

THE ROLE OF  
CLOSTRIDIA  
IN BANTU BEER FERMENTATIONS

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regulations for the degree of Master of Science  
at the University of Cape Town

by

MICHAEL JÜRGEN ALEXANDER IHLENFELDT

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CAPE TOWN,

DECEMBER, 1968.

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S U M M A R Y

1. The occurrence of clostridia in various stages of Bantu beer production was investigated in order to determine their significance as spoilage organisms in the brewing process and in the final product.
  2. The ecology of clostridia was studied by investigating the selective factors of the environment according to the concepts of Beijerinck and Baas Becking. This study was extended by quantitative investigations of the microbial population according to the approach of Hungate.
  3. A new modification of existing techniques for the enumeration of clostridia is described.
  4. Although clostridia were found to occur as part of the normal flora of brewing ingredients and could be detected in all stages of the normal brewing process, they were present in spore-form only. It was found that the environmental factors were not favourable for their growth.
  5. The conditions under which clostridia could occur are discussed.
  6. The strains isolated in this study were grouped using a distance coefficient on the University of Cape Town ICT 1303 computer.
  7. The significance of the two groups that emerged from the numerical study is discussed in relation to the microbial species problem as a whole.
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ABBREVIATIONS

The symbols and code numbers used for malting firms, breweries and malts in the present study are in accordance with a scheme in use in the Bantu Beer Unit of the Council for Scientific and Industrial Research.

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C H A P T E R I

BACKGROUND TO THE INVESTIGATION

There are occasional references to the highly intoxicating properties of certain Bantu beers in the early literature on this subject (Juritz, 1906; Klein, 1908; Doidge, 1910; Hellman, 1934; Fox, 1938). It was suggested by Juritz (1906) and Fox (1938) that these properties might be ascribed to fermentation products such as higher alcohols and related substances formed in a secondary fermentation as a result of the rather poorly controlled brewing procedures at that time.

Clostridia are well known for the production of propanol, butanol, butyric acid, propionic acid and valeric acid, and have been found to produce some of these substances in industrial fermentations that are essentially similar to the brewing process of Bantu beer.

Clostridia have been recorded to be present in Bantu beer by such early workers as Klein (1908) who found "Clostridium putyricum" (sic) in the alcoholic fermentation and considered the environmental conditions to be favourable for the growth of "Bacterium pasteurianum"

Clostridia have been demonstrated to act as spoilage organisms in the production of silage (Gibson et al, 1961) which like Bantu beer has a lactic acid fermentation; and contamination by butyric acid producing organisms has been recorded in the commercial production of lactic acid where starch conversion liquor and grain protein are fermented by Lactobacillus delbrueckii (Schopmeyer, 1954).

As higher alcohols and acids have been periodically detected in beers and fermenting malt mashes by chemists of the Bantu Beer Unit of the Council for Scientific and Industrial Research, the present writer was given the task of investigating the occurrence of clostridia in the brewing process in order to establish their role in the formation of these substances.

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## CHAPTER II

### THE ECOLOGY OF MICRO-ORGANISMS

Ecology has been defined in various ways and is most frequently described as the study of an organism in relation to its environment (Brock, 1966).

The basis of microbial ecological investigations was laid by such early workers as Pasteur, who by the controlled manipulation of environmental factors could obtain bacterial cultures and study their biochemical activities. By means of such techniques, Pasteur in 1863 studied the saccharolytic clostridia (McClung, 1956). This approach led to the development of the enrichment techniques by Beijerinck where, in a mixed population, one organism could be induced to grow preferentially. By this time methods for obtaining pure cultures had been developed, so that specific bacteria could be isolated and their biochemical potentialities studied in vitro.

Using such techniques Beijerinck (1893, 1902) studied the butyric fermentations by saccharolytic anaerobic spore-formers. These he grouped in the genus Granulobacter because of the ability of the cells to form granules under anaerobic conditions. This genus included clostridia viz. Cl. butyricum and Cl. butylicum as well as facultatively anaerobic bacilli, e.g. Bacillus polymyxa. Using similar techniques Omeliansky (1902) studied the cellulolytic clostridia which were eventually brought into pure culture by Khouvine (1923).

Important contributions to the study of microbial ecology were made by Baas Becking (1934, 1947) who clearly understood the value and limitations of enrichment techniques in ecological studies. The principles of Baas Becking's approach have remained relatively unknown until the recent publication by Wood (1965), one of his former collaborators.

Unlike higher plants and animals, micro-organisms (with few exceptions) are ubiquitous in their distribution and their growth and survival depend on the favourable integration of environmental factors. This integration of environmental factors Baas Becking called the "milieu" of the organism. The ubiquity of micro-organisms, as previously observed by Beijerinck and their ability to develop and grow as soon as a favourable milieu is formed was summarised in the so-called "Law" of Baas Becking: "Everything is everywhere and the environment selects" (Alles is overal: maar het milieu selecteert).

It is possible to differentiate between different types of milieu:

1. The "natural milieu" or the conditions under which the organism actually occurs in nature,
2. the "terrestrial milieu" or the conditions in which the organism could grow, whether it occurs in the particular habitat or not, and
3. the "laboratory milieu" or "experimental milieu", i.e. the wider range of conditions under which the organism can be grown in the laboratory.

In the laboratory the milieu can be defined precisely and the "laboratory milieu" may approach the "optimal milieu" of the organism. In nature fluctuating environmental factors are more likely to result in sub-optimal environmental conditions.

The parameters of the milieu of cells that are not in an active state of metabolism, such as spores or resting cells, can be more widely set. This Baas Becking terms the "milieu of latent life" as opposed to the "milieu of active life" in the case of cells that are in an active state of metabolism.

Baas Becking stresses the importance of experimentally delimiting the boundaries of the milieu of an organism. This may be done by measurements as for instance, temperature, pH and rH in an environment in which the microbes have been demonstrated to occur. Such observations can be augmented with pure culture studies, where the effects of the variation of isolated factors can be tested by determining the growth rate of the organism. In this way the minimal, maximal and optimal requirements for growth can be determined for each of the abovementioned factors.

The fermentation of silage may serve as an example: Here the series of events is essentially similar to those of spontaneous sourings in Bantu beer fermentations. Initially, enterobacteria and bacilli, develop which rapidly deplete the environment of oxygen, creating favourable conditions for a lactic fermentation which now ensues. A fall of pH can be recorded in the environment; Cl. sporogenes and Cl. bifermentans which can be demonstrated once conditions have become anaerobic, soon disappear with increasing acidity (Gibson, 1955). Their sensitivity to pH can be confirmed by pure culture studies. If the lactic acid yield is low and the pH does not fall below 4.6, anaerobic spore-formers may develop with the production of butyrate and the utilisation of lactate. Pure culture studies confirmed that

these clostridia were lactate fermenters, viz. Cl. butyricum and Cl. tyrobutyricum (van Beynum and Pette, 1935, 1936).

Chemical changes in the natural environment can thus be correlated with changes in the medium of a pure culture. By studying the physiological potentialities of organisms in pure culture and correlating the data obtained with observations of changes in the natural environment it is possible, as illustrated above, to study the interrelationships of the components of an ecosystem and their relationship to the biocoenosis as a whole.

By determining the milieu it is made possible to predict which organisms occur in a particular habitat, by the degree to which their specific requirements have been met.

A favourable milieu will lead to the establishment of a characteristic flora which may bring about chemical changes in the environment. If conditions are favourable for amylolytic and cellulolytic organisms, the physical structure of the environment may also be altered. Chemical changes are brought about in the milieu by the utilisation of nutrients and the liberation of the end-products of metabolism which may also result in considerable changes in pH and Eh in the environment. These changes may create favourable conditions for other organisms and in this way a succession is established.

In the example given above, enterobacteria and bacilli caused such changes in the environment by the utilisation of oxygen. The changed milieu was favourable for the growth of lactobacilli which in turn altered the environment which now became a favourable milieu for lactate fermenting clostridia.

It is interesting to note that this succession, unlike that of higher plants, results in a more severe environment. Initially, a large number of forms is present which gradually decreases as the medium becomes depleted and end-products accumulate. Under such conditions only the more resistant forms survive.

There is thus a continuous interaction between the organism and its environment. The environment selects by creating favourable conditions for growth for a particular physiological group of organisms which in turn, through their biochemical activities effect changes in that environment, making any particular milieu a transient condition.

The failure of any particular organism to develop in a particular environment can be ascribed to the inhibitive action of one or more limiting factors which can be determined experimentally.

While the approach of Baas Becking has remained relatively unknown, a complementary approach to the ecology of micro-organisms has been developed. This approach has been summarized by Hungate (1962) in his "Criteria for adequacy of ecological analysis".

"Considerations of bacterial ecology deal usually only with qualitative relationships, but quantitative aspects also need consideration. Knowledge of qualitative aspects of a natural microbial community is fairly satisfactory when the following have been answered:

1. What kinds of organisms occur?
2. What activities do they perform and how are these activities interrelated?

Knowledge of quantitative aspects requires, in addition, answers to the Questions:

3. In what numbers does each kind of organism occur?
4. What is the magnitude of its activity, and what factors influence this magnitude?"

In this approach the emphasis is placed on the organisms and not on the environment. The importance of quantitative data and enumerations is stressed and changes in the number of the bacterial population can, in certain circumstances, be used as an indication of the magnitude of its activity.

In the present study this approach was used to augment the study of the milieu. As the present study deals mainly with the autecology of a specific group a complete investigation of the influence of the succession on the milieu would have fallen outside the scope of the present work.

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## CHAPTER III

### THE BREWING PROCESS

#### A

#### Brewing Methods

The brewing of kaffir beer (or Bantu beer, as it is also known) forms an integral part of the culture and customs of the Bantu tribal societies in Southern Africa. There is no common brewing method. Brewing procedures and the ingredients of the beer vary considerably among different tribes. The different types of tribal beer have been described by Juritz (1906), Chapman and Baker (1907) and Doidge (1910). With the rapid urbanisation of the Non-white population in this century the demand for these beers grew rapidly and was frequently met by illicit concoctions which were given descriptive names such as "kill-me-quick" and "skokiaan". The ingredients and methods of preparation of these brews have been described by Hellman (1934).

In order to meet the growing demand for beer by the expanding urban population, the Bantu Beer Industry was established. The equipment conventionally used in the brewing industry could not be applied in this inherently different process so that new brewing techniques had to be developed.

The recommended brewing procedure will be briefly outlined here. It must be borne in mind that many deviations from this scheme exist. Where such deviations are of importance in the understanding of the problem under investigation, they will be described in the relevant section of the text.

In South Africa malted kaffir corn, Sorghum vulgare Pers., is used for the brewing of Bantu beer together with a starchy supplement of either unmalted kaffir corn or maize. In Zambia and Rhodesia, kaffir corn may be replaced by another millet, Pennisetum typhoides Rich., or by Eleusine coracana Gaertn. (Novellie, 1966).

The beer is made by two distinct microbiological processes: a lactic acid fermentation and an alcoholic fermentation.

#### The lactic acid fermentation:

The lactic acid fermentation or souring, is accomplished by maintaining an approximately 10% suspension of malt in water at 48 to 50°C for 16 to 18 hours. During this period an enrichment of homo-fermentative thermobacteria takes place which have been identified as Lactobacillus delbrueckii and L. leichmannii (van Kerken and Ihlenfeldt, unpublished data). This type of fermentation is known as a "spontaneous

souring" and may be used as an inoculum for further lactic fermentations. Lactic acid production is accompanied by a break down of starches by amylases formed during malting. Amylolysis takes place fairly rapidly initially owing to the favourable temperature but begins to fall off when the pH decreases as the souring proceeds. The extent to which this process takes place is primarily dependent on the diastatic power (DP) of the malt used.

The pH at the end of souring is usually in the range of 3.0 to 3.3 and depends on the quantity of acid produced and on the buffering capacity of the malt. In an average souring the lactic acid content varies from 0.8% to 1.6%; it is, however, the pH value that determines the organoleptic acidity.

After souring the bulk of the mash is transferred into cookers but a small amount remains in the vessel to serve as an inoculum for the next batch.

The soured malt in the cooker is diluted with almost two volumes of water. Approximately 10 parts of an adjunct, usually maize grits and less frequently Sorghum meal, are added to every part of malt used for souring. The mixture is boiled for approximately 2 hours and cooled to 60°C when an additional 4 parts of Sorghum malt are added for every 10 parts of brewer's maize grits previously used. This process is known as "mashing" and the malt added is called "conversion malt" to distinguish it from the malt used for souring. The amylases in the conversion malt added further hydrolyse the starches, now gelatinised by boiling. The conversion of starches is incomplete, as the amylases are denatured rapidly at such high temperatures and low pH. Excess sugar production is not desirable, as it would increase the alcohol content of the final product and reduce the viscosity of the beer which would make it less acceptable to the consumer.

The alcoholic fermentation:

When the mash has cooled to approximately 30°C it is pitched with a dried yeast preparation of Saccharomyces cerevisiae. The mash is passed through strainers where the coarser husks are removed and is then pumped into fermentation tanks where a short alcoholic fermentation takes place for 8 to 24 hours at 30°C. The retention time in these tanks depends on the demand for the beer. The legal limit of alcohol in the beer (3% w/v) is difficult to control owing to the continued hydrolysis of starches and because the beer is consumed in an active state of fermentation. During the alcoholic fermentation there is a continued souring action. After the addition of adjunct and boiling the pH may

rise to a value of 4.0, whereas the final product has a pH of 3.3 to 3.5.

Bantu beer contains 4 to 6% total solids, mainly starch. The average protein content is 0.6%. The vitamins present are mainly nicotinic acid, thiamine and riboflavin, averaging 189, 32 and 36 micrograms per 100 ml. of beer, respectively (Novellie, 1966). With the increased use of maize grits as starchy adjunct there has been a considerable drop in vitamin content of the product.

The participation of amylases of mucorales in the hydrolysis of starches as first postulated by Chapman and Baker (1907) for home-brewed beer and frequently quoted in the literature, has been proven not to occur in modern industrial fermentations (Novellie, 1966).

## B                    The brewing process as an environment.

The importance of environmental factors in determining the sequence of ecological events in any particular milieu has been outlined in Chapter II. The most important environmental factors and the significance of micro-environments will be briefly discussed below.

### 1. Hydrogen ion concentration.

Clostridia, even the lactate fermenting forms, are known to be relatively sensitive to low pH values. The growth of Clostridia can generally not be recorded in samples of silage and cheese below pH 4.0. Unless the Clostridia found in Bantu beer are inherently different from those found in the processes previously mentioned, their growth is unlikely to occur in a well-soured beer which has a pH of 3.0 to 3.5. During souring the conditions are, however, different. The pH of a freshly inoculated souring is high enough for the growth of clostridia. A freshly inoculated souring using the recommended 10% inoculum has a pH of approximately 4.5 to 5.5, depending on the buffering capacity of the malt. (See Table VIII, p.27). The pH of a 10% malt suspension in water, as used for a spontaneous souring may vary from 5.5 to 6.5. In many spontaneous sourings and in inoculated sourings where ineffective inocula are used, a long time lag occurs before a drop in pH can be recorded. Under such conditions a fermentation by clostridia is not unlikely to occur.

### 2. Temperature.

In a well controlled souring a temperature of 48 to 50° C is maintained. This temperature is unfavourable for the development of

mesophilic saccharolytic clostridia which have an optimum temperature of 43°C (Henneberg, 1926). The maximum temperature tolerated by these organisms was not investigated.

Thermophilic clostridia have been described by McClung and McCoy (1957) in Bergey's Manual of Determinative Bacteriology and their possible growth in the souring process cannot be excluded.

More favourable temperatures are found in the later alcoholic fermentation but, as was pointed out previously, the low pH at this stage is unfavourable for the growth of clostridia.

### 3. Redox potential.

No data were available on the redox potential in the various stages of the brewing process. This aspect of the environment is investigated in the present study.

### 4. Availability of oxygen.

The growth of clostridia is greatly retarded by the presence of oxygen in the environment unless a low redox potential can be simultaneously established. The effect of oxygen and redox potentials on the growth of anaerobes is further discussed on p. 11.

Oxygen as a gas is poorly soluble in water and its solubility decreases with an increase in temperature. The solubility of oxygen in water at 760 mm atmospheric pressure is only 26.6 mg per litre at 50°C (Lange, 1956) and is probably rapidly utilised by the aerobes that can be demonstrated to be present in the initial stages of spontaneous sourings (van Kerken and Ihlenfeldt, unpublished data). Anaerobic and micro-aerophilic organisms can thus be expected in such sourings.

During the alcoholic fermentation all traces of oxygen will be rapidly removed by the yeast population and, at that stage, the process can be described as completely anaerobic.

### 5. Micro-environments.

Bantu beer is a highly heterogeneous medium in which both liquid and solid phases can be found. Starch grains, and protein granules are mainly responsible for this heterogeneity although larger particles such as husks, rootlets and the remains of coleoptiles may be included in the malt as "roots and shoots" and are found in the lactic acid fermentation before straining.

Very little is known about micro-environments because investigations are hampered by the lack of adequate techniques. Organic particles may form surfaces to which microbes and molecules may adsorb

resulting in distinct micro-environments. The bulk pH and rH as measured in the medium may not necessarily correspond to the values on the surface of a particle.

Whereas, in a well agitated mash relatively homogeneous conditions will prevail, in cases where stirring is inadequate or totally absent major environmental changes can be recorded. The settling of solids and the cooling of the periphery of the fermentation vessel result in considerable environmental changes in various parts of the tank which are reflected in the composition of the flora in such localities. A souring in which such conditions occurred is discussed in Chapter VII.

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CHAPTER IV

METHODS IN THE STUDY OF CLOSTRIDIA

A. Outline of methods used in this study.

1. Introduction.

An overwhelming number of methods and anaerobic techniques have been devised and described for the isolation and purification of clostridia. These have been adequately reviewed by Gibbs and Freame (1965) and Kutzner (1965). The techniques of the French School as used in Prévot's laboratory have been summarized by Lebert and Tardieux (1952).

