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LACTIC ACID BACTERIA OF FERMENTING TEF DOUGH AND FERMENTED KOCHO AND THEIR INHIBITORY EFFECT ON CERTAIN FOOD -BORNE PATHOGENS OR SPOILAGE ORGANISMS

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BY

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DEDICATION

To my grand mother W/o Tisseme Aboye.

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ABSTRACT

Lactic acid fermentation is the commonest and inexpensive traditional household food manufacturing method.

"Injera" and "Kocho" are two major Ethiopian lactic acid fermented foods.

Injera is a pancake-like bread baked from "Tef"(Eragrostis tef) or other cereals' flour fermented for two to three days.

Kocho is a product of "Ensete" plant (Ensete ventricosum) pit-fermented for few weeks to several months. It is then baked and consumed.

Members of the Enterobacteriaceae family initiate tef dough fermentation. They lower the pH from about 6.3 to 4.7. Lactic acid bacteria(LAB)- Leuconostoc, Lactobacillus, Streptococcus and Pediococcus species succeed and further lower the pH to or below 4.00. Species of Bacillus degrade the starch.

Kocho fermentation was found to be initiated by Leuconostoc spp. and the other LAB follow to lower the pH. A properly fermented kocho has a pH of 4-4.5 and contains a large number of LAB. Yeasts and molds were also common at lower pH values in both foods.

Lactic acid bacteria were known to prevent food-borne pathogens and spoilage bacteria from growing in fermenting foods using their antibiotic metabolites.

Nevertheless, inhibitory effect of fermenting tef or fermented kocho on such undesirable organisms was not known.

Hence, in this study effect of these two acidic foods and also their components in broth on certain disease-causing and spoilage bacteria

was determined.

The results showed that tef dough began inhibition after 30 h of fermentation (pH 4.7). This period had maximum nutrient availability and best inhibitory activity among all fermentation periods in tef.

Fermented kocho (pH 4.3) was inhibitory except for *B. cereus*. It also inhibited best at lower concentrations but higher pH values among all agents employed.

Spent media from the LAB inhibited growth of most test bacteria where *Streptococcus* spp. did the best amongst the group.

The pH decline as a result of introducing acetic and/or lactic acid to broth was much higher than any one of the other agents. Therefore, the inhibitory activity of the foods or the LAB was due to antibiotics elaborated by the LAB and some species of *Bacillus*.

Heat treatment of tef dough or kocho extract also seemed to have a promotion effect on antimicrobial potency of the extracts.

Sporeforming bacteria, yeasts and molds survived baking temperature, but much lowered to low populations.

Furthermore, tef dough fermented upto 30-48 h (pH 4.1-4.7) and properly fermented kocho (to about pH 4.3) both baked before consumption were found as safe foods from food-borne infections and spoilage bacteria.

Further studies on these two foods and their microflora were recommended.

CHAPTER ONE

INTRODUCTION AND LITERATURE REVIEW

1.1. FOOD FERMENTATIONS

Food fermentations are complex biochemical transformations of organic substances, brought about by enzymes originating either from the food or microorganisms associated with it (Prescott and Dunn, 1959).

Raw foods harbor a heterogenous group of microorganisms. The degree of contact of different foods with the air, soil, water, animal&human bodies, equipment, and other foods determines the nature of the contaminating flora.

Microorganisms involved in fermentation are bacteria, yeasts and molds (Prescott and Dunn, 1959; Pederson, 1979; Djien, 1982). Nearly all food fermentations are the result of the activities of more than one species of microorganisms.

Fermentation is one of the oldest known methods of preparing and preserving food and fermented foods are generally considered more attractive and desirable than the unfermented raw materials from which they are prepared. In addition, improvements to external properties (such as taste, texture, aroma), nutritional upgrading and extended shelf-life are achieved as the result of fermenting foods. Traditional methods of preparing fermented foods are not complicated

and do not require expensive equipment (Djien, 1982). Fermentation of indigenous foods is, therefore, considered by many to be an effective, inexpensive, and nutritionally beneficial household technology for communities with food scarcity and malnutrition (Jay, 1978; Steinkraus, 1985; Alnwick, 1988).

1.1.1. THE LACTIC ACID FERMENTATION

Lactic acid producing bacteria are, with some exceptions, Gram-positive, catalase negative, non-sporeforming spheres and rods. All require carbohydrates for energy, are unable to synthesize amino acids and growth factors for reproduction, and all produce lactic acid from sugars. These bacteria thrive mostly in microaerophilic to anaerobic conditions (Buchanan and Gibbons, 1974). Their rapid production of lactic acid is known to inhibit the growth of most bacteria isolated from the soil and plant surfaces (Tomkins, et al, 1988; Wong and Chen, 1988). The lactic acid bacteria include species of Lactobacillus, Leuconostoc, Pediococcus, and Streptococcus. Species belonging to Streptococcus and Leuconostoc produce the least acid; the homofermentative species of Lactobacillus produce the greatest amount of acid. Heterofermentative Leuconostoc and Lactobacillus species convert glucose to about 50 % lactic acid, 25% acetic acid and ethyl alcohol, and 25% carbondioxide. This is important in flavor development and in leavening of certain bread-like fermented foods (Jay, 1978; Hasseltine, 1983; Marshall, 1987). In addition to giving the desired aroma, texture, taste and flavor, better palatability and physical appearance, the lactic acid bacteria

are known to produce various metabolites that preserve foods. This directly relates to the production of antimicrobial substances other than acids (Trammer, 1966; Shahani and Ayebo, 1980; Hosono, et al, 1990). These are usually antibiotics which are either bacteriostatic or bactericidal in action (Shoji, 1978; Hastings, et al, 1991; Muriana and Klaenhammer, 1991; Stiles and Hastings, 1991). The actions of these antibiotics are species specific in some cases, and of broad spectrum in others (Schillinger and Lucke, 1989). The antimicrobials so far isolated from lactic acid bacteria include lactocins, lactacins, lacticin, helveticins, diplococcins, leucocins, pediocins, and plantaricins (Schillinger and Lucke, 1989; Stiles and Hastings, 1991).

The antimcrobial behavior of lactic fermentations, therefore, appears to be due to the cumulative effect of the acids, hydrogen-peroxide, carbondioxide (creating anaerobic environment) and the antibiotics. Some of these compounds are volatile or are readily oxidized while others are stable for a short period during the fermentation process.

1.2. TWO COMMON ETHIOPIAN LACTIC ACID FERMENTED FOODS

1.2.1. TEF

Tef (Eragrostis tef) is a fine millet-like grain indigenous to Ethiopia. Tef belongs to the tribe Eragrostea, sub-family Eragrostideae, and family Poaceae. As stated by Ebba (1975) tef has about 35 cultivars of which 21 are white-seeded and 14 brown- or red-seeded. However, all of these cultivars are known to have similar

aminoacid compositions (Lester and Bekele, 1981; Bekele, 1989). Tef is cultivated over a wide range of climatic conditions as well as soil types (Costanza, 1979). According to the report by the Ministry of Agriculture (1983) the production of tef exceeds all other cereal crops cultivated in the country. People also prefer "injera" from tef than from any other source (ICNND, 1959).

TEF FERMENTATION:

Traditionally "injera" (large, flat, thin and round pancake-like fermented bread) is prepared from tef flour mixed with adequate amount of water in large earthen jar.

"Irsho" (left-over batter from previous fermentation or yellowish liquid on top of fermenting dough) is usually added to the dough to enhance the fermentation. The addition of irsho does not alter the composition of the fermentative microflora that initate the process except increasing their population (Gashe, et al, 1982; Gashe, 1987). The addition of irsho ensures that the fermentation will proceed to the desired end.

The dough is allowed to ferment for a period of 24 to 72 h at room temperature (18-22°C) (Table 1).

The first 24 h of fermentation is characterised by excessive gas evolution and rising of dough. When the dough settles and bubbling of gas diminishes, a yellow liquid appears at the surface. This liquid

is known as Irsho and contains sugars as glucose fructose and maltose (Umeta and Faulks, 1988), aminoacids and soluble minerals as well as a large fraction of the microorganisms involved in the fermentation process (Gashe, et al, 1982). Irsho is, however, discarded by housewives because of its slightly higher acidity than the dough. The volume of this liquid continues to increase for upto 48 h. The pH of the dough is reduced from the initial pH of about 6.5 to below 4.0. Gram-negative saccharolytic bacteria including members of the Enterobacteriaceae family are known to reduce the pH to 5-5.5 (Gashe, et al, 1982). Further reduction in pH to 4.0 or below is achieved as the result of the activities of lactic acid bacteria (Gashe, 1985). The lactic acid bacteria that carry out the fermentation process in tef dough belong to the genera Lactobacillus, Leuconostoc, Pediococcus, and Streptococcus.

Before baking, about 10% of the fermented dough is mixed with an equal volume of water and boiled in a container for about 3-5 min to make a thin paste called "Absit". The hot absit is added to the dough and fermentation is allowed to proceed for 30 min to 2 h. It is then baked on an oiled hot earthen plate for 3-5 min.

Yeasts, although present throughout the process, appear in large numbers after the pH has dropped to 5.0 or below. These yeasts become predominant in the liquid layer after complete liquid/solid separation. The fermentative yeasts are also responsible for the rising of the dough during the second stage of fermentation (Gashe,

et al, 1982; Gifawessen and Besrat, 1982).

The strong acidic taste of injera may create complaint of heart burns as the result of consuming injera which has undergone fermentation beyond 48 h. The preferred pH or the appropriate duration for fermentation need to be defined.

1.2.2. ENSETE

Ensete (Ensete ventricosum) one of the leading root crops in Ethiopia is a banana-like perennial which serves as the major carbohydrate source for more than a sixth to a fourth of the population (Shack, 1966; Stanley, 1966; Besrat, et al, 1979; Gashe, 1987).

It is primarily cultivated in Sidamo, southern part of Shoa, Gamo-Gofa, and northern part of Kaffa (Demeke, 1986).

The ensete plant is cultivated at altitudes ranging between 1,500 and 3,000 m. above sea level (Smeds, 1955; Bezuneh, 1966; Bezuneh, et al, 1967; Bezuneh,1984). It takes from 6 to 8 years for an ensete plant to mature and be harvested for food or fiber. The fiber is used for making ropes and sacks, etc. (Shack, 1966).

An average family cultivates 200 to 400 ensete plants in a small plot of land and the consumption per person varies from 10 to 20 plants per year. A mature plant is estimated to yield from 26 to 42 Kg of food. In regions where kocho constitutes the major staple

carbohydrate food, the consumption is estimated as being 0.43 to 0.70 Kg per person per day (Ethiopian Nutrition Institute, 1979). ''Kocho'' and ''bulla'' are the two main fermented ensete products.

KOCHO FERMENTATION:

The inner non-pigmented portions of the trunk and stem are scraped using either large bamboo splinters, shoulder blades from large animals or scythes. The corm is pounded with a multipronged mortar to a pulp. During this process about 60-80 per cent of the long fibers (usually used to make ropes, sacks, etc.) are known to be removed (Girma, et al, 1985; Gashe, 1987).

The pulverized and scraped portions of the stem and corm are then kneaded into a mixture called kocho. This is rolled into small rolls and covered with fresh ensete leaves and left at ambient temperatures to initiate the fermentation for 2 to 5 days or more.

In the mean time a pit size of about 1 m^3 is dug and completely lined with fresh ensete leaves. The kocho which was allowed to ferment at ambient temperature is mixed, kneaded again and placed in the pit. It is then pressed by hand and/or feet and covered with fresh ensete leaves. A portion of the pigmented and cellulosic refuse from the scrapings and pulverized materials is layered on top to create a 2-4 cm cover. Heavy materials such as large stones are then laid on top of the mantle. The whole process of pressing and laying heavy materials is to create air-tight conditions.

