UNIVERSITY OF EDINBURGH

AN INVESTIGATION OF THE LACTIC ACID BACTERIA

by

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Introduction.

It has proved difficult, in this laboratory, to identify the lactic acid bacteria isolated from silage according to the present system of classification. Keddie(1959) stressed this difficulty by his description of a number of lactobacilli isolated from silage, many of which were unidentifiable or apparently atypical of recognized species. Whittenbury(1960) found the same was true of streptococci and leuconostocs isolated from fresh grass and silage. This investigation, a series of studies on lactic acid bacteria isolated from fresh herbage and silage, aims at clarifying this situation. A number of named strains were also examined to assess the relationship of the silage organisms to the known species. This necessitated a general study of the various genera.

This thesis is composed of six sections. The first concerns the reaction of various types of lactic acid bacteria to aerobic and anaerobic environments. The next four sections comprise studies on the classification of enterococci, pediococci, leuconostocs and heterofermentative lactobacilli. The final section is mainly of an applied nature and deals with aspects related to the production of silage.

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Oxygen relationships in the lactic acid bacteria.

INTRODUCTION

Lactic acid bacteria are reputed to prefer anaerobic or microaerophilic conditions for growth and, for this reason, it has been assumed that oxygen is of no importance in their metabolism, energy being obtained only from fermentation.

An exception to this idea has been described by Gunsalus & Sherman(1943) who found that a strain of *Streptococcus faecalis*, which would now be classified as *Str.faecium*, a strain of *Str.durans* and other lactic acid bacteria were only able to utilize glycerol in the presence of oxygen.

The present investigation has shown further instances in which substrate utilisation by various species of lactic acid bacteria requires aerobic conditions. In order to make a rapid assessment of an organism's preference for aerobic or anaerobic conditions, cultures were prepared in soft agar which provides both environments.

Section 1.

Observations on the aerobic behaviour of the organisms led to studies on hydrogen peroxide production, hydrogen peroxide-splitting ability and the question of cytochrome formation; preliminary findings on these topics will be described.

METHODS.

Media

Soft agar medium. This contained: Lab-Lemco,0·5%(w/v); Evans peptone,0·5%(w/v);Tween 80,0·05%(v/v); yeast autolysate (prepared as in Gibson,Stirling,Keddie & Rosenberger,1958), 5·0%(v/v); agar,0·15%; and tap water. Either bromocresol-purple(BCP)

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1·4 ml. of a 1·6%(w/v) alcholic solution, or bromocresol-green(BCG), 2·8 ml. of an 0·4%(w/v) aqueous solution, was added to a litre. Carbohydrates, 0·5%(w/v), were either autoclaved in the medium or prepared as autoclaved or Seitz-filtered distilled water solutions and added to the autoclaved medium. Media used in salt tolerance tests were prepared at double-strength and an equal volume of a salt preparation was added after sterilization. Soft agar with BCP was adjusted to pH 6·8-7·0; that with BCG to pH 5·4.

Agar medium. This had the same composition as the soft agar medium, except that the agar concentration was increased to 1·5%(w/v). The pH was normally adjusted to 6·5.

Inoculum medium. This was similar in composition to the soft agar medium with the following exceptions: agar was omitted and glucose, 0·5%(w/v), was included; the pH was adjusted to 6·5.

Media for detecting hydrogen peroxide formation. Two media were used.

Pyrolusite agar, (MDO agar), based on that of Kneteman(1946), consisted of the agar medium containing 0·5%(w/v) carbohydrate, usually glucose, poured as a plate, to which was added a very thin layer of the same medium containing 4·0%(w/v) pyrolusite, autoclaved in the medium and gently resuspended before pouring. When pyrolusite was unobtainable manganese dioxide, black tech. (Harrington Bros. Ltd.) proved to be a good substitute. The dried plates were inoculated by streaking with a capilliary pipette once across the plate. Hydrogen peroxide formation was indicated by the clearing of the manganese dioxide under and around growth.

Heated-blood dianisidine agar (HBD agar). Originally benzidine was used as the peroxide indicator but, because of its carcinogenic

nature, it was later replaced by o-dianisidine which produced an almost identical dark brown to black colouration in the presence of peroxide and heated-blood. To 90 ml. basal agar cooled to 48°, 5 ml. of a 1:1 mixture of defibrinated ox-blood and sterile tap water was added and the medium was then heated for 15 min. at 100° to denature the blood and destroy the catalase. The o-dianisidine additive was prepared by heating 0·1 g. in 5ml.sterile tap-water for 15 min. at 100°. The mixture of undissolved and dissolved o-dianisidine was then transferred by a wide-mouthed pipette to the medium which was then replaced in a water bath at 48° for a further 15 min. The medium was completed by adding a sterile solution of a carbohydrate, usually glucose, to give a final concentration of 1·0%(w/v).

Agar plates were poured, dried and inoculated by streaking once across the plate with a capillary pipette. Each plate could be inoculated with at least five cultures.

Media for detecting hydrogen peroxide-splitting activity. Three media were regularly used: 1·0%(w/v) glucose basal agar, 0·05%(w/v) glucose basal agar, and 1·0%(w/v) glucose heated-blood agar (HB agar). The latter medium differed from HBD agar only in lacking o-dianisidine. Agar plates were poured, dried and inoculated as before. By using capillary pipettes it was possible to inoculate rapidly a whole series of plates with the one culture. Hydrogen peroxide (10 vol.) was used to detect peroxide-splitting activity. In certain instances glucose was replaced by either fructose, arabinose or xylose, the choice depending on the ability of the organism to utilize these sugars.

Oxygen uptake by cell-suspensions at 30° was measured by conventional Warburg procedures with air as the gas phase. Cells - 15 to 20 mg. cell dry weight - were suspended in 0·07M phosphate

buffer, final pH 6·8. Substrates were added from the side arms after temperature equilibration to give a final concentration of 0·1M in a 3 ml. fluid volume. Centre wells contained 2N KOH.

Cells were harvested from glucose agar at 15-18 hr. and washed twice with sterile tap water.

Organisms

The strains examined were isolated during the course of the investigation or obtained from the National Collection of Type Cultures (NCTC), the National Collection of Dairy Organisms (NCDO), the National Collection of Industrial Bacteria (NCIB), the Institute of Applied Microbiology - Tokyio,- (IAM) and individual investigators.

Incubation Temperature.

Unless otherwise stated all incubations were at 30°.

RESULTS AND DISCUSSION.

Behaviour in soft agar.

The preparation of soft agar cultures used in the study of carbohydrate utilization.

The medium, 90 ml. quantities in bottles, was liquefied by momentarily autoclaving at 10 lb./sq.in., then allowed to cool to 48° in a water bath, and completed by the addition of sterile solutions of carbohydrates. The completed medium was replaced in the water bath or occasionally in an incubator at 50°. It was then distributed in 6-7ml. amounts to sterile 6 x 5/8in. test tubes, the amount being judged visually as the medium was poured directly from the bottle into the tubes. A series of completed media were placed in the one rack in the water bath so that a complete set could be

removed for inoculation with one culture. Immediately prior to inoculation the rack was removed from the bath and the medium allowed to cool to 37-40°, a temperature allowing ample time for inoculation before the agar set. The liquefied medium was inoculated with a capilliary pipette drop of a turbid culture and then tilted two to three times before being allowed to set. The distribution of growth in the tubes indicated that this procedure permitted an adequate distribution of the inoculum throughout the medium.

The addition of methylene blue to the sterile soft agar containing no pH indicator indicated that whilst the top 0·5-1·0 cm. of the medium soon became oxidized, the remainder of the medium remained reduced. Bubbling air through the medium caused the dye to become coloured again, thus confirming that reducing conditions in the medium were responsible for decolourizing the dye. In uninoculated media, reducing conditions persisted at least in the lower half throughout the period of incubation.

