THE TAXONOMY AND PHYSIOLOGY OF THE LACTIC ACID BACTERIA IN SOUTH AFRICAN DRY WINES.

by

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Thesis accepted for the Degree of

DOCTOR OF SCIENCE
(MICROBIOLOGY)

at the University of Stellenbosch.

STELLENBOSCH.
September, 1961.

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CONTENTS.

INTRODUCTION.

It has been known since the inception of the science of microbiology that wines can be infected by bacteria.

In 1873 Pasteur (according to Cruess, 1943), by his famous treatise "Études sur le vin", proved that certain of the European wine diseases such as "pousse" (gaseous lactic spoilage and "tourne" (non-gaseous lactic spoilage) are caused by filamentous, rod-shaped bacteria. Pasteur, however, reportedly did not isolate and study the causative organisms. Experimenting with pure cultures of the wine bacteria began in the eighties only.

Inspired by the first great successes achieved in medical bacteriology Müller-Thurgau, Koch, Möslinger, Seifert (according to Vaughn and Tchelistcheff, 1957) and their contemporaries, endeavoured to elucidate the phenomenon of l-malic acid degradation in wines. Their investigations revealed a secondary bacterial fermentation of malic acid, yielding lactic acid and carbon dioxide as major end-products (malo-lactic fermentation). The beneficial effect of malo-lactic fermentation on the relatively acid wines of Germany and Switzerland was then rapidly realised.

It thus appears that, depending on the bacterial strain(s) and the chemical composition of a wine, the secondary development of lactic acid bacteria may have either a detrimental (lactic spoilage) or beneficial (malo-lactic fermentation) effect on wine quality.

During...

During the past decade the lactic acid bacteria occurring in wines have been subjected to extensive research. These bacteria have now also been found in the wines of France, Portugal, Spain, Italy, Algeria, California, Australia and the Argentine.

In South Africa knowledge of the wine bacteria is limited to reports by Fevrier (1926) and Niehaus (1932) on the symptoms of bacterial spoilage of fortified wines. The results of Niehaus (1932) suggested that the spoilage organisms are of the mannitol-producing, lactic acid type (i.e. "mannitic bacteria"). While it is generally assumed that the bacteria known to occur in South African dry wines are also of this type, no research has been undertaken in this field. It is therefore not known whether all of these wine bacteria should be considered spoilage organisms, or if some of them possess beneficial characteristics such as the ability to affect malo-lactic fermentation.

This study was consequently undertaken to establish basic facts concerning the taxonomy, incidence, nutritional requirements and biochemical activities of the lactic acid bacteria in South African dry wines.

MATERIALS AND METHODS.

Materials.

Four hundred and fifty samples of bottled dry wines (containing 10 to 14 volume per cent ethyl alcohol) were collected at random from wineries, co-operative cellars and depots, representative of the entire South African wine-producing region. These wines could be grouped into three categories:-

(1) Cheap, popular drinking wines (constituting 90 per cent of the samples).

(2) Wines produced primarily for distilling purposes or rebate wines (constituting 5 per cent of the samples).

(3) The more expensive table wines.

As the latter wines are usually aged for some time before being marketed, great care is exercised in their production and microbiological stabilisation. Consequently only a very small percentage of these wines exhibited signs of microbial infection. The wines of the first and second categories, on the other hand, usually receive little or no cellar manipulation. It was therefore not surprising that many of the wines in the first category, and most of those in the second, contained a flocculent sediment or exhibited a definite turbidity indicative of secondary microbial activity.

In addition, eight strains of Gram-positive, catalase-negative, rod-shaped bacteria were received from Dr. J.P. van der Walt, C.S.I.R., Pretoria. All these strains emanated from "off-flavour" rebate wines.

Methods...

Methods.

Media:

Yeast autolysate: Prepared by a modification of the Fornachon (1943) method.

Four hundred and fifty grams of bakers yeast were added to 600 ml. quantities of sterile water, each containing 0.2 gm. magnesium sulphate, 0.1 gm. ammonium chloride, 1.0 gm. potassium dihydrogen phosphate and 15 ml. chloroform. The flasks were plugged with cotton-wool and sealed with thick paper to counter evaporation. After eight days at 45°C, to permit autolysis, the flasks were steamed for 30 minutes in order to coagulate protein. The contents were then filtered, using Theorite No. 5 filter-aid in a Buchner funnel. The clear autolysate was diluted by addition of an equal volume of distilled water to which one ml. of Tween 80 had been added.

This medium contained readily detectable amounts of α-alanine, arginine, lysine, aspartic acid, asparagine, proline, amino-butyric acid, histidine, serine, threonine, methionine, valine, tyrosine, leucine, iso-leucine, phenylalanine, glutamic acid, tryptophane and small amounts of glycine. The final medium invariably had a pH of 5.4 to 5.5 and was sterilised by steaming for 30 minutes.

Yeast autolysate glucose broth: This broth contained 2 per cent (w/v) glucose and was solidified by addition of 2 per cent (w/v) agar.

The selective medium for the isolation and enumeration of lactic acid bacteria: As developed and described by Rogosa, Mitchell and Wiseman (1951).

Gelatin...

Gelatin liquefication: Agar (3% w/v), glucose (0.25% w/v) and gelatin (o.4% w/v) were added to yeast autolysate.

Litmus milk: Bacto litmus milk was employed.

Gas production from glucose: The medium contained 0.4 per cent (w/v) agar and 5 per cent (w/v) glucose in yeast autolysate.

Gas production from malate, citrate and tartrate: Agar 0.4 per cent (w/v) and 0.5 per cent (w/v) organic acid in yeast autolysate constituted the basal medium for these tests. The tests were conducted at pH levels of 3.5, 4.0 and 4.5, obtained by addition of ION potassium hydroxide. In cases where no gas production was observed, the fermentation tests were repeated in this medium without agar.

Catalase activity: Catalase tests were conducted with streak cultures on yeast autolysate glucose agar. As certain cocci give positive catalase tests only on media containing small amounts of utilisable carbohydrate (Felton, Evans and Niven, 1953), catalase production by the isolated cocci was also tested on the appropriate medium of Felton et al. (1953).

Ammonia production from arginine: Glucose (5% w/v) and arginine (0.3% w/v) were added to yeast autolysate.

Nitrate reduction: The medium contained 0.1 per cent (w/v) potassium nitrate and 0.1 per cent (w/v) glucose in yeast autolysate.

Slime production: Yeast autolysate containing 5 per cent (w/v) sucrose was employed.

Oxygen...

Oxygen relationship: In order to obtain a semi-solid medium 0.4 per cent (w/v) agar was added to yeast autolysate glucose broth.

Complete synthetic medium (C.S.-medium): This medium was in some ways similar to that used by Dunn, Shankman, Camien and Block (1947) and had the following composition per 100 ml.:-

Glucose	4.0 gm.
Ammonium chloride	0.6 gm.
Sodium chloride	0.035 gm.
Sodium acetate	1.2 gm.
K H_2PO_4	0.05 gm.
K_2HPO_4	0.05 gm.
$MgSO_4.7H_2O$	0.02 gm.
$FeSO_4.7H_2O$	0.001 gm.
$MnSO_4.4H_2O$	0.001 gm.
Adenine	0.002 gm.
Guanine	0.002 gm.
Uracil	0.002 gm.
Xanthine	0.002 gm.
Thiamine.HCl.	0.1 mg.
Pyridoxine	0.16 mg.
dl-Ca-pantothenate	0.2 mg.
Nicotinic acid	0.2 mg.
Riboflavin	0.2 mg.
Biotin	0.0005 mg.
p-Amino benzoic acid	0.01 mg.
Folic acid	0.0005 mg.
Choline chloride	1.0 mg.
Inositol	2.5 mg.
Vitamin B_{12}	0.1 µg.

Tween 80...

Tween 80	0.2 ml.
l-Malic acid	5.0 mg.
dl α -alanine	20 mg.
Asparagine (natural)	"
l(+)-Arginine.HCl	"
l(-)-Cysteine.HCl	"
l(+)-Glutamic acid	"
Glycine	"
l(-)-Histidine.HCl.H_2O	"
dl-Isoleucine	"
l(-)-Leucine	"
dl-Lysine.HCl.	"
dl-Methionine	"
dl-Phenylalanine	"
l(-)-Proline	"
dl-Serine	"
dl-Threonine	"
dl-Tryptophane	"
l(-)-Tyrosine	"
dl-Valine	"
α-Amino butyric acid	"
dl-Aspartic acid	"

Final pH 5.4 (with conc. HCl).

The medium was distributed in eight ml. quantities in 1.2 x 15 cm. test tubes and sterilised for five minutes at 120°C.

l-Malic acid was incorporated into the medium because of the finding of Skeggs, Nepple, Valentik, Huff and Wright (1950) that it improves the response of L. leichmannii (4797) to vitamin B_{12}.

Isolation...

Isolation:

All samples were examined microscopically. If this examination revealed the presence of bacterial cells, isolation was attempted by application of the method of Fornachon (1943). The medium concerned was modified to contain fructose (0.5% w/v), xylose (0.1% w/v), arabinose (0.1% w/v), as well as glucose (1% w/v). In the cases where no isolates were obtained by this method, circa one ml. of wine, drawn aseptically, was inoculated into a liquid medium allowing selective growth of lactic acid bacteria. If, after incubation for one month at 30°C, no growth had been observed in this medium, it was assumed that no viable cells of lactic acid bacteria were present in the particular samples. However, in all instances where isolation by means of the Fornachon (1943) method failed, no growth occurred in the selective medium.

The younger, recently-infected wines readily yielded bacterial isolates by the above-mentioned method. As experienced by Fornachon (1943), most of the older infected wines were in an advanced stage of spoilage, containing a sediment of non-viable bacterial cells. The non-viability of bacterial cells from these older wines was confirmed by the fact that such cells could be directly stained by Henrici's method (Tanner, 1948).

Subsequent to their purification all isolates were maintained in yeast autolysate glucose broth containing one per cent (w/v) calcium carbonate and transferred monthly.

According to their morphology and the Gram-reaction, some of the isolates appeared to be acetic

acid...

acid bacteria. As the Gram-stain did not suffice as an absolute criterion for distinguishing between lactic acid bacteria and acetic acid bacteria the following procedure was employed:

The isolates were cultured in yeast autolysate glucose broth for three weeks at 30°C, subsequently centrifuged and the culture supernatants analysed for lactic acid by means of paper chromatography.

The differentiation of species:

The isolates were differentiated by means of the following criteria.

Morphology: (1) Gram stain: Hucker's modification (Tanner, 1948).

(2) Cell size and arrangement was measured one week after inoculation in yeast autolysate glucose broth. Due to considerable variation in the growth rate of the different isolates, the determination of cell morphology at a younger stage was ignored.

(3) Motility: hanging drop preparations and Leifson's stain (Manual of Microbiological Methods, 1957).

(4) Spore production: tested by the method of Neisser (Tanner, 1948).

(5) Capsule production: Muir's method (Tanner, 1948).

Cultural characteristics: (1) Growth in yeast autolysate glucose broth.

(2) Growth on yeast autolysate glucose agar slants.

(3) Growth

(3) Growth in yeast autolysate glucose agar (colony type).

(4) Gelatin liquefication.

(5) Growth in litmus milk.

Growth requirements: (1) Oxygen relationship (Manual of Microbiological Methods, 1957).

(2) Optimum temperature, as well as growth at 10°C, 35°C, 40°C and 45°C.

(3) Sodium chloride tolerance in yeast autolysate glucose broth.

(4) Heat survival (Briggs, 1953).

Biochemical characteristics: (1) Gas production from glucose employing the agar-closure technique of Gibson and Abdel Malek (1945).

(2) Optical rotation of the lactic acid produced.

(3) Production of ammonia from arginine using the method of Briggs (1953).

(4) Catalase activity (Manual of Microbiological Methods, 1957).

(5) Nitrate reduction (Tanner, 1948).

Fermentation: (i) Carbohydrate fermentation:-

Fermentation tests were conducted on the following substrates at a concentration of 2 per cent (w/v) in yeast autolysate: L-arabinose, D-xylose, L-rhamnose, D-glucose, D-fructose, D-galactose, D-mannose, D-sucrose, D-maltose, D-lactose, D-melibiose, D-cellobiose, D-melecitose, D-raffinose, D-trehalose, D-mannitol, D-sorbitol, glycerol, inositol, dulcitol, arbutin, salicin, amygdalin, α-methyl-D-glucoside, starch, inulin and dextrin.

Aqueous...

Aqueous solutions of each of these substrates were sterilised and added to an equal volume of sterile, undiluted yeast autolysate immediately before inoculation. Prelimenary tests showed that arabinose, xylose, fructose and maltose could be sterilised without partial destruction only by means of filtration. These sugars were consequently sterile-filtered, through sintered-glass filters.

After the pH of each medium had been measured, using a control tube, the media were inoculated and cultured for sixteen to eighteen days at the optimum temperatures. The pH of each culture was then determined, using a pH-meter and compared with that of the control. A decrease of 0.1 - 0.9 in pH was considered to indicate a weak fermentation, 0.9 - 1.9 a moderate fermentation and a decrease of more than 1.9 indicative of a vigorous fermentation.

(ii) Organic acid fermentation:-

Carbon dioxide production from l-malate, citrate and tartrate was detected by the technique of Gibson and Abdel Malek (1945). As this method may give false negative readings (Keddie, 1959), the results were verified by qualitatively analysing for lactic acid in all cases where no gas production was observed.

In cases where gas production occurred within five days after heavy inoculation by pipette, the fermentation was considered to be vigorous, otherwise the fermentation was considered moderate.

The production of mannitol from fructose: This characteristic served, in conjunction with the gas-production tests, to differentiate between homo- and heterofermentative strains.

The...

The cultures to be tested were inoculated into yeast autolysate containing 0.5 per cent (w/v) fructose. After 20 days at optimum temperatures the cultures were centrifuged, the supernatants evaporated to dryness and the residues extracted with ethyl alcohol. These alcoholic extracts were concentrated and analysed for mannitol.

Slime production: This was investigated on sucrose medium.

The determination of nutritional requirements:

Amino acid and vitamin requirements of the bacterial isolates were assessed by comparing the growth of a specific strain in the complete synthetic medium (C.S.-medium) with that in the synthetic medium from which a specific amino acid or vitamin had been omitted. The media were inoculated with loopfuls (diameter 2 mm.) of three day-old cells (grown in yeast autolysate glucose broth), which had been washed three times with sterile water and suspended in sterile saline solution. The volume of the saline solution used was equal in volume to that of the medium in which the cells had been grown. All cultures were incubated at their optimum temperature until good growth had occurred in the C.S.-medium. Total growths of the cultures were estimated turbimetrically, using a Coleman model 7 photo-nephelometer. The growth in the C.S.-medium was designated as 100 per cent and the growth in the absence of a specific nutritive substance calculated as a percentage of the growth in the C.S.-medium.

If the omission of a nutritive substance induced a growth reduction of 30 per cent or less, the

substance...

substance was considered to be non-essential. When the omission led to a growth reduction of 30 to 69 per cent, the substance was regarded as stimulatory, while substances which caused a growth reduction of 70 per cent or more were termed essential.

Manometric methods:

Oxygen consumption and carbon dioxide evolution was measured at 30°C, using conventional manometric techniques (Umbreit, Burris and Stauffer, 1957). The total volume of liquid in each Warburg vessel was 3.75 ml., made up as follows: 3.0 ml. of a solution of the sugar (1.0% w/v) or organic acid (0.5% w/v) in yeast autolysate, 0.25 ml. of sterile water or 20 per cent potassium hydroxide solution in the centre cup, and 0.5 ml. of a washed cell suspension in the side-arm.

As certain of the organisms proved to be extremely inactive towards the sugars tested, inoculation with dense cell suspensions was necessary to ensure measureable reactions. Due to the absorption of light by such dense cell suspensions, accurate nephelometric standardisation of the different inocula was extremely difficult. Consequently the inocula of any two organisms were only of roughly equal density.

In the cases where gas production was determined under nitrogen or under carbon dioxide, the air in the Warburg vessel was replaced by the required gas for at least five minutes before immersing the vessel in the water bath.

The...

The conducting of large-scale fermentations:

To ensure sufficient amounts of culture fluid for determining the metabolic products of bacterial fermentation, 150 ml. of each medium were used. The basal medium (75 ml. undiluted yeast autolysate plus 60 ml. distilled water) was added to 3.5 x 20 cm. test tubes of 200 ml. capacity and steam sterilised. Ten ml. quantities of sterile-filtered carbohydrate or organic acid solution were then added aseptically. The organic acids were added as their potassium salt buffers to obtain the desired final pH values. Before inoculation the oxygen in the medium was removed by scrubbing with sterile nitrogen for five minutes. Washed cells from a four day-old culture were suspended in enough sterile water to give, after inoculation, a final volume of 150 ml. medium. Subsequent to seeding, nitrogen was again passed through the medium for two to three minutes and the tube then connected to a gas-analysis train. The gas train consisted of three U-tubes, the first of which contained anhydrous magnesium perchlorate (Anhydrone) and served as a drying tube. Carbon dioxide was absorbed in the second tube, containing an amount of Ascarite of known weight. The third tube also contained Ascarite to ensure that no carbon dioxide entered the gas train from the open end. After sixteen to eighteen days at optimum temperature, nitrogen was again bubbled through the fermentation solution. Since the nitrogen now also passed through the gas train it was ensured that all of the carbon dioxide was displaced from the medium and absorbed in the second tube. The medium was next deproteinised, clarified (Neish, 1952) and made

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to a volume of 250 ml. Determination of the non-gaseous products of fermentation was conducted on these solutions.

Methods of analysis:

Optical rotation of the lactic acid produced: Each strain was cultured for two to three weeks in 200 ml. yeast autolysate which contained 5 per cent (w/v) glucose and 3 per cent (w/v) calcium carbonate. After fermentation the culture fluids were heated to 60°C to dissolve calcium lactate, and filtered. Organic acids present in the filtrates were then liberated by acidification to pH 2. Volatile acids were removed by distillation, the acid residues concentrated to a small volume on a water bath (at 50°C) and subjected to ether extraction. The ether extracts were allowed to evaporate at room temperature and the residues dissolved in 20 ml. distilled water each. These aqueous solutions were heated on a water bath to 80°C and small quantities of zinc carbonate added until an excess had been attained. After removal of the excess zinc carbonate the filtrates were again concentrated on a water bath at 50 to 60°C. As soon as crystallisation of zinc lactate could be perceived, ethyl alcohol was added to a concentration of 50 volume per cent. These alcoholic solutions were allowed to stand overnight and the zinc lactate crystals subsequently removed by filtration, employing sintered-glass filters. The crystals were washed with alcohol and dried in a desiccator to constant weight. Water of crystallisation of the zinc lactate was determined by ascertaining the loss of weight after two days at 160°C. Inactive zinc lactate contains 18.2 per cent

water...

water and active zinc lactate 12.9 per cent (Jörgensen, 1956). These results were verified by determining the optical rotation of the lactates by means of a polarimeter.

Qualitative methods: (1) Lactic acid:-

The presence of lactic acid was established by descending chromatography on Whatman No. 1 paper, employing n-butanol : formic acid : water (8 : 1 : 5) as solvent system. After drying at 30°C for one to two hours the papers were sprayed with a solution of bromo-phenol blue (0.04% w/v) in 95 volume per cent ethyl alcohol. The pH of the indicator solution was brought to 6.0 to 6.5 with 0.1N sodium hydroxide before spraying. Organic acids appeared as yellow spots on a blue background.

(2) Mannitol:-

This polihydric alcohol was determined by means of descending chromatography on Whatman No. 1 paper, n-butanol : acetic acid : water (5 : 1 : 2) serving as solvent system. The chromatograms were subsequently dried, sprayed with a 5 per cent solution of silver nitrate containing excess ammonia (S.G. 0.88) and heated to enhance the colour reaction (Hough, 1950).