Fermentation is terminated within three to six months in warmer regions while it may last for several months or years in colder regions. If fermentation under warm conditions is allowed to continue for a prolonged period, the kocho becomes very acidic and occasionally becomes discolored (Gashe, 1987).

The predominant bacteria involved in kocho fermentation are Leuconostoc, Streptococcus, Pediococcus, Lactobacillus species, endospore-forming bacteria, yeasts, and molds (Girma, et al, 1985; Gashe, 1987). Leuconostoc sp. was found to initate the fermentation process. Kocho at 0 h (just before initiation of fermentation) contains about 84 per cent moisture, has a near neutral pH of 6.5 and contains about 1.5 per cent soluble reducing sugars. After 80 days of fermentation the moisture content is reduced to about 60 per cent, the pH to 4.2 and the reducing sugars to 0.3 per cent.

Sporeforming bacilli such as Bacillus cereus, B. subtilis, B. licheniformis, and B. megaterium as well as clostridia participate in the fermentation during the first two weeks of fermentation (Gashe, 1987).

1.3. STATUS OF RESEARCH ON MICROBIOLOGICAL SAFETY OF ETHIOPIAN FERMENTED FOODS

Research on tef and kocho are limited to the microbiogical and biochemical changes taking place during fermentation (Stewart and Asnake, 1966; Besrat, et al, 1979,1980; Gashe, et al, 1982;

Gifawessen, et al, 1982; Girma, et al, 1985, 1989; Gashe, 1987; Umeta, 1986, 1988, 1989).

The antitumor and anticarcinogenic behaviour and potential of lactic cultures, their utilization in the therapy of gastrointestinal disorders, their use in reduction of mutagenic substances in foods and beverages, their production of several limiting nutrients have been known for several years (Vincent,et al, 1959; Fuska, et al, 1976; Schillinger and Lucke, 1989; Hosono et al, 1990; Salminen,1990). Whether the lactic acid bacteria found in association with tef and kocho display simlar characteristics is not known. There is also little data on the survival and growth of pathogenic and spoilage bacteria in the fermenting and fermented foods.

1.4. OBJECTIVES OF THE STUDY:

1. to isolate and identify the microorganisms involved in tef and kocho fermentations.

2. to measure the effects of the extracts(aqueous portions) of fermenting tef and fermented kocho on the growth and survival of selected group of microorganisms which are either associated with food poisoning, food infections or spoilage.

3. to investigate the survival and growth characteristics of

the selected microorganisms introduced into the fermenting foods at time 0 h.

4. to study the effects of acetic and lactic acids and their combinations on the growth and survival of the selected group of microorganisms.

5. to identify those fermentative microorganisms which produce inhibitory substances from the fermenting tef and fermented kocho.

6. to identify the optimum period for production of the inhibitory substance(s) during fermentation.

7. to recommend appropriate time/pH/acidity of fermentation for commencing fermentation and baking of injera.

CHAPTER TWO

MATERIALS AND METHODS

2.1. FOODS EMPLOYED FOR FERMENTATION

2.1.1. TEF

Tef grain (*Eragrostis tef*) was purchased from an open market in Addis Ababa and ground in a local flour mill. One Kg of the flour was thoroughly mixed with 1.8 l of tap water in duplicate 4-litre capacity fermentation jars. The jars were covered with hard paper and allowed to ferment at room temperature (18-22^{0}C) for upto 72 h.

2.1.2. KOCHO

Fermented kocho was bought from an open market in Addis Ababa.

2.2. SAMPLING

Sampling of tef dough was done immediately after mixing the flour with water (0 h) and at 30, 48, and 72 h of fermentation. During sampling, 25 ml aliquot of dough was drawn for dilution as well as subsequent cultivation of the microorganisms. Appropriately diluted samples were pour-plated into the respective culture media.

Appropriately diluted kocho samples were also used for different analyses.

Measurement of pH was carried out during sampling using a pH meter

(Corning, Model 140, USA).

2.3. TEST ORGANISMS

The test organisms selected (food poisoning, food infection or spoilage microorganisms) were the following: Bacillus cereus, Klebsiella species, Pseudomonas aeruginosa, Salmonella species, Shigella species, and Staphylococcus aureus. These test bacteria were obtained from the National Research Institute of Health (NRIH), Addis Ababa.

2.4. CULTURE MEDIA UTILIZED

2.4.1. Assay Broth:

The assay broth used for culturing all the organisms isolated from fermenting tef, fermented kocho and the test bacteria contained the following ingredients (g/l of distilled water): 10, Bactopeptone; 8, 'Lab-Lemco' powder; 10, Tryptose; 20, Dextrose; 2, K_2HPO_4; 2, Ammonium Iron Citrate; 0.2, $MgSO_4.7H_2O$; 0.05, $MnSO_4.2H_2O$; 5, NaCl. Known volume of the dissolved assay broth was distributed into screw-capped test tubes, autoclaved (121^oC, 15 min, 15 lbs/sq in), and then cooled to room temperature before use.

2.4.2. Bacteriological Peptone (OXOID, L 37):

Peptone was used as a diluent at concentration of 0.1%.

2.4.3. Carbohydrate Fermentation Medium:

Carbohydrates (sugars, polyols, etc., at 0.5% concentrations) were

introduced into tubes of Nutrient Broth containing Durham tubes supplemented with bromocresol purple for biochemical characterizations of isolates.

2.4.4. Dextrose Tryptone Agar (OXOID, CM 75):

The medium was composed of (g/l of distilled water): 10, Tryptone (Oxoid,L42); 5, Dextrose; 0.04, Bromocresol purple and 12, Oxoid Agar No.3. Final pH was 6.9.

It was used for isolating Pediococcus species from fermenting tef and fermented kocho. Tiny, pinpoint colonies with large yellowish hallo around them which are Gram-positive and of coccal morphology appearing mostly as diplococci were tentatively identified as pediococci. The colonies were counted and representative samples transferred into tubes for pure culture isolation and characterization. Stock cultures were kept under refrigeration until use.

2.4.5. Egg-Yolk Agar (OXOID, SR 47):

The basal medium was composed of (g/l of distilled water): 2.8, Nutrient Agar and 1, NaCl. This was dissolved and then autoclaved. Ten milliliters of sterile Egg-Yolk emulsion, prepared according to OXOID manual (1972), were added per 100 ml of basal medium following aseptic technique. This was thoroughly mixed and pour-plated. This medium was used to differentiate Bacillus cereus from other bacteria. On this medium colonies of B. cereus appear as large, irregular colonies usually with extended rhizoid-like protrusions and with

clear zones surrounding them.

2.4.6. Medium For Acid And Gas Production:

The medium was used for testing acid and gas production from glucose and was used for characterizing members of the Enterobacteriaceae and spore-forming bacilli isolated from fermenting tef and fermented kocho. The medium contained (g/l of distilled water): 1, Diammonium hydrogen phosphate; 0.2, KCl; 0.2, $MgSO_4$; 0.2, Yeast extract. Into this medium were added 15 ml of a 0.04% (w/v) solution of bromocresol purple. The pH was adjusted to 7.0 before autoclaving. Five milliliters of this solution were placed into each tube containing Durham tube for trapping gases. The tubes were then autoclaved. Stock glucose solution was separately sterilized and appropriate volume aseptically transferred into each tube to give a 0.5% concentration. Tubes were inoculated with the pure cultures and incubated for 7 days. After 7 days of growth, production of acid and gas was detected. Change in color of the medium to yellow and detection of gas was taken as positive result for glucose utilization.

2.4.7. Mac Conkey Agar (OXOID, CM 115):

The components of this medium were (g/l of distilled water): 20, Peptone (Oxoid, L37); 10, Lactose; 1.5, Bile salts No.3 (Oxoid, L56); 5, NaCl; 0.03, Neutral red; 0.001, Crystal violet and 15, Agar No.3 (Oxoid, L13). Final pH was 7.1.

This medium was used to isolate, maintain and purify members of the Enterobacteriaceae family.

2.4.8. Mannitol Salt Agar (OXOID, CM 85):

This medium was composed of (g/l of distilled water): 1,'Lab-Lemco' Beef Extract; 10, Bacto peptone; 10, Mannitol; 75, NaCl; 0.025, Phenol red and 15, Oxoid Agar No.3. Final pH was 7.5.

The medium was used for isolation of Staphylococcus aureus. The organism develops golden-yellow colonies with yellow zones around it.

2.4.9. Methyl Red-Voges-Proskaur Medium (OXOID, CM 43):

The components of this medium were (g/l of distilled water): 5, Peptone (Oxoid,L49); 5, Phosphate buffer and 5, Dextrose. Final pH was 7.5.

This medium was utilized for testing acid as well as Acetyl methyl carbinol productions by members of the Enterobacteriaceae and spore-forming bacilli isolated from the foods. After 3, 5, and 7 days of incubation pH of the medium was monitored. Acetyl methyl carbinol (VP) prduction was detected by mixing 1 ml of the culture with 0.5 ml of alcoholic alpha-naphthol and 0.5 ml of 40% (w/v) potassium hydroxide (Collins, 1976). Development of a red color after 30-60 minutes at room temperature was taken as a positive result for VP prduction test.

For testing acid production, 5 drops of methyl red reagent (0.1 g, methyl red in 300 ml of 95% ethanol made upto 500 ml with distilled water) were added to 5 ml of culture. Development of red color denotes a pH of 4.5 or less and was considered as positive result for strong acid production.

2.4.10. Milk Agar (OXOID, CM 21):

This medium was composed of (g/l of distilled water): 100, Skim milk powder and 20, Agar. Milk Agar plates were streaked with the isolates and incubated at 32°C for 7 and 14 days. Clearing of the medium around and underneath the colonies was taken as positive for hydrolysis of the milk protein, casein.

2.4.11. Nitrate Broth (DIFCO):

The components of this medium were (g/l of distilled water): 5, Peptone; 3, Beef Extract and 1, Potassium nitrate.

A loopful of each pure culture (24 h old in nutrient broth) was inoculated into sterile tubes containing nitrate broth and incubated for three days. After incubation, 5 drops of a sulphanilic acid-acetic acid mixture (8 g, sulphanilic acid in 1 l of 1.5 N acetic acid) and 5 drops of acidic Dimethyl-alpha-naphthyl amine solution (6 ml, aqueous Dimethyl-alpha-naphthylamine mixed with 1 l of 1.5 N acetic acid) were added to each test tube. Positive tubes for nitrate reduction showed development of a red or yellow color or no color even after addition of zinc dust.

2.4.12. Nutrient Broth (OXOID, CM 1):

The medium was composed of (g/l of distilled water): 1, 'Lab-Lemco' powder (Oxoid,L29); 2, Yeast Extract (Oxoid,L20); 5, Peptone (Oxoid,L37) and 5, NaCl. Final pH was 7.4.

This medium was used to grow and maintain all isolates and test bacteria during assays and characterizations.

2.4.13. Potato Dextrose Agar (OXOID, CM 131):

This medium was composed of (g/l of distilled water): 4, Potato Extract; 20, Dextrose and 15, Agar No.1 (Oxoid, L11). Final pH was 5.6.

Yeasts and molds were isolated and maintained using potato dextrose agar.

2.4.14. Pseudomonas Isolation Agar (DIFCO):

The components of this medium were (g/l of distilled water): 20, Bacto Peptone; 1.4, Magnesium chloride; 10, Potassium sulphate; 0.025, Irgasan and 13.6, Bacto Agar. Final pH was 7.0.

It was used to selectively isolate *Pseudomonas aeruginosa* from fermenting foods. Colorless to greenish colonies were taken as typical colonies representing *P. aeruginosa*.

