groups, the variety of fermented animal proteins in Africa is limited. Interests in the fermentation of fish sauces in Africa is very low. Most fermented animal proteins that are available are milk-based. The four common milk-based fermented foods in Africa include yoghurt, kefir, wara and nono. Information is available on the fermentation of yoghurt, kefir and wara. The microorganisms involved in and used for yoghurt production has been identified to include Lactobacillus bulgaricus and Streptococcus thermophilus (Hesseltine, 1965, Hesseltine and Wang (1967); while L.bulgaricus; S.lactis and Saccharomyces cerevisiae have been implicated

in kefir production (Keogh 1978).

2.3. Milk and Milk - Fermented Products

Milk is the first and natural food of the new-born-mammal (Porter 1975). The primitive man who was a food gatherer and hunter became a food producer and domesticator of plants and animal through evolution. At some stage in the course of the human evolution, it was recognised that the milk of some of the domesticated mammals was equally as satisfying in meeting the physiological demands for moisture, energy and nutrient as the human milk (Kroger et. al. 1989). Hence, milk and milk products have always formed a vital component of the diet of human beings and animals even before the dawn of civilization.

There are remarkable pictorial evidences that the cow which is the primary and traditional source of milk has over the ages been used as a beast of burden, object of worship and a source of food by man (Pederson, 1979). For instance, rock drawing in the Libyan desert, believed by Archaeologists to have been made about 9000 BC depict cow worship and the act of milking a cow. Writings, friezes and drawings of the Sumerians of Mesopotamia which go back to about 6000 BC show that milk and milk products were used as food, medicine and sacrificial offerings in the past. There is also evidence that dairying was highly developed among the Summerians and that cows were milked

from behind as some African tribes do today (Pederson, 1979). Evidences in writing and carving in the tomb relics of ancient Egyptians have also shown their interest in dairy as early as 3000 BC. The Egyptians,like the Sumerians, deified the cattle, and their god, Ptah, is revealed as a bull. However, the Egyptians milked their cows from the side. Indians raised dairy cows as early as 2000 BC. These cows, including their urine, were considered holy and the former had a free run of cities and country sides (Pederson 1979). The nomads of Asia-Minor were known to have enjoyed milk and its products, while Greek and Roman records on the use of milk products go back to 1550 BC and 750 BC respectively. The early Romans were even said to have taught cheese-making techniques in the countries they conquered (Keogh, 1978). Milk as a natural diet is mentioned in the Bible (Deuteronomy 32: 14; Genesis 18: 8; Judges 5: 25; 1 Samuel 17: 17).

Other domesticated animals whose milk was used as food apart from cow include buffalo, sheep, goat, horse, camel, yak, zebu, and donkeys (Pederson, 1979, Kroger et al 1989.

Fermented milk preparations, probably discovered accidentally, were the primitive people's way of preserving milk (Porter 1975). The fermentation procedures originated in various areas of the world and differ from area to area, the differences being governed by the environment, habits of the people and the agriculture of

the area to a considerable degree (Pederson 1979).

Nearly every civilization has consumed fermented milk of one type or another. For instance, yoghurt is associated in origin with the peoples of Caucasus and Turkey, leben or leben - raid with the people of Egypt, Syria, Palestine and other areas of the Arabian Penisula; taettemjolk or tettemelk and filbunke, with the Scandinavians; skyr, with the Icelanders; mazun, with the Armenians, cieddu, with the Italians; dahi with the Indians; kefir, and kisselo mleka, with the Balkans; kumias, with the Tartar of Genghi's K'han and Mongolians; and busa, with the natives of Turkestan. The Masai of Kenya also produce fermented milk on which their babies are weaned, and there were also the fermented milk of the Bantus, the Zulus and some other African groups (Pederson, 1979). The procedure of making these milk products was a crude art. There were many "rules of the thumb" methods where ill - defined empirical cultures were used and the outcome of the fermentation had to be accepted. Man, for sometime, did not know what was responsible for the change from raw milk to fermented milk product. Metchnikoff, working in 1907, stated that the Bulgarian bacillus and a "paralactic bacillus" present in "Yahourth" were responsible for "Yahourth's production from milk. His description of the Bulgarian bacillus suggests the organism to be the modern Lactobacillus bulgaricus while the paralactic bacillus is Streptococcus thermophilus of today (Keogh,

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1978). Louis Pasteur was the first man to prove the presence and role of microorganisms in food. He showed in 1837 that the souring of milk was caused by micro - organisms (Jay, 1978). However, the knowledge of the microbiology of fermentation in the last century has led to the production of fermented milk products based on known scientific principles. Milk products have had their quality optimized with the choice microbial cultures and improved conditions of production.

Over eighty fermented milk products are known today (IDF 1984). The amount of milk produced in the world is unkown; so also are the fermented products (kroger et al, 1989). Some of the many common fermented milk products produced scientifically and utilized world-wide include buttermilk, sour cream, cultured buttermilk, kefir, cheese, acidophilus milk and yoghurt. Lactic acid bacteria are basic starter cultures for milk products whether commercially or home produced. These starter cultures may be made up of a single or mixed strains.

Butter, Buttermilk and sour cream are produced from pasteurised cream or milk, inoculated with lactic starter culture and held until a desired amount of acidity is attained. The acidified cream is churned to yield butter which was primarily used for ointment purposes by the beginning of the christian era (Pederson 1979), while the milk that remains after the butter removal gives the butter-milk. Cultured butter milk is obtained commer-

cially when lactic starter is inoculated into skim milk and held until souring occurs, then agitated to give curd which is broken up into fine particles.

Kefir, which originated from the Caucasus mountain, is prepared by inoculating lactic acid (Streptococcus lactis and Lactobacillus bulgaricus) bacteria and yeasts held together by layers of coagulated protein (Kefir grain) into milk to give a product (Kefir) which foams and effervesces like beer (Keogh 1978).

Cheese is the milk curd or coagulum of milk that has been soured by lactic acid bacteria with or without rennet and from which whey portion, of the fermented milk has been removed.

Acidophilus milk is obtained by inoculating intestinal implantable strain of Lactobacillus acidophilus into sterile skim milk and held at 37°C until a smooth curd develops.

Yoghurt has many spelling such as yahourt, yoghourt, yogurt, yeourt, jugurt, yaert and yaoert depending on the language of the area of fermentation. In the Western world, yoghurt is prepared from pasteurised cow's milk inoculated with a mixed culture of Streptococcus thermophilus and Lactobacillus bulgaricus in a 1:1 ratio. In the Middle East and Southern European countries, milk of buffaloes, cows, donkeys, sheep and goats are often used in the local preparation of yoghurt.

2.4. Lactic Acid Bacteria

Gram-positive, catalase negative cocci or rods are considered as lactic acid bacteria.

Some lactic acid bacteria are homofermentative while others are heterofermentative. Some of the homolactics produce acetic and lactic acid when other hexoses apart from glucose are metabolised. All members of the genera Pediococcus and Streptococcus and some Lactobacilli are homofermentative. All members of the genus Leuconostoc and some of the Lactobacilli are heterofermenters. Heterofermentative lactic bacteria produce equal molar amounts of lactic acid, carbon-dioxide and ethanol from hexoses.

Genetic and physiological differences between the homo- and the hetero - fermenting lactic acid results in the *difference in the end product of glucose fermentation*. While the homolactics possess the enzyme aldolase, and hexose isomerase but lack phosphoketolase, thereby using the Embden-Meyerhof - Parnas (EMP) pathway to produce two (2) molecules of lactates per glucose molecule, the heterolactics possess phosphoketolase and lack aldolase and hexose isomerase thereby using the hexose monophosphate or pentose pathway.

The genus Lactobacillus has three sub-genera: Betabacterium, which are mainly heterolactics; Streptobacterium (L. casei and L. plantarum) which produce up to

1.5% of lactic acid with an optimal growth temperature of 30°C and Thermobacteria (L.acidophilus and L. bulgaricus) which can produce up to 3% lactic acid and have an optimal temperature of 40°C (Marth 1974). Lactic acid bacteria, though mesophilic, have some of their members growing at 5°C and some at 45°C. Most can grow at pH range of 4.0 - 4.5 although some can grow at pH 3.2 and some at pH 9.6 They require preformed amino acids, B - vitamins, purines and pyrimidine bases for their growth. They are weakly proteolytic and lipolytic (Stamer, 1976).

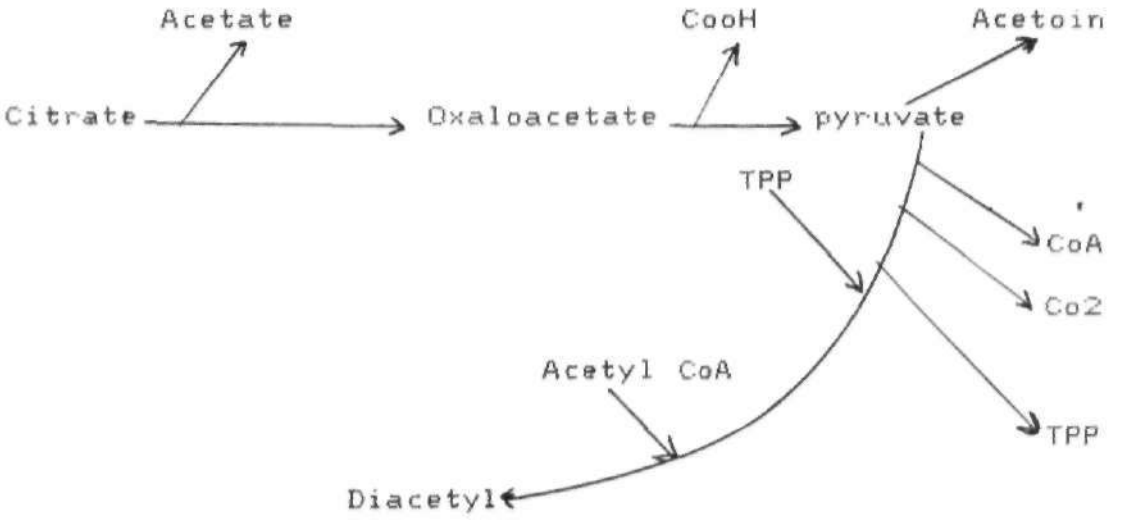
The taxonomy of the lactic acid bacteria were formerly based mainly on gram - reaction, lack of ability to produce enzyme catalase, production of lactic acid of a given configuration and the ability to ferment various carbohydrates. More recently, other properties such as the cell-wall peptidoglycan type, immunologic specificity of enzymes, DNA homology and DNA base composition have come into use.

The cell-wall mucopeptides of bacteria, including the lactics, have been reviewed by Schleifer and Kander (1972), and Williams (1975). While there appears to be a wide variation within most of the lactic acid genera, the homofermentative lactobacilli of the subgenus, Thermobacterium, appears to be the most homogenous, as it has L. - lysine in the peptidoglycan peptide chain and D - aspartic acid as the interbridge peptide. Group N. streptococci have similar wall mucopeptides.

Gasser and Gasser (1971) showed that antisera prepared against NAD - dependant D - lactic dehydrogenases of 3 lactobacilli, reacted against crude extract of almost all other species of lactobacilli containing the enzyme. Extracts of Leuconostoc spp. cross reacted with anti - D - lactic dehydrogenases.

The DNA base composition (expressed as moles % (Guanine + Cytosine) of lactic studied were found to vary from 35 of L. salivarium to 53 of L. fermentum (William 1975). The DNA base composition of heterolactics and homolactics overlap and so it is difficult to group lactics on basis of DNA base composition. Miller et al, (1970, 1971) attempted to group lactics into 3 groups on basis on DNA base composition but found that while group I species were all homofermentators, Group II and III were mixed (homo - and hetero -).

Group N streptococci and Leuconostoc spp have also been observed to produce acetoin and diacetyl from citrate (Jay, 1978) as shown below:



Pyruvate may be produced from lactate and acetyl CoA from Acetate.

2.5. Methods Of Isolation And Enumeration.

Many methods have been designed to isolate and identiofy lactic acid bacteria from fermented milk products.

Streptococcus thermophilus has been isolated from yoghurt by inoculating the fermented milk product on neutral red chalk lactose agar and incubating at 37°C. Other lactic streptococci have been isolated by incubating inoculated red chalk lactose agar at 30°C (Harrigan and McCance, 1976). Lactic acid bacteria have been counted using the modified MRS agar (Hull and Robert 1984).

Enumeration and tests for lactic acid bacteria have been carried out using serial dilution of fermenting samples prepared with 0.1% peptone water. An aliquot of 0.1 ml of the appropriate dilution was pipetted and plated on lactic acid V-8 agar (APHA, 1976) by spreading with a sterile bent glass rod. The plates were incubated anaerobically under a candle jar at 37°C for 48 hours. This provided the low oxygen tension necessary for the growth of lactic organism. This procedure (Lord, 1959) picked and counted green to black colonies with yellow halo as lactics. Enumeration of starter culture organisms in fermented milk products have also been estimated by the pour plate method, using Hansen's yogurt Agar (Porubcan and Sellans, 1973). The plates were incubated at 37°C for 48 to 72 hours after which the colonies were counted.

2.6 The Therapeutic Effects Of Some Fermented Milk Products

Yoghurt has been described as an active bactericidal and protozoicidal milk product (Seneca et al, 1950). It has been used to clear constipation (Ferrer and Boyd, 1955) and has been found to be effective in the treatment of diarrhea caused by a virus or antibiotic treatment. (Niv et al 1963). Acidophilus milks have also been successfully used in the treatment of various gastro-intestinal disorders and skin infections (Gordon et al. 1957 and Silver 1961). These antimicrobial and anti-protozoic properties of these fermented milk products have been attributed to the presence and activities of various species of lactic acid bacteria in the fermented milk products. Several investigators (Mikolajcik and Hamdam, 1975; Shahani et al. 1976, 1977; Pulusani et al. 1979; Rao and Pulusani 1981) have reported that various lactic acid bacteria produce antimicrobial compounds in culture.

Lactobacillus acidophilus cells implanted in the intestines have been observed to control the multiplication of putrefactive organisms which are responsible for intestinal disorders (Ernest, 1984). It was observed to produce substances which suppressed Escherichia coli and pathogenic intestinal bacteria (Lang and Lang, 1973). Pulusani et al (1979), demonstrated that Streptococcus thermophilus ferment skim milk or whole milk to give a product which shows potent antimicrobial activity.

2.7. Preservative Effects Of The Lactics In Foods

Lactic acid bacteria can inhibit growth of food Pathogens and food spoilage microorganisms in some instances (Speck, 1972). This has been demonstrated in country style hams (Karel and Ayres, 1969; Bartholomew and Blumer, 1980a, b) and in numerous dairy products (Goel *et al.* 1971). These lactic acid bacteria presumably produced acids and other metabolites during curing and fermentation processes which inhibited the pathogens. These bacteria have been known to inhibit the spoilage of refrigerated foods. High levels (10^8 - 10^9/g) of *Streptococcus lactis* and *Leuconostoc citrovorum* have been found to retard the growth of Gram-negative bacteria present in ground beef refrigerated at 7°C (Reddy *et al.* 1970). *Streptococcus diacetilactis* has been found to have similar effect in ground beef, milk and cottage cheese (Daly *et al.* 1972) while milk treated with a methanol-acetone extract from Thermophilus milk had its shelf-life increased (Rao and Pulusani, 1981). Mechanically deboned poultry meat had its shelf-life extended with lactic acid bacteria (Raccach *et al.* 1979) while *Lactobacillus bulgaricus* and *Pediococcus cerevisiae* at high levels (10^8 /g) had an inhibitory effect on psychrotrophic flora in refrigerated crab and ground beef.

Daeschel (1989), reviewed some of the inhibitory substances produced by lactic acid bacteria which give

them their preservative properties. These products other than organic acids (lactate and acetate) are antagonistic toward other microorganisms and are produced in much smaller amounts than the organic acids. These include hydrogen-peroxide, diacetyl, bacterocins and secondary reaction products such as hypothiocyanite generated by the action of lactoperoxidase on hydrogen peroxide and thiocyanate and some recently described undefined inhibitory substances such as Microgard and Reuterin. Hydrogen-peroxide is said to be active against Staphylococcus aureus and Pseudomonas spp while Diacetyl (2, 3-butanedione) a metabolic end product of lactic acid bacteria from pyruvate, though known for its buttery aroma it imparts to cultured dairy products, has inhibitory effects on Yeasts, Gram-negative bacteria and non-lactic gram-positive bacteria. Its intense aroma and the relatively large amounts needed to provide preservation has however limited its use as a preservative. Bacterocins which are protein-containing macromolecules which exert a bactericidal mode of action on susceptible bacteria are produced by a large and diverse assortment of bacterial species. colicins, a member of the bacterocins is produced by Escherichia coli, for example. Microgard is a grade 'A skim milk that has been fermented by Propionibacterium shermani and then pasteurised and used as a preservative of cottage cheese. Reuterin is a broad spectrum anti-microbial agent active against yeasts, fungi, protozoa and both gram-positive and gram-negative bacteria. It is

produced by the heterofermentative species of lactic acid bacteria.

Moon et al 1982, tested 43 strains of lactic acid bacteria for inhibitory activity against three strains of Pseudomonas, Vibrio parahaemolyticus and naturally occurring microflora on fresh de-headed shrimp. The degree of inhibitions of lactic acid bacteria against these organisms was found to depend upon the medium used for the cultivation of the lactic acid bacteria prior to testing. Another factor is the presence or absence of lactic acid bacterial cells on the test lawns; filtrates generally are found to be more inhibitory than cell suspensions.

Fermented milk products do not constitute a significant export market world-wide, but it is of considerable economic importance in many countries of the world. In 1907, Metchnikoff published a book entitled "Prolongation of life" where he attributed the good health and the longevity of the Turks and Bulgar to the Bulgarian fermented milk which they consume (Keogh, 1978). Yoghurt was observed to stimulate growth in rats (Hargrove and Alford 1978; Mcdonough et al., 1981). The belief that Yoghurt has special and therapeutic effects (Seneca et al., 1950; Keogh, 1978) and that the lactic acid bacteria present in it produce antimicrobial compounds in culture (Milkolajcik and Hamdan, 1975; shahani et al, 1976; 1977; Pulsani et al, 1979), has led to more attention being paid

to Yoghurt. Hence, in the Western countries since the end of 19th century, increased importance has been attached to their hygienic preparation and to controlling their microbial activity to obtain consistent quality. However, the traditional process of manufacture of milk products has not changed appreciably in Africa and Asia. "Nono" for example is still processed under undefined conditions of temperature and no starter cultures are used.

Chapter 3

MATERIALS AND METHODS

3.1 Collection of Samples

3.1.1 Fully Fermented "Nono"

Three villages (Hanwa, Bomo and Biye) around Zaria town, were chosen for "Nono" samples collection.

Ten samples of fully fermented "nono" (Four samples each from Bomo and Hanwa villages and two from Biye village), were bought from hawkers selceted at random for laboratory analyses. The samples were collected in sterile medical bottles and taken to the laboratory for immediate analyses.

3.1.2 Traditional Processor Monitoring

Five of the above hawkers (two each from both Bomo and Hanwa villages and one from Biye village) were under-studied for their "nono" production methods.

In the traditional preparation of "Nono", milk is obtained from local cow(s) and put into a calabash. The milking procedure is often aided by first allowing the calf to suck the cow in order to stimulate lactation.

The calabash containing the collected milk is then covered with a woven mat (faifai) or another calabash to prevent flies or dirts from settling in and on the milk. The milk is then left at room temperature for about 24 hours to allow for fermentation.

After about 24 hours (depending on the temperature of the environment), the calabash is uncovered and the thin skin on top of the ferment is removed. The thick cream (curd) is broken up and mixed together to give "KINDRIMO". The "kindrimo" is scouped up with the help of wooden spoon into a gourd corked with a dry de-seeded maize cob and shaken vigorously several times for about 30 minutes, removing the cork at intervals, to release the gas formed in the gourd. The mixture is then poured back into the calabash and, using the wooden spoon, the milk butter floating on top of the mixture is collected together, removed and moulded to form the Fulani butter known as "Manshanu". The remaining colloidal mixture in the calabash is known as "Nono", de-fatted fermented milk

A flow-diagram for the traditional preparation of "nono" is given in Fig. 1.

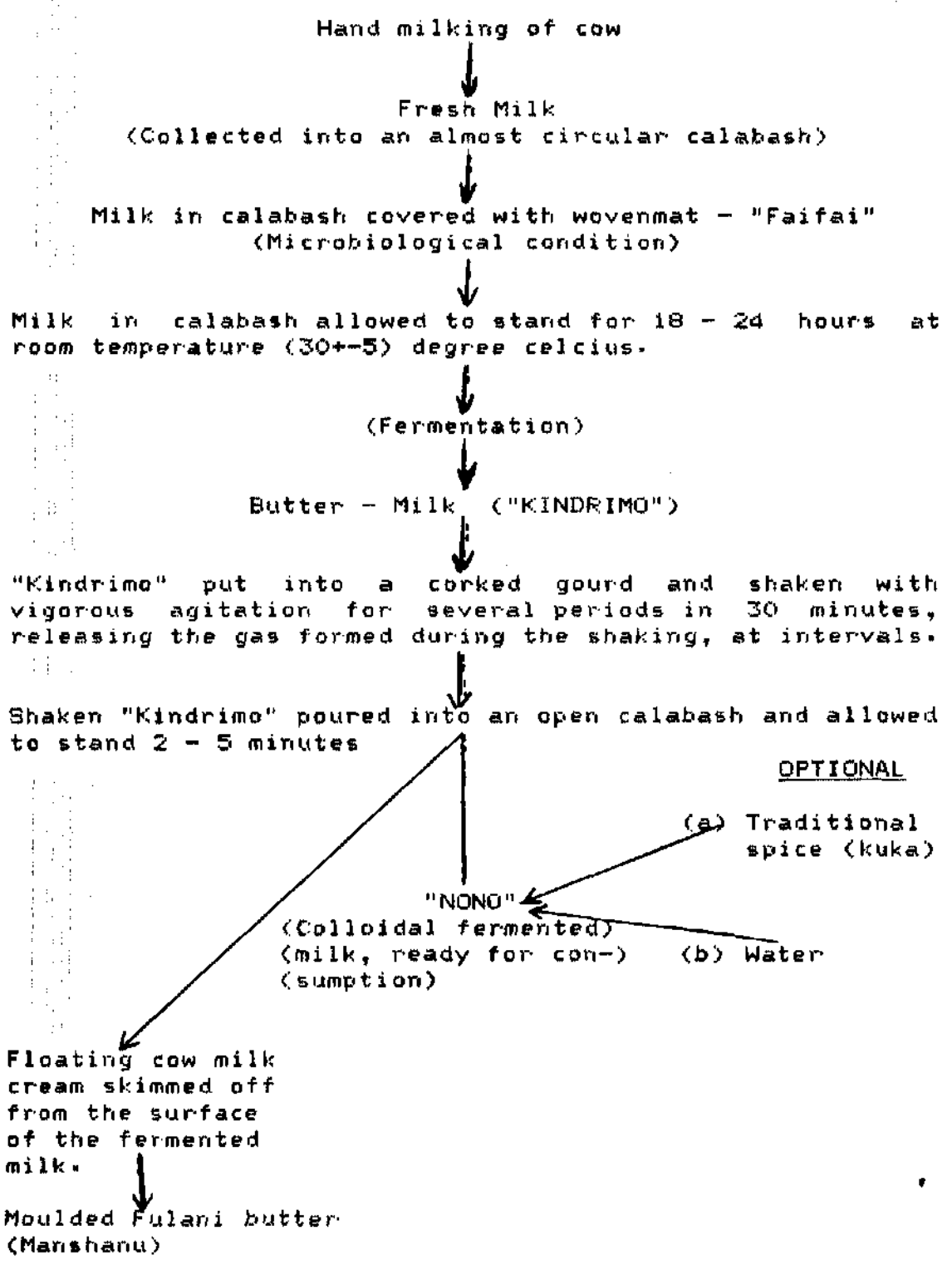


Fig. 1. Traditional preparation of "Nono"

Samples were collected at 6 hour intervals during the various fermentation processes and were subjected to laboratory analyses.

Sampling was repeated ten times.

3.2 Analyses

Samples were subjected to both microbiological and chemical analyses as follows:-

3.2.1 Physico - Chemical Analysis

In fermented dairy products acidity development is important and is usually estimated in terms of pH and Titratable acidity (TTA).

PH: The pH of each sample was taken with a Pye - Unican pH meter model 291 MK 2 supplied with electrode.

Titratable acidity: Ten grams of each sample (fresh milk or "nono") was diluted with 20ml distilled water and 2ml of phenolphtalein was added. The solution was first titrated with 0.1 N NaoH to a persistent pink colour. The amount (%) of lactic acid in the sample was calculated according to AOAC (1984).

X grams fresh milk = Y ml 0.1N NaoH

1 ml 0.1M NaoH = 0.0090 gram lactic acid

% gram lactic acid = Y x 0.0090/X

Diacetyl content: Diacetyl is an important flavour compound in many fermented dairy product. The diacetyl content of each sample was determined by the colorimetric method of Westerfeld (1945). The acetoin content of each sample was first oxidised to diacetyl (since acetoin could not be determined directly because of interfering substances), with a mixture of ferrous sulphate in ferric chloride as follows:-

Ten grams of $FeSO_4 \cdot 7H_2O$ was dissolved in 30ml 50% $FeCl_3$ Two ml of this solution was added to 1ml. of 10N H_2SO_4 and 7ml of sample ("Nono") in a lightly stoppered Universal bottle. The bottle was placed in a boiling water bath for 30 seconds to relieve excessive pressure and then screwed tightly and heated in the boiling water bath for 30 minutes, and then cooled. The content of the bottle was rinsed into a small distillation flask and glass bead was added. The solution was carefully distilled to avoid any trace of iron salt in the distillate by bumping or spraying. Five millilitres of the distillate was used for colour reaction for diacetyl.

Diacetyl was determined with an Evelyns Photo-electric Colorimeter at a wavelength of 530nm. The Colorimeter was standardised as follows:-

A standard solution (approximately 1mg/ml) of diacetyl prepared from the fraction boiling sharply at 88°C was diluted with distilled water to different concen-

trations ranging from 0.01µg/ml, to 30µg/ml. Colour development of the different known concentration of diacetyl and the test solution was achieved by adding 1ml 0.5% creatine solution and 1ml freshly prepared 5% alpha-naphtol to 5ml of each sample in a tube. Five millilitres distilled water was used as blank. Each mixture was allowed to stand for 10 minutes for full colour development. The reagent blank was used to regulate the galvanometer of the colorimeter to 100 while the known different concentrations of diacetyl were read on the colorimeter and the readings used to plot a standard graph. The diacetyl contents of the test solution were extrapolated from the graph (Appendix.III) and recorded.

Samples were collected and analysed in triplicates.

Proximate analysis: In this the total solid, Ash, Protein and Fat contents of each sample was determined by the AOAC (1984) methods.

Total solid: The method of AOAC (1984) was employed. Ten millilitres sample was pipetted into a weighed flat bottom dish. This dish was heated on water - bath for 10 - 15 minutes to expose the maximum surface of the dish bottom to live steam. The dish was then heated in an air oven at 98 - 100°C for about 3 hours. The dish and its contents were dried and cooled in a dessicator to constant weight. The percentage residue formed the total solid.

Ash: The method of AOAC 1984 section 16.035 was employed.

Ten millilitres sample was evaporated to dryness on steam bath in a crucible. The dried sample was ignited in furnace at approximately 550°C until the ash was carbon free (became very white). The crucible and its content was cooled in a dessicator, weighed and the percentage ash was calculated.

Total nitrogen determination: The micro-kjeldahl method of the AOAC (1984) was employed.

Ten millilitres of the sample were transferred to the Kjeldahl digestion flask and 0.7g HgO, 15 gram anhydrous Na_2SO_4 and 25ml H_2SO_4 were added. The mixture was allowed to boil gently for about 2 hours and 200ml water was added and the mixture was cooled to about 25° C. Twenty-five millilitres of 1N Sodium thiosulphate solution was added to the mixture to precipitate mercury. Few granules of zinc was added to the mixture to prevent bumping and 37.5 gram solid NaoH was gently added. The flask was connected to the digest bulb on the condenser with the tip of the condenser immersed in standard acid and 5 - 7 drops indicator in receiver. The flask was rotated to mix the content thoroughly and then heated until all the Ammonia had distilled ($\geq$ 150ml distillate). The receiver was removed, tip of condenser washed and the excess acid in the distillate was titrated with 0.1N NaOH. Correction was made for blank determination of the reagents used by titrating it with 0.1N NaOH. %N = [(ml Standard acid x

Normality acid) − (ml standard NaOH x Normality NaOH)] X 1.4007/gram sample. % Protein = % N x 6.38

Fat determination: Fat was determined by the Rose-Gottliab method of AOAC (1984). Ten ml of samples was pipetted into the fat extracting flask. Two millilitres NH_4OH and 10ml alcohol were added and mixed thoroughly. Twenty-five millilitres of diethyl ether (peroxide free) was added and shaken vigorously for 1 minute. Twenty-five millilitres petroleum ether (boiling range between 30^0 C and 60^0 C) was added and the whole content shaken vigorously for 30 seconds. The flask was centrifuged at about 600 r p m to separate the content and the flask was left to stand until the upper liquid was practically clear. The ether solution was decanted into a weighing flask while the tip and the stopper of the extraction flask were both rinsed with the mixture of equal parts of diethyl ether and petroleum ether into the weighing flask.

The extraction was repeated by adding 15ml diethyl ether and 15ml petroleum ether to the remaining content of the extraction flask. The extract was evaporated on water-bath and dried to constant weight in the oven (102 ± 2)°C. The weighing flask was allowed to cool each time before weighing. After obtaining a constant weight, the fat in the flask was removed completely with 15 - 25ml Petroleum ether and the flask was dried and weighed as before to get the weight of the fat. Loss in wt = wt of fat. Blank determination of the reagents used was

determined to correct the weight of the fat.

Samples were collected and analysed in Triplicates at the Biochemistry laboratory section of the National Animal Production Research Institute (NAPRI) Shika.

Determination of mineral elements: Mineral contents of the samples were determined by the procedures of A.O.A.C. (1984). Thirty millilitres samples were digested using 15ml concentrated nitric acid and 6ml perchloric acid in a Kjeldahl digesting flask. The flask was placed on a water bath at 100°C until the solution in it became colourless. Thirty millilitres concentrated nitric acid was added and the content in the digestion flask was put in a furnace set at about 600°C for 2 hours to ash. The ash was made up to 50ml in a Volumetric flask. Mineral elements of calcium, magnesium, zinc, iron and copper were determined using the Atomic Absorption spectrophotometer while phosphorus was determined using the colorimetric method employing ammonium molybdate while potassium and sodium were determined using the colorimetric method.

3.2.2 Microbiological Studies

Culture media: Media used were Sabouraud Dextrose Agar (SDA) DIFCO containing 30 μg/milli litre chloramphenicol for viable yeast count, deman, Rogosa and sharpe (MRS) Agar (DIFCO) for lactic acid bacteria count and the standard plate count Agar (PCA) DIFCO for total count.

After primary isolation, subsequent purification and storage of the various microorganisms, MRS and PCA continued to be used for lactic acid bacteria while SDA without chloramphenicol was used for yeasts and moulds.

Enumeration of Micro-organisms: For the total bacterial count, serial dilutions from 10^{-1} to 10^{-10} were carried out for each "nono" sample by transferring 1 milli-litre to 9 millilitre sterile physiological saline solution. 0.1ml of suitable dilutions each was plated on six plates of PCA, 6 plates of SDA incorporated with 30Ng/ml chloramphenicol, and 6 plates of MRS Agar. Half of these inoculated plates (three each of PCA, SDA and MRS) were anaerobically incubated while the remaining half were incubated aerobically. These plates were incubated at 37°C for 24 hours and colonies formed per plate were counted and recorded.

Isolation of microoorganisms from "nono": During the enumeration of organisms as described in the last paragraphs, representive colonies were purified on plates of their isolation medium.

Sampling of calabash bowl used for milking for the presence of microorganisms: A milking calabash bowl (which had been washed with water after use the previous day and kept ready for the next day's milking) was sampled for the presence of micro-organisms. Sterile cotton-wool swab dipped in sterile physiological saline was used to

swab the inside of the calabash. The swabs were used to inoculate MRS plates and SDA plates containing 30 µg/ml chloramphenicol. The MRS plates were incubated at 37°C for 24 hours while the SDA plates were incubated at 25°C for 48 hours.

Developing microorganisms were re-isolated for purity and identified.

Characterization of isolates: Isolates were characterized by employing morphological observation and by physiological and biochemical tests. Most of the procedures are described (Lodder and Kreger - Van Rij 1952, Lord 1959, Lodder 1970, Buchanan and Gibbons 1974, Pelczar and Chan 1977, Pederson 1979).

(i) Bacterial isolates: 24 hour old cultures of the bacteria on MRS and PCA plates as appropriate were used for characterization.

Gram - stain was carried out according to the method of Pelczar and Chan (1977). Their motility was also determined using the hanging drop method (Seeley and Vandemark, 1972).

Catalase test: A loopful of the 24 hour old culture (from an appropriate solid medium) of an isolate was added to a drop of hydrogen peroxide on a clean microscope slide in order to test the presence of the enzyme catalase in the culture.

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Gas production indicates that the organism produces the catalase enzyme.

Methyl Red - Voges - Proskaeur test: Oxoid MR - VP (dehydrated medium) was prepared in screw-capped tubes and autoclaved. After autoclaving, two tubes were inoculated with each isolate and incubated at 30°C. After 5 days, 5 drops of methyl red solution (0.2% ethanolic solution) were added to one tube. A positive methyl red test is indicated by a red colour. This shows an acid production with a pH 4.5 or less.

To 1 ml of the culture in the other screw-capped tube, 0.5ml 6% alpha-naphthol and 0.5 ml 16% NaOH were added and the mixture shaken well. Positivity in this VP test is indicated by red colour development within 5 minutes and shows that the organism produced acetylmethyl carbinol from glucose.

Production of gas from glucose: This test is used to differentiate between the homo - and the hetero - fermentative lactic acid bacteria particularly between Leuconostoc and Streptococcus which divide in the same plane. The test helps to detect the carbon-dioxide production from glucose, present in the Gibson's semi-solid medium (Gibson and Abdel - Malek, 1945; Stanier et al. 1964).

The manganese sulphate solution was mixed with the reconstituted skim milk. Yeast extract and glucose were added and the whole mixture was heated in the steamer. Molten Nutrient Agar was added and thoroughly mixed with the mixture while hot, checking and adjusting the pH to 6.5. This semi-solid medium was distributed in test-tubes to a dept of 5-6cm and sterilised by steaming (tyndillization) for 30 minutes on each of 3 succesive days. At the end of the third day, the mixture was cooled, and inoculated by mixing with 0.1ml volume of 18 hour old culture of the isolated MRS broth culture of bacteria. Molten Nutrient Agar at about 50°C was poured into each inoculated tube to give a 2 - 3cm deep layer above the surface of the medium. The agar was left to set and the tubes were incubated at 30°C for 14 days, observing for gas production every 48 hours.

Production of Ammonia form Arginine: This test detects the production of ammonia from arginine by hetero-fermentative lactobacilli. The test distinguishes between hetero-fermentative cocco-bacillary forms of lactobacillus and pediococcus. An Arginine broth medium, composed of MRS broth containing 0.3% (W/V) L-arginine monohydrochloride is used. Each screw-capped tube was filled with 10ml of Arginine broth and sterilized at 121° C for

15 minutes. One-fifth of a millilitre of 24 hour culture in MRS broth was inoculated into the tube which was incubated at 30°C for 14 days. A control experiment was set up with the L. arginine monohydrochloride omitted in the medium. Nesseler's reagent was used as the test reagent for the detection of ammonia in the incubated culture (An orange to brown colour development was accepted as positive while a pale yellow or no colour reaction was accepted as negative).

Digestion of loeffler's serum: Loeffler's serum consisting of 250ml Nutrient broth incorporated with 1% D-glucose and 750ml of sterile serum was prepared. Nutrient broth with 1% glucose was sterilised by steaming at 100°C for 30 minutes, on each of three succesive days. Serum from cow was membrane - filter sterilised and mixed with the sterile Nutrient broth with 1% glucose. This mixture was dispensed aseptically in 7 ml aliquots into bijou bottles which were tightly screwed. The bijou bottles were slopped in an oven and heated slowly to 85°C to coagulate the serum. The medium was sterilised by heating at 85°C for 20 minutes on each of three succesive days.

After the third day of sterilization the serum medium was allowed to cool. 24 hours old culture

of each isolate under test was inoculated onto the serum medium, contained in a bijou bottle, and incubated at 37°C for 14 days. The Bijou bottles were observed daily for liquefaction of the serum.

This test was used to distinguish the coryne-bacterium group from the lactobacilli rods.

Action in litmus milk: A hundred grams powdered skim-milk was dissolved in 1000 ml distilled water. One per cent (1%) of a four per cent (4%) aqueous litmus solution was added to give a pale mauve colour. This was distributed in 10ml aliquots into Universal bottles and sterilised by first passing it through the autoclave at 121° C for 5 minutes and then by steaming for 30 minutes on each of the two following days. Each tube of sterile litmus milk was tested for sterility each time prior to use. One-fifth of a millilitre of 24 hour - old culture was inoculated into each sterile litmus milk and incubated at 37°C for 14 days during which it was observed for change in or loss of colour of the litmus, for clotting of the milk and for gas production.

Citrate fermentation: This test was carried out by inoculating 0.1ml test culture into 0.5ml sterile 10% sodium citrate solution to which 10.5ml sterile reconstituted skim milk has been

added. This citrated milk culture was then poured onto 4ml of molten 2% agar (cooled to 45°C - 50°C) in screw capped tubes. The tube cultures were incubated at 37°C for 24 hours to observe for gas production which is indicative of the fermentation of citrate by the lactic streptococci.

Carbohydrate fermentation: Carbohydrate fermentation pattern of the isolates was studied by API version C kit system. The API 50 CH version C and the API 50 CHL medium No. 5041 version C were used in the study of 49 carbohydrates as substrates.

The API 50 CH version C is a strip which consists of 5 smaller strips (Nos. 1 to 5) each containing 10 numbered tubes with each tube containing a named carbohydrate and its derivatives, (Polyalcools, heterosides, uronic acids), except the first tube of the first smaller strip which contains no carbohydrate and acts as a negative control. The carbohydrate and their derivatives are glycerol, erythritol, D - Arabinose, L-Arabinose, ribose, D-xylose, L-xylose, adonitol, B-Methyl-D-xylosiße, galactose, glucose, fructose, mannose, sorbose, rhamnose, dulcitol, inositol, mannitol, sorbitol, alpha-methyl - D-mannoside, alpha-methyl - D-Glucoside, N - acetyl glucosamine, amygdalin, arbutin, esculin, salicin, celliobiose, maltose,

lactose, melibiose, saccharose/sucrose, trehalose, inulin, melezitose, raffinose, amidon/starch, glycogen, xylitol, gentiobiose, D-turanose, D-lyxose, D-togatose, D-fucose, L-fucose, arabitol, L-arabitol, gluconate, 2-keto gluconate and 5 ketogluconate. The incubation tray (box) of the kit was prepared by filling its honey combed base with about 10mls of water. The strip was then unpacked and placed in the incubation tray which was tilted slightly, ready for the inoculum.

The API 50 CHL medium is a ready-to-use medium that enables the fermentation of the 49 carbohydrates on the API 50 CH strip to be studied. The isolate under test which was usually a Gram-positive cocci or rod, catalase negative and non-sporing, was plated on MRS agar and PCA plates anaerobically at 37°C for 24 hours. This formed the inoculum which was picked with the help of a sterile wire loop into 2ml physiological saline to make a heavy suspension called suspension S. A known number of drops from this heavy inoculum S, (depending on how heavy the inoculum) was transferred to another 5ml physiological saline to form a suspension S2, which had a turbidity equivalent to Mcfarland standard 2 (Kotzekiodu and Roukas 1986). Twice the number of drops of S2 was used to inoculate the API 50 CHL

medium in an ampoule which was aseptically opened. The content of the ampoule was thoroughly mixed and used to inoculate the tubes (not the cupules)or 50 CHL medium. The cupules were overlaid with sterile paraffin oil and incubated aerobically at 37^{o}C for 48 hours taking readings at 24 hours and 48 hours. Bromocresol purple indicator present in the medium changed to yellow to indicate acidification when the carbohydrate substrate was fermented. An exception was the fermentation of Esculin whose fermentation changed the indicator from purple to black. The fermentation results were scored in a semi-quantitative manner, zero was assigned to a negative reaction and 5 to a positive reaction of maximum intensity. Numbers, 1, 2, 3 and 4 were given to the intermediate reactions where numbers 3, 4 and 5 were considered positive. The results were recorded on the result sheets (Appendix.I) to form the biochemical profile of the isolate (microorganism) which was used to type the microorganism on the identification table provided by the manufacturers of the kit. It was used with other profiles for complete identification of the isolate.

Growth of the bacterial isolates in Culture at different temperatures: Yeast Glucose Lemco broth (Naylor and Sharpe 1958) was used as basal medium.

The composition of the broth are as follows: 10 grams of peptone, 10grams of sodium chloride, 5 grams of D-glucose, 3 grams of yeast extract and 15 grams of Agar all of which were dissolved in 1 litre of distilled water.

The ingredients except glucose and agar were dissolved in the distilled water by steaming for 30 minutes to 1 hour. The solution was cooled and adjusted to pH 7.0. The agar was added and boiled to dissolve after which the glucose was added. The mixture was distributed into screw-capped tubes in 10ml aliquot and sterilised by autoclaving at 121°C for 15minutes.

To 10ml of cooled sterile yeast Glucose lemco broth in tubes was added 0.2ml of the 24 hour old pure isolate. Four tubes were inoculated per isolate and incubated one each at the following four temperatures 10°C, 15°C, 40°C and 45°C for growth as a differential test for 7 days. Turbidity of the basal growth medium was used to indicate growth.

Heat resistance at 60 C for 30 minutes: Ten millilitres of 24 hours old MRS broth culture of each isolate in a universal bottle was heated on a waterbath at 60°C for 30 minutes. One-fifth of a millilitre of this heated culture was transferred

to 10ml sterile yeast glucose lemco broth and incubated for growth at 37° C for 7 days. Turbidity of the Yeast Clucose Lemco Broth was used to indicate growth.

Growth in the presence of Sodium Chloride: Two sets of tubes containing 10ml Yeast Clucose Lemco broth were prepared. One set contained 6.5% Nacl and the second set contained 4.0% Nacl (Pederson, 1979). The tubes and their contents were sterilised. Each isolate under test was inoculated into a tube from each set using 0.2ml of 24 hours old pure culture in MRS Broth. The tubes were incubated at 37°C for 7 days and observed for growth.