Changes in pH indicators.

Two indicators, BCP in media at pH 6·0-7·0 and BCG in media at pH 5·0-5·4, were used. In most cases both worked satisfactorily. Sometimes, however, BCG, after changing to a light green-yellow colour, which was shown electrometrically to indicate acid production, was adsorbed by the bacterial growth which became dark green. Occasionally both BCG and BCP were bleached during vigorous fermentation by strong reducing organisms such as Str. faecalis. Doubtful indicator changes which were not accompanied by an increase in growth were checked electrometrically.

Growth behaviour in soft agar when there is a requirement for aerobic conditions.

When a carbohydrate was utilized only in the presence of oxygen, the growth and acid production resulting from its utilization appeared only in the surface layers. A slight amount of non-acid forming growth often appeared in the remainder of the medium and was similar to that appearing in the basal sugar-free medium. As incubation continued, growth increased in the surface layers but not in the lower layers which were, however, often acidified as the acid from the surface layer diffused downwards. Sometimes secondary bands of growth developed below the surface band as the culture became oxygenated during incubation. If growth and acid production were not vigorous, as for instance during the utilization of glycerol by some strains of pediococci, Streptococcus faecium, and Lactobacillus plantarum, the initial indication of acidity which appeared within one to three days, slowly disappeared as the acid diffused through the medium. Such behaviour showed the need for frequent observation in the early stages of incubation. Incubation under an atmosphere of 95% hydrogen/5% carbon dioxide mixture confirmed that if growth and acid production were confined to the surface layer this demonstrated a requirement for oxygen.

Examples of a requirement for oxygen were provided by strains of Str.faecium (Fig.1), pediococci, Lb.plantarum and Aerococcus viridans utilizing glycerol, A.viridans NCTC 8251 and Pediococcus urinae equi IAM 1684 utilizing mannitol and sorbitol, and some leuconostocs and heterofermentative lactobacilli in the initial stages of hexose utilization. The latter observation will be discussed in greater detail later.

Growth behaviour in soft agar when there is a requirement for anaerobic conditions.

When carbohydrates were fermented under anaerobic conditions only (Fig.2), growth and consequent acidity appeared in a sharply defined zone in the lower part of the medium. The height of this zone, dependent on the substrate and the organism, varied from the bottom fifth of the culture to within 1·0 to 0·5 cm. of the surface. In many instances, - e.g. organisms of the Lb.casei-plantarum group utilizing cellobiose, sucrose and glycerol, some unclassified streptococci utilizing lactose, amygdalin, and raffinose - growth and acid production was confined to these areas throughout the period of incubation. But, where a high pH appeared to be a controlling factor, the diffusion of acid permitted growth in areas outside this zone. Examples of this behaviour were observed with Lb.cellobiosus (Figs.3A and 3B) and Pediococcus NCDO 1250 (Fig.4) in a glucose medium initially at pH 6·8-7·0. After the initial stage the area of growth expanded throughout the medium, rapidly in the case of Lb.cellobiosus, slowly in that of the Pediococcus. Both organisms grew uniformly throughout a medium, initially at pH 5·4, without showing an initial preference for anaerobic conditions.

Growth behaviour in soft agar when there is no strict requirement for aerobic or anaerobic conditions.

Growth and acid production appeared uniformly throughout the whole culture and, in the majority of cases, simultaneously (Fig.5). A number of leuconostocs, however, in glucose soft agar showed acid formation and growth initially at the surface but shortly

afterwards growth developed anaerobically and the cultures became uniform in appearance at all depths. This delayed anaerobic growth was thought to be due to the cells in this part of the medium having a longer lag phase than those at the surface; perhaps, as the inoculum was taken from an autoclaved glucose medium, they require to adapt in some way to anaerobic metabolism in a separately sterilized glucose medium.

The occurrence of late adaptive growth and mutant behaviour and the distinction between them.

Sometimes uniform growth and acid production appeared suddenly after 3 to 4 days' incubation with organisms normally fermenting glucose within 24 hr., an instance being a strain of Str.faecium fermenting melibiose. This was thought to be late adaptation and not mutation.

The formation of one or more relatively large acid-producing colonies surrounded by slight non-acid producing growth, similar to that occurring in the sugar-free basal medium, was interpreted as mutant fermentation. Parent cultures were replated two to three times to ensure purity and the test repeated. In every case the mutant behaviour occurred again. The mutant colonies were purified and, after subculture through a glucose medium, re-examined. All behaved as their respective parent strains with the exception of now being able to utilize constitutively the substrate which first indicated their existence.

Initial pH sometimes revealed variation, - e.g. the utilization of raffinose by some strains of Lb.plantarum. The substrate, utilized constitutively in a medium at pH 5·4, was utilized in a mutant fashion at pH 7·0. The mutant utilized the particular substrate constitutively

at the higher pH.

Another type of variation was noted in the fermentation of glycerol by strains of Str.faecalis which utilized glycerol constitutively under aerobic conditions. They were able to ferment glycerol constitutively in the presence of fumarate as a hydrogen acceptor but only in a mutant fashion in the absence of fumarate.

Sometimes the mutants were confined to the aerobic layers (Fig.6), sometimes to the anaerobic regions (Fig.7), but frequently they were distributed throughout the medium (Figs.9,10 and 11). In one instance (Fig.8) variants arose from a thin disc of non-acid producing growth which frequently appears just below the surface in the sugar-free medium. This appeared to be a definite instance of mutant cells appearing during growth in the medium. On a few occasions only a single mutant colony developed (Fig.12).

Occasionally it was difficult to determine whether or not the appearance of a few colonies after 7-14 days' incubation indicated a late mutation or a late adaptation of surviving cells as an inoculum taken from the medium surrounding the colonies did not always give rise to growth in a glucose medium.

Other more complex forms of variation were observed. One was the development of two or three types of colony during the fermentation of di-and tri-saccharides; another was the ability of mutants arising aerobically giving rise to further mutants in the anaerobic portion of the sub-culture.

Weak and slow carbohydrate utilization.

When growth and acid production increased over a number of days but was eventually equivalent to that produced by a rapidly fermented carbohydrate such as glucose, utilization was regarded

as slow. Carbohydrate utilization was judged as weak when growth was only slightly better than that occurring in the sugar-free basal medium and when the pH only dropped 1·0-1·5 units in a medium initially at pH 6·8-7·0. A number of leuconostocs fermented either or both arabinose and xylose in this fashion.

Microaerophilic growth and acid production.

In a sugar-free medium, many organisms produce a thin disc of growth, generally less than 0·5mm. wide, about 0·25 cm. below the surface. Occasionally in a medium containing sugar, acid formation and slightly improved growth arose late in the incubation period in the region of this disc of growth and was restricted to this area. No attempt was made to determine the reasons for this behaviour. Some strains of Lb.buchneri (Fig.14) behaved in this manner with xylose and a number of leuconostocs with various hexoses and di-saccharides.

The effect of temperature on the manner of growth in soft agar in which glucose had been autoclaved.

Some organisms, - i.e. strains of Lb.brevis and Lb.buchneri - which grew aerobically and anaerobically at 30°, only grew in the aerobic layer at 40°. Conversly, some organisms, - e.g. Lb.hilgardii NCIB 8040 and unclassified streptococci - which grew aerobically and anaerobically at 30°, grew only in the anaerobic regions of the medium at 37°. If glucose was replaced by arabinose or xylose, however, no such temperature effect was observed in either case.

A third variation was noted with strains of unclassified streptococci and Lb.fructovorans NCIB 8039. At 30° growth and acid production occurred throughout the medium but as the maximum growth temperature of these organisms was approached, growth and

acid production was gradually restricted to a disc about 2 mm. wide and 1-1·5 cm. below the surface (Figs. 15A and B), and did not develop either above or below this disc on continued incubation.