2.4.15. Rogosa Agar (OXOID, PM 221):

This medium was composed of (g/l of distilled water): 10, Tryptone (Oxoid,L42); 5, Yeast Extract (Oxoid,L20); 20, Dextrose; 1 ml,'Tween 80'; 6, Potassium dihydrogen phosphate; 2, Ammonium citrate; 25, Sodium acetate; 1.32 ml,glacial Acetic acid; 0.575, $MgSO_4.7H_2O$; 0.12, $MnSO_4.2H_2O$; 0.034, $FeSO_4.7H_2O$ and 20, Agar No.3(Oxoid,L13). Final pH was 5.4.

The medium was employed for isolation, purification and maintenance of lactobacilli isolated from fermenting tef dough and fermented kocho. The medium was overlayed to create anaerobic conditions. White to greyish-white, irregular colonies growing in this medium after

incubation for 3-5 days at 32°C and which are rod-shaped and Gram-positive, catalase negative were considered tentatively as lactobacilli.

2.4.16. Saboraud Dextrose Agar (OXOID, CM 41):

This medium was composed of (g/l of distilled water): 10, Mycological Peptone (Oxoid,L40); 40, Dextrose and 15, Agar No.1 (Oxoid,L11). Final pH was 5.6.

Ability to grow on Saboraud dextrose agar was used as one characteristic in the differentiation and identification of *Bacillus* species.

2.4.17. Salmonella-Shigella Agar (OXOID, CM 99):

The components of this medium were (g/l of distilled water): 5, 'Lab-Lemco' Powder; 5, Peptone; 10, Lactose; 8.5, Bile salts; 10, Sodium citrate; 8.5, Sodium thiosulphate; 1, Ferric citrate; 0.00033, Brilliant green; 0.025, Neutral red and 15, Agar. Final pH was 7.0.

Salmonella sp. and *Shigella* sp. were selectively isolated using S-S agar.

2.4.18. Slanetz and Bartley Medium (OXOID, CM 377):

This medium was composed of (g/l of distilled water): 20, Tryptose (Oxoid,L47); 5, Yeast Extract (Oxoid,L21); 2, Dextrose; 4, Disodium phospate $2H_2O$; 0.4, Sodium azide; 0.1, Tetrazolium chloride and 10, Agar No.1 (Oxoid,L11). Final pH was 7.2.

It was used to isolate *Streptococcus* species (especially *Strep*.

faecalis) from the foods. All maroon-colored colonies which are Gram-positive and of coccal morphology were tentatively considered to be Group IV streptococci. Incubation was at 45°C for 24 h.

2.4.19. Starch Agar (DIFCO):

The medium was prepared by adding 0.5% soluble potato starch into nutrient Agar. Duplicate plates were streaked with each of the isolates and incubated at 32°C. At 3 and 5 days of incubation, the plates were flooded with Gram's iodine solution. Presence of a clear zone underneath and around the growth was taken as an indicator of hydrolysis of starch.

2.4.20. Sucrose Gelatin Agar:

This was prepared as follows (g/l of distilled water): 10, Sucrose; 20, Gelatin; 13, Nutrient Broth; 15, Agar. Incubation was at 32°C for 24 h. Typical colonies of Leuconostoc species appeared as moist, runny, droplet-like or frost-like droplet colonies on the surface of the medium. These were confirmed by microscopic observations, too. The cultures were maintained on the same medium.

2.4.21. Thioglycollate Medium (OXOID, CM 173):

This medium was composed of (g/l of distilled water): 5, Yeast Extract (Oxoid,L20); 15, Tryptone (Oxoid, L42); 5.5, Dextrose; 0.5, Sodium thioglycollate; 2.5, NaCl; 0.5, 1-Cystene hydrochloride; 0.001, Resazurin and 0.5, 'Ionagar' No.2. Final pH was 7.1.

The medium was employed to determine if the isolates grew

anaerobically. Icubations were at 32°C. Growth after 3 days was observed. Submerged growth in the tubes was taken as positive for anaerobic growth.

2.4.22. Tryptone Soya Agar (OXOID, CM 131):

The components of this medium were (g/l of distilled water): 15, Tryptone (Oxoid,L42); 5, Soya Peptone (Oxoid,L44); 5, NaCl and 15, Agar No.3 (Oxoid,L13). Final pH was 7.3.

It was used as a general purpose medium for determining total aerbic count, to cultivate spore-formers, and maintain stock cultures of some of the fermentative and test bacteria.

Unless stated other wise, all chemicals used were of analytical grade and were obtained from BDH (England) and Test tube Antibiotic Assay Method as recommended by Kavanagh (1975) was utilized.

2.5. STERILIZATION

Sterilization was carried out at 121°C and 15 lb./sq. in. for 15 min. Heat-labile compounds were filter-sterilized using Carlson HP/EKS filters (Gallen kamp, UK).

2.6. ENRICHMENT FOR SPORE-FORMING MEMBERS

Samples of 25 ml portions of food diluted with 225 ml of diluent were heat-treated at 80°C for 10 min to enrich or select for spore-formers. After appropriate dilution, samples were plated on TSA and incubated at 32°C for 2-5 days.

The following physical and biochemical tests were employed for characterization of the sporeforming Bacillus species as recommended by Starr, et al (1981):

(i). Anaerobic growth;

(ii). Growth at 50 and 65°C;

(iii). Growth in 7% Sodium chloride;

(iv). Production of Catalase;

(v). Acid and gas production from Glucose;

(vi). Nitrate reduction;

(vii). Voges-Proskaur reaction;

(viii).Decomposition of Casein;

(ix). Hydrolysis of starch; and

(X). Growth on Saboraud dextrose agar

2.7. EFFECTS OF VARIOUS COMPONENTS OF FERMENTING TEF AND FERMENTED KOCHO ON TEST ORGANISMS

2.7.1. EFFECT OF FERMENTATION ON THE SURVIVAL AND GROWTH OF THE TEST BACTERIA

The test organisms were thoroughly mixed with fresh tef dough and with fermented kocho to give an initial population of $3x10^3$ and $4x10^3$ CFU/g dry weight of tef and kocho, respectively. At times 0, 30, 48 & 72 h of fermentation 25 ml portions were removed to determine the population of the test bacteria.

2.7.2. EFFECT OF ORGANIC ACIDS ON THE SURVIVAL AND GROWTH OF THE TEST BACTERIA

Nutrient broth containing graded amounts of acetic and lactic acids or their combinations was prepared for culturing the test bacteria. A loopful of cultures from each of the 24 h old test bacteria were inoculated into each tube of assay broth containing the organic acids. Growth was monitored using a spectrophotometer (OD 540 nm) (Spectronic 21, model UVD, Bausch & Lomb, USA).

2.7.3. EFFECT OF SPENT MEDIA OBTAINED AFTER CULTURING LACTIC ACID BACTERIA ON THE SURVIVAL AND GROWTH OF THE TEST BACTERIA

Pure cultures of *Lactobacillus*, *Pediococcus*, *Leuconostoc*, and *Streptococcus* species were grown in the assay broth by incubating tubes at 32^{0}C for 72 h. Graded quantities of the spent media obtained after centrifugation were combined with fresh assay broth contained in tubes following aseptic technique. The tubes were then inoculated with the test bacteria and incubated. Growth was monitored using a spectrophotometer (OD 540 nm).

2.7.4. EFFECT OF FERMENTING TEF DOUGH AND FERMENTED KOCHO EXTRACTS ON THE SURVIVAL AND GROWTH OF THE TEST BACTERIA

Samples of fermenting tef dough and fermented kocho were centrifuged at 5000 rpm for 15 minutes. Extracts from fermenting tef and fermented kocho which has been mixed with half its volume of distilled water were collected aseptically. The clear liquid which appeared on top of the sediment was collected in sterile containers

and used immediately.

Graded amounts of the extracts were introduced into tubes of assay broth. The tubes were inoculated with the test bacteria following asepetic techniques. These were incubated for 24 h at 32°C. Growth was recorded by reading optical density readings at 540 nm using a spectrophotometer.

2.7.5. EFFECT OF HEAT TREATMENT ON INHIBITORY PROPERTY OF EXTRACTS FROM TEF DOUGH AND FERMENTED KOCHO ON THE TEST BACTERIA

Extracts from fermenting tef dough (30 h) and from fermented kocho were collected aseptically in sterile containers and were heat-treated for 5 min at 45, 60, and 80°C. Graded samples of the heat-treated extracts were introduced into tubes containing assay broth. A loopful of 24 h fresh cultures of *P. aeruginosa* and *B. cereus* were inoculated into each of the tubes. The tubes were then incubated for 24 h and absorbance read at 540 nm.

2.7.6. EFFECT OF BAKING TEMPERATURE ON THE SURVIVAL AND GROWTH OF TEST ORGANISMS

A known population of 24 h old culture of each of the test bacteria was inoculated into fermented tef or kocho just before baking. Then portions of the fermenting foods were baked at 92°C for 5 minutes. Microbiological analysis was carried out using the baked products.

2.8. PHYSICAL AND CHEMICAL ANALYSES OF FERMENTING TEF DOUGH AND FERMENTED KOCHO

2.8.1. Determination of Moisture Content:

The method described by Welcher (1975) was modified and employed as follows. Accurately weighed food sample (10 g) was placed in an oven desicator whose temperature was set at 95°C for 24 h. Moisture content was calculated based on differences on weight before and after drying.

2.8.2. Determination of Reducing Sugars:

The method employed was that of Miller (1959). This method uses Dinitrosalicylic acid reagent (DNS). It was prepared by dissolving 8.0 g Dinitrosalicylic acid, 1.6 g Phenol, 0.4 g Sodium sulfite, 160.0 g Potassium sodium tartarate and 8.0 g Sodium hydroxide in 800 ml distilled water. The reagent was kept in brown bottles until use.

To 1 ml portions of the supernatant liquids from tef or kocho 1 ml DNS reagent was added and mixed thoroughly. This was placed in boiling water bath for exactly 5 minutes. After cooling, 5 ml of distilled water was added and the absorbance read at OD 540 nm using a spectrophotometer. The amount of reducing sugars present in the samples was extrapolated from a curve prepared using glucose as a standard.

2.8.3. Determination of Free Aminoacids:

The method outlined by Riemeredes and Klostermayer (1976) was

modified and utilized. Ninhydrin solution was prepared by dissolving 5 g of Ninhydrin in 188 ml of Methyl cellosolve. This was then combined with 62 ml of 0.1% $SnCl_2$ in sodium acetate buffer, pH 5.5. The buffer was pepared by combining 5.44 g of sodium acetate, trihydrated, in 100 ml of distilled water and 100 ml of glacial acetic acid. The final volume was raised to 1 l after pH adjustment.

To 1 ml aliquots of the clear supernatants (food samples) obtained by centrifuging, 1 ml of the ninhydrin solution and 1 ml of the acetate buffer were mixed in a tube and thoroughly mixed. The tube was immersed in a boiling water for 10 minutes. The color that developed was measured with a spectrophotometer at 570 nm. A standard curve prepared using aspartic acid served for estimating total soluble amino acids present in the samples.

CHAPTER THREE

RESULTS

3.1. MAJOR EVENTS IN TEF FERMENTATION

The major events taking place during tef fermentation are summarized in Table 1.

Table 1 shows that it is the Gram-negative saccharolytic bacteria including certain members of the family Enterobacteriaceae which were responsible for initiating the fermentation, dough rising and for reduction in pH from 6.3 to 5.8. Dough rising and evolution of gas in the fermenting dough were major characteristics associated with their activities and lasted for 18 h.