The types of techniques employed vary greatly with the nature of the problem investigated. In the present study combinations of techniques were used which will be briefly outlined here and their source of reference stated. The scheme followed was obtained by trial and error and was found to give the most rapid and reliable results in the analysis of Bantu beer.

More difficult was the selection of suitable media. Clostridia do not form part of the dominant flora of malt and beer and are greatly outnumbered by bacilli and enterobacteria. In both these groups facultative anaerobes can be found which frequently interfere in the isolation of clostridia (Kutzner, 1965).

Gentian violet, sodium azide, sorbic acid, sulphonamides and various antibiotics have been used to increase the selectivity of media and to suppress the accompanying flora. Most of these substances have been shown to be insufficiently selective and even to inhibit anaerobic spore formers, (Mossel et al., 1956; Gibbs and Freame, 1965; Narayan, 1966). Gentian violet, if used in the right concentration, does not seriously inhibit clostridia but is not very effective in suppressing aerobic organisms. Similarly, clostridia have been shown to be sensitive to sodium thiosulphate, which is frequently included with ferric citrate in differential media to test for hydrogen sulphide production (Gibbs and Freame, 1965).

Alternatively, aerobes can be suppressed by lowering the redox potential of the medium. Cystain, ascorbic acid, thioglycollic acid and iron salts in the reduced state are most frequently used for this purpose. Such media have the advantage that they make the "aerobic" growth of clostridia possible, as the reductants remove the hydrogen peroxide which is produced by obligate anaerobes in the presence of oxygen (Kutzner, 1965).

The media used in the present study were selected for the following attributes:

1. They must support the growth of both proteolytic and saccharolytic forms.
2. Interference from facultative anaerobes must be minimal.
3. They must give reproducible results.

In the present study attempts were made to increase the selectivity of media using gentian violet and thioglycollic acid. Since the counts obtained with these substances were much lower than with other methods, they were considered unsuitable and were not further investigated. The selection of media is described below in Section B.

## 2. Material.

Malt, bran and brewers' grits were collected from various breweries and maltsters mentioned throughout the text. These samples were treated aseptically whenever possible and stored at room temperature in the laboratory.

## 3. Preparation of Diluents.

The most effective diluent has been shown to be a solution containing peptone (Gibbs and Freame, 1965). A 0.1% peptone solution was used in this study with the addition of 0.1% agar to obtain a better suspension. The diluent was made up in 90 ml. quantities and sterilised in screw-cap bottles to prevent the access of air after sterilisation and maintain anaerobic conditions. Ten-fold dilutions were prepared. A 10% (w/v) suspension of malt, bran and brewers' grits was used for further dilutions.

## 4. Enumerations.

All samples were enumerated by the Most Probable Number Technique (MPN). Enumerations were carried out in test-tubes when solid or semi-solid media were used, or in McCartney bottles if broth was used. Triplicate or quintuplicate samples were incubated for each dilution, depending on the accuracy desired and the time available.

In each case two series were incubated. A pasteurised series was used to determine the total number of spores present. An un-pasteurised series was interpreted to represent the sum of spores and vegetative cells in the original sample. From this the number of vegetative cells in the sample could be calculated.

Samples were pasteurised in a water-bath at 75°C for 30 minutes (Gibbs and Freame, 1965).

#### 5. Preparation of pure cultures.

Pure cultures were prepared by streaking pasteurised samples on RCM agar (Gibbs and Freame, 1965). Plates were incubated in anaerobic jars under 95% Nitrogen and 5% CO<sub>2</sub>. To remove traces of oxygen a mixture of 3g pyrogallol, 10g diatomaceous earth and 2g Na<sub>2</sub>CO<sub>3</sub> was prepared in a mortar and paper envelopes containing approximately 2g of this mixture were placed in each inverted Petri dish. After 3 days' incubation at 37°C, colonies from such plates were restreaked on the same medium. This procedure was repeated at least four times until pure cultures were obtained.

If no pure cultures could be obtained by this method, shake cultures in Burri tubes were used as described by Gibbs and Freame (1965) using VF basal medium (Lebert and Tardieux, 1952). Shake cultures of every isolate were tested in this medium for aerobic contamination. Aerobes grow near the surface or throughout the medium. The growth of anaerobes is restricted to the bottom of the tube.

Spore forming strains giving a negative catalase reaction were tested for the presence of cytochromes by the benzidine test of Deibel and Evans (1960). All strains giving a negative catalase and benzidine reaction were taken to be clostridia.

#### 6. Maintenance of stock cultures.

Pure cultures were grown in RCM broth (see below) until good sporulation had taken place. Cultures were then centrifuged and the spore sediment washed twice, using N saline: A concentrated spore suspension was inoculated into sterile soil. Such cultures were allowed to dry at room temperature and were then stored at 4°C.

Alternatively, strains were grown on RCM agar slopes in an anaerobic jar. When adequate growth had taken place, the tubes were removed from the jar and the cotton-wool plugs were trimmed to the level of the rim of the test tube. A few crystals of pyrogallol and two drops of an N/10 Sodium hydroxide solution were placed on the surface of the trimmed plug. The tubes were then sealed with a rubber bung (Kutzner, 1965). The cultures were stored at 4°C. If it was found that large numbers of spores were formed, the bung and plug were removed and replaced by a sterile cotton-wool plug.

B

Experimental.

1. The selection of media.

In order to select an efficient medium and obtain information about the flora of malt and beer simultaneously, enumeration studies were conducted on malt and beer of different origins using different media.

Media Tested:

1. Re-enforced clostridial Medium. (RCM)

(Hirsch and Grinstead, 1954; Gibbs and Freame, 1965).

Peptone	10g
Oxoid lab-lemco	10g
Hydrated Sodium acetate	5g
Difco Yeast extract	1.5g
Soluble Starch	1g
L-cystein HCl	0.5g
Distilled water	1000ml.

pH adjusted to 7.1 to 7.2.

Dispensed in McCartney bottles and sterilised at 15 lbs. overpressure for 15 minutes.

Two per cent agar was added to obtain a solid medium.

Differential Re-enforced clostridial Medium. (DRCM)

A 4% solution of sodium sulphite and a 7% solution of Ferric citrate was filter-sterilised separately and stored at 4°C.

On the day of use equal volumes were mixed and 2ml. of the mixture were added to 100ml. of RCM broth.

The medium was dispensed in McCartney bottles.

Semi-solid RCM

To the RCM broth described above 0.2% agar was added. The medium was incubated with Top-layer agar. (See below).

The medium was dispensed in test-tubes containing dilutions of the sample to be enumerated.

2. Liver Medium. (Kutzner according to van Kerken, personal communication).

Skin Milk	1000ml.
Ox liver (minced)	50g
Potatoes (grated)	100g
Glucose	10g

Milk, liver and potatoes were sterilised at 15 lbs. overpressure for 1 hour and passed through a sieve. Glucose was added and the pH adjusted to 6.5. The medium was dispensed in test-tubes and sterilised at 15 lbs. overpressure for 15 minutes.

3. Viande-Foie (VF broth) Adapted from Lebert and Tardieux (1952).

Lean beef (minced)	1300g
Ox liver (minced)	500g
Pepsin (Merck, DAB6 = 1:35000)	1g
Distilled water	9000ml.

All fat was removed from the beef before mincing. The minced liver and beef were added to 6 l. of water in a water-bath at 47°C. Then 3 l. of water at 60°C were added with 75ml. pure concentrated HCl. Pepsin was dissolved in 30ml distilled water and added to the mixture. A temperature of 48 to 50°C was maintained for 20 hours with intermittent stirring. The mixture was then boiled, filtered and sterilised at 15 lbs. overpressure for 30 minutes.

Before use the pH was adjusted to 7.6.

The medium was dispensed in test-tubes and re-sterilised at 15 lbs. overpressure for 15 minutes.

4. VF deep agar.

Agar was added to VF broth in a concentration of 1.5% and sterilised at 15 lbs. overpressure for 15 minutes.

5. Gelatin Medium (Rosenberger, 1951).

Gelatin	120g
Bacto-tryptone	15g
Yeast extract	5g
Cystein HCl	0.5g
Resazurin	5.0mg.
Distilled water	1000ml.

pH adjusted to 7.0.

The medium was sterilised at 15 lbs. overpressure for 15 minutes and dispensed into test-tubes containing dilutions of the sample to be enumerated. Top-layer agar was used.

6. Acetate-lactate Medium (Rosenberger, 1951).

This medium was not used extensively as it is selective for lactate fermenters only. A full description is therefore not given.

7. Top-layer agar. (Rosenberger, 1951).

Thioglycollic acid	0.5ml
Cystein HCl	0.5g
Resazurin	5.0mg
Agar	10g
Distilled water	1000ml.
pH adjusted to 7.0.	

The medium was sterilised at 15 lbs. overpressure for 15 minutes.

This medium was used to overpour semi-solid media to obtain anaerobic conditions. Top-layer agar was used to overpour Acetate-lactate medium, gelatin medium and semi-solid RCM in test-tubes.

Enumeration studies were conducted on sourings and beers of different origins. A two-hour old souring pH 3.3 from Brewery B at 43.8°C was compared with a 1½-hour old souring of Brewery D. The pH of this sample was 3.6 and the souring temperature 51.2°C. Samples were enumerated as described above, in the following media.

RCM broth.

Liver medium.

Gelatin medium with Top-layer agar.

Acetate-lactate medium with Top-layer agar.

Similarly, the final beers from Breweries B and E were examined using RCM semi-solid medium with Top-layer agar, DRCM broth and VF deep agar. The medium last mentioned was incubated in Burri-tubes.

The results are summarised in Tables I and II.

In order to ascertain whether RCM medium also supported the growth of proteolytic forms, 1 ml. aliquots were withdrawn from the enumeration series of semi-solid RCM, pasteurised and inoculated into gelatin medium. As the same sample had been previously enumerated on gelatin the results could be compared. (See Table II).

Tubes in which gas-formation could be detected were selected from the unpasteurised series. Streak plates on RCM agar were incubated both aerobically and anaerobically and the presence of aerobes and anaerobes was recorded. (See Table III).

Pure cultures of aerobes were re-inoculated into the original medium and the tubes examined after 3 days.

Results and Discussion.

TABLE I

Clostridia in Industrial Sourings

Medium	Approximate number of cells per ml.			
	Brewery B		Brewery D	
	unpast.	past.	unpast.	past.
RCM broth	$10^2$	$10^3$	$10^4$	$10^4$
Liver medium	$10^2$	$10^2$	$10^3$	10
Gelatin medium with top-layer agar	$10^2$	$10^2$	$10^3$	$10^3$
Acetate-lactate medium with top-layer agar	$10^2$	10	$10^2$	10

TABLE II

Clostridia in Beer

Medium	Number of cells per ml.			
	Brewery B		Brewery E	
	unpast.	past.	unpast.	past.
1 DRCM broth	$4 \times 10^2$	$3 \times 10^2$	$4 \times 10^2$	$5 \times 10^2$
2 RCM broth	$3 \times 10^2$	$5 \times 10^2$	$5 \times 10^2$	$4 \times 10^2$
3 RCM semi-solid agar	$5 \times 10^3$	$1 \times 10^3$	$1 \times 10^3$	$2 \times 10^3$
4 Liver medium with top-layer	$1 \times 10^3$	$4 \times 10^3$	$1 \times 10^4$	$2 \times 10^3$
5 Gelatin with top-layer	$2 \times 10^2$	$1 \times 10^2$	less than 10	less than 10
6 VF deep agar	$2 \times 10^1$	$2 \times 10^2$	$2 \times 10^2$	$5 \times 10^2$
7 No. 2 (above) transferred to gelatin with top-layer	$3 \times 10^3$	$3 \times 10^2$	less than 10	0

TABLE III

The occurrence of aerobes and anaerobes in enumeration experiments

Medium	Total No. of tubes tested	Number of tubes showing colonies	
		Aerobic	Anaerobic
RCM semi-solid	20	4	20
RCM broth	20	10	19
Liver medium	20	4	16
Gelatin	5	0	5
Acetate-lactate	10	1	10

No significant differences in pasteurised and unpasteurised counts could be detected, thus indicating that clostridia were present in spore-form only. Slightly higher counts were obtained with RCM semi-solid medium and with liver-medium than with any of the other media used. See Table II. Liver-medium, however, had the disadvantage of supporting the growth of aerobes as can be seen in Table III. Slightly lower counts were obtained with DRCM and RCM broth, the latter also supporting the growth of aerobes.

RCM semi-solid medium seemed to support the growth of proteolytic strains more readily than gelatin medium. Higher counts were obtained when pasteurised samples from the RCM enumeration series were inoculated into gelatin than if gelatin was used for the primary isolation. (Compare Table II, Nos. 3, 5 and 7).

Gelatin medium was occasionally used as a comparison in later studies and the results obtained agreed very closely with those set out here. Relatively low counts were obtained with VF deep agar and acetate-lactate medium. These media were found to be too selective to be used in a study of this nature. RCM in the semi-solid form gave the highest counts and the least interference from aerobic organisms. It was, therefore, chosen for all further routine enumerations.

Aerobes that did occur were brought into pure culture and identified as belonging to the genera Aerobacter and Bacillus. If such pure cultures were re-inoculated into RCM semi-solid agar very little gas production was recorded. The behaviour of such strains was different from the highly aerogenic fermentation of clostridia and their presence could therefore always be detected in enumeration series.

The merits of the various media tested are shortly outlined below.

Semi-solid RCM with Top-layer Agar.

This medium clearly gave the best results (see p.18 ) and was, therefore, used for all subsequent enumerations. The medium was easy to prepare and did not favour the growth of aerobes.

RCM broth and DRCM broth.

Both media frequently supported the growth of aerobes which could frequently be detected in the form of a pellicle. Lower counts were realised than with the semi-solid medium. The media were not used in further experiments.

Liver medium.

The medium was excluded on account of the high level of aerobic contamination even when incubated with anaerobic top-layer agar.

It was found to give excellent results when clostridia were recovered from stock cultures in sterile soil and was subsequently used for this purpose.

Acetate-lactate medium and gelatin medium.

These media were excluded as they were too selective for specific physiological groups and did not give a true reflection of the clostridial flora in sourings and beer.

VF deep agar.

Very low counts were realised possibly because the medium contains no fermentable carbohydrates which would favour the growth of saccharolytic clostridia usually dominant on malt. The medium was not used extensively, although it gave reproducible results.

Semi-solid RCM, the medium chosen, gave excellent results. Unfortunately it did not lend itself to direct colony counts which are more accurate than enumerations by the MPN technique because of the excessive gas production by the organisms that were enumerated.

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CHAPTER V

THE FLORA OF BANTU BEER AND ITS  
INGREDIENTS

A. The flora of kaffir corn

Five representative samples of kaffir corn were investigated. Samples were taken aseptically from unopened bags at 3 different maltsters. A suspension of the micro-organisms on the material was made by shaking 10g of kaffir corn in 90 ml. of sterile agar-peptone for 5 minutes. Dilutions were prepared from the supernatant and enumerated in semi-solid RCM.

Less than 10 clostridia were present per gram of the original sample in all cases. Clostridia are present in very small numbers on kaffir corn only. Similarly, it was found that bacilli, enterobacteria and lactobacilli did not occur on kaffir corn in large numbers.

B. The flora of malt.

In the course of the present study a large number of malts were investigated. Microbes occur in very large numbers on kaffir corn malt. Before experiments were conducted with any particular malt, the flora of representative samples was investigated routinely. Results obtained for clostridia are summarised in Table IV below. In most cases enterobacteria and lactobacilli were enumerated as well. See Table VIII, p. 27a; Fig. 3, p. 48; Fig. 5, p. 50.

TABLE IV  
Number of clostridia per gram of malt.

Reference No.	Maltster	MPN of cells per gram	
		unpast.	past.
M66/19	E	$2 \times 10^8$	$1 \times 10^5$
M66/25	B	$3 \times 10^3$	$4 \times 10^3$
M66/27	E	$5 \times 10^2$	$4 \times 10^2$
M67/104	E	$2 \times 10^3$	$2 \times 10^3$
M67/152	B	$1 \times 10^3$	$8 \times 10^3$
M68/113	A	$2 \times 10^4$	$2 \times 10^4$
M68/114	C	$2 \times 10^5$	$2 \times 10^5$
M68/143	E	$3 \times 10^5$	$4 \times 10^5$
-	E	$4 \times 10^6$	$3 \times 10^5$
-	E	$5 \times 10^6$	$4 \times 10^3$

Clostridia could be detected on all malts investigated and these proved to be almost exclusively saccharolytic forms. Samples obtained from maltster E frequently showed a slightly higher count than malts of other origins.

Clostridia can be considered to be a normal component of the flora of malt. The slightly higher counts given by unpasteurised samples in some instances, may indicate that vegetative cells occur on malt. In most cases, however, clostridia occur as spores only.

C. The flora of grits:

Six representative samples of brewers' maize grits from different breweries were investigated. Grits were studied that had been obtained from Breweries D, B and E and were enumerated in semi-solid RCM.

The results are tabulated below:

TABLE V

Number of clostridia per gram of brewers' grits.

Brewery	No. of sample	<u>MPN of cells per gram</u>	
		unpast..	past.
D	D1	less than 10	less than 10
	D2	less than 10	less than 10
	D3	less than 10	less than 10
B	B1	$2 \times 10^1$	$4 \times 10^1$
	B2	less than 10	less than 10
	B3	less than 10	less than 10
E	E1	less than 10	less than 10
	E2	less than 10	less than 10

On the average, a very small number of cells per gram of grits was recorded. As the accuracy of the Most Probable Number Technique is very low at this level, the exact counts obtained are not given if less than 10 cells per gram were recorded. It was found that enterobacteria, lactobacilli and bacilli occurred in very small numbers on the same samples.

The low counts obtained may be ascribed to the polishing of the grain where the microbial flora is removed with the fused pericarp and testa of the caryopsis.

Clearly, the number of cells introduced with Brewer's grits into the mash is insignificant.