The effect of varying salt concentrations on glucose utilization.

Three variations were observed. The first of these variations concerned *A.viridans* NCTC 8251 and *P.urinae equi* IAM 1684. These organisms were able to proliferate and form acid only in the surface layers when the salt concentration was close to the maximum tolerated. The second variation was the great reduction in the number of cells of the inoculum capable of initiating growth as the salt concentration was increased (Figs. 16A, B and C). The third was observed with some pediococci which only grew in the middle zone of the medium at salt concentrations approaching the maximum tolerated (Fig.17). At the highest salt concentrations tolerated, some strains of pediococci produced only two or three acid-forming colonies. These colonies appeared to have arisen from survivors of the physiological conditions rather than from mutants, as, on subsequent transfer to a medium containing a similar concentration of salt, no growth or only a few colonies appeared.

Carbohydrate utilization by Lb.brevis and Lb.buchneri in soft agar.

Soft agar revealed growth variations dependent on the type of carbohydrate, and on whether the carbohydrate was autoclaved in the medium or separately sterilized by Seitz-filtering or autoclaving before adding to the autoclaved medium.

In soft agar (pH 6·8-7·0), to which glucose or gluconate was added after sterilization, aerobic growth appeared within 24 hr. and anaerobic growth 2-3 days later. Sometimes anaerobic growth was

delayed for 7 days or longer though lowering the initial pH to 5·4 hastened its appearance. In the early stages of utilization, therefore, oxygen appeared to be taking the place of some of the hydrogen acceptors normally involved in fermentation. That this was possible seemed to be confirmed by the fact that the appearance of surface growth was delayed under anaerobic cultural conditions and that cell-suspensions of these organisms took up oxygen actively on glucose, gluconate and fructose when examined by the Warburg mannometric technique.

In an attempt to improve anaerobic growth, a number of substances which might be used as hydrogen acceptors were added to the glucose medium. Methylene blue had a slightly beneficial effect but was inhibitory when added in significant amounts. Diacetyl had a markedly beneficial effect, both hastening the appearance and increasing the density of growth. Fructose, 0·2%(w/v), improved growth significantly over that occurring in an 0·2%(w/v) fructose medium. Sodium fumarate had no effect. A marked improvement was also obtained by autoclaving the glucose in the medium, anaerobic growth frequently appearing at the same time as aerobic growth although it never reached the same density.

With separately sterilized arabinose and xylose, providing these substances were vigorously utilized, growth appeared within 24 hr. and was distributed uniformly throughout the medium.

In a fructose medium, growth appeared throughout within 24 hr. but, whereas aerobic growth and acidity were marked, anaerobic growth and acidity were slight. Anaerobic growth increased on incubation but did not become as dense as the aerobic growth; it was

improved, however, by adding diacetyl, by increasing the concentration of fructose to 2.0%(w/v), by adding glucose, 0·5%(w/v), or by autoclaving the fructose in the medium.

With all other carbohydrates utilized the type of growth was similar to that observed with glucose.

An interpretation of variation in aerobic and anaerobic growth.

The pathway of the heterolactic fermentation has been demonstrated in Ln.mesenteroides (DeMoss, Bard & Gunsalus, 1951, Gunsalus & Gibbs, 1952, and Hurwitz, 1958) and indicated in the heterofermentative lactobacilli (Gibbs, Sokatch & Gunsalus, 1955). More recently Eltz & Vandemark (1960) have shown that many of the enzymes involved in the fermentative pathway of Ln.mesenteroides are also present in the extracts of fructose grown cells of Lb.brevis. Some of the results obtained by Eltz & Vandemark, who used a medium containing separately sterilized fructose for growing Lb.brevis, are used in interpreting the varying growth behaviour.

During the heterofermentative dissimilation of glucose three stages of oxidation occur involving the reduction of either triphosphopyridine nucleotide (TPN) or diphosphopyridine nucleotide (DPN). They are the dehydrogenation of glucose-6-phosphate, 6-phosphogluconate and 3-phosphoglyceraldehyde. During gluconate dissimilation only the latter two stages occur. During arabinose fermentation, however, only one of these oxidative steps takes place, the dehydrogenation of 3-phosphoglyceraldehyde.

Eltz & Vandemark have demonstrated that the dehydrogenation of the triosephosphate by Lb.brevis was specifically the function of DPN in the presence of adenosinetriphosphate (ATP) and that pyruvate was reduced only by reduced DPN(DPNH). Therefore, as anaerobic

acid production occurs in arabinose soft agar and is not improved or diminished by the addition of glucose, it is assumed that the initial failure to utilize glucose and gluconate anaerobically is not caused by the organisms' inability to oxidize 3-phosphoglyceraldehyde or reduce pyruvate, or by an inhibitory substance in glucose and gluconate preparations.

The inability to ferment may, therefore, be due to the failure of the oxidation of DPNH and reduced TPN(TPNH) by either acetaldehyde dehydrogenase or ethanol dehydrogenase. Eltz & Vandemark demonstrated the presence of ethanol dehydrogenase in Lb.brevis, DPNH and TPNH being oxidized in the presence of acetaldehyde, but were unable to demonstrate the presence of acetaldehyde dehydrogenase previously shown to occur in Ln.mesenteroides by Hurwitz(1958). It seems that the absence of this enzyme or its failure to function may be responsible for the initial failure of anaerobic growth. The observation that anaerobic growth also failed in gluconate soft agar appears to be circumstantial evidence confirming this suggestion. As one mol. of TPNH or DPNH requires to be oxidized per mol. of gluconate, the remaining mol. of DPNH being oxidized by lactic acid dehydrogenase, the absence or non-functioning of acetaldehyde dehydrogenase would account for the failure of anaerobic growth.

The appearance, then, of aerobic growth but not of anaerobic growth in the early stages of glucose and gluconate utilization suggests that the DPNH not oxidized by lactic acid dehydrogenase and TPNH are oxidized by oxygen via DPNH and TPNH oxidases or peroxidases. Eltz & Vandemark observed that TPNH and DPNH were rapidly oxidized under aerobic conditions by cell extracts of Lb.brevis.

The slight anaerobic growth occurring at the same time as aerobic growth in the fructose medium may be explained by the ability of these organisms to utilize fructose as a hydrogen acceptor. This was demonstrated with extracts of Lb.brevis by Eltz & Vandemark, fructose being reduced to mannitol with DPNH as the hydrogen donor. Assuming that both ethanol and acetaldehyde dehydrogenses were not functioning, 2 mol. of fructose would be reduced in the utilization of 1 mol. of fructose, which would account for the slight anaerobic growth and the marked improvement of anaerobic growth, in many instances, when the concentration of fructose was increased to 2·0%(w/v).

The occurrence of dense aerobic growth in fructose soft agar suggests that oxygen is acting as a hydrogen acceptor as postulated in the utilization of glucose and gluconate.

The greater amount and the more rapid appearance of anaerobic growth in soft agar containing either glucose, gluconate, or fructose which had been autoclaved in the medium, suggests that some substance is produced by this treatment which either induces acetaldehyde dehydrogenase or counteracts some substance preventing its formation or activity.

Carbohydrate utilization by some leuconostocs.

A number of non-dextran forming leuconostocs, isolated from fresh grass, grew in a manner closely similar to Lb.brevis and Lb.buchneri with glucose, fructose and anabinose, though anaerobic growth in the glucose and fructose medium was more dense and earlier in appearing. Frequently the anaerobic growth in the glucose medium was separated from aerobic growth by a zone of no growth (Fig.18). This zone was later shown to be caused by hydrogen

peroxide, formed at the surface, diffusing downwards and inhibiting the initiation of anaerobic growth in that region. Sometimes the zone diminished on further incubation (Fig.19). Autoclaving sugars in the medium did not obviously alter the manner of growth.