Growth in alkaline pH: A set of yeast Glucose Lemco broth (basal medium) was adjusted to pH 9.2 and another set adjusted to pH 9.6 using 0.1N NaOH. One-fifth of a millilitre of 24 hour old pure culture of isolate in MRS broth was inoculated into a tube each from each set and incubated at 37°C for 7 days. Turbidity of the basal medium was used to indicate growth.

(ii) Yeast isolates: Identification of yeasts were carried out by means of conventional morphological observations as well as by physiological tests.

Details of the methods are available (Lodder and Kreger - Van Rij 1952, Lodder 1970).

Preparation of slide culture: A Petri-dish containing a filter paper disc at the base on which a U-shaped glass rod carrying two clean miroscope slides was sterilised. Four drops of sterile 20% solution of glycerol was aseptically added to the filter paper to keep the slide culture from drying out during incubation. Molten malt agar was pipetted with sterile Pasteur pipette and aseptically added to the surface of the slides to form a very thin layer of agar medium. This agar medium was streaked with the yeast isolate, covered with a sterile cover slip and incubated at 25°C for 7 days with the Petri-dish cover in place. The slide was examined under the compound microscope (low power) for the colonial and morphological characteristics of the yeast.

To test a culture for pseudomycelia formation, it was inoculated into corn meal agar plate containing 1% Tween 80 and incubated for 24 hours at 25°C. After 24 hours, the plates were uncovered and placed directly under the x10 and x40 objective lenses of the compound microscope to observe the morphological characteristics of the isolates.

Formation of Pellicle in malt extract broth: Malt extract broth composed of malt extract 30.0 grams, mycological peptone 50.0grams and made up to one litre with distilled water was prepared and distributed in 10mls aliquots into universal containers and then sterilised at 121°C for 15 minutes. A sterile wire loop was used to transfer each yeast culture from its SDA plate to a tube of malt extract broth which was then incubated at 25° C for two weeks.

Utilization of ethanol as carbon source: A basal medium (Lodder and Kreger - Van Rij, 1952; Lodder 1970), containing 1 gram of Ammonium sulphate, 1 gram potassium dihydrogen phosphate 0.5 grams of magnesium sulphate hydrated ($MgSo4.7H_2O$), was all dissolved in a litre of distilled water.

The basal medium was distributed in 5ml amount in test-tubes and sterilized at 121°C for 15 minutes. 0.3ml membrane filter sterilized 50% ethanol and one drop of membrane filtered sterilised 50% solution of yeast extract were added to give the ethanol broth. 24 hour-old culture of the yeast isolate under test taken from a malt agar plate was inoculated into a tube of ethanol broth and into another tube of the same medium but with ethanol omitted. This was to avoid false

positive growth since yeast extract was present in the medium. The tubes were incubated at 25°C for 3 weeks and examined at frequent intervals for growth.

Growth on 0.5% Sodium acetate agar: Nutrient Agar containing 0.5% anhydrous sodium acetate formed 0.5 sodium acetate agar whose slants were prepared in universal bottles and inoculated with 24 hour old yeast culture (Pederson, 1979). These slants were incubated at 25°C for 4 weeks and any growth occuring were taken, stained appropriately and then examined for spore.

Ability to grow at 25°C and 37°C: Twenty-four hour old pure cultures of the isolates were inoculated onto Malt Agar plates (2 plates per isolate). One plate was incubated at 25°C and another at 37°C for 2 - 5 days observing every 24 hours for growth.

Ability to grow in 10% Nacl (W/V): Ten percent Sodium chloride Agar was prepared by adding 100 gram Sodium chloride to 10 gram Yeast extract and 20 gram Agar in 1 litre of distilled water. The mixture was boiled to dissolve the materials and autoclaved to sterilise it at 121°C for 15 minutes after which it was cooled and aseptically distributed in about 10 mls portion into sterile plates.

These 10% sodium chloride Agar plates were allowed to cool and solidify and then inoculated with 24 hour old pure cultures of yeasts isolates under test (A plate per isolate). The plates were incubated at 25°C for 7 days for growth.

Carbohydrate fermentation: Nineteen (19) carbohydrates fermentations by each isolate was studied using the API kit consisting of API 20cAUX and API 20c medium. This kit was brought out of the refrigerator (4°C) where it was stored and placed at room temperature 2 hours before use to warm up to room temperature.

A provided incubation tray in the API kit was prepared by filling the honey combed wells at the bottom of the tray with sterile distilled water. The strip containing the different types of carbohydrate in its cupules except the first cupule which contained no substrate and was used as control, was placed in the incubation tray. The carbohydrates are glucose, glycerol, 2-keto -D - gluconate, L - arabinose, D - Xylose, adonitol, xylitol, galactose, inositol, sorbitol, alpha - Methyl - D - glucoside, N - acetyl - D - glucosamine, cellobiose, lactose, maltose, saccharose/sucrose, trehalose, melezitose and raffinose.

The pure yeast cultures of the isolates to be tested were re-isolated on SDA - chlrolamphenicol Agar plates incubated at 30°C for 48 hours to form the inocula. An inoculum was transferred to a sterile 2ml physiological saline (suspension medium) to give a thick suspension, equivalent to a 2 McFarland turbidity as indicated in the API kit manual. Three drops from this suspension was transferred to an aseptically opened API 20c medium and mixed thoroughly using Pasteur pipette. The cupules of the strip in the incubation tray was filled with the suspension in API 20c medium avoiding bubble formation. The tray was covered and incubated at 30°C for 72 hours observing for more turbidity compared to the control, every 24 hours. The reactions obtained were recorded and compared to the identification table to aid the identification of the isolate.

Identification of miroorganisms: Identification of bacteria and yeast isolates using the characteristics determined previously, were made by reference to Bergey's manual of Determinative Bacteriology (Buchanan and Gibbons, 1974); Laboratory methods in food and Diary Microbiology (Harrigan and McCance, 1976) Identification scheme for the lactic acid and bacteria (Sharpe, 1979), The yeast, a taxonomic study (Lodder and kreger-

van Rij 1952) and The Yeasts: taxonomic studies (Lodder, 1970).

Some physiological studies on the predominant micro-flora of "nono": Some physiological aspects of some of the dominant isolates in the fermentation mixture were studied. The microorganisms were Streptococcus cremoris, treptococcus lactis, Streptococcus diacetilactis, Lactobacillus plantarum, Lactobacillus brevis, Lactobacillus cellobiosus, Candida pseudotropicalis and Saccharomyces cerevisiae.

Effect of temperature on growth: The effect of temperature on the growth of the pure isolates was determined. 30ml of MRS broth and malt extract broth were separately dispensed into 100ml conical flasks and sterilised by auto-claving at 121^{0}C for 15 minutes. They were inoculated with their respective pure isolates. The flasks were incubated at 15^{0}C, 20^{0}C, 25^{0}C, 30^{0}C, 35^{0}C and 40^{0}C Growth was assessed every 6 hours for 48 hours by measuring the turbidity at 540nm using a Bauch and Lomb spectrophotometer 20 (Harrigan and McCance, 1976).

Effect of pH on growth: A double strength of MRS broth (for the Lactics) and malt extract broth (for the Yeasts) were prepared and 15ml dispensed

into conical flasks. The McILvaine's citric acid - sodium dihydrogen phosphate buffer series (Hales, 1958), was used. One-tenth of a millilitre citric acid and 0.2ml Na HPO were used to prepare the Acetate buffer solutions. The pH of the broth in each flask was adjusted to the desired pH which ranged from 2 - 6, by adding 15ml of the appropriate buffer (V/V) to each flask. This made the broth media single strength. A few drops of 0.1 N Hcl were added to adjust to the required pH values. The pH of the broth after autoclaving was checked by aseptically taking 5ml of the broth and measuring the pH. The broth in the flasks were then inoculated and incubated at 30°C for 48 hours.

Bacterial growth was determined colorimetrically every 6 hours for 48 hours at 540nm using a Sequois-Turner digital spectro photometer model 390. Uninoculated media was used as blank. The growth of yeasts was assess by the dry cell weight method. The culture flasks were manually shakened to suspend the yeast cells before aseptically withdrawing 10 ml aliquots of the growing yeasts using sterile pipettes. The harvested cells (10 ml) were centrifuged at 800 g for 10 minutes, washed two times with 10 ml distilled water before being dried to constant weight in a pre-weighed

filter paper at 60°c. Dried weight of the cells were calculated and used as an index of growth expressed as milligrams/10 ml culture. 540nm while the growth of yeasts was obtained by the dry weight method.

3.2.3. Pure Culture Studies On "Nono" Fermentation

Milk samples pasteurised using the Short Time High Temperature - STHT pasteurisation (140°C - 142°C/3sec) by the Sona Dairies Nigeria Limited, Otta, were used for the studies. The milk samples were brought to the Laboratory in sealed sachets as marketted by the manufacturers. The samples were plated on PCA and MRS plates to confirm their sterility before being used for fermentation studies.

The lactic acid bacteria used as starter cultures were plated on MRS agar plates and incubated anaerobically at 37°C for 18 hours while the yeasts were plated on SDA plates and incubated at 25°C for 48 hours. By means of an inoculation loop, each culture was harvested into 10mls sterile physiological saline to give an absorbance of 0.05 at 540nm. The suspensions were used as sources of the inoculum of each culture.

The pure microorganisms singly and in different combinations used are as listed in Table I below.

Table I Microorganisms used as starter culture singly and in combination in pure culture studies on "nono" fermentation

Flask No.	Microorganism(s) used as starter culture.
A	Streptococcus cremoris
B	Streptococcus lactis
C	Streptococcus diacetilactis
D	Lactobacillus brevis
E	Saccharomyces cerevisiae
F	Streptococcus cremoris +Lactobacillus brevis
G	Streptococcus diacetilactis + Lactobacillus brevis
H	Streptococcus cremoris + Saccharomyces cerevisiae
I	Streptococcus diacetilactis + Saccharomyces cerevisiae
J	Lactobacillus brevis + Saccharomyces cerevisiae
K	Strept. cremoris + Lacto. brevis + S.cerevisiae
L	Strept. diacetilactis + Lacto. brevis +S.cerevisiae

Use of starter culture: For use of starter cultures, organisms were inoculated singly and in combinations into 99ml of pasteurised milk. Single inoculation consisted of 1ml of suspensions prepared previously. For mixture of two different combinations, 0.5ml of each microorganism was used and 0.33ml of each microorganism was used for a mixture of three different combinations. Also, uninoculated 99ml pasteurised milk was prepared as control. All the 13 flasks (Table1) were incubated at 30°C for 48 hours. Samples were taken every 6 hours and analysed for

pH, titratable acidity, diacetyl content and acceptability.

Effect of temperature: Fermentation of "nono" takes 18 - 36 hours (Makinde 1979), depending on the temperature of the environment. Its fermentation is known to take longer during the harmattan season than during the hot season. The temperature at which "nono" could best be produced with a mixture of Streptococcus diacetilactis, Lactobacillus brevis and Saccharomyces cerevisiae as starter culture in pasteurised milk was examined. Six sterile conical flasks had 99ml Pasteurised milk aseptically transferred into them and 0.33ml each of the organisms forming the starter culture were added. A flask each was incubated for 24 hours at 15°C, 20°C, 25°C, 30°C, and 37° C Samples were collected every 6 hours for pH, titratable acidity, diacetyl content acceptability tests.

Effects of closed and opened systems: "Nono" is produced in partially closed calabashes. The effect of aerobically and anaerobically incubating milk to produce "nono" with a selected starter culture inoculum was examined. Two 500ml bell-jars fixed with taps at their lower ends were sterilised and used for this experiment. One of the jars was filled with 99ml pasteurised milk and 1 ml inocula (0.33ml each of Streptococcus diacetilactis, Lactobacillus brevis and Saccharomyces cerevisiae) and the mouth (located at the top) of the jar was left open. This

formed the open system. The other jar was filled to the brim with 495ml pasteurised milk and 5ml inocula (1.66ml each of Streptococcus diacetilactis, Lactobacillus brevis and Saccharomyces cerevisiae) with a closed top to form the closed system. The jars were incubated at 30° c for 48 hours and samples were collected every 6 hours for pH, titratable acidity, diacetyl content and acceptability tests.

Effect of stationary and agitated fermentation: In the traditional production of "Nono", no agitation is allowed throughout the fermentation period. Industrially however, in the production of yoghurt, agitation is known to make the reaction faster and increases flavour (Pederson 1979). Stationary and agitated fermentations were carried out observing for optimum effect. Two sterile conical flasks were prepared with 99ml of pasteurised milk put into each and 1ml (0.33ml of each of Streptococcus diacetilactis, Lactobacillus brevis and Saccharomyces cerevisiae) added. Two other uninoculated conical flasks containing 99mls pasteurised milk were also prepared as control. One inoculated conical flask and an uninoculated one were incubated at 30°C for 48 hours unagitated while the other inoculated conical flask and its control were put onto the orbital shaker incubator at 30°C for 48 hours and shaken at 30 r.p.m. Samples were taken from the 4 flasks every 6 hours for pH, titratable acidity, diacetyl content and acceptability tests. Acceptability tests were carried

out at the end of 48 hours on the collected samples using sensory evaluatioon. The collected samples were kept in the Deep Freezer prior to acceptability test.

Sensory Evaluation: A panel of eight people who were familiar with "nono" was chosen for the sensory evaluation. Each sample was assesed for colour, odour, taste, viscosity and overall acceptabillity, using a four-point scale (Appendix. II), which was set out for each attribute, on a score sheet attached to the sample. The score per attribute was a mean value of the scores of the eight panelists. For example if two of the eight panelists scored a sample as white brown for colour, four scored for dirty white and two scored the same sample as pure white, total score for the attribute-colour-by the eight panelist is twenty-two. Mean value for the attribute is 2.75 which is approximately 3 and which can be translated as dirty-white. Acceptability of the product was scored in relation to the "nono" processed naturally at 25°C - 30°C.

3.3 Effect of Storage Temperature on Quality of "Nono"

Some baches of fully fermented "nono" were stored at 4°C and 30°C. The batch kept at 30°C was observed for 48 hours with sampling every 12 hours while that stored at 4° C was observed for 12 days with sampling every 3 days. The collected samples were analysed for pH, titratable acidity, diacetyl content and acceptability test and also, the total bacterial, the total lactic acid and the total yeast counts.

3.4. Other Microbiological Studies on "Nono"

Different unknown microbiological and biochemical activities are likely to be involved in the natural fermentation of milk to "nono". It is possible that some microorganisms present in "nono" antagonise themselves while there might be some synergistic reactions between others. Some of the lactic acid bacteria involved in this natural fermentation may possibly be used as probiotics. It is also possible that some of these organisms are phage susceptible. The ecological balance of the microorganisms in producing "nono" similar in aesthetic qualities in most areas of production is unknown. This work has therefore investigated a few of the possible interactions that might be involved between microorganisms in the natural production of "nono" from milk.

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3.4.1. The Inhibition Of Some Organisms By Lactics Isolated From "Nono":

Yoghurt and milk, are said to prolong life (Keogh, 1978). The ways this is brought about is still unknown. Also, these lactics flourish in the presence of some other organisms to ferment milk. It is possible that the starter culture responsible for milk fermentation in Yoghurt (and so forth) production are capable of inhibiting other microorganisms especially those that could be dangerous to human health. The inhibition of Escherichia coli, Staphylococcus aureus, Pseudomonas aeruginosa, Micrococcus sp and Serratia spp obtained from the Department of Microbiology, Ahmadu Bello University, Zaria by the lactics isolated from "nono"was tested and employed the cup well assay technique. Single and mixtures of the lactics were inoculated into pasteurised milk and incubated at 37^{0}C for 24 hours. The fermented milk was then centrifuged at 3000 r.p.m. for 20 minutes and the supernatant solution was seitz filtered into sterile bottle. 15ml Nutrient Agar was inoculated with 0.1ml of the test organisms in Petri-dishes. The agar was allowed to solidify. Six wells of uniform size about 5mm diameter were made on the solidified agar and 0.2ml portion of the lactic culture filtrates were each introduced into a well. The Petri-dishes were incubated at 37^{0}C for 24 hours and examined for zones of inhibition.

3.4.2. Effects Of The Lactic Acid Bacteria Isolated From "nono" On Each Other:

It is necessary to observe the effect of the lactics on the growth of each other inorder to have a better understanding on the fermentation technology of "nono". Broth cultures of the lactic acid bacteria (Streptococcus lactis, Streptococcus cremoris, Streptococcus diacetilactis, Lactobacillus plantarum) were prepared in MRS broth. One-fifth of a millilitre culture of a lactic was dropped on a plate each and spread around and allowed to dry. This plate was divided into sections. One-twentieth of a millilitre of the culture of the lactic to be tested for antibiotic production was dropped on each sector and labelled. The plates were incubated for 48 hours and zones of inhibition measured.

3.4.3 Screening For The Presence Of Bacteriophage In "Nono":

The possibility of the presence of phages in "nono" which could affect the lactics of "nono" was examined. Bacteriophage was enriched for, by first centrifugging "nono" aseptically at 3000 r.p.m. for 20 minutes. 1ml of the supernatant solution was added to 5 ml of 18 hours old MRS broth culture of the lactic to be tested. The broth was incubated at 37°C for 24 hours after which it was centrifuged at 3000 r.p.m for 20 minutes and seitz filtered using 0.45 m millipore filter. Half of a millilitre filtrate was dropped on the lawn culture of the lactic under test in lactic agar plates. These plates

were incubated at 37°C for 48 hours and observed for the presence of plagues.

Chapter 4.

RESULTS

4.1 Traditional Production Of "Nono"

The method used for "Nono" production in the various locations were found to be generally similar. The traditional production methods are illustrated in Fig. 1. Slight differences were observed in the taste, and colour of the products from the various sampling localities.

4.2 Microbial Physico-Chemical Nutritional Analysis of "Nono"

4.2.1 Microbial Count of Market "Nono"

The microbial counts of the "nono" produced by each of the five processors in the three "nono" producing villages are shown in Table 2. The microflora was found to consist predominantly of bacteria ($1.6 \times 10^{9} - 9.0 \times 10^{11}$) and yeasts ($1.5 \times 10^{7} - 1.0 \times 10^{9}$).

TABLE 2. Microbial counts of market "nono" in three villages around Zaria, Nigeria.

Village Name	Processor Code	No. of Sample	Range of Microbial count/ml: Total aerobic Plate count	Lactic acid bacteria count	Yeast counts
Hanwa	H1	10	$4.5 - 4.9 \times 10^{11}$	$3.4 - 4.9 \times 10^{10}$	$9.7 \times 10^{8} - 1.0 \times 10^{9}$
	H2	10	$1.6 - 1.9 \times 10^{11}$	$6.7 - 8.5 \times 10^{9}$	$6.2 - 7.1 \times 10^{8}$
	H3	10	$2.3 - 2.9 \times 10^{11}$	$1.6 - 2.9 \times 10^{10}$	$5.5 - 9.1 \times 10^{8}$
	H4	10	$1.1 - 1.5 \times 10^{11}$	$1.3 - 9.5 \times 10^{10}$	$3.4 - 6.4 \times 10^{8}$
Mean($\bar{x}$)			2.60×10^{11}	3.855×10^{10}	8.8415×10^{8}
Standard Deviation(SD)			1.33×10^{11}	3.73×10^{10}	9.93×10^{8}
Standard Error of Mean SE($\bar{x}$)			2.103×10^{11}	5.90×10^{9}	1.57×10^{8}
Bomo	B1	10	$1.6 \times 10^{9} - 1.6 \times 10^{10}$	$5.3 \times 10^{8} - 7.6 \times 10^{9}$	$1.5 - 1.6 \times 10^{7}$
	B2	10	$3.0 - 3.7 \times 10^{9}$	$1.3 - 1.4 \times 10^{9}$	$6.0 - 8.5 \times 10^{7}$
	B3	10	$2.6 - 2.9 \times 10^{9}$	$5.6 - 7.5 \times 10^{8}$	$4.2 - 4.5 \times 10^{7}$
	B4	10	$2.1 - 3.2 \times 10^{9}$	$1.1 - 2.0 \times 10^{8}$	$1.6 - 1.8 \times 10^{7}$
Mean($\bar{x}$)			4.134×10^{9}	1.314×10^{9}	3.624×10^{7}
Standard Deviation(SD)			3.035×10^{9}	1.806×10^{9}	2.266×10^{7}
Standard Error of Mean SE($\bar{x}$)			4.80×10^{8}	2.86×10^{8}	3.583×10^{8}
Biye	Y1	10	$1.4 \times 4.8 \times 10^{10}$	$1.4 - 1.8 \times 10^{10}$	$1.5 - 5.0 \times 10^{7}$
	Y2	10	$1.2 - 9.0 \times 10^{11}$	$9.8 \times 10^{9} - 1.9 \times 10^{10}$	$9.0 \times 10^{7} - 2.6 \times 10^{8}$
Mean($\bar{x}$)			2.998×10^{11}	1.523×10^{10}	1.032×10^{8}
Standard Deviation (SD)			3.311×10^{11}	2.831×10^{9}	8.643×10^{7}
Standard Error of Mean SE($\bar{x}$)			5.235×10^{10}	4.476×10^{8}	1.367×10^{7}

4.2.2 Physico-Chemical Analyses of "Nono"

Table 3 shows the pH, titratable acidity and the diacetyl contents of the one hundred samples of "nono" purchased from the three villages around Zaria. The pH of the samples fall into the range 4.0 - 4.3. The lactic acid contents of the "nono" samples were also similar (0.54 ± 0.001 to 0.90 ± 0.02 % lactic acid/100 g).

However, there were wide variations among the diacetyl contents of the various samples (1.3 ± 0.01 to 9.4 ± 0.02 µg/ml).

Table 3: pH, TTA and Diacetyl contents of market "nono" from three different villages.

Village Name	Processor Code	No. of Samples	pH	TTA (%. lactic Acid/100g)	Diacetyl (µg/ml)
Hanwa	H1	10	4.1 ± 0.1	0.67 ± 0.01	1.44 ± 0.0
	H2	10	4.2 ± 0.1	0.90 ± 0.02	1.70 ± 0.0
	H3	10	4.4 ± 0.1	0.58 ± 0.01	5.12 ± 0.0
	H4	10	4.3 ± 0.2	0.62 ± 0.02	4.72 ± 0.0
Mean($\bar{x}$)			4.245	0.693	3.245
Standard Deviation (SD)			0.148	0.127	1.705
Standard Error of Mean SE($\bar{x}$)			0.023	0.020	0.270
Bomo	B1	10	4.3 ± 0.1	0.59 ± 0.01	5.40 ± 0.0
	B2	10	4.3 ± 0.1	0.54 ± 0.01	9.40 ± 0.0
	B3	10	4.3 ± 0.1	0.63 ± 0.01	8.00 ± 0.0
	B4	10	4.3 ± 0.2	0.61 ± 0.02	4.20 ± 0.0
Mean($\bar{x}$)			4.30	0.593	6.783
Standard Deviation (SD)			0.093	0.035	2.05
Standard Error of Mean SE($\bar{x}$)			0.015	0.006	0.323
Biye	Y1	10	4.3 ± 0.1	0.75 ± 0.01	1.30 ± 0.0
	Y2	10	4.3 ± 0.1	0.64 ± 0.01	3:30 ± 0.0
Mean($\bar{x}$)			4.3	0.695	2.305
Standard Deviation (SD)			0.065	0.057	1.024
Standard Error of Mean SE($\bar{x}$)			0.015	0.013	0.229

Proximate and mineral elements contents of the prepared "nono" samples produced by the ten processors in the three different villages are shown in Table 4.

TABLE 4 Proximate and Mineral Elements Analysis of Market "Nono" Purchased from three Villages (Wet Weight).

Village name	Processor code	Total Nitrogen (%)	Total solid (%)	Fat (%)	Ash (%)	Crude Protein (%)	Ca	Mg	Zn	Fe	K	Na	Cu	P
							(			mg/100ml				)
Hanwa	H1	0.34	5.87	0.8	0.41	2.17	14.5	2.4	0.3	0.0	26.0	12.0	0	15.8
	H2	0.62	10.68	1.5	0.72	3.96	26.0	6.2	0.5	0.9	55.0	20.0	0	44.6
	H3	0.42	6.22	0.4	0.39	2.68	28.9	4.0	0.5	0.5	38.0	14.0	0	28.8
	H4	0.26	4.00	0.3	0.26	1.66	18.5	2.9	0.4	0.1	26.0	12.0	0	18.9
Mean($\bar{x}$)		0.41	6.65	0.74	0.45	2.61	21.97	3.88	0.41	0.38	36.3	14.5	0	27.0
Standard Deviation(SD)		0.17	2.90	0.53	0.20	1.09	6.40	1.74	0.14	0.37	13.5	4.42	0	12.3
Standard Error of Mean SE($\bar{x}$)		0.27	0.46	0.08	0.03	0.17	1.01	0.27	0.02	0.06	2.14	0.70	0	1.9
Bomo	B1	0.24	3.31	0.4	0.26	1.53	19.0	2.9	0.4	0.3	24.0	11.6	0	15.9
	B2	0.39	4.79	0.8	0.36	2.49	19.7	3.0	0.4	0.9	25.0	12.4	0	22.7
	B3	0.34	4.83	0.2	0.33	2.17	25.5	3.5	0.5	0.5	32.0	12.0	0	24.9
	B4	0.31	5.33	2.0	0.27	1.98	17.7	2.5	0.4	0.4	20.0	8.0	0	19.7
Mean($\bar{x}$)		0.32	5.39	0.9	0.31	2.05	20.5	3.0	0.43	0.53	25.5	11.0	0	20.9
Standard Deviation (SD)		0.10	5.12	0.8	0.08	0.71	4.0	0.7	0.12	0.26	6.5	2.4	0	3.8
Standard Error of Mean SE($\bar{x}$)		0.15	0.81	0.1	0.01	0.11	0.63	0.11	0.02	0.04	1.0	0.4	0	0.6
Biye	Y1	0.09	12.98	1.6	0.88	5.68	27.5	6.3	0.7	0.5	59.0	24.0	0	16.3
	Y2	0.18	2.83	0.3	0.22	1.15	13.5	2.5	0.3	0.4	24.0	8.0	0	15.8
Mean($\bar{x}$)		0.14	12.60	1.0	0.91	3.41	20.5	4.4	0.5	0.5	41.5	16.0	0	16.0
Standard Deviation (SD)		0.06	22.2	0.7	1.71	2.54	7.6	2.1	0.2	0.1	18.5	8.5	0	3.0
Standard Error of Mean SE($\bar{x}$)		0.01	3.50	0.1	0.27	0.40	1.2	0.3	0.04	0.02	2.3	1.3	0	0.5

There are wide variations in the proximate and the mineral elements contents of the samples. The total nitrogen (Wet Weight) ranged from 0.09% from a processor at Biye Village to 0.62% from a processor at Hanwa Village. The total solid, Fat, Ash, and Crude protein ranged from (2.83-12.98%); (0.2-1.6%); (0.22-0.88%); and (1.15-5.68%) respectively. The mineral elements examined and estimated as mg/100ml showed Potassium (K) as having the highest value of (20-59) mg/100ml, followed by phosphorus (15.8-44.6) mg/100ml; calcium (13.5-28)mg/100ml, and sodium (8-24)mg/100ml, in "nono". Iron and zinc though present were low in value. While iron content ranged between (0-09)mg/100ml, zinc content was between (0.3-0.7)mg/100ml. None of the "nono" samples contained traceable copper.

4.2.3 Bacteria Isolated from Market "Nono"

Table 5 shows the morphological and biochemical characteristics of the bacteria isolated from purchased market "nono" samples. The bacterial flora was found to consist of a large number of lactic acid bacteria and Bacillus species. The bacteria include Streptococcus lactis, Streptococcus cremoris, Streptococcus diacetilactic, Lactobacillus plantarum, Lactobacillus brevis, Lactobacillus cellobiosus and Bacillus spp.

TABLE 5: Results of the morphological, biochemical and physiological tests on bacteria isolated from market "nono"

ISOLATES																							
	H_{11}	H_{21}	H_{31}	H_{12}	H_{22}	H_{32}	H_{13}	H_{23}	H_{33}	H_{14}	H_{24}	H_{34}	H_{15}	H_{25}	H_{35}	H_{16}	H_{26}	H_{36}	H_{17}	H_{27}	H_{37}
	H_{41}	B_{11}	B_{21}	H_{42}	B_{12}	B_{22}	H_{43}	B_{13}	B_{23}	H_{44}	B_{14}	B_{24}	H_{45}	B_{15}	B_{25}	H_{46}	B_{16}	B_{26}	H_{47}	B_{17}	B_{27}
	B_{31}	B_{41}		B_{32}	B_{42}		B_{33}	B_{43}		B_{34}	B_{44}		B_{35}	B_{45}		B_{36}	B_{46}		B_{37}	B_{47}	
	Y_{11}	Y_{21}		Y_{12}	Y_{22}		Y_{13}	Y_{23}		Y_{14}	Y_{24}		Y_{15}	Y_{25}		Y_{16}	Y_{26}		Y_{17}	Y_{27}	
Identified Micro-organisms	S. lactis			S. cremoris			S. diaceti-lactis			L. plantarum			L. brevis			L. cellobiosus			Bacillus spp				

KEY: Isolate codes:

H_{11} -H_{17} ; H_{21} -H_{27}) = Isolate from processors H1, H2, H3, H4 from "Hanwa" Village respectively.
H_{31} -H_{37} ; H_{41} -H_{47})

B_{11} -B_{17} ; B_{21} -B_{27}) = Isoiate from processors B1, B2, B3, B4 from "Bomo" Village respectively.
B_{31} -B_{37} ; B_{41} -B_{47})

Y_{11} -Y_{17} ; Y_{21} -Y_{27} ; = Isolate from processors Y1 and Y2 from "Biye" Village respectively.

See Appendix iv. for details of the identification.

4.2.4 Yeasts Isolated From Market "Nono"

Table 6 shows the characteristics of yeasts isolates obtained from "nono". These include Candida pseudotropicalis, Candida tropicalis, Endomyces spp, Trichosporon beigelii and Saccharomyces cerevisiae

TABLE 6 Results of)the morphological and biochemical tests on Yeast Isolates from market "nono"

Isolates	H_{101}	H_{201}	H_{102}	H_{202}	H_{103}	H_{203}	H_{104}	H_{204}	H_{105}	H_{205}
	H_{301}	H_{401}	H_{302}	H_{402}	H_{303}	H_{403}	H_{304}	H_{404}	H_{305}	H_{405}
	B_{101}	B_{201}	B_{102}	B_{202}	B_{103}	B_{203}	B_{104}	B_{204}	B_{105}	B_{205}
	B_{301}	B_{401}	B_{302}	B_{402}	B_{303}	B_{403}	B_{304}	B_{404}	B_{305}	B_{405}
	Y_{101}	Y_{201}	Y_{102}	Y_{202}	Y_{103}	Y_{203}	Y_{104}	Y_{204}	Y_{105}	Y_{205}

Identified microorganisms	Candida pseudotro-picalis	Candida tropicalis	Endomyces spp	Saccharo-myces cerevisae	Tricho-sporon beigelii

KEY: ISOLATES CODES

H_{101} - H_{105} ; H_{201} -H_{205} ; H_{301} -H_{305} ; H_{401} -H_{405} = Isolates from processors H_1, H_2, H_3, and H_4 from "Hanwa" Village respectively.

B_{101} - B_{105} ; B_{201} -B_{205} ; B_{301} -B_{305} ; B_{401} -B_{405} = Yeast isolates from processors B_1, B_2, B_3, B_4, from "Bomo" village respectively.

Y_{101} -Y_{105} ; Y_{201} -Y_{205} = Yeast isolates from processors Y_1 and Y_2 from "Biye" village respectively.

See Appendix v. for detailed identification.

4.3 Microbial and Physico Chemical Changes During The Traditional Preparation Of "Nono"

The results of the investigations carried out on the traditional fermentation of "nono" from five different processors, in the three different villages around Zaria, Nigeria, and from ten processing batches for each processor, are as follows:

4.3.1 Traditional Fermentation At "Hanwa" Village

Figure 2 shows changes in the pH and titratable acidity (TTA) of the fermenting milk during "nono" production by the two processors in "Hanwa" village. The changes in pH and TTA of the fermenting milk by the two processors were similar in pattern. However, slight differences were observed in pH (±0.2) and TTA (±0.12) at some points in the fermentation. Generally, the pH of the fermenting milk decreased from 6.8 ± 0.2 to 4.4 ± 0.1 during the 24 hours fermenting period. Correspondingly, the TTA of the fermenting milk increased from 0.8 ± 0.02% to 1.28 ± 0.02% during the same period.

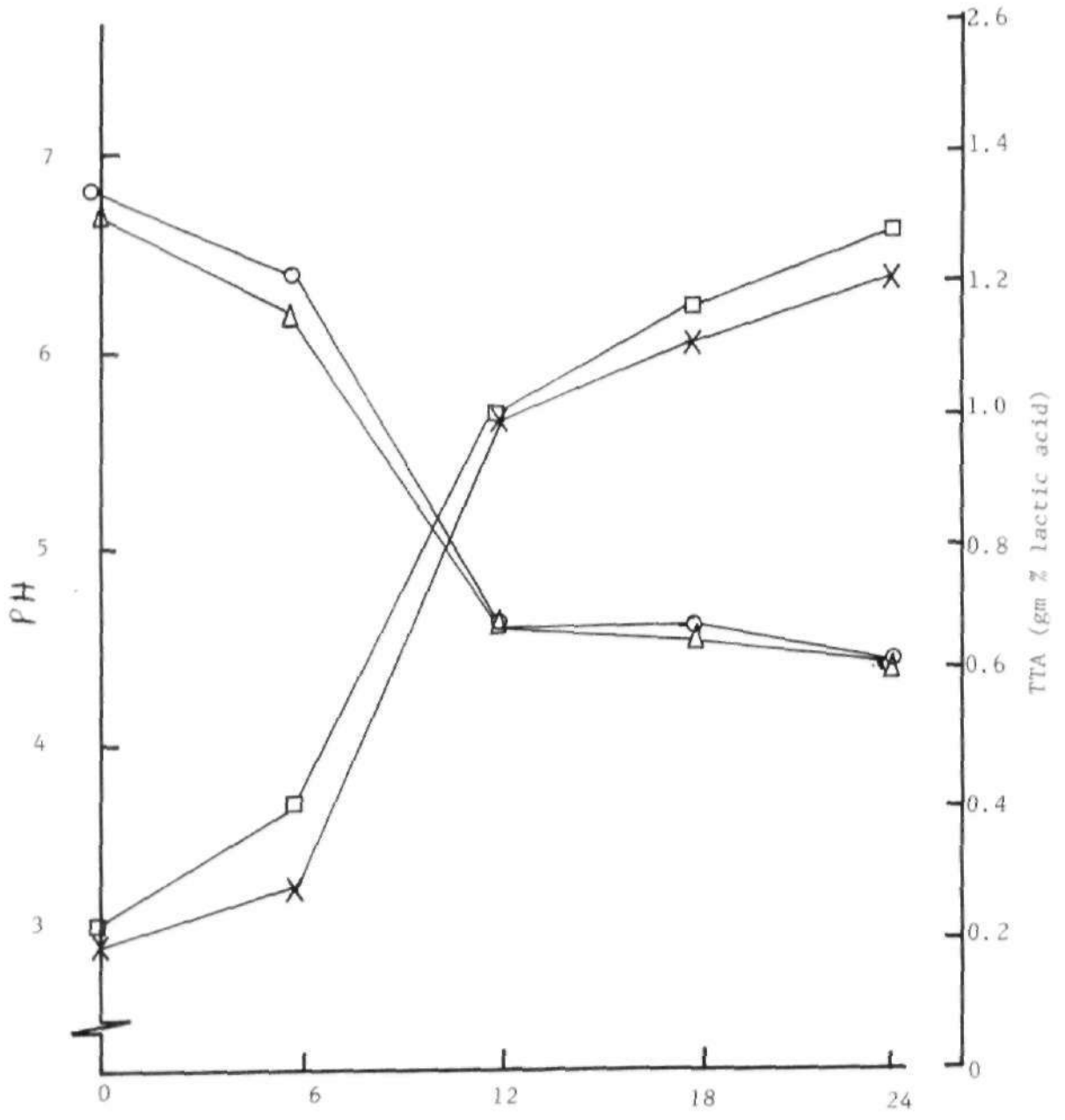


FIGURE 2: Changes in pH and TTA during traditional preparation of "nono" by two processors in "Hanwa" Village

The changes in the microbial contents of "nono" prepared by each of the two processors in "Hanwa" village are shown in Figures 3 and 4. Similar patterns of increase were found in the microbial counts of "nono" produced by both processors. Total aerobic bacterial counts were highest throughout the fermentation, followed by the lactic acid bacteria count. Though, the yeasts were found in relatively low numbers (they persisted throughout the fermentation.

The diacetyl contents also increased appreciably from zero at the beginning of the fermentation to 3.6µg/ml at 24 hours of fermentation (Figure 5).