D. The flora of bran.

Bran was only used extensively in Brewery B. The flora of this brewery was exceptional, in that large numbers of proteolytic clostridia occurred in the beer which were usually absent in the products of other breweries. (See Table II, Nos. 5 and 7).

Samples of bran, collected at Brewery B, were enumerated in gelatin medium and RCM as previously described.

TABLE VI

Number of clostridia per gram of bran.

Sample	MPN of cells per gram			
	Gelatin		RCM	
	unpast.	past.	unpast.	past.
B1	$9 \times 10^2$	$7 \times 10^2$	$1 \times 10^3$	$2 \times 10^3$
B2	$1 \times 10^3$	$2 \times 10^3$	$3 \times 10^3$	$4 \times 10^3$
B3	$6 \times 10^2$	$7 \times 10^2$	$9 \times 10^2$	$1 \times 10^3$

All samples of bran contained large numbers of proteolytic clostridia which accounts for their presence in beer from Brewery B. RCM was included as a control, and the counts obtained indicate that the proteolytic strains were not greatly outnumbered by saccharolytic forms.

E. The flora of Beer.

1. Enumeration experiments.

The number of clostridia present in the final product were determined by different methods and have been summarised in Table II, Page 17. Routine enumerations on beer samples were frequently carried out and special attention was paid to so-called "bad beers" which were frequently sent to the laboratory for investigation. The results obtained in such determinations were very uniform. The same

number of clostridia per ml. were found to be present in pasteurised and unpasteurised samples, clearly indicating that they were present in spore-form only. The number of clostridia present in so-called "bad beers" agreed closely with the acceptable products.

Approximately 70 beer samples were investigated in the course of this study and the uniform counts obtained do not warrant tabulation. On an average  $10^2$  to  $10^4$  cells were demonstrated to be present per ml. of beer which is equivalent to the approximate number of cells present on malt.

If spoilage can be attributed to the growth of clostridia, these organisms should be present in large numbers and should be visible microscopically. This was found to be the case once only. See Chapter VII.

## 2. Enrichment experiments.

The recovery of clostridia from sourings of Brewery B was attempted with specially designed media as higher alcohols such as butanol and propanol had been reported to be present by chemists of the Bantu Beer Unit of the C.S.I.R. As a control, sourings of a brewery in which no such flavours had been detected were included in the enrichment series.

Initially the presence of propionic acid was suspected in the souring of Brewery B. Enrichments were therefore designed with the purpose of recovering propionic acid producing clostridia.

### Experimental Procedures.

A three-hour old souring, pH 3.2, from Brewery B was used for the enrichments. The basal medium had the following composition:

Bacto-peptone	3g
Yeast-extract	4g
phosphate buffer (pH. 7.1)	5 ml.
saturated $\text{CaSO}_4$ solution	2 ml.
Cystein HCl	0.2g
$\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ .	0.05g
$\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ .	0.01g
distilled water	1000 ml.

A series of enrichment media was prepared by the addition of various carbon sources in the concentration of 3g per 100 ml. basal medium. The carbon sources consisted of amino-acids ( $\beta$ - alanine, asparagine, serine, threonine), carboxylic acids (lactic and succinic acid) and alcohols (ethylene glycol and propylene glycol) after Gaston

and Stadtman (1963). The pH was adjusted to pH 7.0 in all cases. The selective action of propionic acid was also investigated. The acid was added in concentrations of 0.2 and 0.4% to the succinate and lactate medium, to sterile converted malt and pasteurised samples of Brewery B sourings. The cultures were incubated anaerobically in 200 ml. quantities in glass-stoppered bottles at 37°C.

Enrichments that showed signs of growth were subcultured twice in the same medium. Inocula were plated onto solid medium of the same composition and were brought into pure culture on RCM agar.

#### Results.

Clostridia were recovered from all enrichment media with the exception of those containing serine and threonine as carbon-sources. The strains isolated could be morphologically divided into 7 groups on the bases of cell and spore morphology and seemed very similar to strains recovered in enumeration techniques. Strains were selected from these studies for computer analysis. See Table XVII p. 61.

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CHAPTER VI

PHYSIOLOGICAL STUDIES

A. pH as limiting factor

1. Studies with pure cultures.

In order to determine whether pH was limiting the growth of clostridia in spontaneous sourings and beers, the strains previously isolated were tested for their ability to grow at various pH values.

A more concentrated RCM broth was prepared which, on the addition of 10% buffer, would give the normal final concentration. To 180 ml. portions of this medium 20 ml. of phthalate buffer was added and batches of the medium were adjusted to pH 3.0, 3.5, 4.0 and 4.5.

A thin layer of molten paraffin wax (MP52 - 55°C) was used to obtain an anaerobic seal after inoculation. The tubes were incubated at 37°C and read after 1, 2, 4 and 10 days.

The results are briefly outlined in Table VII. For the detailed reaction of each strain refer to Table XVIII .

TABLE VII

The growth of 77 isolates at different pH values.

Growth recorded	Total number of strains.											
	pH 3.0		pH 3.5		pH 4.0		pH 4.5		pH 5.0		pH 5.5	
	S	P	S	P	S	P	S	P	S	P	S	P
After 1 day	0	0	0	0	9W	2W	18	10	58	12	59	16
After 2 days	0	0	3W	0	13	4	41	10	56	15	61	16
After 4 days	0	0	4W	0	21	4	42	10	57	15	61	16
After 10 days	0	0	4	0	24	4	43	10	57	15	61	16

P = proteolytic

W weak growth

S = saccharolytic

The pH minimum of growth of clostridia from Bantu beer lies between the values of 3.0 and 3.5. At this pH growth is very much slower than at higher values and therefore not optimal. Growth was only detected after 2 days' incubation. In general, the proteolytic strains

were as sensitive to low pH values as saccharolytic clostridia. See Table VII and Fig. 1.

Whereas, from the above data, it seems highly unlikely that clostridia will be responsible for spoilage of a normal beer (pH 3.0 - 3.5) this possibility cannot be excluded in spontaneous sourings where a fall in pH can only be recorded after 2 to 3 hours.

## 2. The pH stat.

It had been found in routine laboratory experiments that material withdrawn from a pH stat gave better results when used as an inoculum than material derived from a previous souring. In the pH stat, sterile NaOH is added aseptically and automatically, to a fermenting mash to maintain the hydrogen ion concentration at a predetermined value.

Experiments were conducted at pH 4.0 and pH 5.0. It was found that material derived from a pH 5.0 stat gave better results than an inoculum from a pH 4.0 stat.

As clostridia had shown rapid growth at pH 4.5 and pH 5.0 in previous experiments, it was thought possible that they might show an increase in numbers in the pH stat at pH 5.0.

### Experimental.

The same malt, M66/19 (Maltster E), was used in all experiments. The culture was transferred into fresh malt after 3 days before the medium became exhausted. Samples were taken after inoculation and again before the next transfer after 3 days. All samples were withdrawn aseptically after vigorous stirring for 3 minutes. Sterile  $\text{CaCO}_3$  was added to raise the pH of the sample to 5.3 and serial dilutions were prepared.

Aliquots were plated onto pre-dried McConkey agar and acetate-agar (Sharpe, 1960) for the enumeration of enterobacteria and lactobacilli respectively. Lactobacilli were incubated in an atmosphere of 95% Nitrogen 5%  $\text{CO}_2$  at 37°C and counted after 3 days.

Inocula were obtained from actively growing routine sourings that were subcultured in a fresh malt suspension using a 10% inoculum after 12 hours.

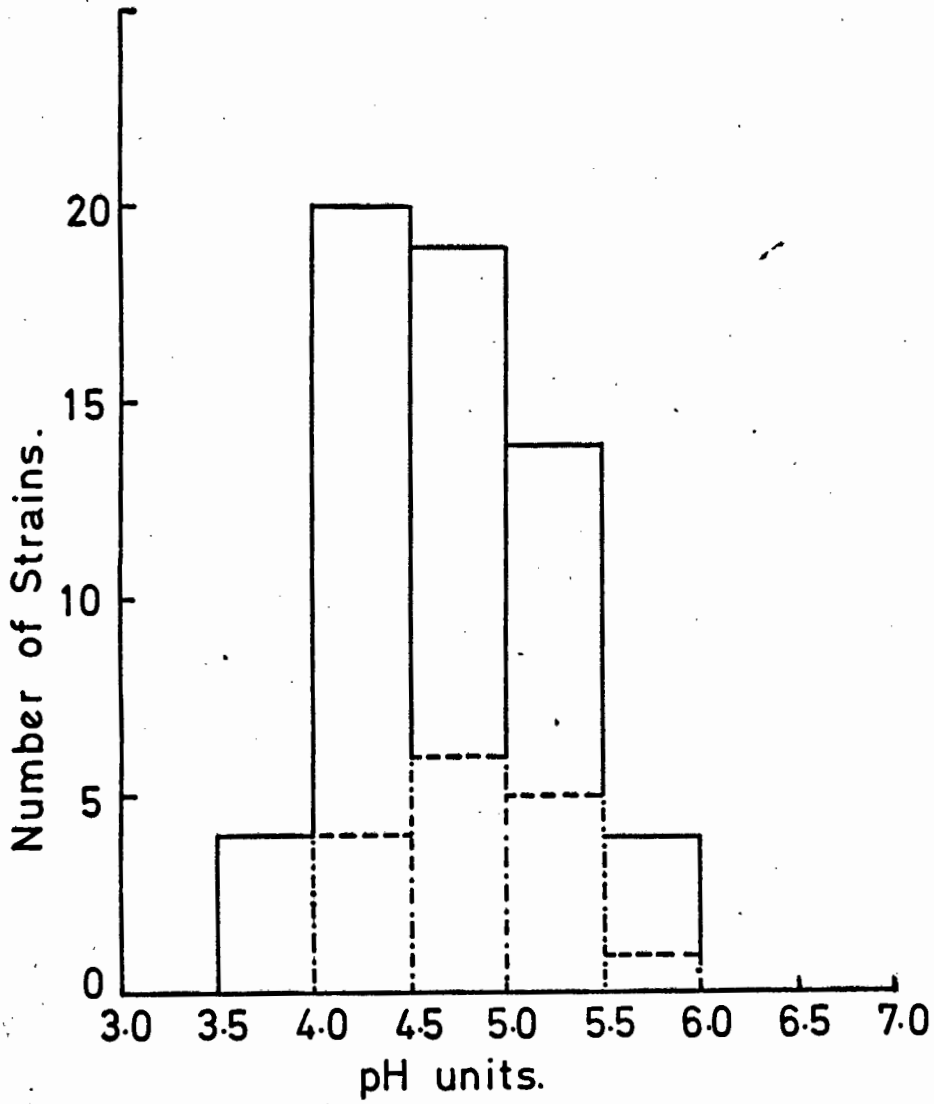


FIGURE:1. Histogram showing pH Minima of 77 Strains.

Solid line = Saccharolytic Strains.

Broken line = Proteolytic Strains.

TABLE VIII

The flora of Malt M66/19 and its development in the pH stat.

Origin of Sample	Time of Sampling	PH	Number of organisms present per ml. sample			
			Lactobacilli	Enterobacteria	Clostridia unpasteurised	Clostridia pasteurised
Routine souring (5% malt suspension)	After inoculation	4.3	$7 \times 10^6$ *	$5 \times 10^5$	$2 \times 10^5$	$3 \times 10^3$
	Second transfer (after 12 hrs.)	3.1	$5 \times 10^8$ *	0	$8 \times 10^2$	$8 \times 10^2$
pH 4.0 stat (20% malt suspension)	After inoculation	5.3	$2 \times 10^6$ *	$2 \times 10^4$	$1 \times 10^2$	$2 \times 10^4$
	After 3 days	4.0	$1 \times 10$	0	$5 \times 10^2$	$2 \times 10^2$
pH 5.0 stat (20% malt suspension)	After inoculation	5.3	$7 \times 10^7$ *	$2 \times 10^6$	$5 \times 10^6$	$8 \times 10^3$
	After 3 days	5.0	$2 \times 10^7$ *	$3 \times 10^4$	$2 \times 10^4$	$1 \times 10^4$
Malt M66/19 (5% malt suspension)	-	6.0	$1 \times 10^{4+}$	$1 \times 10^6$	$5 \times 10^6$	$2 \times 10^4$
Malt M66/19 (20% malt suspension)	-	5.8	$5 \times 10^{4+}$	$7 \times 10^6$	$4 \times 10^2$	$2 \times 10^4$

\* The lactic acid bacteria encountered consisted almost entirely of thermobacteria.

+ The lactic acid bacteria encountered consisted almost entirely of pediococci and betabacteria.

The co-operation of Dr. A.E. v. Kerken in the enumeration of lactobacilli and enterobacteria is gratefully acknowledged.

### Results:

The counts obtained for clostridia, enterobacteria and lactobacilli from the inoculum, pH stat and the malt used are listed in Table VIII.

The enterobacteria formed slimy lactose fermenting colonies on McConkey's agar and were identified as belonging to the genus Aerobacter. Whereas no thermobacteria were demonstrated to be present on malt they formed the dominant group in all inoculated sourings. Betabacteria and pediococci could not be demonstrated in such sourings although they were the dominant organisms on malt.

### Discussion:

A higher survival rate of lactobacilli could be recorded at pH 5.0 than at pH 4.0, as no decrease in the viable count was detectable after 3 days at the higher pH value. This may have been accompanied by greater acid production, although the amount of NaOH consumed was not recorded. Pediococci, streptobacteria and heterofermentative forms did not develop. Enterobacteria did not show an increase in any experiment. In one instance, they could still be demonstrated after 3 days at pH 5.0. This survival of enterobacteria after 3 days could not be demonstrated in subsequent experiments at the same pH value.

The malt was highly contaminated with clostridia. These must have been present partly in a vegetative form as can be seen by the large differences obtained between pasteurised and unpasteurised counts of malt and in sourings after inoculation. After 3 days the same counts were realised for pasteurised and unpasteurised samples and those can be ascribed to the presence of spores only.

At no time during the experiments could an increase in the number of clostridia be detected. Clearly pH could not have been limiting in this experiment as the vast majority of strains had grown at this pH value in the pure culture studies previously recorded in this chapter (p. 25).

The failure of clostridia to grow could be attributed to the following factors:

1. The high lactate concentration.
2. The depletion of certain nutrients by the actively growing lactobacilli.
3. The relatively high temperature (48°C).
4. The production of peroxide.

Lactic acid bacteria have been shown to produce hydrogen peroxide under aerobic conditions. (Hewitt, 1950; Thimann, 1963). The growth of lactobacilli may have raised the redox potential to an unfavourable level for the germination and subsequent multiplication of clostridia by the production of  $H_2O_2$ .

Naturally, a combination of the abovementioned factors cannot be excluded.

B. Growth in sterile sourings.

In order to test whether a depletion of nutrients became limiting to the growth of clostridia or, whether the lactate concentration was inhibiting their development the following experiment was conducted.

Methods.

Approximately 200 ml. of souring derived from a pH 5.0 stat were sterilised at 15 lbs. overpressure for 15 minutes and transferred aseptically to 50 ml. glass stoppered bottles. Material from a normal, 24-hour old routine souring at pH 3.2 was divided equally and one portion adjusted to pH 5.0 with 10% KOH. Both portions were sterilised as above and transferred aseptically to similar bottles. The glass stoppered bottles were filled completely. Before inoculation the flasks were boiled in a water-bath to expel dissolved air.

Duplicate flasks were inoculated with approximately 1g of malt M66/19. The flasks were incubated at 37°C.

Results and Discussion.

With the exception of the unadjusted normal souring, an aerogenic fermentation could be recorded after 1 day in all cases. No fermentation was observed in the uninoculated controls showing that the sterilisation had been effective.

Clearly, pH 3.2 must have been too low for the development of clostridia that occurred on this particular malt. Essential nutrients could not have been depleted as the growth of clostridia took place in all sterile sourings where the pH had been adjusted to 5.0, nor could the lactate concentration have been restricting their development.

Six strains were isolated and brought into pure culture. These were included as strains (OTU's) 1, 2, 3, 4, 5 and 6 in the computer study. It is interesting to note that five of these strains proved to be lactate-fermenters. See feature 9, Table XVIII, p. 71.

### C. Temperature as limiting factor.

In order to test the effect of pH as limiting factor all strains isolated in the course of this study were tested for their ability to grow at 15°C, 37°C, 40°C, 45°C, 48°C, 50°C, 52°C and 54°C. In this way the approximate maximum growth temperature could be determined. The low temperature was included to differentiate between mesophilic and thermophilic strains. Temperature differences have been shown to be useful criteria in the taxonomy of lactobacilli (Briggs, 1953). Rapid growth at low temperatures may be an important attribute of strains that cause spoilage of the final product.

#### Methods.

Strains were incubated in a water-bath using a thermostat of an accuracy of 0.5°C. The maximum incubation period at high temperatures was 3 days. Strains were incubated at 15°C for 2 weeks. A vigorously growing strain in VF broth was used to inoculate RCM broth which had been heated to dispel dissolved air and an air-tight seal was made with paraffin wax. (Melting point 52 to 55°C).

#### Results.

The detailed results of every strain were used in the computer analysis in Chapter IX and are recorded as features 35 and 58 in Table XVIII. (For a key to the scoring see Table XVII). The results are summarised in the form of a Histogram in Fig. 2.

#### Discussion.

The maximum growth temperature of most strains lies slightly below the recommended souring temperature range of 48 to 50°C. The majority of proteolytic strains have their temperature maximum within this range and only two of the 77 strains tested were able to grow above this temperature. It is thus unlikely that temperature is limiting these forms, although it must be borne in mind that the maximum temperature is not optimal for the growth of these organisms.

Fifty-three of the 77 strains were able to grow at 45°C and at higher temperatures. Of the 24 strains that grew at 15°C, 21 were able to grow at 45°C and above this temperature. It is thus impossible to arrange the strains in mesophilic and thermophilic groups, possibly because these terms are arbitrarily defined.