One strain, however, S 70, was unusual in that anaerobic growth was always very slight with any carbohydrate as an energy source, including glucose, fructose and arabinose, either sterilized separately or autoclaved in the medium. Citrate improved anaerobic growth slightly but insignificantly. Diacetyl, however, markedly improved growth and anaerobic acid production. As aerobic growth was always more dense than anaerobic growth and as cell suspensions were active in taking up oxygen on glucose, gluconate and fructose, it would seem that either or both DPNH and TPNH oxidases are of significance in the carbohydrate metabolism of this organism.

Use of terms in subsequent descriptions of growth in soft agar.

As the variations in growth observed in soft agar may be of use in taxonomic work on lactic acid bacteria, a series of terms, with brief definitions based on the earlier detailed descriptions, are set out in Table 1., and will be used in the subsequent discussions. The types of growth are illustrated diagrammatically (Fig.20).

The advantages of using a soft agar medium rather than a liquid medium.

The ability of lactic acid bacteria to gain or lose the property of fermenting various carbohydrates has been frequently reported. This variability has generally been attributed to the organisms rather than to the methods used. Observations recorded during this investigation on behaviour in soft agar and liquid media, suggest that the methods rather than the organisms are responsible for variability.

Soft agar has many advantages as a test medium apart from giving constant results. It provides aerobic, partially aerobic, and anaerobic conditions, thus offering a varying environment. Its semi-solid nature allows organisms to develop and remain in that part of the medium most suitable for their growth. Potentially toxic products, such as peroxide, are allowed to diffuse away from the growth. In soft agar it is possible to distinguish between aerobic, anaerobic, slow, weak, late adaptive and microaerophilic utilization, and to determine when utilization is only the property of a few mutant cells. In liquid medium, on the other hand, it is impossible to distinguish between all the types of utilization mentioned.

Shallow liquid cultures are frequently used in testing substrate utilization by lactic acid bacteria. In this investigation variable results in liquid media were obtained if a substrate was used aerobically only, anaerobically only, or mutantly. In the case of glycerol utilization by some strains of Str.faecium, growth and acid production was very slight and only after 6-8 days incubation was acidity obvious. Occasionally the changes in the medium were slight enough to suggest that the reaction was doubtful. In soft agar cultures, however, improved surface growth and marked acidity were often detected in the surface regions within 2-3 days. When growth and acid production with glycerol was weak in soft agar, it was rarely observed at all in liquid medium. The much weaker reaction in the liquid medium was thought to be due to the inability of glycerol utilizing cells to remain in the most favourable part of the medium - the surface - as they were able to do in soft agar. Consequently the resulting acid and growth were diluted through the

medium and utilization was not obvious as it was in soft agar.

When growth and acid formation only took place in an anaerobic environment, shallow liquid cultures incubated aerobically frequently failed to support fermentation but did so if incubated anaerobically. In deep liquid cultures incubated aerobically, fermentation occurred more frequently indicating that sufficiently reduced conditions for growth occur more often than in shallow media. In soft agar, growth and fermentation always took place as anaerobic conditions were always provided.

When a substrate was used mutantly - only a few colonies appearing in soft agar - liquid media frequently did not support fermentation. This suggests that an insufficient number of mutant cells, or of cells giving rise to mutants, are transferred to initiate a positive reaction. Heavy inocula produced a greater number of positive reactions than a light inocula except when anaerobic conditions were also required.

To sum up, variable reactions occur in a liquid medium if utilization is weak and strictly aerobic, anaerobic or mutant. Soft agar, however, gives constant results. It also indicates the conditions required for utilization, - i.e. aerobic or anaerobic - and the type of utilization occurring, - i.e. weak or mutant.

Hydrogen peroxide production.

The many examples encountered of lactic acid bacteria with a requirement, or initial preference, for aerobic conditions led to a study of their ability to produce hydrogen peroxide. The group of non-dextran producing leuconostocs referred to earlier were the first organisms examined, as they were difficult to maintain on aerobically incubated slopes; Strain S 70 in particular produced

very slight growth and died before producing much or any indication of acidity.

By the use of glucose MDO agar, it was found that all of these leuconostocs produced peroxide and that the presence of a peroxide-destroying substance, pyrolusite in this instance, greatly improved the surface growth which now remained viable for longer periods.

A large number of dextran-forming leuconostocs were next examined and were found to produce no detectable peroxide. Therefore, as such a test might have taxonomic significance and in order to determine the relationship of the manner of growth to peroxide production, a wide range of lactic acid bacteria were tested on MDO and HBD agar. HBD agar was employed in preference to the commonly used fresh lysed-blood medium as it was found that on the latter many organisms which formed no peroxide produced a dark brown growth in the absence or presence of dianisidine. When examining organisms which were inhibited by blood, heated-blood was replaced by haematin, 100 ug/ml of medium.

The results suggested that peroxide formation may be of taxonomic significance, as will be discussed in later sections. With respect to the second object, the relationship between peroxide production and preference for aerobic conditions, no clear-cut correlation was obtained. Many organisms with a requirement for aerobic conditions produced peroxide and many did not; some which grew preferentially anaerobically produced peroxide and some did not; and some organisms, growing equally well aerobically and anaerobically, produced peroxide and some did not. A number of organisms growing preferentially aerobically but not producing detectable peroxide - e.g. <u>Lb.brevis</u> and <u>Lb.buchneri</u> - took up oxygen actively on glucose. This suggests

that peroxidases are possessed by these particular organisms. Flavoprotein peroxidases have been previously described in lactic acid bacteria by Dolin(1957) and Strittmatter(1959).

In the majority of cases the results obtained on MDO agar were confirmed on HBD agar. There were a few instances, however, of organisms producing a strong peroxide reaction on MDO agar and a negative or slight reaction on HBD agar. This behaviour was confirmed in repeated tests. Eventually it was discovered that these organisms possessed a strong peroxide-splitting activity when grown on the heated-blood medium but not when grown on the pyrolusite medium. A note on this topic has already been published (Whittenbury,1960).

Hydrogen peroxide-splitting activity.

The ability of lactic acid bacteria to split peroxide was examined further. The test medium was composed of 1·0%(w/v) glucose agar and 1·0%(w/v) glucose HB agar. In certain instances glucose was replaced by other sugars. Plates, poured so that one half contained glucose agar and the other half HB agar, were inoculated by streaking across the two halves. Incubation was for two days except in the case of slow growing organisms. Peroxide-splitting activity was detected by the effervescence resulting from the addition of hydrogen peroxide (10 vol.). In no instance was peroxide split by growth on the glucose agar except in the immediate vicinity of HB agar. The sterile media did not split peroxide.

If organisms were able to split peroxide when grown on HB agar, the reaction varied from a continuous bubbling to an explosive effervescence. At first it appeared that organisms belonging to genera Lactobacillus, Leuconostoc and Pediococcus but not Streptococcus

were capable of this reaction, but in repeated experiments it was observed that some strains of Str.faecalis produced a few bubbles initially, though they failed to cause a continuous breakdown of the peroxide. Later it was found that a vigorous effervescence ensued if the growth of these Str.faecalis strains was heaped and peroxide added, or if the growth was scraped off and placed in peroxide. A search for further peroxide-splitting organisms using this method revealed that a number of Str.faecalis, Lb.plantarum and Lb.casei strains had been originally overlooked.

Anaerobic cultivation, in the majority of cases, had no effect on the peroxide-splitting ability of the organisms. Some Lb.brevis strains, however, were either less active or unable to split peroxide when grown anaerobically and one Lb.viridescens strain, unable to split peroxide or to grow well aerobically at 30°, split peroxide actively after being grown anaerobically on HB agar.