(3) Amino acids:-

The amino acids contained in 20 ml. of yeast autolysate medium were partially freed from interfering substances by adsorbtion on Dowex 50 W (H) x 8 resin and subsequent elution with 5 N Ammonium hydroxide, as described by du Plessis (1960). After concentration of

the ...

the eluent fraction a suitable amount was spotted on Whatman 3 MM paper and subjected to two-dimensional chromatography. Butanone : propionic acid : water (15 : 5 : 6) (Clayton and Strong, 1954) and n-butanol : acetone : water : dicyclohexylamine (10 : 10 : 5 : 2) (Hardy, Holland and Naylor, 1955) served as first and second dimensional solvents respectively. The chromatograms were dried at 75°C and sprayed with a solution of ninhydrin (0.25%) in 95 volume per cent ethyl alcohol containing 7 per cent acetic acid (du Plessis, 1960).

Quantitative methods: (1) Analytical procedures as described by Neish (1952) were employed in the following determinations:-

Carbon dioxide: by absorption on Ascarite.

Residual sugar: by the Anthrone and/or copper reduction method.

Glycerol: by colorimetric determination of the formaldehyde formed on periodate oxidation, after separation by partition chromatography on a Celite 535 column.

Fermentation acids: by partition chromatography on a silica gel column.

2,3-Butanediol: by colorimetric determination of acetaldehyde formed on periodate oxidation, after separation by partition chromatography on a Celite 535 column.

Ethyl alcohol: through oxidation by acid dichromate, followed by measurement of the excess dichromate.

Acetoin...

Acetoin plus diacetyl: colorimetrically with an equal mixture of creatine and alkaline 1-naphtol.

Diacetyl: colorimetrically with hydroxylamine hydrochloride in the presence of urea in strong acid medium.

(2) Mannitol:-

No suitable method for the determination of mannitol in the presence of glycerol and residual fructose could be found. Methods devising the crystallisation of mannitol from a definite volume of fermentation solution (Coyne and Raistrick, 1931, Gayon and Dubourg, 1894, 1901) were found to be too inaccurate. The following indirect method was thus developed.

Total polihydroxy alcohols, mannitol and glycerol, were determined colorimetrically as glycerol, by the periodate oxidation method (Neish, 1952), a correction being applied for the residual fructose. Glycerol was then separated from interfering substances by partition chromatography and determined, as already described. The difference between these two values thus represents the amount of mannitol present, expressed as glycerol. In order to ascertain the mannitol content, the difference between the two values was multiplied by 1.98. This is warranted since one gram of glycerol gives the same amount of formaldehyde on periodate oxidation as 1.98 grams of mannitol.

(3) l-Malic acid:-

After the last of the fermentation acids (lactic acid) had been eluted from the chromatographic

column according to the Neish (1952) method, the packing was flushed with 20 ml. chloroform and the residual malic acid eluted with 75 ml. of 50 volume per cent n-butanol in chloroform, saturated with 0.01 N hydrochloric acid.

Elution of the residual malic acid was first attempted with 50 volume per cent n-butanol in benzene. Considerably larger amounts of butanol/benzene, which impeded accurate titration of the eluent solutions, were then necessary for quantitative elution.

(4) Citric acid:-

As in the case of l-malic acid, and for the same reason, a n-butanol-chloroform mixture was preferred as eluent for residual citric acid. As less tailing occurred when the eluents were saturated with a stronger acid, 0.02 N Sulphuric acid was used. This solvent system could not be used for the chromatographic determination of the fermentation acids as formic acid is known to decompose in the presence of strong acid (Neish, 1949). Consequently the residual citric acid was determined on a different column.

Six grams of silica gel (Ramsey and Patterson) were well mixed with 3.0 ml. of 0.04N sulfuric acid, slurried in chloroform and packed into a column 1.8 cm. in diameter and containing a sintered-glass disc to support the packing. The silica gel was compressed into a column about 4 cm. long by placing a thick filter paper disc on top of it and then ramming it down with a glass plunger. A 0.5 gm. sample of dry silica was slurried in chloroform and packed on top of the wet

silica...

silica, using another filter paper disc. The sample to be analysed was treated and brought on the column as described by Neish (1952). After the fermentation acids had been eluted with 75 ml. of 35 volume per cent n-butanol in chloroform, the residual citric acid was eluted with 90 ml. of 50 volume per cent n-butanol in chloroform and determined by titration, under nitrogen, with 0.01N sodium hydroxide.

THE OCCURRENCE OF LACTIC ACID BACTERIA IN WINE.

It became apparent from the exact observations of Pasteur (according to Cruess, 1943) that certain of the wine bacteria are of the lactic acid type.

With the advent of pure culture methods the wine bacteria were subjected to intensive research. The information which came to light from these investigations facilitated the determination of the taxonomic relationship of these organisms.

Several morphologically different bacteria were encountered by the earlier investigators; Kramer found a bacillus, Boersch a sarcina and Aderhold a diplococcus (according to Cruess, 1943). In a series of papers Gayon and Dubourg (1894, 1901) reported the isolation of a threadlike mannite-producing organism from wine. A mannite-forming organism isolated by Mazé and Perrier (1903) was reported to resemble closely the "mannitic ferment" of Gayon and Dubourg (1894, 1901). When Seifert (according to Vaughn and Tchelistcheff, 1957) described the malic acid degrading Micrococcus malolacticus the important role of the lactic acid producing cocci in European wine-making became evident.

After isolating and studying a great number of bacteria from both sound and spoiled wines, Müller-Thurgau and Osterwalder (1913) reported on the characteristics of four new species; Bacterium mannitopoeum, B. gracile, Micrococcus variococcus and M. acidovorax. In later papers these investigators described three other

organisms...

organisms; B. intermedium from Swiss red wine and B. gayoni from Algerian wine (1918), and the tartrate-fermenting B. tartarophthorum (1919).

The work of Arena (according to Vaughn and Tchelistcheff, 1957) yielded two more hitherto unnamed species; B. acidovorax and M. multivorax.

Berry and Vaughn (1952) isolated from Californian wine tartrate-decomposing bacteria; subsequently identified as strains of Lactobacillus plantarum.

According to Luthi (1957) certain streptococci are involved in bringing about the malady known as "ropiness" of wine. Streptococci were also isolated from wine by Hochstrasser (according to Vaughn and Tchelistcheff, 1957), who described S. malolacticus and S. malolacticus var. mucilaginosus.

It is known that, apart from several species of Lactobacillus, the heterofermentative cocci Leuconostoc mesenteroides and L. dextranicum are also to be found in the wines of California, (Vaughn, 1955).

Fornachon's (1957) investigations on malo-lactic fermentation revealed that organisms resembling descriptions of Lactobacillus hilgardii, L. brevis, L. buchneri and L. fermenti occur frequently in Australian dry wines. A coccus resembling M. variococcus (Müller-Thurgau and Osterwalder, 1913) was also encountered.

Recently Ingraham, Vaughn and Cooke (1960), continuing the study of the bacteria in Californian wines, isolated a considerable number of both homo- and heterofermentative bacilli as well as cocci.

It...

It is evident that the lactic acid producing rods encountered by the earlier investigators were placed in the genus Bacterium. Most of these organisms are synonymous with species of the genus Lactobacillus, or closely related genera.

Charlton, Nelson and Werkman (1934), on the strength of morphological evidence, concluded that B. gracile is coccoid and therefore belongs to the genus Leuconostoc. This opinion was shared by Pederson (1938) and recently conceded by Peynaud (1955).

Bacterium gayoni and B. intermedium are, according to Breed, Murray and Hitchens (1948), both synonymous with L. fermenti. Vaughn (1955) suggested that B. acidovorax is a synonym for L. plantarum. The possibility that B. tartarophthorum may also be a lactobacillus was considered but its present status is still in doubt.

Breed et al (1948) considered Micrococcus acidovorax synonymous with M. luteus. However, as emphasised by Vaughn (1955), the taxonomic position of the wine cocci is still obscure.

The taxonomy and incidence of lactic acid bacteria in South African dry wines.

In order to establish the taxonomy and incidence of lactic acid bacteria in South African dry wines, the said number of samples were analysed microbiologically. This analysis yielded 64 lactic acid producing bacterial strains which could tentatively be divided into two main groups:

1. Homofermentative...

1. Homofermentative strains. (No gas from glucose, fructose not reduced to mannitol).
2. Heterofermentative strains. (Gas produced from glucose, fructose reduced to mannitol).

On account of their morphology, optimum temperature and fermentation reactions, etc., the strains of group 1 could be subdivided into three types and those of group 2 into four types.

As the literature concerning the taxonomy of the lactic acid bacteria lacks agreement and completeness in many respects, thus impeding the differentiation and identification of species, a full description of the characteristics of the isolated strains is given below.

1. The homofermentative group:

(i) Type I (17 cultures):

Morphology:- Rods, 0.2 µ - 0.5 µ x 0.8 - 4 µ, occurring singly, in pairs and in chains which may measure 22.5 µ or longer. Non-motile, Gram positive, non-capsulated, non-sporeforming.

Figure 1: Cells of a homofermentative Type I culture after one week in yeast autolysate glucose broth (x 1800).

Yeast...

Yeast autolysate glucose agar colonies:- Surface colonies white, circular, smooth with entire margins and slightly convex elevation. Subsurface colonies mostly spindle-shaped.

Yeast autolysate glucose agar slant:- Visible growth after two to three days incubation. Growth limited, effuse. Streaks exhibit greyish-white colour.

Yeast autolysate glucose broth:- The medium becomes uniformly turbid after 24 to 48 hours incubation and exhibits a pronounced silky waviness when shaken gently.

Growth on sucrose medium:- No slime production.

Litmus milk:- Some strains produce small amounts of acid.

Catalase activity:- Negative.

Fermentation:- Vigorous acid production from glucose, fructose, galactose, mannose and trehalose. Moderate acid production from maltose, sucrose, cellobiose, mannitol, sorbitol, arbutin, salicin, amygdalin and α-methyl glucoside. Slight acid production from glycerol. Most strains also produce moderate amounts of acid from melecitose. No acid from xylose, arabinose, rhamnose, lactose, melibiose, raffinose, inositol, dulcitol, starch, inulin and dextrin.

Type of lactic acid produced:- Laevo-rotary.

Reduction of nitrate:- Negative.

Production of ammonia from arginine:- Most strains give weak positive tests.

Oxygen...

Oxygen relationship:- Microaerophilic.

Temperature relations:- Optimum temperature, 35 - 37°C. Growth at 10°, 45° and 48°C.

Salt tolerance:- Good growth in 4 per cent (w/v) sodium chloride, most strains exhibit weak growth at 6 per cent.

Heat survival:- Does not survive 60°C for ninety minutes.

Taxonomic considerations:- If optimum temperature and pentose fermentation, which according to Pederson (1938) are the most important differential characteristics, are considered, these organisms agree with the description of Lactobacillus leichmannii Bergey et al, 1925 (see Breed, Murray and Smith, 1957). This view is further substantiated by the finding of Rogosa, Wiseman, Mitchell, Disraely and Beaman (1953) that L. leichmannii produces laevo-rotary lactic acid. Although none of the strains of L. leichmannii studied by Rogosa et al (1953) could ferment galactose, the original strain described by Henneberg (1903) could bring about a weak galactose fermentation. The production of ammonia from arginine is also characteristic of L. leichmannii (Rogosa and Sharp, 1959).

(ii) Type II (Five cultures):

Morphology:- Rods, 0.2 - 0.6 µ x 0.6 - 4 µ, occurring singly, in pairs or chains which may measure 20 µ or longer. Non-motile, Gram-positive, non-capsulated, non-sporeforming.

Figure 2...

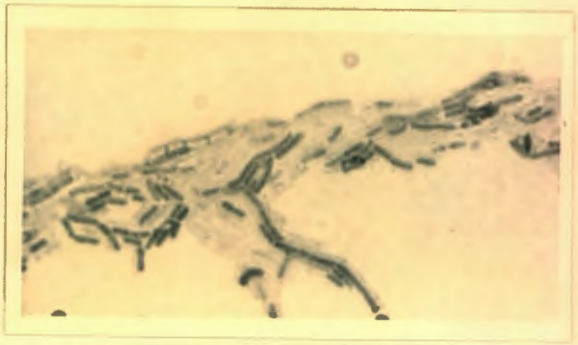


Figure 2: Cells of a homofermentative Type II culture after one week in yeast autolysate glucose broth (x 1800).

Yeast autolysate glucose agar colonies:- Surface colonies white, circular, smooth with entire margins and slightly convex elevation. Subsurface colonies mostly spindle-shaped or irregular.

Yeast autolysate glucose agar slant:- Visible growth after two to three days incubation. Growth limited, effuse. Streak whitish to grey.

Yeast autolysate glucose broth:- Uniform turbidity after 24 to 48 hours incubation and shows pronounced silky waviness when shaken gently.

Growth on sucrose medium:- No slime production.

Litmus milk:- Slightly acid.

Catalase activity:- Negative.

Fermentation:- Vigorous acid production from glucose, fructose, galactose, mannose, trehalose and salicin. Moderate acid production from xylose, maltose, sucrose, cellobiose, melecitose, mannitol, sorbitol, arbutin, amygdalin and α-methyl glucoside.

Slight...

Slight acid production from glycerol. No acid from arabinose, rhamnose, lactose, melibiose, raffinose, inositol, dulcitol, starch, inulin or dextrin.

Type of lactic acid produced:- Laevo-rotary.

Reduction of nitrate:- Negative.

Production of ammonia from arginine:- Weakly positive.

Oxygen relationship:- Microaerophilic.

Temperature relations:- Optimum temperature 33 - 37°C. Growth at 10°C, 45° and 48°C.

Salt tolerance:- All strains exhibit good growth in 4 per cent (w/v) sodium chloride, weak growth at 6 per cent.

Heat survival:- No survival after ninety minutes at 60°C.

Taxonomic considerations:- It is evident that these organisms differ from those of Type I in but one respect; the fermentation of xylose. It therefore seems logical to consider these Type II organisms as xylose-fermenting strains of L. leichmannii.

(iii) Type III (17 cultures):

Morphology:- Spheres, 0.2 - 0.7 µ in diameter, occurring singly, in pairs, threes and tetrods. Non-motile, Gram-positive, non-capsulated, non-sporeforming.

Figure 3...

Figure 3: Cells of a homofermentative Type III culture after one week in yeast autolysate glucose broth (x 1800).

Yeast autolysate glucose agar colonies:- Colonies small, develop slowly. Surface colonies circular, white, smooth, with entire margins and umbonate elevation. Subsurface colonies smaller, smooth, spindle-shaped or irregular.

Yeast autolysate glucose agar slant:- Visible growth after four to five days incubation. Growth limited, effuse.

Yeast autolysate glucose broth:- Moderate turbidity after two to three days incubation.

Growth on sucrose medium:- No slime production.

Litmus milk:- No change.

Catalase activity:- Negative.

Fermentation...

Fermentation:- Vigorous acid production from trehalose. Moderate acid production from glucose, fructose, galactose, maltose, mannose, sucrose, cellobiose, arbutin, salicin, amygdalin and α-methyl glucoside. No acid from xylose, arabinose, rhamnose, lactose, melibiose, melecitose, raffinose, mannitol, sorbitol, glycerol, inositol, dulcitol, starch, inulin or dextrin.

Type of lactic acid produced:- Optically inactive.

Reduction of nitrate:- Negative.

Production of ammonia from arginine:- Negative.

Oxygen relationship:- Microaerophilic.

Temperature relations:- Optimum temperature 25 - 28°C. Growth at 10°C, no growth above 35°C.

Salt tolerance:- Good growth in 2 per cent (w/v) sodium chloride, some strains show weak growth in 4 per cent.

Heat survival:- No survival after ninety minutes at 60°C.

Taxonomic considerations:- As the main metabolic product of these cocci is lactic acid, these organisms belong to the family Lactobacillaceae Winslow et al, 1917 (see Breed et al, 1957). According to Pederson (1949) such Gram-positive, homofermentative, comparatively high acid producing, catalase-negative cocci, which occur in tetrods, singly and in pairs, should be excluded from the genera Streptococcus and Leuconostoc and placed in the genus Pediococcus Balcke 1884 emend. Mees 1934 (see Breed et al, 1957) with P. cerevisiae

as...

as the type-species. After examining a large number of cocci from fermenting vegetables, Pederson (1949) found that although many strains showed differences with regard to carbon compounds fermented, in no case could any significant trend be demonstrated. It was thus concluded that all of the strains belonged to one species.

Keeping in mind these findings and the characteristics of the wine cocci, it seems that these organisms should, to all appearances, be considered strains of P. cerevisiae. The homofermentative cocci isolated from Californian wine by Ingraham et al (1960), were also tentatively placed in this genus.

2. The heterofermentative group:

(i) Type I (two cultures):

Morphology:- Rods, 0.3 - 0.8 /u x 1.5 - 6 /u, occurring singly and in pairs. Moderate tendency to form chains which may be 20 /u or longer. Non-motile, Gram-positive, non-capsulated, non-sporeforming.

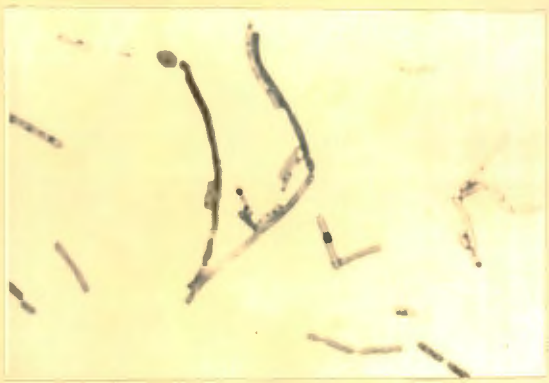


Figure 4: Cells of a heterofermentative Type I culture after one week in yeast autolysate glucose broth (x 1800).

Yeast...

Yeast autolysate glucose agar colonies:- Colonies mostly subsurface, white, smooth, irregular or spindle-shaped. Some subsurface colonies with slightly filamentous borders.

Yeast autolysate glucose agar slant:- Visible growth after two to three days incubation. Growth very scant, effuse.

Yeast autolysate glucose broth:- Uniform turbidity after 24 to 48 hours incubation and shows a silky waviness when shaken gently.

Growth on sucrose medium:- No slime production.

Litmus milk:- No change.

Catalase activity:- Negative.

Fermentation:- Vigorous fermentation of arabinose. Moderate acid production from glucose, fructose, galactose, maltose, sucrose, lactose, melibiose, melecitose, raffinose and mannitol. Slight acid production from sorbitol. No acid from xylose, rhamnose, mannose, trehalose, glycerol, inositol, dulcitol, arbutin, salicin, amygdalin, cellobiose, α-methyl glucoside, starch, inulin and dextrin.

Type of lactic acid produced:- Optically inactive.

Reduction of nitrate:- Negative.

Production of ammonia from arginine:- Positive.

Oxygen relationship:- Microaerophilic.

Temperature relations:- Optimum temperature 37°C. Growth at 10° and 45°C.

Salt...

Salt tolerance:- Good growth in 4 per cent (w/v) sodium chloride, no growth in 6 per cent.

Heat survival:- No survival after ninety minutes at 60°C.

Taxonomic considerations:- The characteristics of these isolates are in good agreement with those of certain strains of Lactobacillus buchneri (Henneberg) Bergey et al, 1923 (see Breed et al, 1957), studied by Rogosa et al (1953). Breed, et al (1948) had defined L. buchneri as fermenting both xylose and arabinose. Of the ninety strains studied by Rogosa et al (1953), only 14 per cent fermented both these substrates. The remaining 86 per cent fermented arabinose but not xylose, as did these wine strains.

Certain of the strains isolated by Pederson (1929 a, b, 1930) from spoiled tomato products and sauerkraut were designated L. mannitopoeus which, according to Breed et al (1948), is synonymous with L. buchneri. Although these isolates fermented both arabinose and xylose, only approximately half as much acid was formed from xylose as compared to arabinose.

Of all the heterofermentative lactobacilli studied by Pederson (1929 a, b, 1930) and Rogosa et al, (1953), only isolates which agreed with the description of L. buchneri fermented melecitose. According to Rogosa and Sharp (1959) this fermentation is characteristic of L. buchneri.