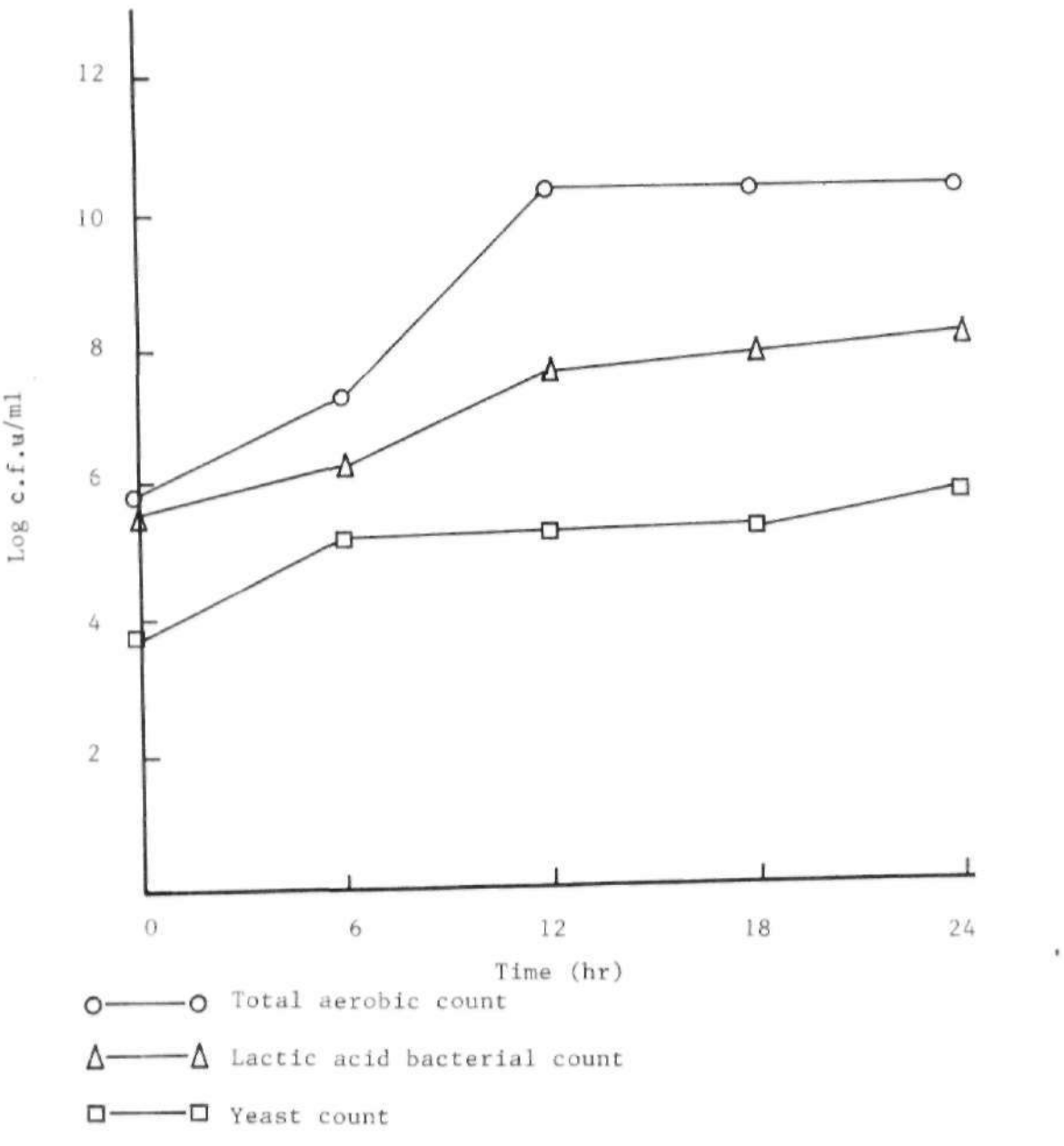


FIGURE 3: Microbial changes during traditional preparation of "nono" by processor H_1 in "Hanwa" village

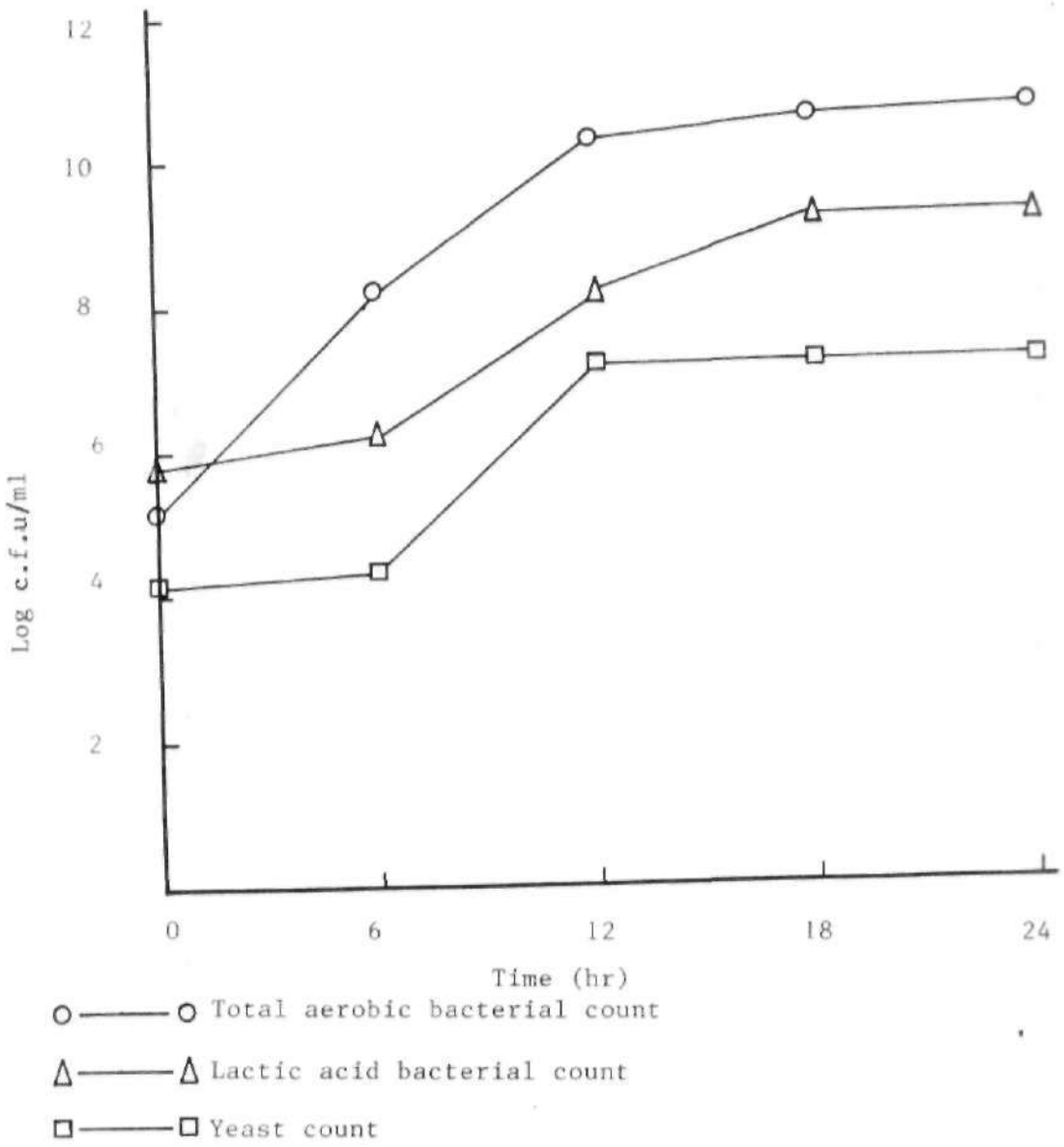


FIGURE 4: Microbial changes during traditional preparation of "nono" by processor H_2 in "Hanwa" Village

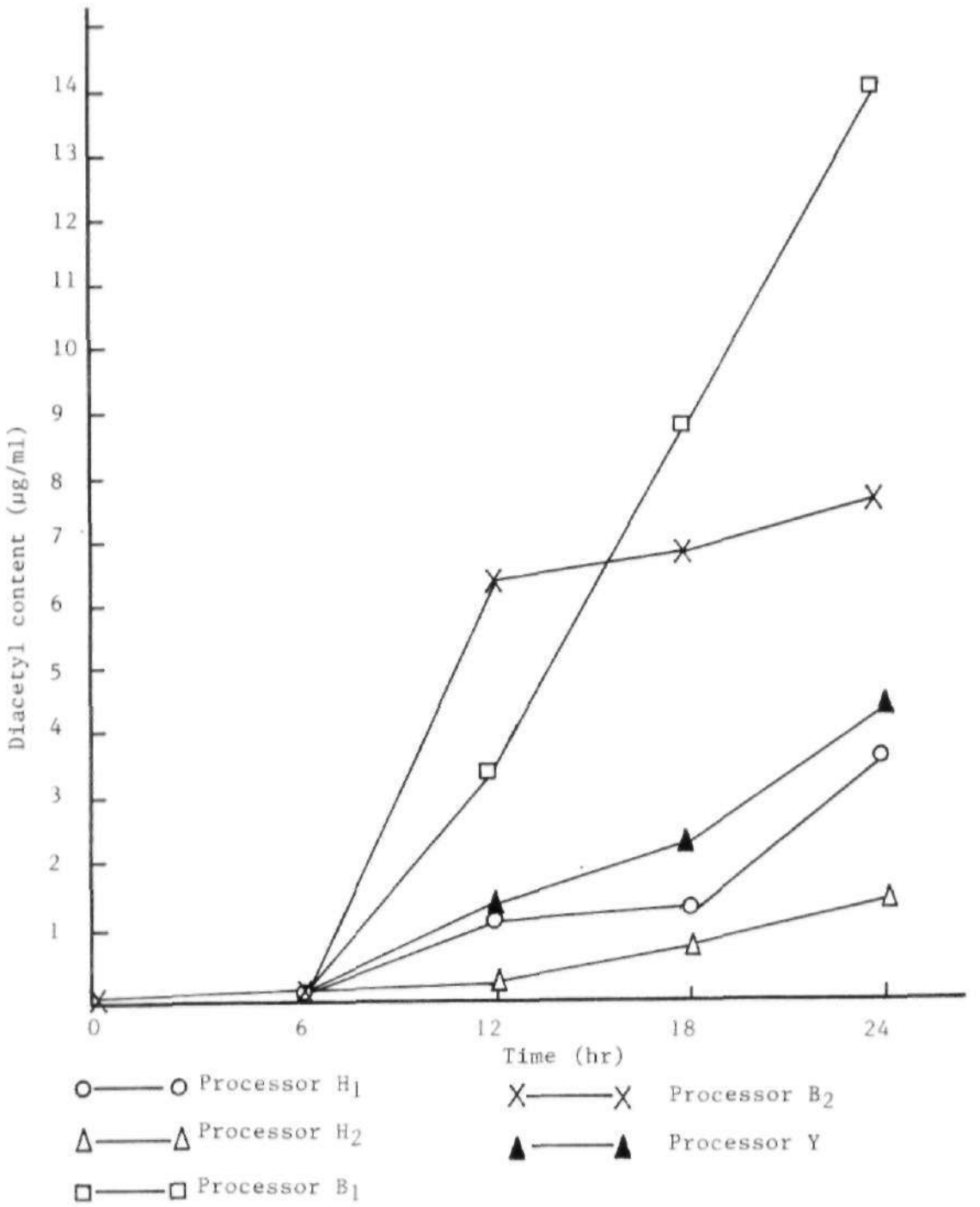


FIGURE 5: Diacetyl content (µg/ml) of traditionally processed "nono" by processors from the three different villages

4.3.2 Traditional Fermentation At "Bomo" Village

Figure 6 shows the changes in the pH and TTA of the fermenting milk during the production of "nono" by two processors in "Bomo" village. Little differences were observed in the production of the two processors. The pH of the fermenting milk decreased from 6.90 ± 0.20 at the beginning to 4.3 ± 0.05 during the 24 hours fermentation period. Moreover, acid production increased from 0.18 ± 0.1% lactic acid to 1.02 ± 0.01% lactic acid during the same period.

Figures 7 and 8 show changes in the microbial counts (1.4×10^{4}) to (2.8×10^{12}) of the fermenting milk during processing by the two processors of "Bomo" village. The pattern of increase of the microorganisms during the fermentation were as observed in the case of "Hanwa" village.

Figure 5 shows the changes in the diacetyl content (0 - 14.0μg/ml.) of milk during fermentation at "Bomo" village by the two processors. There was increase in the diacetyl content of the milk as fermentation progressed. There was an appreciable difference between the diacetyl content of "nono" produced at "Hanwa" and "Bomo" villages.

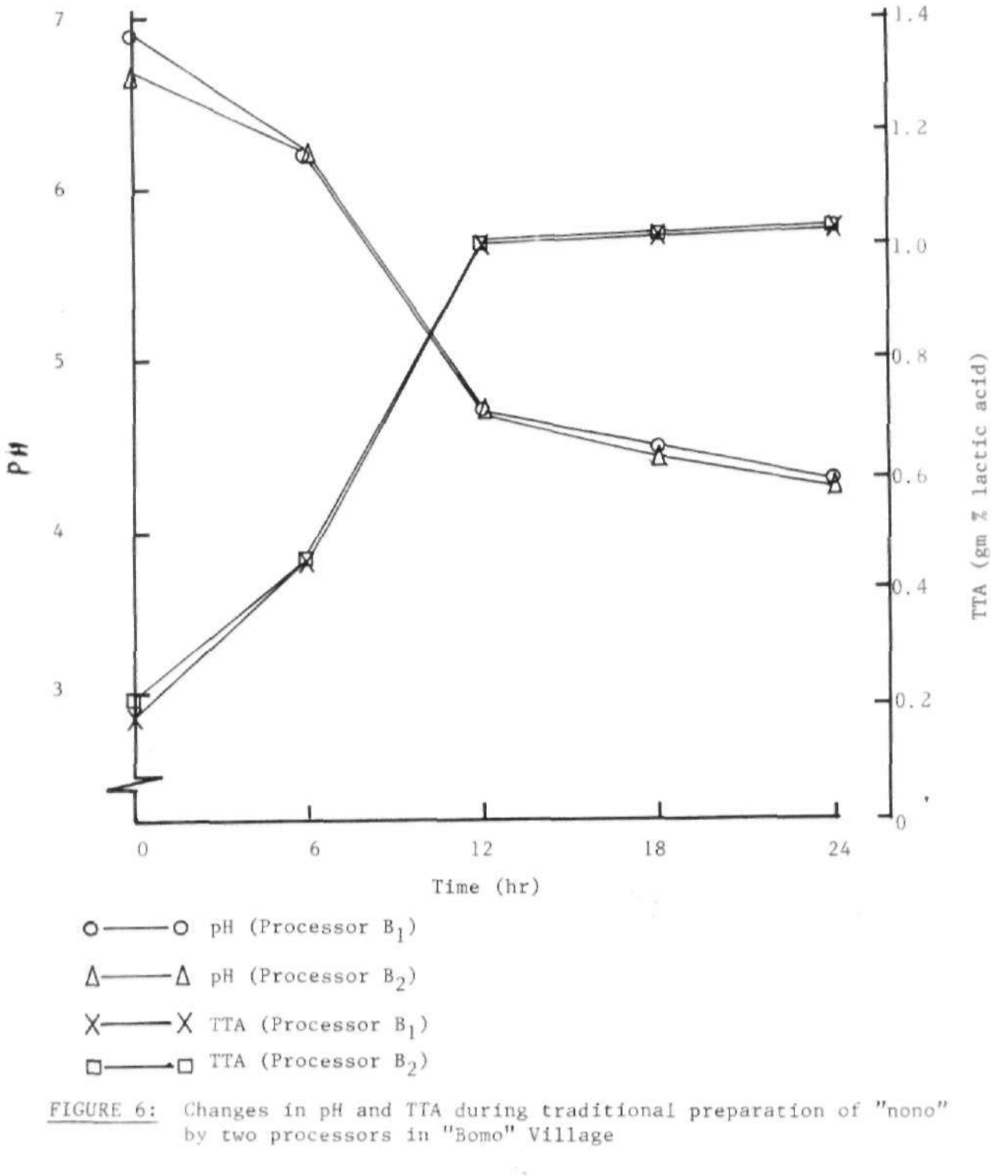


FIGURE 6: Changes in pH and TTA during traditional preparation of "nono" by two processors in "Bomo" Village

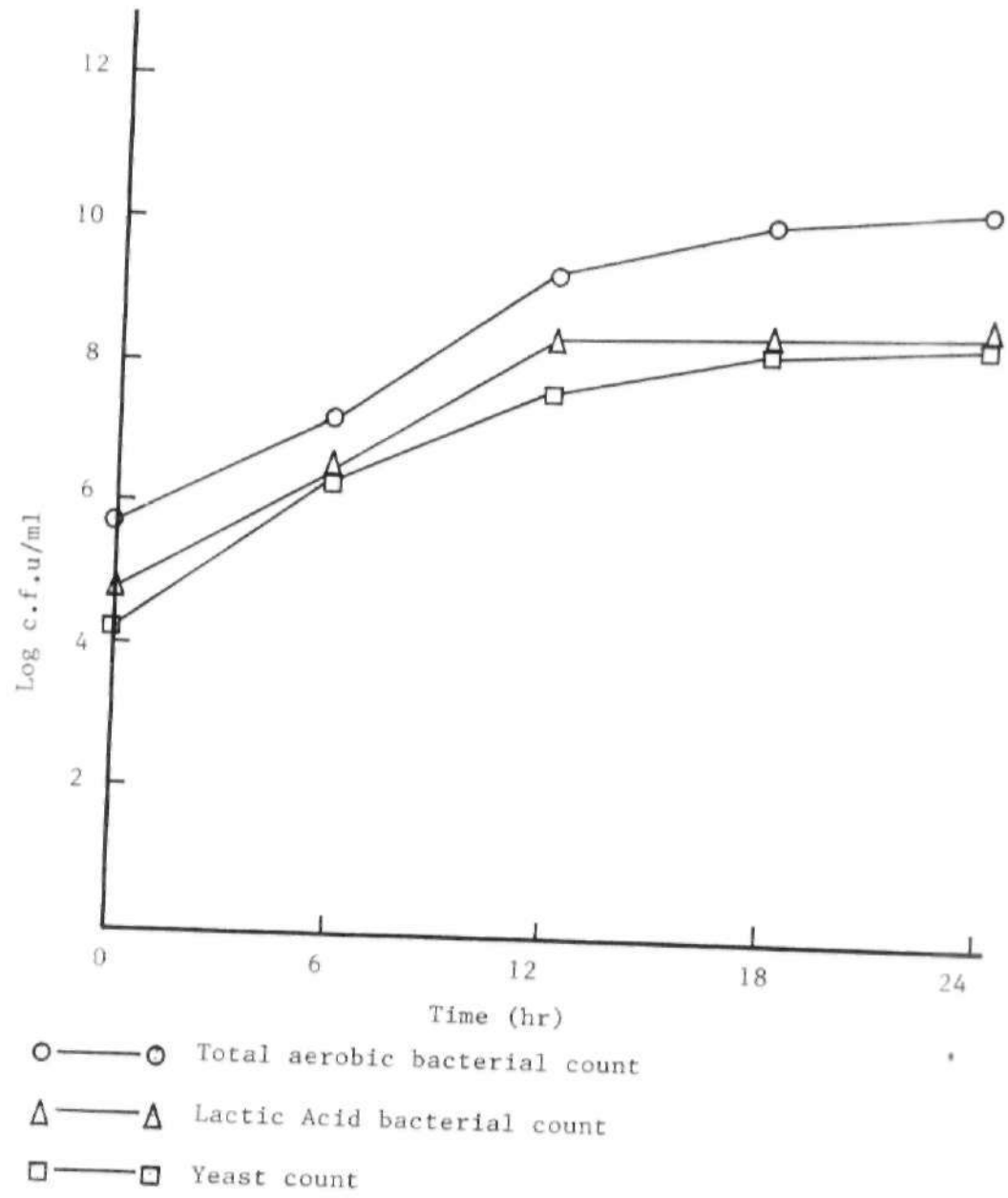


FIGURE 7: Microbial changes during traditional preparation of "nono" by processor B_1 in "Bomo" Village

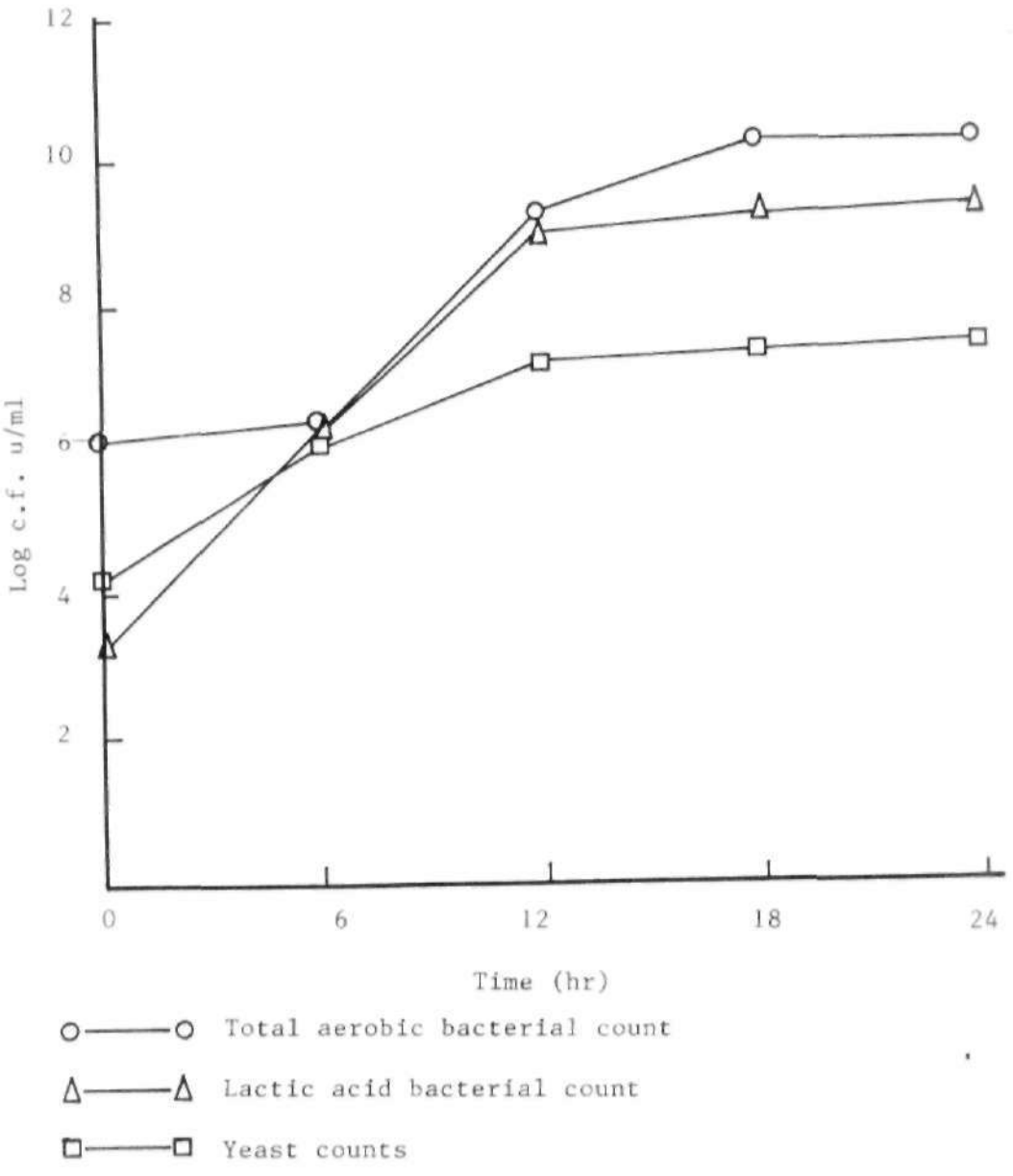


FIGURE 8: Microbial changes during traditional preparation of "nono" by processor B_2 in "Bomo" Village

4.3.3 Traditional Fermentation At "Biye" Village

Figure 9 shows the changes in the pH and the TTA of the fermenting milk during the production of "nono" at Biye village. The pH of the fermenting milk decreased from 6.8 ± 0.1 at the initial stage of fermentation to 4.4 ± 0.05 at the end of the 24 hours fermentation. TTA also correspondingly increased during the same period.

Figure 10 shows the changes in the microbial load of the fermenting milk during "nono" production at "Biye" village (1.4×10^{4}) - (9.0×10^{10}). The pattern of increase of the microbial load was similar to those of "nono" processed at Hanwa and Bomo villages.

Figure 5 shows the diacetyl content of "nono" during processing at "Biye" village. The diacetyl content (0 - 5.65µg/ml.) increased with the period of fermentation and it ranged quantitatively to those of "Hanwa" village.

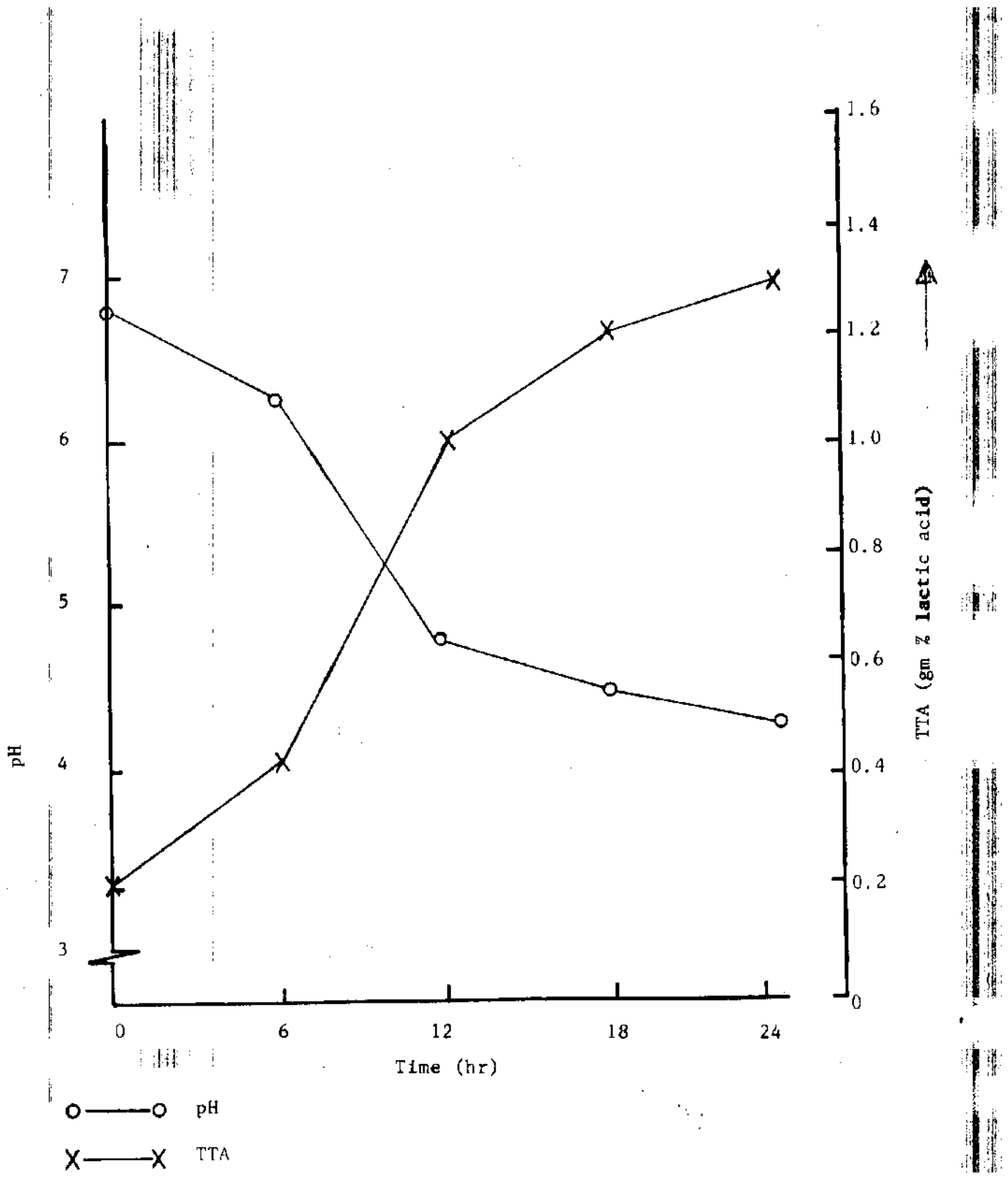


FIGURE 9: Changes in pH and TTA during traditional preparation of "nono" by a processor in "Biye" Village

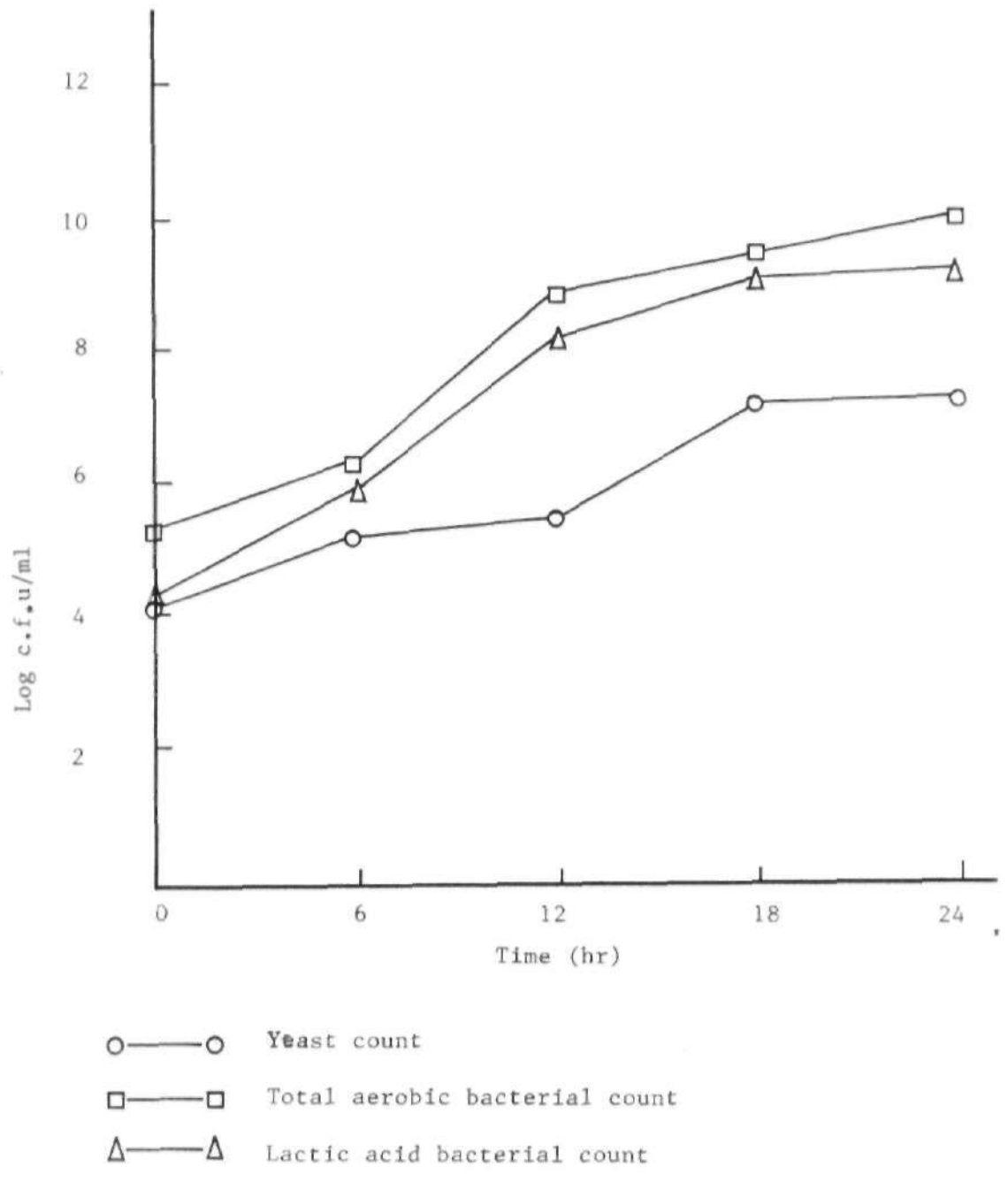


FIGURE 10: Microbial changes during traditional preparation of "nono" by processor Y in "Biye" Village

4.3.4 Microorganisms Isolated From Fermenting Milk During "Nono" Production

Tables 5-16 show the cultural, morphological and biochemical characteristics of major microorganisms isolated at different periods of the fermentation process. The spectrum of the microorganisms isolated include Bacillus spp., Lactobacillus plantarum, Lactobacillus brevis, Lactobacillus cellobiosus, Streptococcus lactis, Streptococcus cremoris, Streptococcus diacetilactis and some yeasts identified as Saccharomyces cerevisiae, Endomyces spp., Candida pseudotropicalis, Candida tropicalis and Trichosporon beigelii.

Morphological observation of colonies developing on isolation media showed that Streptococcus species were the predominant lactic acid bacteria at the initial stage of the fermentation. Lactobacillus species were not really observed before the 12 hours of fermentation. Similar morphological observation showed that the yeasts which were low in number at the beginning of the fermentation increased appreciably at the end of fermentation. In general, the isolated microorganisms resembled those that were found in the market "nono".

TABLE 7 Results of Cultural, morphological and Biochemical characteristics of bacteria isolated from fermenting milk at Zero hours of fermentation in the production of "nono"

ISOLATES				
	H_{111} H_{211} H_{311}	H_{112} H_{212} H_{312}	H_{113} H_{213} H_{313}	H_{114} H_{214} H_{314}
	H_{411} B_{111} B_{211}	H_{412} B_{112} B_{212}	H_{413} B_{113} B_{213}	H_{414} B_{114} B_{214}
	B_{311} B_{411}	B_{312} B_{412}	B_{313} B_{413}	B_{314} B_{414}
	Y_{111} Y_{211}	Y_{112} Y_{212}	Y_{113} Y_{213}	Y_{114} Y_{214}

Identified bacteria	Strept. lactis	Strept. cremoris	Strept. diacetilactis	Bacillus sp

KEY

H_{111} - H_{114} ; H_{211} - H_{214} ; H_{311} - H_{314} ; H_{411} - H_{414} = Isolates from processo H_1, H_2, H_3 H_4, from "Hanwa" Village respectively.

B_{111} - B_{114} -; B_{211} - B_{214} ; B_{311} - B_{314} ; B_{411} - B_{414} = Isolate from processo B_1, B_2, B_3, and B_4 from "Bomo" Village respectively.

Y_{111} - Y_{114} ; Y_{211} - Y_{214} = Isolate from processors Y_1, and Y_2 from "Biy Village respectively.

See Appendix vi. for detailed identification.

TABLE 8: Results of the Morphological and Biochemical tests of Yeasts isolated from fermenting milk at zero hour of fermentation in production of "nono"

ISOLATES									
	H_{117}	H_{217}	H_{317}	H_{118}	H_{218}	H_{318}	H_{119}	H_{219}	H_{319}
	H_{417}	B_{117}	B_{217}	H_{418}	B_{118}	B_{218}	H_{419}	B_{119}	B_{219}
	B_{317}	B_{417}		B_{318}	B_{418}		B_{319}	B_{419}	
	Y_{117}	Y_{217}		Y_{118}	Y_{218}		Y_{119}	Y_{219}	

Identified Yeast	Candida pseudo-tropicalis	Candida tropicalis	Saccharomyces cerevisiae

KEY

$H_{117} - H_{119}$; $H_{217} - H_{219}$; $H_{317} - H_{319}$; $H_{417} - H_{419}$ = Isolates from processors H_1, H_2, H_3, H_4 from "Hanwa" Village respectively.

$B_{117} - B_{119}$; $B_{217} - B_{219}$, $B_{317} - B_{319}$ $B_{417} - B_{419}$ = Isolates from processors B_1, B_2, B_3, and B_4 from "Bomo" Village respectively.

$Y_{117} - Y_{119}$; $Y_{217} - Y_{219}$ = Isolate from processors Y_1, and Y_2 from "Biye" Village respectively.

See Appendix vii. for detailed identification.

TABLE 9 Results of Cultural, morphological and Biochemical characteristics of bacteria isolated from fermenting milk at 6 hours of fermentation in the production of "nono"

ISOLATES	H_{121} H_{221} H_{321} H_{421} B_{121} B_{221} B_{321} B_{421} Y_{121} Y_{221}	H_{122} H_{222} H_{322} H_{422} B_{122} B_{222} B_{322} B_{422} Y_{122} Y_{222}	H_{123} H_{223} H_{323} H_{423} B_{123} B_{223} B_{323} B_{423} Y_{123} Y_{223}	H_{124} H_{224} H_{324} H_{424} B_{124} B_{224} B_{324} B_{424} Y_{124} Y_{224}
Identified bacteria	Strept. lactis	Strept. cremoris	Strept. diacetilactis	Bacillus spp.

KEY

$H_{121} - H_{124}$; $H_{221} - H_{224}$; $H_{321} - H_{324}$; $H_{421} - H_{424}$ = Isolates from processors H_1 , H_2, H_3 H_4 from "Hanwa" Village respectively.

$B_{121} - B_{124}$ $B_{221} - B_{224}$ $B_{321} - B_{324}$ $B_{421} - B_{424}$ = Isolate from processors B_1, B_2, B_3, and B_4 from "Bomo" Village respectively.

$Y_{121} - Y_{124}$; $Y_{221} - Y_{224}$ = Isolate from processors Y_1 , and Y_2 from "Biye" Village respectively.

See Appendix viii. for detailed identification.

TABLE 10: Results of the Morphological and Biochemical tests of Yeasts isolated from fermenting milk at 6 hours of fermentation in production of "nono"

ISOLATES									
	H_{127}	H_{227}	H_{327}	H_{128}	H_{228}	H_{328}	H_{129}	H_{229}	H_{329}
	H_{427}	B_{127}	B_{227}	H_{428}	B_{126}	B_{228}	H_{429}	B_{129}	B_{229}
	B_{327}	B_{427}		B_{328}	B_{428}		B_{329}	B_{429}	
	Y_{127}	Y_{227}		Y_{128}	Y_{228}		Y_{129}	Y_{229}	
Identified Yeast	Candida pseudo-tropicalis		Candida tropicalis			Saccharomyces cerevisiae			

KEY

H_{127} - H_{129}; H_{227} - H_{229}; H_{327} - H_{329}; H_{427} - H_{429} = Isolates from processors H_1, H_2, H_3, H_4 from "Hanwa" Village respectively.

B_{127} - B_{129}; B_{227} - B_{229}; B_{327} - B_{329}; B_{427} - B_{429} = Isolates from processors B_1, B_2, B_3, and B_4 from "Bomo" Village respectively.

Y_{127} - Y_{129}; Y_{227} - Y_{229} = Isolate from processors Y_1, and Y_2 from "Biye" Village respectively.

See Appendix ix. for detailed identification.

TABLE 11: Results of the cultural, morphological and biochemical characteristics on bacteria isolated from fermented milk at 12 hours of fermentation in the production of "nono".

ISOLATES																					
	H_{131}	H_{231}	H_{331}	H_{132}	H_{232}	H_{332}	H_{133}	H_{233}	H_{333}	H_{134}	H_{234}	H_{334}	H_{135}	H_{235}	H_{335}	H_{136}	H_{236}	H_{336}	H_{137}	H_{237}	H_{33}
	H_{431}	B_{131}	B_{231}	H_{432}	B_{132}	B_{232}	H_{433}	B_{133}	B_{233}	H_{434}	B_{134}	B_{234}	H_{435}	B_{135}	B_{235}	H_{436}	B_{136}	B_{236}	H_{437}	B_{137}	B_{23}
	B_{331}	B_{431}		B_{332}	B_{432}		B_{333}	B_{433}		B_{334}	B_{434}		B_{335}	B_{435}		B_{336}	B_{436}		B_{337}	B_{437}	
	Y_{131}	Y_{231}		Y_{132}	Y_{232}		Y_{133}	Y_{233}		Y_{134}	Y_{234}		Y_{135}	Y_{235}		Y_{136}	Y_{236}		Y_{137}	Y_{237}	
Identified Micro-organisms	S. lactis			S. cremoris			S. diaceti-lactis			L. plantarum			L. brevis			L. cellobiosus			Bacillus spp		

KEY: Isolate codes:

H_{131}-H_{137} ; H_{231}-H_{237}) = Isolate from processors H1, H2, H3, H4 from "Hanwa" Village respectively.
H_{331}-H_{337} ; H_{431}-H_{437})

B_{131}-B_{137} ; B_{231}-B_{237}) = Isolate from processors B1, B2, B3, B4 from "Bomo" Village respectively.
B_{331}-B_{337} ; B_{431}-B_{437})

Y_{131}-Y_{137} ; Y_{231}-Y_{237} ; = Isolate from processors Y1 and Y2 from "Biye" Village respectively.

See Appendix x. for details of the identification.

TABLE 12: Results of the Morphological and Biochemical tests of Yeasts isolated from fermenting milk at 12 hours of fermentation in production of "nono"

ISOLATES	H_{131y} H_{431y} B_{331y} Y_{131y}	H_{231y} B_{131y} B_{431y} Y_{231y}	H_{331y} B_{231y}	H_{132y} H_{432y} B_{332y} Y_{132y}	H_{232y} B_{132y} B_{432y} Y_{232y}	H_{332y} B_{232y}	H_{133y} H_{433y} B_{333y} Y_{133y}	H_{233y} B_{133y} B_{433y} Y_{233y}	H_{333y} B_{233y}
Identified Yeast	Candida pseudo-tropicalis		Candida tropicalis				Saccharomyces cerevisiae		

KEY

H_{131y} - H_{133y}; H_{231y} - H_{233y}; H_{331y} - H_{333y}; H_{431y} - H_{433y} = Isolates from processors H_1, H_2, H_3, H_4 from "Hanwa" Village respectively.

B_{131y} - B_{133y}; B_{231y} - B_{233y}, B_{331y} - B_{333y}, B_{431y} - B_{433y} = Isolates from processors B_1, B_2, B_3, and B_4 from "Bomo" Village respectively.

Y_{131y} - Y_{133y}; Y_{231y} - Y_{233y} = Isolate from processors Y_1, and Y_2 from "Biye" Village respectively.

See Appendix xi for details of the identification.

TABLE 13: Results of the cultural, morphological and biochemical characteristics on bacteria isolated from fermented milk at 18 hours of fermentation in the production of "nono".

ISOLATES	H_{141} H_{441} B_{341} Y_{141}	H_{241} B_{141} B_{441} Y_{241}	H_{341} B_{241}	H_{142} H_{442} B_{342} Y_{142}	H_{242} B_{142} B_{442} Y_{242}	H_{342} B_{242}	H_{143} H_{443} B_{343} Y_{143}	H_{243} B_{143} B_{443} Y_{243}	H_{343} B_{243}	H_{144} H_{444} B_{344} Y_{144}	H_{244} B_{144} B_{444} Y_{244}	H_{344} B_{244}	H_{145} H_{445} B_{345} Y_{145}	H_{245} B_{145} B_{445} Y_{245}	H_{345} B_{245}	H_{146} H_{446} B_{346} Y_{146}	H_{246} B_{146} B_{446} Y_{246}	H_{346} B_{246}	H_{147} H_{447} B_{347} Y_{147}	H_{247} B_{147} B_{447} Y_{247}	H B
Identified Micro-organisms	S. lactis		S. cremoris			S. diaceti-lactis				L. plantarum			L. brevis			L. cellobiosus			Bacillus spp			

KEY: Isolate codes:

H_{141}-H_{147}; H_{241}-H_{247})
H_{341}-H_{347}; H_{441}-H_{447}) = Isolate from processors H1, H2, H3, H4 from "Hanwa" Village respectively.

B_{141}-B_{147}; B_{241}-B_{247})
B_{341}-B_{347}; B_{441}-B_{447}) = Isolate from processors B1, B2, B3, B4 from "Bomo" Village respectively.

Y_{141}-Y_{147}; Y_{241}-Y_{247}; = Isolate from processors Y1 and Y2 from "Biye" Village respectively.

See Appendix xii. for details of the identification.

TABLE 14. **Results of the morphological and biochemical tests of Yeast Isolated from fermenting milk at 18 hours of fermentation in the production of "nono"**

Isolates	H_{141y} H_{241y} H_{142y} H_{242y} H_{143y} H_{243y} H_{144y} H_{244y} H_{145y} H_{245y} H_{341y} H_{441y} H_{342y} H_{442y} H_{343y} H_{443y} H_{344y} H_{444y} H_{345y} H_{445y} B_{141y} B_{241y} B_{142y} B_{242y} B_{143y} B_{243y} B_{144y} B_{244y} B_{145y} B_{245y} B_{341y} B_{441y} B_{342y} B_{442y} B_{343y} B_{443y} B_{344y} B_{444y} B_{345y} B_{445y} Y_{141y} Y_{241y} Y_{142y} Y_{242y} Y_{143y} Y_{243y} Y_{144y} Y_{244y} Y_{145y} Y_{245y}
06 Identified microorganisms	Candida pseudotropicalis; Candida tropicalis; Endomyces spp; Saccharomyces cerevisae; Trichosporon beigelii

KEY: ISOLATES CODES

H_{141y} – H_{145y}; H_{241y} – H_{245y}; H_{341y} – H_{345y}; H_{441y} – H_{445y} = Isolates from processors H_1, H_2, H_3, and H_4 from "Hanwa" Village respectively.

B_{141y} – B_{145y}; B_{241y} – B_{245y}; B_{341y} – B_{345y}; B_{441y} – B_{445y} = Yeast isolates from processors B_1, B_2, B_3, B_4 from "Bomo" village respectively.

Y_{141y} – Y_{145y}; Y_{241y} – Y_{245y} = Yeast isolates from processors Y_1 and Y_2 from "Biye" village respectively.

See Appendix xiii. for details of the identification

TABLE 15: Results of the cultural, morphological and biochemical characteristics on bacteria isolated from fermented milk at 24 hours of fermentation in the production of "nono".

	H_{151}	H_{251}	H_{351}	H_{152}	H_{252}	H_{352}	H_{153}	H_{253}	H_{353}	H_{154}	H_{254}	H_{354}	H_{155}	H_{255}	H_{355}	H_{156}	H_{256}	H_{356}	H_{157}	H_{257}	H_{35}
	H_{451}	B_{151}	B_{251}	H_{452}	B_{152}	B_{252}	H_{453}	B_{153}	B_{253}	H_{454}	B_{154}	B_{254}	H_{455}	B_{155}	B_{255}	H_{456}	B_{156}	B_{256}	H_{457}	B_{157}	B_{25}
ISOLATES	B_{351}	B_{451}		B_{352}	B_{452}		B_{353}	B_{453}		B_{354}	B_{454}		B_{355}	B_{455}		B_{356}	B_{456}		B_{357}	B_{457}	
	Y_{151}	Y_{251}		Y_{152}	Y_{252}		Y_{153}	Y_{253}		Y_{154}	Y_{254}		Y_{155}	Y_{255}		Y_{156}	Y_{256}		Y_{157}	Y_{257}	
Identified Micro-organisms	S. lactis		S. cremoris			S. diaceti-lactis			L. plantarum			L. brevis			L. cellobiosus			Bacillus spp		

KEY: Isolate codes:

H_{151}-H_{157}; H_{251}-H_{257}) = Isolate from processors H1, H2, H3, H4 from "Hanwa" Village respectively.
H_{351}-H_{357}; H_{451}-H_{457})

B_{151}-B_{157}; B_{251}-B_{257}) = Isoiate from processors B1, B2, B3, B4 from "Bomo" Village respectively.
B_{351}-B_{357}; B_{451}-B_{457})

Y_{151}-Y_{157}; Y_{251}-Y_{257}; = Isolate from processors Y1 and Y2 from "Biye" Village respectively.

See Appendix xiv. for details of the identification.