Even before the pH dropped to 5.8 or less, lactic acid bacteria appeared in large numbers. The first to appear in abundance were species of Leuconostoc, Streptococcus and Pediococcus. These were followed by Lactobacillus species (Table 2). The lactic acid bacteria lowered the pH to about 4.0 after 48 h of tef fermentation. During the active involvment of the lactic acid bacteria in the fermentation, a yellowish liquid appeared on top of the dough. The yellowish liquid which appears on top of the dough is known as "irsho". Yeasts and molds appeared in large numbers during the last stage of the fermentation when the pH was less than 4.5. Sporeforming members (Bacillus species) were present though not in

TABLE 1

MAJOR EVENTS IN TEF FERMENTATION

FERMENTATION TIME(h)	pH	PHYSICAL CHANGES IN TEF DOUGH	DOMINANT MICROFLORA
6	6.0	Beginning of gas evolution & dough rising	Gram-negative,aerogenic bacteria including members of the family Enterobacteriaceae
18	5.8	Dough rising completed	Members of the family Enterobacteriaceae
30	4.7	Appearance of liquid layer; dough began to settle; substantial reduction of gas evolution	<u>Pediococcus</u> sp. and <u>Leuconostoc</u> sp. <u>Streptococcus</u> sp. and <u>Lactobacillus</u> sp.
48	4.1	Maximum volume of liquid layer;complete settlement of dough; small amount of gas evolution	<u>Pediococcus</u> sp., <u>Leuconostoc</u> sp.,and <u>Lactobacillus</u> sp.
72	4.0	Confluent growth of film yeasts on the liquid layer	<u>Pediococcus</u> sp., <u>Lactobacillus</u> sp., <u>Leuconostoc</u> sp., yeasts and molds

large numbers as the lactics during the entire period of the fermentation.

3.2. MICROBIAL DYNAMICS OF FERMENTING TEF

The tef flour contained Gram-negative saccharolytic bacteria, Lactobacillus, Pediococcus, Leuconostoc, Streptococcus species, sporeforming bacteria and yeasts and molds.

The most abundant group initially were the saccharolytic members, leuconostoc and streptococci (Table 2). The saccharolytic members remained as the most dominant group during the first portion of the fermentation. As can be seen in Table 2, their population increased ($4x10^6$ CFU/g of tef flour at 18 h) until the pH fell to or below 5.8 and became inhibitory to their growth. Thereafter, their population declined. The saccharolytic members contributed a larger share to the total count for the first 18 h (Table 2).

Species of Pediococcus, Leuconostoc and Streptococcus appeared in large numbers after the 6th h of fermentation. The population increased by about 10^3-10^5 times at 18 h of fermentation. The population of Pediococcus, Leuconostoc and Streptococcus species stood at $6.5x10^8$, $3.4x10^8$ and $4.3x10^6$ CFU/g of flour at 30 h of fermentation. The population of Streptococcus and Leuconostoc species declined after the pH of the dough dropped to below 4.7. On the other hand, Pediococcus species showed population increment in the dough even when the pH was between 4.1 and 4.7. The pediococci started to

show decline in population after the pH dropped below 4.1.

Lactobacillus species had the least population during the first 18 h of fermentation when the pH was greater than 5.8. Its population, however, increased dramatically and at time 30 h, pH 4.7, it had reached $3x10^8$ CFU/g of flour. Their population continued to increase till the end of the fermentation. After the pH of the dough dropped to 4.1, lactobacilli became the single most abundant fermentative bacteria.

Of the sporeforming members, Bacillus species were found in modest numbers throughout the fermentation period.

The yeasts were present in fairly low numbers initially, but, their population increased to $6x10^7$ CFU/g of flour at 30 h of fermentation (pH 4.7). Further increase in population ($1x10^9$ CFU/g of flour) occured up to 72 h of fermentation. The abundance of molds was not determined due to lack of precision of the method used to estimate the microflora.

3.3. PHYSICAL AND CHEMICAL CHANGES DURING TEF FERMENTATION

Reducing sugars in the fermenting tef increased from about 32 mg/g at time 0 h to 73.8 mg/g dry weight of fermenting tef at 30 h of fermentation (Table 3). After 30 h of fermentation, the concentration of reducing sugars declined moderately. This decrease in sugar content was closely associated with declines in pH values through

TABLE 2

MICROBIAL DYNAMICS OF FERMENTING TEF

Fermentation Time (h)	p^H	ESTIMATED NUMBER OF ORGANISMS PER g DRY WT. OF TEF FLOUR							
		Enterobacteriaceae	*Lactobacillus species*	*Pediococcus species*	*Leuconostoc species*	*Streptococcus species*	*Bacillus species*	Yeasts	Total count
0	6.3	8.2×10^1	2.2×10^1	6.8×10^1	1.5×10^1	8.0×10^1	<10	<10	3.5×10^2
6	6.0	3.1×10^5	5.0×10^1	2.5×10^2	1.3×10^4	4.0×10^3	0.6×10^1	6.0×10^1	3.3×10^5
18	5.8	4.0×10^6	5.0×10^2	3.6×10^7	5.4×10^7	1.4×10^6	4.0×10^3	2.0×10^3	5.9×10^7
30	4.7	2.0×10^6	3.0×10^8	6.5×10^8	3.4×10^8	4.3×10^6	6.0×10^4	6.0×10^7	3.7×10^8
48	4.1	8.0×10^5	1.6×10^9	1.8×10^9	9.0×10^7	1.0×10^6	6.0×10^5	8.0×10^8	2.7×10^9
72	4.0	1.0×10^3	2.4×10^9	1.0×10^9	9.0×10^6	1.0×10^5	2.0×10^5	1.0×10^9	4.4×10^9

TABLE 3

PHYSICAL AND CHEMICAL CHANGES DURING TEF FERMENTATION

FERMENTATION TIME(h)	CONTENT/ g DRY WT. OF TEF FLOUR			
	pH	MOISTURE (per cent)	REDUCING SUGARS (mg)	FREE AMINOACIDS (mg)
0	6.3	65.50±2.5	32.4±1.2	3.8±0.1
6	6.0	66.00±2.9	69.2±1.5	4.0±0.1
18	5.8	67.60±3.5	72.0±1.5	4.5±0.2
30	4.7	68.00±3.0	73.8±2.0	5.8±0.3
48	4.1	67.40±2.6	70.2±1.1	5.2±0.2
72	4.0	66.40±2.2	34.2±1.0	4.3±0.1

time (pH 6.3 at time 0 h to 4.7 at time 30 h) and increase in the microbial load. The free aminoacid and water-soluble peptides also continued to increase up to 30 h of fermentation. Fermentation beyond 30 h brought about decreases in both reducing sugars and in the aminoacids and water soluble peptides. The moisture content showed the least variation.

3.4. MOISTURE,SUGAR, AND AMINO ACID CONTENTS OF FERMENTED KOCHO

The moisture content, reducing sugars, free amino acids and water-soluble peptides in kocho are shown in Table 4. As can be seen from Table 4, the amount of reducing sugars available in fermented kocho was very low (0.73 mg/g dry weight of kocho). The free amino acids and soluble protein content was 5.6 mg/g dry weight of kocho.

3.5. MICROBIAL COMPOSITION OF FERMENTED KOCHO

The pH of the kocho purchased from the market was relatively acidic (pH 4.3). The microflora present included lactic acid bacteria (*Lactobacillus*, *Pediococcus*, *Leuconostoc* and *Streptococcus* species), members of the families Bacillaceae, Actinomycetaceae and Streptomycetaceae as well as yeasts and molds (Table 5).

The dominant organisms were *Lactobacillus* spp. followed by species of *Pediococcus*. This was followed by yeasts and molds and by the spore-forming organisms. *Streptococcus* spp. were present in low numbers. On the other hand, Gram-negative saccharolytic bacteria including members of the Enterobacteriaceae family were absent.

TABLE 4

MOISTURE, SUGAR, AND AMINOACID CONTENT OF FERMENTED KOCHO

FERMENTATION TIME (h)	pH	CONTENT PER g. DRY WT. OF KOCHO		
		MOISTURE (per cent)	REDUCING SUGARS (mg.)	FREE AMINOACIDS (mg.)
Purchased fermented	4.3	44.0±3.5	0.73±0.06	5.6±0.3

TABLE 5

MICROBIAL COMPOSITION OF FERMENTED KOCHO

pH	ESTIMATED NUMBER OF ORGANISMS PER g DRY WT. OF KOCHO							
	Enterobacteriaceae	*Lactobacillus* species	*Pediococcus* species	*Leuconostoc* species	*Streptococcus* species	Spore-forming bacteria	Yeasts	Total count
4.3	----	5.2×10^6	2.3×10^6	1.9×10^4	<10	1.4×10^4	2.8×10^5	7.8×10^6

---- : No growth

3.6. SURVIVAL AND GROWTH OF THE TEST BACTERIA IN FERMENTING TEF

The test bacteria were able to grow and show population increments till the pH of the fermenting dough dropped to 4.7 (Table 6). Though the degree of inhibition of growth was variable, all were found to survive up to the last phase of the fermentation period (72 h, pH, 4.0). It appeared that the least affected of the test bacteria was *Salmonella* sp. which showed an elevated but a stable population of $2x10^4$ CFU/g even at pH 4.0. The most affected of the test bacteria were *Klebsiella* sp. and *Pseudomonas aeruginosa*.

3.7. SURVIVAL AND GROWTH OF THE TEST BACTERIA IN FERMENTED KOCHO

As can be seen in Table 7, *Shigella* sp. showed modest increment in population till the pH dropped to about 4.2. On the other hand, *B. cereus* survived the 72 h fermentation without significant drop in population. At the end of 72 h in the kocho, the test bacteria either died (*Klebsiella* sp. ,*Staph* . *aureus* and *Salmonella* sp.) or had only few survivors (*Shigella* sp. and *P. aeruginosa*).

3.8. SURVIVAL AND GROWTH OF THE TEST BACTERIA IN ASSAY BROTH SUPPLEMENTED WITH ACETIC AND LACTIC ACIDS

The most common organic acids produced in fermenting tef (Umeta, 1989) and kocho (Urga, Personal communications) are lactic and acetic acids. The two acids together make up about 97 per cent of the total organic acid produced by the fermentative microorganisms in both foods. Concentrations of acids greater than 1µl of acid/ml of broth were sufficient to retard growth of the test bacteria (Fig. 1a

TABLE 6

SURVIVAL AND GROWTH PATTERN OF THE TEST BACTERIA

IN FERMENTING TEF

Fermentation Time (h)	p^H	ESTIMATED POPULATION OF TEST BACTERIA (CFU/g DRY WT. OF TEF FLOUR)				
		Klebsiella species	*Staph. aureus*	*Salmonella species*	*Pseudomonas aeruginosa*	*Bacillus cereus*
0	6.3	4.0×10^3	4.0×10^3	4.0×10^3	4.0×10^3	4.0×10^3
30	4.7	5.6×10^7	2.8×10^4	8.9×10^4	4.0×10^4	3.0×10^5
48	4.1	6.0×10^5	1.2×10^3	5.0×10^4	1.3×10^3	1.5×10^3
72	4.0	< 10	4.0×10^1	2.0×10^4	< 10	5.6×10^2

ND: Not Determined

TABLE 7

SURVIVAL AND GROWTH PATTERN OF THE TEST BACTERIA IN KOCHO

Fermentation Time (h)	pH	ESTIMATED POPULATION OF TEST BACTERIA (CFU/g DRY WT. OF KOCHO)					
		Klebsiella species	*Staph. aureus*	*Salmonella species*	*Shigella species*	*Pseudomonas aeruginosa*	*Bacillus cereus*
0	4.3	3.0×10^3	3.0×10^3	3.0×10^3	3.0×10^3	3.0×10^3	3.0×10^3
30	4.2	-	< 10	< 10	7.4×10^4	< 10	1.1×10^5
48	4.2	-	-	< 10	< 10	< 10	1.6×10^4
72	4.1	-	-	-	< 10	< 10	4.0×10^3

- : No growth

-1c). Addition of 1 μl acetic acid per ml of broth was sufficient to lower the pH to about 6.4. At slightly greater than 1 μl of acetic or 1.5 μl of lactic acid/ml of broth, when pH was between 6.2-6.5, there was complete inhibition of growth. The most sensitive to the acetic acid treatment were Salmonella sp., Shigella sp. Staph. aureus and B. cereus; while P. aeruginosa and Klebsiella species were slightly resistant. On the other hand, when lactic acid was used, inhibition started for most of the test bacteria when the concentration exceeded 1.5-2 μl of lactic acid/ml broth. Salmonella and Shigella spp. and B.cereus were the most affected while S. aureus, Klebsiella sp. and P.aeruginosa slightly better survived. Nevertheless, the organisms did not die even after the addition of 5-6μl of acid or combinations of acids into the broth. Combining the two acids together did not improve the effectivness of the acids on the test bacteria.