(ii) Type II (16 cultures):

Morphology:- Rods, 0.3 - 0.7 µ x 1.2 - 3.8 µ,

occurring...

occurring singly, in pairs or in long filaments of 25 /u or longer. Non-motile, Gram-positive, non-capsulated, non-sporeforming.

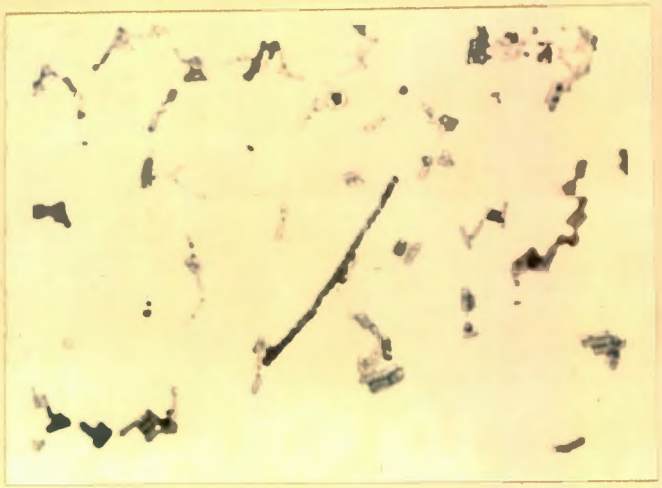


Figure 5: Cells of a heterofermentative Type II culture after one week in yeast autolysate glucose broth (x 1800).

Yeast autolysate glucose agar colonies:- Colonies mostly subsurface, white, spindle-shaped or irregular with entire margins. Some subsurface colonies with slightly filamentous margins.

Yeast autolysate glucose agar slant:- Visible growth after three to four days incubation. Growth limited, effuse. Streak cream-coloured.

Yeast autolysate glucose broth:- Moderate turbidity after two to four days, more pronounced in the depths of the medium. After a few more days the turbidity clears, leaving a flocculent sediment.

Growth on sucrose medium:- No slime production.

Litmus...

Litmus milk:- No change.

Catalase activity:- Negative.

Fermentation:- Vigorous acid production from xylose. Moderate acid production from glucose, fructose, galactose, maltose, sucrose, melibiose, raffinose and α-methyl glucoside. Slight acid production from lactose. No acid from arabinose, rhamnose, mannose, melecitose, trehalose, cellobiose, mannitol, sorbitol, glycerol, inositol, dulcitol, arbutin, salicin, amygdalin, starch, inulin or dextrin.

Type of lactic acid produced:- Optically inactive.

Reduction of nitrate:- Negative.

Production of ammonia from arginine:- Positive.

Oxygen relationship:- Microaerophilic to facultative anaerobic.

Temperature relations:- Optimum temperature 28 - 32°C. Growth at 10°C, no growth at 45°C.

Salt tolerance:- Good growth in 4 per cent (w/v) sodium chloride, no growth in 6 per cent.

Heat survival:- No survival after ninety minutes at 60°C.

Taxonomic considerations:- The fact that these isolates ferment xylose vigorously but fail to ferment arabinose and mannose, accentuates the similarity between these organisms and Lactobacillus hilgardii (Douglas and Cruess), Vaughn, Douglas and Fornachon (1949).

The characteristics of these isolates were

compared...

compared with those of a type-strain of L. hilgardii, received from Dr. J.C.M. Fornachon, and found to agree in practically all respects.

(iii) Type III (four cultures):

Morphology:- Rods, 0.5 - 0.8 µ x 1.5 - 6 µ, occurring singly and in pairs. A minimum of filaments are produced. Non-motile, Gram-positive, non-capsulated, non-sporeforming.

Figure 6: Cells of heterofermentative Type III culture after one week in yeast autolysate glucose broth (x 1800).

Yeast autolysate glucose agar colonies:- Colonies mostly subsurface, irregular, white, smooth. Some colonies possess filamentous margins.

Yeast autolysate glucose agar slant:- Visible growth after two to three days incubation. Growth limited, effuse. Streak white to cream-coloured.

Yeast...

Yeast autolysate glucose broth:- Moderate turbidity after two to three days incubation, more pronounced in the depths of the medium. After a few more days the turbidity clears to leave a flocculent sediment. One strain showed a tendency to produce a slight flakiness in the medium.

Growth on sucrose medium:- No slime production.

Litmus milk:- No change.

Catalase activity:- Negative.

Fermentation:- Vigorous acid production from xylose, arabinose and glucose. Moderate acid production from fructose, galactose, maltose, sucrose, melibiose, raffinose and α-methyl glucoside. Slight acid production from lactose. Some strains produce a slight acidity from mannitol. No acid from rhamnose, mannose, melecitose, trehalose, cellobiose, sorbitol, glycerol, inositol, dulcitol, arbutin, salicin, amygdalin, starch, inulin and dextrin.

Type of lactic acid produced:- Optically inactive.

Reduction of nitrate:- Negative.

Production of ammonia from arginine:- Positive.

Oxygen relationship:- Microaerophilic.

Temperature relations:- Optimum temperature 28 - 30°C. Growth at 10°C, no growth at 45°C.

Salt tolerance:- Growth in 2 per cent (w/v) sodium chloride, no growth in 4 per cent.

Heat survival:- No survival after ninety minutes at 60°C.

Taxonomic...

Taxonomic considerations:- The characteristics of these isolates closely resemble the descriptions by Breed et al (1948) and Rogosa et al (1953) of Lactobacillus brevis (Orla-Jensen) Bergey et al, 1934 (see Breed et al, 1957).

It is of interest to note that these isolates failed to ferment mannose, as did all the strains of L. brevis studied by Rogosa et al (1953).

(iv) Type IV (four cultures):

Morphology:- Rods, 0.4 - 0.8 μ x 1 - 4 μ, occurring singly, in pairs and in long filaments. Individual filaments up to 50 μ have been observed. Non-motile, Gram-positive, non-capsulated, non-sporeforming.

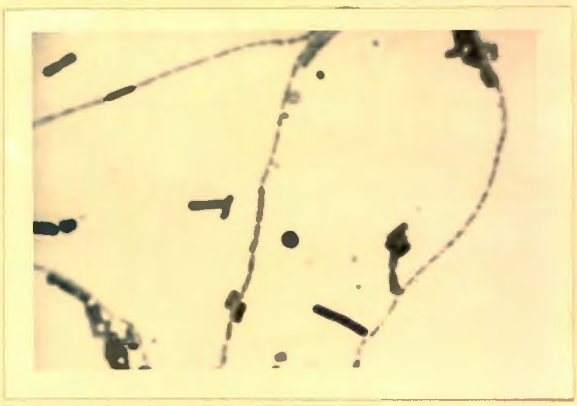


Figure 7: Cells of a heterofermentative Type IV culture after one week in yeast autolysate glucose broth (x 1800).

Yeast autolysate glucose agar colonies:- Surface colonies white with flat elevation; some possess slightly filamentous margins. Subsurface colonies

white...

white, mostly irregular in shape.

Yeast autolysate agar slant:- Visible growth after two to three days incubation. Growth limited, effuse. Streak white to creamish-white.

Yeast autolysate glucose broth:- Uniform turbidity after one to three days incubation. Exhibits a pronounced silky waviness when shaken gently. After a few more days the turbidity clears and leaves a flocculent sediment.

Growth on sucrose medium:- No slime production.

Litmus milk:- No change.

Catalase activity:- Negative.

Fermentation:- Vigorous acid production from xylose, glucose, fructose and mannose. Moderate acid production from galactose, maltose, sucrose, melibiose, melecitose, raffinose, cellobiose, mannitol, arbutin, salicin, amygdalin and α-methyl glucoside. Slight acid production from lactose and sorbitol. No acid from arabinose, rhamnose, trehalose, glycerol, inositol, dulcitol, starch or dextrin.

Type of lactic acid produced:- Optically inactive.

Reduction of nitrate:- Negative.

Oxygen relationship:- Microaerophilic.

Temperature relations:- Optimum temperature 30°C. Growth at 10°C and at 45°C.

Salt tolerance:- Good growth in 4 per cent (w/v) sodium chloride, most strains grow weakly in 6 per cent.

Heat...

Heat survival:- No survival after ninety minutes at 60°C.

Taxonomic considerations:- The taxonomic position of this group of isolates is not quite clear. The isolates resemble L. buchneri when pentose and melecitose fermentation is considered. However, these organisms ferment several more carbohydrates than the strains of L. buchneri studied by Rogosa et al (1953), or the heterofermentative Type I organisms, and have a lower optimum temperature. It therefore seems that the taxonomic position of this group depends upon which of these characteristics have the greatest differential value. If the power of melecitose fermentation, as such, is a valid criterion for distinguishing L. buchneri (Rogosa and Sharp, 1959), then these isolates must be considered strains of this species. If, on the other hand, greater differential value is attached to the wider range of carbohydrates fermented and the lower optimum temperature, the isolates must be considered as strains of an unknown species of Lactobacillus.

A summary of the main differential characteristics of the isolated bacterial strains is given in Table 1.

Table 1....

Table 1: Differential characteristics of the lactic acid bacteria occurring in South African dry wines.

Group.	Organism.	Opt. temp. (°C).	Optical rotation of lactic acid.	Production of NH_3 from arginine.	Fermentation.																			
					d-xylose	l-arabinose	d-mannose	d-sucrose	d-lactose	d-melibiose	d-cellobiose	d-melecitose	d-raffinose	d-trenalose	d-mannitol	d-sorbitol	glycerol	arbutin	salicin	amygdalin	α-meth.glucoside	l-malic acid	citric acid
Homo-fermentative.	L.leichmannii (Type I)	35-37	l	±	-	-	++	+	-	-	+	x	-	++	+	+	±	+	+	+	+	++	++
	L. leichmannii (Type II)	32-37	l	±	++	-	++	+	-	-	+	+	-	++	+	+	±	+	++	+	+	++	++
	P. cerevisiae	25-28	dl	-	-	-	+	+	-	-	+	-	-	++	-	-	-	+	+	+	+	++	-
Hetero-fermentative.	L. buchneri	35-37	dl	+	-	++	-	+	+	+	-	+	+	-	+	±	-	-	-	-	-	±	-
	L. hilgardii	28-32	dl	+	++	-	-	+	±	+	-	-	+	-	-	-	-	-	-	-	+	++	±
	L. brevis	28-30	dl	+	++	++	-	+	±	+	-	-	+	-	x	-	-	-	-	-	+	++	±
	Lactobacillus sp.	30-32	dl	+	++	±	++	+	±	+	+	+	+	-	+	±	-	+	+	+	+	++	++

All strains fermented glucose, fructose, galactose and maltose, while none fermented rhamnose, inositol, dulcitol, starch, inulin, dextrin or tartaric acid.

++ Vigorous fermentation (final pH 3.0 - 3.5)
+ Moderate fermentation (" " 3.5 - 4.5)
± Weak fermentation (" " 4.5 - 5.4)
x Fermented by most strains tested.

In order to relate the pH readings to total acidity, as certain investigators have done (Pederson, 1929 a, b, 1930, 1936, 1938; Rogosa et al, 1953), the buffer curve of the basal medium (yeast autolysate) is presented in Figure 8.

The relative frequencies with which the different species occurred are presented in Table 2. The wines from which the 64 bacterial strains were isolated emanated from thirty different cellars. The percentage frequency (in Table 2) was calculated on the basis of the number of cellars, out of this total of thirty, in the wines of which a specific species was encountered.

Table 2: The source and incidence of the isolated bacterial species.

Species.	Source.		No. of cellars represented by wines.	% Frequency.
	White dry wine.	Red dry wine.		
L. leichmannii (Type I)	16	-	2	6.67
L. leichmannii (Type II)	5	-	1	3.33
P. cerevisiae	16	1	17	56.67
L. buchneri	2	-	1	3.33
L. hilgardii	15	1	12	40.00
L. brevis	3	1	3	10.00
Lactobaccillus sp.	4	-	3	10.00
Total	61	3	-	-

Pediococcus...

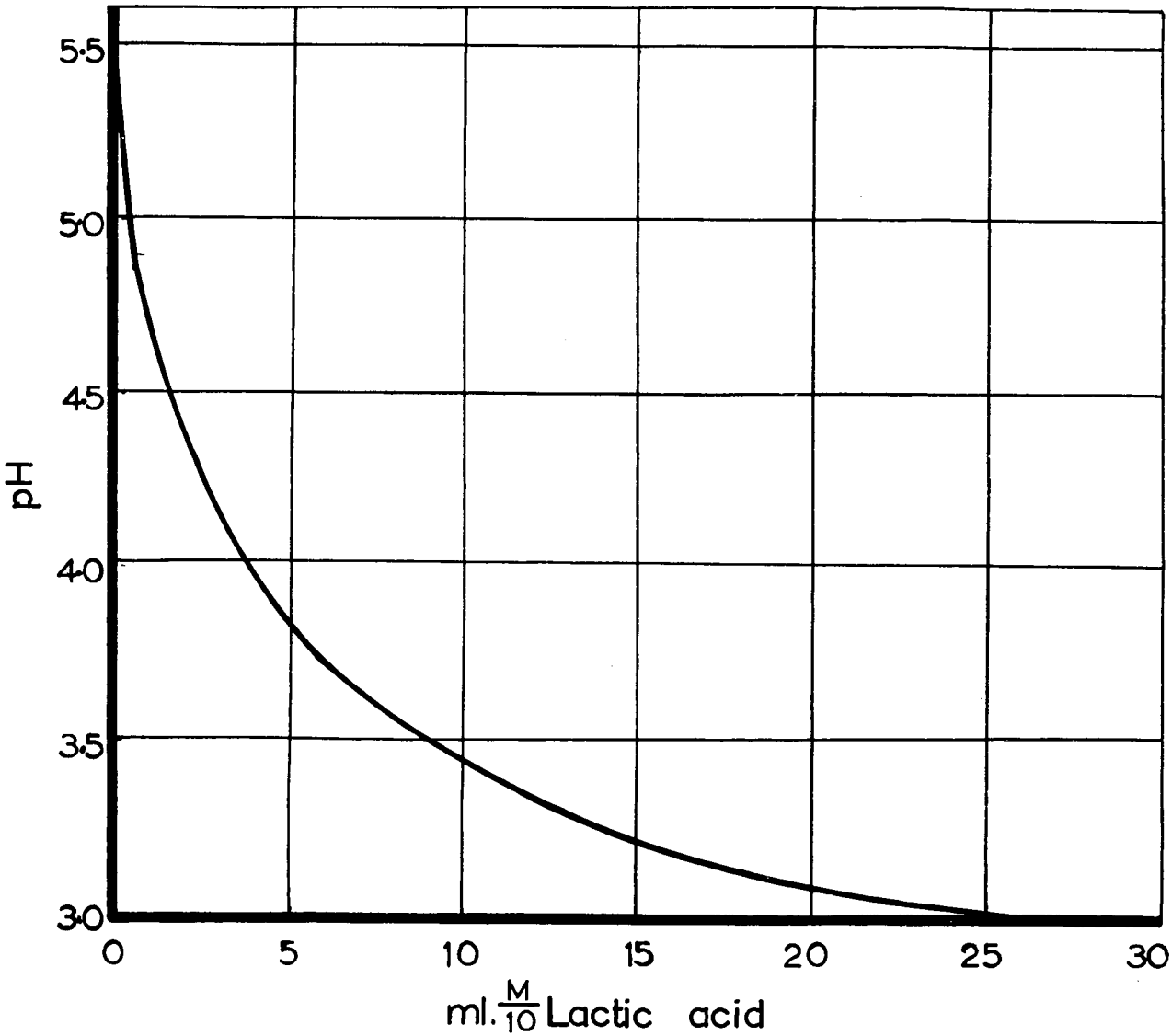


Fig.8: Buffer curve of yeast autolysate medium.

Pediococcus cerevisiae and L. hilgardii have, as indicated in Table 2, an exceptionally high incidence in South African dry wines. Both of these organisms, as well as L. brevis, were encountered in Australian wines by Fornachon (1957). Lactobacillus hilgardii, although not accepted by Bergey's Mannual (Breed et al, 1957) is considered by Vaughn (1955) to be next in importance, among the lacto-bacilli, to L. plantarum and L. brevis in the spoilage of Californian table wines. Lactobacillus buchneri was defined by Breed et al (1948) as being synonymous with B. mannitopoeum, the organism described by Müller-Thurgau and Osterwalder (1913).

According to available information L. leichmannii has not been encountered in the wines of other countries.

NUTRITIONAL REQUIREMENTS OF THE LACTIC ACID BACTERIA.

For cell synthesis all living organisms require a utilisable source of energy consisting of nitrogen and carbon containing compounds, as well as inorganic salts. The required substances must be supplied in appropriate concentrations in an environment favourable for growth of the organism. Considering these requirements, the lactic acid bacteria are among the most complex organisms so far studied.

Vitamin requirements.

Orla-Jensen, Otte and Snog-Kjaer (1936) showed that riboflavin and one or more other "activators" are necessary for growth of certain lactic acid bacteria. They concluded from tentative evidence that one of these "activators" was pantothenic acid. Snell, Strong and Peterson (1938, 1939) substantiated this assumption by employing purified preparations of pantothenic acid. They found this vitamin, as well as nicotinic acid, essential for growth of two Lactobacillus species in a hydrolysed casein medium. Additional factors were necessary for other species.

Using a basal medium containing glucose, hydrolysed casein, inorganic salts, vitamin B_1 and the ether-soluble fraction of yeast extract, Wood, Anderson and Werkman (according to Wood, Geiger and Werkman, 1940), confirmed the mentioned assumption of Orla-Jensen et al (1936). The activity of the acid and alkali labile, ether soluble factor for the lactic acid bacteria had

previously...

previously been demonstrated by Snell, Tatum and Peterson (1937).

The strains studied by Möller (1938, 1939) required crystalline vitamin B6 in addition to the ether soluble factor. Biotin proved to be essential for some strains, while thiamine, nicotinic acid, riboflavin, β-alanine and certain unknown factors also had an effect.

According to Orla-Jensen et al (1936) thiamine is not important in the nutrition of lactic acid bacteria. However, Wood et al (1940) found that their hetero-fermentative strains required thiamine in addition to riboflavin and the ether-soluble fraction of yeast extract.

Other factors which have been found essential for some lactic acid bacteria include, amongst others, p-amino benzoic acid (Snell, 1948) and folic acid (Mitchell, Snell and Williams, 1941).

Studies in this field during the last decade have greatly enhanced our knowledge of nutrition and metabolism. Several of the growth factors, amongst others pyridoxal, pyridoxanine, folinic acid, lipoic acid and pantethine, were discovered through such studies. Others such as pantothenic acid and folic acid were discovered independently, but our knowledge of them greatly increased by making use of the lactic acid bacteria (Snell, 1952).

Specific strains of lactic acid bacteria are currently employed in the determination of biologically active compounds such as vitamins (A. V. C., 1951), amino acids (Schweigert, Guthneck, Kraybill and Greenwood,

1949) and pyrimidines (Merrifield and Dunn, 1950), and have proved to be extremely useful aids in biochemical analysis.

Intensive studies have been made of the vitamin requirements of the lactic acid bacteria from various other sources, including milk (Snell, 1948), the human mouth (Rogosa et al, 1953), the rumen (Ford, Perry and Briggs, 1958), brewery material (Russel, Bhandari and Walker, 1954; Moore and Rainbow, 1955; Williamson, 1959) and apple cider (Carr, according to Luthi, 1959). A survey of the literature failed to yield data on the nutrition of the wine lactic acid bacteria.

Knowledge of the vitamin requirements of these lactic acid bacteria seems essential to an understanding of the mechanism of their cell metabolism. Such knowledge may also prove to be of value in the selection of a measure to control bacterial contamination of wines.

According to Rogosa and Sharp (1959) data on the vitamin requirements can be advantageously employed as additional criteria for the differentiation of Lactobacillus species.

A comparison of the requirements of twelve selected strains from S.A. dry wines with those of other lactic acid bacteria:

In order to gain an impression of the vitamin requirements of the wine lactic acid bacteria, twelve strains were selected and the determinations made, employing the synthetic medium, as described. All the

strains grew relatively well in the "complete" medium. The results are presented in Table 3.