When the initial pH of HB agar was varied from 7·0-4·5, the peroxide-splitting activity was not affected, provided growth occurred. Variation in sugar concentration also had no effect. On media containing low concentrations of sugar, however, a further type of peroxide-splitting activity was encountered in some leuconostocs and pediococci which were incapable of splitting peroxide when grown on HB agar or glucose agar containing 1·0%(w/v) glucose. This reaction, which was weak and took place regardless of the presence of heated-blood, will be described later.

The nature of the stimulation by heated-blood was examined further. Haematin, prepared by dissolving haematin hydrochloride in tap water containing a drop of 0·1 N NaOH, and added to glucose agar to yield a final concentration of 100 ug/ml., promoted a similar peroxide-splitting activity to that occurring on the HB medium.

Protoporphyrin IX in the presence of ferrous and ferric salts, however, did not.

Washed cell-suspensions of representative strains, harvested from aerobically incubated 1·0%(w/v) glucose agar at 15-18 hr., were examined for their ability to form a peroxide-splitting enzyme in the presence of haematin. The cells were resuspended in tap water to give a final concentration of approximately 10 mgm. dry weight per ml. Haematin was added to give final concentrations of 5,50, and 100 ugm/ml. These preparations were tested for peroxide-splitting activity at different times at room temperature. The results (Table 2) indicated that only the organisms capable of splitting peroxide when grown on HB agar were able to form a peroxide-splitting enzyme in the presence of haematin. Cell-suspensions heated at 100° for 2 min. before or after the addition of haematin failed to develop a peroxide-splitting activity. Unheated cell-suspensions of a Str.faecalis strain which was able to form a peroxide-splitting enzyme when grown on HB and haematin agar, also failed to develop a detectable activity. As the reaction of this and similar strains was comparatively weak on HB or haematin agar it is possible that the maximum time of incubation (2 hr.) of the cell-suspensions was insufficient to produce a visibly detectable reaction. The results of this experiment suggest that certain lactic acid bacteria constitutively form a heat-sensitive apo-enzyme, conceivably apo-catalase, of a peroxide-splitting enzyme.

A weak peroxide-splitting activity, occurring in the absence of heated-blood or added haematin, has already been described for certain lactic acid bacteria. Felton, Evans & Niven(1953) observed that pediococci grown on a medium containing 0·05%(w/v) glucose

possessed a catalase activity which was apparently sensitive to acidity and not detected on media containing high concentrations of sugar. A similar type of activity has been recorded for strains of Lb.plantarum (Dacre & Sharpe, 1956, Langston & Bouma, 1960a) Ln.mesenteroides (Langston & Bouma, 1960b) and Str.faecalis (Langston, Gutierrez & Bouma, 1961). A similar property, already reported (Whittenbury 1960), was observed with some strains of dextran-forming Ln.mesenteroides and a number of pediococci. This type of peroxide-splitting activity was most pronounced with cultures grown on a sugar-free medium or on 0·05%(w/v) glucose agar slopes incubated aerobically overnight. All the leuconostocs and some of the pediococci failed to split peroxide on 0·5%(w/v) glucose agar. The remaining strains of pediococci were able to split peroxide fairly actively when grown on 0·5%(w/v) glucose agar but not on 1·0%(w/v) glucose agar. Haematin or heated-blood did not appear to affect the reaction. Lowering the initial pH of the 0·05%(w/v) glucose medium affected peroxide-splitting activity. At pH 6·0 all of these organisms were able to split peroxide but at pH 4·5 - all strains grew - only the pediococci capable of splitting peroxide when grown on 0·5%(w/v) glucose agar continued to split peroxide, though less vigorously than at pH 6·0. Anaerobically grown cultures of the leuconostocs were unable to split peroxide except for one strain which produced a very weak and questionable reaction after being allowed to stand in contact with the atmosphere for 1 hr. Anaerobically grown cultures of the pediococci were just as active in splitting peroxide as the aerobically grown cultures.

A comparison of the two types of peroxide-splitting activity.

From the results obtained so far it seems clear that two types of peroxide-splitting activity are present in the lactic acid bacteria,

a haematin-requiring activity not affected by the concentration of glucose or other sugar tested and a less vigorous activity not requiring the presence of haematin, apparently sensitive to pH and only regularly observed on a medium containing little or no sugar.

Investigating the latter type of activity in pediococci, Delwiche(1961) found that 10^{-2} M-sodium azide and potassium cyanide failed to affect peroxide splitting activity which was, however, inactivated by heating for 3 min. at 90°. No haematin or flavin spectrum could be observed in enzyme preparations. He also found that the activity was not increased by the addition of flavin or inhibited by acriflavin. These results indicate that this enzyme is not a haem-containing catalase, and that it is probably not a flavin-containing enzyme.

The peroxide-splitting activity of haematin-requiring organisms examined here was found to be completely inhibited by 10^{-3} M-cyanide and azide and almost completely by 10^{-4} M-cyanide and azide, indicating that the enzyme was very similar to catalase. Cell-suspensions or broken cell extracts of the pediococci and leuconostocs which split peroxide in the absence of haematin continued to split peroxide in the presence of 10^{-1} M-azide and cyanide. Table 3 summarizes some of the differences between the two peroxide-splitting activities. In order to avoid confusion which is developing in the literature on the subject of catalase activity in lactic acid bacteria, it is proposed to refer to organisms producing a peroxide-splitting activity only in the presence of haematin, or heated-blood, as being apo-catalase positive and to organisms possessing the other type of activity as being pseudocatalase positive until such time as their biochemical nature is more clearly understood.

The relationship between peroxide production and peroxide-splitting activity.

The most outstanding example of organisms benefiting from the possession of apo-catalase was provided by the group of non-dextran forming leuconostocs, referred to previously, which exhibited a preference for aerobic conditions but were sensitive to the peroxide which they formed aerobically. Overall, however, apo-catalase formation was not correlated with peroxide production. Many organisms which did not produce detectable peroxide were apo-catalase positive or pseudocatalase positive and many peroxide-forming organisms were apo-catalase negative and pseudocatalase negative.

In view of the fact that apo-catalase may be of significance in the survival of the organisms, particularly of the leuconostocs mentioned above, an investigation was made of their ability to form a peroxide-splitting enzyme in what may be considered to be their natural environment. Herbage was the environment from which the leuconostocs had been isolated and it was assumed that if they proliferate there, grass juices must be available to them, the juices being released either by damage to or death of the leaves.

The ability of a number of apo-catalase positive and negative organisms to split peroxide when grown on whole milk agar and on media containing various plant extracts was tested. The plant-extract medium was water agar to which was added filter-sterilized or autoclaved extracts of leaves of ryegrass, cocksfoot, clover, and similarly treated extracts of turnip root and potato tuber, the final concentration of the extract being approximately 25-50%(v/v). The apo-catalase positive organisms split peroxide when grown on

medium containing the filter sterilized or autoclaved grass and clover extracts but did not do so when grown on media containing the other extracts or on milk agar. An examination of pseudocatalase positive organisms revealed their ability to split peroxide on these media. This activity could be related to low sugar content, as the addition of increasingly large amounts of glucose inhibited the action of these organisms but not that of the apo-catalase positive organisms. It seems reasonable to suppose, then, that the leuconostocs discussed above can and may naturally exist aerobically on herbage, without succumbing to peroxide, by utilizing an iron porphyrin released with the plant juices.