3.9. SURVIVAL AND GROWTH OF THE TEST BACTERIA IN BROTH CONTAINING EXTRACTS FROM FERMENTING TEF

As shown in Figs. 2a-2c, there was moderate decrease in pH with increasing amounts of extracts introduced into the broth (a drop of about 1.6-2 pH units). The extract stimulated growth of most of the test bacteria when used at concentrations below 100 μl/ml of broth. Concentrations above 100 μl/ml of broth inhibited growth of the test bacteria to varying degrees. Extract obtained at 30 h of fermentation was generally better in inhibiting growth of the test bacteria than those obtained at 48 and 72 h of fermentation. The most resistant test bacteria to such kinds of treatment were B. cereus and

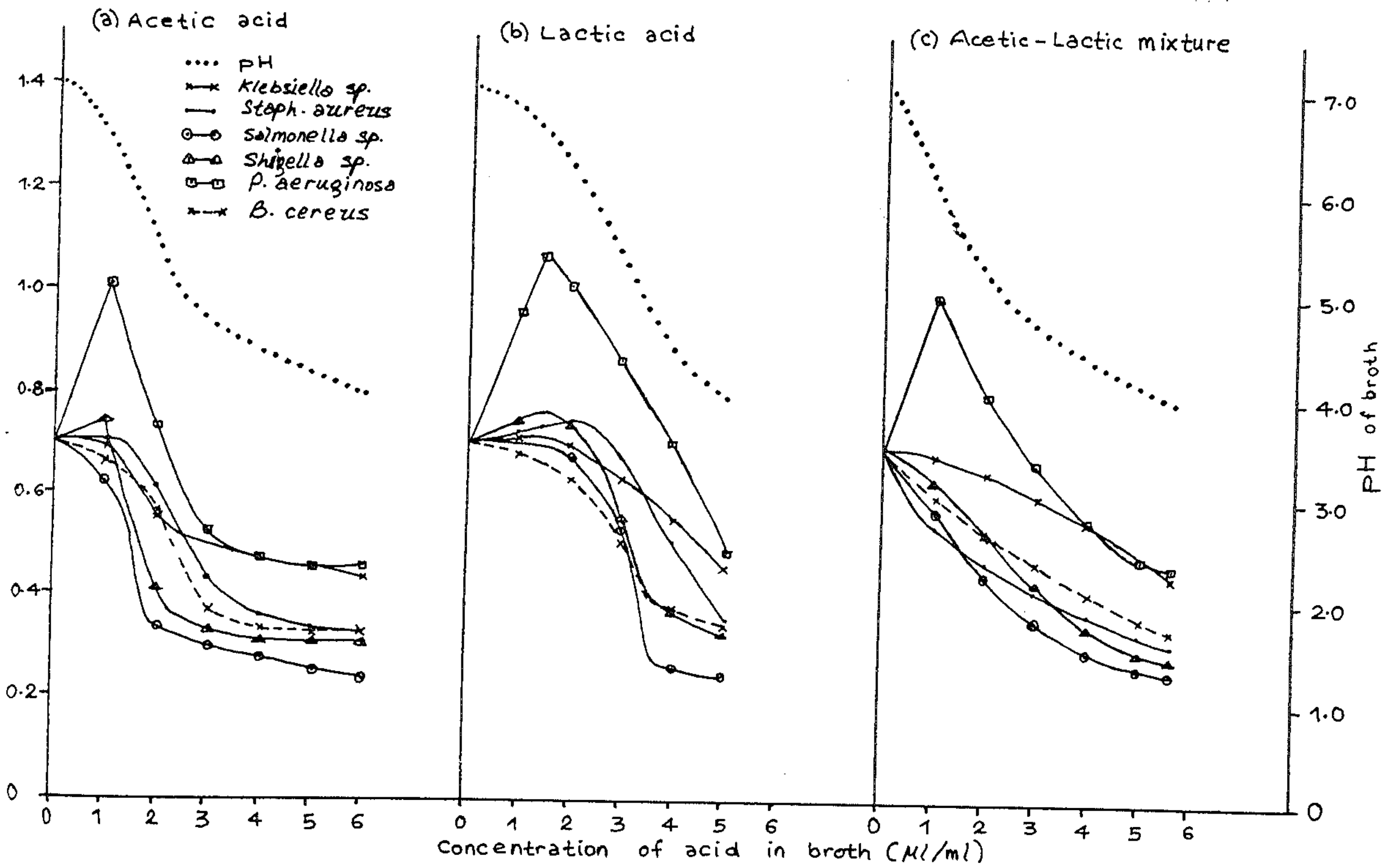


FIG. 1. SURVIVAL AND GROWTH OF THE TEST BACTERIA IN BROTH SUPPLEMENTED WITH ACETIC AND LACTIC ACIDS AND THEIR COMBINATIONS.

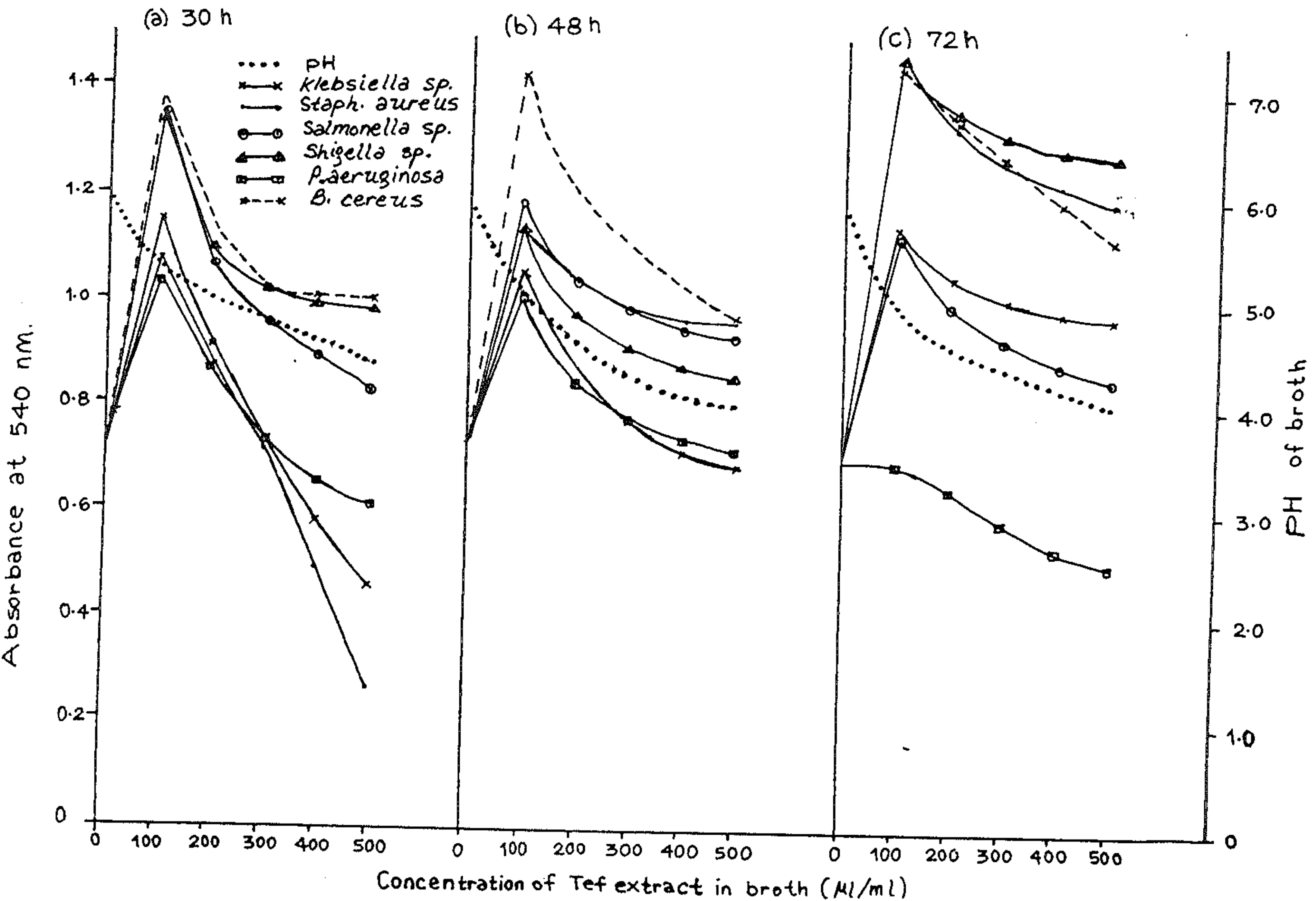


FIG. 2. SURVIVAL AND GROWTH OF THE TEST BACTERIA IN BROTH CONTAINING EXTRACTS FROM FERMENTING TEF DOUGH

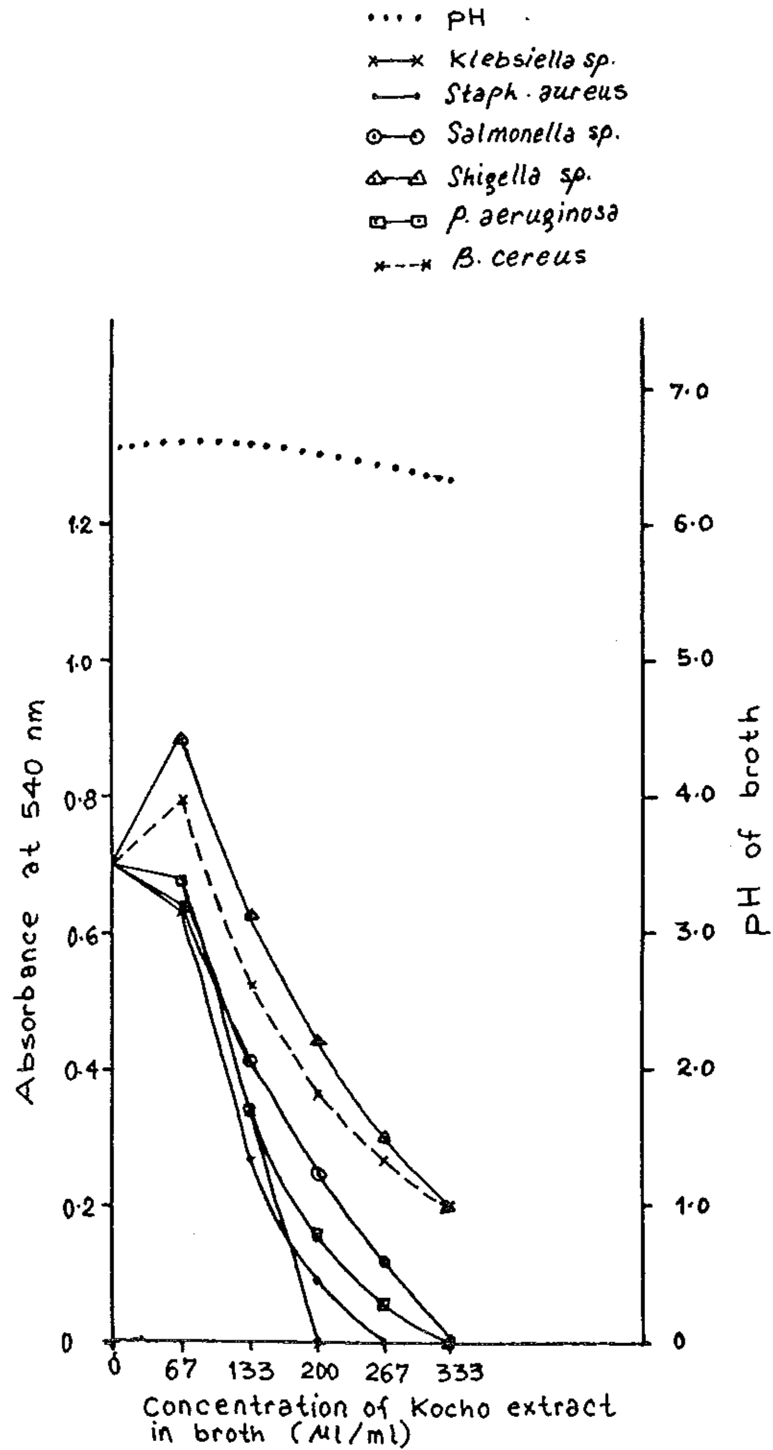


FIG. 3. SURVIVAL AND GROWTH OF THE TEST BACTERIA IN BROTH CONTAINING EXTRACTS FROM FERMENTED KOCHO.