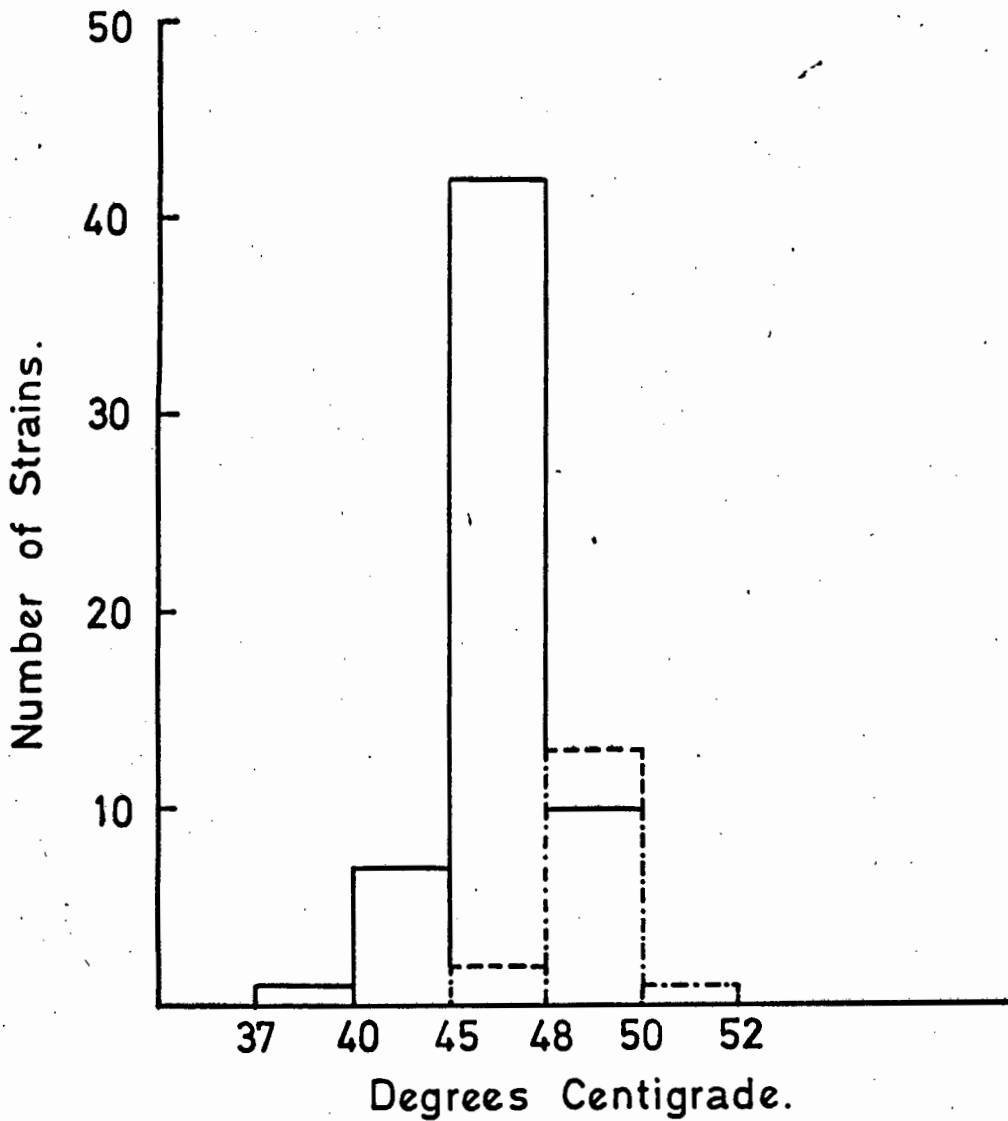


FIGURE: 2. Histogram showing the Maximum Growth Temperatures of 77 Strains.

Solid line = Saccharolytic Strains.

Broken line = Proteolytic Strains.

D. Oxidation - reduction potential as limiting factor.

The oxidation-reduction potential was investigated in normal and strictly anaerobic sourings.

Methods.

Experiment I.

A series of spontaneous sourings were conducted under nitrogen to obtain strictly anaerobic conditions. The souring was constantly stirred in a 5 litre vessel at 50°C and was charged with nitrogen which had been passed through vanadous oxide and amalgamated zinc to remove all traces of oxygen (Stauffer, 1958). The pH was automatically recorded and the Eh was read at regular intervals. Four sourings were conducted under these conditions using a 10% (w/w) malt suspension of Malt 168/143.

Experiment II.

Normal spontaneous sourings were conducted using the same malt as in the previous experiment. Sourings were conducted in 1 litre Erlenmeyer flasks and in Kjeldahl flasks. As unstable readings were obtained, 0.5 ml. of a 0.05% methylene blue solution in water was added per 80 ml. souring. Sourings were stirred with the normal rotary stirrer in Erlenmeyer flasks. Magnetic stirrers were used for sourings in Kjeldahl flasks.

Results:

Experiment I.

In a typical experiment the Eh values varied between Eh +139mV at pH 6.25 and Eh +159mV at pH 6.20 during the first 4 hours, after which an increase to Eh +177mV at pH 6.19 could be recorded. A decrease in Eh to +168mV at pH 5.81 was recorded after 5 hours. After 14 hours an Eh of +389mV at pH 3.33 was reached. Unfortunately, these experiments were not repeatable although a similar trend was observable in most cases.

Experiment II.

A summary of the results is given in Table IX.

TABLE IX.

Oxidation-reduction values obtained with malt M68/143 in spontaneous sourings at 50°C.

30g (total wt.) in Kjeldahl flasks.					1150g (total wt.) in Erlenmeyer flask.				
Age (minutes)	pH	mV		rH	Age (minutes)	pH	mV		rH
		E	Eh				E	Eh	
35	6.15	-80 <sup>o</sup>	+143	16.8	25	6.08	-130 <sup>o</sup>	+93	15.1
70	6.15	-80 <sup>o</sup>	+68	14.5	35	6.15	-144 <sup>o</sup>	+79	14.8
105	6.15	-140*	+83	14.3	95	6.13	-155*	+68	14.4
135	6.15	-195	+28	14.9	115	6.15	-155	+68	14.4
225	6.23	-193	+30	13.2	130	6.15	-155	+68	14.4
1400	3.48	180	+43	13.4	210	6.15	-140	+83	14.9

$$Eh = E + k$$

Where k, a constant, is equal to +223mV for this Eh meter.

Eh redox potential

E measured potential difference

$$rH = \frac{2. Eh}{0.1984 \times T} + 2.pH$$

\* methylene blue added.

Discussion.

The importance of redox potentials for the growth of anaerobes has been previously mentioned in Chapter IV, where it was pointed out that anaerobes may be capable of growth in the presence of oxygen if the medium has a low redox potential.

Eh and rH measurements were initially applied to simple, reversible and defined chemical systems. Later these measurements were adopted for bacterial cultures where the nature of the chemical system is essentially different. The chemical changes in a bacterial culture are irreversible. Furthermore, a medium such as Bantu beer has many known and unknown chemical systems each with its own redox characteristics.

Starka (1968) points out that for these reasons the relationship between Eh and pH cannot be exactly established in a biological system and that rH measurements under such conditions have little meaning.

The above data must therefore be interpreted with great caution. The most that can be said is that redox potentials represent a trend in the metabolic activities of the culture as a whole.

It was found with pneumococci and other catalase negative forms that very high redox potentials of 400 to 500mV could be detected when these were grown in the presence of oxygen. This high redox potential was shown to be a result of the accumulation of peroxide as an immediate fall in Eh was recorded when catalase was added to the system (Thimann, 1963).

The redox requirements of a strict anaerobe, Cl. perfringens, are given by Starka (1968). The following Eh values were found to be limiting for the growth of the organism at the corresponding pH levels:

<u>pH</u>	<u>Eh</u> (+mV)
6.0	106
6.2	131
6.4	160
6.6	150
6.8	114
7.0	90
7.2	80
7.4	35 - 70
7.8	0 - 30

These values fall well within the limits set in normal spontaneous sourings. See Table IX. It would therefore appear that the Eh is not limiting in spontaneous sourings, although it must be borne in mind that Eh determinations only became repeatable when methylene blue had been added to the system as a mediator, which tends to poise the Eh at a certain level.

For strictly comparable results the limiting Eh should be determined for different clostridia in a Sorghum malt medium. Even if the chemical nature of this medium is unknown it would bear a closer relationship to conditions in the natural environment than a synthetic medium.

The higher readings obtained in Experiment I than in Experiment II are difficult to explain. Under strictly anaerobic conditions a lower redox potential should be recorded than if oxygen is admitted to the system.

In conclusion, it should be pointed out that there does not appear to be an appreciable accumulation of peroxide in sourings, because the Eh is relatively low. The experiments outlined above should be repeated and the repeatability of results tested before any deductions can be made with any degree of certainty.

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CHAPTER VII

STUDIES CONDUCTED IN A BREWERY

A. Description of the brewery and the nature of the brewing process.

An extensive investigation of the souring process was carried out in Brewery D.

This brewery was one of the oldest and the last of its kind on the Witwatersrand and had a unique method of brewing. The brewing process was not well controlled so that it seemed likely that clostridia might occur in the initial souring process and, indeed, gas production had been recorded on several occasions in the souring tanks. An extensive survey was carried out with the Chemical Engineering Staff of the Bantu Beer Unit in order to obtain detailed descriptions of the brewing process before the brewery ceased production in 1967. This description may therefore be of some historical interest.

The brewing procedures differed considerably from those outlined in Chapter III and are briefly described below:

Kaffir corn malt and brewers' grits were mixed in the ratio 1:3, in an open vessel with about 900 gallons of hot water up to a final volume of 1500 gallons. This mixture, termed the "premix", had a temperature of 74 to 80°C and consisted of 30 to 40% solids.

Immediately after mixing, the premix was pumped into a receiving souring vessel through a piping system consisting of a central closed vessel and an attached swivel-arm distribution pipe.

The open, receiving souring vessel contained a residue of the previous souring which served as an inoculum for the new batch and formed about 7% of the total volume. The mixture was stirred until the receiving vessel had been almost filled. This mixture was allowed to stand for about 2 hours at 58 to 63°C while the hydrolysis of starches took place. This process is known as "prewashing" to differentiate it from normal "mashing" which takes place after the lactic acid fermentation, as described in Chapter III.

The temperature was then lowered to about 50 to 52°C by the addition of 400 gallons of cold water. The mixture was stirred for about 10 minutes and allowed to sour for 6 to 10 hours. After this time the souring was again stirred before the transfer of the mixture to the fermentation tanks of the alcoholic fermentation.

Whereas relatively homogeneous conditions exist throughout the mash in a normal, well-agitated souring, the inadequate stirring in this particular fermentation has important implications:

The settling of all particulate matter results in a gradation of the physical structure of the environment, from a liquid at the surface to a relatively compact mass of dense material at the bottom of the vessel. As a result of the cooling of the periphery of the tank temperature gradients are formed. These changes in environmental conditions in various parts of the tank can be expected to be reflected in the composition of the microbial flora in such areas.

The production of gas in this particular souring may be ascribed to such differences in environmental factors. It had been observed in this brewery that gas was usually evolved at the periphery of the vessel, the region where the lowest temperature was recorded.

#### B. The flora of souring.

The composition of the microbial flora and the succession of micro-organisms was investigated in two experiments. In the first experiment (I) the souring was stirred for three minutes before sampling; in the second experiment (II) the normal brewing schedule was followed.

#### Experiment I.

The following samples were taken on the 13/2/67, from the intermittently stirred mash from a tank marked "34".

- Sample 1. Malt used for this particular souring.
- Sample 2. Brewers' grits used for this souring.
- Sample 3. Withdrawn after transfer from premix and inoculation in the souring tank.
- Sample 4. Withdrawn 2 hours later after the addition of tap water and stirring.
- Sample 5. Withdrawn 4 hours after the previous sample and after thorough agitation.
- Sample 6. Withdrawn shortly before the end of souring; 9 hours after inoculation, after thorough stirring.

## Experiment II

The following samples were taken from tank "32" on the 16/2/67. The samples were always taken from the same point in the vessel in an area where the temperature was estimated to be in the normal souring range (48 - 52°C). This point was 2 ft. below the surface and 2 ft. from the side of the vessel. Precautions were taken to minimize interference with the normal brewing procedure.

- Sample 7. Malt used for this souring.
- Sample 8. Brewers' grits used for this souring.
- Sample 9. Sample from the premix.
- Sample 10. Withdrawn directly after transfer from the premix and inoculation in souring tank.
- Sample 11. Withdrawn 1 <sup>3</sup>/<sub>4</sub> hours after inoculation and directly after dilution of this premash with tap water and stirring.
- Sample 12. Withdrawn 2 <sup>3</sup>/<sub>4</sub> hours after inoculation.
- Sample 13. Withdrawn 3 <sup>3</sup>/<sub>4</sub> hours after inoculation.
- Sample 14. Withdrawn 6 hours after inoculation.
- Sample 15. Withdrawn 8 <sup>1</sup>/<sub>2</sub> hours after inoculation.
- Sample 16. Withdrawn directly at the end of souring after vigorous agitation, 9 hours after inoculation.

In order to obtain a comprehensive picture of the souring process, figures obtained for yeasts and the LLP group (Leuconostoc, Lactobacillus and Pediococcus) are included. These enumerations were carried out with the collaboration of Dr. A.E. van Kerken of the Bantu Beer Unit. The pH was determined by Mr. R. van Bellinghen of the Bantu Beer Unit.

The results of Experiment I are summarised in Table X and Experiment II in Table XI.

Samples from tanks in which an aerogenic fermentation became evident were examined microscopically and large numbers of yeasts were found to be present. These yeasts were brought into pure culture on acidified MY agar (Wickerham, 1951) and were identified. See Table XII.

TABLE X

SUMMARY OF THE SAMPLES FROM AN INTERMITTENTLY STIRRED SOURING AND THE ORGANISMS RECOVERED (Experiment I)  
(COUNTS GIVEN AS NUMBERS PER ml SOURING)

Sample No.	Description	pH	temp.	titr. ml 0.5N NaOH /50 ml souring	Enterobacteria	Total count agar (37°C)		Clostridia		Yeasts	LLP-group	Strains of LLP-group recovered	
						Unpast.	Past.	Unpast.	Past.			Species	Numbers of isolates identified
1	Malt	-	-	-	$6 \times 10^6$	$1 \times 10^8$	$4 \times 10^6$	$9.2 \times 10^3$	$1.6 \times 10^4$	-	$4 \times 10^4$	<u>L. fermenti</u> <u>Ped. cerevisiae</u>	5 3
2	Grits	-	-	-	-	$1 \times 10^3$	30	absent	absent	-	less than 10/ml	-	-
3	Directly after transfer to souring vessel	5.8	62.5°C	-	less than 100/ml	$1 \times 10^7$	$1 \times 10^6$	$9.2 \times 10^3$	$2.2 \times 10^3$	less than 100/ml	$2 \times 10^7$	<u>L. fermenti</u> <u>L. delbrueckii</u>	5 5
4	2 hours after A3 After 'pre-mashing', dilution and stirring	5.62	60°C	0.45	less than 10/ml	$1 \times 10^7$	$3 \times 10^5$	$9.2 \times 10^3$	$1.6 \times 10^4$	20	$1 \times 10^7$	<u>L. fermenti</u> <u>Ped. cerevisiae</u> <u>L. delbrueckii</u>	1 1 8
5	6 hours after A3 (thoroughly mixed)	3.62	53.5°C	5.72	less than 10/ml	$5 \times 10^7$	$1 \times 10^6$	-	-	600	$3 \times 10^8$	<u>L. delbrueckii</u>	14
6	9 hours after A3 End of souring (thoroughly mixed)	3.42	52.5°C	8.54	less than 10/ml	$5 \times 10^5$	$5 \times 10^5$	$1.1 \times 10^3$	$1.9 \times 10^3$	less than 10/ml	$2 \times 10^8$	<u>L. delbrueckii</u>	10

TABLE XI.