As mentioned originally, the ability of lactic acid bacteria to form a peroxide-splitting enzyme in the presence of haematin was first noted when organisms producing a strong peroxide reaction on MDO agar failed to do so on HBD agar. Subsequently, however, a large number of organisms, despite their ability to split peroxide, were found to continue to produce peroxide on HBD agar, even on continued subculture on haematin or HB agar. When glycerol was substituted for glucose, a further group of organisms, those utilizing glycerol only in the presence of oxygen and including the pseudocatalase positive types, were found to form peroxide, though still able to split added peroxide. It appears, therefore, that not all the peroxide produced by these organisms comes into contact with peroxide-destroying enzymes. Assuming that such enzymes are situated either in the cell or in the cell membrane, peroxide, if formed inside the cell, should be destroyed peroxidatively or catalatically before diffusing to the outside environment. However, as it accumulates outside the cells it appears that peroxide is probably formed at the

membrane surface being destroyed as it diffused into the cell but not as it diffused away from the cell. The suggestion that peroxide may be formed at the membrane is supported to some extent by the findings of Weibull, Beckman & Bergström(1959) and Brown, Jeffery & Salton (unpublished data quoted by Salton,1961). In the first instance all but a trace of DPNH oxidase was found in the membrane fraction of Bacillus megaterium and in the second instance a similar finding was noted for a strain of Pseudomonas. If peroxide-destroying enzymes are situated within the cell cytoplasm, as catalase apparently is in B.megaterium (Weibull et al.1959), their major function may be to protect cell contents from invading peroxide. Such a phenomenon may explain why the amounts of peroxide formed, if any, by catalase-possessing bacteria do not appear to bear any relationship to their catalase content. Behaviour of the lactic acid bacteria on MDO agar substantiates this idea to some extent. The peroxide-sensitive strains grew well provided that the peroxide formed by them was not allowed to accumulate in the immediate vicinity of growth. Pyrolusite, being insoluble in this medium, can only protect the organisms from external concentrations of peroxide. This suggests, therefore, that in the absence of pyrolusite, the peroxide concentration increases in the immediate vicinity of the cell so slowing down the diffusion rate of peroxide away from the cell and causing inhibitory amounts to be retained within the cell.

The ability of whole cells to take up haematin.

The question arose as to whether or not the ability of lactic acid bacteria to form a peroxide-splitting enzyme on heated-blood and haematin media was related directly to their ability to take up haematin.

Washed cell-suspensions of representative organisms, grown on glucose agar, HB agar and haematin agar, were centrifuged and tested for haemin by the use of various benzidine-peroxide reagents. Three reagents were used; (1) the benzidine-base and (2) benzidine-hydrochloride reagents used by Deible & Evans(1960) and (3) a reagent made up of equal volumes of hydrogen peroxide (1 vol.) and an alcoholic solution of benzidine (0·1% w/v).

Unheated cells were examined first (Table 4) and only the third reagent gave any indication of the presence of haemin. Some of the peroxide-splitting organisms harvested from HB agar gave a light green-blue colour which rapidly faded, the cells apparently being able to decolourize the oxidized dye. Cells heated by being immersed in boiling water for 2 min. gave more strongly positive results but again only with the third reagent. Irrespective of their ability to form the peroxide-splitting enzyme, all organisms harvested from haematin and HB agar gave a strong deep blue colouration with the exception of the enterococci which only gave a light green-blue colouration. Of the cells harvested from glucose agar, the three strains of apo-catalase positive pediococci produced a blue colour. This may indicate that these organisms have either synthesized haemin or another iron porphyrin or that they have taken up traces of haemin from the medium, the constituents of which gave a negative result. If either was the case, the pediococci were not able to utilize the porphyrin to provide visible evidence of catalase-like activity. These results may be of doubtful validity as Hepler(1952) found that substances unrelated to haemin produced a similar reaction. But the fact that, with the exception of the three pediococci already mentioned, only organisms grown on the HB or haematin medium gave positive results, suggests that

haematin was the substance responsible for the positive reaction, and that the lactic acid bacteria examined take up haematin, regardless of their ability to produce a catalase-like enzyme.

Preliminary investigations on the ability of lactic acid bacteria to form cytochromes.

The ability of an organism to take up haemin and form catalase has been recorded previously (Jensen,1957). This organism, a streptomycin resistant mutant of *Micrococcus pyogenes var. aureus* (*Staphylococcus aureus*), was unable to synthesise haemin and, therefore, was catalase and cytochrome negative. Provided with haemin, however, it produced colonies similar in size to the parent, and was cytochrome positive as well as catalase positive. Jensen(1957) concluded that this mutant, though unable to synthesise haemin, still formed the apo-enzymes of catalase and the cytochromes. As some of the lactic acid bacteria seemed to be similar to this mutant in that they formed a peroxide-splitting enzyme when provided with haematin, a preliminary investigation was made of their ability to form cytochromes as well when grown on haematin or on HB agar. Representatives of apo-catalase positive and negative leuconostocs, lactobacilli, pediococci and streptococci were examined.

A number of physiological tests gave negative results. Dense cell-suspensions did not reduce 2:3:5 triphenyl-tetrazolium-chloride (0·01%w/v) in the presence of succinate (0·1%w/v) nor was succinate (0·5%w/v) used as an energy source by aerobically incubated cultures. None were capable of giving a cytochrome *c*-cytochrome oxidase reaction with Nadi reagent. Nitrate was not reduced to nitrite in media allowed to become acid or maintained at or slightly above

neutrality. The fact that many of the organisms continued to produce peroxide in spite of their ability to form a peroxide-splitting enzyme strongly suggested that cytochromes were not formed or, if formed, were not functioning significantly in those particular organisms.

Finally, cell-suspensions of a number of organisms were sent to Dr.E.F.Hartree who kindly agreed to examine them for cytochrome content using the low temperature method (Keilin & Hartree 1949, 1950).

Four of the apo-catalase positive strains were found to possess cytochromes when grown on the HB agar but not when grown on the glucose agar (Table 5). It appears that, if heavier concentrations of cells had been provided, all may have been seen to possess both a and b type cytochromes, as in the case of the strain of Str.faecalis examined, H69D5, which possessed the cytochromes a_1, a_2, and b_1.

The apo-catalase negative strain examined formed very slight amounts of cytochrome b_1 on both the HB and glucose agar, possibly indicating an ability to synthesise slight amounts of cytochrome, but this needs confirmation by further investigation.

The two pseudocatalase positive strains, grown on 0·05%(w/v) glucose agar only, neither formed cytochromes nor contained any haemin demonstrable as pyridine haemochromogen.

It appears from these preliminary findings that some lactic acid bacteria form cytochromes under certain cultural conditions. Whether they are functional, vestigial, or even artefacts due to the methods of culture, remains to be determined.

Earlier findings on behaviour with respect to oxygen and the

ability to form haem-enzymes, suggest that lactic acid bacteria may have developed from haemin synthesizing, aerobically respiring organisms but now have an impaired respiratory system.

CONCLUSIONS

1. This investigation has shown that a preference for anaerobic or microaerophilic conditions for growth is far from universal in the lactic acid bacteria. Some preferred aerobic conditions and some required strictly aerobic conditions when utilizing certain substrates. Others required or preferred anaerobic conditions and others did not exhibit a preference for either aerobic or anaerobic conditions. Soft agar, shown to be useful for demonstrating these requirements, was also incidentally useful in showing other variations in growth behaviour; variations which may explain the often reported variable fermentative behaviour of some lactic acid bacteria.
2. Lactic acid bacteria differed in their ability to form hydrogen peroxide. Some produced peroxide and some did not, regardless of their preferences for aerobic or anaerobic conditions.
3. Two types of peroxide-splitting activity were detected though never in the same organism. One type was dependent on the presence of haematin in the medium. The other type was apparently due to a non-haem enzyme which was acid-sensitive. There was no correlation between peroxide-splitting activity and a preference for aerobic or anaerobic conditions, or between peroxide-splitting activity and the ability to form peroxide.
4. Of the few organisms examined, some appeared capable of forming cytochromes when grown on heated-blood agar. One organism showed traces of a cytochrome when grown on glucose agar. The significance

of these observations has yet to be determined.