Table 3: Vitamin requirements of twelve strains of lactic acid bacteria from South African dry wines.

Species.	Homofermentative.				Heterofermentative.							
	L. leichmannii.		*P. cerevisiae.*		*L. buchneri.*		*L. brevis.*		*L. hilgardii.*		Lactobac. sp.	
Strain No.	1	2	1	2	1	2	1	2	1	2	1	2
Nicotinic acid	+	+	+	+	+	+	+	+	+	+	+	+
Ca-pantothenate	+	+	+	+	+	+	+	+	+	+	+	+
Riboflavin	+	+	+	+	+	+	+	+	+	+	+	+
Thiamine	-	-	-	S	+	+	+	+	+	+	+	+
Pyridoxine	+	+	+	+	-	-	S	S	S	S	S	S
Vitamin B_{12}	-	-	+	+	S	S	S	-	-	-	S	S
Folic acid	+	+	S	-	-	-	-	-	-	-	S	+
Biotin	-	-	+	S	-	-	S	-	-	-	S	S
p-Amino benzoic acid	-	-	+	S	-	-	-	-	-	-	-	-
Inositol	-	-	-	-	-	-	-	-	-	-	-	-
Choline chloride	-	-	-	-	-	-	-	-	-	-	-	-
Incubation time (hours)	72	72	168	168	72	72	72	72	72	72	86	86

\+ Requirement.

S Stimulatory.

\- No exogenous requirement.

Nicotinic...

Nicotinic acid, riboflavin and calcium pantothenate were required by all strains tested. In addition to these the eight heterofermentative strains required thiamine and the four homofermentative strains pyridoxine. The omission of any one of these essential vitamins from the complete medium led to a growth reduction of at least 95 per cent. The omission of inositol and/or choline chloride, on the other hand, had no adverse effect on the growth of any of the organisms under these conditions.

The finding that all strains required pantothenic acid is in accord with the results of Cheldelin, Hoag and Sarett (1945), Rogosa et al (1953), Russel et al (1954), Moore and Rainbow (1955), Ford et al (1958) and Williamson (1959) with lactic acid bacteria from other sources. These facts emphasise the importance of pantothenic acid in the nutrition of the lactic acid bacteria. According to Russel et al (1954) the components of pantothenic acid, whether supplied separately or together, cannot displace the intact vitamin in the nutrition of these bacteria.

It was shown by Novelli and Lipman (1947) that 90 per cent of the pantothenic acid utilised by L. arabinosus can be traced to coenzyme A; a pantothenic acid derivative. Coenzyme A was subsequently found to be a general constituent of living organisms, being present in all the micro-organisms tested including species of Lactobacillus, Propionibacterium, Escherichia, Proteus and Clostridium. A stimulation of pyruvic acid oxidation by pantothenic acid in pantothenic acid deficient organisms was demonstrated by the work of Novelli and Lipman (1947).

Many...

Many of the lactic acid bacteria studied by Shankman, Camien, Block, Merrifield and Dunn (1947), Rogosa et al (1953), Moore and Rainbow (1955), Ford et al (1958) and Williamson (1959) require nicotinic acid in addition, as do the wine strains examined here. Certain strains have been reported which could, however, dispense completely with exogenous nicotinic acid (Shankman et al, 1947; Russel et al, 1954).

Nicotinic acid is utilised by washed cells of L. arabinosus 17 - 5 in the synthesis of cozymase (Hughes and Williamson, 1950); a finding which stresses the importance of nicotinic acid in the nutrition of these bacteria.

The requirement for riboflavin by all strains tested make the wine lactic acid bacteria different from those isolated from brewery products, only a fractional percentage of which required this vitamin (Russel et al, 1954; Moore and Rainbow, 1955; Williamson, 1959). Most of the rumen lactobacilli, on the other hand, require riboflavin (Ford et al, 1958). Different species of the lactic acid bacteria, and even different strains of a single species are known to vary markedly with respect to their requirement for this vitamin. Riboflavin, in the form of the coenzymes derived from it, serves as a catalyst for hydrogen transfer (Snell, 1951).

The results obtained in this investigation with thiamine are in agreement with those of Rogosa et al (1953), who observed that all strains requiring this vitamin are heterofermentative. This fact may

represent...

represent one of the fundamental differences between homo- and heterofermentative lactic acid bacteria as thiamine, after being converted to cocarboxylase or thiamine pyrophosphate through phosphorylation, exerts its catalytic effects in the decarboxylation of certain α-keto acids, including pyruvic acid (Snell, 1951).

Biotin, essential for the growth of most milk organisms (Snell, 1948) and the strains studied by Rogosa, Tittsler and Geib (1947), is not required by the wine lactobacilli or those from the mentioned other sources, but is required by two strains of Pediococcus cerevisiae, as indicated in Table 3. The results of Ochoa, Mehler, Blanchard, Jukes, Hoffmann and Regan (1947), indicate that biotin is involved in the synthesis of enzyme systems mediating the fixation of carbon dioxide. It has been suggested that the relationship of biotin to such enzyme systems is less direct than that of a prosthetic group or a component of a prosthetic group (Blanchard, Korkes, del Campillo and Ochoa, 1950).

Barring the strains of L. buchneri, pyridoxine was stimulatory to all heterofermentative strains and essential for all of the homofermentative strains tested (Table 3). The present evidence indicates that one of the primary roles of pyridoxine in metabolism is to catalyse reactions involved in the synthesis of amino acids. Pyridoxine (as pyridoxal phosphate) is also an essential component of enzymes which catalyse the transamination reaction and the decarboxylation of several amino acids (Snell, 1951).

No...

No organisms are known to require both folic acid and p-amino benzoic acid. Folic acid is synthesised by L. arabinosus when the organism is grown in the presence of excess p-amino benzoic acid (Sarett, 1947). The interchangeability of thymine and folic acid as growth factors for various lactic acid bacteria, supports the view that folic acid functions in the synthesis of thymine, which in turn is utilised in the synthesis of nucleic acid (Stokes, 1944). The significance of p-amino benzoic acid is apparent from its role as a constituent of the folic acid molecule.

The fact that the wine lactobacilli do not require choline chloride, inositol or p-amino benzoic acid make them similar in this respect to most of the many strains studied by Rogosa et al (1947) and Shankman et al, 1947).

Additional taxonomic considerations:

The xylose non-fermenting (Strain 1) and xylose fermenting (Strain 2) strains of L. leichmannii exhibit identical vitamin nutrition patterns (Table 3). This finding strengthens the view that these strains belong to one species.

These strains of L. leichmannii could dispense with exogenous vitamin B_{12}, in spite of the fact that certain strains of this organism are employed in the biological determination of vitamin B_{12} (Skeggs et al, 1950). It is, however, doubtful whether a requirement for this vitamin can be considered characteristic of L. leichmannii, as Russel et al (1954) also

described...

described strains of this organism for which vitamin B12 was non-essential.

Rogosa and Sharp (1959) stated that L. leichmannii can dispense completely with exogenous riboflavin. The wine strains studied here all required riboflavin, as did a beer strain (Strain EE4) of L. leichmannii encountered by Russel et al (1954).

The vitamin requirements of the wine pediococci are in fairly good agreement with those of the strains of P. cerevisiae studied by Jensen and Seeley (1954).

Most of the strains of L. buchneri require riboflavin (Rogosa and Sharp, 1959) as do the two wine strains studied here. Riboflavin was non-essential for the growth of most of the brewery strains of L. buchneri studied by Russel et al (1954).

Rogosa and Sharp (1959) maintained that L. brevis does not require riboflavin and that this organism is the only known heterofermentative lactobacillus for which folic acid is indispensable. This view is not substantiated by the results in Table 3. Furthermore, the type-strain of L. hilgardii (received from Dr. J.C.M. Fornachon) required folic acid in addition to the vitamins required by these (South African) wine strains of this organism.

Correlation of vitamin requirements and biochemical characteristics:

Rogosa et al (1947), after studying approximately 250 strains of Lactobacillus, made the following

observations:...

observations:-

(i) Of 47 strains which grew in the absence of folic acid, 45 fermented melibiose or raffinose or inulin or arabinose or xylose.

(ii) All strains which grew without folic acid also grew well in the absence of pyridoxine, but the converse was not true.

(iii) All gas-forming strains and also those which fermented either arabinose or xylose, grew well in the absence of pyridoxine.

All of the wine lactobacilli which could dispense with folic acid fermented melibiose, raffinose and either xylose or arabinose or both (Tables 1 and 3).

The wine-strains of L. leichmannii (Table 3) required both folic acid and pyridoxine, thus differing in this respect from the strains studied by Rogosa et al (1947).

The gas forming strains all grew well in the absence of pyridoxine, as did all the strains which fermented xylose or arabinose or both, except for the xylose fermenting strain of L. leichmannii.

These results seem to suggest that vitamin requirements, as such, should be used only as additional criteria for the identification of species of the lactic acid bacteria. These requirements are possibly influenced to a varying extent by the substrate (through competition and natural selection) from which the isolates emanate.

Amino...

Amino acid requirements.

Unlike the vitamins, amino acids are major structural components of the cell.

Orla-Jensen et al (1936) studied the amino acid requirements of a large number of lactobacilli. As a determination of essential amino acids the work was not conclusive since the semi-synthetic basal medium used contained whey, which was probably not free from traces of amino acids.

The results of Snell et al (1937) and Wood et al (1940) indicated that both homo- and hetero-fermentative lactobacilli require a complex mixture of amino acids for growth.

The amino acid requirements of 23 strains of lactic acid bacteria were investigated by Dunn et al (1947), while Jensen et al (1954) studied those of the pediococci. Except in the case of those strains employed in the biological determination of amino acids (Snell, 1945; Schweigert et al, 1949; Schweigert, Guthneck and Scheid, 1950; Skeggs, Driscoll, Taylor and Wright, 1953), the amino acid requirements of the lactic acid have not been intensively studied.

The number and identity of the amino acids required by lactic acid bacteria are highly dependent upon the vitamins present in the medium (Stokes and Gunness, 1945; Snell, 1952).

The lactic acid bacteria from South African fortified wines studied by Niehaus (1932) could not grow in normal, sound wines. When, however, these

bacteria...

bacteria were inoculated into the same wines which had been left on a yeast sediment for a fortnight, growth readily occurred. Burroughs (1955) encountered a more or less analogous phenomenon in the production of apple ciders and showed that the growth of lactic acid bacteria in bottled ciders is directly related to the extent to which yeast autolysis had been allowed to occur during fermentation and storage. Burroughs and Carr (1956) established a definite correlation between the growth of these bacteria and the amino acid content of the ciders.

Although many sound wines probably contain sufficient vitamins to uphold growth of the lactic acid bacteria (Castor, 1953), the amino acid content possibly determines, at least in some instances, its susceptibility to this type of bacterial infection. Apart from contributing to our understanding of metabolism, knowledge of the amino acid requirements of the wine lactic acid bacteria may contribute to an understanding as to why some wines made from specific grape varieties (e.g. Hanepoot) seem more susceptible to infection by these bacteria.

A comparison of the requirements of twelve selected strains from S.A. dry wines with those of other lactic acid bacteria:

The amino acid requirements of the selected twelve bacterial strains are presented in Table 4.

Table 4...

Table 4: Amino acid requirements of twelve strains of lactic acid bacteria from South African dry wines.

Species.	Homo-fermentative.				Heterofermentative.							
	L. leich-mannii.		P. cerevi-siae.		L. buch-neri.		L. brevis.		L. hil-gardii.		Lacto-bac. sp.	
Strain No.	1	2	1	2	1	2	1	2	1	2	1	2
Glutamic acid	+	+	+	+	+	+	+	+	+	+	+	+
Valine	+	+	+	+	+	+	+	+	+	+	+	+
Arginine	+	+	+	+	+	+	+	+	+	+	+	+
Leucine	+	+	+	+	+	+	+	+	+	+	+	+
Iso-leucine	+	+	+	+	+	+	+	+	+	+	+	+
Methionine	+	+	+	+	S	S	+	S	+	S	S	S
Threonine	S	S	+	+	-	-	-	-	-	-	-	-
Lysine	-	-	S	S	-	-	-	-	-	-	-	-
Asparagine	-	-	S	-	-	-	-	-	-	-	-	-
Aspartic acid	-	-	+	+	-	-	-	-	-	-	-	-
Tryptophane	+	+	+	+	+	+	+	+	-	-	-	-
Serine	+	+	+	+	S	S	S	+	+	-	+	+
α-Amino butyric acid	-	-	-	-	-	-	-	-	-	-	-	-
Proline	-	-	+	+	S	S	-	+	S	S	+	S
Histidine	S	S	+	+	S	S	-	S	S	-	-	-
Glycine	S	-	+	+	-	-	-	-	-	-	-	-
α-Alanine	-	-	+	-	+	+	-	-	-	S	S	-
Phenylalanine	+	+	+	+	S	S	-	+	S	-	+	S
Tyrosine	+	+	+	+	-	-	+	+	-	-	+	+
Cysteine	+	+	+	+	+	+	-	+	S	S	-	S
Incubation time(hours)	72	72	168	168	72	72	72	72	72	72	86	86

\+ Requirement.

S Stimulatory.

\- No exogenous requirement.

The number of essential amino acids ranged from five (L. hilgardii Strain 2) to seventeen (P. cerevisiae Strain 1). Glutamic acid, valine, arginine, leucine

and...

and iso-leucine were required by all strains, while methionine was either essential or stimulatory. Only α-amino butyric acid had no effect whatsoever on growth of any of the strains. The number of strains requiring the following amino acids are shown in parenthesis: tryptophane (8), serine (8), tyrosine (8), cysteine (7), phenylalanine (6), proline (4), α-alanine (3), histidine (2), threonine (2), glycine (2), aspartic acid (2), lysine (0) and asparagine (0).

Glutamic acid and valine were also found to be essential for the growth of all of the 23 strains of lactic acid bacteria studied by Dunn et al (1947). Of these 23 strains, nineteen required leucine, while iso-leucine was essential for twenty of the strains.

The amino acid requirements of the two strains of P. cerevisiae agree closely with those of the strains studied by Jensen and Seeley (1954). These wine strains of L. leichmannii exhibit amino acid requirements fairly similar to that of L. leichmannii ATCC 4797 (Skeggs et al, 1953). The type strain of L. hilgardii, tested under these conditions, required phenylalanine and cysteine in addition to the amino acids required by L. hilgardii Strain 2. There is, on the other hand, only slight similarity between the amino acid requirements of the wine strains of L. buchneri and L. brevis and the requirements established for other strains of these organisms (Wood et al, 1940; Dunn et al, 1947).

It appears that the amino acid requirements of the lactic acid bacteria are influenced by the presence of substances other than vitamins. In this

respect...

respect Carr (1959) reported tentative evidence that 3-methyl-2,3-dihydroxybutyric acid and 2-methyl-2,3-dihydroxybutyric acid, thought to be excreted by yeasts, can be used by certain lactic acid bacteria instead of valine.

Of the organisms occurring in South African dry wines (Table 2) the pediococci, although having the most exacting nutritional requirements, exhibit the highest incidence. Of the lactobacilli, however, L. hilgardii occurs the most frequently and requires the smallest number of vitamins and amino acids.

The results presented in Tables 3 and 4 suggest that these wine bacteria may be readily employed in the biological determination of most of the vitamins and amino acids which had been termed essential.

Gaseous requirements of some of the isolates from South African dry wines.

The influence of carbon dioxide on the rate of gas production from glucose:

This influence was ascertained by comparing the rate of gas production under aerobic conditions, in an atmosphere of nitrogen and in an atmosphere of carbon dioxide. The most typical strain of each species (see Table 1) was selected for this purpose, gas production being determined manometrically.

All the strains tested evolved gas more rapidly under aerobic conditions than under nitrogen.

In...

In the presence of carbon dioxide the two homofermentative organisms evolved gas at a rate more or less similar to that under nitrogen. The heterofermentative organisms, under carbon dioxide, initially produced gas from glucose at a rate slightly less than under aerobic conditions. After two to six hours, however, the cells under carbon dioxide invariably exhibited a sudden sharp increase in gas production.

The extent to which these conditions influenced the rate of gas production by L. buchneri is represented by Figure 9, which is considered typical for all of the heterofermentative strains tested.

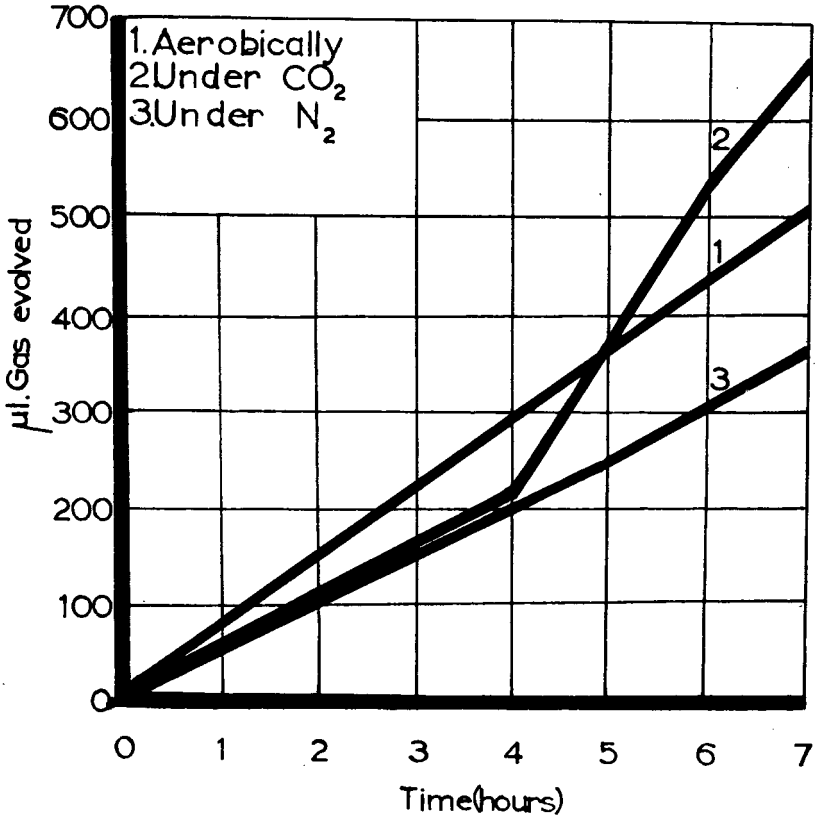


Fig.9: Gas production from glucose by L. buchneri, under three different conditions.

SOME PHYSIOLOGICAL CHARACTERISTICS OF THE ISOLATED SPECIES COMPARED WITH THOSE OF OTHER LACTIC ACID BACTERIA.

South African wines contaminated by lactic acid bacteria usually exhibit high volatile acid contents, and are organoleptically inferior. The products responsible for these defects are imparted to the wine by the reaction of these bacteria with the utilisable substances contained in wines. Several of the carbohydrates, polialcohols, glycosides and organic acids accessible to these bacteria (Table I) are known to occur in sound, normal wines.

The hexoses, glucose and fructose, may occur in dry wines in small quantities as an aftermath of incomplete alcoholic fermentation. Certain of the pentoses, not fermentable by yeasts, have also been found in dry wines. l-Arabinose has been encountered in quantities of up to 1.26 gm. per liter, while small amounts of d-xylose is also usually found (Vogt, 1953). Other utilisable substances which are known to occur in wines include l-malic acid, citric acid and small quantities of glycerol (Theron and Niehaus, 1938).

In order to acquire more knowledge of the changes brought about in wines by the lactic acid bacteria in the presence of these wine components, seven bacterial strains, representing five known species and one of uncertain identity, were selected for further study. In each case the most typical strain (Strain 1) of the species was taken, the only exception being

L. leichmannii...

L. leichmannii where two strains were selected, Strain 1 and a xylose-fermenting variant (Strain 2) of which only its xylose fermentation was studied.