SUMMARY OF THE UNSTIRRED SOURING AND THE ORGANISMS RECOVERED (Experiment II)  
(COUNTS EXPRESSED AS NUMBERS PER ml SOURING)

Sample No.	Description	Age (h/min)	pH (room temp.)	temp.	titr. ml 0.5N NaOH / 50 ml souring	Enterobacteria	Total count agar (37°C)		Clostridia		Yeasts	LLP-group	Strains of LLP-group recovered	
							Unpast.	Past.	Unpast.	Past.			Species	Number of isolates identified
7	Malt	-				1x10 <sup>7</sup>	3x10 <sup>8</sup>	8x10 <sup>4</sup>	5x10 <sup>2</sup>	4.3x10 <sup>2</sup>	-	3x10 <sup>5</sup>	<u>L. buchneri</u> <u>L. fermenti</u> <u>Ped. cerevisiae</u>	1 9 2
8	Grits	-				1x10 <sup>3</sup>	2x10 <sup>4</sup>	8x10 <sup>4</sup>	absent	absent	-	less than 10/ml	<u>Ped. cerevisiae</u>	1
9	Material from premix	0		65°C	-	400	1x10 <sup>6</sup>	10 <sup>6</sup>	1x10 <sup>2</sup>	1x10 <sup>2</sup>	170	1x10 <sup>5</sup>	<u>L. cellobiosus</u> <u>L. fermenti</u> <u>Ped. cerevisiae</u>	2 6 2
10	After inoculation	0/40	5.83	63°C	0.38	200	9x10 <sup>5</sup>	10 <sup>6</sup>	1x10 <sup>2</sup>	1x10 <sup>2</sup>	20	1x10 <sup>7</sup>	<u>L. fermenti</u> <u>Ped. cerevisiae</u> <u>L. delbrueckii</u>	2 1 7
11	After prewashing, dilution and stirring	2/25	5.70	58.5°C	0.48	200	2x10 <sup>5</sup>	10 <sup>5</sup>	1x10 <sup>2</sup>	1x10 <sup>2</sup>	520	6x10 <sup>6</sup>	<u>L. fermenti</u> <u>L. delbrueckii</u>	1 9
12	2 ft. below surface unstirred	3/25	4.69	59.5°C	1.13	less than 100/ml	7x10 <sup>5</sup>	10 <sup>5</sup>	10	10	470	6x10 <sup>7</sup>	<u>L. delbrueckii</u>	11
13	Ditto	4/25	3.92	46°C	2.56	less than 100/ml	1x10 <sup>5</sup>	10 <sup>5</sup>	1x10 <sup>2</sup>	1x10 <sup>2</sup>	240	1x10 <sup>8</sup>	<u>L. delbrueckii</u>	10
14	Ditto	6/35	3.53	48.75°C	5.42	less than 10/ml	5x10 <sup>5</sup>	10 <sup>5</sup>	1x10 <sup>2</sup>	1x10 <sup>2</sup>	40	1x10 <sup>8</sup>	<u>L. delbrueckii</u>	10
15	Ditto	10/05	3.36	49.5°C	7.40	less than 10/ml	1x10 <sup>5</sup>	10 <sup>5</sup>	1x10 <sup>2</sup>	5x10 <sup>2</sup>	110	4x10 <sup>7</sup>	<u>L. delbrueckii</u>	11
16	End of souring Thoroughly mixed	10/25	3.38	50°C	8.37	less than 10/ml	1x10 <sup>5</sup>	10 <sup>5</sup>	1x10 <sup>2</sup>	1x10 <sup>2</sup>	10	1x10 <sup>8</sup>	<u>L. delbrueckii</u>	10

TABLE XII

YEASTS RECOVERED FROM GAS-PRODUCING REGIONS IN SOURING TANKS

TANK NO.	SOURCE	SPECIES RECOVERED
32	1 ft. from bottom among settled solids	2 x <u>Saccharomyces cerevisiae</u> 1 x <u>Saccharomyces italicus</u>
33	Sampled through valve at base of tank	2 x <u>Saccharomyces cerevisiae</u> 1 x <u>Candida robusta</u> (asporogenous <u>S. cerevisiae</u> )

## Discussion.

In both experiments fluctuations in temperatures could be recorded showing a greater variation in the second experiment. The falling pH and increasing titration figure indicate a normal rate of acid production. The relatively higher final pH value and the higher titration value in the first experiment may be attributed to a greater buffering capacity of the brewing mixture as compared to the souring of the second experiment.

The "premix" has no equivalent in the normal brewing procedure. The high temperature at this stage should bring about a gelatinisation of starches which facilitates its simultaneous and subsequent conversion to sugars. The elevated temperatures also have a partial sterilisation effect as is indicated by the decrease in the number of enterobacteria and a decrease in differences between unpasteurised and pasteurised counts. This is especially evident in Table XI where no differences between unpasteurised and pasteurised counts could be detected from material derived from the "premix". These organisms were, therefore, present in the form of spores only.

Whereas heterofermentative lactobacilli and pediococci were dominant in the initial stages of souring, they could no longer be detected in later stages because of the rapid multiplication of Lactobacillus delbrueckii as soon as the temperature had been lowered by the addition of cold water. L. delbrueckii is thus the dominant organism in these sourings and shows maximum lactic acid production when no further increase in numbers can be detected, i.e. when the culture has reached the stationary phase. Unlike enterobacteria and vegetative cells of bacilli and clostridia, the lactobacilli show a relative insensitivity to the high temperatures of the "premix" and the "premashing" stage.

Large numbers of lactobacilli can still be demonstrated in the final stages of souring and these therefore make an effective inoculum when the residue is promptly used for this purpose for the next batch.

No gas-formation occurred in the experiments described above. It can be seen from Tables X and XI that the number of yeasts remained relatively constant. In cases where an aerogenic fermentation was observed large numbers of yeasts were found to be present microscopically especially in the sediment. These were identified as Saccharomyces cerevisiae, Saccharomyces italicus and as Candida robusta and were traced to the distribution system, especially the central vessel described in the preceding section. This vessel remained half-filled after every transfer of the premix. The residue was frequently in a state of

vigorous fermentation and served as an inoculum for subsequent sourings. The sporadic occurrence of an alcoholic fermentation may thus be related to the retention time in this vessel or the time that elapses between any two sourings. A short time interval would result in the introduction of a small number of yeasts only, and a normal fermentation would ensue as was the case in experiments I and II outlined above. If a long time elapsed between sourings the introduction of a large number of yeasts would ensure their development in the areas of the tank that had cooled sufficiently.

C. The occurrence of clostridia and their characteristics.

Four isolates of clostridia from Experiment II were brought into pure culture and identified. These strains, C94 to C97, were identical in their fermentation patterns and agree closely to the description of Cl. butyricum as given by Kutzner (1963). If the characters of these strains are compared with descriptions given in the seventh edition of Bergey's Manual they can be placed between Cl. butyricum, Cl. beijerincki and Cl. butylicum.

Strain C92 proved to be the only clostridium in the present study to have been isolated from material where it was present in sufficiently large numbers to be visible microscopically. The strain occurred in the residue of a tank that must have been out of use for a considerable time. The pH of the material from which the strain was isolated was 5.4.

The physiological and morphological properties of these strains are summarised in Table XIII.

It is highly unlikely that this organism could have contributed to the spoilage of normal beer as it was unable to grow at low pH values. Similarly, the conditions during souring are near the limits of physiological tolerance of this strain, as can be seen when comparing its pH and temperature requirements in pure culture with the conditions during souring.

The characteristics of strains C92} isolated from the disused tank, agree closely with the description of Cl. thermoaceticum as given in Bergey's Manual except for its inability to grow at elevated temperatures. It could perhaps be classified as a mesophilic form of this species. This strain is included as strain (OTU) 76 in the numerical analysis described in Chapter IX.

TABLE XIII

Physiological characteristics of clostridia from Brewery D.

Strain	C 92 (OTU 76)	C94 - 97
Morphology	spores terminal, round, swelling cell	spores oblong, sub-terminal, distending the cell
Presence of granulose	-	+(4)
Pigment production	-	-(4)
Fermentation of		
<u>cellobiose</u>	++	+(4)
<u>cellulose</u>	-	-(4)
<u>fructose</u>	++	++(4)
<u>galactose</u>	+	++(4)
<u>glucose</u>	+++	+++ (4)
<u>glycerol</u>	-	+++ (3)-(1)
<u>acetate and glycerol</u>	-	+++ (4)
<u>inulin</u>	+	-(4)
<u>lactate</u>	-	++(4)
<u>lactose</u>	+	++(4)
<u>maltose</u>	++	+(4)
<u>mannitol</u>	-	+(4)
<u>acetate and mannitol</u>	-	+++ (3)+(1)
<u>raffinose</u>	+	++(4)
<u>starch</u>	+	++(4)
<u>salicin</u>	++	+(3)-(1)
<u>sucrose</u>	++	+(2)-(2)
<u>tartrate</u>	-	NR
<u>xylose</u>	+	++(4)
<u>sorbitol</u>	-	+(1)-(3)
<u>dulcitol</u>	-	-(4)
Production of		
<u>H<sub>2</sub>S</u>	-	-(4)
<u>Indole</u>	-	-(4)
<u>acetyl-methyl-carbinol</u>	-	-(4)
Reduction of NO <sub>3</sub>	-	-(4)
Liquefaction of gelatin	-	-(4)
Growth at	48°C	-(4)
(pH 7.2)	45°C	+(1)-(3)
	40°C	+++ (4)
	15°C	++(1)-(3)
Growth at	pH 5.5	+++ (4)
(37° C)	pH 5.0	+++ (4)
	pH 4.5	++(2)-(2)
	pH 4.0	-(4)
Reaction in litmus milk	ARGC	ARGC

+++ strong reaction  
 ++ moderate reaction  
 + weak reaction  
 - no reaction

NR not recorded  
 ARGC acid, reduction, gas, coagulation

## CHAPTER VIII

### CLOSTRIDIA IN SPONTANEOUS SOURINGS

Spontaneous sourings are brought about by maintaining an approximately 10% Sorghum malt suspension at 48 to 50°C for several hours. Under such conditions an enrichment of thermobacteria is expected to occur. Such spontaneous sourings are used as inocula for further lactic fermentations and, with the marketing of so-called "quick souring" malts, mainly by Maltster E, they have in some instances been used as the sole means of lactic acid generation.

As may be expected such sourings are not always successful. The success of a spontaneous souring depends to a large extent on the nature of the flora initially present on the malt. The enrichment of lactobacilli with low acid-producing capacities, in the absence of other forms, will result in a slow lactic acid generation with a poor acid yield.

It had been found by de Schaapdrijver and van Bellinghen (1968, unpublished data) that malts could be divided into two groups by their capacity for acid-production in spontaneous sourings at 50°C. The first group had a slow rate of acid-production with relatively low lactic acid yields. The second group produced 2 to 3 times as much lactic acid under the same experimental conditions. The reasons for this different behaviour of malts was not given by the authors.

The ecology of spontaneously fermenting malt mashes was investigated extensively. Population studies of the more important groups of micro-organisms that had been shown to occur in such sourings, in previous experiments, were correlated with chemical changes in the environment.

As all microbiological samples had to be investigated immediately and as the whole souring process lasted for approximately 20 to 30 hours, these studies could only be undertaken with the collaboration of many workers. The microbial analyses were conducted with Dr. A.E. van Kerken and Miss J. de Villiers of the Microbiology Research Group and chemical analyses were carried out by staff of the Analytical Section of the Bantu Beer Unit.

Five malts were investigated and sourings with each malt were conducted in duplicate. As the behaviour of clostridia was the same in every experiment, only two sourings are discussed in detail in the present study. Other spontaneous sourings are only discussed in as far as the series of ecological events may influence the development of clostridia.

In experiment S7, a "quick souring malt" (M68/143) from Maltster E was allowed to sour spontaneously. The succession of micro-organisms in this experiment was compared with the succession in a similar experiment (S2) using a "slow souring malt" (M68/114) obtained from Maltster C.

#### Methods.

A 10% (w/w) suspension of each malt was allowed to sour in a 5 litre round bottom flask in a water-bath at 50°C. The souring was continuously stirred with a rotary stirrer. Samples were withdrawn aseptically, examined microscopically and processed immediately.

The following analytical tests were performed on each sample by the Staff of the B.B.U., according to their Tentative Methods of Analysis:-

- (i) The pH at room temperature;
- (ii) The titrable acidity expressed as ml. N NaOH per 100 g sample when titrated to an end-point of pH 6.3;
- (iii) The volatile acidity expressed as ml. N NaOH per 100 g sample;
- (iv) The percentage lactic acid (w/v);
- (v) The reducing sugars expressed in ml. 0.05 N  $\text{Na}_2\text{S}_2\text{O}_3$ ;
- (vi) A specific glucose determination using glucose oxidase. The amount of glucose is given as g glucose/100 ml. sample. This can be expressed in ml. 0.05 N  $\text{Na}_2\text{S}_2\text{O}_3$  by multiplying the percentage glucose by a factor of 0.645;
- (vii) The approximate ratio of glucose : fructose : maltose as determined by thin layer chromatography;
- (viii) The percentage (w/v) of soluble and insoluble nitrogen.

#### Results:

Of the five malts investigated, three consistently gave high lactic acid yields (M67/104; M68/113; M68/143).

One Malt (M68/114) with low acid yields was investigated. The fifth malt (M67/152) gave a low yield in one experiment and a high yield in the replicate.

The changes in the microbial population are given graphically with pH changes in Fig. 3, p.48 and Fig. 5, p.50. The chemical changes during souring are given in Fig. 4, p.49 and Fig. 6, p.51. Additional chemical data are listed in Table XIV, p.52 and Table XV, p.53.

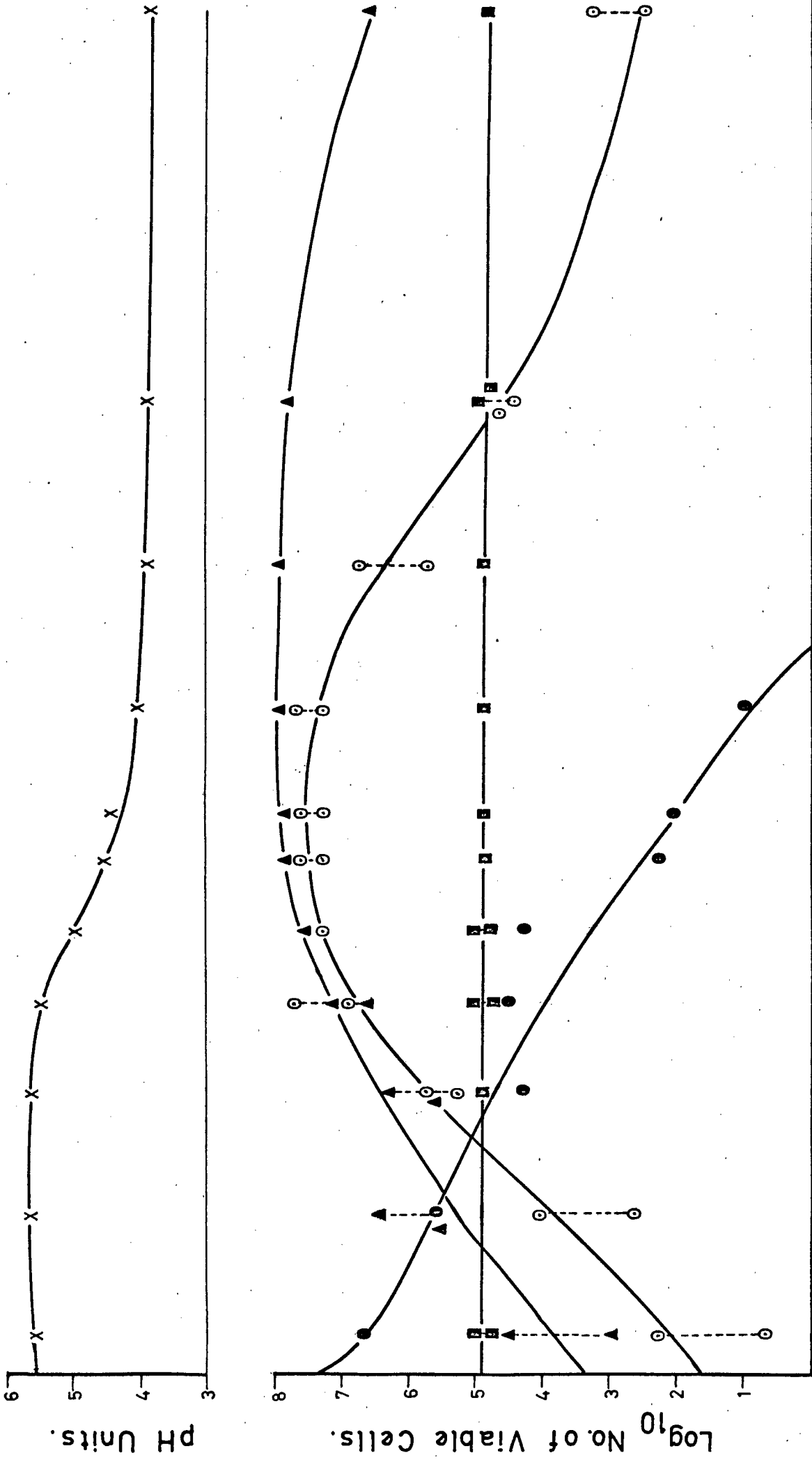


FIG:3. Experiment S2. Spontaneous Souring with Malt M68/114. Population & pH Changes.

KEY:  $\blacktriangle$  = 1/10 % Glucose Specific.  
 $\circ$  = % Lactic Acid Specific.  
 $\ominus$  = Titrable Acidity.  
 $\times$  = Volatile Acidity.

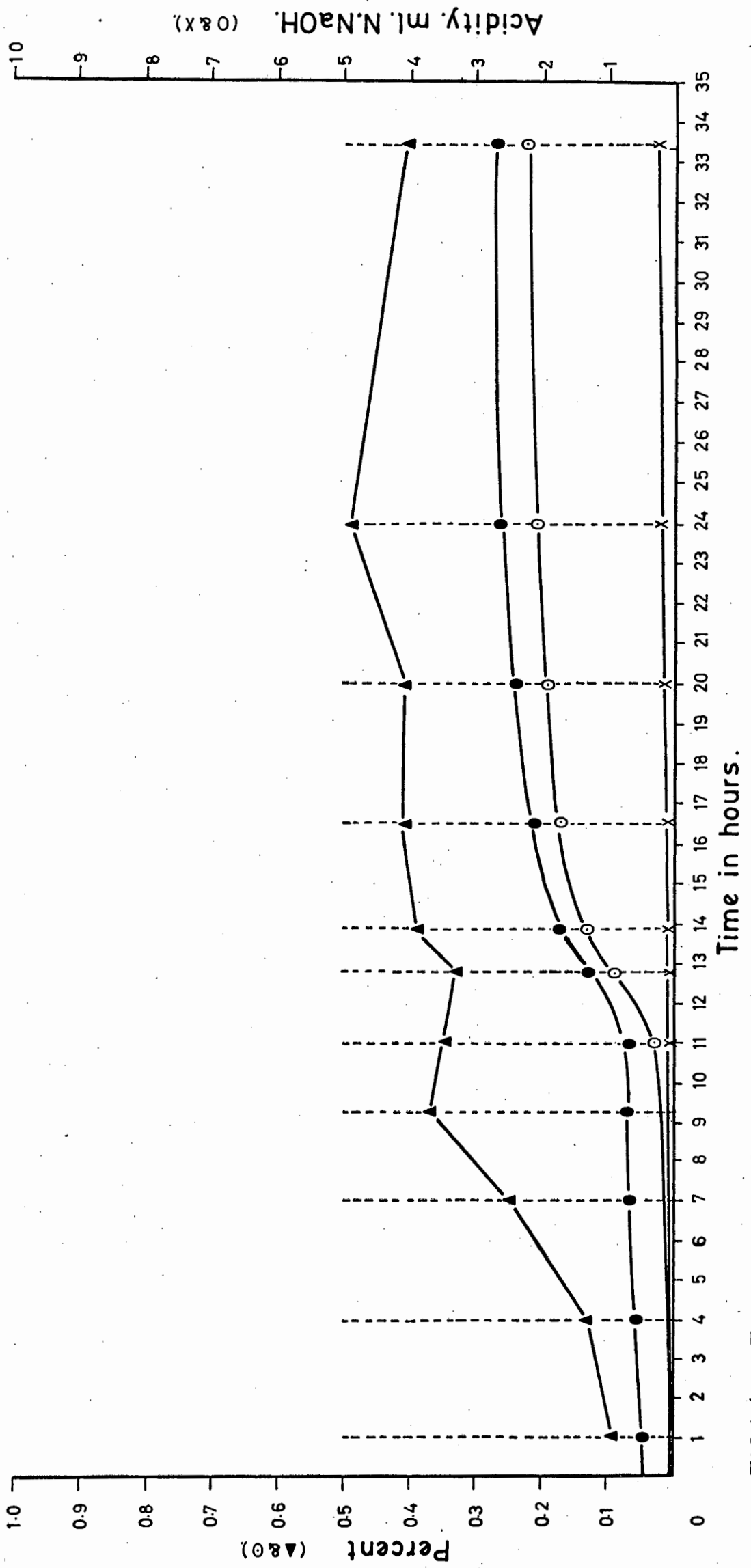


FIG: 4. Experiment S2. Chemical Changes in a Spontaneous Souring with Malt M68/114.