TABLE 16. Results of the morphological and biochemical tests of Yeast Isolated from fermenting milk at 24 hours of fermentation in the production of "nono"

Isolates	H_{151y}	H_{251y}	H_{152y}	H_{252y}	H_{153y}	H_{253y}	H_{154y}	H_{254y}	H_{155y}	H_{255y}
	H_{351y}	H_{451y}	H_{352y}	H_{452y}	H_{353y}	H_{453y}	H_{354y}	H_{454y}	H_{355y}	H_{455y}
	B_{151y}	B_{251y}	B_{152y}	B_{252y}	B_{153y}	B_{253y}	B_{154y}	B_{254y}	B_{155y}	B_{255y}
	B_{351y}	B_{451y}	B_{352y}	B_{452y}	B_{353y}	B_{453y}	B_{354y}	B_{454y}	B_{355y}	B_{455y}
	Y_{151y}	Y_{251y}	Y_{152y}	Y_{252y}	Y_{153y}	Y_{253y}	Y_{154y}	Y_{254y}	Y_{155y}	Y_{255y}

Identified microorganisms	Candida pseudotropicalis	Candida tropicalis	Endomyces spp	Saccharomyces cerevisae	Trichosporon beigelii

KEY: ISOLATES CODES

H_{151y} - H_{155y}; H_{251y} -H_{255y}; H_{351y} -H_{355y}; H_{451y} -H_{455y} = Isolates from processors H_1, H_2, H_3, and H_4 from "Hanwa" Village respectively.

B_{151y} - B_{155y}; B_{251y} -B_{255y}; B_{351y} -B_{355y}; B_{451y} -B_{455y} = Yeast isolates from processors B_1, B_2, B_3, B_4, from "Bomo" village respectively.

Y_{151y} -Y_{155y}; Y_{251y} -Y_{255y} = Yeast isolates from processors Y_1 and Y_2 from "Biye" village respectively.

See Appendix xv. for details of the identification.

4.3.5 Microorganisms Isolated From The Calabash Used In The Processing of "Nono"

The microorganisms isolated from the calabash used in the processing of "nono" were the same as those that were isolated from "nono". (Tables 17).

Table 17:

Microorganisms Isolated From Calabashes Used In The Collection of Milk and In Milk Fermentation to Produce "Nono"

BACTERIA	:	Streptococcus lactis, Streptococcus cremoris, Streptococcus diacetilactis, Lactobacillus plantarum, Lactobacillus cellobiosus, Lactobacillus brevis, Bacillus spp.
YEASTS	:	Endomyces spp, Candida tropicalis, Candida pseudotropicalis, Saccharomyces cerevisiae, Trichosporon beigelii

4.4 Physiological Studies On Some Selected Isolates of "Nono"

The effects of temperature and pH on some selected isolates of "nono" are shown in Figures 11-25. The isolates investigated include Lactobacillus cellobiosus, Lactobacillus brevis, Lactobacillus plantarum, Streptococcus lactis, Streptococcus diacetilactis, Streptococcus cremoris, Saccharomyces cerevisiae and Candida tropicalis. These pure culture experiments were carried out to show the optimum temperature and pH for growth of these selected isolates.

4.4.1 Effect Of Temperature On The Growth Of The Isolates

All the Lactobacillus species grew best at a temperature of 40°C, followed by growth at 35°C. While growth was good and similar at 30°C and 25°C, it was poor at 15°C and 20°C (Fig. 11-13). All Streptococcus species grew best at a temperature of 35°C, growth at 25°C and 30°C were comparable. Their growth was poor at 15°C and 20°C and there was no growth at all at 40°C (Fig. 14-16). Yeasts grew best at 25°C followed by growth at 20°C. Growth at 40°C was poor while growth at 30°C and 35°C were good and similar (Fig. 17 and 18).

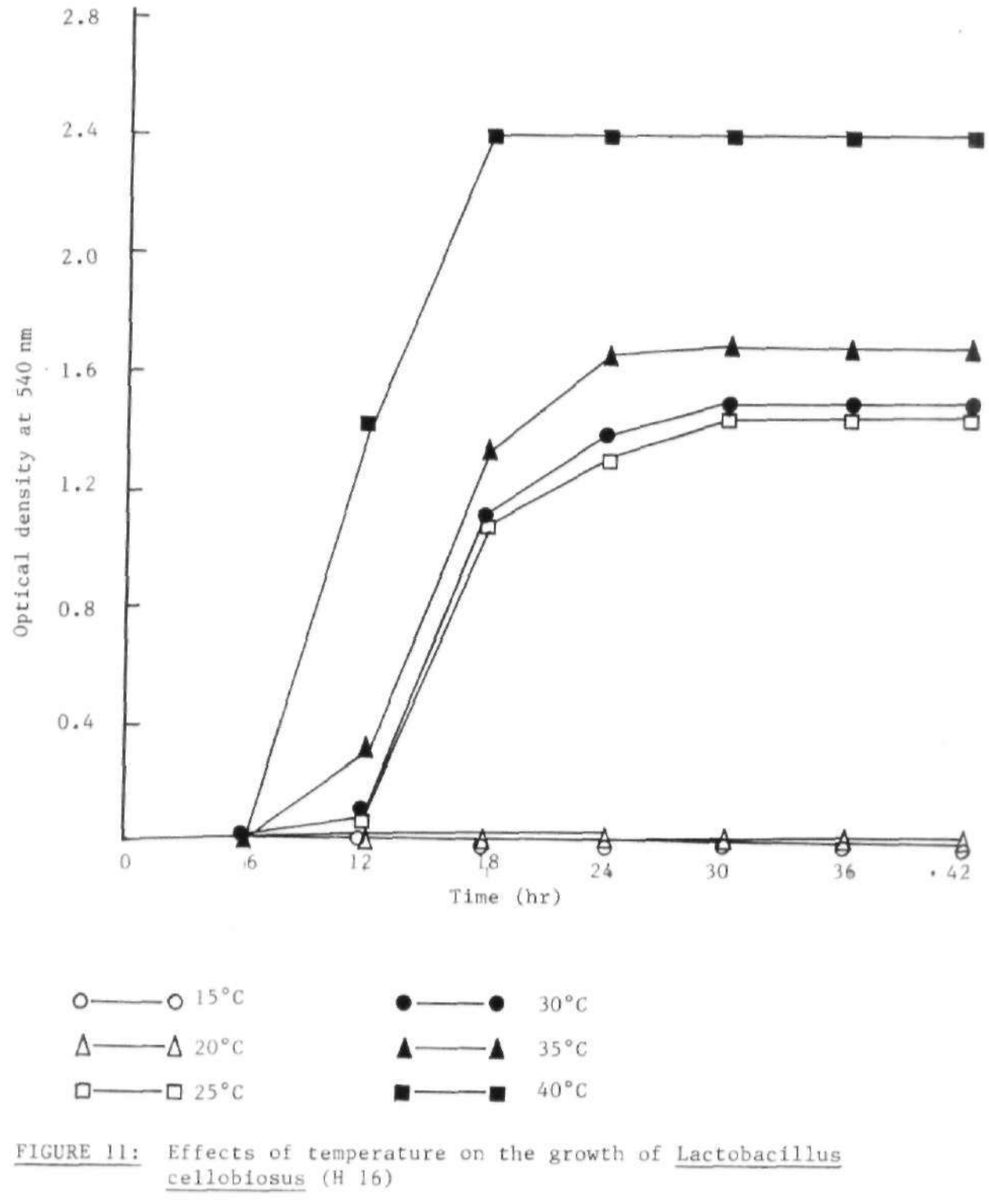


FIGURE 11: Effects of temperature on the growth of Lactobacillus cellobiosus (H 16)

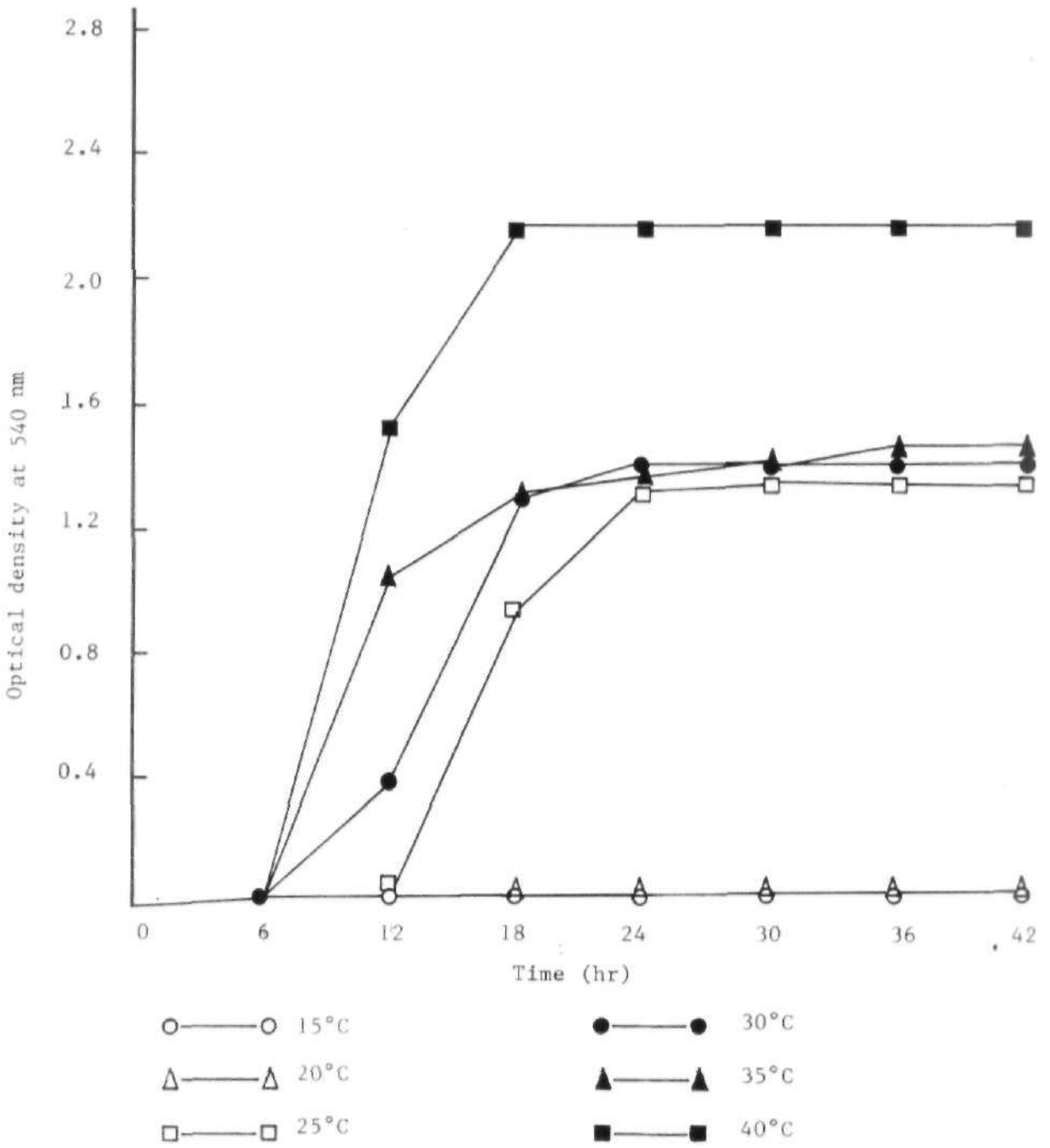


FIGURE 12: Effects of temperature on the growth of Lactobacillus brevis (H 15)

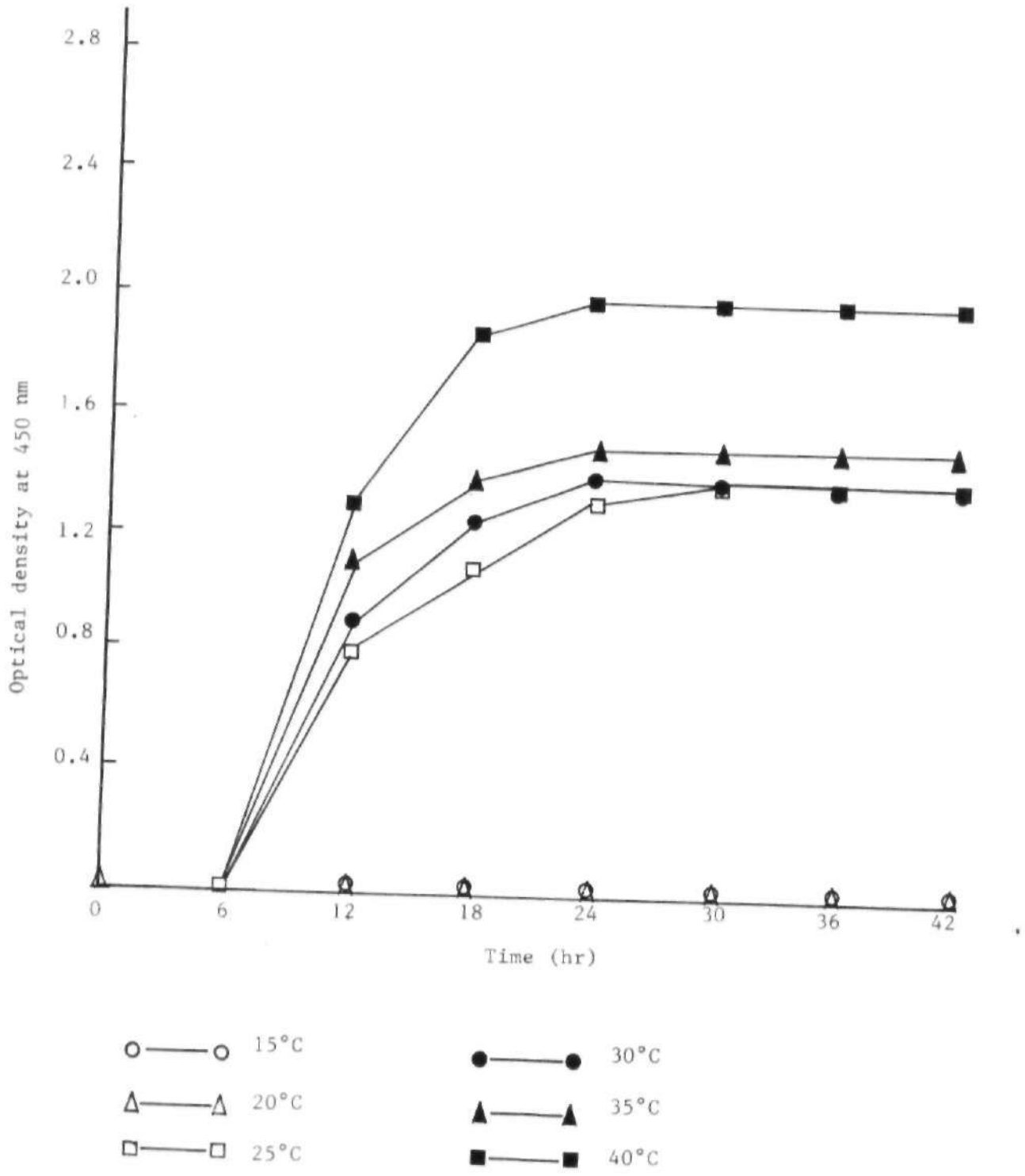


FIGURE 13: Effects of temperature on the growth of Lactobacillus plantarum (H 14).

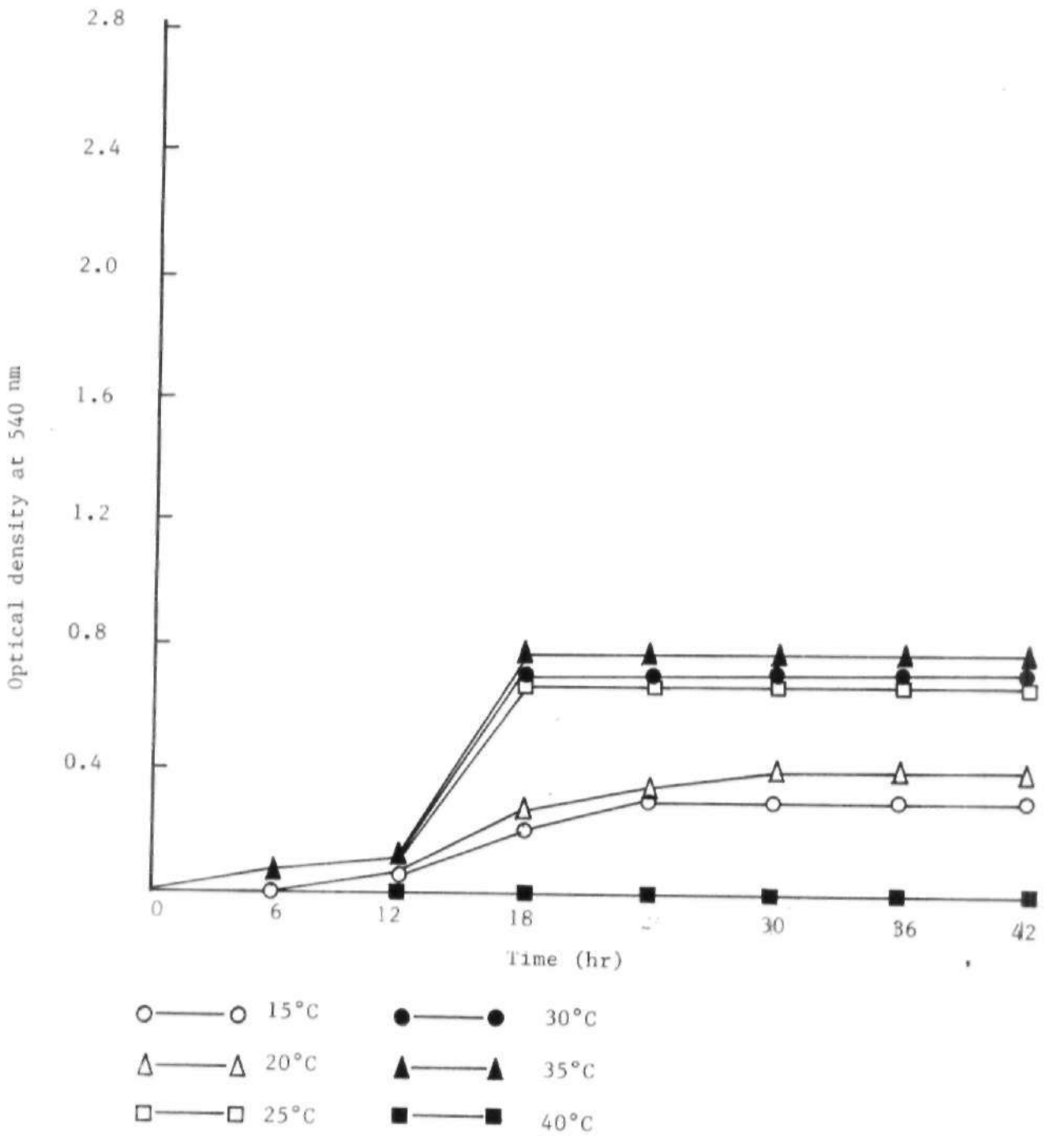


FIGURE 14: Effects of temperature on the growth of Streptococcus diacetilactis (H 13)

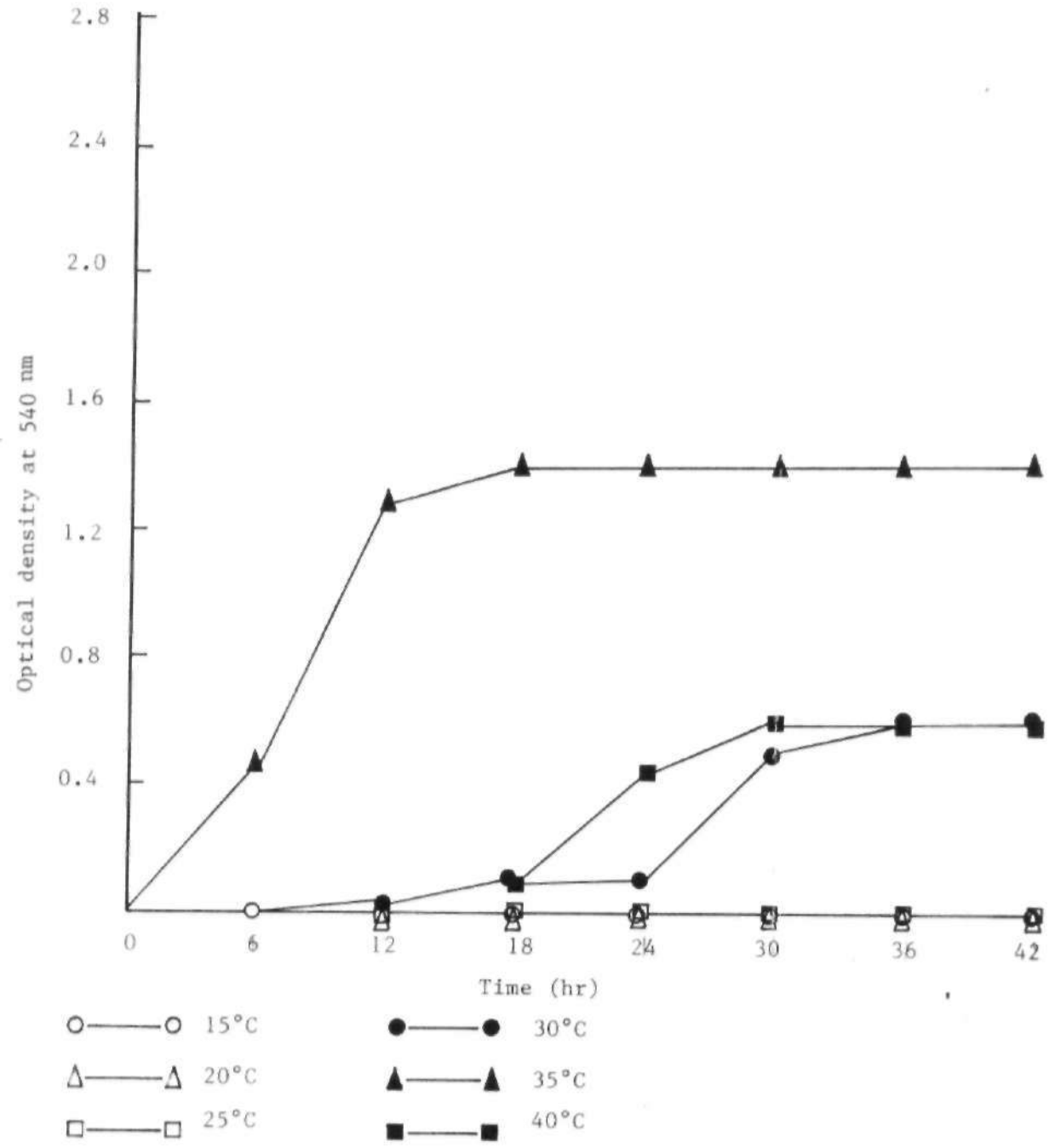


FIGURE 15: Effects of temperature on the growth of Streptococcus cremoris (H 12)

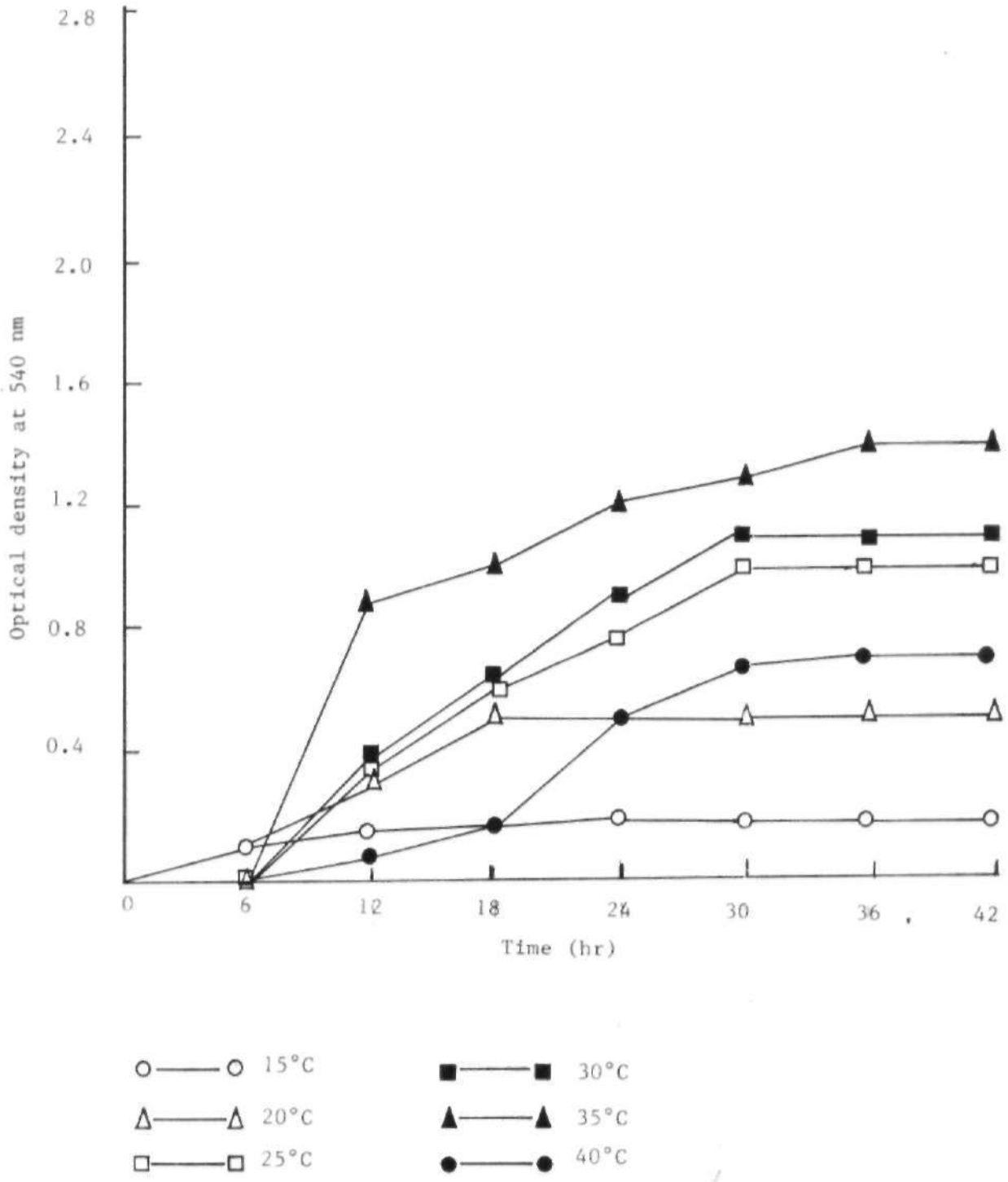


FIGURE 16: Effects of temperature on the growth of Streptococcus lactis (H 11)

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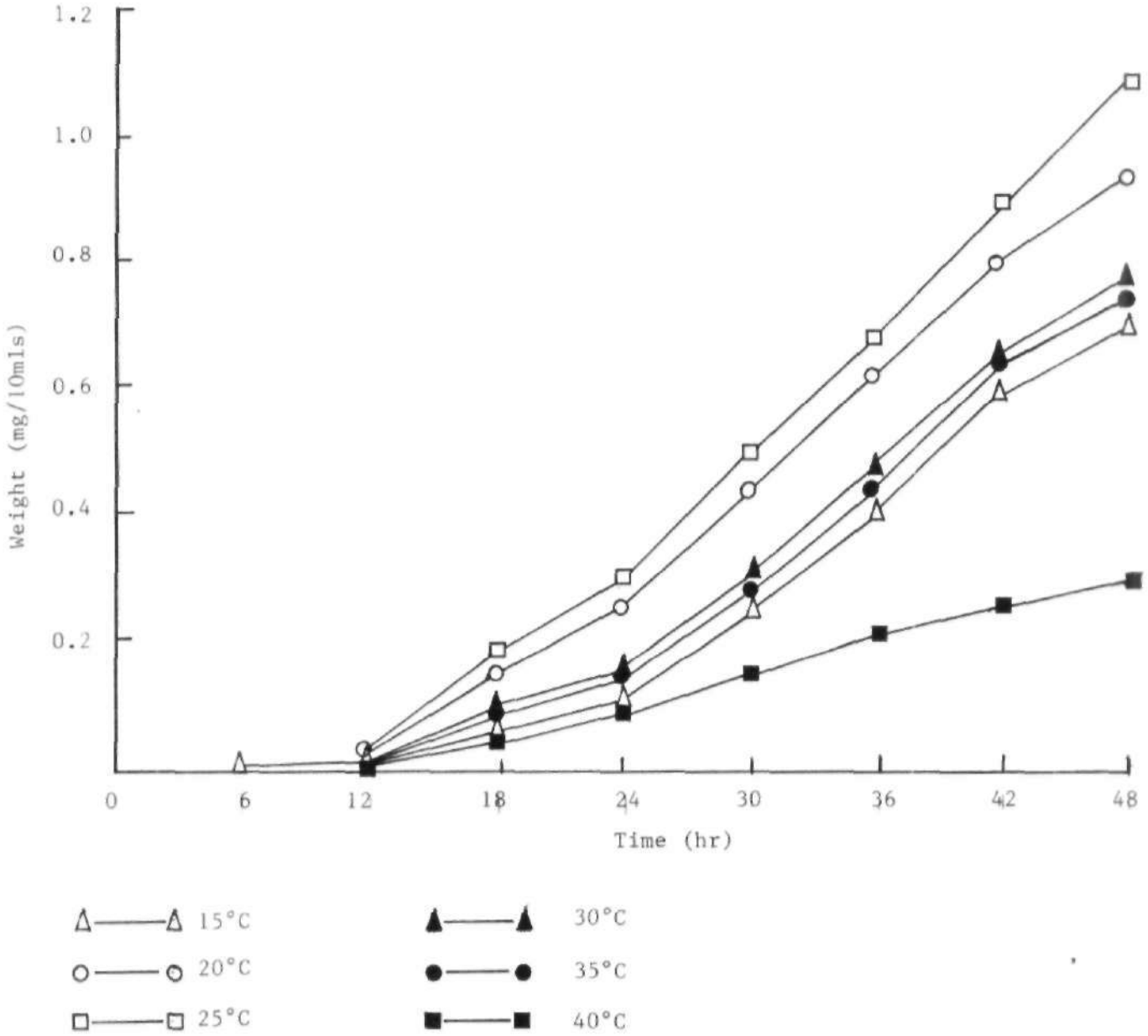


FIGURE 17: Effects of temperature on the growth of Candida pseudotropicalis (H. 117

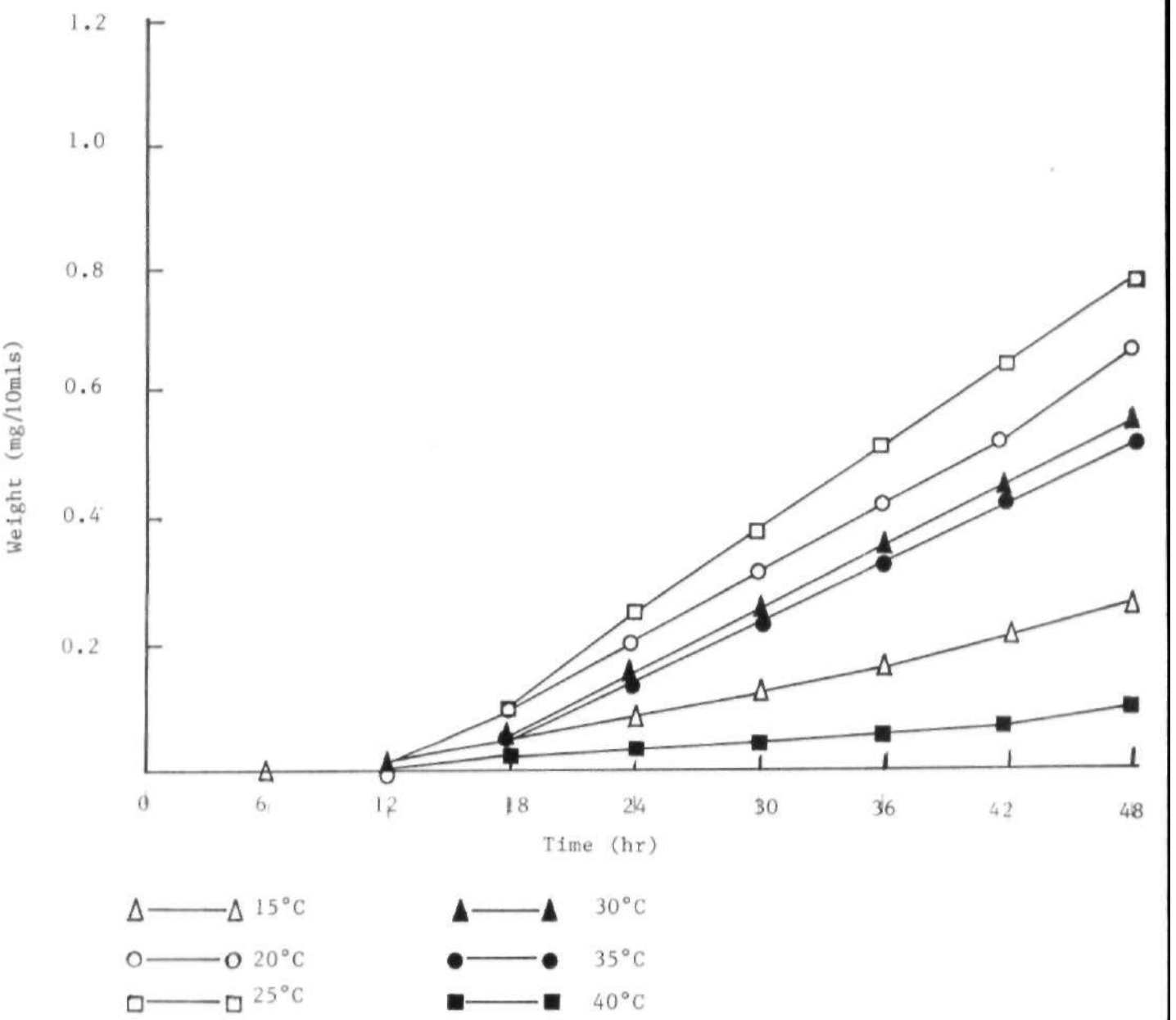


FIGURE 18: Effects of temperature on the growth of Saccharomyces cerevisiae (H 119)

4.4.2 Effect Of pH On The Growth Of The Isolates

Lactobacillus plantarum and Lactobacillus brevis both had their best growth at pH 5 and followed by their growth at pH 4 (Figs. 20 and 21). Lactobacillus cellobiosus, however, grew best between pH 3 and pH 4 followed by growth at pH 5. It had a very poor growth at pH 8 (Fig 19).

Streptococcus diacetilactis and Streptococcus lactis both grew best at pH 6 followed by growth at pH 5 (Figs. 22 and 24). However, Streptococcus cremoris preferred a pH of 6 followed by growth at pH 7 (Fig. 23). All the Streptococcus specie grew at pH 8 but did not grow at low pH of 3 and 4.

Both yeast strains (Candida pseudotropicalis and Saccharomyces cerevisiae preferred acidic pH for growth, and grew best at pH 4 and pH 3. The growth at pH 8 was the least and the growth at pH 2 and 5 was more than growth at pH 6 and 7 (Figs. 25 and 26).

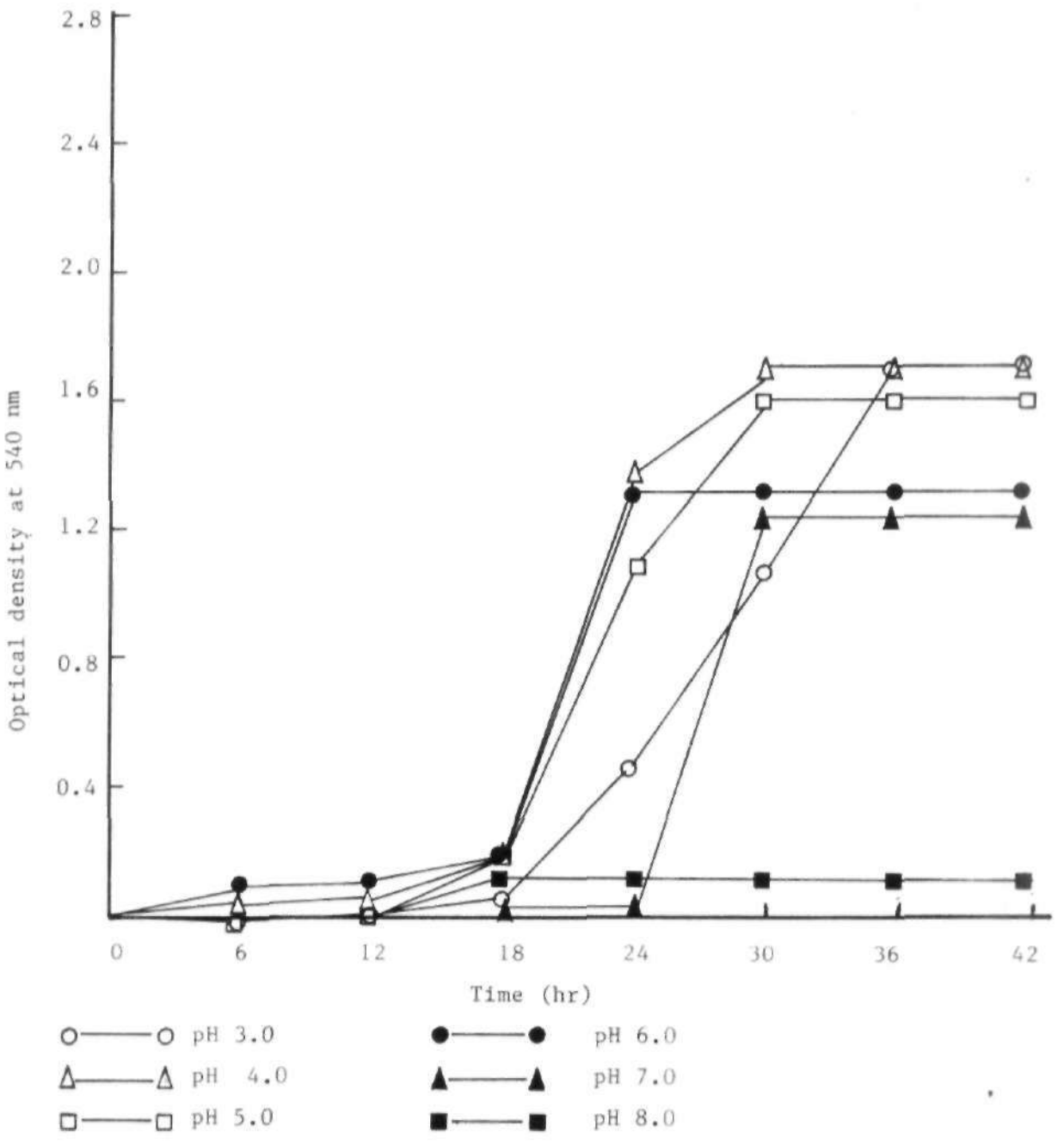


FIGURE 19: Effects of pH on the growth of Lactobacillus cellobiosus (H 16)

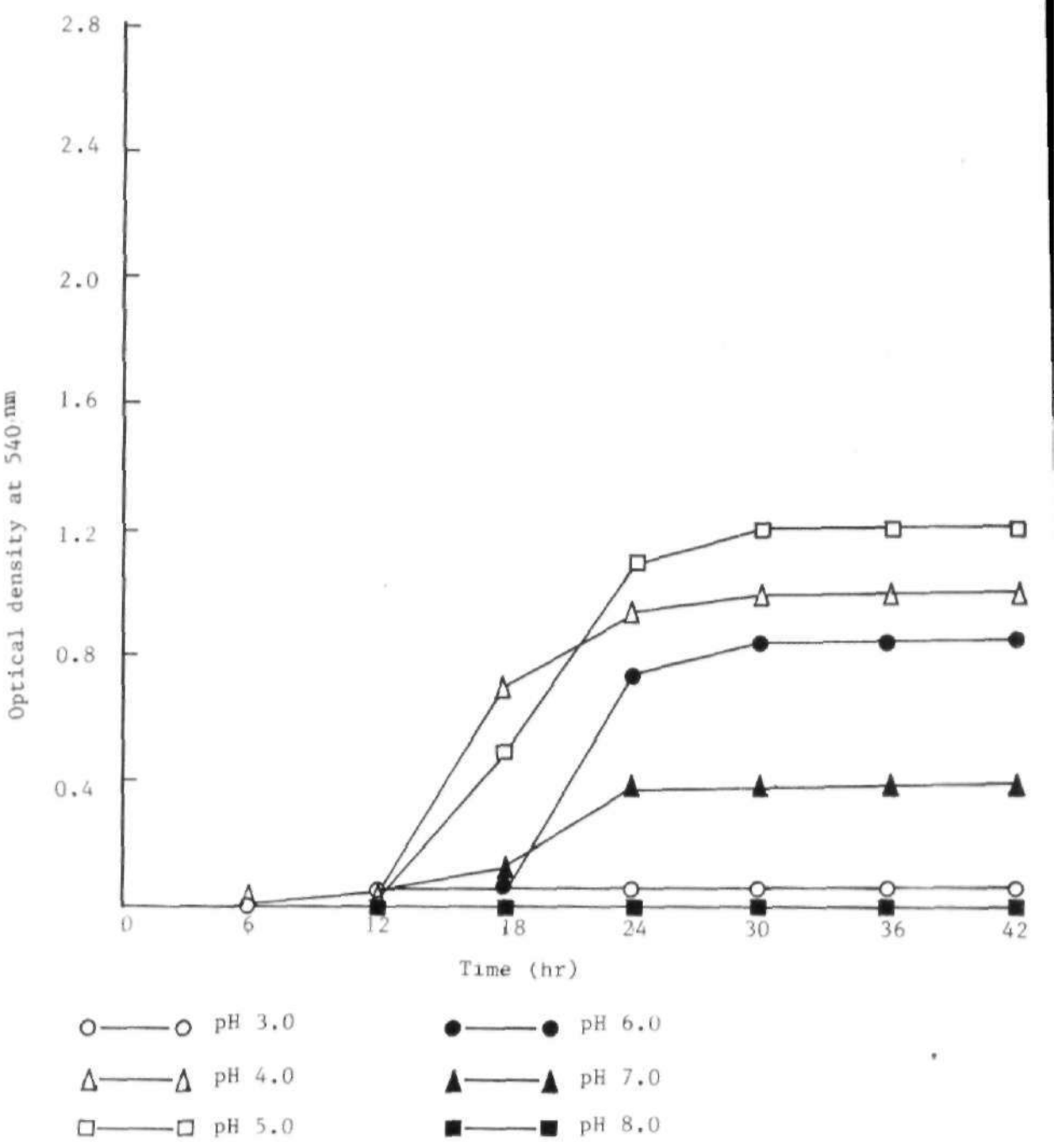


FIGURE 20: Effects of pH on the growth of Lactobacillus brevis (H 15)