Shigella sp. and the most sensitive were Pseudomonas aeruginosa, Klebsiella sp. and Staph. aureus when 30 h extract was employed. The rest of the bacteria showed intermediate responses.

3.10. SURVIVAL AND GROWTH OF THE TEST BACTERIA IN BROTH CONTAINING EXTRACTS FROM FERMENTED KOCHO

Result of the study on the survival and growth of the test bacteria in broth containing graded quantities of extract from fermented kocho is shown in Fig. 3. Introduction of large amounts of the extract (333 µl/ml of broth) into the broth did not bring about drastic change in pH of the broth (a drop of 0.2 pH units). Growth of Shigella species and B. cereus was stimulated when the concentration of the extract was less than 67 µl/ml of broth. On the other hand growth of the rest of the test bacteria was inhibited at concentrations of 67 µl/ml of broth. Increase in extract concentration beyond 200 µl/ml of broth was toxic to the test bacteria with the exception of B. cereus and Shigella sp. which were inhibited from growing but were able to survive even in broth supplemented with 333 µl/ml of broth.

3.11. SURVIVAL AND GROWTH OF THE TEST BACTERIA IN BROTH CONTAINING SPENT MEDIA FROM BROTH CULTURES OF LACTOBACILLUS, LEUCONOSTOC, PEDIOCOCCUS AND STREPTOCOCCUS SPECIES

Survival and growth of the test bacteria in broth into which were added graded concentrations of spent media from the lactic acid bacteria isolated from fermenting tef and fermented kocho was

evaluated. The spent media were obtained by growing Lactobacillus, Leuconostoc, Pediococcus, and Streptococcus spp. in broth separately (Fig. 4).

As can be seen in Fig. 4a-4d, the drop in pH of the broth as the result of the addition of spent media from Lactobacillus and Leuconostoc spp (a drop in pH from about 6.0 to 5.4) was very small. For broth receiving spent media from Pediococcus and Streptococcus species the change was big (a drop in pH from about 6.2 to 4.7). Spent media from broth containing Lactobacillus species when used at or more than 300 µl/ml of broth and Leuconostoc and Pediococcus spp. at 200 µl/ml of broth and Streptococcus sp. at 100 µl/ml of broth, inhibited growth of the test bacteria (Fig. 4a-4d). The spent medium from Lactobacillus sp. when used at 500 µl/ml of broth was lethal to P. aeruginosa and Staphylococcus aureus. On the other hand, the spent medium from Streptococcus sp. was lethal to Klebsiella and Shigella species and Pseudomonas aeruginosa including Staph. aureus when used at 500 µl/ml of broth. Spent medium from Leuconostoc sp. stimulated growth when used at concentrations below 200 µl/ml of broth. However, when used at 500 µl/ml of broth, it inhibited the growth of Staph. aureus, Salmonella sp. and Bacillus cereus more than the rest of the test bacteria. At 500 µl of spent medium from Pediococcus sp./ml of broth, P. aeruginosa, and Klebsiella species were found to be more sensitive than Staph. aureus, Salmonella sp. and B. cereus. The most resistant was Shigella species.

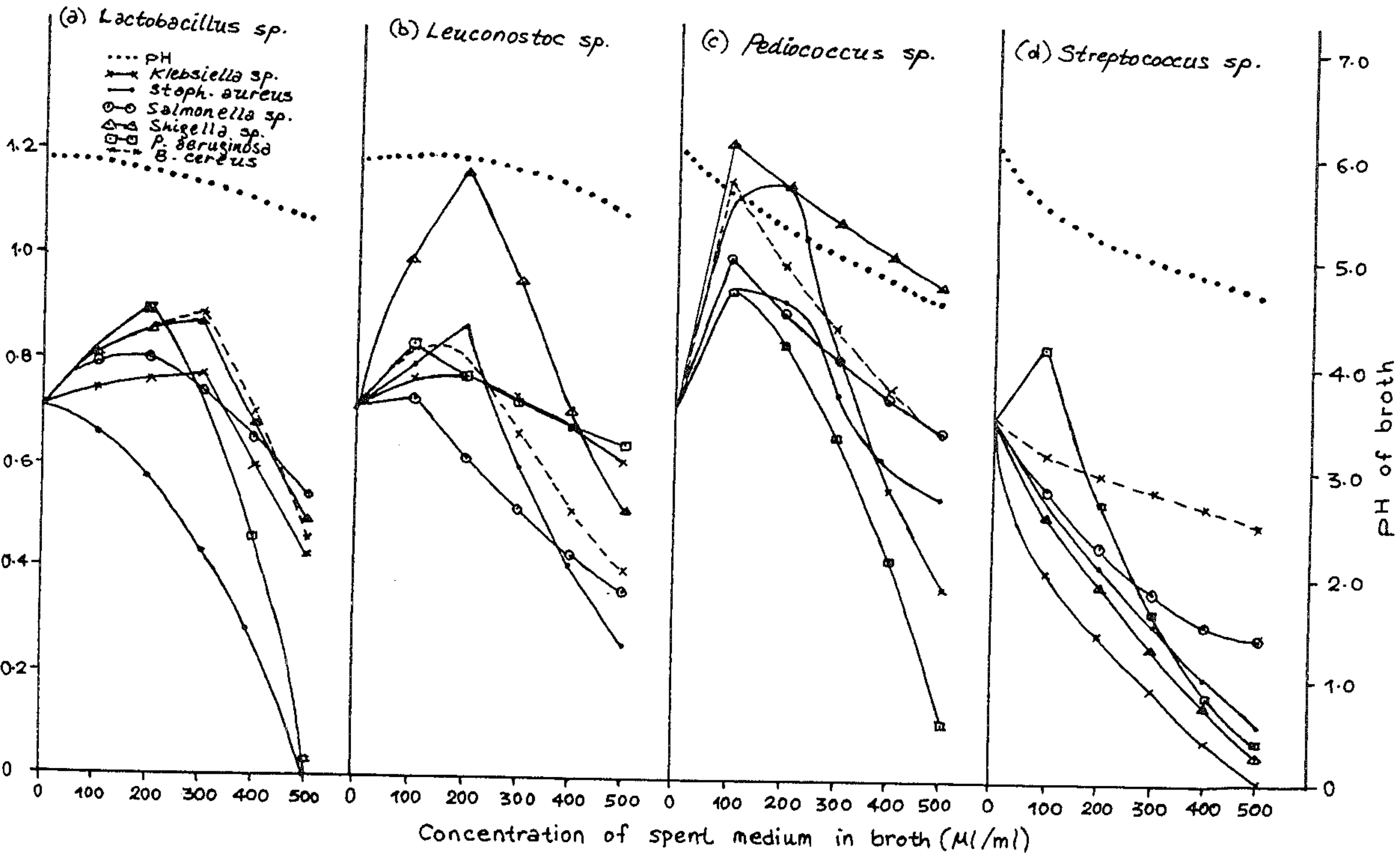


FIG. 4. SURVIVAL AND GROWTH OF THE TEST BACTERIA IN BROTH CONTAINING SPENT MEDIA FROM LACTIC ACID BACTERIA ISOLATED FROM TEF AND KOCHO.

The pH changes along introducing increasing concentrations of the spent media, tef or kocho extracts to broth were lower than that for the organic acids alone or in combination (Fig. 5).

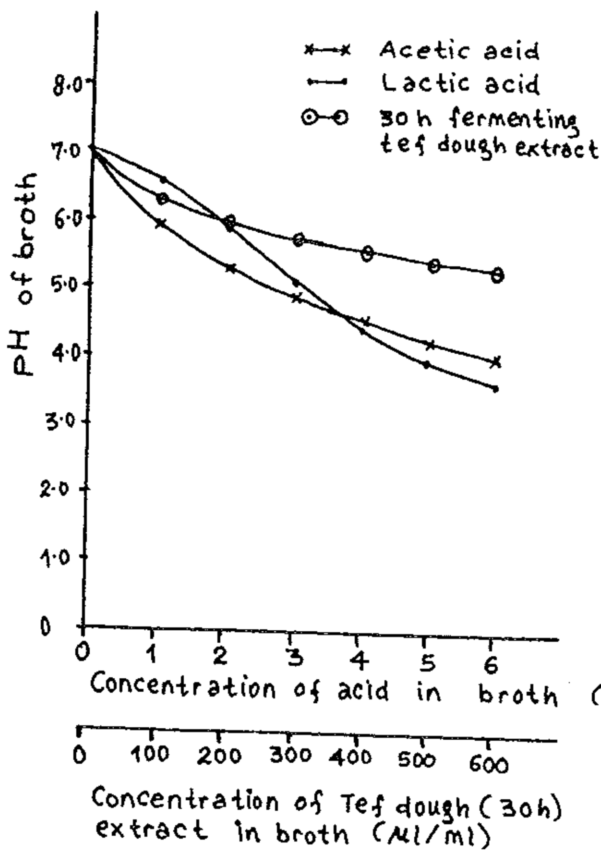
3.12. EFFECT OF HEAT-TREATMENT ON THE INHIBITORY PROPERTY OF EXTRACTS FROM FERMENTING TEF

A study was conducted to check the effect of heat treated extract from 30 h fermented tef on P. aeruginosa and B. cereus. Twenty-four h cultures of P. aeruginosa and B. cereus were introduced into tubes of fresh assay broth containing graded concentrations of the heat treated extracts. As can be seen from Fig 6, the extracts inhibited the growth of P. aeruginosa at all concentrations used (up to 500 µl/ml of broth). There was stimulation of growth of B. cereus by those extracts heat treated below 80°C when used at concentrations less than 100 µl/ml of broth. The heat treatment did not affect the potency of the extracts on the two bacteria. Pseudomonas aeruginosa was more sensitive to the heat treated extract than Bacillus cereus.