KEY.

- X = pH
- ▲ = Pediococci.
- = Clostridia.
- = Enterobacteria.
- = Bacillus.
- T = Thermobacteria.

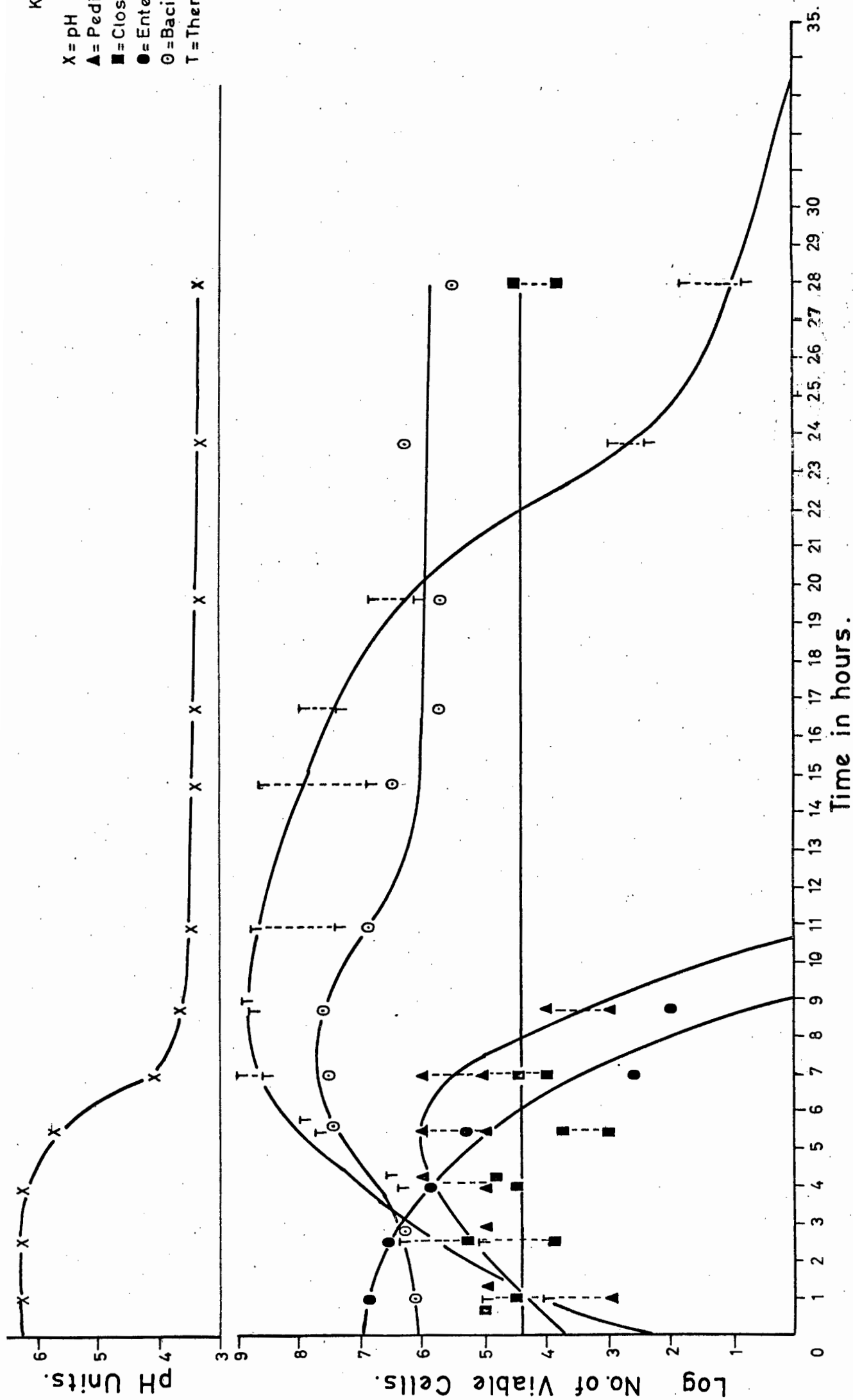


FIG:5. Experiment S7. Spontaneous Souring with Malt M68/143. Population & pH Changes.

KEY:  $\Delta$  = 1/10% Glucose Specific.  
 $\odot$  = % Lactic Acid Specific.  
 $\bullet$  = Titrable Acidity.  
X = Volatile Acidity.

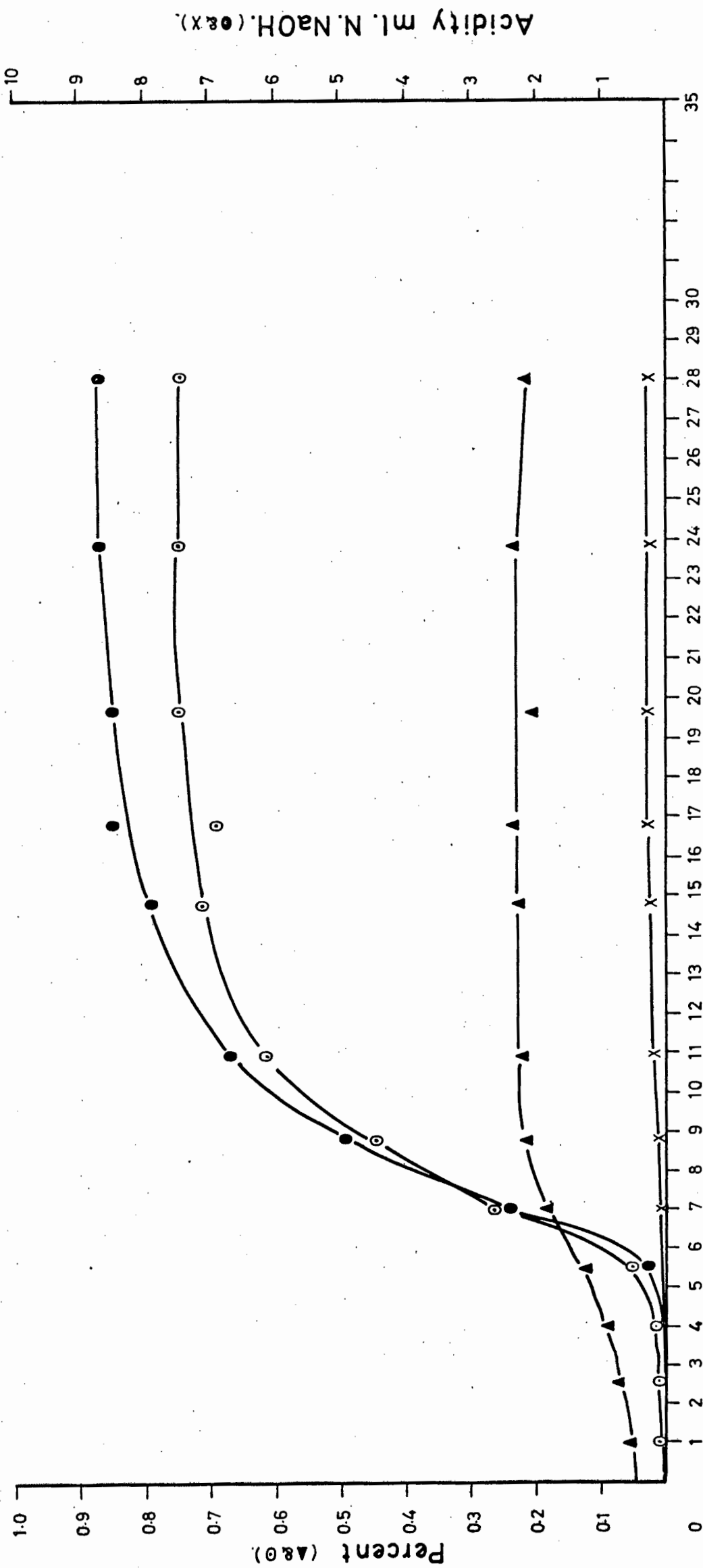


FIG: 6. Experiment S7. Chemical Changes in a Spontaneous Souring with Malt M68/143.

TABLE XIV

## EXPERIMENT S2 - CHEMICAL DATA

Sample No.	S2/1	S2/2	S2/3	S2/4	S2/5	S2/6	S2/7	S2/8	S2/9	S2/10	S2/11
Age (hours/minutes)	1	4	7	9/15	11	12/40	13/50	16/30	20	24	33/30
Reducing sugars	1.05	1.64	2.45	2.88	2.90	3.19	3.33	3.27	3.34	3.52	3.54
% Glucose (specific)	0.87	1.37	2.46	3.70	3.53	3.38	3.84	4.09	4.06	4.89	4.02
<u>Chromatograms</u> (T.L.C.)											
Glucose $\frac{x}{10}$	4	5	6	7	7	6	6	5	5	5	5
fructose $\frac{y}{10}$	†	†	†	†	†	†	†	†	†	†	†
maltose $\frac{z}{10}$	1	2	3	4	3	3	3	3	2	1	1
<u>% Nitrogen</u>											
Soluble N	0.08	0.08	0.09	0.09	0.09	0.09	0.09	0.10	0.11	0.11	0.11
Ins. sol. N	1.75	1.79	2.16	2.40	2.81	2.98	3.25	3.23	3.06	-	-

TABLE XV  
EXPERIMENT S7 CHEMICAL DATA

Sample No.	S7/1	S7/2	S7/3	S7/4	S7/5	S7/6	S7/7	S7/8	S7/9	S7/10	S7/11	S7/12
Age (hours/minutes)	1	2/35	4/0	5/30	7/00	8/45	10/55	14/45	16/45	19/40	24/00	33/15
Reducing sugars	0.71	0.94	1.12	1.34	1.67	1.80	1.65	1.53	1.52	1.52	1.52	1.71
% Glucose (specific)	0.59	0.79	0.99	1.30	1.87	2.17	2.20	2.22	2.38	2.06	2.35	2.19
<u>Chromatograms</u>												
<u>Ratio</u>												
Glucose	1	1½	2½	3	3½	4	4½	5	5½	5½	5½	5½
fructose	1/4	1/4	1/4	1/4	0	0	0	0	0	0	0	0
maltose	½	1	½	2	2½	2	1½	1¼	1¼	1	1	1

## Discussion.

In all spontaneous sourings conducted with "quick souring"malts, non-motile rods were observed microscopically and pure cultures of these organisms were identified as Lactobacillus delbrueckii. Motile bacilli and pediococci were nearly always seen in the initial stages of souring with this type of malt, but they were eventually outnumbered by the thermobacteria and could no longer be detected microscopically after a few hours.

In spontaneous sourings conducted with "slow souring"malts, no thermobacteria could be seen. Pediococci and motile rods were detected microscopically and seemed to form the dominant flora. They were usually still visible after 1 day. The motile rods were isolated and identified as Bacillus coagulans.

These microscopic observations were confirmed by the results obtained in enumeration experiments. In Experiment S7, conducted with a quick souring malt, the rapid multiplication of thermobacteria was accompanied by a rapid fall in pH. See Fig. 5. Small increases in the numbers of pediococci and bacilli were recorded, decreasing rapidly in numbers after 9 hours, when the pH became unfavourable. The number of bacilli reached a constant level after 15 hours when a pH of 3.3 had been reached. At this low pH the bacilli were present as spores only as the same values were obtained for unpasteurised and pasteurised counts.

In Experiment S2 a slow souring malt was used. No thermobacteria could be detected in enumeration experiments. Bacilli decreased very slowly in numbers and large numbers of pediococci could still be detected after 35 hours. A slow decrease in pH was detected reaching a minimum of only 3.9 after 20 hours.

A high lactic acid content of 0.7%, after 15 hours, was recorded in Experiment S7. In Experiment S2 only 0.2% lactic acid was recorded after the same time. See Fig. 4 and Fig. 6. In both experiments the maximum rate of lactic acid production took place just before the culture of lactic acid bacteria reached the stationary phase as is shown by increases in the titrable acidity and in the specific lactic acid present.

No significant changes could be detected in the soluble Nitrogen content in Experiment S2. See Table XIV.

The amount of sugar present in the mash results from two concurrent processes: Sugar production by the enzymatic activity of the malt and its simultaneous utilisation by the microbial flora. Reducing sugars were present throughout the souring process and never became

limiting. See Table XIV and Table XV. The small differences obtained between values for the total reducing sugars and values for the specific glucose determination, indicate that mainly glucose was produced. This is confirmed by the semi-quantitative, chromatographic analysis where mainly glucose and only small quantities of fructose and maltose could be detected. See Table XIV and Table XV.

In no cases could vegetative clostridia be found in sourings, not even if slow souring malts were used where the pH remained favourable almost throughout the process. The inactivity of clostridia is confirmed by the low values obtained for the volatile acidity, as volatile acids are the main end-product of clostridial fermentations. The small increases in the volatile acidity may be ascribed to the activity of the bacillus rather than to the homofermentative pediococci and thermobacteria.

In general, it can be said that such spontaneous sourings are an inefficient means of acid-production on an industrial scale. Lower yields of lactic acid are obtained after a longer period than is the case in an inoculated souring. Inocula from such spontaneous sourings will give unpredictable results because of the sporadic development of thermobacteria. It was expected that the conditions in spontaneous sourings would form a favourable milieu for clostridia. This was shown not to be the case in the present study.

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## CHAPTER IX

### THE CLASSIFICATION OF CLOSTRIDIA

A.

#### Introduction.

Attempts were made to identify the strains that had been isolated and brought into pure culture in the course of investigations described in the preceding Chapters. When it was found that existing keys gave unsatisfactory results, it was attempted to group the strains by means of "phenetic" or "numerical" taxonomic techniques.

#### 1. Existing keys and classifications.

The anaerobic bacteria have been somewhat neglected by taxonomic investigators. A reason for this may be the difficulties experienced in isolating and handling these organisms because of rather laborious anaerobic techniques. Whereas keys exist that have found general acceptance for most aerobic groups of bacteria, artificial though these groups may be; such keys do not exist for the genus Clostridium.

There are several good keys that deal with species in selected applied fields. There are numerous keys to clostridia of medical importance. Buttiaux and Beerens (1955) have devised a key to species causing spoilage in canned foodstuffs. Oakley (1956) is more comprehensive and assigns species to physiological groups.

Apart from these minor keys two systems exist that claim to be more comprehensive. McClung and McCoy (1957) in the 7th edition of Bergey's Manual of Determinative Bacteriology list 93 species, while Prévot (1957, 1966) elevates the genus Clostridium (sensu Bergey) to class status comprising 3 orders, 5 families, 10 genera and approximately 200 species.

These keys proved to be rather unsatisfactory. The strains could be assigned to species by following the key, but more often than not they agreed poorly with the species description.

#### 2. The choice of data.

It has been pointed out by Sneath (1957 a and b) and by Sokal and Sneath (1963) that the tests chosen for numerical analyses should cover as wide a field as possible; that the number of attributes should be large and based on the nature of the problem to be solved. Ecological data from the previous chapters, such as maximum growth temperatures and lowest pH values at which growth could take place were thus included in this study, as it was hoped that strains that could be of importance in the beer fermentations might emerge, as distinct groups having these

features in common.

As morphological attributes are widely used in the taxonomy of clostridia such data were included as well as numerous physiological tests. In this study the fermentation of 18 carbohydrate sources was examined. Kutzner (1963) working on strains of Cl. butyricum and Cl. tyrobutyricum that normally did not ferment glycerol and mannitol found that in the presence of acetate such fermentation could sometimes take place. All strains were tested for this ability.

The nature of the remaining tests is self-explanatory. The tests are summarised in Table XVI.

### 3. The scoring of attributes.

The scoring of attributes presented some theoretical problems which are briefly outlined below. Quantitative data were avoided as much as possible as there are certain logical objections to such multistate scoring, especially if an arbitrary scale is used (Colless, 1967). For example, initially all fermentation tests were recorded as 3 state data in the following way:

No fermentation (0). The pH did not fall below 5 after  
14 days' incubation.

Weak fermentation (1) A pH value above 4 and below 5 was  
recorded after incubation for 14 days.

Strong fermentation (2) The pH fell to a value below 4 after  
14 days' incubation,

where (0), (1) and (2) represent the figures recorded on the score-sheet. Thus 3 cultures having pH values of 3.9, 4.0 and 4.9 after 14 days are recorded as (1), (2) and (2) respectively.

This gives rise to a gross distortion of the results, as the first two values are much closer than the second and third.