5. These preliminary experiments suggest that lactic acid bacteria may have developed from haemin-synthesizing, aerobically-respiring organisms, but now have an impaired respiratory system.

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Table 1.

Brief definitions of terms and symbols which will be used in subsequent descriptions of growth and acid production in soft agar by lactic acid bacteria.

Term.	Definition.
Aerobic (A)	Acid-forming growth occurring at the surface of the medium.
Preferentially aerobic (PA)	Acid-forming growth initially restricted to the surface but occurring later and less densely in the remainder of the medium.
Anaerobic (An)	Acid-producing growth occurring in a zone below the oxygenated portion of the medium.
Preferentially anaerobic (PAn)	Acid-forming growth initially restricted to a zone below the oxygenated portion of the medium.
Aerobic and anaerobic (AAn)	Acid-forming growth appearing uniformly and simultaneously throughout the medium, not affected by the presence or absence of oxygen.
Microaerophilic (M)	Acid-forming growth appearing as a thin disc below the surface but in the oxygenated portion of the medium.
Mutant (Mu)	Acid formation restricted to a few colonies which are frequently surrounded by slight non-acid forming growth.

Table 2.

Peroxide splitting activity of unheated cells in the presence of haematin.

	Activity on heated-blood agar.	Peroxide split after different times (min.)								
		0	5	10	15	0	5	10	15	20
		5 ug/ml. haematin				50 ug/ml. haematin				
Lb.plantarum NCIB 5914	+	-	+	++	++	-	++	+++	+++	+++
Lb.plantarum NCIB 6105	-	-	-	-	-	-	-	-	-	-
Lb.helveticus NCIB 4113 (Lb.casei)	-	-	-	-	-	-	-	-	-	-
Lb.brevis NCIB 947	+	-	+	++	++	-	++	+++	+++	+++
Lb.buchneri NCIB 8007	-	-	-	-	-	-	-	-	-	-
Non-dextran forming leuconostoc S70	+	-	-	+	+	-	+	+	++	+++
Ln.mesenteroides NCDO 538	-	-	-	-	-	-	-	-	-	-
Str.faecalis H69D5	(+)	-	-	-	-	-	-	-	-	-
Str.faecium HGH511	-	-	-	-	-	-	-	-	-	-
Ln.mesenteroides NCIB 8018 (pediococcus)	+	-	+	++	++	-	++	+++	+++	+++
Pediococcus RW 5	-	-	-	-	-	-	-	-	-	-

(+) signifies weak +

+, ++, +++ indicate intensity

Table 3.

Characteristics of the two peroxide splitting-enzymes.

	Type	
	"Apo-catalase"	"Pseudocatalase"
Haematin required.	+	-
Sensitive to acidity.	-	+
Inhibited by 1×10^{-1} M-azide.	+	-
Inhibited by 1×10^{-1} M-cyanide.	+	-
Heat sensitive.	+	+

Table 4.

Haematin content of lactic acid bacteria.

Organism	Peroxide split on heated-blood agar.	Reaction with benzidine-peroxide reagent (3).			
		Unheated cells		Heated cells	
		A	B	A	B
Lb.plantarum NCIB 5914	+	-	(+)	-	+
Lb.plantarum NCIB 6105	-	-	-	-	+
Lb.helveticus NCIB 4113 (Lb.casei)	-	-	-	-	+
Lb.brevis NCIB 947	+	-	(+)	-	+
Lb.buchneri NCIB 8007	-	-	-	-	+
Non-dextran forming leuconostoc, S 70	+	-	-	-	+
Ln.mesenteroides NCDO 538	-	-	-	-	+
Str.faecalis H69D5	(+)	-	-	-	(+)
Str.faecalis S41	(+)	-	-	-	(+)
Str.faecalis NCIB 370	(+)	-	-	-	(+)
Str.faecium S 43	-	-	-	-	(+)
Str.faecium HGH 511	-	-	-	-	(+)
Str.faecium IMEC	-	-	-	-	(+)
Ln.mesenteroides NCIB 8018 (pediococcus)	+	-	(+)	+	+
Pediococcus S5	+	-	(+)	+	+
Pediococcus S7	+	-	(+)	+	+
Pediococcus RW5	-	-	-	-	+

Reaction with benzidine-peroxide.
- \+ Deep blue colour
- (+) Light green-blue colour
- \- Colourless

A - Cells grown on 1·0%(w/v) glucose agar

B - Cells grown on 1·0%(w/v) glucose, haematin agar. (Cells grown on 1·0%(w/v) glucose heated-blood agar gave same result.)

Table 5.

Cytochrome content of some lactic acid bacteria.

Group	Organism	Culture	Cytochromes detected after addition of $Na_2S_2O_4$	Formation of pyridine haemochromogen
Apo-catalase positive	*Lb.plantarum* NCIB 5914	A	None	Minute amount
		B	a_2 b_1	
	Lb.brevis NCIB 947	A	None	None
		B	b_1	
	Ln.mesenteroides NCIB 8018 (pedicoccus)	A	None	None
		B	None	Very small amount.
	Non-dextran forming leuconostoc RW 1.	A	None	None
		B	b_1 a_2 (very weak bands)	
	Str.faecalis H69D5	A	None	None
		B	b_1 a_1 a_2	
Apo-catalase negative and pseudocatalase negative.	*Lb.plantarum* NCIB 6105	A	b_1) very weak	
		B	b_1) bands.	
Pseudo-catalase positive.	*Ln.citrovorum* NCIB 7837 (pediococcus)	C	None	None
	Ln.mesenteroides RW 66	C	None	None

A. Cells harvested from 1·0%(w/v) glucose agar

B. Cells harvested from 1·0%(w/v) glucose heated-blood agar.

C. Cells harvested from 0·05%(w/v) glucose agar.

EXPLANATION OF PLATE 1

Fig. 1. Str.faecium utilizing glycerol aerobically at 48 hr.

Fig. 2. Strain of the Lb.plantarum-casei group utilizing cellobiose anaerobically at 72 hr.

Fig. 3A. Lb.cellobiosus utilizing glucose anaerobically at 24 hr.

Fig. 3B. Lb.cellobiosus utilizing glucose at 36 hr. Growth beginning to develop in upper part of the medium.

Fig. 4. Pediococcus NCDO 1250 utilizing glucose anaerobically at 48 hr.

Fig. 5. Lb.fermenti utilizing glucose aerobically and anaerobically at 24 hr.

Fig. 6. Strain of Lb.plantarum-casei group utilizing sucrose aerobically and mutantly at 4 days.

Fig. 7. Strain of Lb.plantarum-casei group utilizing cellobiose anaerobically and mutantly at 5 days. Surface growth is non-acid producing.

Fig. 8. Strain of Lb.plantarum-casei group utilizing mannitol microaerophilically and mutantly at 1 week.

Fig. 9. Str. faecium utilizing sorbitol aerobically, anaerobically and mutantly at 6 days.

Fig. 10. A strain of pediococcus utilizing lactose aerobically, anaerobically and mutantly at 5 days.

Fig. 11. A strain of Lb.plantarum-casei group producing a few mutant colonies with cellobiose at 5 days. Sub-surface disc is non-acid producing.

Fig. 12. A strain of Lb.plantarum-casei group producing a single mutant colony with sucrose at 1 week.

PLATE 1.