Preliminary tests indicated that glycerol is not utilised at pH levels below 4.7. The utilisation of this compound was not further investigated as it is extremely doubtful whether it is of importance at the low pH values of dry wines.

Carbohydrate metabolism.

All of the selected strains could utilise d-glucose and d-fructose, while only three could utilise l-arabinose and four d-xylose.

Oxygen consumption and carbon dioxide evolution during the utilisation of these carbohydrates by washed resting cell suspensions were studied manometrically at pH 4.7 to 4.8. Prior to their application in the Warburg experiments the cells were grown in yeast autolysate containing a mixture of small amounts of all these sugars.

Manometric results:

The results obtained in this study are represented by Figures 10 to 15. From these the following observations can be made:-

(i) Cell suspensions of the heterofermentative organisms consumed more oxygen during glucose utilisation as compared with fructose utilisation. The reverse holds true for the two homofermentative organisms L. leichmannii and P. cerevisiae.

(ii) All...

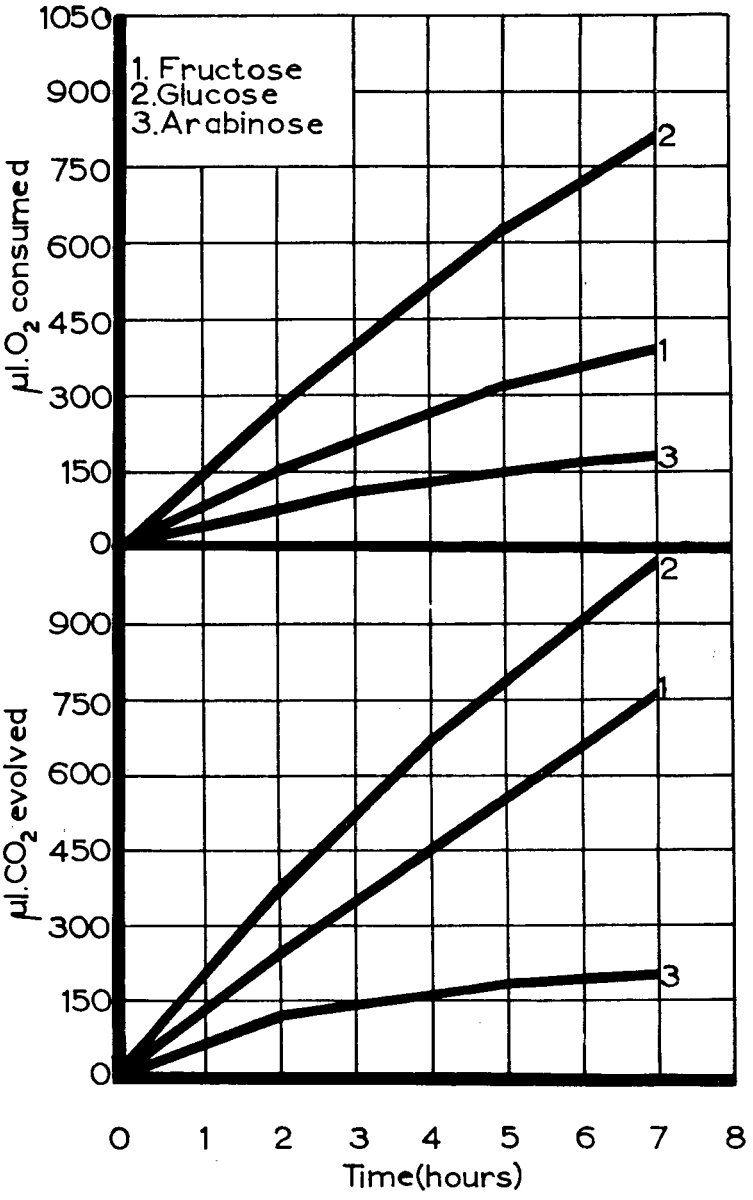


Fig.10: Oxygen consumption and carbon dioxide evolution during utilization of fructose, glucose and arabinose by cell suspensions of L.buchneri.

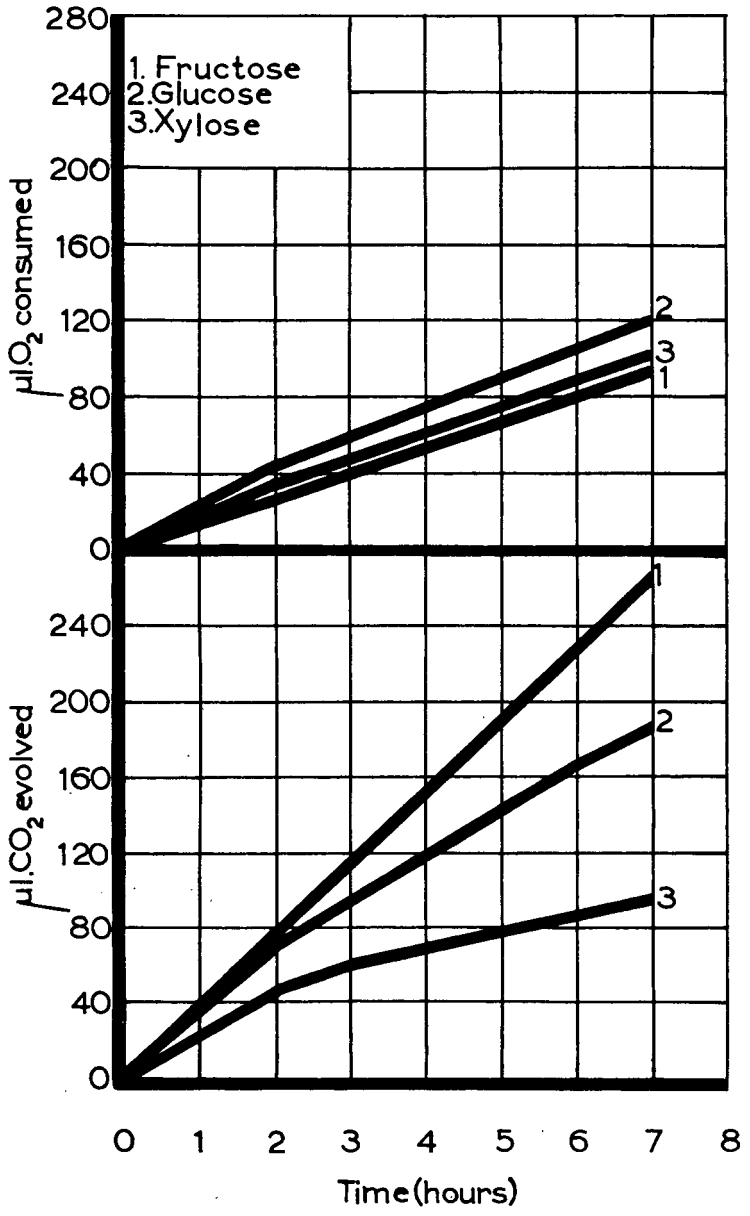


Fig.11: Oxygen consumption and carbon dioxide evolution during utilization of fructose, glucose and xylose by cell suspensions of L.hilgardii

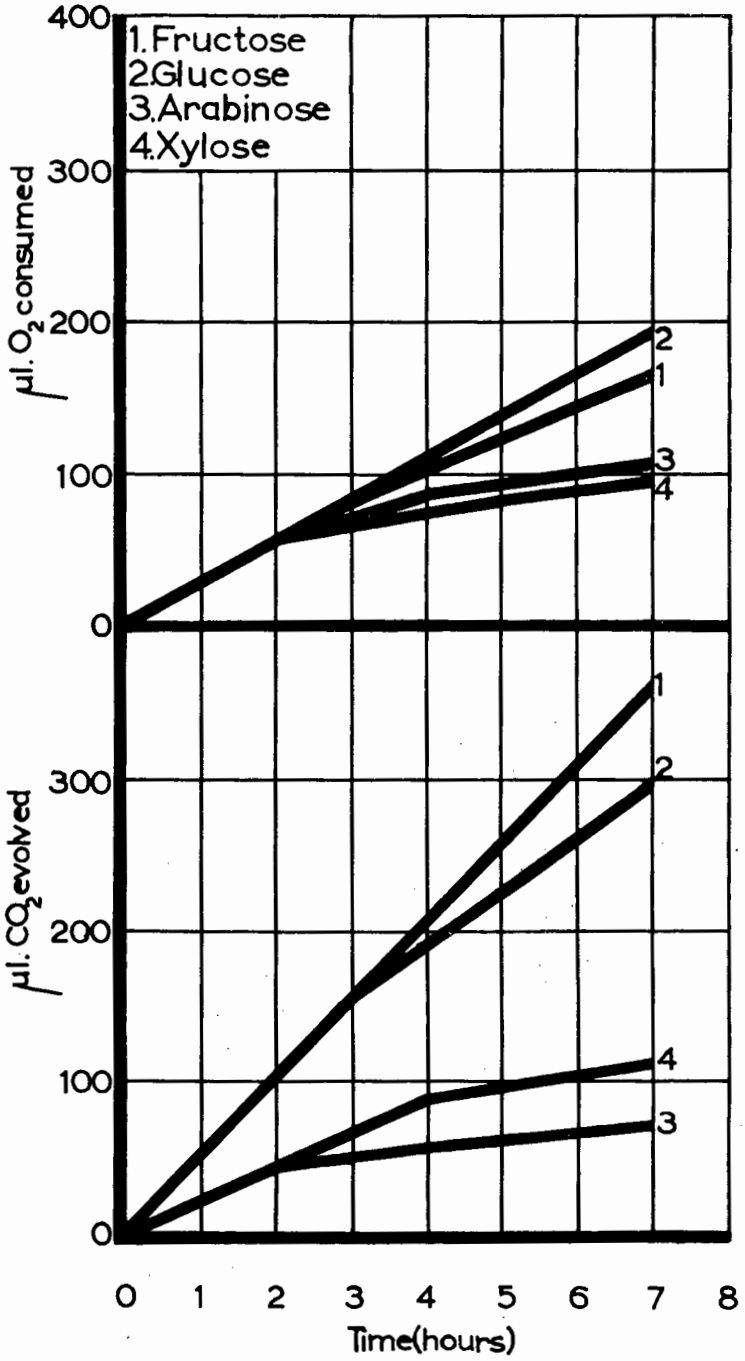


Fig. 12: Oxygen consumption and carbon dioxide evolution during utilization of fructose, glucose, arabinose and xylose by cell suspensions of a Lactobacillus species.

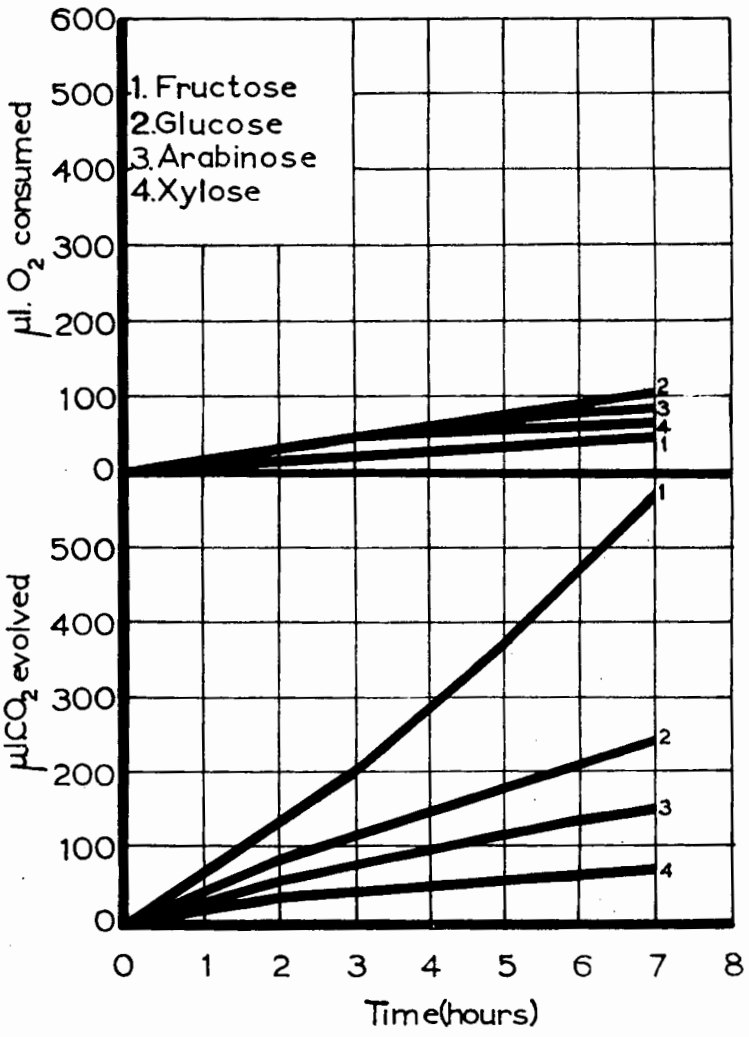


Fig.13: Oxygen consumption and carbon dioxide evolution during utilization of fructose, glucose, arabinose and xylose by cell suspensions of L.brevis.

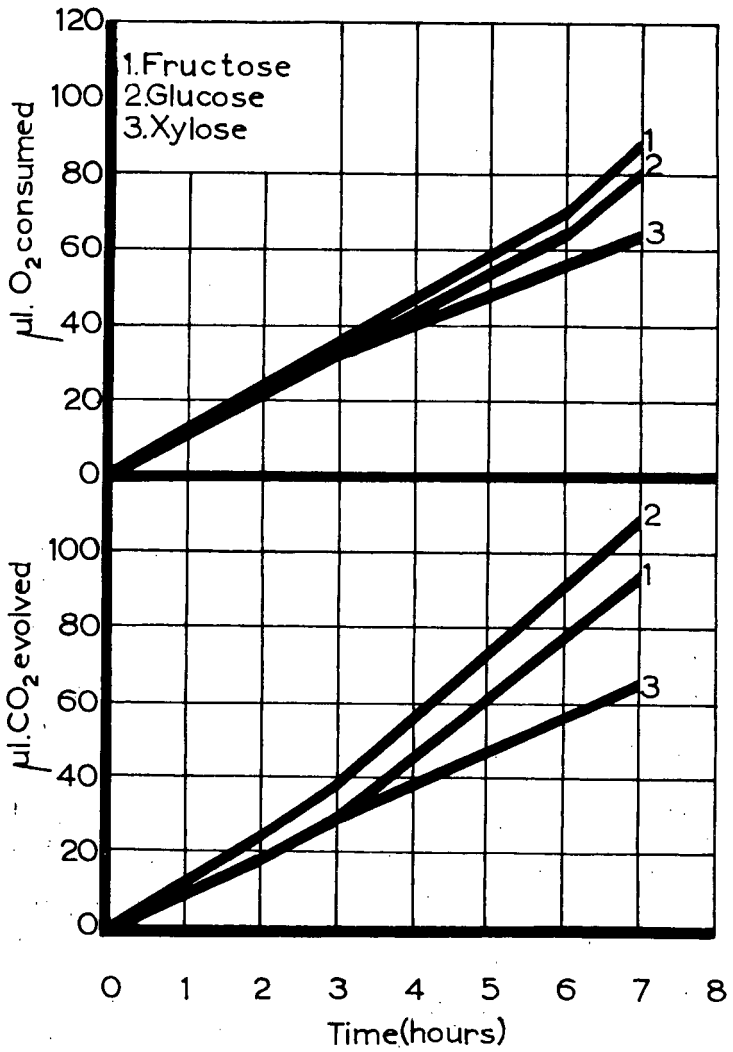


Fig.14: Oxygen consumption and carbon dioxide evolution during utilization of fructose and glucose by cell suspensions of L.leichmannii and during utilization of xylose by cell suspensions of L.leichmannii strain 2.

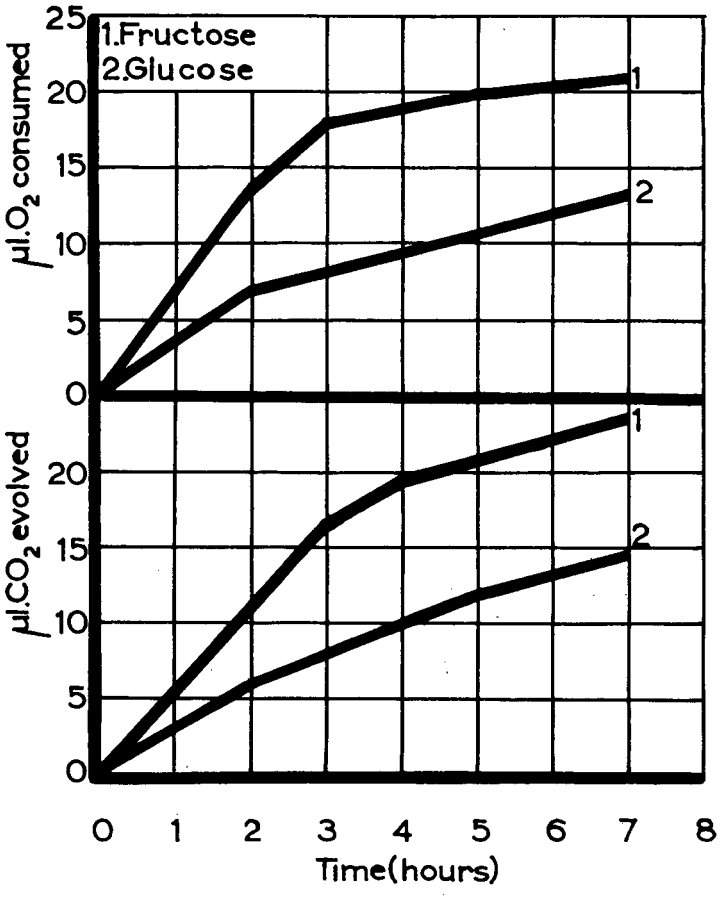


Fig.15: Oxygen consumption and carbon dioxide evolution during utilization of fructose and glucose by cell suspensions of P.cerevisiae.

(ii) All the heterofermentative strains evolved carbon dioxide at a faster rate from fructose than from glucose, except L. buchneri for which the reverse was true.

(iii) The homofermentative L. leichmannii evolved carbon dioxide at a faster rate from glucose than from fructose, the reverse being true for P. cerevisiae.

(iv) With L. brevis the rate of oxygen consumption and carbon dioxide evolution during l-arabinose utilisation was of the same order as that during d-xylose utilisation. The organism designated Lactobacillus sp., consumed oxygen at a more or less similar rate during utilisation of these two pentoses, but produced considerably less carbon dioxide from arabinose than from xylose.

(v) In all cases where both pentoses and hexoses were utilised, carbon dioxide was produced at a considerably faster rate from the hexoses than from pentoses.

(vi) The small amounts of oxygen consumed and carbon dioxide evolved by P. cerevisiae reflect the relatively inactive nature of this organism.

In order to investigate the dissimilation of the carbohydrates by growing cultures of the seven selected bacterial strains, large-scale fermentations were conducted on these substrates (at pH 4.7 to 4.8), (see Page 14). The number of strains used in the different experiments varied according to their ability to ferment the substrates employed (see Table 1).

Fermentative...

Fermentative dissimilation of sugars:

Hexoses: (i) d-Glucose:- The results of Pederson (1929) showed that as much as 88 per cent of the glucose fermented by the homofermentative L. plantarum is converted to lactic acid. In addition only traces of acetic acid and ethyl alcohol were found. On the other hand it appears that the product-patterns of glucose fermentation by the heterofermentative lactic acid bacteria vary in degree from carbon dioxide, ethyl alcohol and lactic acid in equimolar quantities (Peterson and Fred, 1920; Pederson, 1929) to complex mixtures including, in addition, substantial quantities of acetic acid and glycerol (Nelson and Werkman, 1935).

Data representing the fermentative dissimilation of glucose by six lactic acid bacteria from South African dry wines are given in Table 5.

Table 5...

Table 5: Fermentative dissimilation of d-glucose by six lactic acid bacteria from South African dry wines.