Data were only scored according to such an arbitrary scale if no other satisfactory solution could be found, e.g. attributes 58, 59, 60, 61. See Table XVI.

The fermentation of carbohydrates is usually accompanied by a fall of pH in the medium and is measured as such in fermentation tests after a given time of incubation. This method was also used in this study. But it must be pointed out that it is a poor indication of the extent to which fermentation has taken place in the case of clostridia. A strain producing mainly neutral products during fermentation, viz. alcohols will not lower the pH to such a degree as will a predominantly acid-producing strain. Gas production is not always indicative of fermentation

as most clostridia produce a considerable amount of gas in the basal medium. The most reliable method would be the quantitative analysis of the end-products of fermentation, but this is too time-consuming and therefore impracticable.

Care was taken not to weight attributes by a duplication in scoring, e.g. acid-production was not scored in milk, as lactose was one of the carbohydrate sources included in the fermentation series. Clotting ability in litmus milk, however, bore no relation to such an ability in cystein milk and was, therefore, scored separately.

4. Data used in this study.

The following is a summary of the attributes chosen for this study:

TABLE XVI

A key to the tests used.

<u>Attributes</u>	<u>No. of attribute</u>	<u>State of attribute</u>
<u>Fermentation of the following carbohydrates and other carbon sources.</u>		
cellobiose	1	2
cellulose	2	2
fructose	3	2
galactose	4	2
glucose	5	2
glycerol	6	2
acetate-glycerol	7	2
inulin	8	2
acetate-lactate	9	2
lactose	10	2
maltose	11	2
mannitol	12	2
acetate-mannitol	13	2
raffinose	14	2
starch	15	2
salicin	16	2
sucrose	17	2
xylose	18	2
sorbitol	19	2
dulcitol	20	2

Production of:

indole	21	2
acetyl-methyl-carbinol	22	2

<u>Reduction of nitrate to nitrite</u>	23	2
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<u>Reduction of nitrite</u>	24	2
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<u>Proteolysis of gelatin</u>	25	2
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Reaction in litmus milk:

reduction of litmus	26	2
clotting	27	2
stormy clot	28	2

Reaction in cystein milk:

curdling	29	2
clotting	30	2
stormy clot	31	2
digestion	32	2

<u>Production of lecithinase</u>	33	2
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<u>Production of lipases</u>	34	2
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Growth temperatures (See attribute 58)

growth at 15°C	35	2
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<u>Gas production in VF agar stabs</u>	36	2
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<u>Motility</u>	37	2
-----------------	----	---

<u>Gram stain after 10 hrs.</u>	38	2
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after 24 hrs:	39	2
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Production of the following compounds:

acetone	40	2
acetic acid	41	2
butyric acid	42	2
caproic acid	43	2
ethyl alcohol	44	2
butanol	45	2
iso-propanol	46	2

Morphological characters:

inclusion bodies	47	2
granulose	48	2

cells with rounded ends	49	2
clostridia swollen	50	2
filament formation	51	2
spores terminal	52	2
spores subterminal	53	2
spores central	54	2
pigment production	55	2
colony flat (0) or raised (1)	56	2
<u>Production of H<sub>2</sub>S:</u>	57	3
negative (0)		
blackening in form of a deposit (1)		
blackening of the whole tube (2)		
<u>Growth temperatures:</u>		
growth at 37°C (0)	58	4
growth at 40°C (1)		
growth at 45°C (2)		
growth at 48°C (3)		
<u>Lowest pH value at which growth takes place:</u>		
pH 3.5 (0)	59	5
pH 4.0 (1)		
pH 4.5 (2)		
pH 5.0 (3)		
pH 5.5 (4)		
<u>Sodium chloride tolerance:</u>	60	3
No growth in 4% NaCl (0)		
growth in 4% NaCl (1)		
growth in 8% NaCl (2)		
<u>Degree of anaerobiosis:</u>		
growth in VF deep agar stabs	61	4
no growth (0)		
growth less than 1 cm. from surface (1)		
growth ceases between 1 and 2 cm from surface (2)		
growth ceases below 2 cm from surface (3)		
<u>Colony type:</u>	62	3
regular margin (0)		
rhizoid margin (1)		
lobed margin (2)		

5. The choice of strains.

The following named strains were obtained from the National Collection of Dairy Organisms (NCDO), National Institute for Research in Dairying, Reading, England, and these were included in this study as points of reference.

NCDO Catalogue Number	No. of strain in this study	Organism
1758	66	<u>Clostridium beijerinckii</u>
935	67	<u>Clostridium bifermentans</u>
1710	68	<u>Clostridium sporogenes</u>
1715	72	<u>Clostridium tyrobutyricum</u>
856	73	<u>Clostridium butyricum</u>

Strain 77, Cl. acetobutylicum, was obtained from National Chemical Products Ltd., Germiston (NCP strain 210).

The origins of all strains used in the computer analysis are given below in Table XVII.

TABLE XVII

The origin of strains used in this study.

No. of strain (OTU)	Original designation	Origin
1	A1	Clostridia growing in sourings that had been sterilised previously. Sourings were derived from the pH 5.0 stat. See p.27a.
2	A2	
3	A3	
4	A4	
5	A5	
6	A6	
7	J8	Isolated from enumeration series of sourings in Brewery D. (Experiment I). See Table X, p.39.
8	J9	
9	J10	
10	J11	
11	J12	
12	J13	
13	J14	
14	J15	

No. of strain (OTU)	Original designation	Origin.
15	J16	
16	J17	
17	J19	
18	J20	
19	J21	
20	J22	Isolated from enumeration series of sourings in Brewery D. (Experiment II). See Table XI , p. 40.
21	J23	
22	J24	
23	J25	
24	J26	
25	J28	
26	J29	
27	J32	
28	J35	
29	J37	
30	J38	
31	X7A	Isolated from freeze-dried beer by Dr. A.E. van Kerken (February, 1965)
32	X7Ta	
33	X7Tb	
34	X7Te	
35	X7G	
36	X9T(1)	
37	X10a	
38	X11a	
39	X12a	
40	X13a	
41	C14	Beer from Brewery B. Enumeration in RCM semi-solid agar. Table II, p. 17.
42	C15	
43	C16	
44	C12	
45	C18	
46	C20	
47	C21	Souring from Brewery B. Enumeration in liver medium. Table I, p. 17.
48	C23	
49	C24	
50	C25	
51	C26	
52	C27	Souring from Brewery B. Enumeration in RCM broth. Table I, p. 17.
53	C30	

No. of strain (OTU)	Original designation	Origin.
54	C31	Souring from Brewery B. Enumeration
55	C33	in gelatin medium. Table I, p. 17.
56	C34	
57	C35	
58	C36	
59	C37	Same origin as OTU's 63 to 64 below.
60	C39	Beer from Brewery B. VF medium. Table II
61	C40	Souring from Brewery D. RCM semi- (p. 17.
62	C41	solid medium. Table I, p. 17.
63	C42	Souring from Brewery D. Acetate-lactate
64	C43	medium. Table I, p. 17.
65	C44	Enrichment with $\beta$ -alanine (p. 23).
66	C48	<u>Cl. beijerinckii.</u>
67	C49	<u>Cl. bifermentans.</u>
68	C50	<u>Cl. sporogenes.</u>
69	C55	Enrichment with ethylene glycol as
70	C64	carbon source. (p. 23)
71	C66	
72	C71	<u>Cl. tyrobutyricum.</u>
73	C72	<u>Cl. butyricum.</u>
74	C78	The pH5.0 stat. Table VIII, p. 27a.
75	C81	
76	C92	Disused tank in Brewery D, p. 43.
77	C93	<u>Cl. acetobutylicum.</u>

## 6. Theoretical considerations.

It was attempted in this study to group 77 strains phenetically using the 62 attributes described above. As is customary in Adansonian techniques all features or attributes were, a priori, considered to be of equal importance. "Resemblance" or "Difference" between strains was calculated in terms of a "distance coefficient" as described by Cain and Harrison (1958) and reviewed by Sokal and Sneath (1963), Williams and Dale (1965) and Colless (1967).

Taxonomic Distance can be defined as the Pythagorean distance between two OTU's (Operational Taxonomic Units, i.e. the strains selected for this study) as represented by points in a p-dimensional

hyperspace where  $p$  represents the co-ordinates in such a space; each co-ordinate representing a quantitative value of each attribute. Distances between such points are expressed as MCD (Mean Character Difference) values and are plotted in a matrix.

Heterogeneity analysis and Homogeneity:

As can be expected, data presented for classification must be heterogeneous to some degree. This can be determined by the function.

$$H_t = \frac{1}{p} \sum_{j=1}^p \frac{a_{jr}}{a_{jr \max}}$$

where  $H_t$  represents the heterogeneity for 2-state data. In this function the actual number of rare states  $a_{jr}$  is divided by a hypothetical maximally heterogeneous case with the same number of included members for the  $j$ th of  $p$  attributes.

In the present study, where 2-state as well as quantitative data were used the function had to be amended as follows (cf. Hall, 1967).

$$H_{tq} = \frac{1}{p} \sum_{jt=1}^{pt} \frac{a_{jtr}}{a_{jtr \max}} + \frac{1}{p} \sum_{jq=1}^{pq} \frac{s_{jqn}}{s_{jqn \max}}$$

where  $t$  and  $q$  refer to two-state and quantitative data respectively.

$S_{jqn}$  refers to the standard deviation of attribute  $j$  for a set of  $n$  members.

$S_{jqn \max}$  refers to a hypothetical group of  $n$  members having maximal heterogeneity.

Heterogeneity can be converted to Homogeneity ( $H_{qm}$ ) by the following formula:

$$H_{qm} = 1 - H_{tq}$$

The calculation of MCD values.

The MCD value between two OTU's is equal to the average Heterogeneity value between such OTU's.

$$MCD = H_{tq \text{ av}} = H_{tq \text{ pairs}}$$

In the method outlined above both negative and positive matches are used in the calculation of MCD values between two OTU's. The acceptability of negative matches has been discussed by Colless (1967) and Hill et al. (1961).

Proteolytic clostridia are known to differ from saccharolytic strains by their general inability to ferment sugars. If one considers the proteolytic group on its own, it is possible that a particular carbohydrate is not fermented by two organisms for different reasons, viz. a sugar may not be attacked because of the lack of more than one enzyme, or because of a permeability effect. Bearing this in mind, it would seem that negative matches are not necessarily acceptable whereas positive matches and a positive and negative comparison are valid. For these reasons Sokal and Sneath (1963) suggest that negative matches should be omitted in Adansonian analyses conducted with bacteria.

Positive matches are usually considered to be sound (Hill et al, 1961; Sokal and Sneath 1963), but surely the same reasoning can be employed here. A carbohydrate may be fermented by different pathways in two organisms or by structurally different enzymes or isozymes.

Essentially these are problems to which no satisfactory answers have yet been found and it may be pointed out that all tests are essentially arbitrary observations of certain superficial characters and not of the causes that have led to their expression.

Distance coefficients as used in this study take positive and negative matches into account and are not generally used in Adansonian analyses of bacteria.

## B.

### Methods.

#### 1. The inoculum.

Strains were recovered from soil or RCM slopes and inoculated into liver medium as previously described in Chapter IV.

After good growth had taken place, a tube of VF broth was inoculated and incubated for 24 hrs. at 37°C in an anaerobic jar. This was used as an inoculum for the following tests:

#### 2. Physiological tests.

Various methods have been described for conducting fermentation tests of anaerobic bacteria, that vary not only in the composition of the medium but also in the way anaerobiosis is achieved (Hungate, 1950; Lebert and Tardieux, 1952; Spray, 1936).

A simple method has been described by Buttiaux and Beerens (1955). Anaerobiosis is achieved in a semi-solid medium by lowering the redox potential with cystein and boiling the medium to expel air before use.

This method, however, gave spurious results. It has been pointed

by Stüssi et al. (1962) that agar may be utilised as carbon-source by many saccharolytic clostridia. The medium described by Buttiaux and Beerens (1955) was therefore modified by omitting the agar. Now, however, it was clearly not anaerobic enough for growth to take place, even after boiling to expel air. However, repeatable results were obtained if the medium was sealed with paraffin wax after inoculation as has been described by Stüssi et al. (1962).

The basal medium that was eventually used was adapted from Buttiaux and Beerens (1955) and had the following composition:

Bacto Tryptone	10g
Sodium chloride	5g
beef extract	3g
yeast extract	5g
cystein HCl	0.4g
Distilled water	1000 ml.

The pH was adjusted to 7.4. The medium was dispensed in 9 ml. quantities and sterilised. To this 1 ml. of the filter-sterilised carbon source was added aseptically to give a final concentration of 0.5%. Acetate was added aseptically in the form of the sodium salt to give the same final concentration. (Attributes 7 and 13). Starch and inulin were sterilised in an autoclave and were added to the basal medium in a 0.5% concentration.

A drop in pH to a value below 5.0 after 14 days' incubation at 37°C was scored as a positive reaction.

The fermentation of lactate was tested in the medium described by Bryant and Burkey (1956). An increase in pH after 14 days to a value above pH 7.5 was recorded as a positive reaction.

The reduction of nitrate was tested after 14 days in the basal medium with 0.2% glucose and 0.5% sodium nitrate, by the addition of 1 ml. of a 0.5% naphthylamine solution in dilute acetic acid, followed by 1 ml. of a 0.3% sulphanilic acid solution in dilute acetic acid. A red colour denoted the reduction of nitrate to nitrite. If no colour developed, a knife-point of zinc dust was added, to reduce nitrates to nitrite. If again no colour developed the nitrite stage must have been missed and nitrite was taken to have been further reduced. If colour was produced on the addition of zinc dust, nitrates were not reduced by the test organism.

Liquefaction of gelatin was detected in the basal medium with 0.2% glucose and 15% gelatin. Liquefaction was recorded after 1, 3 and 14 days.

The reactions in litmus milk (attributes 26 to 28) were recorded after 14 days. Difco powdered litmus milk was used.

The reactions in cystein milk (attributes 29 to 32) were recorded after 14 days. 0.08% cystein was added to skimmed milk and the pH adjusted to 7.4 before sterilisation.

The production of lecithinase and lipase was recorded on the medium described by Willis (1965) after 3 days' incubation in an anaerobic jar.

The production of  $H_2S$  was recorded after 1, 3, and 14 days' incubation in the basal medium, containing 0.02% ferrous sulphate and 0.3% sodium thiosulphate that had been filter-sterilised separately and added aseptically to the basal medium.

Temperature and pH studies described in a previous chapter were included in this analysis.

Growth in 4% and 8% NaCl was recorded after 14 days' incubation. This test was carried out in the basal medium with sodium chloride.

The degree of anaerobiosis (attribute 61) was recorded in VF deep agar by measuring the height from the surface of the stab where growth could be visibly detected after 7 days.

#### The analysis of fermentation products.

The production of indole (attribute 21) was detected after 3 days in the basal medium by the Ehrlich reaction. Acetyl-methyl-carbinol (attribute 22) was detected after 3 days in the basal medium plus 1% glucose by the Barrit reaction.

For the analysis of all other fermentation products (attributes 41-46) the following procedure was adopted:

The principle of the Ivan-Hall marble tube was used for fermentations on a larger scale. A 200 ml. conical flask was provided with a long neck with a constriction at its base above which was a side-arm, large enough to hold a marble. The flask was filled with basal VF medium plus 1% glucose to the level of the side-arm and sterilised. Before use the medium was steamed for 20 minutes to expel air, cooled and inoculated. The marble was then removed from the side-arm by tilting the flask. It came to rest on the constriction, thus sealing the medium from the atmosphere. The flask was incubated aerobically at 37°C.

After 2 to 3 days when the fermentation was complete and no more gas was formed, the liquid was decanted and centrifuged. The supernatant was then used for the analysis.

The pH of the fluid was adjusted to pH 7.5 to 8.0 and was distilled till 50 ml. distillate was collected which was used for the analysis of alcohols and acetone.

The residual fluid was cooled and acidified with concentrated sulphuric acid to pH 1 to 2 and steam distilled until 50 ml. distillate was obtained. This was used in the analysis of volatile acids.

#### Analysis of the alkaline distillate.

Acetone was determined according to the method described by Neish (1950).

Isopropanol was determined by its oxidation with acid sodium dichromate to acetone (Neish, 1950). Isopropanol present could be calculated from the difference between total acetone present after oxidation and the acetone present before oxidation.

Ethanol was determined according to the method of Lebert and Tardieux (1952).

Other alcohols were determined by the oxidation with acid potassium dichromate to their corresponding acids as described by van der Lek (1930). The oxidised alcohols were steam-distilled and the distillate containing the volatile acids was proceeded with as described below.

#### Analysis of the acid distillate

The volatile acids contained in the distillate were determined by paper chromatography according to the method of Kennedy and Barker (1951). It must be pointed out that this method does not differentiate between primary and secondary alcohols originally present.

### 3. Morphological observations.

RCM agar slopes were inoculated and incubated in an anaerobic jar for 10 hours. Material from these slopes was used for tests 47 to 54.

Granulose (attribute 48) was tested with Gram's Iodine Solution. Gram stains (attributes 38 and 39) were made on material from such agar slopes.

Motility (attribute 37) was determined microscopically on material derived from basal VF medium which was used as the original inoculum.

Attributes 55, 62 and 66 were tested on RCM agar plates after incubation at 37°C for 36 hours in an anaerobic jar.

#### 4. Scoring.

Two-state data were scored as 0, if the test was negative, or as 1, if positive. Quantitative data were scored as indicated in Table XVII. In cases where there was a logical bar, i.e. if there was a dependence of one attribute on another, such an attribute was scored as NC (No Comparison) if the independent attribute was positive. NC's were also used to avoid weighting of any particular feature.

#### 5. The Computation of Data.

The work was conducted using the ICT 1301 computer at the University of Cape Town. The computer programme was written in Manchester Autocode (MAC) and was kindly made available for this study by Dr. A.V. Hall of the Bolus Herbarium, University of Cape Town.