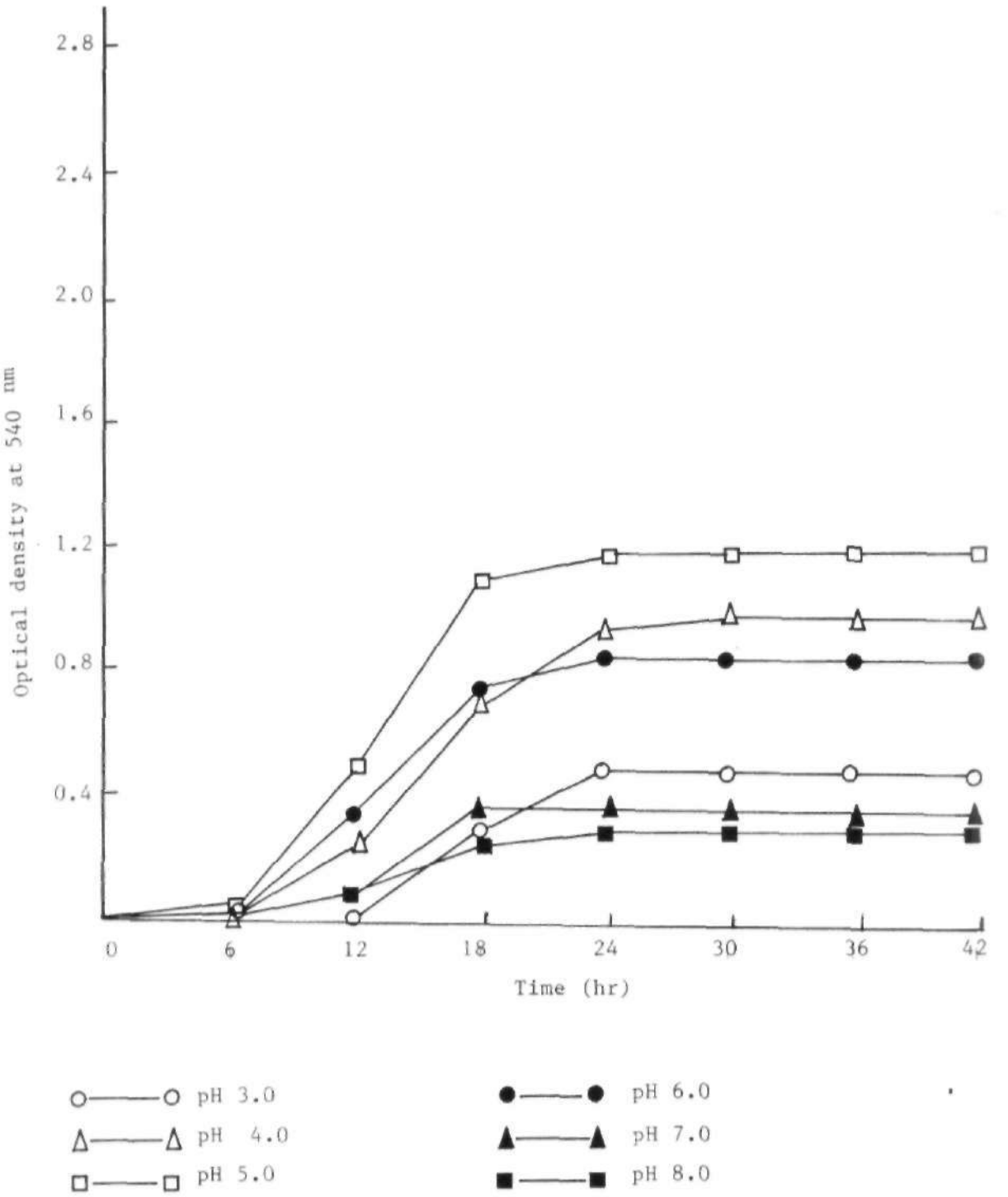


FIGURE 21: Effects of pH on the growth of Lactobacillus plantarum (H 14)

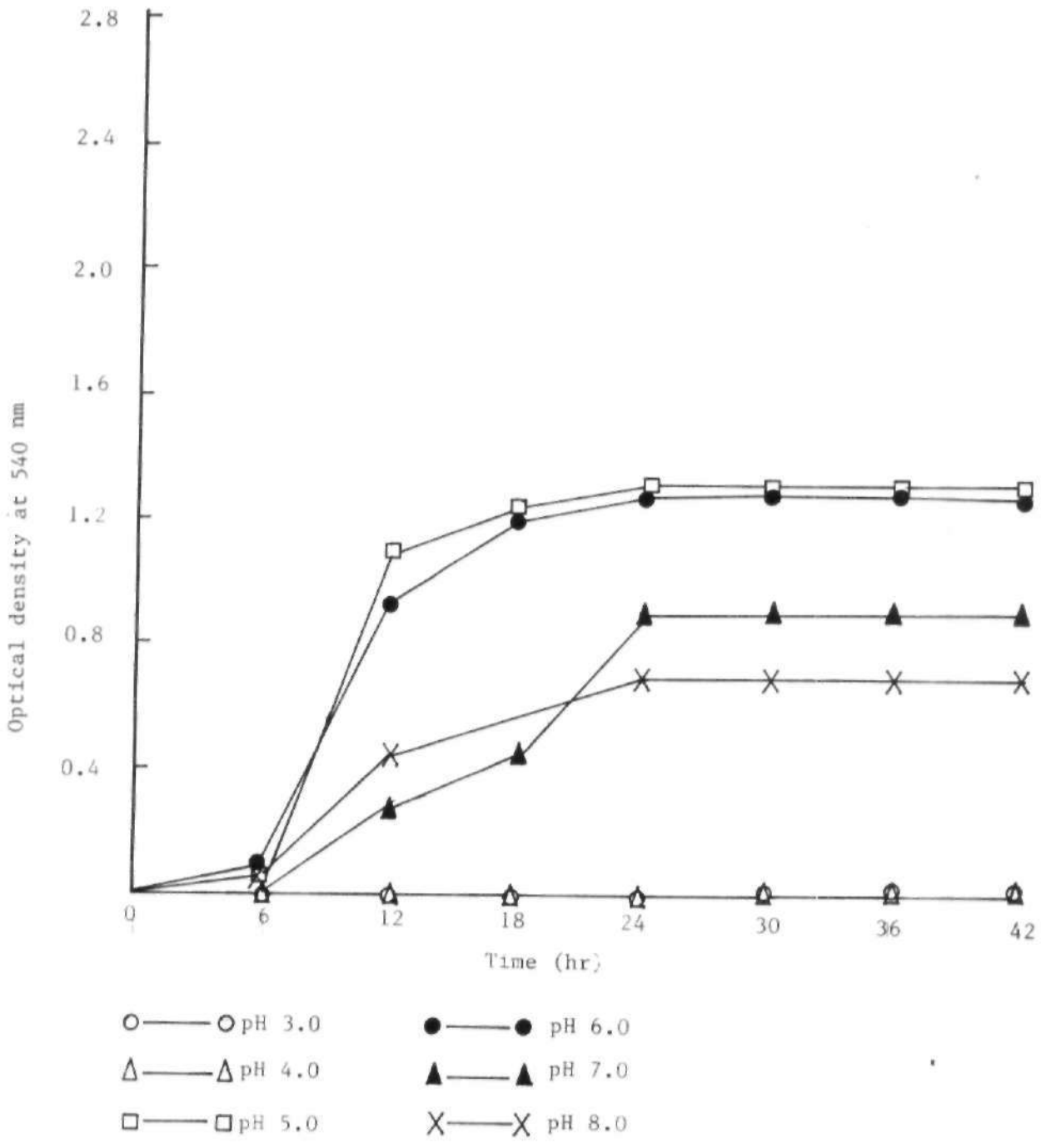


FIGURE 22: Effects of pH on the growth of Streptococcus diacetilactis (H 13)

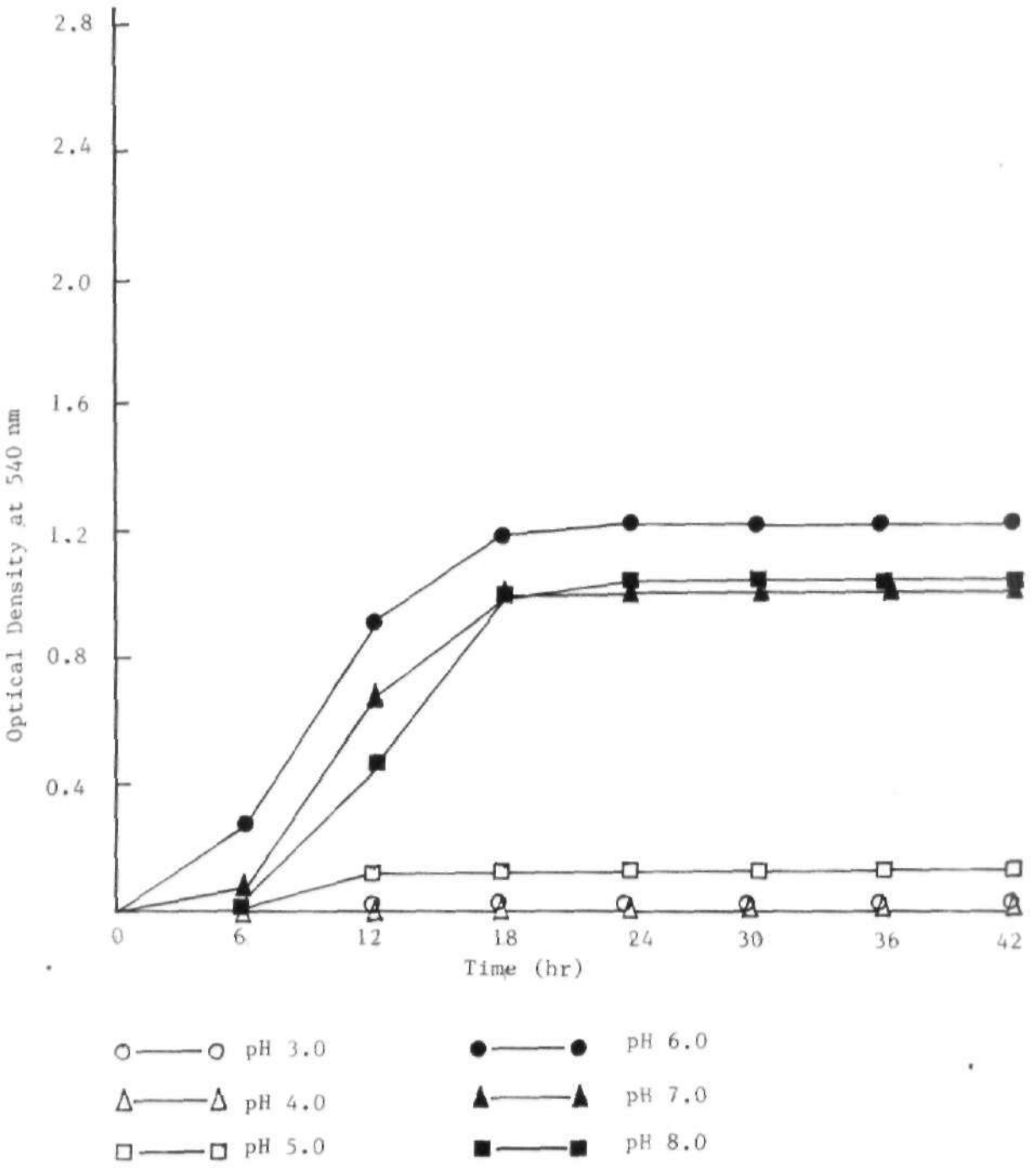


FIGURE 23: Effects of pH on the growth of Streptococcus cremoris (H 12)

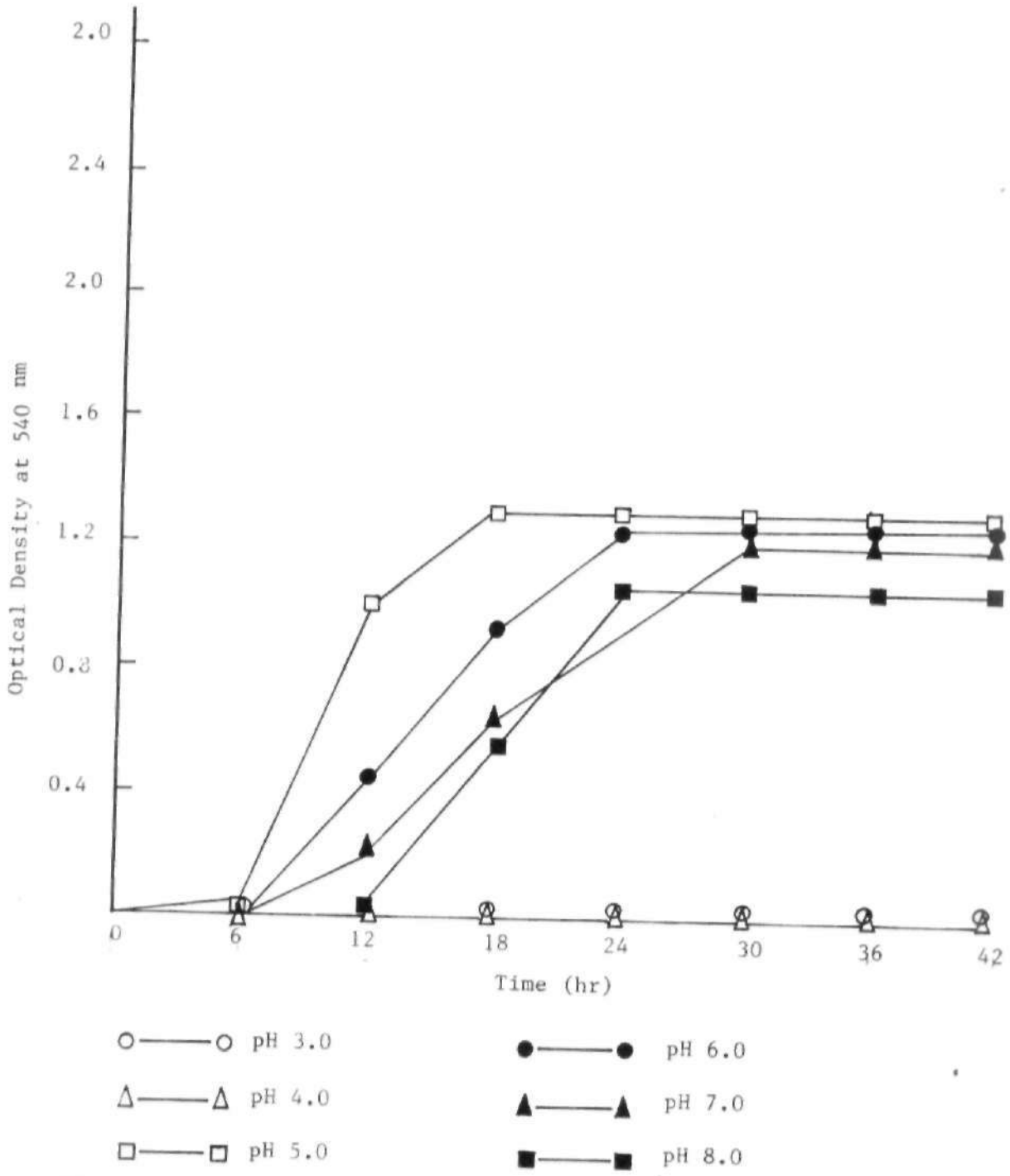


FIGURE 24: Effects of pH on the growth of Streptococcus lactis (H 11)

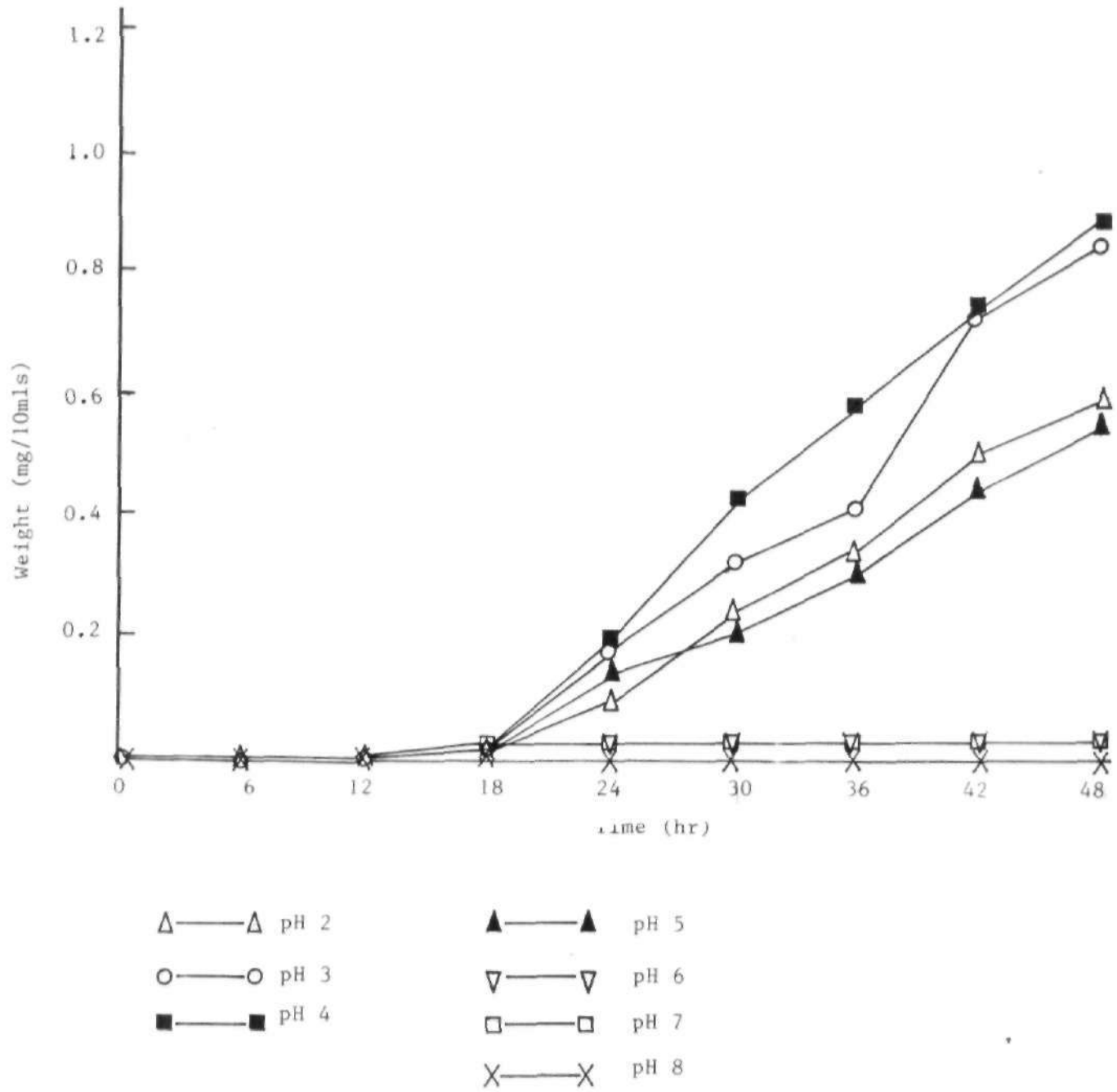


FIGURE 25: Effects of pH on the growth of Candida Pseudotropicals (H 117)

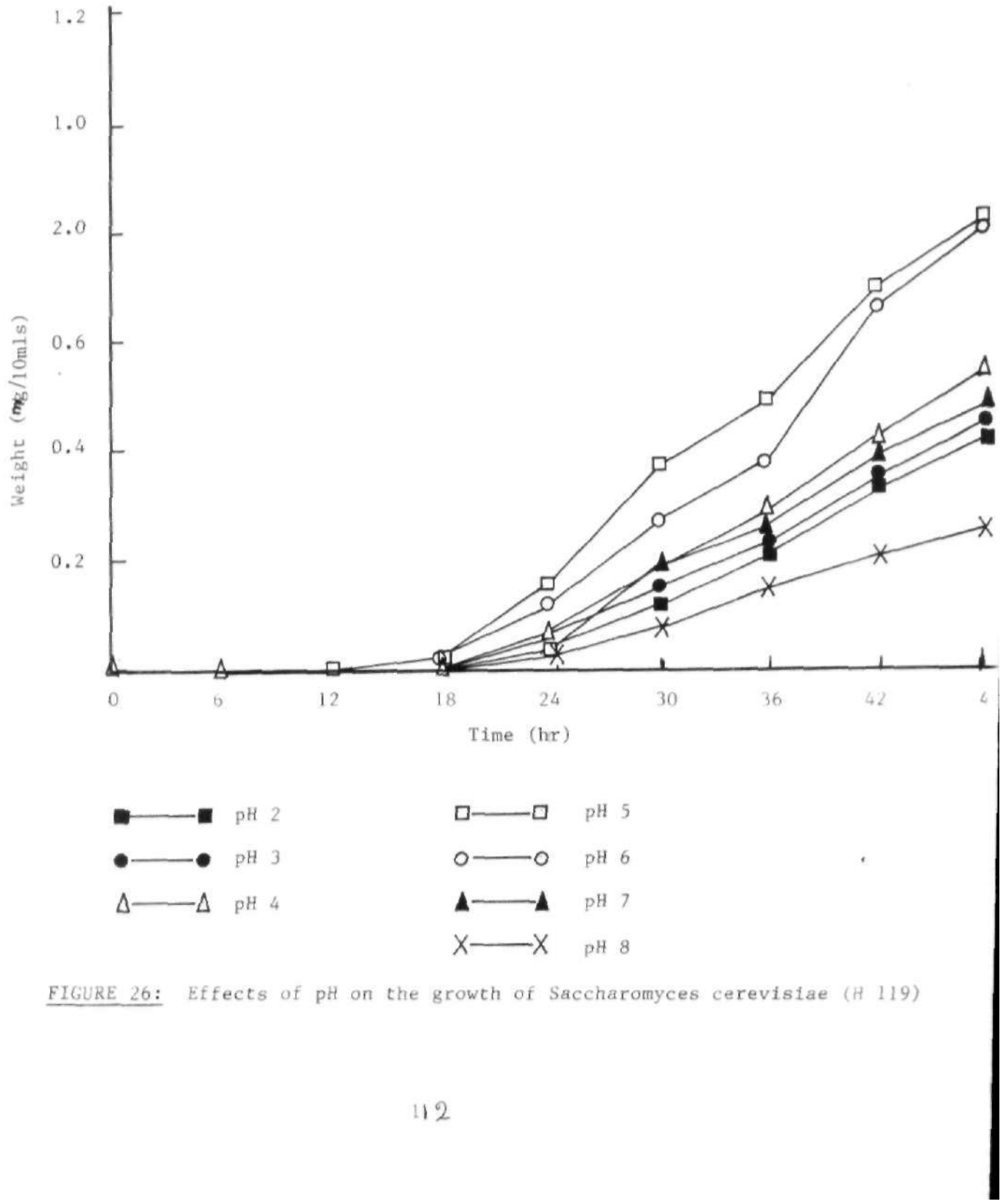


FIGURE 26: Effects of pH on the growth of Saccharomyces cerevisiae (H 119)

4.5 Optimization Studies On "Nono" Production

The use of the following parameters to optimize "nono" was investigated.

4.5.1 The Influence of Temperature on Microbial and Physico Chemical Changes Occuring In "Nono" Fermentation

The results of the changes in the pH, TTA and Diacetyl content and acceptability of "nono" fermented at different temperatures are as shown in Figs. 27-29 and Table 18. It was observed that the pH during "nono" fermentation decreased from pH 7 at zero hour of fermentation to between 5.1 at temperature of 15°C to pH 4.2 at temperature of fermentation of 37°C within 24 hours of fermentation. The decrease in pH increased with increase in the temperature of fermentation (Fig. 27). The TTA of "nono" produced at 25°C, 30°C and 37°C of production were comparable, while that of "nono" produced at 15°C was the lowest (Fig. 28). The higher the TTA, the lower the pH. Within 24 hours of fermentation, the pH was at 5.1 and the TTA was about 0.5% lactic acid at 15°C while the pH was 4.7 and the TTA was about 1.04% lactic acid (Figs 27 and 28).

Figure 29 shows that the least amount of diacetyl was produced at 15 C while the highest was produced at 37°C.

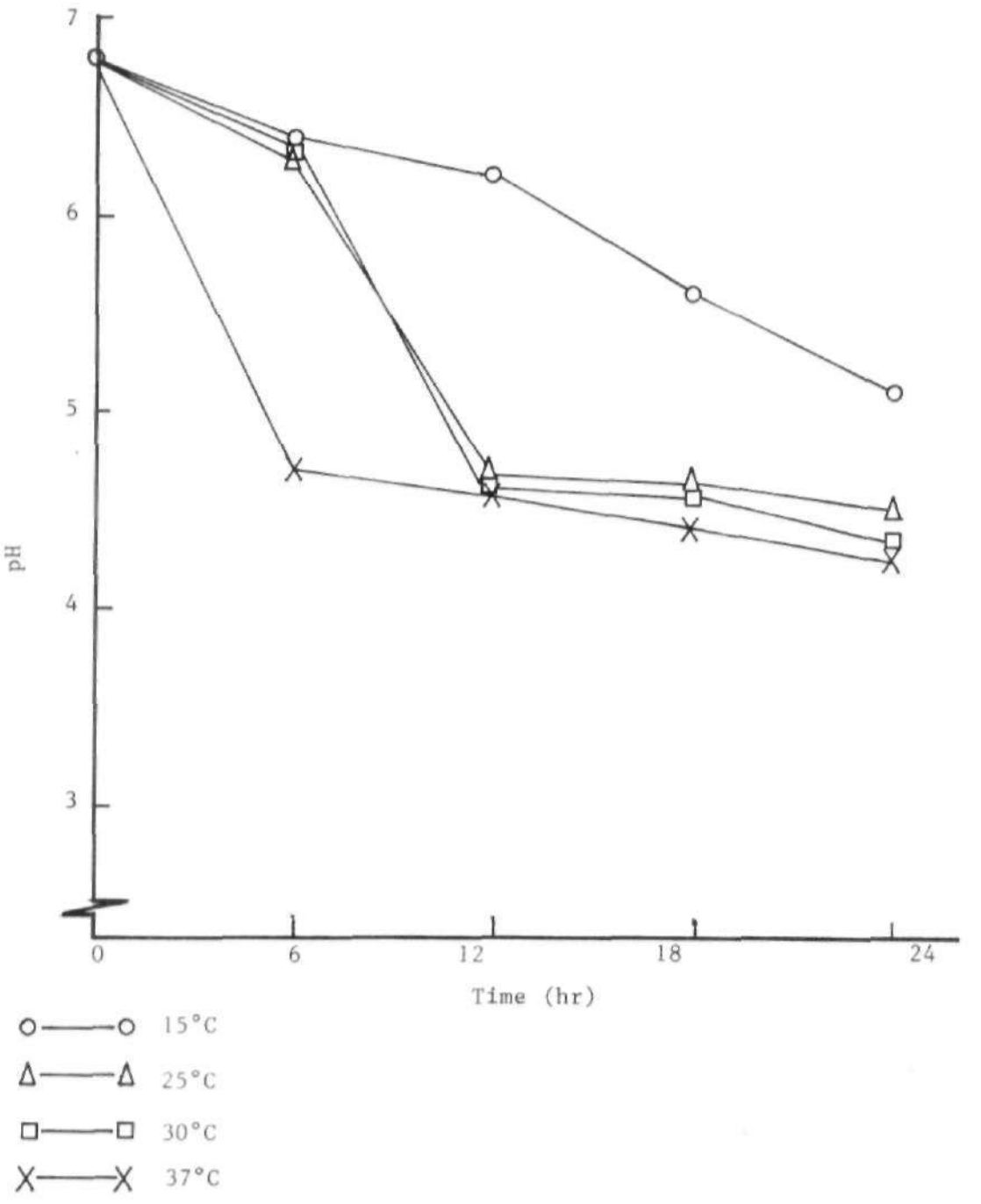


FIGURE 27: Effects of the temperature of fermentation on the changes in pH during "nono" production.

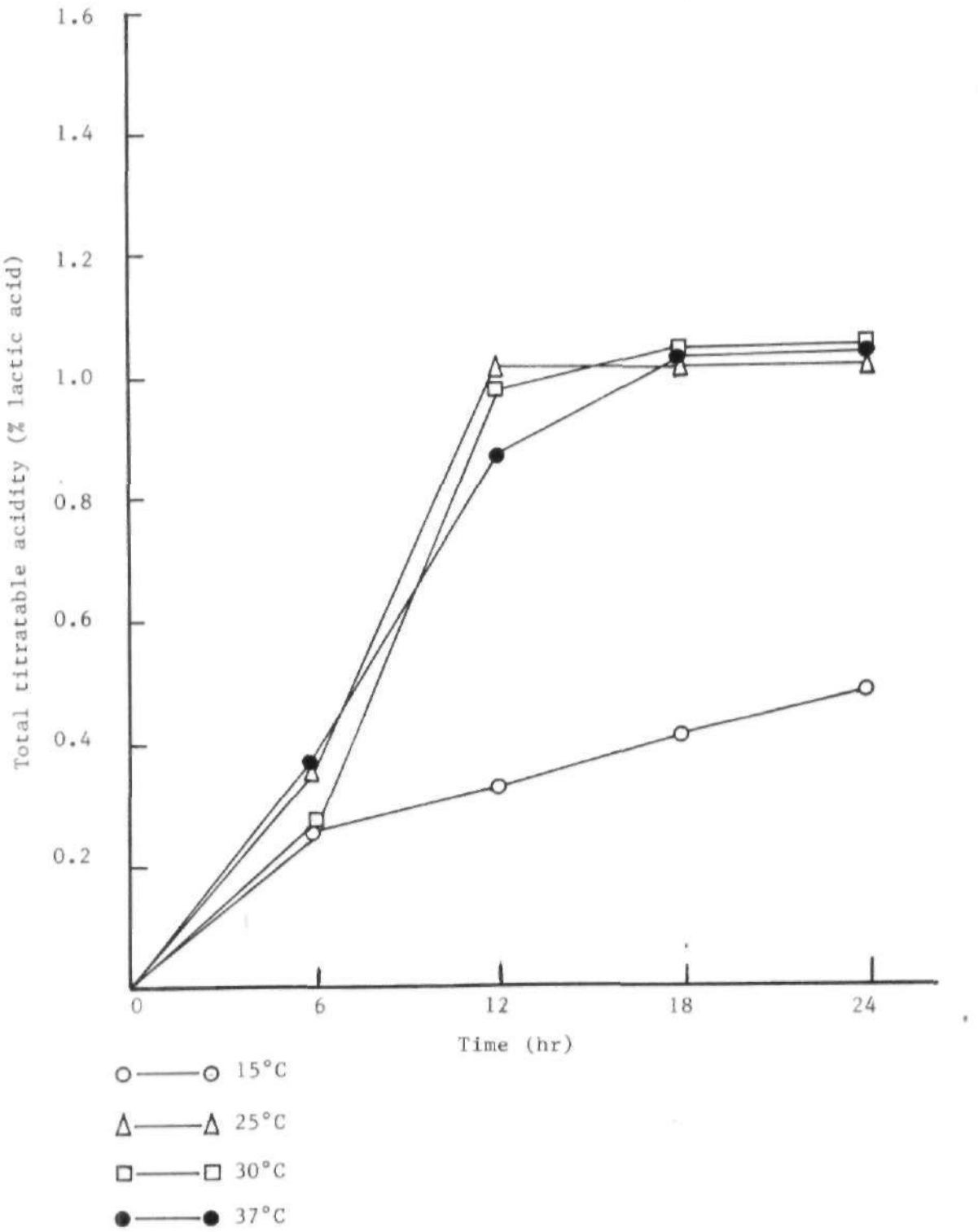


FIGURE 28: Effects of temperature of fermentation on the changes in the total titratable acidity (TTA) during "nono" production.

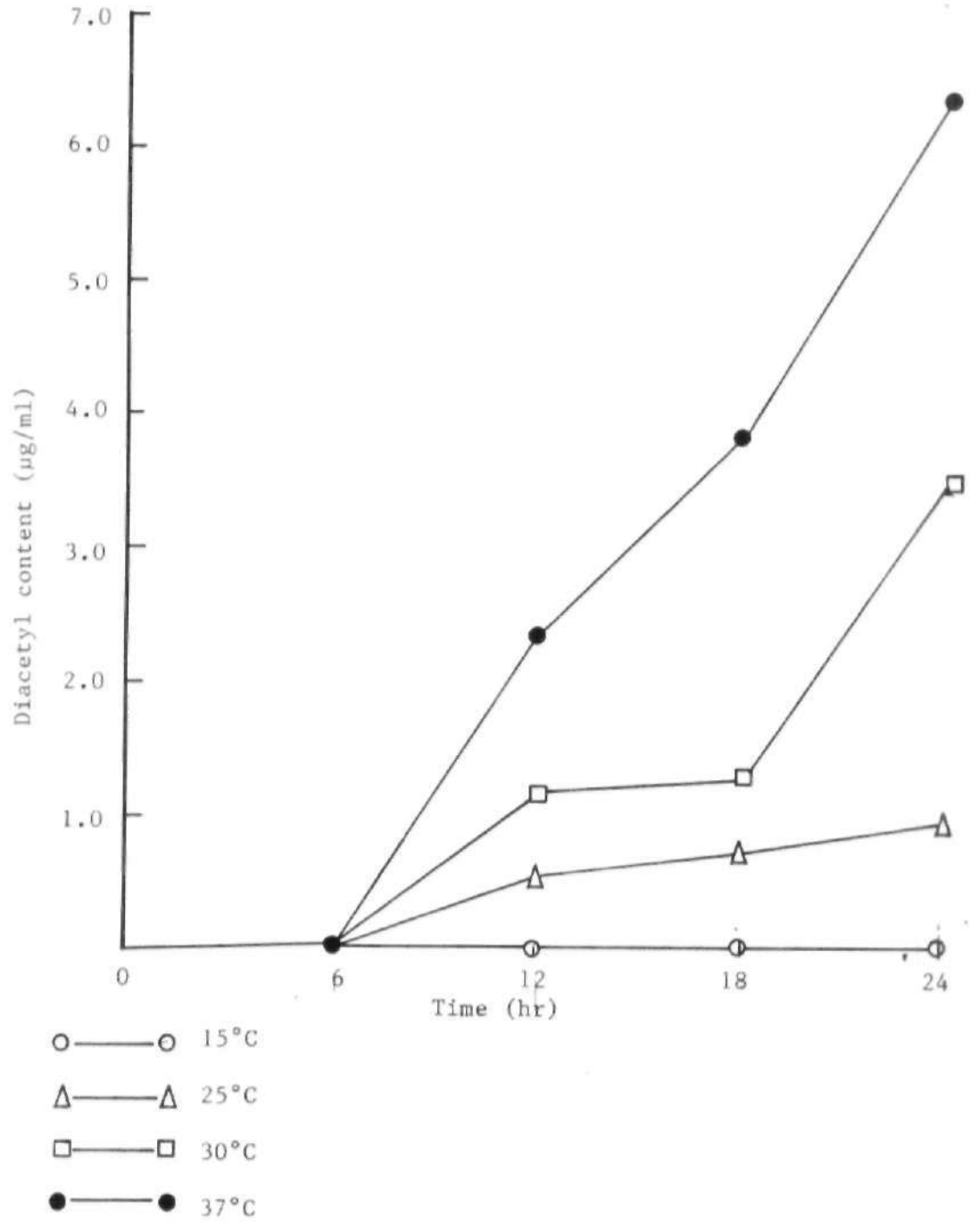


FIGURE 29: Effects of temperature of fermentation on Diacetyl content during "nono" production.

A taste panel assessment of "nono" in terms of colour, odour, taste, viscosity, texture and overall acceptability showed that "nono" produced at temperatures 25° C and 30° C were the best in overall acceptability. "Nono" produced at 37° C was less acceptable while that produced at 15° C had very low acceptability (Table 18). The results presented in the table are corresponding descriptions of the mean values derived from the panelists' scores.

TABLE 18 Qualities of "nono" processed at different temperatures (means of Sensory rating)

Quality attribute	Fermentation Temperature			
	15°C	25°C	30° C	37° C
Colour	White	Dirty white	Dirty white	Dirty white
Odour	Not pronounced	Pronounced	Pronounced	Very pronounced
Taste	Not sour	Moderately sour	Moderately sour	Very sour
Viscosity	Watery	Thick curd	Thick curd	Thick rough curd
Texture	Smooth	Coarse	Coarse	Very coarse
Overall acceptability	Poor	Good	Good	Fair

The "nono" produced from single strain cultures of Strept. cremoris, Strept. diacetilactis and Strept lactis had fair acceptability while "nono" produced using single strains of Lactobacillus brevis, Lactobacillus cellobiosus and Saccharomyces cerevisiae had poor acceptability (Table 19).

Table 19: Qualities of "nono" processed by the use of different starter cultures at room temperature (means of Sensory evaluation)

Microorganisms used as starter culture	Q U A L I T I E S Colour	Odour	Viscosity	Taste	Texture	Overall acceptability
Streptococcus lactis	b	c	i	k	p	r
Streptococcus diacetilactis	b	e	i	l	p	r
Streptococcus cremoris	b	c	i	k	p	r
Lactobacillus brevis	b	c	h	l	p	q
Lactobacillus cellobiosus	b	c	h	l	p	q
Saccharomyces cerevisiae	a	c	g	k	p	q
S. cremoris + L. brevis	a	c	j	m	q	s
S. diacetilactis + L. brevis	a	e	j	m	q	t
S. cremoris + S. cerevisiae	a	c	j	l	o	q
S. diacetilactis + S. cerevisiae	a	e	j	l	o	q
L. brevis + Saccharomyces cerevisiae	a	c	h	l	n	q
S. cremoris + L. brevis + S. cerevisiae	a	d	j	m	o	r
S. diacetilactis + L. brevis + S. cerevisiae	a	f	j	m	o	t

KEY: Mean scores correspond to the descriptions below.

a. => Dirty White b. => White c. => Not pronounced d. => Slightly pronounced

e. > Pronounced f. => Very pronounced g. => Watery h. => Slightly watery

i. => Slight curd j. => Thick curd k. => Not sour l. => Slightly sour

m. => Sour n. => Very coarse o. => Coarse p. => Smooth

4.5.2 The Effects of Single Starter Culture On Diacetyl-Content, pH and TTA Changes During "Nono" Production At Room Temperature

The TTA for the Streptoccocus strains were approximately similar and the highest (range 0.7 - 0.8gm % lactic acid). The TTA for the Lactobacilli were also fairly similar and intermediate (0.36 - 0.38 gm % lactic acid) whereas the TTA for S. cerevisiae were consistently low during the fermentation with a final level of about 0.2gm % lactic acid (Fig. 30).

The pH effects of the single starter culture during "Nono" production were similar for the lacto-bacillus tested. The pH dropped from 6.8 to around 4.6 within 24 hours while the pH for the Streptococci were slightly higher, giving a reading around pH 5 within the same period. The pH effect of Saccharomyces cerevisiae on milk during "Nono" production showed that the pH dropped just from 6.8 to 5.5 during the 24 hours of fermentation (Fig. 31).

Only Streptococcus diacetilactis showed appreciable production of diacetyl among the bacteria strains tested. By 24 hours, the level of diacetyl it had produced was 4μg/ml. Streptococcus lactis, Streptococcus cremoris and Saccharomyces cerevisiae also produced diacetyl (2μg/ml, 1.4 μg/ml and 0.4μg/ml respectively) but their production was much less than that of Strept. diacetilactis. (Fig. 32).