	Homofermentative.				Heterofermentative.							
	L. leichmannii		P. cerevisiae.		L. brevis		L. hilgardii		Lactob. sp.		L. buchneri	
	mMol/l.	% ** Carbon.	mMol/l.	% Carbon.	mMol./l.	% Carbon.	mMol./l.	% Carbon.	mMol./l.	% Carbon.	mMol./l.	% Carbon
Residual glucose *	61.48		78.14		10.82		32.67		44.44		13.38	
Glucose fermented	38.52		21.86		89.18		67.33		55.56		86.62	
Products formed:												
Carbon dioxide	6.49	2.81	5.64	4.30	82.46	15.41	67.44	16.69	50.77	15.23	86.56	16.66
Ethyl alcohol	6.33	5.48	4.83	7.37	58.33	21.80	63.80	31.59	46.08	27.65	73.33	28.22
Acetic acid	1.34	1.16	1.52	2.32	26.88	10.05	10.81	5.35	3.09	1.85	16.19	6.23
Lactic acid	67.49	87.60	34.87	79.76	69.03	38.70	55.29	41.06	58.20	52.38	62.75	36.22
Glycerol	1.45	1.88	1.40	3.20	20.19	11.32	6.28	4.66	1.04	0.94	16.70	9.64
Acetoin	0.10	0.17	0.08	0.24	-	-	-	-	-	-	-	-
Diacetyl	0.06	0.10	0.05	0.15	-	-	-	-	-	-	-	-
% Carbon recovery		99.2		97.3		97.3		99.4		98.1		97.0

* Glucose added - 100 mMol/l.

** Calculation for % Carbon.

Example for CO_2: $\frac{6.49 \times 12 \text{ (C in } CO_2) \times 100}{38.52 \times 72 \text{ (C in glucose)}}$

$= \frac{6.49 \times 12 \times 100}{38.52 \times 72}$

$= 2.81$

The homofermentative organisms, especially P. cerevisiae, utilised a relatively small amount of the added glucose. In both cases 80 per cent or more of the carbon dissimilated is accounted for as lactic acid; a fact which makes these two organisms similar in this respect to the homofermentative strains studied by Pederson (1929).

Our present knowledge of the mechanism of homofermentation is based primarily on the results of studies employing isotopic glucose. (Gibbs, Dumrose, Bennett and Bubeck, 1950; Gibbs, Sokatch and Gunsalus, 1955). The results of these investigations imply that the homofermentative lactic acid bacteria fermentatively dissimilate glucose via a classical Embden-Meyerhof glycolytic route.

The homofermentative organisms appear to possess at least some carboxylase activity, as reflected by the small amounts of carbon dioxide produced during fermentation. In both cases the molar quantity of carbon dioxide is more or less equal to that of the acetic acid plus the ethyl alcohol.

The formation of small amounts of glycerol is seen as evidence supporting the view of Gibbs et al (1950) that dihydroxyacetone or dihydroxyacetone diphosphate is probably formed as an intermediate during homofermentation of glucose.

The presence of the neutral volatile products acetoin and diacetyl among the end products of the homofermentative organisms, is of considerable interest to the enologist. These products are known to be the major causes of off-flavours and odours

developed in concentrated orange juice and have also been associated with "beer sickness" (Vaughn and Tchelistcheff, 1957).

Diacetyl production seems to be characteristic of P. cerevisiae (Jensen and Seeley, 1954), as for many homofermentative lactobacilli (Christensen and Pederson, 1958. Moreover, Rowatt (1951) has shown that L. plantarum produces acetoin and carbon dioxide from pyruvate. This reaction is known to occur in numerous bacteria, amongst others Clostridium acetobutylicum (Wilson, Peterson and Fred, 1927), Aerobacter aerogenes (Silverman and Werkman, 1941) and Micrococcus pyogenes var. aureus (Watt, 1949).

The heterofermentative organisms, especially L. brevis and L. buchneri, dissimilated considerable quantities of the added glucose. These two organisms, whilst producing relatively greater amounts of acetic acid than the other organisms tested, produced comparatively more glycerol. The quantity of glycerol produced was in most cases less than that of the acetic acid. The heterofermentative strains of Nelson and Werkman (1935) produced, during anaerobic dissimilation of glucose, quantities of glycerol equivalent to twice that of the acetic acid. Under the conditions in which these fermentations were carried out, the heterofermentative wine lactobacilli produced these compounds in approximately a 1 : 1 ratio. However, the data in Table 5 alone does not warrant the assumption that acetic acid and glycerol are produced in a fixed ratio during the fermentation of glucose by heterofermentative lactobacilli.

In...

In contrast to the homofermentative organisms, only 36 per cent (L. buchneri) to 52 per cent (Lactobacillus sp.) of the carbon dissimilated by the heterofermentative organisms is accounted for as lactic acid.

The mechanism of glucose fermentation by the heterofermentative lactic acid bacteria was elucidated mainly by the investigations of De Moss, Bard and Gunsalus (1951) and studies employing a tracer technique (Gunsalus and Gibbs, 1952; Gibbs et al, 1955). The results obtained by these investigators indicate that these bacteria lack aldolase and that their fermentative pattern conforms to the present concept of an anaerobic hexosemonophosphate pathway as the mechanism of glucose fermentation.

With all four heterofermentative organisms the carbon dioxide evolved per mole of substrate dissimilated approaches a ratio of 1 : 1. This result supports the view that the first step in the heterofermentation of glucose is the splitting of the hexose molecule to yield carbon dioxide and ribulose-5-phosphate (Eltz and Vandemark, 1960).

The results of Gunsalus and Gibbs (1952) with Leuconostoc mesenteroides indicated that the 5-carbon compound is subsequently split to yield a 3-carbon and a 2-carbon intermediate. Furthermore, Eltz and Vandemark (1960) have shown that sonic extracts of fructose-grown cells of L. brevis actively dissimilate ribose-5-phosphate, in the presence of glutathione, phosphate, magnesium and thiamine pyrophosphate, to acetyl phosphate and glyceraldehyde-3-

phosphate...

phosphate. Last-named investigators were able to demonstrate in these sonic extracts the presence of triosephosphate dehydrogenase, phosphoglycerate kinase and lactic acid dehydrogenase. This finding, together with the evidence in the literature for the presence of enolase (Stone and Werkman, 1937) implicate the classical route of lactate formation from glyceraldehyde-3-phosphate. Other enzymatic data indicate that glycerol is formed through reduction of dihydroxyacetone phosphate to α-glycerol phosphate by a DPN-linked α-glycerol phosphate dehydrogenase, followed by hydrolysis of the latter by a specific phosphatase (Schlenck, 1951). Since aldolase could not be demonstrated in the heterofermentative lactic acid bacteria (De Moss et al, 1951; Eltz and Vandemark, 1960) and since no formation of a symmetrical 3-carbon compound could be shown during hetero-lactic fermentation (Gibbs et al, 1955), the mechanism of glycerol formation by these bacteria remains to be determined.

Eltz and Vandemark (1960) showed acetokinase to be present in extracts of L. brevis to catalyse the formation of acetate from acetyl phosphate. It is known that acetate added during hetero-lactic fermentation is reduced to ethanol (Gunsalus and Gibbs, 1952). Since Eltz and Vandemark (1960) demonstrated the presence of ethanol dehydrogenase in L. brevis this finding does not exclude the possibility that ethanol may arise via other mechanisms. However, no route of acetaldehyde formation from acetic acetate or acetyl phosphate could reportedly be shown in this organism.

If,...

If, as indicated by the results in Table 5, the first step in the fermentative dissimilation of glucose by the heterofermentative wine lactobacilli is the liberation of carbon dioxide from the hexose molecule, the molar quantity of the ribulose-5-phosphate formed to substrate utilised should also approach a 1 : 1 ratio. Furthermore, the theoretical molar quantity of ribulose-5-phosphate required for the formation of the end products, as found, can be calculated; assuming that the 5-carbon intermediate is split into a 3-carbon compound, which yields lactate and glycerol, and a 2-carbon compound which yields acetate and ethanol. Theoretically the total of the molar quantities of 3-carbon end products (lactate + glycerol), and that of the 2-carbon end products (acetate + ethanol) should then be equal to the amount of ribulose-5-phosphate.

The data obtained with the heterofermentative wine lactobacilli (Table 5) were analysed in this manner and the results are presented in Table 6.

Table 6...

Table 6: The molar amounts of total 2-carbon and total 3-carbon compounds produced during dissimilation of d-glucose by the four heterofermentative lactobacilli.

	L. buchneri mMol/l.	L. brevis mMol/l.	L. hilgardii mMol/l.	Lactob. sp. mMol/l.
Glucose utilised	86.62	89.18	67.33	55.56
Lactate + glycerol	79.45	89.22	61.57	59.24
Acetate + ethanol	89.52	85.21	74.61	49.17
Ri-5-P (theoretically)	84.49	87.22	68.09	54.22
$\frac{\text{Ri-5-P}}{\text{Glucose utilised}}$	0.98	0.98	1.01	0.98

In this scheme the theoretical amount of 5-carbon compound formed is represented by two values; lactate + glycerol and acetate + ethanol. The theoretical amount of ribulose-5-phosphate in Table 6 represents the average of these two values. It is evident from Table 6 that the molar ratio of ribulose-5-phosphate to substrate utilised approaches 1 : 1 in all four cases. These results indicate, as do the amounts of carbon dioxide produced, that the heterofermentative wine lactobacilli use the hexosemonophosphate pathway for glucose dissimilation.

It is of interest that no acetoin or diacetyl was produced from glucose by any of the heterofermentative organisms. The strain of L. brevis

studied...

studied by Walker (1959), while producing no acetoin from pyruvate under ordinary conditions, appeared to possess a latent acetoin-forming system demonstrateable only in the presence of inhibitors of the suitable pathways.

(ii) d-Fructose:- The fermentation of this hexose by the homofermentative L. plantarum differs very little from its fermentation of glucose (Pederson, 1929).

The heterofermentative lactic acid bacteria are unique in their ability to form mannitol from fructose. Yields of mannitol accounting for as much as 70 per cent of the fructose fermented were found by Peterson and Fred (1920).

Data obtained for the fermentative dissimilation of fructose by the wine lactic acid bacteria isolated during this investigation are given in Table 7.

Table 7...

Table 7: Fermentative dissimilation of d-fructose by the six lactic acid bacteria from South African dry wines.

	Homofermentative.				Heterofermentative.							
	L. leichmannii.		P. cerevisiae.		L. brevis.		L. hilgardii.		Lactob. sp.		L. buchneri.	
	mMol/l.	% Carbon.	mMol/l.	% Carbon.	mMol/l.	% Carbon.	mMol/l.	% Carbon.	mMol/l.	% Carbon.	mMol/l.	% Carbon.
Residual fructose*	70.83		87.39		8.07		1.82		24.10		21.21	
Fructose fermented	29.17		12.61		91.93		98.18		75.90		78.79	
Products formed:												
Mannitol	-	-	-	-	39.94	43.45	51.60	52.56	52.96	69.78	49.08	62.29
Carbon dioxide	9.41	5.38	7.20	9.52	48.92	8.87	45.96	7.80	18.95	4.16	29.39	6.22
Ethyl alcohol	8.58	9.81	4.75	12.56	37.01	13.42	28.76	9.76	6.88	3.02	9.17	3.88
Acetic acid	1.86	2.13	1.49	3.94	16.91	6.13	14.37	4.88	9.60	4.22	19.40	8.21
Lactic acid	47.50	81.42	17.53	69.51	42.68	23.21	28.42	14.47	21.02	13.85	26.84	17.03
Glycerol	1.45	2.49	0.54	2.14	1.79	0.97	12.61	6.42	1.80	1.19	0.40	0.25
Acetoin	0.12	0.27	0.09	0.48	-	-	-	-	-	-	-	-
Diacetyl	0.05	0.11	0.08	0.42	-	-	-	-	-	-	-	-
% Carbon recovery		101.6		98.6		96.1		95.9		96.2		97.9

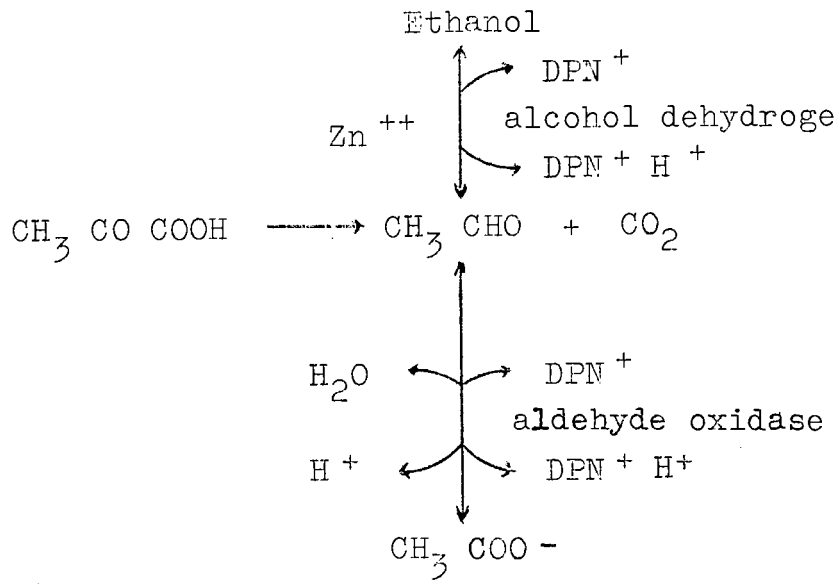
* Fructose added - 100 mMol/l.

No...

No mannitol production by the homofermentative organisms could be demonstrated.

Although the fermentation of fructose by the two homofermentative organisms agrees qualitatively with that of glucose (Table 5), quantitative differences exist. Comparatively less of the added sugar was utilised while relatively more carbon dioxide, ethanol and acetic acid were produced.

The molar amount of carbon dioxide produced by the two homofermentative organisms was approximately equal to that of the acetic acid plus the ethanol, as in the fermentation of glucose by these organisms. These results indicate that acetic acid and ethanol are probably formed from pyruvate via a common precursor by means of the following reaction:-



The...

The results obtained with fructose as well as those for glucose fermentation by the homofermentative lactic acid bacteria, are compatable with an Embden-Meyerhof glycolytic route.

It is evident from Table 7 that, in contrast to the homofermentative strains, the heterofermentative organisms produced considerable amounts of mannitol. The quantity of mannitol formed accounted for from 45.5 per cent (L. brevis) to 69.8 per cent (Lactobacillus sp.) of the fructose fermented.

All the heterofermentative organisms except L. buchneri utilised greater amounts of the added fructose as compared to glucose, a fact which is in accord with the manometric data (Figures 11 to 15).

Lactobacillus hilgardii formed considerably more glycerol from fructose than the other heterofermentative organisms which produced but trace amounts of this compound. No acetoin or diacetyl could be demonstrated among the end products of these fermentations.

Studies by Nelson and Werkman (1936,1940) indicated that fructose acts as its own hydrogen acceptor during fermentation and is reduced to mannitol. Eltz and Vandemark (1960) demonstrated, in sonic extracts of fructose-grown cells of L. brevis, the presence of a DPN-specific mannitol dehydrogenase which catalyses the reduction of fructose but not of glucose. They also presented evidence for the presence in these extracts of fructokinase, hexosephosphate isomerase,

glucose-...

glucose-6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase which, in the presence of adenosine triphosphate and suitable hydrogen acceptors, affect the overall oxidation of fructose to carbon dioxide and ribulose-5-phosphate. These results indicate that the utilised fructose which is not reduced to mannitol, is dissimilated via the hexosemonophosphate pathway.

The molar ratio of carbon dioxide evolved to fructose oxidised, i.e. molar quantity of fructose fermented minus that of the mannitol formed, should accordingly approach 1 : 1. The theoretical molar quantity of ribulose-5-phosphate necessary to yield the 2-carbon and 3-carbon compounds found (Table 7) can also be calculated as in the case of glucose fermentation (Table 6).

Table 8: The molar amounts of carbon dioxide, total 2-carbon and total 3-carbon compounds produced during fermentative dissimilation of d-fructose by the four heterofermentative lactobacilli.

	L. buchneri. mMol/l.	L. brevis. mMol/l.	L. hilgardii. mMol/l.	Lactob. sp. mMol/l.
Fructose oxidised	29.71	51.99	46.58	22.94
Carbon dioxide	29.39	48.92	45.96	18.95
Acetate + ethanol	28.57	53.92	43.13	16.48
Lactate + glycerol	27.24	44.47	41.03	22.82
Ri-5-P (theoretically)	27.91	49.20	42.08	19.65
$\frac{CO_2}{\text{Fructose oxidised}}$	0.99	0.94	0.99	0.83
$\frac{\text{Ri-5-P}}{\text{Fructose oxidised}}$	0.94	0.95	0.90	0.86

The...

The theoretical amount of Ri-5-P in this case was calculated as in Table 6.

It is evident that the molar ratio of carbon dioxide evolved, as well as that of the theoretical quantity of ribulose-5-phosphate to fructose oxidised, approaches 1 : 1 in all four instances. These results are in agreement with the view that the dissimilated fructose not reduced to mannitol is fermented via the hexosemonophosphate pathway.

Pentoses: (i) d-Xylose:- It is known that many of the lactic acid bacteria from European wines are able to utilise this pentose in addition to glucose and fructose (Müller-Thurgau and Osterwalder, 1913, 1918). For some time xylose fermentation was associated with studies involving spoiled wines (Weinstein and Rettger, 1932).

Fred, Peterson and Anderson (1921) showed that both homo- and heterofermentative lactobacilli produce one mole each of lactic acid and acetic acid per mole of xylose utilised. Weinstein and Rettger (1932) reported that 88 to 90 per cent of the xylose utilised by L. pentoaceticus (syn. L. brevis) was converted to volatile and non-volatile acids.

Table 9 shows the results obtained for the fermentation of xylose by four lactobacilli from South African dry wines.

Table 9...

Table 9: Fermentative dissimilation of d-xylose by four lactobacilli from South African dry wines.

	Homo-fermentative.				Heterofermentative.			
	L. leichmannii. (Strain 2)		L. brevis.		L. hilgardii.		Lactob. sp.	
	mMol/l.	% Carbon.	mMol/l.	% Carbon.	mMol/l.	% Carbon.	mMol/l.	% Carbon.
Residual xylose [x]	13.44		25.24		22.85		19.52	
Xylose fermented	86.56		74.76		77.15		80.48	
Products formed:								
Carbon dioxide	15.52	3.59	17.46	4.67	7.85	2.04	10.68	2.65
Ethyl alcohol	7.19	3.32	6.35	3.40	3.96	2.05	4.79	2.38
Acetic acid	80.06	37.00	67.64	36.19	74.55	38.65	77.62	38.58
Lactic acid	75.41	52.27	56.94	45.70	69.67	54.18	74.32	55.41
Glycerol	1.81	1.26	5.44	4.37	2.17	1.69	1.27	0.95
Diacetyl	0.02	0.02	-	-	-	-	-	-
% Carbon recovery		97.5		94.3		98.6		100.0

[x] Xylose added - 100 mMol/l.

It...

It appears from these data that the organisms produce from xylose small amounts of products other than volatile and non-volatile acids. However, acetic acid and lactic acid accounted for from 81.9 per cent (L. brevis) to 94 per cent (Lactobacillus sp.) of the xylose utilised.

The presence of small amounts of carbon dioxide among the end products are in accordance with results obtained with other lactobacilli, both homo- and heterofermentative (Fred et al, 1921). It is noteworthy that the homofermentative L. leichmannii Strain 2 produced more carbon dioxide from xylose than two of the three heterofermentative lactobacilli. This fact and the similar product yields, suggests a xylose fermentation pathway essentially common to both the homo- and heterofermentative lactobacilli.

Results of tracer studies on xylose fermentation (Lampen, Gest and Sowden, 1951; Gest and Lampen, 1952) strongly supported the hypothesis that the pentose molecule is cleaved into 2-carbon and 3-carbon units, as implied by the results of Fred et al (1921), and indicated that cleavage occurs between the second and third carbons. The results of Bernstein (1953) confirmed this view.