3.13. EFFECT OF HEAT-TREATMENT ON THE INHIBITORY PROPERTY OF EXTRACTS FROM FERMENTED KOCHO

Survival and growth of P. aeruginosa and B. cereus in broth containing heat-treated extracts of fermented kocho was also determined. As shown in Fig. 7, the activity of the extract was not affected by heat treatment. It however stimulated growth when 45°C heat-treated extract was used on P. aeruginosa and B. cereus. At higher temperatures of heat-treatment, however, it killed the cells

(a) Acids versus Tef extract



(b) Acids versus spent media

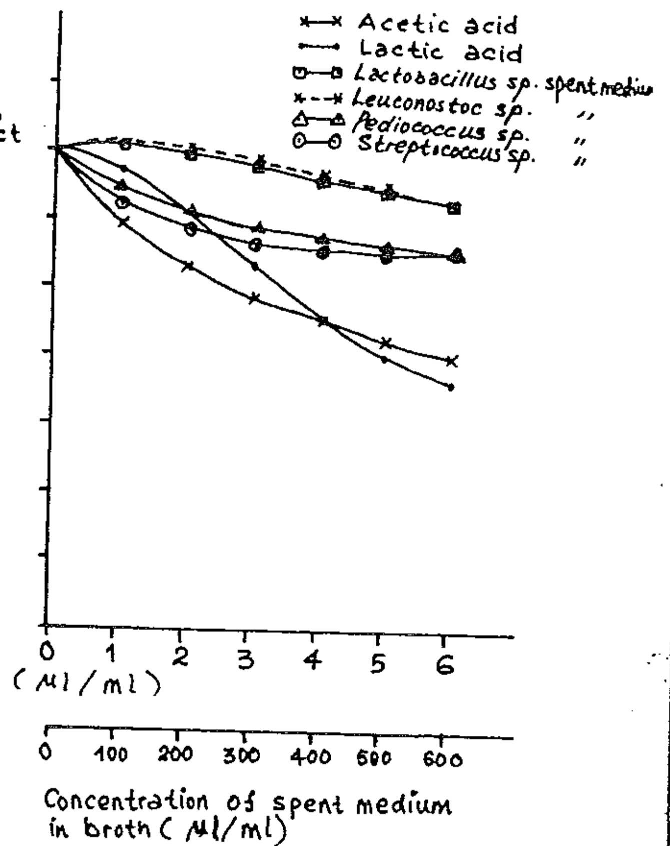


FIG. 5. CHANGES IN pH VALUES ALONG INCREASING CONCENTRATIONS OF ACIDS, FERMENTING TEF EXTRACTS OR SPENT MEDIA FROM LACTIC ACID BACTERIA IN BROTH

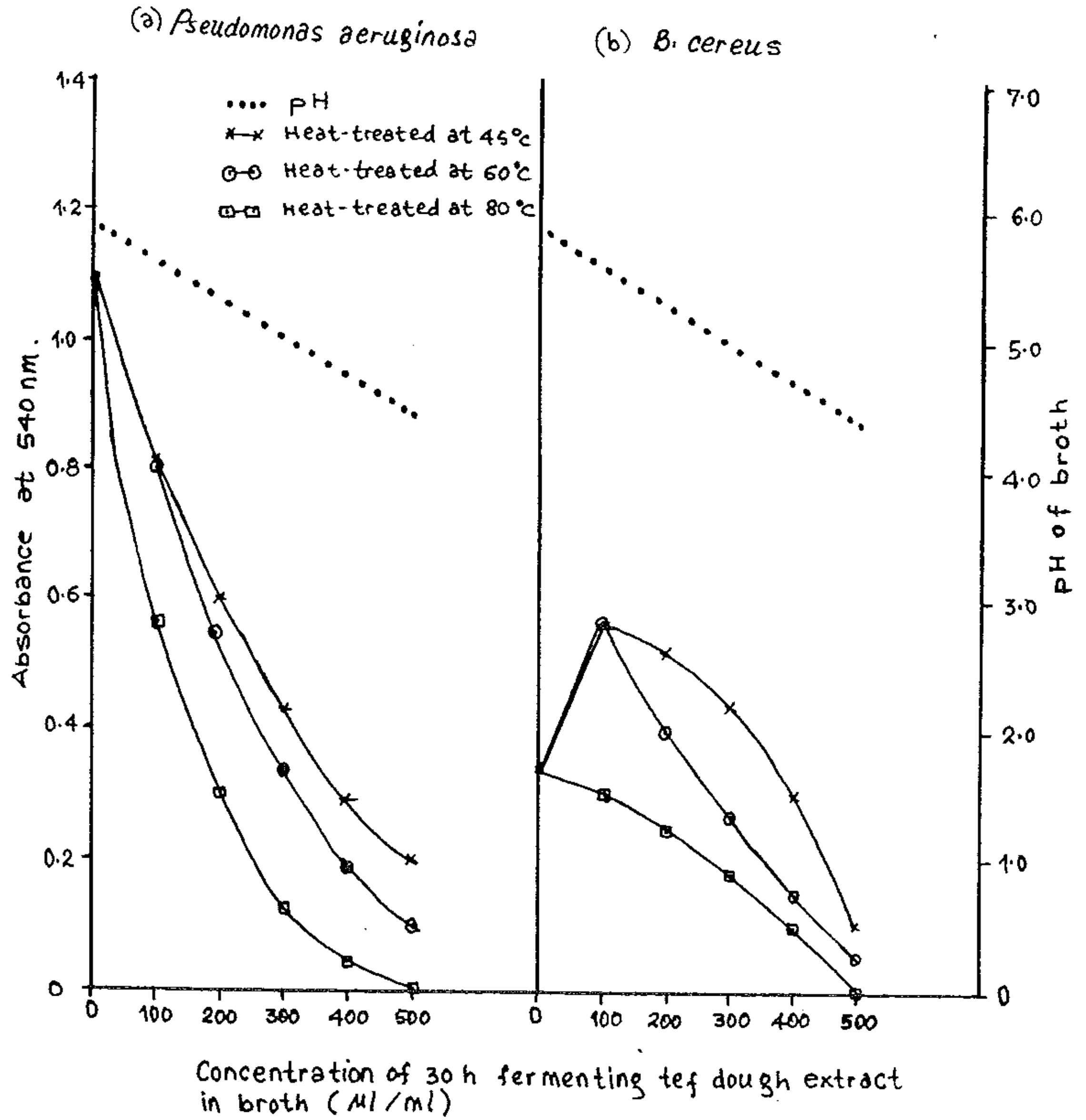


FIG. 6. EFFECT OF HEAT TREATMENT ON THE INHIBITORY PROPERTY OF 30 h FERMENTING TEF DOUGH EXTRACTS

when used at 100-300 µl/ml of broth.

3.14. EFFECT OF BAKING-TEMPERATURE ON THE SURVIVAL AND GROWTH OF THE TEST BACTERIA INTRODUCED INTO TEF AND KOCHO

The fate of a known population of the test bacteria introduced into fermented tef and kocho just before baking was monitored. Injera and pancake from kocho were baked in an oven (baking temperature 92-95°C for 3-5 min) following the traditional methods. As can be seen in Table 8, all of the test bacteria except B. cereus were not able to withstand the baking temperature for the specified time. Some yeasts and molds, however, survived the baking temperature.

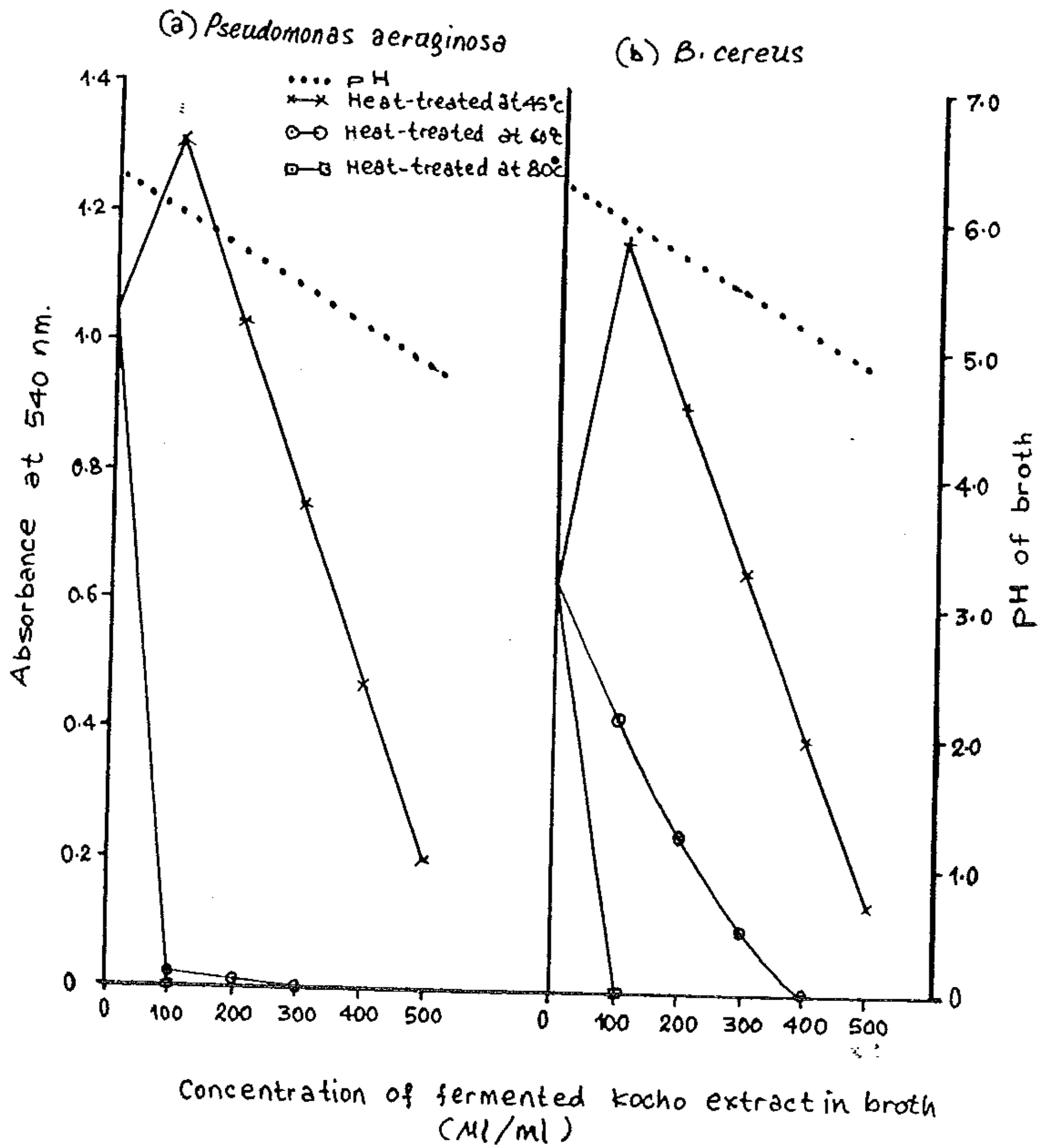


FIG. 7. EFFECT OF HEAT TREATMENT ON THE INHIBITORY PROPERTY OF FERMENTED KOCHO EXTRACTS

TABLE 8

MICROBIAL COUNT OF THE TEST ORGANISMS AFTER BAKING OF TEF DOUGH AND KOCHO

Food Item	ESTIMATED POPULATION OF TEST ORGANISMS (CFU/g DRY WT. OF TEF OR KOCHO)							
	Klebsiella species	*Staph. aureus*	*Salmonella species*	*Shigella species*	*Pseudomonas aeruginosa*	*Bacillus cereus*	Yeasts	Molds
Injera	NG	NG	NG	NG	NG	0.5×10^1	3.0×10^3	1.5×10^2
Pancake from Kocho	NG	NG	NG	NG	NG	1.5×10^3	1.7×10^3	7.5×10^1

NG : No growth

CHAPTER FOUR

DISCUSSION AND CONCLUSION

4.1. MICROBIAL COMPOSITION OF FERMENTING TEF

Unlike fermented cereals such as idli (India), puto (Philippens), khobaz arabec (Syria), kubani (Yemen), kenkey and banku (Ghana), pozol (Mexico) and sour bread doughs, the initiators of tef fermentation were saccharolytic Gram-negative bacteria which are mostly associated with the plants, soil or animals which come in contact with it (Mukherjee et al, 1965; Djien, 1982; Gashe et al, 1982; Gashe, 1985). These bacteria were responsible for decreasing the pH of the fermenting dough from 6.3 at time 0 h to 5.8 at 18 h (Tables 1&2). As the result of their activities, conditions were made favourable for the lactic acid bacterial group to succeed them. Leuconostoc species which was present in large numbers throughout most part of the fermentation period is also known to initiate fermentations in certain types of foods (Mukherjee et al, 1965; Pederson, 1979; Gashe, 1987). Strong acid producers belonging to genera Pediococcus and Lactobacillus were abundant during the latter portion of the fermentation (Table 1).