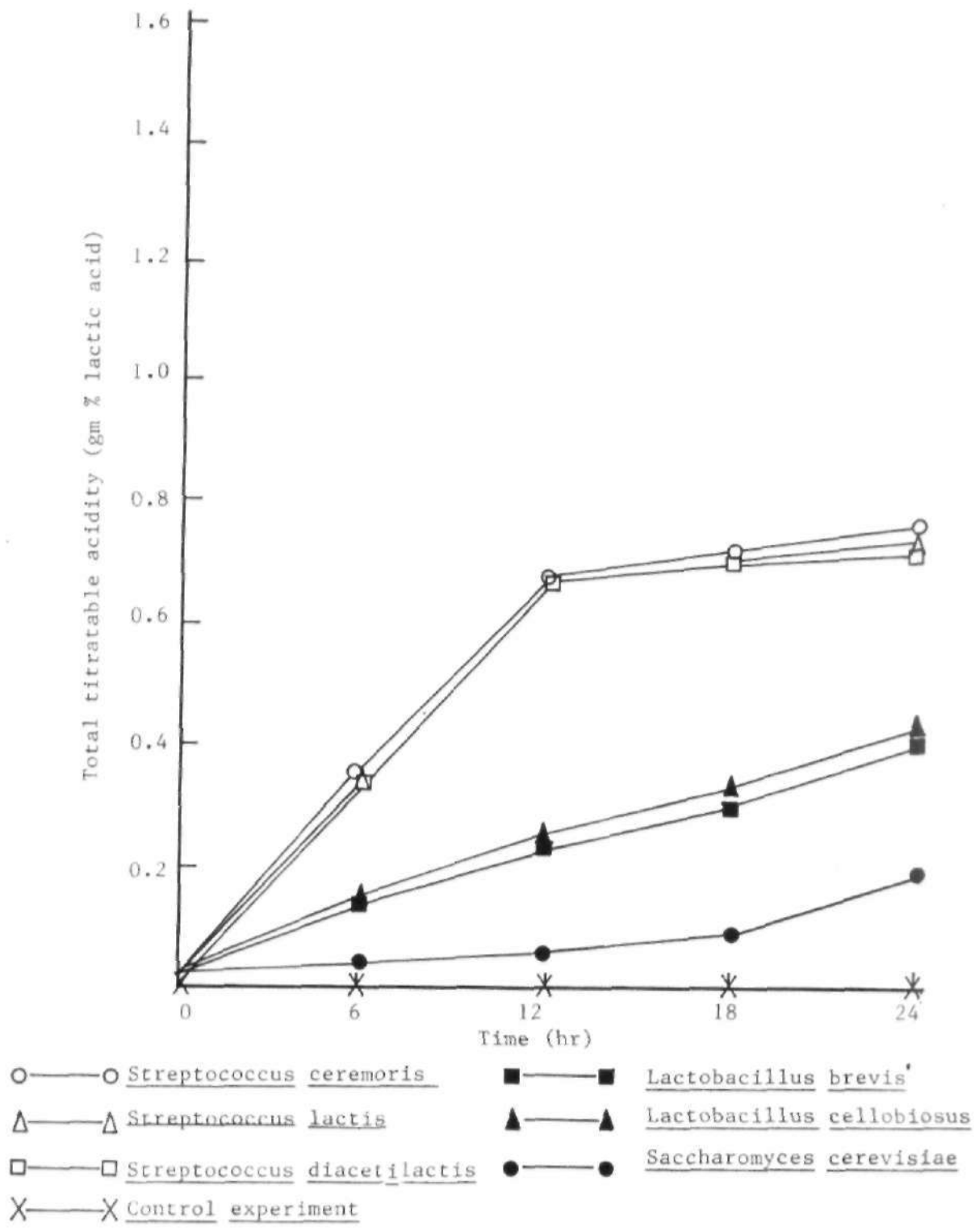


FIGURE 30: Effects of single starter culture on the total titratable acidity changes during "nono" production at room temperature

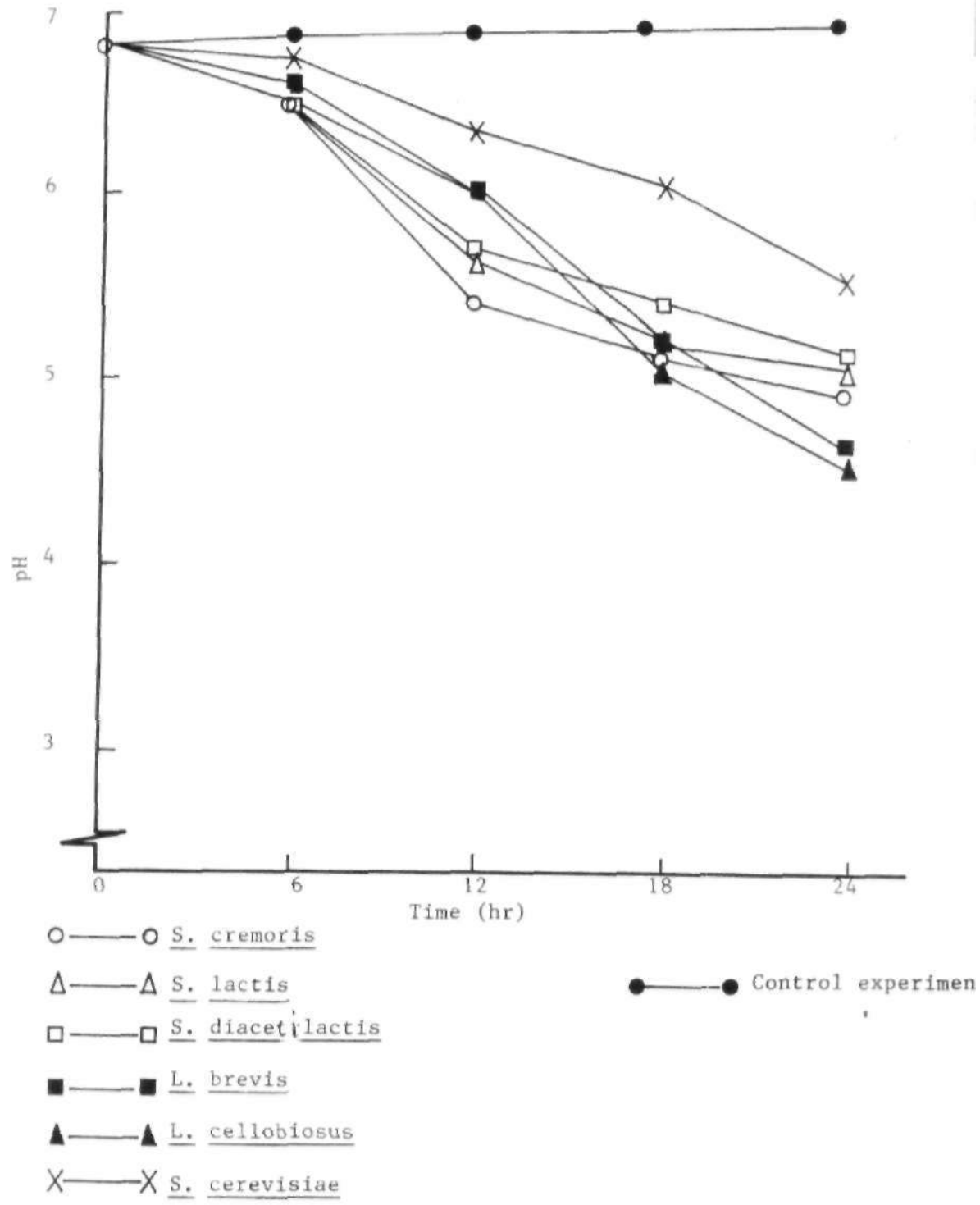


FIGURE 31: Effects of single starter culture organisms on pH changes during "nono" production at room temperature

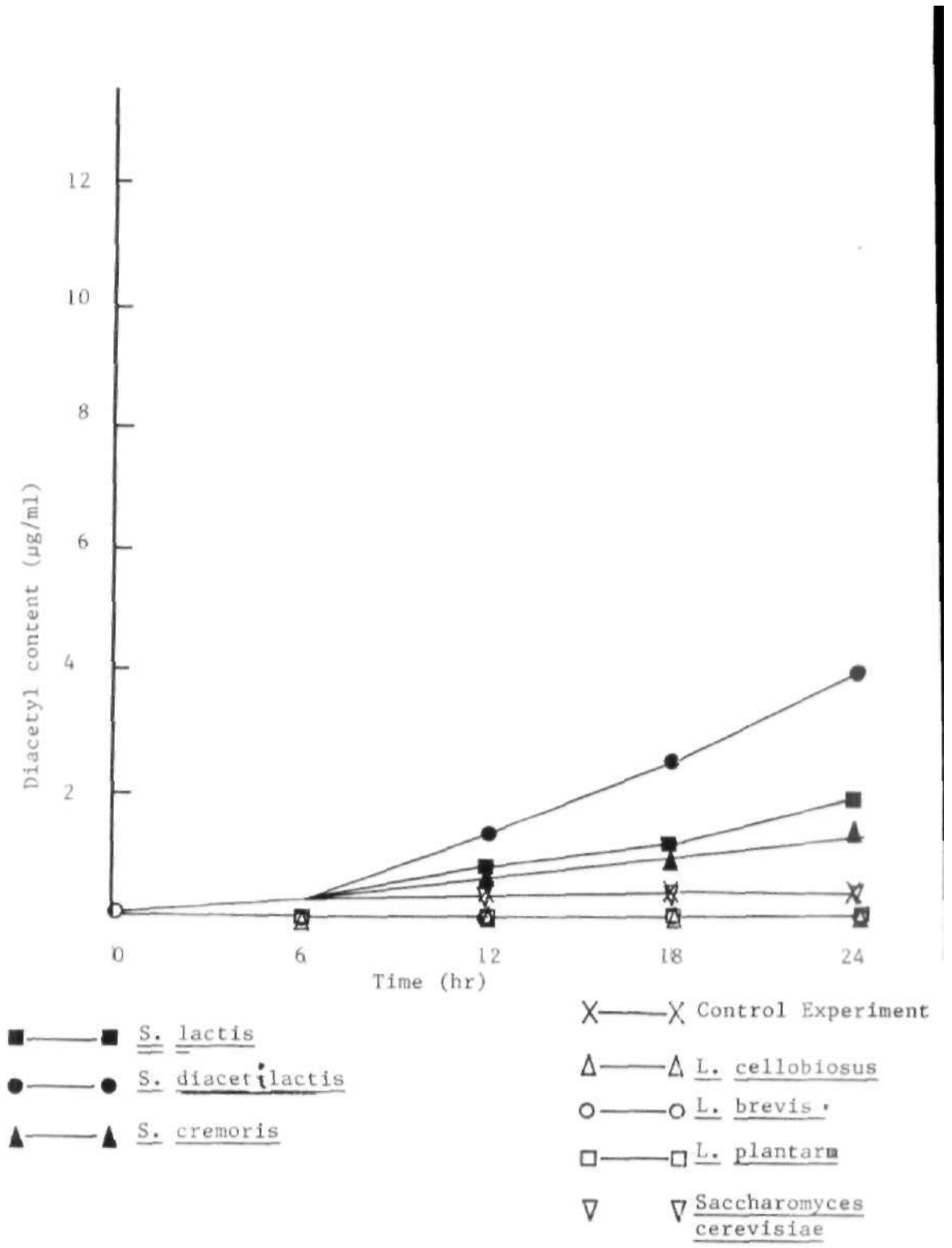


FIGURE 32: Effects of single starter culture on diacetyl content of milk during "nono" production at room temperature

4.5.3 The Effects of Mixed Starter Cultures on Diacetyl Content, pH and TTA Changes During "Nono" Production At Room Temperature

The combination of Streptococcus cremoris and Lactobacillus brevis (Fig.33), gave the highest pH drop (pH 6.9 to pH 4.4), closely followed by the combination of Streptococcus diacetilactis and Lactobacillus brevis (pH 6.9 to pH 4.5). The combinations of Streptococcus cremoris and Saccharomyces cerevisiae and Streptococcus diacetilactis nd Saccharomyces cerevisiae gave the pH drop of (6.9 to 4.7) while the combination of Lactobacillus brevis + Saccharomyces cerevisiae gave the least pH drop.

Similarly, the TTA production was highest for the combination of Streptococcus cremoris and Lactobacillus brevis, closely followed by the combination of Streptococcus diacetilactis and Lactobacillus brevis. The combinations of Streptococcus cremoris and Saccharomyces cerevisiae; Streptococcus diacetilactis and Saccharomyces cerevisiae produced the least but similar amounts of TTA (Fig. 34).

The diacetyl production was highest for the two combinations involving Streptococcus diacetilactis, (Streptococcus diacetilactis; Lactobacillus brevis and Streptococcus diacetilactis and Saccharomyces cerevisiae and least for the combination of Lactobacillus brevis and Saccharomyces cerevisiae (Fig. 35).

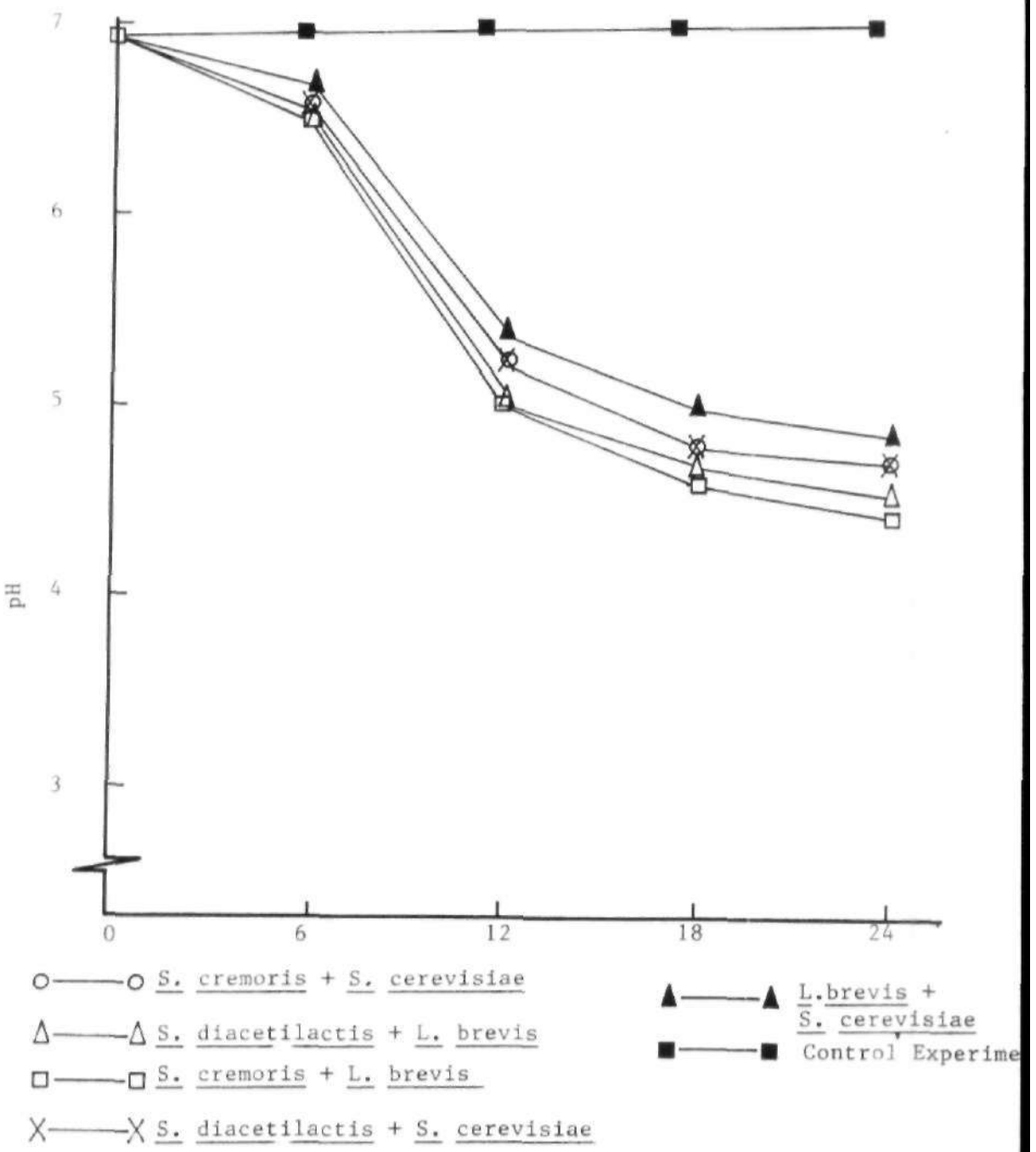


FIGURE 33: The effects of mixed starter culture on pH changes during "nono" production at room temperature

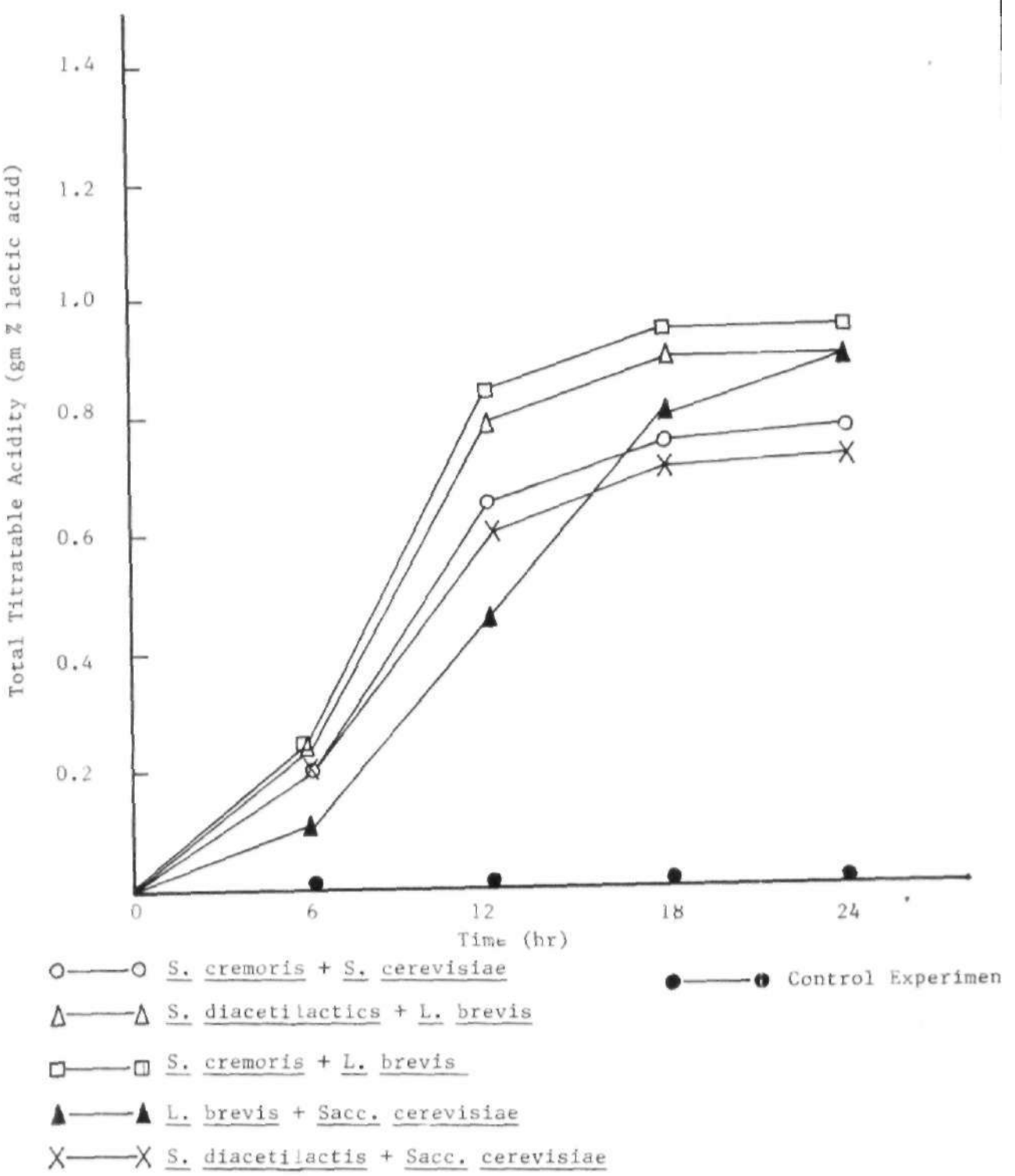


FIGURE 34: The effects of mixed starter culture of TTA changes during "nono" production at room-temperature

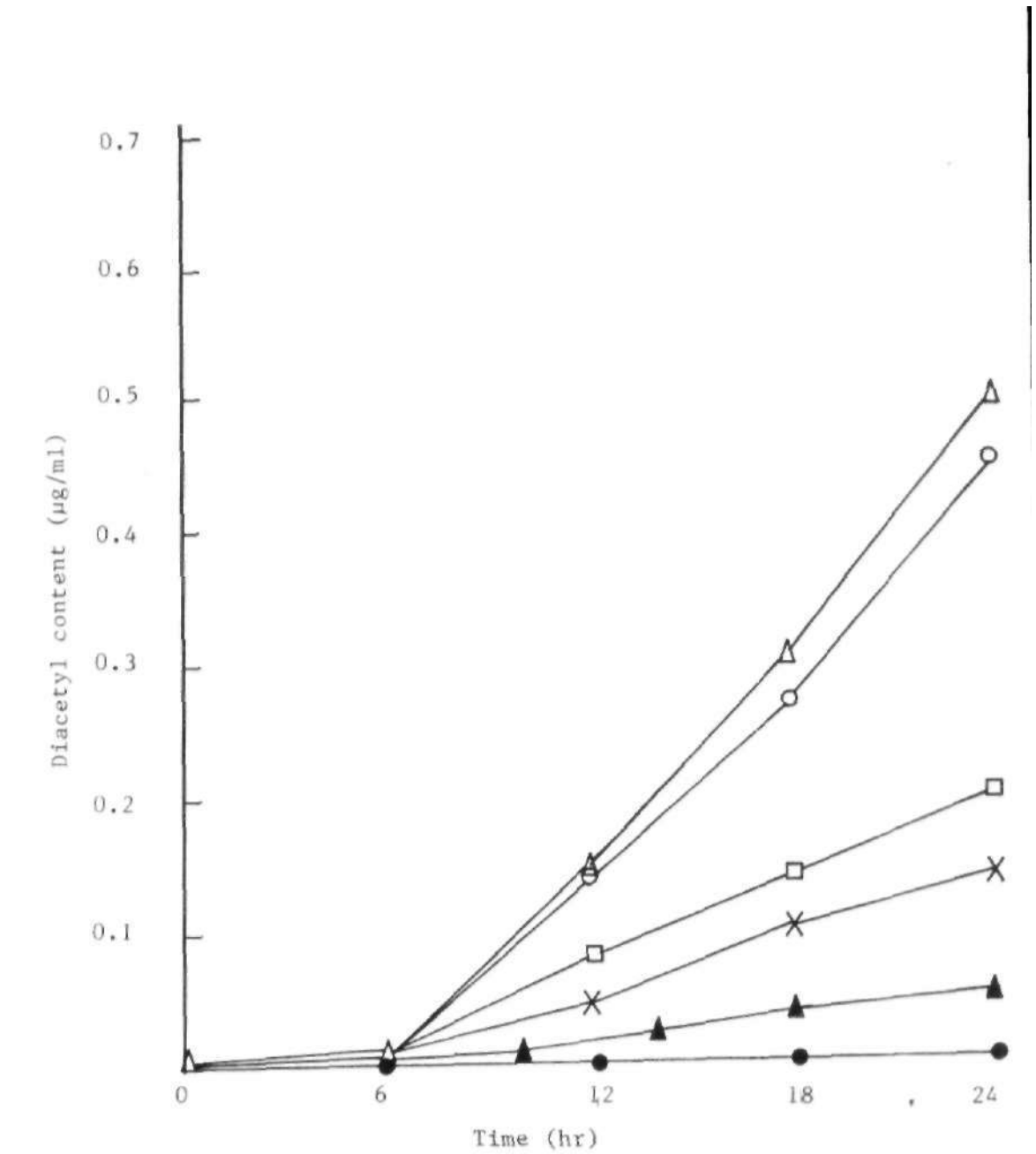


FIGURE 35: The effects of mixture starter culture on diacetyl content during "nono" fermentation.

While "Nono" produced from the combination of Streptococcus cremoris and Lactobacillus brevis, Streptococcus diacetilactis and Lactobacillus brevis was very well accepted, "nono" produced from the combination of Streptococcus cremoris and Saccharomyces cerevisiae; Streptococcus diacetilactis and Saccharomyces cerevisiae; Lactobacillus brevis and Saccharomyces cerevisiae were rated poorly (Table 19).

The effects on the changes of pH, TTA and diacetyl content of combinations of Strept. diacetilactis, + Lactobacillus brevis + Saccharomyces cerevisiae as starter culture for the production of "nono" is shown in Fig. 36. While the pH dropped from 6.9 to 4.3 within 24 hours of fermentation, the TTA increased from 0 to 1.2% lactic acid. The diacetyl content also increased from 0 to approximately 0.5㎍/ml. The TTA and the diacetyl produced by this combination of starter culture was higher than those of any other combination of two microorganisms, as starter culture. The "nono" produced from this mixture of starter culture had good acceptability (Table 18).

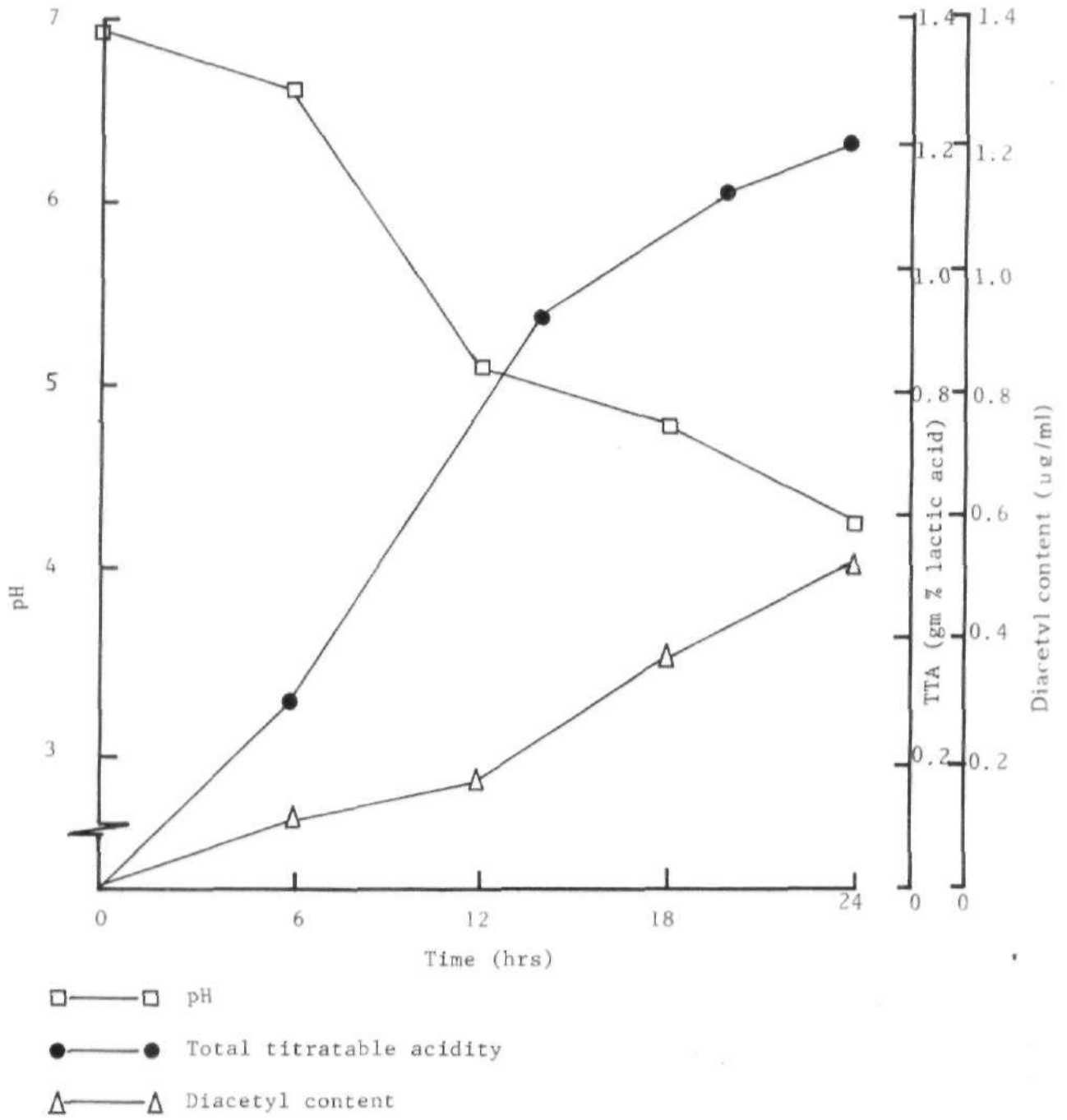


FIGURE 36: Changes in pH, TTA and Diacetyl content of milk fermented with mixture of S. diacetilactics, L. brevis and Saccharomyces cerevisiae as starter culture at 30°C.

4.5.4 The Effects of Closed and Opened System During "Nono" Production

The effects of closed and opened system on the pH, TTA and diacetyl content during "nono" production showed some slight differences. While the "nono" fermented under the closed system was a bit less sour, it was more frothy than the "nono" fermented under the opened system. Both "nono" produced were quite acceptable (Table 20). The pH in the closed system was a bit higher than that of the opened system, the TTA was also less than that of the opened system but the diacetyl content was more than that of the opened system (Fig. 37) at about 24 hour of fermentation.

TABLE 20 Qualities of "nono" processed under closed and opened system of fermentation at room temperature (* mean of sensory evaluation)

Qualities	Opened system	Closed Sytem
Colour	Dirty White	Dirty White
Odour	Pronounced	Pronounced
Viscosity	Thick curd	Slightly thick curd
Taste	sour	Slightly sour
Texture	Smooth	Slightly smooth
Overall acceptability	Good	Good

* Mean scores correspond to the description.

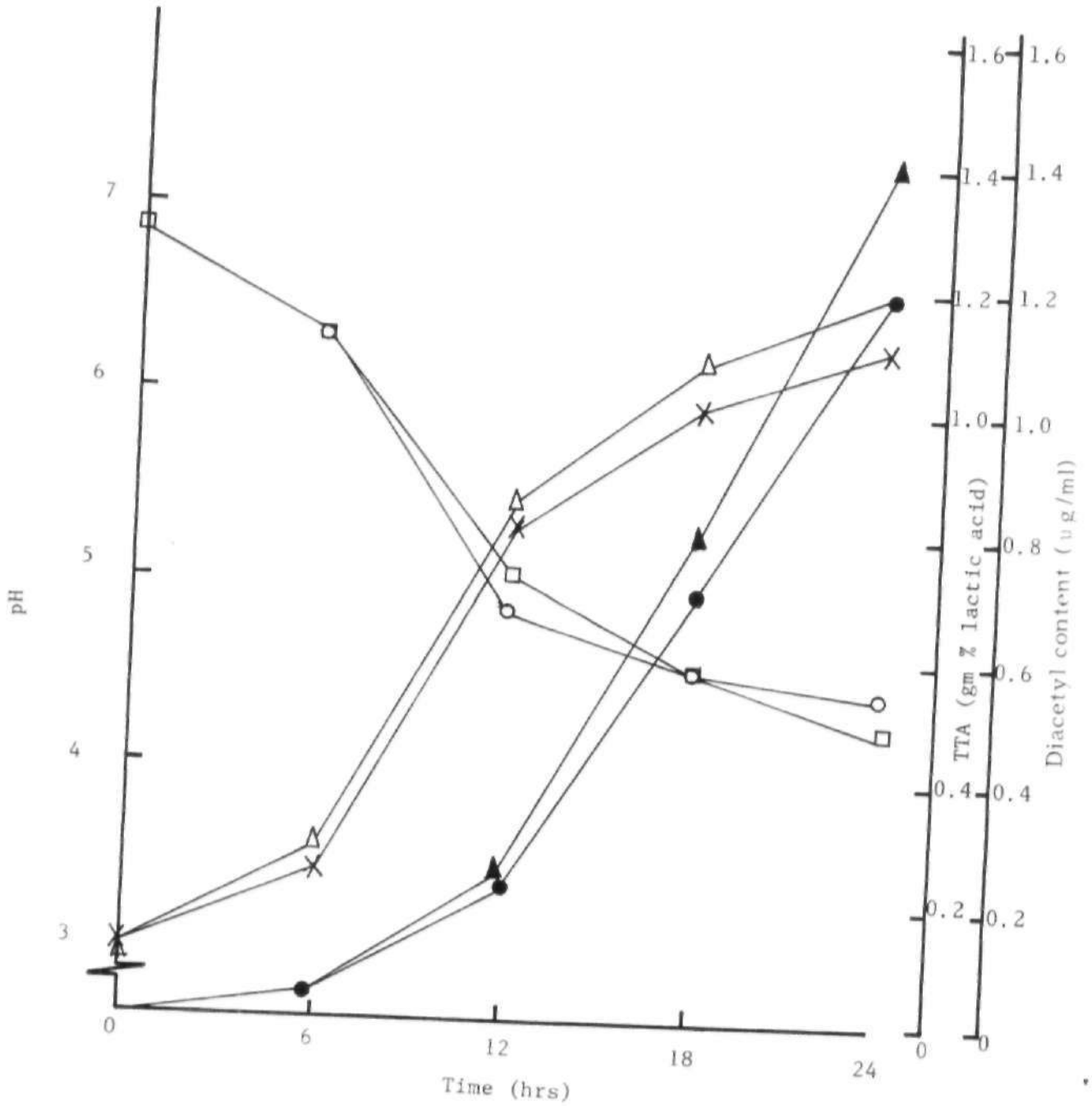


FIGURE 37: Effects of closed and open systems on the fermentation of "nono"

4.5.5 The Effects of Stationary and Agitated Fermentation in "Nono" Production

While the "nono" produced under agitated fermentation seemed more watery or less Viscous (Table 21), it also had more gas than the "nono" produced under stationary fermentation. The diacetyl content and the TTA of the agitated fermented "nono" were higher and the pH was less than the "nono" produced under stationary condition (Fig. 38). Both of these differently produced "nono" had good acceptability although the "nono" produced under stationary condition seemed more acceptable than that produced under agitation (Table 21).

TABLE 21 Qualities of "nono" processed under stationary and agitated condition of fermentation at room temperature.(* mean of sensory evaluation).

Qualities	Stationary condition	Agitated condition
Colour	Dirty White	Dirty White
Odour	Pronounced	Pronounced
Viscosity	Thick curd	Slightly thick curd
Taste	sour	Sour
Texture	Smooth	Coarse
Overall acceptability	Good	Fair

* Mean scores correspond to the descriptions.

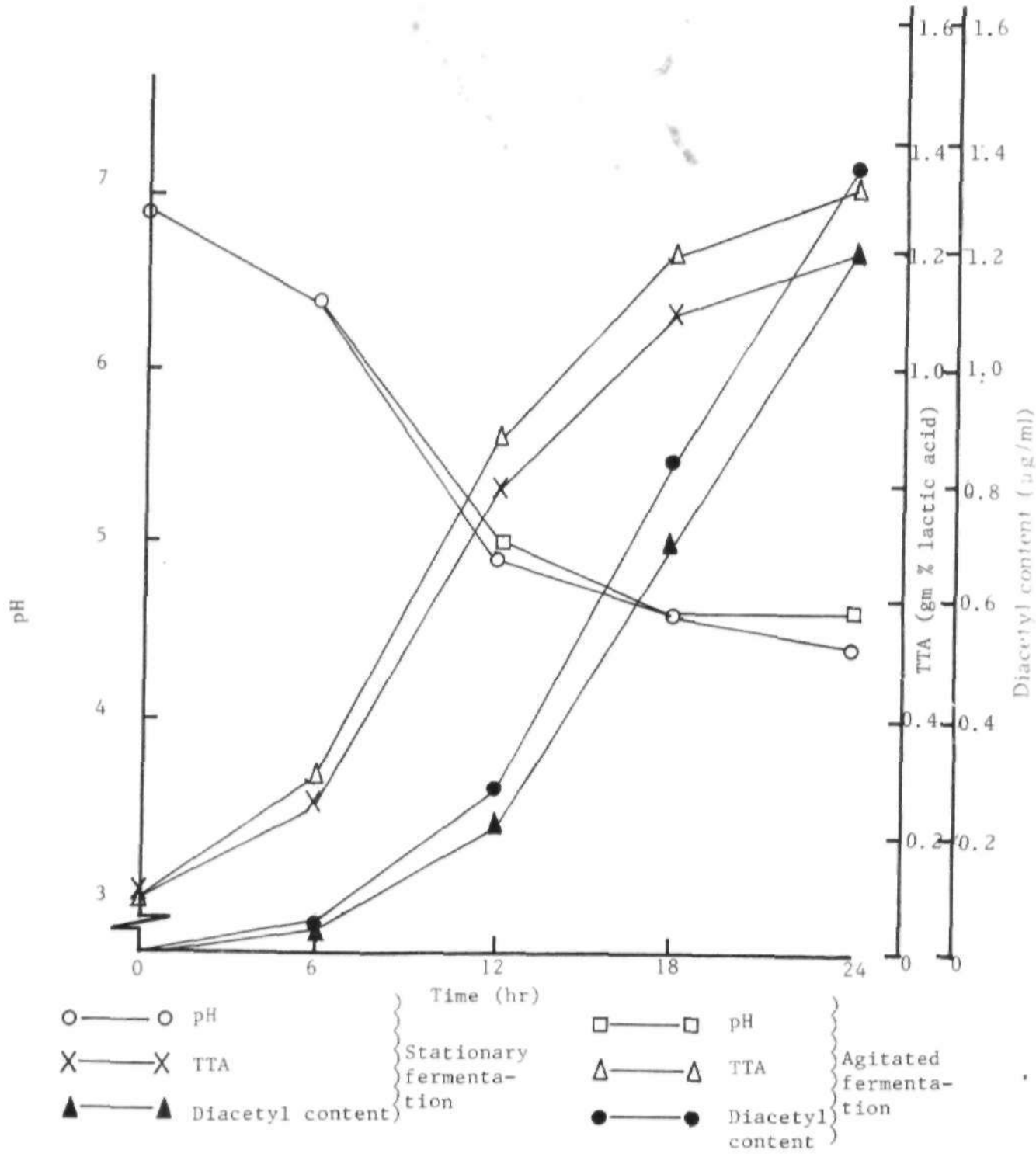


FIGURE 38: The effects of agitated and stationary condition of fermentation on "nono"

4.6 The Effect of Storage Temperature On "Nono"

Figures 39 and 40 show the effects of storage temperature on the total aerobic bacterial count, yeast count, lactic acid bacterial count, pH, TTA and diacetyl content of traditionally produced "nono".

"Nono" stored at 25°C for 48 hours was observed to increase in its microbial count (Total Aerobic Bacteria, Lactic Acid Bacteria and Yeast) within 24 hours of storage but decreased by 36 hours and further decreased by 48 hours (Fig. 39). At 48 hours of storage, the microbial populations in "nono" were less than the population in "nono" at zero hours of storage. The pH, decreased gradually from 4.5 at zero hour of storage to 4.1 at 36 hours of storage but the pH increased to 4.4 by 48 hours of storage. While the TTA increased with increase in time of storage (0.55 - 1.07 % lactic acid), the diacetyl content was correspondingly decreasing (1.2-0.18 µg/ml).

The total aerobic microbial count of "nono" stored at 4°C increased in the first 3 days of storage. Subsequently it decreased by the 6th, up till the 12th day. (Fig. 40). While the lactic acid bacteria count decreased with increase in the length of storage, the yeast count increased till the 6th day, after which there was a decrease by the 9th day and a further decrease by the 12th day. The yeast counts appeared to remain stable through out the storage period.

The pH was almost constant, with a pH of 4.5 at zero hour of storage, pH of 4.4 from the 3rd to the 9th day and a pH of 4.5 by the 12th day of storage. The TTA increased by the 3rd day of storage (0.63 % lactic acid) and remained almost constant till the 9th day (0.62 % lactic acid) after which it decreased to give a 0.57 % lactic acid by the 12th day. The diacetyl content increased steeply from day zero of storage to day 3 (Fig. 40) but decreased gradually after that to give a diacetyl content of 1.2μg/ml by the 12th day.

A taste panel assessment revealed that "nono" kept at 25° C was still quite acceptable at 24 hours of storage after which it was scored poor acceptability (Table 22).

"Nono" stored at 4°C was acceptable even by the 12th day of storage although the texture and viscosity had changed (Table 22).

TABLE 22 Qualities of traditionally produced "nono" stored at temperatures of 25 c and 4 c(means of sensory ratings

Temperature of storage	Time of storage	QUALITIES					Overall acceptability
		Colour	Flavour	Taste	Viscosity	Texture	
25° C	0 hours	a	c	e	i	m	p
	12 hours	a	c	e	i	m	p
	24 hours	a	b	f	g	k	o
	36 hours	a	b	f	h	k	n
	48 hours	a	b	f	h	j	n
4°C	0 days	a	c	e	i	m	p
	3 days	a	d	e	i	m	p
	6 days	a	c	e	g	m	p
	9 days	a	c	e	g	l	k
	12 days	a	c	e	g	l	p

KEY: Mean scores correspond to the descriptions below.

a. => Dirty white b. => Slightly pronounced c. => Pronounced d. => Very pronounced

e. => Sour f. => Very sour g. => Slightly watery h. => Watery

i. => Thick j. => Coarse k. => Slightly coarse l. => Slightly smooth

m. => Smooth n. => Poor o. => Fair p. => Good

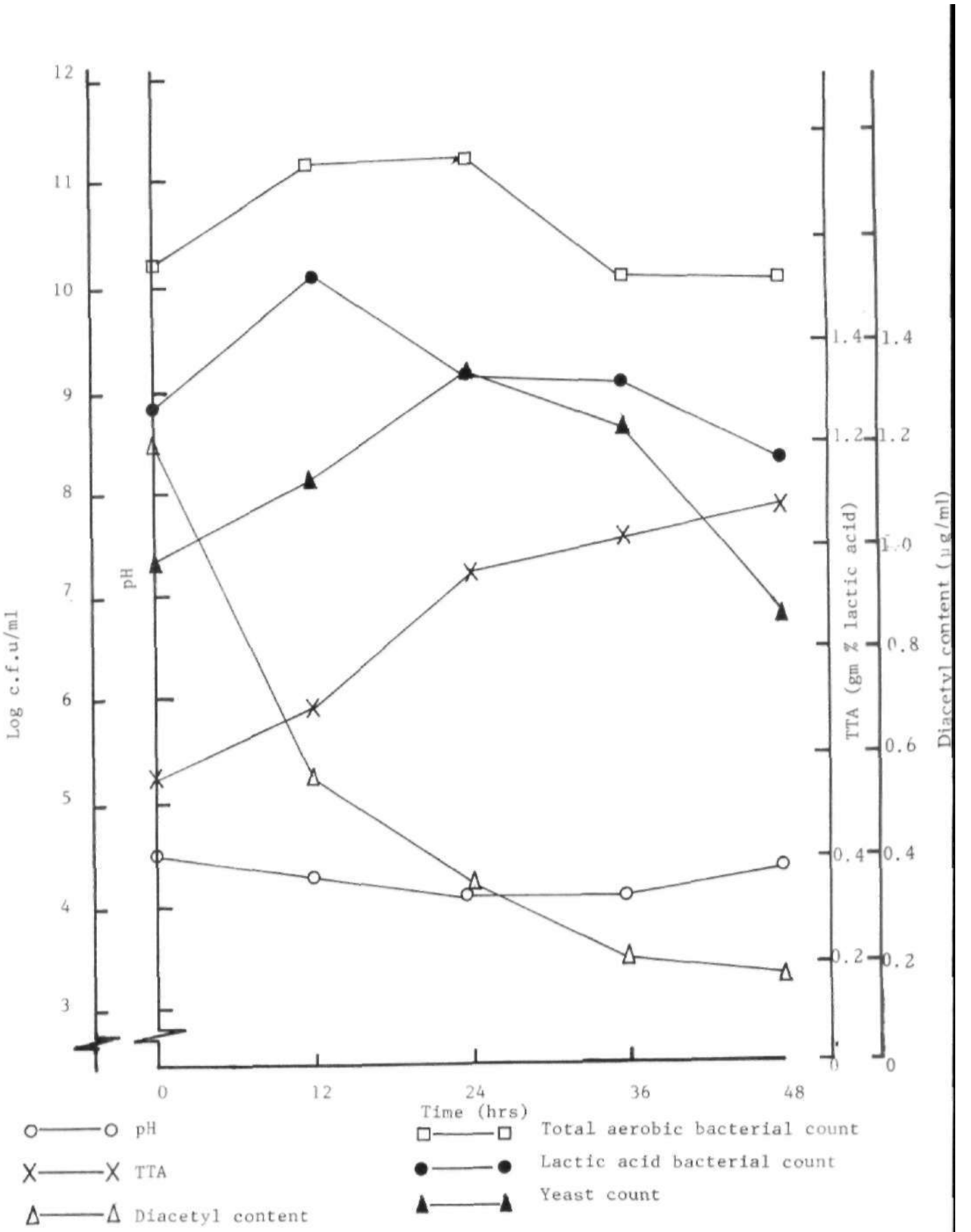


FIGURE 39: The effects of storing "nono" at 25°C

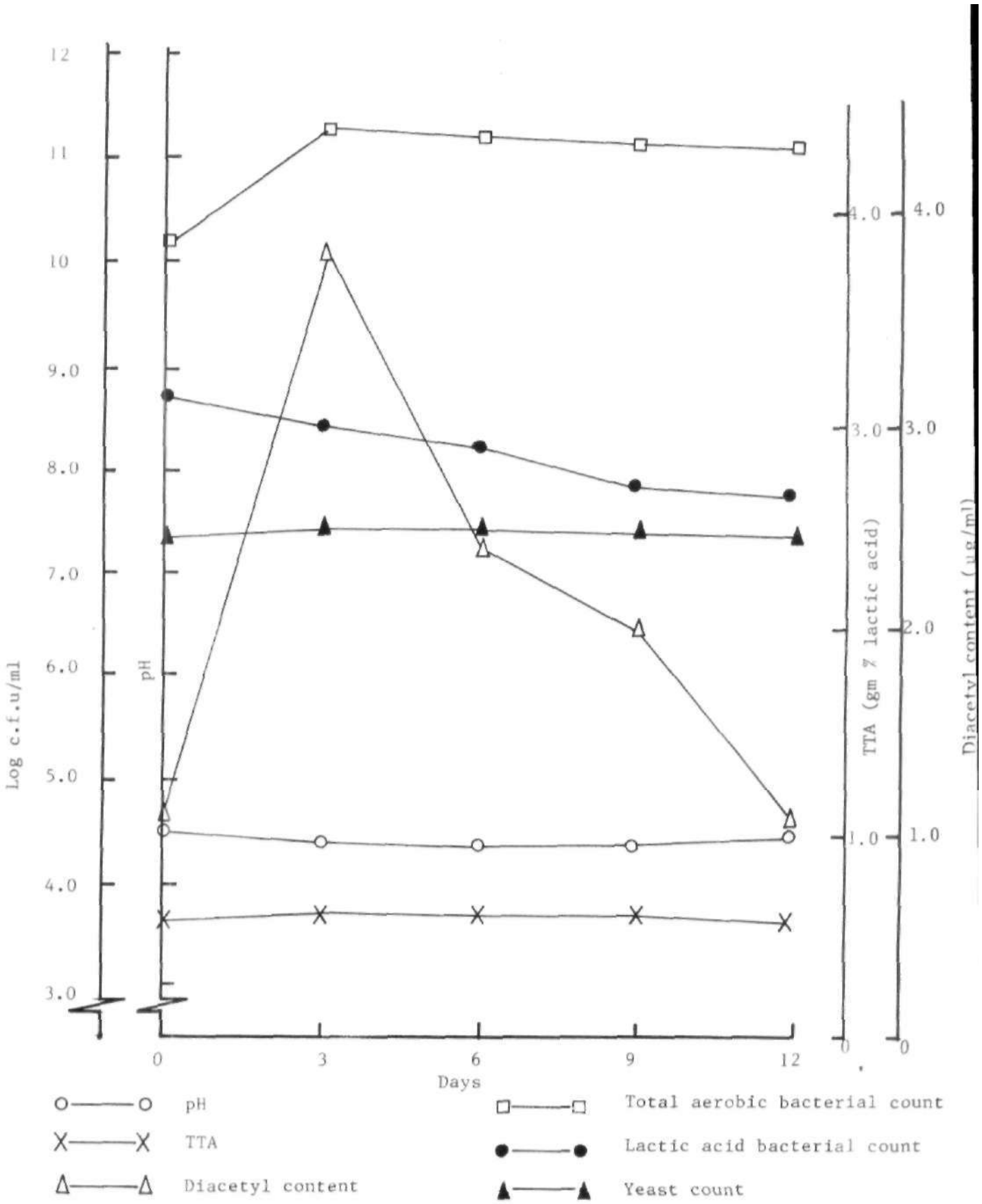


FIGURE 40: The effects of storing "nono" at 4°C

4.7 Other Microbiological Studies On "Nono"

The results of the effects of lactic acid bacteria isolated from "nono" on some spoilage organisms and on one another and the test for the presence of phages in "nono" are shown below.