After the matrix of MCD values had been calculated, the group with the smallest heterogeneity value was found and recorded. All heterogeneity values of the OTU's linked in this way were replaced by a larger value, and values were found for such trial links of this group with other members. These were again recorded in the working matrix, and the case with the next minimum heterogeneity value was determined. Alternative choices sometimes presented themselves, and these were selected by manual control.

From these data a dendrogram was constructed. See Figure 7.

#### C.

#### Results.

The results of the 62 tests performed on the 77 strains in this study, are recorded in Table XVIII, p. 71.

A dendrogram was constructed from these linkages and reproduced here as Fig. 7, p. 70. Two distinct groups emerged from the analysis and gave a good separation in the dendrogram.

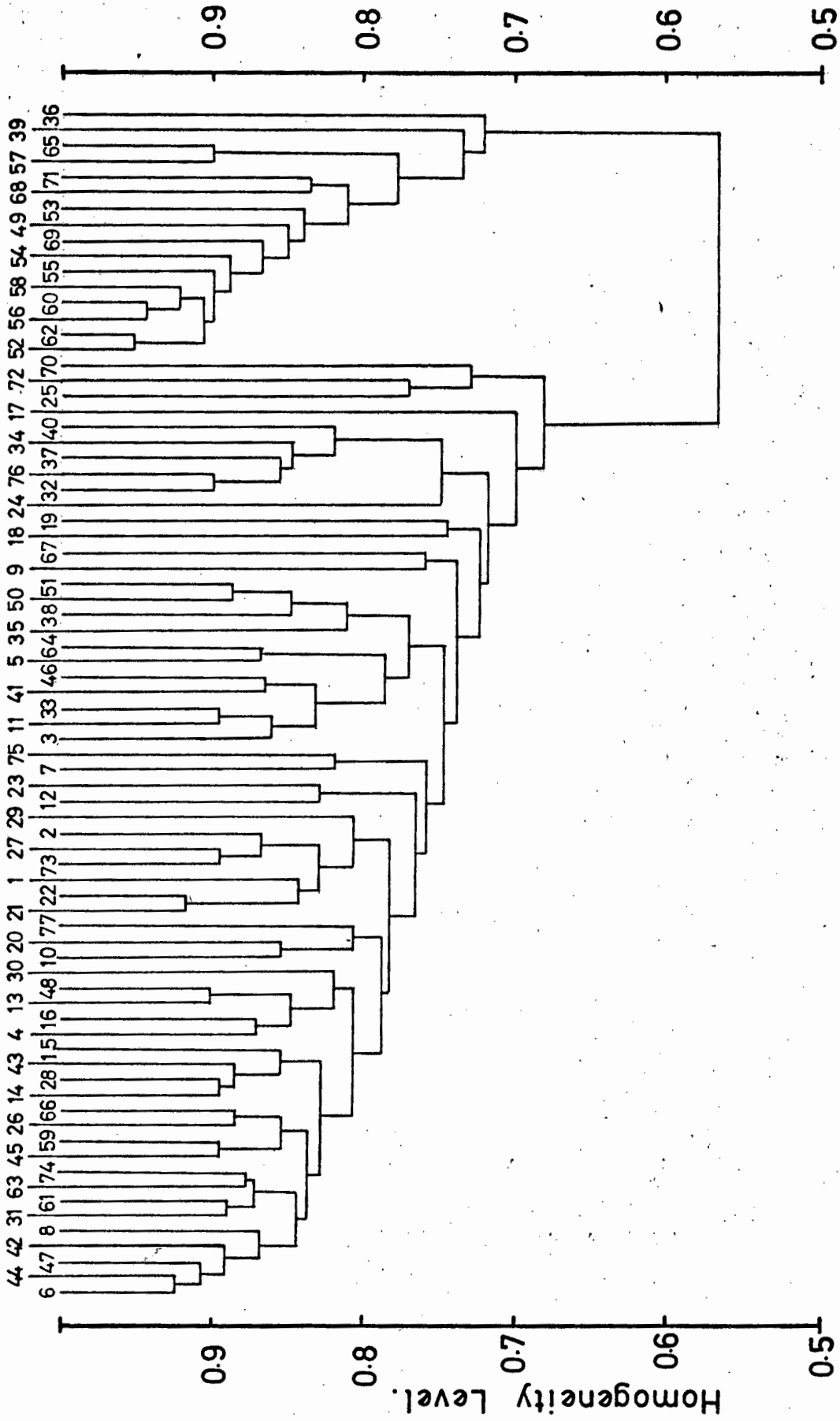


FIGURE:7. Dendrogram relating 77 Strains using 62 Characters.

KNOWN STRAINS.

- 66. *Cl. beijerinckii.* 72. *Cl. tyrobutyricum.*
- 67. *Cl. bifermentans.* 73. *Cl. butyricum.*
- 68. *Cl. sporogenes* 77. *Cl. acetobutylicum.*





D.

Discussion.

In order to discuss the results obtained in the computer analysis the species concept and reasons for variability in micro-organisms must be examined more closely.

Whereas in higher organisms Linnaean taxonomy seems to have given reasonably satisfactory results its application to bacteriology, it is generally conceded, has resulted in a certain amount of chaos. The question comes to mind whether this is an inherent fault in orthodox taxonomy or a result of its application to bacteriology.

Species and Speciation in higher Organisms.

In pre-Darwinian times the species was an essentially static entity with a continuity of form within such a group except for a small "sporting" variation between members. With the advent of the theory of evolution the species concept became somewhat more pliable, as it was postulated that new species could arise by the selection of hereditary variants which were endowed with some advantage of reproduction or survival over their competitors.

Genetics has revealed to us that such hereditary variations are brought about by random mutations in a population and that these are brought together by the process of recombination in sexually reproducing forms. Isolating mechanisms, however, exist that limit such mutations to groups sharing a common gene-pool in a given population, i.e. to species that are reproductively isolated. This has resulted in the geneticist's definition of a species as "a potentially interbreeding group". Such potentially interbreeding groups may, however, be still reproductively isolated in nature. This isolation may be due to geographical barriers or different flowering times in plants. Different species may be sexually incompatible or, if capable of interbreeding, produce sterile or non-viable off-spring and are genetically isolated in this way.

Reproductively isolated organisms are therefore also in effect genetically isolated and one species does not contribute to the gene-pool of another.

Before the advent of genetics and experimental taxonomy species were entirely defined in morphological terms and this is to a large degree still the case to-day. But as morphology is an expression of the genotype, a break in the morphological continuum is in practice frequently accompanied by a disruption in the reproductive and genetic continuum. If morphological criteria are to be used in taxonomy,

genetic isolation is by itself not enough evidence in delimiting a species. A  $3n$  individual in a population of higher plants is incapable of genetic exchange with a  $2n$  member and thus genetically isolated but such individuals may be morphologically indistinguishable. Species must be defined by a combination of the above criteria and not by any one of them. It is still very much a matter of judgment of the taxonomist as to what constitutes a species.

This may serve as a very brief review of our knowledge of speciation and the species concept in higher organisms. The following points must be stressed.

- (i) Species are in practice mainly delimited by using morphological criteria.
- (ii) Effectual and not necessarily potential genetic exchange within a group in nature can be used to delimit a species.
- (iii) The taxonomist deals mainly with individual organisms constituting a group.

#### Genetic variation and speciation in bacteria.

In bacteria, as in higher organisms, genetic variability is a result of random spontaneous mutations. This has been demonstrated for the fermentation of sugars by Ryan (1952). But, as a result of the haploid nature of the bacterium, such mutations must immediately be expressed and exposed to the selective forces of the medium.

Recombination in bacteria can be brought about in different ways.

##### (i) Bacterial sexuality.

This term is perhaps somewhat a misnomer as bacterial "sexuality" is quite unlike the sexuality of higher organisms; in that genetic transfer between two conjugating cells is always partial, resulting in a merozygote. This is not followed by "crossing over" in a reduction division, but hereditary factors are passed onto the progeny by a "copy choice replication" mechanism. The episomal nature of the sex-determining factor has no equivalent among higher organisms (de Long, 1967).

##### (ii) Transduction.

Transduction by temperate phage never results in the transfer of more than 1 or 2 closely associated genetic markers to the host bacterium and are usually close to the point of attachment of the phage to the bacterial genophore.

(iii) Transformation.

Here again rarely more than one genetic determinant is incorporated by a bacterium and this process only takes place in actively growing cultures.

As in higher organisms certain isolating mechanisms exist. Fertility factors may affect pairing ability in bacterial sexuality. Differences in structure of the genetic material may make incorporation impossible in all three processes outlined above. Bacteriophages are often extremely specific. Intraspecific and intrageneric transduction has, however, been recorded among the enterobacteria (Ravin, 1960). Similarly transformation among species of the genera Haemophilus and Streptococcus has been recorded.

The bacterial species.

Three points were noted above, which characterise the approach of the systematist of higher organisms, but the microbial taxonomist has a fundamentally different approach.

Whereas stable morphological attributes exist in bacteria, these are in fact very few. We know that cocci do not mutate into rods, probably because they are genetically determined by many interdependent hereditary factors. But as so few morphological criteria exist, there has been a general shift of emphasis to physiological and biochemical attributes, many of which are exceedingly labile and may be changed by single gene mutations. Strains obtained from enrichment techniques were often described on the basis of a biochemical reaction for which the organism was selected, often without checking the ability of existing isolates to perform the same physiological feat. The result has been a large number of monotypic species and a bad correlation of characters between them, some tests being applied to one species and different tests to others.

Whereas the taxonomist of higher organisms can study the effective reproductive isolation in nature, the bacteriologist can only do so in the laboratory and his knowledge of genetic exchange in the natural environment remains largely hypothetical and based on inference from indirect techniques.

Furthermore, the bacteriologist does not deal with individual bacteria but with populations, the so-called "pure culture" of millions of cells, which he puts through various tests usually bearing no relation to the environmental conditions from which the organism was isolated. In fact, Shimwell and Carr (1960) point out that we are classifying mixed and

composite cultures which may vary in cell composition according to the tests applied. Shimwell (1959) found that "pure cultures" of Acetobacter soon became mixtures of "species" and suggests that bacteria are continually evolving at a fairly rapid rate, possibly as a result of their short generation time and the immediate expression of any mutation that arises.

In this study on clostridia two distinct groups were formed. Quoting the first member of each group, these can be designated as group 6 and group 52. Neither group can be satisfactorily subdivided, as there is no significant drop in Homogeneity between members in either of these groups. Group 6 consists exclusively of saccharolytic clostridia and group 52 of proteolytic strains.

The difference in Homogeneity level considered significant is essentially a matter of judgment of the individual worker. In this case, however, it is quite clear that there are only two groups, as differences in homogeneity within each group are exceedingly small.

A gap in the phenotypic continuum does exist and one could thus consider each group as a species but, as has been pointed out, this may not coincide with such a break in nature. The problem solved would be one of rank only. Subspecific classifications would become necessary to define the properties of the strains more closely, as the number of predictive features in such a large group is low.

The groups obtained can be likened to the "biotypes" of Winogradsky (1952) which form distinct groups from which the "species" in Bergey's Manual differ in minor secondary characteristics. Such a "biotype" forms a natural group as opposed to the artificial bacterial species. This has been demonstrated by de Ley (1963) using comparative biochemical techniques and is confirmed here by numerical taxonomic means. This study thus confirms the presence of two "biotypes" corresponding to the saccharolytic and proteolytic groups.

Clearly, for practical purposes, a classification into such broad groups is inadequate. A classification based on the isolate with some numerical code to indicate the physiological attributes of each strain and recorded on computer cards was suggested by Cowan (1962). Alternatively, reference strains could be selected using defined physiological variants of a given biotype and the taxonomic distance or percentage similarity of unknown strains could be calculated from such "indicators". In a study of this nature this could be the best solution even if the "species problem" as such remains unsolved.

CHAPTER X

DISCUSSION OF ECOLOGICAL DATA

The physiological potentialities of clostridia occurring on malt and the environmental factors in various stages of the brewing process were investigated and have been discussed in preceding chapters. On the whole, it can be said that the environmental conditions during the brewing process and in the beer are not favourable for the growth of clostridia. Nevertheless, they were found to occur in an instance, in a disused souring tank and have been recorded by Klein (1908) and van der Walt (1956) in Bantu Beer. Their physiological potentialities and the degree to which these have been met by the environment will be briefly discussed here.

Clostridia were shown to be practically absent on kaffir corn, yet occurred in relatively large numbers on most malts. It is probable that they multiply during the malting process, where the rapid respiration of the germinating kaffir corn may create sufficiently anaerobic conditions. The relatively high malting temperature of 30°C falls well within the range of growth of clostridia occurring on malt.

pH

In a normal, well-soured beer the low pH prevents the clostridia from growing. Nevertheless, there is one report of clostridia occurring in spoilt beer by van der Walt (1956). Unfortunately, the pH of the sample investigated is not given. The author observed "motile clostridia" microscopically and isolated clostridia by enrichment techniques. Pure cultures were identified as Cl. butyricum. The beer must have had an abnormally high pH which would have made the growth of clostridia possible. Alternatively, the results could be explained by the growth of spores normally present in beer in the enrichment medium. If this were the case, the motile cells observed could possibly have been bacilli.

Spoilt beer samples were frequently brought to the laboratory for investigation by the present writer. Clostridia could never be detected microscopically.

In Chapter VI it was found that a few strains could grow at pH<sup>4.0</sup> after incubating for 1 day. Such strains could possibly proliferate in the initial stages of spontaneous sourings where a relatively high pH is maintained for a long period, especially if no thermobacteria develop. In spontaneous sourings other factors seem to be unfavourable, as the growth of clostridia could never be recorded. From Table XVIII it can be seen that growth at low pH is very seldom linked to a high maximum

growth temperature. This is a probable explanation for their failure to develop in spontaneous sourings.

In the pH stat a favourable pH is maintained for even longer periods than in spontaneous sourings. Here additional factors, such as the high lactate content of the medium could restrict the growth of clostridia. Antagonistic effects between clostridia and lactobacilli have been described by Gibson et al. (1961). The authors found that the growth of clostridia in silage was erratic and unpredictable even if a relatively high number of spores could be demonstrated initially.

Henneberg (1909 and 1926) whose valuable contribution to the knowledge of industrial fermentations must be acknowledged, observed that clostridia multiplied in grain washes only sporadically and that more vigorous butyric fermentations occurred if the culture was contaminated by bacilli. When clostridia were associated with lactobacilli, restrictive influences on their development were observed, but in the presence of bacilli an enhancement of growth of the clostridia could be recorded.

#### Temperature.

From the studies of maximum and minimum temperatures for growth it became evident that the strains could not be grouped into thermophilic and mesophilic groups. The maximum temperature of most strains was slightly below the souring temperature. Most proteolytic strains had a maximum within the temperatures of souring but, as these are rarely found in beers, they cannot be considered as a major factor of possible interference in the souring process. Proteolytic clostridia occur in large numbers on bran. Bran, however, is only used by one brewery (Brewery C).

#### Time.

If the limit of growth of clostridia is taken as pH 4.0 where the growth of some strains could be recorded after only 1 day, it becomes evident that their growth in spontaneous sourings is unlikely to occur, as these pH values are reached after a relatively short time of 13 hours in a slow souring and 7½ hours in a quick souring. See Fig. 3 and Fig. 5. It is, however, difficult to compare spontaneous sourings at 50°C with growth at different pH values at 37°C. It may well be that an unfavourable pH coupled with an unfavourable growth temperature near the maximal limit tolerated by the organisms would result in no growth at all; whereas some growth may be recorded when anyone of the two factors mentioned is near the optimum.

### Nutritional requirements and redox potential.

Nutritional requirements of clostridia were shown to be met by the malt mash, as sterile mashes and sourings supported the growth of clostridia. This could be expected, as the more complex growth requirements of lactobacilli are met by the same medium. Carbon sources do not limit the growth of the mixed population in spontaneous sourings. The production of glucose by the malt amylases exceeds the rate of utilisation of this sugar by the population. If however, a malt of low diastatic power is used, conditions would become more favourable for starch fermenting clostridia. The competition of thermobacteria and pediococci would virtually be eliminated, as these do not ferment starch to any extent. Only the starch fermenting Bacillus coagulans would be able to grow in such environmental conditions; and, it was pointed out earlier, that the growth of this organism may enhance the growth of clostridia.

Redox potentials, as far as could be ascertained, fall within the limits required for the growth of clostridia. It was pointed out in Chapter VI that the interpretation of such data must be done with caution.

### General.

From the above discussion it would appear that the conditions of beer brewing embrace the range of tolerance of clostridia, although these conditions are not optimal for their growth. In Chapter II the milieu was defined as the integration of environmental factors. It is impossible to study such an integration of environmental factors in the laboratory in a single experiment, without creating exceedingly complex experimental conditions. In the present study only single factors were studied.

When studying the minimal pH for growth and maximum growth temperatures, it would have been important to choose a medium bearing a close chemical resemblance to the natural environment, i.e. a Sorghum malt mash. Unfortunately, this approach had to be abandoned, as malt mashes vary considerably from batch to batch. In order to compare results of different strains tested in the computer study a semi-defined medium was used in order to maintain standard conditions.

Furthermore, it must be pointed out that the study of isolated factors in the laboratory need not bear a strict relationship to the natural environment where different factors vary independently of each other. Thus it may be found that the maximum temperature for growth of a certain strain may be different at an optimal pH of 7.0 and at a sub-optimal pH of 5.0. This may explain the behaviour of clostridia in Bantu beer, where not any one factor prevents the growth of clostridia,

but a combination of sub-optimal conditions. It is this combination or integration of sub-optimal factors that prevents the proliferation of clostridia. In fact, it is the "milieu" that is unfavourable for "active-life". It is for this reason that their numbers remain constant in the form of viable spores (so-called "latent-life").

Although it is not unlikely that clostridia cause spoilage of home-brewed beer and did so in factories with poorly controlled brewing procedures as described by Klein (1908) earlier this century; spoilage by clostridia does not occur in modern breweries, even in cases where the brewing process is not very well controlled.



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