According to enzymatic data recently obtained with L. plantarum (syn. L. pentosus) xylose is fermentatively dissimilated via the following pathway (see Heath, Hurwitz, Horecker and Ginsburg, 1958 b):-

d-xylose...

d-xylose $\underset{\text{xyloisomerase}}{\rightleftharpoons}$ d-xylulose

(Mitsuhashi and Lampen, 1953)

$\underset{\text{xylulosekinase, ATP.}}{\rightarrow}$ xylulose-5-P.

(Stumpf and Horecker, 1956)

$\underset{\text{thiamine pyrophosphate}}{\overset{\text{phosphoketolase, Pi}}{\rightarrow}}$ Acetylphosphate + d-glyceraldehyde phosphate.

(Heath et al, 1958 b)

It was also shown by Heath et al (1958 b) that crude extracts of this organism contain acetokinase to catalise the formation of acetate from acetyl phosphate. The triose phosphate is reportedly converted to lactic acid via the Embden-Meyerhof route.

The molar ratio of acetic acid to lactic acid in Table 9 approaches 1 : 1 with all four wine lactobacilli. These results are thus compatible with the existing data mentioned above. However, the scheme proposed by Heath et al (1958 b) for xylose fermentation by L. plantarum does not elucidate the mechanism by which glycerol, ethanol and carbon dioxide are formed.

Diacetyl was produced from xylose only by the homofermentative organism. No acetoin production could be demonstrated.

(ii) l-Arabinose:- The lactobacilli studied by Fred et al. (1921), including both homo- and heterofermentative types, fermented l-arabinose to end

products...

products similar to those produced from d-xylose.

Peterson (1929) showed that certain lactobacilli from spoiled tomato products dissimilate l-arabinose to form acetic acid and lactic acid in equimolar quantities.

Data representing the dissimilation of arabinose by three heterofermentative lactobacilli from South African dry wines are given in Table 10.

Table 10: Fermentative dissimilation of l-arabinose by three heterofermentative lactobacilli from South African dry wines.

	L. buchneri.		L. brevis.		Lactob. sp.	
	mMol/l.	% Carbon.	mMol/l.	% Carbon.	mMol/l.	% Carbon.
Residual arabinose *	17.85		30.06		91.38	
Arabinose fermented	82.15		69.94		8.62	
Products formed:						
Carbon dioxide	6.89	1.68	12.12	3.47	4.63	10.74
Ethyl alcohol	5.21	2.54	5.78	3.31	1.92	8.91
Acetic acid	76.10	37.06	63.14	36.11	8.23	38.19
Lactic acid	75.04	54.81	57.64	49.45	6.47	45.03
Glycerol	1.09	0.80	4.62	3.96	-	-
% Carbon recovery		96.9		96.3		102.9

* Arabinose added - 100 mMol/l.

It appears that L. buchneri and L. brevis vigorously fermented l-arabinose. The organism designated Lactobacillus sp. fermented d-xylose vigorously (Table 9), but utilised only a fractional part of the

added...

added arabinose. l-Arabinose fermentation by L. brevis, on the other hand, resembled closely its fermentation of d-xylose, both in end product yield and amount of pentose dissimilated.

The results of Fred et al (1921) implied that the lactobacilli ferment l-arabinose and d-xylose by essentially similar mechanisms. This view was substantiated by the results of isotopic studies (Rappaport, Barker and Hassid, 1951).

Recent investigations on the enzymatic constitution of arabinose-grown cells of L. plantarum, have elucidated the mechanism of l-arabinose fermentation by the lactobacilli. The results of these studies indicate that the fermentation proceeds according to the following reactions (see Heath et al, 1958 b):-

l-arabinose $\xrightleftharpoons{\text{l-arabinoseisomerase}}$ l-ribulose
(Heath, Horecker, Smyrniotis and Takagi, 1958 a)

$\xrightarrow{\text{ribulokinase, ATP.}}$ l-ribulose-5-P.
(Burma and Horecker, 1958 a).

$\xrightleftharpoons{\text{l-Ri-5-P 4-epimerase}}$ d-xylulose-5-P.
(Burma and Horecker, 1958 b).

According to Heath et al (1958 a) xylulose-5-phosphate is subsequently converted, through phosphoketolase action, to acetyl phosphate and triose-phosphate. These compounds are then converted to acetate and lactate respectively, as in the case of

d-xylose...

d-xylose fermentation by this organism, making xylulose-5-phosphate the key intermediate in pentose fermentation by L. plantarum.

This scheme, as that established for d-xylose fermentation, does not elucidate the mechanism by which carbon dioxide, ethanol and glycerol are formed during pentose fermentation.

Glycerol is presumably formed from the 3-carbon intermediate by a mechanism similar to that functioning during hexose fermentation by these organisms.

There seems to be two possibilities as to the way in which the ethanol may arise during pentose fermentation:-

(i) Through the reductive decarboxylation of the intermediate pyruvate formed in the Embden-Meyerhof route, and

(ii) From the 2-carbon intermediate, as in the hexose monophosphate pathway.

However, the considerable amounts of carbon dioxide encountered during pentose fermentation (Tables 9 and 10) suggest that ethanol arises by reaction (i).

The rest of the carbon dioxide encountered possibly arises from the secondary fermentation of lactate.

Heath et al (1958 b) concluded that pentose fermentation is utilised primarily for the production of energy. They demonstrated that one equivalent of ATP is consumed during fermentation, while three equivalents of ATP are produced through phosphoketolase

action...

action. The net yield of two equivalents of ATP per mole of sugar fermented is the same as that derived from the fermentation of hexose.

The high amounts of acetic acid produced from pentose by the wine lactobacilli are significant, since a sudden increase in the volatile acidity of a wine is usually among the first symptoms of this kind of bacterial contamination.

Degradation of organic acids.

l-Malic acid (malo-lactic fermentation):

It has been known for many years that the fixed acid content of wines can be decreased through bacterial action.

The earlier workers Nöllner, Nicklés and Pasteur (according to Vaughn and Tchelistcheff, 1957) were primarily interested in the decomposition of tartrates. Consequently, it was not until after the investigations of Müller-Thurgau (according to Vaughn and Tchlistcheff, 1957), that proper attention was given to the fermentation of malic acid in wines. Soon afterwards Koch (according to Luthi, 1957) succeeded in isolating a bacterial strain with the assistance of which he could artificially reduce the malic acid content of wine. Most of the organisms studied by Müller-Thurgau and Osterwalder (1913) could ferment l-malic acid, yielding lactic acid and carbon dioxide.

The...

The malo-lactic fermentation is known to have a definite beneficial effect on the quality of many European wines. Ferre (according to Suverkrop and Tchelistcheff, 1949) indicated that this fermentation during the early stages of storage was largely responsible for the quality of wines from the French Burgundy district. Ribereau-Gayon (according to Vaughn and Tchelistcheff, 1957) confirmed that the malo-lactic fermentation is desirable for the "Grand Vins" of this wine-producing region. In Northern Portugal the successful production of the "Vinhos Verdes" is dependent on a vigorous malic acid fermentation (Gomez, da Silva, Babo and Guimaraes, 1956 a). These investigators (1956 b) were also able to demonstrate that wines which had been deacidified by the use of selected bacterial strains are superior to those produced by spontaneous bacterial fermentation. It is thus understandable why malo-lactic fermentation is not only considered desirable in many European wine-producing areas, but is fostered as the second fermentation.

The mechanism of the malo-lactic fermentation was elucidated by the isolation, first from pigeon liver (Ochoa, Mehler and Kornberg, 1947) and later from l-malic acid adapted cells of L. arabinosus (Korkes, del Campillo and Ochoa, 1950), of a "malic" enzyme, mediating the conversion of l-malic acid to lactic acid and carbon dioxide. Evidence presented by last-named investigators indicated that in L. arabinosus this reaction is a DPN-linked dismutation between l-malate and pyruvate, resulting from

the...

the interaction of lactic dehydrogenase with a DPN-specific "malic" enzyme as shown by the following reactions:-

$$\text{l-malate} + \text{DPN}_{ox} \xrightleftharpoons{Mn\ ++} \text{Pyruvate} + CO_2 + \text{DPN}_{red.}$$
("malic" enzyme)

$$\text{Pyruvate} + \text{DPN}_{red.} \rightleftharpoons \text{lactate} + \text{DPN}_{ox.}$$
(lactic dehydrogenase).

$$\underline{\text{Sum:}}\ \text{l-malate} \xrightleftharpoons{(Mn\ ++,\ DPN)} \text{lactate} + CO_2.$$

The reversibility of this reaction was demonstrated with isotopic carbon dioxide, but its equilibrium position is overwhelmingly in favour of decarboxylation.

Jerchel, Flesch and Bauer (according to Luthi, 1959) recapitulated the work on the decomposition of l-malic acid, using purified enzyme extracts from both L. arabinosus and B. gracile (syn. Leuconostoc mesenteroides). They found that there were two pathways by which l-malic acid could be decomposed, depending upon the method used for the lysis of the cells. By careful treatment, using the method of Korkes et al (1950), the breakdown proceeds directly from pyruvic to lactic acid. By using other enzyme preparations, a further intermediate (oxalacetic acid) could be obtained. This intermediate was subsequently converted to pyruvic and lactic acid.

Schanderl (1950) was apparently one of the first to note that the malic acid breakdown reaction

is...

is in reality endothermic. A satisfactory explanation of this reaction is as yet lacking. As a result of various reports claiming an increase in ammonia-nitrogen in wine following malo-lactic fermentation, the possibility of a concomitant breakdown of higher nitrogen compounds has been suggested (Schanderl, 1950). Luthi (1959), on the other hand, while convinced that higher nitrogen compounds do play a role, does not believe that there is an accumulation of ammonia during this acid breakdown.

Malic acid decomposition has been reported in L. plantarum, L. brevis, L. buchneri, L. fermenti and L. hilgardii (Vaughn et al, 1949; Vaughn, 1955). It is thus clear that malo-lactic fermentation is a characteristic widely distributed in the genus Lactobacillus.

Preliminary tests with the agar-closure technique indicated the presence of the malic enzyme in the lactic acid bacteria from South African dry wines. To verify this finding and to gain an impression of the speed of the reaction, oxygen consumption and carbon dioxide evolution during l-malic acid utilisation by resting cell suspensions of six of these bacteria were studied manometrically. This study was conducted at pH levels of 3.9 and 4.4. The results obtained at pH 3.9 are presented in Table 11 and Figure 16.

Table 11...

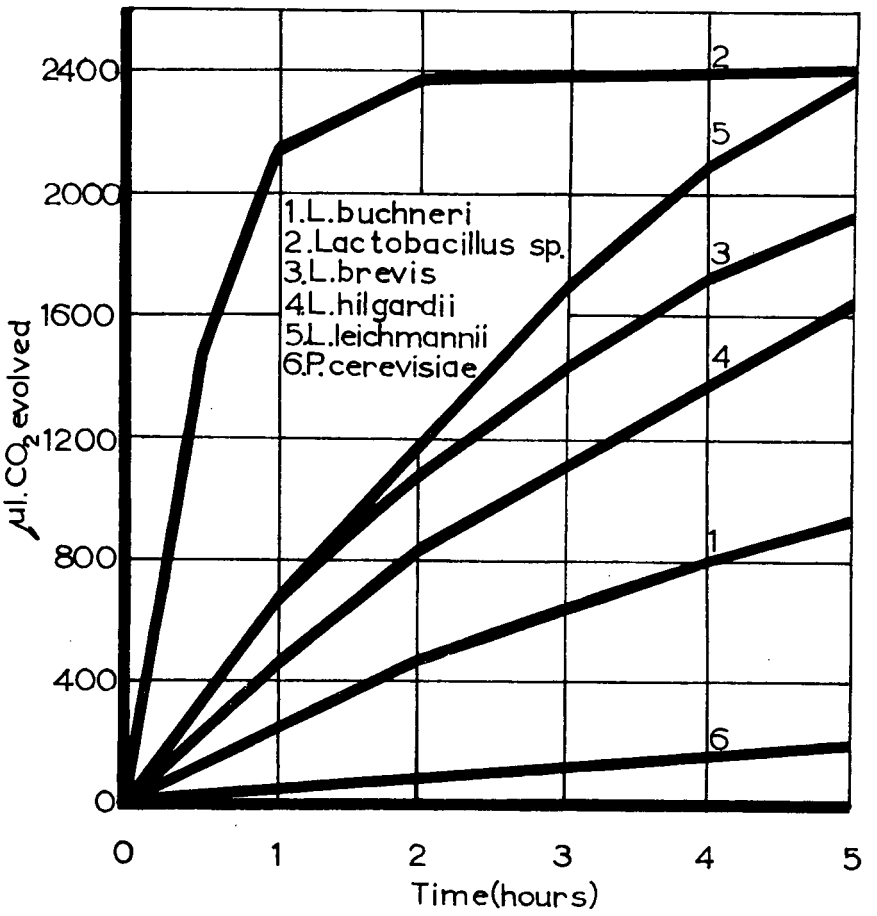


Fig.16: Decarboxylation of l-malic acid, at pH 3·9, by malate adapted cell suspensions of lactic acid bacteria from wine.

Table 11: Oxygen consumption (μ l) during l-malic acid utilisation at pH 3.9, by l-malate adapted cells of six lactic acid bacteria from South African dry wines.

Time. (hours)	Homo-fermentative.		Heterofermentative.			
	L. leich-mannii.	P. cerevi-siae.	L. brevis.	L. hil-gardii.	L. buch-neri.	Lactob. sp.
1	12.57	2.51	6.05	14.58	23.63	24.48
2	18.09	3.01	19.62	22.12	47.26	36.15
3	22.61	3.51	29.67	29.16	70.38	42.32
4	27.63	4.52	39.23	34.19	91.49	47.03
5	31.92	5.00	50.29	41.73	113.10	53.54

Considerable quantities of carbon dioxide were evolved during l-malic acid utilisation while comparatively small amounts of oxygen were consumed. Cell suspensions which were not adapted to malate evolved only small amounts of carbon dioxide, demonstrating the presence of malic enzyme in these organisms and confirming the adaptable nature thereof.

All the organisms tested evolved less carbon dioxide and consumed more oxygen at pH 4.4 than at pH 3.9. It is obvious that the optimum pH for

malic...

malic acid decomposition by these organisms is considerably lower than that for sugar utilisation.

In order to study the dissimilation of l-malic acid by growing cultures of these wine lactic acid bacteria, fermentation tests were conducted on this acid at pH 3.9. Material balances for these fermentations are given in Table 12.

Table 12: Fermentative dissimilation of l-malic acid, at pH 3.9, by six malate-adapted lactic acid bacteria from South African dry wines.

	Homofermentative.				Heterofermentative.							
	L. leichmannii.		P. cerevisiae.		L. brevis.		L. hilgardii.		L. buchneri.		Lactob. sp.	
	mMol/l.	% Carbon.	mMol/l.	% Carbon.	mMol/l.	% Carbon.	mMol/l.	% Carbon.	mMol/l.	% Carbon.	mMol/l.	% Carbon.
Residual malic acid *	21.00		18.09		17.40		20.76		41.42		15.37	
l-Malic acid fermented	29.00		31.91		32.60		29.24		8.58		34.63	
Products formed:												
Lactic acid	23.36	60.41	28.33	66.59	27.59	63.47	26.79	68.72	8.50	74.30	30.13	65.25
Carbon dioxide	29.04	25.04	31.80	24.91	34.85	26.73	28.12	24.04	7.82	22.79	36.53	26.37
Acetic acid	1.07	1.85	-	-	1.84	2.82	1.01	1.73	0.07	0.41	1.34	1.94
Ethyl alcohol	6.02	10.38	4.02	6.30	5.05	7.75	2.43	4.16	0.68	3.96	4.85	7.00
Acetoin	0.20	0.69	-	-	-	-	-	-	-	-	-	-
Diacetyl	0.08	0.28	0.06	0.19	-	-	-	-	-	-	-	-
% Carbon recovery		98.7		98.0		100.8		98.7		101.5		100.6

* l-Malic acid added - 50 mMol/l.

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It appears from these data that the organisms fermented the major part (58 to 73%) of the added acid, except in the case of L. buchneri where only 17 per cent was dissimilated.

The molar ratio of carbon dioxide evolved to l-malic acid fermented approaches 1 : 1 with all six organisms. These results are therefore compatible with the equation established for l-malic acid breakdown in L. arabinosus (Korkes et al, 1950).

All the organisms formed small amounts of ethanol, presumably from the intermediary pyruvate. Small quantities of acetic acid were produced by all the organisms except P. cerevisiae. These results, as those of the sugar fermentations, do not reveal whether the acetic acid is produced via pyruvate or by the secondary fermentation of lactic acid.

Both homofermentative organisms produced diacetyl from l-malic acid while L. leichmannii formed acetoin in addition. Vaughn and Tchelistcheff (1957) already suspected the production of minute amounts of these substances during malo-lactic fermentation.

It is clear from the evidence obtained in these fermentation experiments that there is no clear-cut differentiation between "spoilage" bacteria and "malo-lactic" bacteria. An organism may cause either spoilage or malo-lactic fermentation, depending on the conditions existing in the contaminated wine.

Blanchard et al (1950) have shown that amino acids and glucose are required for maximum malic enzyme formation by L. arabinosus. Vitamins were not

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required for induction if the bacterial cells were harvested from a medium sufficient in these substances. However, cells grown in media deficient in biotin or nicotinic acid required these vitamins for optimal enzyme induction. Recently Deal and Lichstein (1961) demonstrated that the nutritional requirements for malic enzyme synthesis by this organism closely paralleled those necessary for growth.

The presence of malo-lactic bacteria in South African dry wines suggests that, contrary to general belief, malo-lactic fermentation very likely occurs in at least some of these wines. It may be added that this fermentation has been demonstrated in the dry wines of both Australia (Fornachon, 1957) and California (Suverkrop and Tchelistcheff, 1949), two countries possessing wine-producing conditions similar in many respects to those existing in South Africa.

Citric acid:

Bacterial decomposition of citric acid, like the malo-lactic fermentation, has been known for many years. The work of Müller-Thurgau and Osterwalder (1913) indicated that this phenomenon leads to the formation of volatile acid.

Deffner (according to Peynaud, 1956) studied citric acid fermentation by certain lactic acid bacteria in neutral media. Under these conditions acetic acid, formic acid, succinic acid, carbon dioxide and traces of alcohol and acetaldehyde were found as end products. Under the same conditions

oxalacetic...

oxalacetic acid gave rise to the same products and in the same proportions, except for acetic acid of which one molecule less was found. These results caused the author to suggest that during the first stage of the acid breakdown the citric acid molecule is split to yield oxalacetic and acetic acids.

On account of results obtained with Betacoccus cremoris and Streptococcus citrophilus, van Beynum and Pette (1939) concluded that pyruvic acid probably is an intermediate in the citric acid fermentation.

It is known that Streptococcus paracitrovorus is unable to utilise citrate as sole source of carbon for growth, but that in the presence of readily fermentable carbohydrates the acid is rapidly attacked (Slade and Werkman, 1940). It was demonstrated soon afterwards that cell suspensions of this organism grown in the presence of citrate and lactose, subsequently fermented citric acid in the absence of carbohydrate (Slade and Werkman, 1941). These results were interpreted as suggesting that the presence of citrate in the growth medium was responsible for the formation of the enzymes necessary for the fermentation of this acid. By studying the fermentation of citric acid, as well as that of pyruvic and oxalacetic acids, Slade and Werkman (1941) obtained data confirming the view that citric acid breakdown by the lactic acid bacteria proceeds via oxalacetic and pyruvic acids.

The...

The predominant products of citric acid fermentation, in neutral media, by three enterococci and two homofermentative lactobacilli were shown to be acetic acid and carbon dioxide (Campbell and Gunsalus, 1944). Formic acid and lactic acid accounted for most of the remaining carbon. Traces of acetylmethylcarbinol and ethanol were also produced.

Charpentie, Ribereau-Gayon and Peynaud (according to Peynaud, 1956) and Charpentie (according to Peynaud, 1956) studied the fermentation of citric acid, both in neutral media and in wines enriched with the acid. The end products of the fermentation were reported to be carbon dioxide, acetic acid, small quantities of lactic acid, acetoin and 2,3-butylene glycol. Apparently no formic acid was encountered under these conditions.