4.7.1 Effects of Lactic Acid Bacteria On Other Microorganisms

The result is as shown in Table 23. The lactic singly and in mixtures had no inhibitory effects on the growth of Serratia spp., Micrococcus spp. and Pseudomonas aeruginosa. However, Streptococcus cremoris and Lactobacillus plantarum singly had inhibitory effects on E. coli with Lactobacillus plantarum having higher inhibitory effect. The mixture of Streptococcus lactis and Lactobacillus plantarum and mixture of Lactobacillus cellobiosus and Lactobacillus plantarum also had appreciable inhibitory effect on fermentation, on E. coli and on S. aureus. The inhibitory effect of the mixture of Lactobacillus plantarum and Lactobacillus cellobiosus on Escherichia coli. was the greatest. Mixtures of lactics had higher inhibitory effects on S. aureus growth than their single cultures but the inhibitory effect of mixture, of Streptococcus lactis and Streptococcus diacetilactis was greatest.

Table 23:

The inhibitory effect of the lactics isolated from "nono" on some other microorganisms

	Diameter of inhibition zone in mm excluding a well diameter 5mm.												
Test organism:	a	b	c	d	e	f	g	h	i	j	k	l	m
Escherichia coli	0	15	0	0	0	18	14	0	0	0	18	0	20
Serratia spp	0	0	0	0	0	0	0	0	0	0	0	0	0
Micrococcus spp	0	0	0	0	0	0	0	0	0	0	0	0	0
Pseudomonas aeruginosa	0	0	0	0	0	0	0	0	0	0	0	0	0
Staphylococcus aureus	8	12	12	8	8	0	13	14	10	11	7.5	10	9

Key: a - m are cell filtrates of the lactics used where

(a) = *Streptococcus lactis*

(b) = *Streptococcus cremoris*

(c) = *Streptococcus diacetilactis*

(d) = *Lactobacillus cellobiosus*

(e) = *Lactobacillus brevis*

(f) = *Lactobacillus plantarum*

(g) = a + b ; (h) = a + c ; (i) = a + d ; (j) = a + e ; (k) = a + f ;

(l) = d + e ; (m) = d + f.

4.7.2 Effects Of The Lactic Acid Bacteria Isolated from "Nono", on one another

It was observed that there was no inhibitory zone on any of the sections. There were thicker growths on where two isolates were dropped together which gave an indication that the organisms grown together probably stimulated each other's growth.

4.7.3 Screening for the Presence of Bacteriophage in "Nono"

No plagues which would have been an indication of the presence of bacteriophages in "nono" were observed after incubation.

Chapter 5

DISCUSSION

Fermentation is an ancient method of milk preservation which has yielded many well - known food products today. These include yoghurt, acidophilus milk, cheese, butter, kefir and kumiss among so many. These products are well known. In addition their processing technology and the microoganisms involved in their production are well established.

In this study, investigations were carried out on the production of "nono" by fermentation of milk in Zaria, northern Nigeria. The microorganisms implicated in this investigation are the lactic acid bacteria: S. lactis, S. cremoris, S. diacetilactis, L. cellobiosus, L. brevis, L. plantarum and the yeasts C. pseudotropicalis, C. tropicalis and T. beigelii. The well known species such as Lactobacillus bulgaricus and Streptococcus thermophilus associated with the production of yoghurt, Lactobacillus acidophilus used in the production of butter milk (Pederson, 1979), all similar to "nono" in preparation, were not isolated in this study. However, the cooler mountainous area of Europe and Asia with temperature similar to the northern temperate Asia climate have a preparation similar to yoghurt and not using the well known strains but others such as Streptococcus lactis, Leuconostoc cremoris and Lactobacillus casei. Lactobacillus bulgaricus may grow slowly if the

fermentation is allowed to continue for a long time (Pederson, 1979). Moreover, Streptococcus lactis and Lactobacillus bulgaricus are known to antagonise each other in milk fermentation though the antagonism subside with time. It is likely that environmental influence and antagonism between the strains were responsible for the relative absence of some well known strains in "nono".

There are, however, products with wide acceptance in Eastern Europe and Westen Asia which have similar microorganisms to those of "nono" in their preparation. For example, Kefir,which contains about 1% acid and 1% alcohol from southern Russia, Turkey and the Balkan, is prepared from the fermentation of milk by a variety of microoragnisms. These include L. bulgaricus, L. casei, L. brevis and Torulopis holmii (Pederson, 1979). Moreover kumiss, a milk-fermented preparation from the Asiatic peoples of the plains of Western Asia contains about 0.7 - 1.3% (and rarely 2%) acid, and often retains its CO_2 which gives it exhilarating effect. Its production is brought about by the fermentation of milk by a mixture of Strept. lactis, Lacto. casei and yeasts (Pederson, 1979).

"Nono" bought from the market and ready for consumption (market "nono") in the three different villages showed very high microbial load and the same pattern, with the total aerobic bacterial counts being higher than the yeast count and no mould was detected (Table 2). The high microbial load is not surprising,

since the starting material (milk) is almost a complete diet (Porter, 1975) and its biological value changes little with fermentation (Alms, 1981).

Though the pH and the total titratable acidity (Table 3) of the market "nono" samples were around the same range, the diacetyl content varied greatly, probably owing to the presence of the different loads of aroma-producing microorganisms in the milk. The wide variations also observed in the proximate and mineral element contents of market "nono" (Table 4) might be due in part to the effects of additives used in "nono" immediately after fermentation.

The processing of "nono" in the three villages around Zaria, were studied. The methods used and the changes occurring during the processing were found to be similar (Figs. 1-10). Such similarities in food processing methods in the same regions of Africa have been reported from cassava products (Odunfa, 1988) and even in fermented milk products in different regions all over the world (Keogh, 1978; Pederson, 1979). However, there could be slight variations in the type of milk used in fermentation. Usually, milk is pooled from different cows. These cows' milk composition could be affected by herds, feed, breed, ages, stages of lactation and colostrum level (Porter, 1975; Mclean et al. , 1984). This could contribute to the difference in texture, colour

and taste of the fermented products. Another source of variation observed in "nono" from the different villages could be the slight differences in the number and types of microorganisms in the milk used for fermentation and the length of fermentation. The number and types of microoganisms depend on the time of the year and area of exposure (Radmore et al., 1988). These are known to influence the acidity and the quality of milk-fermented products (Pederson, 1979).

The presence and the amount of water and or additive used in "nono" after processing could also lead to variation in "nono". The addition of water could dilute the "nono" affecting the texture, viscosity, pH, the total titratable acidity and even the flavour. A known additive called Kuka (cream of tartar of Baobab fruit), often used on "nono" has a pH of 3.2 and lowers the pH of "nono" on addition (Makinde 1979). This additive, being a white substance, could affect the colour of "nono", making it look whiter. Its total titratable acidity, viscosity and flavour could also be affected. Kuka is known to lower the micobial load of "nono" (Makinde, 1979) and therefore, could lead to variation in its shelf life.

None of the milk used in the traditional fermentation process underwent pasteurisation or exposure to any source of heat or light. This non-pasteurisation in the traditional method of fermentation possibly protects the heat labile nutrients, in particular, the water soluble

vitamins (Harris and Karmas, 1973; Rolls and Porter, 1973) and other growth stimulatory factors in yoghurt (Hargrove and Alford, 1980). Also, heat treatment of milk could affect the viscosity of Yoghurt (Parnell-clunies et al., 1986). Although pasteurisation temperature has been observed to improve milk viscosity, HTST treatment of milk produces Yoghurt of low viscosity.

The predominant spectrum of microorganisms isolated from market "nono" samples include bacteria such as Bacillus spp., Streptococcus lactis, Streptococcus cremoris, Streptococcus diacetilactis, Lactobacillus plantarum, Lactobacillus brevis, Lactobacillus cellobiosus (Table 5) and yeast, made up of Candida tropicalis, Candida pseudotropicalis, Saccharomyces cerevisiae, Trichosporon beigelii, and Endomyces species (Table 6)

Bacillus spp have been found to be associated with many traditionally fermented foods in Nigeria. These include Ogi (Akinrele, 1966; 1970); Ogi-baba (Adeyele, 1986) Gari (Okafor, 1977); Fufu, Lafun (Oyewole and Odunfa 1988), Legume-based fermented foods such as Ogiri, Iru (Oyewole and Odunfa, 1986), and Daddawa (Ogbadu and Okagbue 1988). Bacillus spp have also been associated with other fermented foods of the world such as fish sauce (Saisithi et al. 1966), Corn meal (Nanson and Fields, 1982) and many differently fermented milk products (Pederson, 1979). However, not many species of Bacillus

have been found to participate in the fermentation of such foods (Pederson, 1979). Generally, it is only in legume-based food that Bacillus spp are exclusively important for obtaining desirable products. In this study, their role is not clear but since they may produce proteolytic enzyme, which will hydrolyse casein in milk, their activity may lead to milk spoilage.

Also, the presence of Bacillus spp. in milk produces undesirable changes just as the presence of many other genera of bacteria, such as Proteus, Serratia, Salmonella, Shigella, Brucella and Pseudomonas and Yeast (Pederson, 1979). Some of the undesirable effects may result in food-poisoning illnesses. Though many species of Bacillus are known to be involved in food poisoning (Jay, 1978), and survive at both acidic and alkaline pH (Geopfert et al.1972), and also have long range of growth temperature (10^{0} C - 50^{0} C), the production of the antimicrobial substances by the lactics and the removal of carbohydrates in milk (Goel et al., 1971; Hurst, 1972; Bartholomew and Blumer, 1980 a: b:and Daeschel, 1989) may not favour the toxin production of Bacillus in "nono".

Lactic acid bacteria have also been involved in some African fermented foods. These include mainly the products of acidic processes such as found in products of fermented cassava (Okafor, 1977; Okafor and Oyolu, 1984; Oyewole and Odunfa, 1988); in fermented vegetables (Etchells et al., 1975;Kotzekidou and Roukas, 1986) and

fermented milk products (keogh, 1978; Pederson, 1979 and Kroger et al ., 1989). In this study, the lactic acid bacteria isolated in "nono" and in the production of "nono" were similar to those always found in acidic fermented foods. The streptococcus species (S. cremoris and S.diacetilactis isolated are very well known starter organisms in natural or controlled fermented milk and fermented products (Keogh 1978; Pederson, 1979). Of the three species of Lactobacillus encountered in this study, only Lactobacillus brevis is a common strain of milk and fermented milk (Pederson, 1979). Both Lactobacillus cellobiosus and Lactobacillus plantarum are very commonly found in many foods (Buchanan and Gibbons, 1974). L. plantarum has been observed to have its origin in plant materials (Pederson, 1979; Buchanan and Gibbons, 1974 and Harrigan and McCance, 1976). Lactobacillus cellobiosus on the other hand has been associated with vegetable fermentation but more often with the oral cavity (Buchanan and Gibbons 1974). However, all the three species of Lactobacillus have been found to become established in natural fermentation, especially of vegetables (Etchells et al. 1975; Kotzekiodu and Roukas, 1986).

The presence of these lactic acid bacteria in milk is not surprising. They probably arose from the environment (the air, the soil and the grass). The animals milked often are usually tethered on the bare ground where they lie on the soil and eat grass. These

animals also go grazing, eating grass. They are usually not cleaned up before being milked and often, the ewe is allowed to stimulate lactation when milking is about to take place. However, L. cellobiosus, being a normal inhabitant of the oral cavity (Buchanan and Gibbons, 1974), could possibly be involved in the digestion of food in which milk and its products could be included . An investigation to know if this strain (L. cellobiosus) could be of use in milk fermentation could be carried out.

All the lactic acid bacteria isolated from "nono" were mesophilic (Fig. 11 - 16). The three Streptococcus strains (Strept. lactis, Strept. diacetilactis and Strept. cremoris) grew best at 35°C (fig. 14 - 16), while their growth at 30° C and 25°C were similar and next to the growth at 35°C. Growth of Strept. cremoris and Strept. diacetilactis was poor at 40°C. Various workers have given the optimal temperature for the growth of Strept.lactis as between 30°C and 40°C. While Buchanan and Gibson (1974) have the optimal temperature at 30° C, Dohn and Rahn, (1939) observed it to be 40°C or slightly higher. All the three strains of streptococcus isolated gave high total titratable acidity (fig. 30) at room temperature (25° C - 30°C). Golding et al. (1943), observed that 30° C was the temperature at which streptococcus produce acid most rapidly.

The optimum temperature of growth of the three most commonly found Lactobacillus specie in "nono" was at 40 c followed by growth at 35°C (fig. 11 - 13). This agrees with Marth (1974), who gave the optimum temperature of growth of Lactobacillus spp to be between 30°C and 40° C. In this study, Lactobacillus cellobiosus and Lactobacillus brevis gave a low total titratable acidity (fig. 30), while that of Lactobacillus plantarum was a bit higher. This also agrees with the views of Buchanan and Gibbons (1974), that Lactobacillus cellobiosus and Lactobacillus brevis give little acid in milk.

In this study, the pH of the naturally and traditionally fermented milk was found to drop from around 7 to around 4.3 ± 0.5 (fig. 26). The lactic streptococci (figs. 22-24) were observed to grow very well at neutral and near neutral pH (pH 5 - pH 8) while the Lactobacillus (figs. 19-21) grew well in acidic pH range (pH 3 - pH 5). This indicates that the lactic streptococci probably started off the reaction in the milk and lowered the pH enough for the Lactobacillus specie to start growing to further lower the pH. Similar association has been observed by many workers. Suhaimi et.al. (1987), observed that not only did an association of lactic acid bacteria readily adapt to alkaline pH values, they were also capable of growing at pH 10.0 and rapidly producing lactic acid down to a final of pH 4.0. While the lactic streptococcus present in the enriched association were

capable of growing at pH 9.5, the lactobacilli were only capable of surviving at such pH values. However, Bautista et al. (1966), reported that the lactobacilli stimulate the growth of the streptococcus by liberating essential amino acids from the milk proteins.

The three most dominant strains of streptococcus observed in this study are all homofermenters whose end product of glucose fermentation have been reported to be lactic, acetic and formic acids, Carbon - dioxide, ethanol, glycerol, diacetyl, acetoin and 2,3 - butanediol under anaerobic condition (Platt and Foster, 1958) and acetaldehyde (Keenan et al., 1966), but mainly lactic acid. However, the hetero fermenters produce equal molar amounts of lactic acid, carbon-dioxide and ethanol from hexoses. Of the lactobacillus species isolated, L. plantarum is homofermentative while L. cellobiosus and L. brevis are heterofermenters (Buchanan and Gibbons, 1974). The end products of the fermentation in this study were not examined but the presence of alcohol, carbon - dioxide and other volatile organic compounds could not be ruled out.

At the initial stage of fermentation in this study, only Streptococci strains were isolated, (Table 11). the streptococci strains probably had to lower the pH of the medium (milk) to allow the Lactobacillus to grow. The yeast counts were low at the beginning of the fermentation but were mainly Saccharomyces cerevisae, Candida

pseudotropicalis and Candida tropicalis. The appreciable increase in the number of yeast probably was due to more available easily digestable nutrients in form of lactic acid formed from the break - down of lactose by the lactic acid bacteria and an acidic medium of growth. Lactic acid bacteria are believed to provide an acidic environment for growth of yeast while the yeast provide vitamins and other growth factors for the growth of lactic acid bacteria (Nout 1980). Endomyces spp and Trichosporon beigelii settled into the fermenting medium at about 18 hours of fermentation probably as spoilage organisms (Pederson 1979).

Almost the same effects, microbiologically and biochemically, were obtained by processing "nono" at 25°C, 30°C and 37°C (Figs. 27 - 29). However, the taste panel assessment favoured the "nono" production at temperatures 25°C and 30°C most. The total titrable acidity production at these two temperatures were found to be highest among the temperatures tested. It was probable that the lactic acid content at 37°C was easily converted to other end products, the temperature of 37° C favouring higher enzymatic reaction. These end products probably changed the taste of "nono" slightly. The most acceptable temperatures of fermentation of 25°C and 30°C happen to be the range of temperature at which "nono" is traditionally produced and lactic streptococci best produce acid (Golding et al. 1943).

The use of single starter culture (Fig. 30 - Fig. 32; Table 19) in milk for the production of "nono" did not produce the exact desired effect; however, the use of some mixed starter culture of Streptococcus strain and a lactobacillus strain in milk, produced "nono" that was acceptable and similar to naturally produced "nono" (Figs. 33 - 35; Table 19). The inter-relationship between Streptococcus and Lactobacillus have been known for long (Bautista et al. 1966) and have often been associated together in many fermented milk products (Keogh 1978; Pederson, 1979). Keogh (1978), stated that acidophilus milk contains only Lactobacillus acidophilus as starter culture but in an attempt to improve its flavour, Strept. cremoris or Strept. lactis and an aroma organism such as Strept. diacetilactis or Leuconostoc or alternatively with L. bulgaricus and S. thermophilus were inoculated in a quantity of milk and mixed with L. acidophilus as starter culture.

"Nono" is a very acidic product with a strong flavour. The consistent isolation of Saccharomyces cerevisiae and lactose fermenting yeast - Candida pseudotropicalis may suggest that ethanol may be formed in "nono". Although no attempt was made to detect ethanol in this study, it is conceivable that glucose and or galactose produced by hydrolysis of lactose by the yeast will be fermented by S. cerevisiae. This speculation appears to be valid because a mixed starter culture

containing S. cerevisiae was shown in this study to produce the most acceptable "nono". Apparently, the ethanol may even react with other substances to produce substances like esters which might even improve flavour.

Both closed and opened systems of "nono" production using the most acceptable mixed culture (Strept. strain, Lactobacillus strain and Saccharomyces cerevisiae) produced acceptable "nono" (Fig. 37, Table 20) but the product of the opened system processing was more acceptable. This is probably due to the fact that the "nono" obtained under closed system had very strong flavour, produced more gas and was less sour. It appears that the microoganisms involved in the fermentation were more active in the closed than in the opened system and their activities resulted in the production of more gases which were undesirable. Similarly, although these organisms were constantly isolated, localities may have effect on them. It is well known that geographical factors may influence the species of organisms that can be found in foods. Different types of "nono" can be produced commmercially with different mixtures of microorganisms.

Stationary and agitated modes of fermentation appeared to produce similar types of "nono" (Fig. 38 and Table 21). The "nono" produced under agitation was more watery and had higher diacetyl content and higher total titratable acidity than that produced under stationary condition. This could be due to increased reaction under

agitation. This affected the viscosity which is one of the appreciated qualities of "nono".

Preservation of fermented milk is difficult in tropical countries where refrigeration facilities are not adequate. "Nono" processed and stored at ambient temperature (25° C ± 4°C) had less bacterial load than "nono" processed at ambient temperature but stored at 4° C. This is probably due to the growth of psychrotrophic bacteria at the favourable temperature of 4° C. Such psychrotrophic organisms have been reported to occur in some fermented milk products e.g cultured milk products e.t.c (Jay, 1978; Pederson, 1979; Hogarty and Frank, 1982). Further work would be required to determine whether some of the streptococcus species isolated from "nono" are psychrotrophic bacteria relatively. The total titratable acidity of "nono" stored at 25° C increased rapidly while its pH correspondingly decreased compared to "nono" store at 4°C whose titratable acidity increased slightly with slight fall in pH (Fig. 39 and 40). These reactions might be due to the effects of some psychrotrophic lactic acid bacteria. These produce in milk slow increase in acidity and slight fall in pH with rapid proteolytic changes in milk without any curdling effect (Sudarsanam and Srinivasan 1987). While the mesophiles in "nono" stored at 25°C continue their activities, those in "nono" stored at 4° C may be inactivated or may have their reactions slowed down, while the psychrophiles take over.

"Nono" samples stored at 4°C were more acceptable (pH 4.4, diacetyl content 1.1 µg/ ml) than "nono" stored at 25° C (pH 4.4, diacetyl 0.84 µg/ml) probably because the "nono" stored at 25°C was too acidic and had pronounced flavour (Figs 39 and 40). Another possible reason is that rancidity changes would occur more readily "nono" stored at 25°C.

The diacetyl content of "nono" stored at 4° C rose steeply within the first three days of storage (Fig. 40), then fell gradually from the 6th day to give the same amount of diacetyl content on the 12th day as that of fresh "nono" about to be stored. The reason for the sharp rise is not clear but more work should be done on psychrophiles to find if they contribute to increase in diacetyl content. The decline can be explained because some psychrotrophic lactic streptococcus occuring in fermented milk products can produce diacetyl reductase and consequently reduce the diacetyl content of the product even at low temperature (Seitz et al. 1963, Hogarty and Frank 1982). There might have been natural selection of mutants in which streptococcus which lack reductase have been selected out. Those with and without have been present one time or the other (fluctuate) in the system.

That lactic acid bacteria produce antimicrobial and antiprotozoic metabolites is becoming well documented (Daeschel, 1989). It was not surprising therefore that

the lactic acid bacteria tested against other microorganisms inhibited some of them like Escherichia coli and Staphylococcus aureus (Table 2.3). This is in agreement with the findings of other workers such as Hurst (1972), Daly et al. (1972), and Moon et al. (1982). The lack of inhibitory effects of the lactics on the growth of some of the microorganisms like Serratia spp., Micrococcus spp. and Pseudomonas aeruginosa might be due to the medium used for the cultivation of the lactic acid bacteria prior to testing (Moon et al. 1982). This medium is said to determine the degree of inhibitors of lactic acid bacteria. Moreover, the addition of 2% level of a fermentable carbohydrate to the medium of growth supports the production of antimicrobial substances (Rao and Pulusani, 1981).

The lactic acid bacteria seemed to have had some synergistic effects on one another as there were heavier growths where any two lactics met on the growth medium. The pulling together of their elaborated anti-microbial metabolites probably increased the concentration of such metabolites in the medium of growth leading to stronger inhibition of any other organisms except the lactics. Thus, the competion for the nutrients and other materials for growth in the medium will be reduced. The probability of a metabolite produced by one strain of the lactics stimulating the growth of another cannot be ruled out. For example, Streptococcus species probably reduces the pH

of milk as a medium of growth for the Lactobacillus species to grow while yeast will thrive better in a medium whose lactose has been converted to lactic acid by the lactics. The lactic and acetic acids produced by the lactics in weakly buffered media give synergistic interactions between the two acids.

No phages were found in "nono" in this work. This probably means that "nono" samples examined have no bacteriaophage infections. Some gastro-intestinal disorders could be caused by virus (Gordon et al. 1957 and Silver et al. 1961) in food. Presence of phages may not only prevent the lactics from giving the desired end product from the fermentation of milk, it could totally destroy the lactics.

A general inference that can be made from this study is that microorganisms needed for the manufacture of "nono" could be a mixture of Streptococcus lactis or Streptococcus cremoris or Streptococcus diacetilactis preferrably, with Lactobacillus brevis. The presence of yeast Saccharomyces cerevisiae is probably an asset as it might give flavour to "nono". The role of Lactobacillus cellobiosus in the fermentation should be investigated further. Also, the effect of changing the inoculum ratio of the microorganisms found to be suitable would need to be determined. There are many dairy products manufacturing companies in the country, especially in the Northern part of Nigeria and Yoghurt is one of the

manufactured products. Lyophilised strains of Streptococcus thermophilus and Lactobacillus bulgaricus with powdered milk are usually imported into the country and used as raw materials for yoghurt production. Cows from which milk can be extracted are available in the Northern part of Nigeria. A development of the inocula is all that is needed to turn "nono" production into large scale instead of the about 4 litres production per day of a typical "nono" manufacturer/producer. Also, foreign exchange could be saved on importation of these raw materials.

Large scale manufacture of "nono", its packaging and distribution would not only help to retain a good protein source; it would also help to standardise "nono" production and place its name among other fermented milks known world wide.

Chapter 6

SUMMARY

Microbiological and related investigations have been carried out on "nono", a Nigerian fermented milk product, with the primary purpose of determining the microbial and physicochemical changes which occur in the fermentation, the microorganisms involved, suitable starter cultures and other conditions for optimizing the fermentation and for preserving the product.

This studies show that pH dropped from 6.8 $\pm$ 0.1 to 4.3 $\pm$ 0.1 during the traditional production of "nono". There is a corresponding increase in the titratable acidity. "Nono" is therefore an acid food.

Analyses of fully prepared "nono" showed that there were slight increases in the content of minerals which occur in milk. the following proportions of minerals were found in "nono": K (24.0 - 59.0 mg/100ml), Ca (13.5 - 28.9 mg/100ml), Mg (2.4 - 6.4 mg/100ml), Zn (0.3 - 0.7 mg/100ml), Fe (0.0 - 0.9 mg/100ml), Na (8.0 - 24.0 mg/100ml), P (15.8 - 44.6 mg/100ml). Copper was not detected in "nono" samples examined.

Crude protein was relatively high (1.2 - 5.7)% in "nono". This indicates that the product is suitable as a dietary source of protein. An increase in fat was also observed.

Many microorganisms were isolated from "nono" but only Streptococcus cremoris, Streptococcus lactis, Streptococcus diacetilactis, Lactobacillus cellobiosus, Lactobacillus brevis, and Saccharomyces cerevisiae were incriminated in its fermentation. Raw milk could not be fermented into "nono" with pure cultures of single strains of the above microorganisms but a combination of a Streptococcus and a Lactobacillus gave a product similar to "nono". The most acceptable starter culture for "nono" production was a combination of Streptococcus diacetilactis, Lactobacillus brevis and Saccharomyces cerevisiae.

Of four temperatures tested, 25°C and 30°C were suitable for production of acceptable "nono"; 15°C and 37° C were unsuitable. Although stationary and agitated fermentations yielded acceptable products, the stationary method should be preferred because agitation is a mechanical process and would increase costs in a commercial operation. The open system of fermentation would also be preferable to the closed system of "nono" fermentation.

"Nono" could be stored at 4°C with retention of its microbiological, biochemical and aesthetic values, although its texture and its viscosity would slightly change.

Some lactic acid bacteria isolated from "nono" were inhibitory to the growth of Staphylococcus aureus and

Escherichia coli. If the lactics produce antimicrobial agents during their activity in "nono" production, the agents would be useful for safeguarding the product from growth of the two organisms which could constitute health hazards.

No bacteriophage was detected in the "nono" samples examined in this study. Thus the potentially useful starters isolated from these studies are relatively free from lytic bacteriophages and can be propagated and used for large scale production of "nono".

The shelf-life of "nono" which could be maintained at 4°C for up to 12 days can be extended or improved by packaging the product with sterile material. The packaging would facilitate distribution, sale and possible introduction of "nono" to other parts of the world.

Overall, this thesis has provided considerable information on the microbiological and physico-chemical aspects of "nono" fermentation and on the nutritional value of the fermented product. It has established some possible methods for optimizing and enhancing its acceptability and for prolonging its shelf-life. Briefly, they are: use of suitable starter cultures consisting of Streptococci and Lactobacilli, use of a suitable range of temperatures (25°C - 30°C), an open or closed fermentation system without agitation and preservation at 4°C for at least 12 days. These findings provide a sufficient

background for a pilot-scale development study of "nono" fermentation and this is suggested for further work.

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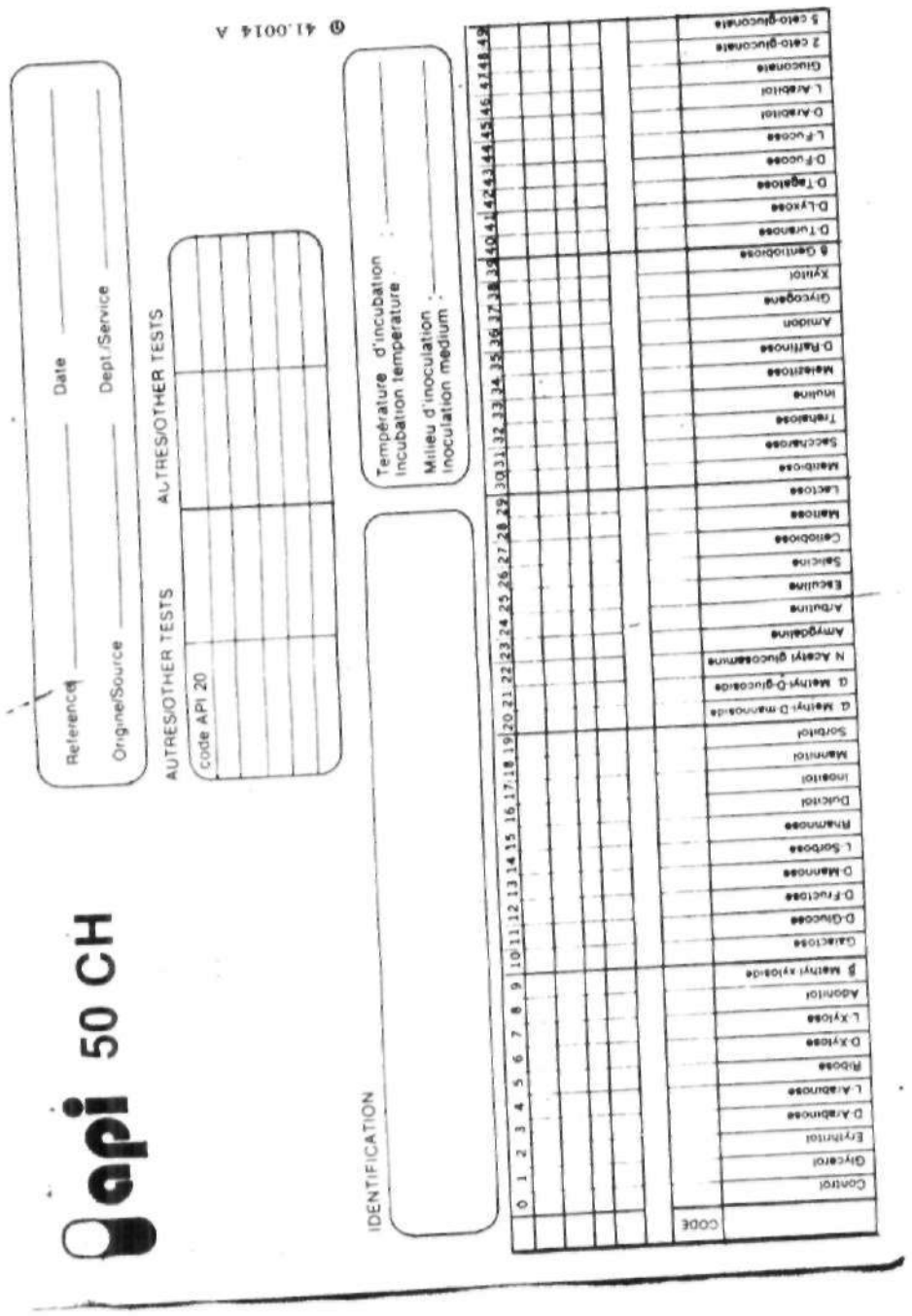
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Appendix I: A.P.I. 50CH Identification result form.



APPENDIX II: SENSORY-EVALUATION SCORE SHEET.

DEPARTMENT OF MICROBIOLOGY,
AHMADU BELLO UNIVERSITY, ZARIA.

NAME: _____ DATE: _____

SAMPLE: _____

Please taste each sample and circle the one that best describes the attributes of the products and answer the questions. Rinse your mouth after tasting each sample.

COLOUR

(1) Whitish Brown (2) Yellowish White

(3) Dirty White (4) Pure White

ODOUR

(1) Very Pronounced (2) Moderately Pronounced

(3) Slightly Pronounced (4) Pronounced

VISCOSITY/THICKNESS

(1) Very watery (2) Slightly Watery

(3) Slightly Thick (4) Very Thick

TEXTURE/SMOOTHNESS

(1) Very Coarse (2) Slightly Coarse

(3) Slightly Smooth (4) Very Smooth

TASTE/ACIDITY

(1) Not Sour (2) Slightly Sour

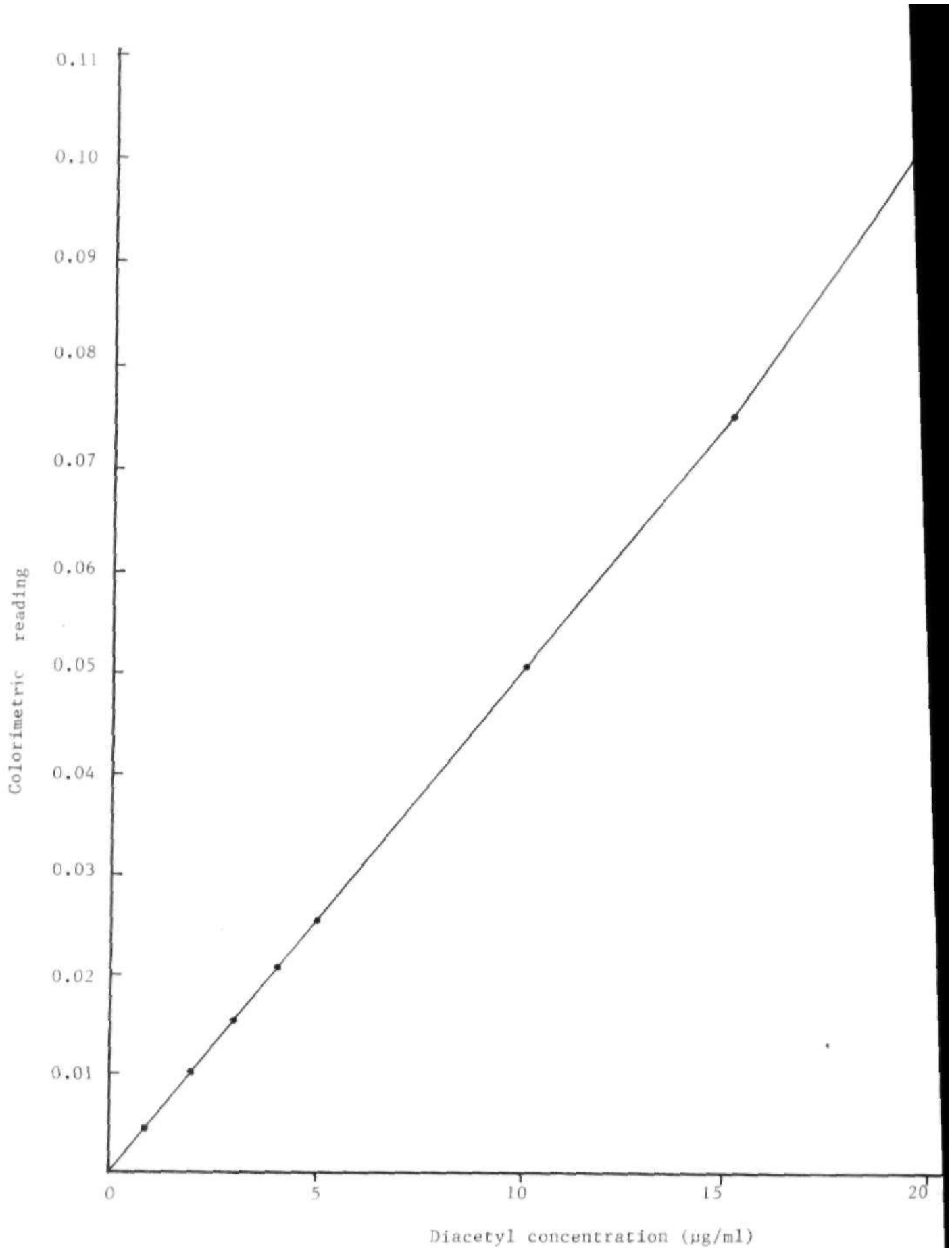
(3) Moderately Sour (4) Very Sour

OVERALL QUALITY or ACCEPTABILITY

(1) Very poor (2) Poor

(3) Fair (4) Good

(5) Very Good



APPENDIX III: Standard graph: "The relationship of colour intensity at 540nm to the amount of diacetyl in solution.

APPENDIX. IV

TABLE 5: Results of the morphological, biochemical and physiological tests on bacteria isolated from market "nono"

TESTS	H_{11} H_{21} H_{31} H_{41} B_{11} B_{21} B_{31} B_{41} Y_{11} Y_{21}	H_{12} H_{22} H_{32} H_{42} B_{12} B_{22} B_{32} B_{42} Y_{12} Y_{22}	H_{13} H_{23} H_{33} H B_{13} B_{23} B_{33} B Y_{13} Y_{23}	H_{14} H_{24} H_{34} H_{44} B_{14} B_{24} B_{34} B_{44} Y_{14} Y_{24}	H_{15} H_{25} H_{35} H_{45} B_{15} B_{25} B_{35} B_{45} Y_{15} Y_{25}	H_{16} H_{26} H_{36} H_{46} B_{16} B_{26} B_{36} B_{46} Y_{16} Y_{26}	H_{17} H_{27} H_{37} H_{47} B_{17} B_{27} B_{37} B_{47} Y_{17} Y_{27}
Gram-reaction and morphological characteristics	Gram-positive cocci diving in one plane to appear in twos and short chains	Gram-positive cocci in short and long chains	Gram-positive cocci in twos and short chains	Gram-positive rods	Gram-positive rods in short chains	Gram-positive rods in twos and and short chains	Gram-positive rod singly arranged
Growth and pigmentation on PCA plates	Non-Pigmented poor growth	Non-Pigmented poor growth	Non-pigmented poor growth	Non-pigmented poor growth	Poor growth	Poor growth	Good growth
Growth and pigmentation on MRS Agar	No Pigmentation. Poor growth	No Pigmentation. Poor growth	No Pigmentation. Poor growth	No Pigmentation. Poor growth	No Pigmentation. Poor growth	No Pigmentation. Poor growth	No Pigmentation. Good growth
Spore formation	-	-	-	-	-	-	+
Catalase test	-	-	-	-	-	-	-
Production of CO_2 gas from Glucose in Gibson's semi-solid medium	-	-	-	-	+	+	
Production of Ammonia from Arginine	+	-	+	-	+	+	
Digestion of Loeffler's Serum medium	-	-	-	-	+	+	
Growth at 15°C	+	+	+	+	+	-	
Growth at 45°C	-	-	-	-	-	+	
Growth at 10°C	+	+	+				
Heat resistance at 60 for 30minutes	-	-	-				
Growth in the presence of 6.5% NaCl	-	-	-				
Growth at pH 9.6	-	-	-				
Reduction of litmus before clotting in litmus milk	+	+	+				

Action on litmus milk	Acid clot reaction	Acid clot reaction	Acid clot reaction			
Haemolysis on Horse blood agar	-	-	-			
* Growth at 40 °c	+	-	+			
Growth in the presence of 4% NaCl	+	-	+			
* Growth at pH 9.2	+	-	+			
Gas from citrate in Semi-solid citrate Milk Agar	-	-	+			
** Acetoin Production (Voges-Proskauer reaction)	-	-	+			
SUGAR FERMENTATION						
1. L - Arabinose	-	-	-	-	+	+
2. Ribose	-	-	+	+	+	+
3. D - Xylose	-	-	+	-	-	-
4. Galactose	+	+	+	+	+	+
5. Glucose	+	+	+	+	+	+
6. Fructose	+	+	+	+	+	+
7. Mannose	+	+	+	+	+	+
8. Mannitol	-	-	-	+	-	-
9. Sorbitol	-	-	-	+	-	-
10. &-Methyl-D-Mannoside	-	-	-	+	-	-
11. N-Acetyl Glucosamine	-	+	+	+	-	-
12. Amigdalin	-	-	-	+	-	-
13. Arbutin	-	-	+	+	-	-
14. Esculin	+	+	+	+	-	+
15. Salicin	-	-	+	+	-	-
16. Cellobiose	+	-	+	+	+	+
17. Maltose	+	+	+	+	+	+
18. Lactose	+	+	+	+	+	+
19. Melibiose	-	-	-	+	+	+
20. Saccharose/sucrose	-	-	-	+	+	+
21. Trehalose	-	-	+	+	+	+
22. Melezitose	-	-	-	+	-	-
23. Raffinose	-	-	-	+	+	+
24. Amidon/Starch	-	-	-	+	-	-
25. Gentiobiose	-	-	+	+	-	-
26. Gluconate	-	-	-	+	+	+
27. 5 Keto Gluconate	-	-	-	-	-	+

Identified Micro-organisms	Strept. lactis	Strept. cremoris	Strept diacetilactis	Lacto. plantarum	Lacto. brevis	Lacto cellobiosus	Bacillus spp

KEY: Isolate codes:

$H_{11}-H_{17}$; $H_{21}-H_{27}$; $H_{31}-H_{37}$; $H_{41}-H_{47}$ = Isolate from processors H1, H2, H3, H4 from "Hausa" Village respectively.
$B_{11}-B_{17}$; $B_{21}-B_{27}$; $B_{31}-B_{37}$; $B_{41}-B_{47}$ = Isolate from processors B1, B2, B3, B4 from "Bono" Village respectively.
$Y_{11}-Y_{17}$; $Y_{21}-Y_{27}$; = Isolate from processors Y1 and Y2 from "Biye" Village respectively.
* Yeast Glucose- Lemco broath was used as basal medium for the test.
* * Sterile Skim- Milk was used as growth medium for the test.