At 30 h of fermentation, when irsho formation has begun high populations of Streptococcus, Pediococcus and Leuconostoc species were present in the fermenting dough. The Lactobacillus species were

low initially but appeared in significant numbers after 30 h of fermentation (Table 2). This indicates that a succession of microorganisms is taking place during the fermentation and then association appeared to be of a metabiotic type.

The Bacillus species found in association with the fermenting dough were strong amylase producers. Their presence at the earlier parts of the fermentation is suspected to have contributed to the increased sugar concentration in the dough (Table 3). The sporeformers can tolerate and even grow at pH values of about 4-4.5. Their population in the dough increased until the pH dropped to below 4.1 and what was isolated thereafter were the spores and not the vegetative cells (Table 2).

Yeasts and molds appeared in large numbers on top of the liquid layer (irsho) of fermenting tef after the pH dropped to or below 4.7. The work of Gifawessen, et al, (1982) showed that the dominant yeasts of fermenting tef dough were species of Torulopsis, Saccharomyces, Candida and Pichia.

4.2. MICROBIAL COMPOSITION OF FERMENTED KOCHO

Kocho on the other hand, requires months rather than hours to ferment (Besrat et al, 1979; Girma et al, 1985; Gashe, 1987). A properly fermented kocho has a pH of 4-4.5 and contains a large number of lactic acid bacteria (Gashe, 1987). The initiators of fermentation have been found to be Streptococcus and Leuconostoc species.

Pediococcus species often succeeds this group, but, is always overtaken by *Lactobacillus* species in the latter part of the fermentation (Gashe, 1987).

In this study the most abundant members in the fermented kocho were *Lactobacillus* and *Pediococcus* species followed by *Leuconostoc* and *Streptococcus* species (Table 5). Yeasts, *Bacillus* species and molds were present in significant numbers (Table 5).

In both types of foods, it is the succession of microorganisms which brings about the desired changes. This is not unusual, because, most traditionally fermented foods are invariably carried out by mixed cultures. This type of fermentation will require the use of mixed culture as starter cultures rather than using one or two cultures as in fermentation of milk products (Pederson, 1979; Djien, 1982).

The rapid proliferation of lactic acid bacteria and their acid tolerance are the foremost features accounting for their competitive dominance and inhibition of undesirable microflora in acidic foods (Johnson, *et al*, 1982; Booth, 1985; Bender, 1987; McDonald, 1990; Parrot, *et al*, 1990).

4.3. SURVIVAL AND GROWTH OF THE TEST BACTERIA UNDER VARIOUS CONDITIONS

The test bacteria survived and grew in the fermenting tef till the pH dropped to about 4.7. Though acidity below 4.7 was inhibitory to

growth of all of the test bacteria, they were able to survive even at the lowest pH for over 24 h.

When the populations of Staph. aureus and Salmonella sp. at similar pH (pH 4.0) were compared with that of the survival estimates of Meaza, et al (1989), the values obtained in this study were higher than that of the previous work. This dicsripancy may be due to the slightly higher inoculum density of the test bacteria in this work.

Since the pH of the kocho into which these bacteria were introduced was 4.3, most were killed immediately or inhibited from growing. The exception was Bacillus species which survived the treatment in the form of endospores (Table 7).

The reduced pH in both types of foods was due to the presence of organic acids. For example at about 30 h fermentation, when the pH of the fermenting tef was about 4.7, the quantity of organic acid (acetic plus lactic) present is estimated to be 1.7 μl/g dry weight of dough (Umeta and Faulks, 1989). Addition of 1 μl of acetic or lactic acid into one ml of broth reduced its pH by about 0.4-1.1 pH units (from pH 7.0 to 5.9-6.6). Concentration of acid beyond 1 μl/ml of broth reduced the pH significantly and inhibited growth of the test bacteria effectively (Fig. 5a).

When the test bacteria were introduced into broth containing extracts from fermenting tef or kocho, growth was inhibited; however, the drop

in pH was not great when compared to the drop in pH effected by the addition of pure organic acids (acetic, lactic or both) into the broth (Figs. 5a & 5b).

Tef extract (100 ul/ml of broth) did not reduce the pH of the broth as much as the pure acids when used at 1 ul or less in the broth. Under such conditions, the pH drops only by 0.7 pH units (from pH 6 to 5.3). However, inhibition is effected at concentrations greater than 100 ul/ml of broth. This indicates that it is not pH or acidity which alone is inhibiting the growth of the test bacteria. Increased fermentation periods beyond 30 h in tef increased the acidity (reduced the pH), but, inhibition was reduced (Fig. 2a-c).

In the case of kocho, the pH drop as the result of the addition of even 333 ul extract/ml of broth was very low (from pH 6.5 to 6.3); nevertheless, growth inhibition was strong when the extract was used at concentrations beyond 67 ul/ml of broth. Except B. cereus and Shigella sp., the rest of the test bacteria died when 200-333 ul of kocho extract/ml of broth was used. This inhibition by the extract from fermenting tef and fermented kocho must be due to the action of microbial metabolites. Metabolic by-products of lactic acid bacteria are known to inhibit various groups of microorganisms (Wheater, 1951; Trammer, 1966; Pulsani, 1979; Wong, 1988; Schillinger, 1989; Gilliland, 1990; Lukasova, 1990; Hastings, 1991).

Study was carried out to determine if each of the lactic acid

bacteria isolated from tef and kocho could produce inhibitory substances when grown separately in broth. The study showed that the spent medium from Lactobacillus species was inhibitory to growth of the test bacteria when its concentration exceeded 300 µl/ml of broth (pH 5.7 or less in broth). The spent media from Leuconostoc and Pediococcus species at 200 µl/ml of broth and that from Streptococcus species at 100 µl/ml of broth also inhibited the growth of the test bacteria (Fig.4.). The most affected test organisms by spent medium from Lactobacillus species were Staph. aureus and P. aeruginosa. Spent medium from Leuconostoc species inhibited growth of Staph. aureus and Salmonella much more than the other test bacteria. Spent medium from Pediococcus species inhibited growth of P. aeruginosa and Klebsiella species more than the rest test bacteria. On the other hand, spent medium from Streptococcus species inhibited all of the test bacteria and the most sensitive were Klebsiella sp., Salmonella sp., P. aeruginosa and Staph. aureus. The change in pH with increase in concentration of the spent medium was very small for Lactobacillus and Leuconostoc species (a drop in pH of about 0.5 pH units) (Figs. 4a & 4b). However, spent media from Pediococcus and Streptococcus species caused pH change by 1.5 pH units (Figs. 4c & 4d). Though the drop in pH was similar (about 1.5 pH units) for broth receiving spent media from Pediococcus and Streptococcus species, the survival of the test bacteria was much higher when it was from Pediococcus species.

Acidification usually exerts a marked control of enzymatic activities (Moat, 1979). This in turn affects cell division. Most organisms are

capable of utilizing lactic acid in lower concentrations, but not acetic acid. That must be why acetic acid was a stronger inhibitor than lactic acid in this study. However, at higher concentrations because of the pH rather than the type of acid, bacteria can not tolerate the effect so they die immediately (Kabayashi et al, 1982).

Bacillus species produce antibiotics such as mycobacillin, subtilin, bacillin, subsporin, polymyxin, colistin, gatavalin, laterosporin and laterosporamine (Katz, 1974). Bacillin and subtilin have been found to prevent growth of certain sporeforming bacteria (Berdy,1974; Pederson,1979; Hastings,1991). Therefore, kocho which contains relatively higher number as well as kinds of Bacillus species than tef may contain higher amount of inhibitory substances that can act on the test bacteria. That may also be the reason why the extract from kocho was toxic to most of the test bacteria.

The extract from the foods and the spent media from each of the lactic acid bacteria isolated inhibited the test bacteria at pH values higher than those of the organic acids. This indicates that inhibition of growth was not solely due to acid production. Lactic cultures are known for their inhibitory activities in mixed cultures against a variety of pathogenic, disease-causing and spoilage microflora common to foods (Trammer, 1966; Tagg, 1976; Shoji, 1978; Pulsani, 1979; Barefoot, 1983; Ahn, 1990; Lindgren, 1990; Lukasova, 1990; Lewus, 1991). These antagonisms are also the result of rapid acidification, production of H_2O_2, antibiotics and antibiotic-like

substances such as bacteriocins (nisin, lactacins, diplococcin, lactocin, helveticin, pediocins, lacticin, plantaricins). The production of CO_2 as a by-product during fermentation also serves to maintain the environment anaerobic, inhibitory to the aerobes. The antibiotics were, however, found to be more important than the other factors (DeKlerk, 1961; DeClerk, 1967; Daeschael, 1985; Stiles, 1991).

Pathogenic and spoilage microorganisms introduced into fermenting foods may be inhibited from growing by lactic acid bacteria. But, the present study also showed that inhibition was not complete and nor was its effect directed equally to all species of bacteria. Hence, it could not be used as a single effective method in controlling growth of pathogens and spoilage organisms. This conclusion was drawn because the spores of *Bacillus* species, some spores of yeasts and molds as well as some vegetative cells of *Staph. aureus* and *Salmonella* species survived the various treatments (Tables 6,7&8).

Some antibiotic-like substances from lactic acid bacteria are known to retain their potency even after heat-treatment for 30-60 min at 100°C (Hastings, *et al*, 1991; Stiles and Hastings, 1991). In this study, too, heat-treatment up to 80°C for 5 min did not affect the potency of the antimicribial agent(s) produced (Figs. 6 & 7). In fact there was slight amplification of potency. The amplification of inhibitory effects by heat could be attributed to the accumulation of denatured and so toxic substances lethal to the test bacteria and/or

activation of some ions by heat (Thimann, 1963; Moat, 1979).

The survivors of the fermentation process were killed during the baking of injera from tef or pancake from kocho (Table 8). Those which survived were spores from _B_. _cereus_ and fungi. Some of the microorganisms associated with the fermenting foods could produce toxic but thermostable substances (Tartakow and Vorperian, 1981; Doyle, 1988; Newsome, 1988). The presence and fate of this kind of compounds has not been investigated in this study.

CHAPTER FIVE

RECOMMENDATIONS

Based on the findings of this study the following recommendations are drawn:

(1) Study on cereal-based (such as wheat, barley, sorghum, corn or combinations) fermented foods should be done exhaustively to uderstand the nature of the microorganisms involved.

(2) Investigation into the development of starter culture for tef fermentation needs to be carried out.

(3) Thorough study on the types of yeasts involved in primary and secondary fermentations of tef must be carried out.

(4) Standardization on acidity, pH and extent of fermentation for tef must be formulated. Tef dough fermented up to 30-48 h (pH 4.1-4.7) and properly fermented kocho (to about pH 4.3) both baked before consumption were found as safe foods from food-borne pathogens and spoilage bacteria.

(5) Studies on isolation and elucidation of the chemical structures along with searching for the uses of the microbial metabolites as preservatives and/or the organisms themselves as starters in such foods as meat, fish, vegetables, and the likes should be encouraged.

(6) Most of the spore-forming bacteria encountered in both foods have a variety of enzymes such as amylases, pectinases, etc.

Therefore, study on their use for industrial purposes must be investigated.

(7) Some of the test bacteria have the potential of producing thermostable but poisonous by products; their fate needs to be determined.

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