Preliminary tests, both by the agar-closure technique and paper chromatography, had already indicated that most of the lactic acid bacteria from South African dry wines can decompose citric acid in addition to l-malic acid. In order to study the utilisation of citric acid and l-malic acid by resting cell suspensions of the organisms concerned, manometric determinations were simultaneously conducted at the same two pH levels. The manometric data confirmed that P. cerevisiae and L. buchneri are unable to dissimilate citric acid. Oxygen consumption and carbon dioxide evolution during citric acid utilisation by the other four organisms (at pH 3.9), are indicated in Table 13 and Figure 17.

Table 13...

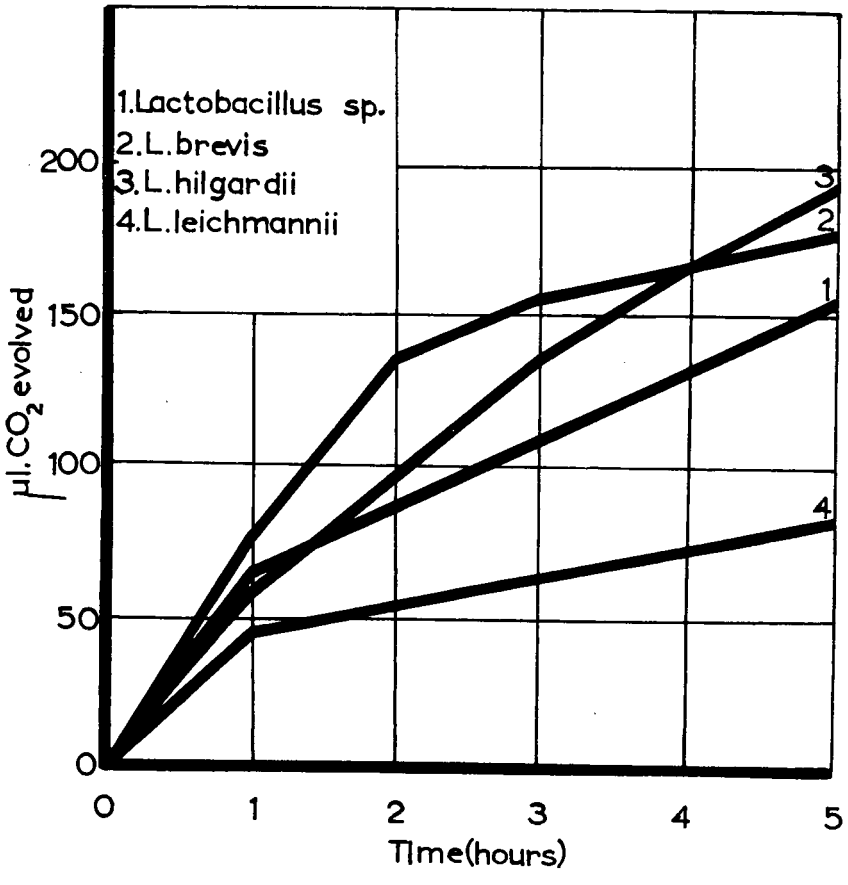

Fig.17: Carbon dioxide production by citrate-adapted cell suspensions of lactobacilli from wine, during citric acid utilization at pH 3·9

Table 13: Oxygen consumption (μ l) by citrate-adapted resting cell suspensions of four lactobacilli from South African dry wines, during citric acid utilisation at pH 3.9.

Time (hours)	Homofermentative.	Heterofermentative.		
	L. leichmannii.	L. brevis	L. hilgardii.	Lactob. sp.
1	13.96	9.00	10.49	13.98
2	18.97	18.48	20.47	22.97
3	20.98	26.46	26.96	30.96
4	23.97	31.48	35.95	36.45
5	27.12	36.96	44.00	41.94

It appears from these data that the oxygen consumption by each of these organisms during citric acid utilisation was very similar, both in rate and quantity, to that during l-malic acid utilisation (Table 11). It is also evident that carbon dioxide was produced at a much faster rate from l-malic acid than from citric acid.

Cell suspensions of these organisms which had not been citrate-adapted could evolve only small quantities of carbon dioxide from citric acid, suggesting that the citric acid degrading enzymes are adaptable in nature.

Lactobacillus brevis and L. hilgardii produced carbon dioxide from citric acid at a faster rate

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at pH 3.9 than at pH 4.4, while the reverse was true for the other two organisms.

As in the case of l-malic acid, the dissimilation of citric acid by growing cultures of the organisms concerned was studied at pH 3.9.

Table 14: Fermentative dissimilation of citric acid at pH 3.9, by four citrate-adapted lactobacilli.

	Homofermentative.				Heterofermentative.			
	L. leichmannii.		L. brevis.		L. hilgardii.		Lactob. sp.	
	mMol/l.	% Carbon.	mMol/l.	% Carbon.	mMol/l.	% Carbon.	mMol/l.	% Carbon.
Residual citric acid*	24.65		41.46		42.44		28.38	
Citric acid fermented	25.35		8.54		7.56		21.62	
Products formed:								
Carbon dioxide	28.25	18.57	8.40	16.39	7.72	17.02	27.66	21.32
Acetic acid	26.18	34.43	9.41	36.73	6.82	30.07	24.78	38.21
Formic acid	4.86	3.20	1.21	2.36	1.07	2.36	5.21	4.02
Lactic acid	16.57	32.68	7.43	43.50	6.30	41.67	12.53	28.98
Succinic acid	0.31	0.82	0.10	0.78	0.12	1.06	0.67	2.07
Ethyl alcohol	2.82	3.71	0.84	3.28	0.68	3.00	2.61	4.02
Acetoin	0.65	1.71	-	-	-	-	-	-
% Carbon recovery		95.1		103.0		95.2		98.6

* Citric acid added - 50 mMol/l.

Lactobacillus...

Lactobacillus leichmannii and the organism designated Lactobacillus sp. dissimilated considerably more of the added citric acid than did the other two organisms.

The homofermentative L. leichmannii produced considerably more acetoin from citric acid than from the other substrates tested. No diacetyl or 2,3-butylene glycol formation could be demonstrated under these conditions.

Except for the small quantities of succinic acid, the end product yields obtained here were essentially the same as those obtained in neutral media by Campbell and Gunsalus (1944) with other lactic acid bacteria; considerable quantitative differences were however observed. In such neutral cultures the lactobacilli studied by these authors formed lactic acid accounting for only six to eight per cent of the citric acid fermented. The wine lactobacilli, under these acid conditions, produced considerably more lactic acid, accounting for from 29 per cent (Lactobacillus sp.) to 43.5 per cent (L. brevis) of the citric acid fermented. This finding is not unexpected as it is known that acid conditions favour the formation of lactic acid (Gunsalus and Niven, 1942).

It was suggested by Campbell and Gunsalus (1944) that the formic acid produced from citric acid by lactic acid bacteria arises from the intermediary pyruvate by the phosphoroclastic reaction, since formic acid is known to be formed from pyruvate and from glucose by certain streptococci (Barron

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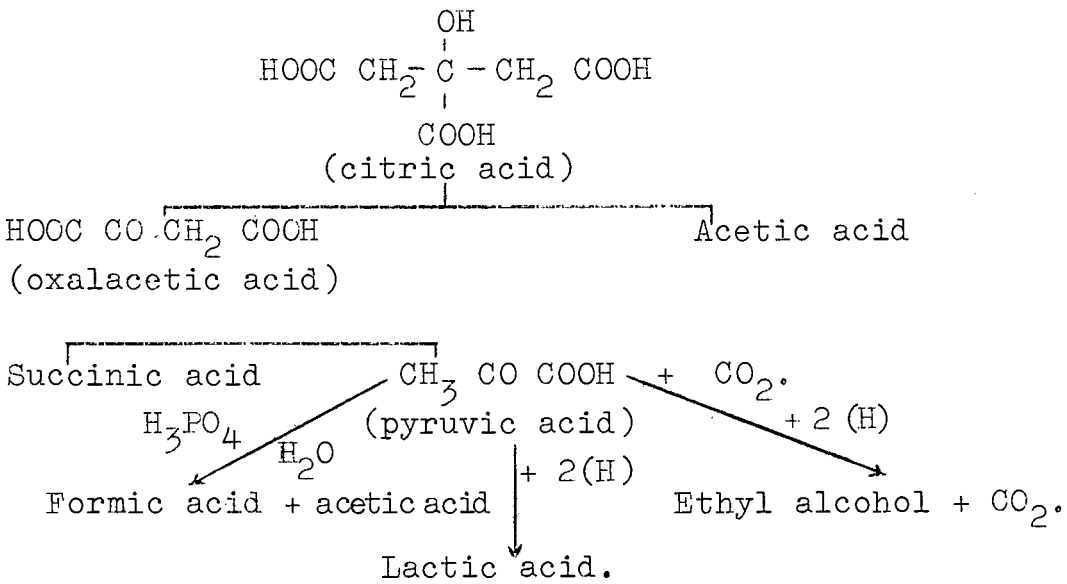
and Lyman, 1939; Gunsalus and Niven, 1942). It was subsequently shown by Utter and Werkman (1944) that this reaction, which also occurs in Escherichia coli, is in reality a phosphoroclastic split of pyruvate into acetyl phosphate and formic acid according to the equation:-

$$CH_3\ CO\ COOH + H_3PO_4 \rightleftharpoons CH_3\ CO\ (OPO_3H_2) + HCOOH.$$

Keeping in mind that oxalacetic acid (Deffner, according to Peynaud, 1956; Slade and Werkman, 1941) and pyruvic acid (Slade and Werkman, 1941) are known to be intermediates in the decomposition of citric acid by lactic acid bacteria, the following scheme is suggested for citric acid fermentation by the wine lactobacilli.



The acetoin formed by L. leichmannii presumably arises from the intermediary pyruvate by the reaction 2 Pyruvate ⟶ Acetoin + 2 CO_2, as in L. plantarum (Rowatt, 1951).

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It is assumed that the small amounts of succinic acid are formed from the intermediary oxalacetate, as in S. paracitrovorus (Slade and Werkman, 1941).

According to the proposed scheme one mole of citric acid fermented yields one mole of intermediary oxalacetic acid. The theoretical quantity of oxalacetic acid required for the formation of the end products as indicated in the scheme, may be calculated by adding the molar amount of succinic acid to (i) the molar amount of carbon dioxide formed from oxalacetate, or (ii) the molar quantity of intermediary pyruvate. It is evident from the proposed scheme that the amount of carbon dioxide formed from oxalacetate is found by subtracting the ethanol, or the ethanol plus twice the amount of acetoin (in the case of L. leichmannii), from the total quantity of carbon dioxide produced. The molar quantity of pyruvic acid may be calculated from the amounts of end products arising from it. Thus, the value obtained by adding the molar quantities of formic acid, lactic acid and ethanol represents the intermediary pyruvic acid. In the case of L. leichmannii twice the molar quantity of acetoin found must also be added.

To further satisfy the suggested scheme, the value obtained by subtracting the molar amount of formic acid found from that of the acetic acid should equal the quantity of citric acid fermented.

The data obtained with the four wine lactobacilli (Table 14) were analysed accordingly and the

results are given in Table 15.

Table 15: The molar amounts of oxalacetic acid (theoretically) and that of acetic acid minus formic acid produced, and their ratios to citric acid fermented by four lactobacilli from wine.

	L. leichmannii. mMol/l.	L. brevis. mMol/l.	L. hilgardii. mMol/l.	Lactob. sp. mMol/l.
Citric acid fermented	25.35	8.54	7.56	21.62
Oxalacetic acid (theor.) according to:				
(i) CO_2 produced	24.44	7.66	7.16	25.72
(ii) Pyruvic acid required	25.86	9.58	8.17	21.02
Average $\left(\frac{(i)+(ii)}{2}\right)$	25.15	8.62	7.67	23.37
Acetic acid - formic acid	21.32	8.20	5.75	19.57
$\frac{\text{Oxalacetic acid (av.)}}{\text{Citric acid fermented}}$	0.99	1.01	1.02	1.08
$\frac{\text{Acetic acid - formic acid}}{\text{Citric acid fermented}}$	0.84	0.96	0.76	0.91

It is evident from Table 15 that the results obtained are in good agreement with the theoretical values, and are therefore compatible with the proposed scheme for the homo- and heterofermentative dissimilation of citric acid by the wine lactobacilli.

It was shown by Korkes et al (1950) that the malic enzyme can also decarboxylate oxalacetic acid. Several of the bacterial strains studied here, although grown in a medium containing both l-malate and citric

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acid, thus possessing the malic enzyme, were unable to degrade citric acid. The limiting step in the citric acid breakdown in such cases therefore appears to be the initial splitting of the citric acid molecule into acetic acid and oxalacetic acid.

CONCLUSIONS.

Contamination of South African dry wines by lactic acid bacteria is more common than is generally realised. This is especially true of the lower-priced drinking wines and of rebate wines.

The causative organisms, homo- and hetero-fermentative rods as well as homofermentative cocci, can be divided into seven distinct types representing at least five species of two genera of the lactic acid bacteria (family Lactobacillaceae). All the rod-shaped forms belong to the genus Lactobacillus. The cocci, which in many ways resemble the spherical forms known to occur in beers and in fermenting vegetables, in turn belong to the genus Pediococcus.

Of the lactic acid bacteria found in South African dry wines P. cerevisiae and L. hilgardii exhibit by far the highest incidence.

All the lactic acid bacteria isolated and especially P. cerevisiae, exhibit complex nutritional requirements. Among the vitamins nicotinic acid, calcium-pantothenate and riboflavin are the most important. The heterofermentative lactobacilli furthermore require thiamine, while pyridoxine seems to play a special role in the nutrition of these homofermentative lactic acid bacteria. In addition all the bacteria require a complex mixture of amino acids, of which glutamic acid, valine, arginine, leucine and iso-leucine have the greatest influence on growth.

There appears to be no definite correlation between the complexity of the nutritional requirements

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of an organism and its frequency of occurrence in dry wines.

All the wine lactic acid bacteria studied are able to utilise both d-glucose and d-fructose. The homofermentative organisms, in all probability, dissimilate these two sugars via the Embden-Meyerhof glycolytic route. These homofermentative strains do not produce mannitol from fructose.

The results obtained for glucose fermentation by the heterofermentative lactobacilli, on the other hand, are compatible with a hexosemonophosphate pathway. During fructose fermentation by the heterofermentative strains a substantial fraction of the dissimilated hexose is reduced to mannitol. The fraction of the utilised hexose not reduced to mannitol is probably dissimilated via the same pathway by which glucose is fermented.

Many of the wine lactic acid bacteria are able to utilise d-xylose or l-arabinose or both, in addition to glucose and fructose. The results obtained for pentose fermentation suggest that the pentose molecule is split into 3-carbon and 2-carbon fragments which yield lactic acid and acetic acid respectively. This pentose fermentation pathway functions in both homo- and heterofermentative organisms. It seems possible that the fermentation, by these organisms, of the pentoses contained in dry wines may contribute to the high volatile acid content by which such contaminated wines are usually characterised.

The production of malic enzyme can be induced in the wine lactic acid bacteria. It may therefore be surmised that, depending on the l-malic acid content of the wines, at least some malo-lactic fermentation can take place in South African dry wines.

Citric acid is degraded by most of the wine lactobacilli. The enzymes which catalyse this reaction are adaptable, as in the case of the malic enzyme. The citric acid in young wines is probably another important source from which acetic acid is formed during bacterial contamination. With the lactic acid bacteria which possess the malic enzyme, but are unable to dissimilate citric acid, the limiting step in the reaction appears to be the initial cleavage of the citrate molecule to yield oxalacetic acid and acetic acid.

One or both of the neutral volatile compounds, acetoin and diacetyl, are produced by these homofermentative wine lactic acid bacteria from the utilisable carbohydrates and organic acids contained in dry wines. The production of these compounds by the heterofermentative strains on the other hand could not be demonstrated. These facts suggest that the homofermentative lactic acid bacteria are probably responsible, at least to some extent, for the "off-flavour" of some South African dry wines.

The lactic acid bacteria occurring in South African dry wines are in many ways similar to those occurring in the wines of Europe, California and Australia.

SUMMARY.

By employing an enrichment technique, 64 strains of lactic acid bacteria were isolated from South African dry wines. The isolates included homofermentative cocci as well as both homo- and heterofermentative rods.

The cocci closely resembled the description of Pediococcus cerevisiae. All the homofermentative rods corresponded, more or less, to the descriptions of Lactobacillus leichmannii. Among the heterofermentative isolates, L. hilgardii, L. brevis, L. buchneri and an unidentified Lactobacillus species could be distinguished.

A study was made of the amino acid and vitamin requirements of twelve representative strains of the wine lactic acid bacteria. A completely synthetic medium was employed for this purpose. Growth in the presence and absence of a specific nutritive substance was measured nephelometrically. All the strains required nicotinic acid, riboflavin, calcium-pantothenate and thiamine or pyridoxine. In addition at least five amino acids, glutamic acid, valine, arginine, leucine and iso-leucine were required.

Gas-production from glucose by the heterofermentative lactobacilli was markedly stimulated by the presence of carbon dioxide.

The physiology of seven selected strains was further investigated by studying the effect of both

resting...

resting cell suspensions and growing cultures on the utilisable carbohydrates and organic acids contained in dry wines. Most of the strains utilised fructose at a faster rate than glucose, as reflected by the manometric data.

Material balances were calculated for the fermentations of the mentioned substrates. When glucose and fructose were fermented by the homofermentative strains, 70 per cent or more of the sugar dissimilated was accounted for as lactic acid. With glucose as substrate the heterofermentative strains produced substantial quantities of acetic acid, ethanol and glycerol, in addition to lactic acid. The carbon dioxide produced by the heterofermentative strains during glucose fermentation was more or less equal in molar quantity to the sum of the 2-carbon end products. The sum of the 2-carbon end products was in turn more or less equal to that of the 3-carbon end products, indicating a three-way split of the glucose molecule. Fructose fermentation by the heterofermentative organisms yielded considerable amounts of mannitol in addition to the products formed from glucose. The molar quantity of carbon dioxide produced from fructose was also approximately equal to that of the 2-carbon and the 3-carbon end products, as in the glucose fermentations.

Fermentation of the pentoses d-xylose and l-arabinose by both homo- and heterofermentative organisms, yielded equimolar quantities of lactic acid and acetic acid.

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The presence of malic enzyme was demonstrated in all the species encountered. l-Malic acid was fermentatively dissimilated by all strains to yield lactic acid, carbon dioxide and small amounts of ethanol. In addition most strains produced small amounts of acetic acid.

Citric acid was degraded by most of the strains. The main products of citric acid degradation was shown to be carbon dioxide, acetic acid and lactic acid, with formic acid and ethanol accounting for most of the remaining carbon. A scheme is proposed for the fermentative dissimilation of citric acid by the wine lactobacilli.

One or both of the neutral volatile compounds acetoin and diacetyl, was produced in small amounts by the homofermentative organisms from all the substrates tested. The production of these compounds by the heterofermentative organisms could not be demonstrated.

ACKNOWLEDGMENTS.

I wish to acknowledge my indebtedness to the following:-

My promotor, Dr. J.A. van Zyl, Head of the Section for Wine Microbiology of the Viticultural-Oenological Research Institute for valuable criticism and comment.

My examiners Professors H.A. Louw and I.S. Perold, respectively of the departments of Microbiology and Agricultural Biochemistry of the Faculty of Agriculture, University of Stellenbosch, for valuable suggestions and highly appreciated interest.

Mr. Paul du Toit, Director of the V.O.R.I. and through him the Department of Agricultural Technical Services for generous allowance of scope and facility to carry out this work.

The various members of the Staff of the V.O.R.I. who at some time or another kindly rendered their assistance.

Mr. C.T. de Waal, Senior Lecturer in Oenology to the Department of Viticulture-Enology of the Faculty of Agriculture, University of Stellenbosch, for centering my interest on this particular subject.

To all the above-mentioned I gratefully tender my sincere thanks and appreciation.

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