APPENDIX. V

TABLE 6 Results of the morphological and biochemical tests on Yeast Isolates from market "nono"

Isolates	H_{101} H_{201} H_{102} H_{202} H_{103} H_{203} H_{104} H_{204} H_{105} H_{205} H_{301} H_{401} H_{302} H_{402} H_{303} H_{403} H_{304} H_{404} H_{305} H_{405} B_{101} B_{201} B_{102} B_{202} B_{103} B_{203} B_{104} B_{204} B_{105} B_{205} B_{301} B_{401} B_{302} B_{402} B_{303} B_{403} B_{304} B_{404} B_{305} B_{405} Y_{101} Y_{201} Y_{102} Y_{202} Y_{103} Y_{203} Y_{104} Y_{204} Y_{105} Y_{205}					
Morphology on Corn meal Agar to which Tween 80 was added	Pseudomycelia with blastospores	Pseudomycelia	True mycelium with athrospores	Unicellular spherical but mainly ovoid. Sometimes a pseudomycelium.	True mycelium with arthrospores
Formation of pellicle on malt broth	No Pellicle. Film formed at the top of broth	No Pellicle. Film formed at the top of broth	Disintegrating pellicle	No Pellicle. Ring formed	No Pellicle. Rough film at top of broth
Spore formation on sodium acetate agar	-	-	+	+	+
Colonial characteristics on Sabouraud Dextrose Agar	White-Cream coloured growth	White-Cream coloured growth	Yeast-like; white in colour and butyrous when old.	Shiny to dull cream coloured circular	Brownish, fluffy, mold-like growth
Utilization of ethanol as Carbon-Source	+	+	+	+	+
<u>SUGAR FERMENTATION</u>					
Glucose	+	+	+	+	+
Glycerol	+	-	+	-	-
2-Keto-D-Gluconate	+	-	-	-	+
L-Arabinose	-	-	-	-	+
D - Xylose	+	+	+	-	+
Adonitol	-	+	-	-	+
Galactose	+	+	+	+	+
Inositol	-	-	-	-	-
Sorbitol	-	+	+	-	-

α-Methyl-D-glucoside	-	+	-	-	+
N-Acetyl-D-Glucosamine	-	+	-	-	+
Cellobiose	-	-	-	-	+
Lactose	+	-	-	-	+
Maltose	-	+	-	-	+
Saccharose/Sucrose	+	+	-	+	+
Trehalose	-	+	-	-	-
Melezitose	-	+	-	-	+
Raffinose	+	-	-	+	-

Identified microorganisms	Candida pseudotropicalis	Candida tropicalis	Endomyces spp	Saccharomyces cerevisae	Trichosporon beigelii

KEY: ISOLATES CODES

H_{101} - H_{105} ; H_{201} -H_{205} ; H_{301} -H_{305} ; H_{401} -H_{405} = Isolates from processors H_1, H_2, H_3, and H_4 from "Hanma" Village respectively.

B_{101} - B_{105} ; B_{201} -B_{205} ; B_{301} -B_{305} ; B_{401} -B_{405} = Yeast isolates from processors B_1, B_2, B_3, B_4, from "Bomo" village respectively.

Y_{101} -Y_{105} ; Y_{201} -Y_{205} = Yeast isolates from processors Y_1 and Y_2 from "Biyo" village respectively.

TABLE 7 Results of Cultural, morphological and Biochemical characteristics of bacteria isolated from fermenting milk at Zero hours of fermentation in the production of "nono"

ISOLATES	H_{111} H_{211} H_{311} H_{411} B_{111} B_{211} B_{311} B_{411} Y_{111} Y_{211}	H_{112} H_{212} H_{312} H_{412} B_{112} B_{212} B_{312} B_{412} Y_{112} Y_{212}	H_{113} H_{213} H_{313} H_{413} B_{113} B_{213} B_{313} B_{413} Y_{113} Y_{213}	H_{114} H_{214} H_{314} H_{414} B_{114} B_{214} B_{314} B_{414} Y_{114} Y_{214}
Gram-reaction and morphological characteristics	Gram-positive cocci in twos and short chains	Gram-positive cocci in short and long chains	Gram positive cocci in twos and short chains	Gram positive rods singly arranged
Growth and pigmentation on PCA plates	Non-pigmented Poor growth	Non-pigmented poor growth	Non-pigmented poor growth	Non-pigmented good growth
Growth and pigmentation on MRS Agar	No pigmentation. Poor growth	No pigmentation. Poor growth	No pigmentation. Poor growth	No pigmentation. Good growth
Spore formation	-	-	-	+
Production of CO_2 gas from Glucose in Gibson's semi-solid medium	-	-	-	+
Production of Ammonia from Arginine	-	-	-	
Digestion of Loeffler's serum medium	-	-	-	
Growth at 15°C	+	+	+	
Growth at 45°C	-	-	-	
SUGAR FERMENTATION				
1. l- Arabinose	-	-	-	
2. Ribose	-	-	+	
3. D - Xylose	-	-	+	
4. Galactose	+	+	+	
5. Glucose	+	+	+	
6. Fructose	+	+	+	
7. Mannose	+	+	+	
8. Mannitol	-	-	-	
9. Sorbitol	-	-	-	
10. δ - Methyl - D - Mannoside	-	-	-	

11. N-Acetyl - Gluco-Samine	-	+	+
12. Amygdalin	-	-	-
13. Arabutin	-	-	+
14. Esculin	+	+	+
15. Salicin	-	-	+
16. Cellobiose	+	-	+
17. Maltose	+	+	+
18. Lactose	+	+	+
19. Melibiose	-	-	-
20. Saccharose/ Sucrose	-	-	-
21. Trehalose	-	-	+
22. Melezitose	-	-	-
23. Raffinose	-	-	-
24. Amidon/Stach	-	-	-
25. Gentiobiose	-	-	+
26. Gluconate	-	-	-
27. 5 Keto Gluconate	-	-	-
Growth at 10°C	+	+	+
Heat Resistance at 60°C for 30 minutes	-	-	-
Growth in presence of 6.5% NaCl	-	-	-
Growth at pH 9.6	-	-	-
Reduction of litmus before clothing in litmus milk	+	+	+
Action in litmus milk	Acid clot reaction	Acid clot reaction	Acid clot reaction
Haemolysis on Horse blood agar	-	-	-
Growth at 40°C	+	-	+
* Growth in the presence of 4% NaCl	+	-	+
* Growth at pH 9.2	+	-	+
Gas from citrate in semi-solid citrate milk Agar	-	-	+
** Acetoin production (Voges - Proskauer reaction)	-	-	+

Identified bacteria	Strept. lactis	Strept. cremoris	Strept. diacetilactis	Bacillus spp.

KEY:

* Yeast Glucose iemco broth was used as basal medium for the test

** Sterile skim - milk was used as growth medium for the test.

H_{111} - H_{414} ; H_{112} - H_{412} ; H_{113} - H_{413} ; H_{114} - H_{414} = Isolates from processors H_1, H_2, H_3, H_4 from "Hanwa" Village respectively.

B_{111} - B_{414} ; B_{112} - B_{412} ; B_{113} - B_{413} + B_{114} - B_{414} = Isolate from processors B_1, B_2, B_3, and B_4 from "Bomo" Village respectively.

Y_{111} - Y_{411} ; Y_{112} - Y_{412} = Isolate from processors Y_1, and Y_2 from "Biye" Village respectively.

TABLE 8: Results of the Morphological and Biochemical tests of yeasts isolated from fermenting milk at zero hour of fermentation in production of "nono"

ISOLATES	H_{117} H_{217} H_{317} H_{417} B_{117} B_{217} B_{317} B_{417} Y_{117} Y_{217}	H_{118} H_{218} H_{318} H_{418} B_{118} B_{218} B_{318} B_{418} Y_{118} Y_{218}	H_{119} H_{219} H_{319} H_{419} B_{119} B_{219} B_{319} B_{419} Y_{119} Y_{219}
Morphology on corn meal Agar with added tween 80	Pseudomycelia with blastospores present	Pseudomycelia present	Ovoid cells with pseudomycelia present
Formation of pellicle on malt broth	No pellicle. Forms film at the top of the broth	No pellicle. Form film at top of the broth	No pellicle There is ring formation
Colonial characteristics on Sabouraud Dextrose Agar	Dull white to cream colour	Dull white Butter texture	Shiny white to cream colour
Spore formation on sodium acetate Agar	-	-	+
Utilization of ethanol as Carbon-Source	+	+	+
SUGAR FERMENTATION			
Glucose	+	+	+
Glycerol	+	-	-
2-Keto-D-Gluconate	-	+	-
D - Xylose	+	+	-
Adonitol	-	+	-
Galactose	+	+	+
Sorbitol	-	+	-
α-Methyl-D-Gluconate	-	+	-
N-Acetyl-D-Glucosamine	-	+	-
Lactose	+	-	-
Maltose	-	+	+
Saccharose/Sucrose	+	+	+
Trehalose	-	+	-
Melezitose	-	+	-
Raffinose	-	-	+
Identified Yeast	Candida pseudo-tropicalis	Candida tropicalis	Saccharomyces cerevisiae

KEY

H_{117} - H_{119} ; H_{217} - H_{219} ; H_{317} - H_{319} ; H_{417} - H_{419} = Isolates from processors

H_1, H_2, H_3 H_4 from "Hanwa" Village respectively.

B_{117} - B_{119} ; B_{217} - B_{219} ; B_{317} - B_{319} ; B_{417} - B_{419} = Isolates from processors

B_1, B_2, B_3, and B_4 from "Bona" Village respectively.

Y_{117} - Y_{119} ; Y_{217} - Y_{219} = Isolate from processors Y , and Y from "Biye" Village respectively.

TABLE 9 Results of Cultural, morphological and Biochemical characteristics of bacteria isolated from fermenting milk at 6 hours of fermentation in the production of "nono"

ISOLATES	H_{121} H_{221} H_{321} H_{421} B_{121} B_{221} B_{321} B_{421} Y_{121} Y_{221}	H_{122} H_{222} H_{322} H_{422} B_{122} B_{222} B_{322} B_{422} Y_{122} Y_{222}	H_{123} H_{223} H_{323} H_{423} B_{123} B_{223} B_{323} B_{423} Y_{123} Y_{223}	H_{124} H_{224} H_{324} H_{424} B_{124} B_{224} B_{324} B_{424} Y_{124} Y_{224}
Gram-reaction and morphological characteristics	Gram-positive cocci in twos and short chains	Gram-positive cocci in short and long chains	Gram positive cocci in twos and short chains	Gram positive rods singly arranged
Growth and pigmentation on PCA plates	Non-pigmented Poor growth	Non-pigmented poor growth	Non-pigmented poor growth	Non-pigmented good growth
Growth and pigmentation on MRS Agar	No pigmentation. Poor growth	No pigmentation. Poor growth	No pigmentation. Poor growth	No pigmentation. Good growth
Spore formation	-	-	-	+
Production of CO_2 gas from Glucose in Gibson's semi-solid medium	-	-	-	+
Production of Ammonia from Arginine	+	-	+	
Digestion of Loeffler's serum medium	-	-	-	
Growth at 15°C	+	+	+	
Growth at 45°C	-	-	-	
SUGAR FERMENTATION				
1. L- Arabinose	-	-	-	
2. Ribose	-	-	+	
3. D - Xylose	-	-	+	
4. Galactose	+	+	+	
5. Glucose	+	+	+	
6. Fructose	+	+	+	
7. Mannose	+	+	+	
8. Mannitol	-	-	-	
9. Sorbitol	-	-	-	
10. δ - Methyl - D - Mannoside	-	-	-	

11. N-Acetyl - Gluco-Samine	-	+	+
12. Amygdalin	-	-	-
13. Arabutin	-	-	+
14. Esculin	+	+	+
15. Salicin	-	-	+
16. Cellobiose	+	-	+
17. Maltose	+	+	+
18. Lactose	+	+	+
19. Melibiose	-	-	-
20. Saccharose/ Sucrose	-	-	-
21. Trehalose	-	-	+
22. Melezitose	-	-	-
23. Raffinose	-	-	-
24. Amidon/Stach	-	-	-
25. Gentiobiose	-	-	+
26. Gluconate	-	-	-
27. 5 Keto Gluconate	-	-	-
Growth at 10°C	+	+	+
Heat Resistance at 60°C for 30 minutes	-	-	-
Growth in presence of 6.5% NaCl	-	-	-
Growth at pH 9.6	-	-	-
Reduction of litmus before clothing in litmus milk	+	+	+
Action in litmus milk	Acid clot reaction	Acid clot reaction	Acid clot reaction
Haemolysis on Horse blood agar	-	-	-
Growth at 45°C	+	-	+
* Growth in the presence of 4% NaCl	+	-	+
* Growth at pH 9.2	+	-	+
Gas from citrate in semi-solid citrate milk Agar	-	-	+
** Acetoin production (Voges - Proskauer reaction)	-	-	+
Identified bacteria	Strept. lactis	Strept. cremoris	Strept. diacetilactis

Bacillus spp.	

KEY:

* *Yeast Glucose Lemco broth was used as basal medium for the test*

** Sterile skim - milk was used as growth medium for the test.

H_{121} - H_{124} ; H_{221} - H_{224} ; H_{321} - H_{324} ; H_{421} - H_{424} = Isolates from processors H_1, H_2, H_3 H_4 from "Hanma" Village respectively.

B_{121} - B_{124} ; B_{221} - B_{224} ; B_{321} - B_{324} ; B_{421} - B_{424} = Isolate from processors B_1, B_2, B_3, and B_4 from "Bomo" Village respectively.

Y_{121} - Y_{124} ; Y_{221} - Y_{224} = Isolate from processors Y_1, and Y_2 from "Biye" Village respectively.

TABLE 10: Results of the Morphological and Biochemical tests of Yeasts isolated from fermenting milk at 6 hours of fermentation in production of "nono"

ISOLATES	H_{127} H_{227} H_{327} H_{427} B_{127} B_{227} B_{327} B_{427} Y_{127} Y_{227}	H_{128} H_{228} H_{328} H_{428} B_{128} B_{228} B_{328} B_{428} Y_{128} Y_{228}	H_{129} H_{229} H_{329} H_{429} B_{129} B_{229} B_{329} B_{429} Y_{129} Y_{229}
Morphology on corn meal Agar with added tween 80	Pseudomycelia with blastospores present	Pseudomycelia present	Ovoid cells with pseudomycelia present
Formation of pellicle on malt broth	No pellicle. Forms film at the top of the broth	No pellicle. Form film at top of the broth	No pellicle There is ring formation
Colonial characteristics on Sabouraud Dextrose Agar	Dull white to cream colour	Dull white Butter texture	Shiny white to cream colour
Spore formation on sodium acetate Agar	-	-	+
Utilization of ethanol as Carbon-Source	+	+	+
SUGAR FERMENTATION			
Glucose	+	+	+
Glycerol	+	-	-
2-Keto-D-Gluconate	-	+	-
D - Xylose	+	+	-
Adonitol	-	+	-
Galactose	+	+	+
Sorbitol	-	+	-
&-Methyl-D-Gluconate	-	+	-
N-Acetyl-D-Glucosamine	-	+	-
Lactose	+	-	-
Maltose	-	+	+
Saccharose/Sucrose	+	+	+
Trehalose	-	+	-
Melezitose	-	+	-
Raffinose	-	-	+
Identified Yeast	Candida pseudo-tropicalis	Candida tropicalis	Saccharomyces cerevisiae

KEY

H_{127} - H_{129} ; H_{227} - H_{229} ; H_{327} - H_{329} ; H_{427} - H_{429} = isolates from processors

H_1, H_2, H_3 H_4 from "Hanma" Village respectively.

B_{127} - B_{129} ; B_{227} - B_{229} ; B_{327} - B_{329} ; B_{427} - B_{429} = isolates from processors

B_1, B_2, B_3, and B_4 from "Bomo" Village respectively.

Y_{127} - Y_{129} ; Y_{227} - Y_{229} = isolate from processor 2, and Y from "Biye" Village respectively.

APPENDIX. X

TABLE III: Results of the cultural, morphological and biochemical characteristics on bacteria isolated from fermenting milk at 12 hours of fermentation in in the production of "nono"

TESTS	H 131 H 231 H 331 H 132 H 232 H 332 H 133 H 233 H 333 H 134 H 234 H 334 H 135 H 235 H 335 H 136 H 236 H 336 H 137 H 237 H 337 H 931 B 131 B 231 H 931 B 132 B 232 H 933 B 133 B 233 H 934 B 134 B 234 H 935 B 135 B 235 H 936 B 136 B 236 H 937 B 137 B 237 B 331 B 931 Y 131 B 932 B 932 Y 132 B 933 B 934 B 934 B 934 B 935 B 935 B 936 B 936 B 937 B 937 Y 231 Y 232 Y 133 Y 233 Y 134 Y 234 Y 135 Y 235 Y 136 Y 236 Y 137 Y 237
Gram-reaction and morphological characteristics	Gram-positive cocci diving in one plane to appear in twos and short chains; Gram-positive cocci in short and long chains; Gram-positive cocci in twos and short chains; Gram-positive rods; Gram-positive rods in short cabins; Gram-positive rods in twos and and short chains; Gram-positive rod singly arranged
Growth and pigmentation on PCA plates	Non-Pigmented poor growth; Non-Pigmented poor growth; Non-pigmented poor growth; Non-pigmented poor growth; Poor growth; Poor growth; Good growth
Growth and pigmentation on MRS Agar	No Pigmentation. Poor growth; No Pigmentation. Poor growth; No Pigmentation. Poor growth; No Pigmentation. Poor growth; No Pigmentation. Poor growth; No Pigmentation. Poor growth; No Pigmentation. Goo growth
Spore Formation	- - - - - - +
Catalase test	- - - - - - -
Production of CO_2 gas from Glucose in Gibson's semi-solid medium	- - - - + +
Production of Ammonia from Arginine	- - - - + +
Digestion of Loeffler's Serum medium	- - - - + +
Growth at 15°C	+ + + + + -
Growth at 45°C	- - - - - +
Growth at 10°C	+ + +
Heat resistance at 60 for 30minutes	- - -
Growth in the presence of 6.5% NaCl	- - -
Growth at pH 9.6	- - -
Reduction of litmus before clotting in litmus milk	+ + +

Action in litmus milk	Acid clot reaction	Acid clot reaction	Acid clot reaction			
Haemolysis on Horse blood agar	-	-	-			
* Growth at 40°C	+	-	+			
Growth in the presence of 4% NaCl	+	-	+			
* Growth at pH 9.2	+	-	+			
Gas from citrate in Semi-solid citrate Milk Agar	-	-	+			
** Acetoin Production (Voges-Proskauer reaction)	-	-	+			
SUGAR FERMENTATION						
1. L - Arabinose	-	-	-	-	+	+
2. Ribose	-	-	+	+	+	+
3. D - Xylose	-	-	+	-	-	-
4. Galactose	+	+	+	+	+	+
5. Glucose	+	+	+	+	+	+
6. Fructose	+	+	+	+	+	+
7. Mannose	+	+	+	+	+	+
8. Mannitol	-	-	-	+	-	-
9. Sorbitol	-	-	-	+	-	-
10. &-Methyl-D-Mannoside	-	-	-	+	-	-
11. N-Acetyl Glucosamine	-	+	+	+	-	-
12. Amigdalin	-	-	-	+	-	-
13. Arbutin	-	-	+	+	-	-
14. Esculin	+	+	+	+	-	+
15. Salicin	-	-	+	+	-	-
16. Cellobiose	+	-	+	+	+	+
17. Maltose	+	+	+	+	+	+
18. Lactose	+	+	+	+	+	+
19. Melibiose	-	-	-	+	+	+
20. Saccharose/sucrose	-	-	-	+	+	+
21. Trenalose	-	-	+	+	+	+
22. Melezitose	-	-	-	+	-	-
23. Raffinose	-	-	-	+	+	+
24. Amidon/Starch	-	-	-	+	-	-
25. Gentiobiose	-	-	+	+	-	-
26. Gluconate	-	-	-	+	+	+
27. 5 Keto Gluconate	-	-	-	-	-	+

Identified Micro-organisms	Strept. lactis	Strept. cremoris	Strept diaceti-lactis	Lacto-plantarum	Lacto-brevis	Lacto cellobiosus	Bacillus spp

KEY: Isolate codes

$H_{(1)}$-$H_{(37)}$; $H_{(1)}$-$H_{(37)}$; $H_{(1)}$-$H_{(37)}$; $H_{(1)}$-$H_{(37)}$ = Isolate from processors H1, H2, H3, H4 from "Hanma" Village respectively.
$B_{(1)}$-$B_{(37)}$; $B_{(1)}$-$B_{(37)}$; $B_{(1)}$-$B_{(37)}$; $B_{(1)}$-$B_{(37)}$ = Isolate from processors B1, B2, B3, B4 from "Bono" Village respectively.
$Y_{(1)}$-$Y_{(37)}$; $Y_{(1)}$-$Y_{(37)}$; = Isolate from processors Y1 and Y2 from "Biye" Village respectively.
* Yeast Glucose- Lemco broath was used as basal medium for the test.
* * Sterile Skim- Milk was used as growth medium for the test.

TABLE 12: Results of the Morphological and Biochemical tests of yeasts isolated from fermenting milk at 12 hours of fermentation in production of 'nono'

ISOLATES	H_{131y} H_{231y} H_{331y} H_{431y} B_{131y} B_{231y} B_{331y} B_{431y} Y_{131y} Y_{231y}	H_{132y} H_{232y} H_{332y} H_{432y} B_{132y} B_{232y} B_{332y} B_{432y} Y_{132y} Y_{232y}	H_{133y} H_{233y} H_{333y} H_{433y} B_{133y} B_{233y} B_{333y} B_{433y} Y_{133y} Y_{233y}
Morphology on corn meal Agar with added tween 80	Pseudomycelia with blastospores present	Pseudomycelia present	Ovoid cells with pseudomycelia present
Formation of pellicle on milk broth	No pellicle. Forms film at the top of the broth	No pellicle. Form film at top of the broth	No pellicle There is ring formation
Colonial characteristics on Sabouraud Dextrose Agar	Dull white to cream colour	Dull white Butter texture	Shiny white to cream colour
Spore formation on sodium acetate Agar	-	-	+
Utilization of ethanol as Carbon-Source	+	+	+
SUGAR FERMENTATION			
Glucose	+	+	+
Glycerol	+	-	-
2-Keto-D-Gluconate	-	+	-
D - Xylose	+	+	-
Adonitol	-	+	-
Galactose	+	+	+
Sorbitol	-	+	-
A-Methyl-D-Glucoside	-	+	-
N-Acetyl-D-Glucosamine	-	+	-
Lactose	+	-	-
Maltose	-	+	+
Saccharose/Sucrose	+	+	+
Trehalose	-	+	-
Melezitose	-	+	-
Raffinose	-	-	+
Identified Yeast	*Candida pseudo-tropicalis*	*Candida tropicalis*	*Saccharomyces cerevisiae*

KEY

H_{131y} - H_{333y} ; H_{231y} - H_{233y} ; H_{331y} - H_{333y} ; H_{431y} - H_{433y} = Isolates from processors

H_1, H_2, H_3 H_4 from "Hanwa" Village respectively.

B_{131y} - B_{333y} ; B_{231y} - B_{233y} ; B_{331y} - B_{333y} ; B_{431y} - B_{433y} = Isolates from processors

B_1, B_2, B_3, and B_4 from "Bomo" Village respectively.

Y_{131y} - Y_{133y} ; Y_{231y} - Y_{233y} = Isolate from processors Y_1, and Y_2 from "Biye" Village respectively.

APPENDIX. XII

TABLE 13: Results of the cultural, morphological and biochemical characteristics on bacteria isolated from fermenting milk at 18 hours of fermentation in the production of "nono"

TESTS	H141 H241 H341 H142 H242 H342 H143 H243 H343 H144 H244 H344 H145 H245 H345 H146 H246 H346 H147 H247 H347 H941 B141 B241 H442 B142 B242 H443 B143 B243 H444 B144 B244 H445 B145 B245 H446 B146 B246 H447 B147 B247 B341 B441 Y141 B342 B442 Y142 B343 B443 B344 B444 B345 B445 B346 B446 B347 B447 Y241 Y242 Y143 Y243 Y144 Y244 Y145 Y245 Y146 Y246 Y147 Y247						
Gram-reaction and morphological characteristics	Gram-positive cocci diving in one plane to appear in twos and short chains	Gram-positive cocci in short and long chains	Gram-positive cocci in twos and short chains	Gram-positive rods	Gram-positive rods in short cahins	Gram-positive rods in twos and and short chains	Gram-positive rod singly arranged
Growth and pigmentation on PCA plates	Non-Pigmented poor growth	Non-Pigmented poor growth	Non-pigmented poor growth	Non-pigmented poor growth	Poor growth	Poor growth	Good growth
Growth and pigmentation on MRS Agar	No Pigmentation. Poor growth	No Pigmentation. Poor growth	No Pigmentation. Poor growth	No Pigmentation. Poor growth	No Pigmentation. Poor growth	No Pigmentation. Poor growth	No Pigmentation. Goo growth
Spore formation	-	-	-	-	-	-	+
Catalase test	-	-	-	-	-	-	-
Production of CO_2 gas from Glucose in Gibson's semi-solid medium	-	-	-	-	+	+	
Production of Ammonia from Arginine	-	-	-	-	+	+	
Digestion of Loeffler's Serum medium	-	-	-	-	+	+	
Growth at 15°C	+	+	+	+	+	-	
Growth at 45°C	-	-	-	-	-	+	
Growth at 10°C	+	+	+				
Heat resistance at 60 for 30minutes	-	-	-				
Growth in the presence of 6.5% NaCl	-	-	-				
Growth at pH 9.6	-	-	-				
Reduction of litmus before clotting in litmus milk	+	+	+				

A ion in litmus milk	Acid clot reaction	Acid clot reaction	Acid clot reaction			
Haemolysis on Horse blood agar	-	-	-			
* Growth at 46°C	+	-	+			
Growth in the presence of 4% NaCl	+	-	+			
* Growth at pH 9.2	+	-	+			
Gas from citrate in Semi-solid citrate Milk Agar	-	-	+			
** Acetoin Production (Voges-Proskauer reaction)	-	-	+			
SUGAR FERMENTATION						
1. L - Arabinose	-	-	-	-	+	+
2. Ribose	-	-	+	+	+	+
3. D - Xylose	-	-	+	-	-	-
4. Galactose	+	+	+	+	+	+
5. Glucose	+	+	+	+	+	+
6. Fructose	+	+	+	+	+	+
7. Mannose	+	+	+	+	+	+
8. Mannitol	-	-	-	+	-	-
9. Sorbitol	-	-	-	+	-	-
10. &-Methyl-D-Mannoside	-	-	-	+	-	-
11. N-Acetyl Glucosamine	-	+	+	+	-	-
12. Amigdalin	-	-	-	+	-	-
13. Arbutin	-	-	+	+	-	-
14. Esculin	+	+	+	+	-	+
15. Salicin	-	-	+	+	-	-
16. Cellobiose	+	-	+	+	+	+
17. Maltose	+	+	+	+	+	+
18. Lactose	+	+	+	+	+	+
19. Melibiose	-	-	-	+	+	+
20. Saccharose/sucrose	-	-	-	+	+	+
21. Trehalose	-	-	+	+	+	+
22. Melezitose	-	-	-	+	-	-
23. Raffinose	-	-	-	+	+	+
24. Amidon/Starch	-	-	-	+	-	-
25. Gentiobiose	-	-	+	+	-	-
26. Glucobate	-	-	-	+	+	+
27. 5 Keto Gluconate	-	-	-	-	-	+

Identified Micro-organisms	Strept. lactis	Strept. cremoris	Strept diacetilactis	Lacto. plantarum	Lacto. brevis	Lacto cellobiosus	Bacillus spp

KEY: Isolate codes:

H_{141}-H_{147}; H_{241}-H_{247}; H_{341}-H_{347}; H_{441}-H_{447} = Isolate from processors H1, H2, H3, H4 from "Hanwa" Village respectively.
B_{141}-B_{147}; B_{241}-B_{247}; B_{341}-B_{347}; B_{441}-B_{447} = Isolate from processors B1, B2, B3, B4 from "Bono" Village respectively.
Y_{141}-Y_{147}; Y_{241}-Y_{247} = Isolate from processors Y1 and Y2 from "Biye" Village respectively.
* Yeast Glucose- Lemco broth was used as basal medium for the test.
* * Sterile Skim- Milk was used as growth medium for the test.

TABLE 14: Results of the Morphological and Biochemical tests of Yeasts isolated from fermenting milk at 18 hours of fermentation in production of "nono"

Isolates	H_{141y} H_{241y} H_{341y} H_{441y} B_{141y} B_{241y} B_{341y} B_{441y} Y_{141y} Y_{241y}	H_{142y} H_{242y} H_{342y} H_{442y} B_{142y} B_{242y} B_{342y} B_{442y} Y_{142y} Y_{242y}	H_{143y} H_{243y} H_{343y} H_{443y} B_{143y} B_{243y} B_{343y} B_{443y} Y_{143y} Y_{243y}	H_{144y} H_{244y} H_{344y} H_{444y} B_{144y} B_{244y} B_{344y} B_{444y} Y_{144y} Y_{244y}	H_{145y} H_{245y} H_{345y} H_{445y} B_{145y} B_{245y} B_{345y} B_{445y} Y_{145y} Y_{245y}
Morphology on Corn meal Agar to which Tween 80 was added	Pseudomycelia with blastospores	Pseudomycelia	True mycelium with athrospores	Unicellular spherical but mainly ovoid. Sometimes a pseudomycelium.	True mycelium with arthrospores
Formation of pellicle on malt broth	No Pellicle. Film formed at the top of broth	No Pellicle. Film formed at the top of broth	Disintegrating pellicle	No Pellicle. Ring formed	No Pellicle. Rough film at top of broth
Spore formation on sodium acetate agar	-	-	+	+	+
Colonial characteristics on Sabouraud Dextrose Agar	White-Cream coloured growth	White-Cream coloured growth	Yeast-like; white in colour and butyrous when old.	Shiny to dull cream coloured circular	Brownish, fluffy, mold-like growth
Utilization of ethanol as Carbon-Source	+	+	+	+	+
SUGAR FERMENTATION					
Glucose	+	+	+	+	+
Glycerol	+	-	+	-	-
2-Keto-D-Gluconate	+	-	-	-	+
L-Arabinose	-	-	-	-	+
D - Xylose	+	+	+	-	+
Adonitol	-	+	-	-	+
Galactose	+	+	+	+	+
Inositol	-	-	-	-	-
Sorbitol	-	+	+	-	-

6-Methyl-D-glucoside	-	+	-	-	+
N-Acetyl-D-Glucosamine	-	+	-	-	+
Cellobiose	-	-	-	-	+
Lactose	+	-	-	-	+
Maltose	-	+	-	-	+
Saccharose/Sucrose	+	+	-	+	+
Trehalose	-	+	-	-	-
Melezitose	-	+	-	-	+
Raffinose	+	-	-	+	-

Identified microorganisms	Candida pseudotropicalis	Candida tropicalis	Endomyces spp	Saccharomyces cerevisae	Trichosporon beigelii

KEY: ISOLATES CODES

H_{141y} - H_{145y} ; H_{241y} -H_{245y} ; H_{341y} -H_{345y} ; H_{441y} -H_{445y} = Isolates from processors H_1, H_2, H_3, and H_4 from "Hanma" Village respectively.

B_{141y} - B_{145y} ; B_{241y} -B_{245y} ; B_{341y} -B_{345y} ; B_{441y} -B_{445y} = Yeast isolates from processors B_1, B_2, B_3, B_4 from "Bomo" village respectively.

Y_{141y} -Y_{145y} ; Y_{241y} -Y_{245y} = Yeast isolates from processors Y_1 and Y_2 from "Biye" village respectively.

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APPENDIX. XIV

TABLE 15: Results of the cultural, morphological and biochemical characteristics on bacteria isolated from fermenting milk at 24 hours of fermentation in the production of "nono"

	H151 H251 H351 H152 H252 H352 H153 H253 H353 H154 H254 H354 H155 H255 H355 H156 H256 H356 H157 H257 H357 H451 B151 B251 H452 B152 B252 H453 B153 B253 H454 B154 B254 H455 B155 B255 H456 B156 B256 H457 B157 B25						
TESTS	B351 B451 Y151	B352 B452 Y152	B353 B453	B354 B454	B355 B455	B356 B456	B357 B457
	Y251	Y252	Y153 Y253	Y154 Y254	Y155 Y255	Y156 Y256	Y157 Y257
Gram-reaction and morphological characteristics	Gram-positive cocci diving in one plane to appear in twos and short chains	Gram-positive cocci in short and long chains	Gram-positive cocci in twos and short chains	Gram-positive rods	Gram-positive rods in short tahins	Gram-positive rods in twos and and short chains	Gram-positive rod singly arranged
Growth and pigmentation on PCA plates	Non-Pigmented poor growth	Non-Pigmented poor growth	Non-pigmented poor growth	Non-pigmented poor growth	Poor growth	Poor growth	Good growth
Growth and pigmentation on MRS Agar	No Pigmentation. Poor growth	No Pigmentation. Poor growth	No Pigmentation. Poor growth	No Pigmentation. Poor growth	No Pigmentation. Poor growth	No Pigmentation. Poor growth	No Pigmentation. Goo growth
Spore formation	-	-	-	-	-	-	+
Catalase test	-	-	-	-	-	-	-
Production of CO_2 gas from Glutose in Gibson's semi-solid medium	-	-	-	-	+	+	
Production of Ammonia from Arginine	-	-	-	-	+	+	
Digestion of loeffler's Serum medium	-	-	-	-	+	+	
Growth at 15°c	+	+	+	+	+	-	
Growth at 45°c	-	-	-	-	-	+	
Growth at 10°c	+	+	+				
Heat resistance at 60 for 30minutes	-	-	-				
Growth in the presence of 6.5% NaCl	-	-	-				
Growth at pH 9.6	-	-	-				
Reduction of litmus before clotting in litmus milk	+	+	+				

Identified Micro-organisms	Strept. lactis	Strept. cremoris	Strept diaceti-lactis	Lacto. plantarum	Lacto. brevis	Lacto cellobiosus	Bacillus spp
Milk Haemolysis on Horse blood agar	-	-	-				
* Growth at 40°C	+	-	+				
Growth in the presence of 4% NaCl	+	-	+				
* Growth at pH 9.2	+	-	+				
Gas from citrate in Semi-solid citrate Milk Agar	-	-	+				
** Acetoin Production (Voges-Proskauer reaction)	-	-	+				
SUGAR FERMENTATION							
1. L - Arabinose	-	-	-	-	+	+	
2. Ribose	-	-	+	+	+	+	
3. D - Xylose	-	-	+	-	-	-	
4. Galactose	+	+	+	+	+	+	
5. Glucose	+	+	+	+	+	+	
6. Fructose	+	+	+	+	+	+	
7. Mannose	+	+	+	+	+	+	
8. Mannitol	-	-	-	+	-	-	
9. Sorbitol	-	-	-	+	-	-	
10. &-Methyl-D-Mannoside	-	-	-	+	-	-	
11. N-Acetyl Glucosamine	-	+	+	+	-	-	
12. Amigdalin	-	-	-	+	-	-	
13. Arbutin	-	-	+	+	-	-	
14. Esculin	+	+	+	+	-	+	
15. Salicin	-	-	+	+	-	-	
16. Cellobiose	+	-	+	+	+	+	
17. Maltose	+	+	+	+	+	+	
18. Lactose	+	+	+	+	+	+	
19. Melibiose	-	-	-	+	+	+	
20. Saccharose/sucrose	-	-	-	+	+	+	
21. Trenalose	-	-	+	+	+	+	
22. Melezitose	-	-	-	+	-	-	
23. Raffinose	-	-	-	+	+	+	
24. Amidon/Starch	-	-	-	+	-	-	
25. Gentiobiose	-	-	+	+	-	-	
26. Gluconate	-	-	-	+	+	+	
27. 5 Keto Gluconate	-	-	-	-	-	+	

KEY: Isolate codes:

H_{151}-H_{157}; H_{251}-H_{257}; H_{351}-H_{357}; H_{451}-H_{457} = Isolate from processors H1, H2, H3, H4 from "Hanwa" Village respectively.
B_{151}-B_{157}; B_{251}-B_{257}; B_{351}-B_{357}; B_{451}-B_{457} = Isolate from processors B1, B2, B3, B4 from "Bono" Village respectively.
Y_{151}-Y_{157}; Y_{251}-Y_{257} = Isolate from processors Y1 and Y2 from "Biye" Village respectively.

* Yeast Glucose- Lemco broath was used as basal medium for the test.

* * Sterile Skim- Milk was used as growth medium for the test.

TABLE 16: Results of the Morphological and Biochemical tests of Yeasts isolated from fermenting milk at 24 hours of fermentation in production of "nono"

Isolates	H_{151y} H_{251y} H_{351y} H_{451y} B_{151y} B_{251y} B_{351y} B_{451y} Y_{151y} Y_{251y}	H_{152y} H_{252y} H_{352y} H_{452y} B_{152y} B_{252y} B_{352y} B_{452y} Y_{152y} Y_{252y}	H_{153y} H_{253y} H_{353y} H_{453y} B_{153y} B_{253y} B_{353y} B_{453y} Y_{153y} Y_{253y}	H_{154y} H_{254y} H_{354y} H_{454y} B_{154y} B_{254y} B_{354y} B_{454y} Y_{154y} Y_{254y}	H_{155y} H_{255y} H_{355y} H_{455y} B_{155y} B_{255y} B_{355y} B_{455y} Y_{155y} Y_{255y}
Morphology on Corn meal Agar to which Tween 80 was added	Pseudomycelia with blastospores	Pseudomycelia	True mycelium with arthrospores	Unicellular spherical but mainly ovoid. Sometimes a pseudomycelium.	True mycelium with arthrospores
Formation of pellicle on malt broth	No Pellicle. Film formed at the top of broth	No Pellicle. Film formed at the top of broth	Disintegrating pellicle	No Pellicle. Ring formed	No Pellicle. Rough film at top of broth
Spore formation on sodium acetate agar	-	-	+	+	+
Colonial characteristics on Sabouraud Dextrose Agar	White-Cream coloured growth	White-Cream coloured growth	Yeast-like; white in colour and butyrous when old.	Shiny to dull cream coloured circular	Brownish, fluffy, mold-like growth
Utilization of ethanol as Carbon-Source	+	+	+	+	+
SUGAR FERMENTATION					
Glucose	+	+	+	+	+
Glycerol	+	-	+	-	-
2-Keto-D-Gluconate	+	-	-	-	+
L-Arabinose	-	-	-	-	+
D - Xylose	+	+	+	-	+
Adonitol	-	+	-	-	+
Galactose	+	+	+	+	+
Inositol	-	-	-	-	-
Sorbitol	-	+	+	-	-

6-Methyl-D-glucoside	-	+	-	-	+
N-Acetyl-D-Glucosamine	-	+	-	-	+
Cellobiose	-	-	-	-	+
Lactose	+	-	-	-	+
Maltose	-	+	-	-	+
Saccharose/Sucrose	+	+	-	+	+
Trehalose	-	+	-	-	-
Melezitose	-	+	-	-	+
Raffinose	+	-	-	+	-

Identified microorganisms	Candida pseudotropicalis	Candida tropicalis	Endomyces spp	Saccharomyces cerevisae	Trichosporon beigelii

KEY: ISOLATES CODES

H_{151y} - H_{155y} ; H_{251y} -H_{255y} ; H_{351y} -H_{355y} ; H_{451y} -H_{455y} = Isolates from processors H_1, H_2, H_3, and H_4 from "Hanma" Village respectively.

B_{151y} - B_{155y} ; B_{251y} -B_{255y} ; B_{351y} -B_{355y} ; B_{451y} -B_{455y} = Yeast isolates from processors B_1, B_2, B_3, B_4 from "Bomo" village respectively.

Y_{151y} -Y_{155y} ; Y_{251y} -Y_{255y} = Yeast isolates from processors Y_1 and Y_2 from "Biye" village respectively.