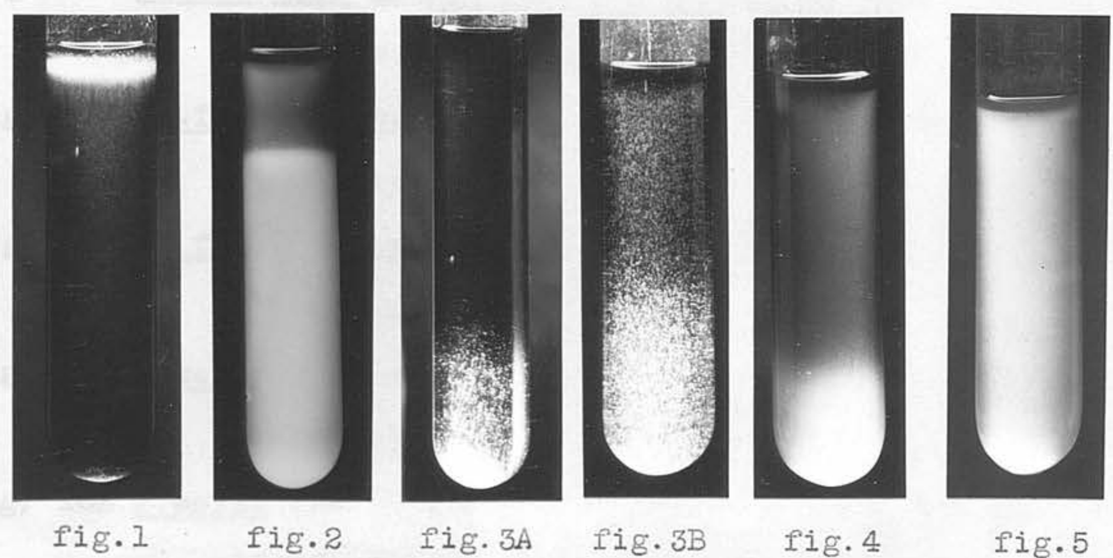

fig.1 fig.2 fig.3A fig.3B fig.4 fig.5

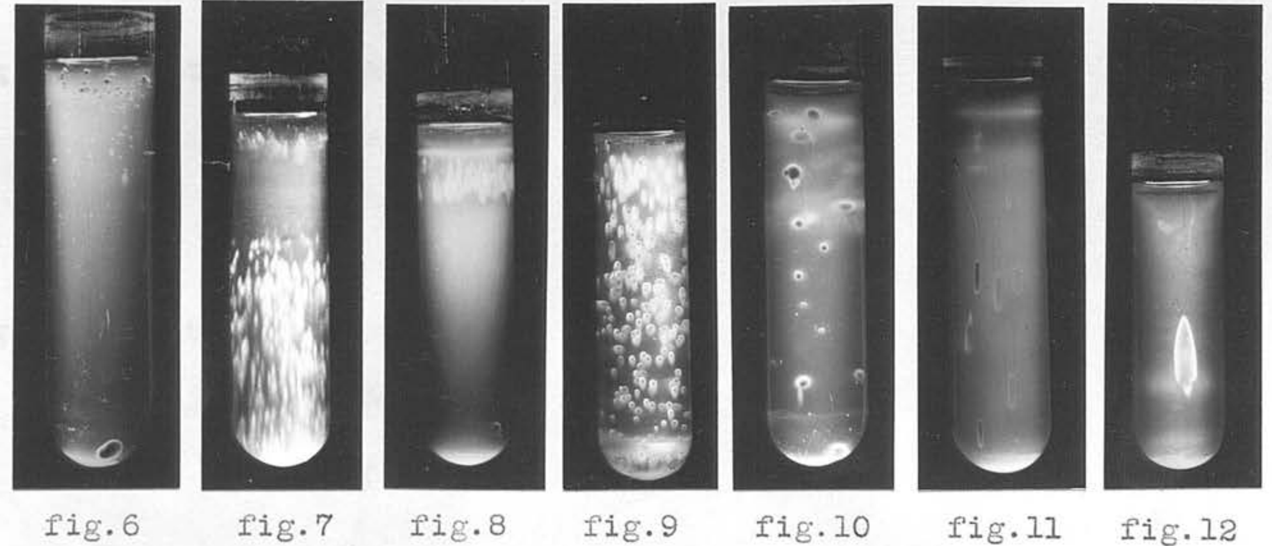

fig.6 fig.7 fig.8 fig.9 fig.10 fig.11 fig.12

EXPLANATION OF PLATE 2.

Fig. 14. Lb. buchneri growing and producing acid microaerophilically at 72 hr. in xylose soft agar.

Fig. 15A. Lb. fructovorans NCIB 8039. Growth distribution at 35° at 3 days in glucose soft agar.

Fig. 15B. Lb. fructovorans NCIB 8039. Growth distribution at 37° at 3 days in glucose soft agar.

Fig. 16A. P. soyae IAM 1673 utilizing glucose in the presence of salt, 5·0%(w/v), at 4 days.

Fig. 16B. P. soyae IAM 1673 utilizing glucose in the presence of salt, 15·0%(w/v), at 4 days.

Fig. 16C. P. soyae IAM 1673 utilizing glucose in the presence of salt, 18·0%(w/v), at 1 week.

Fig. 17. A strain of pediococcus utilizing glucose in the presence of salt, 10·0%(w/v), at 5 days.

Fig. 18. A strain of non-dextran forming leuconostoc utilizing glucose aerobically and anaerobically at 3 days. Aerobic growth is separated from anaerobic growth by a zone of inhibition due to hydrogen peroxide.

Fig. 19. A strain of non-dextran forming leuconostoc utilizing glucose at 5 days. The zone of inhibition has been reduced.

PLATE 2

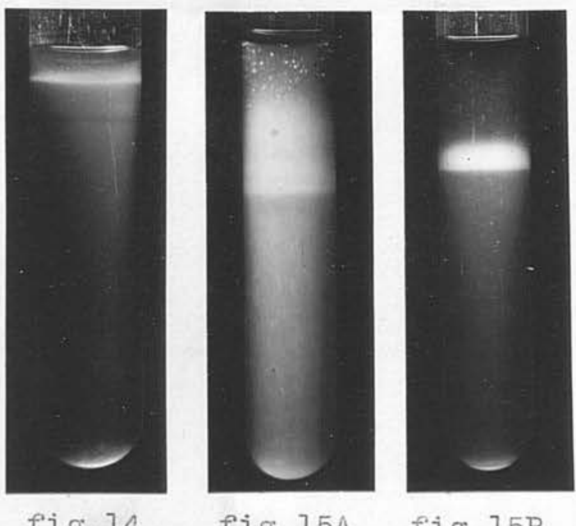

fig.14 fig.15A fig.15B

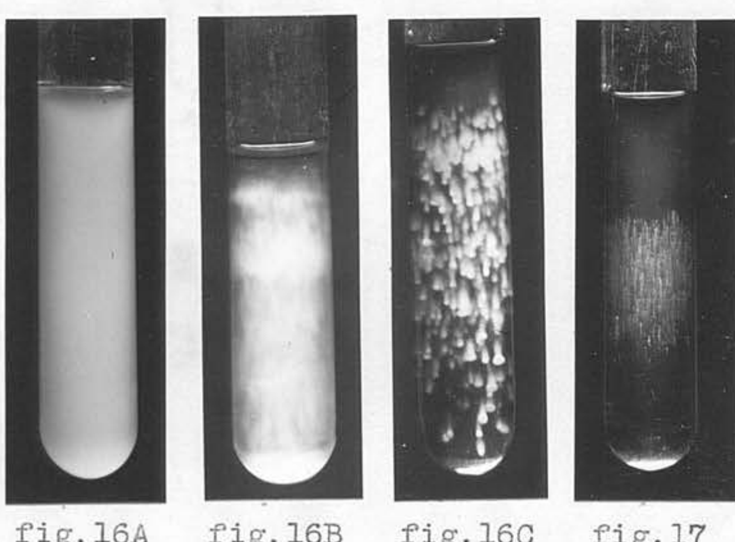

fig.16A fig.16B fig.16C fig.17

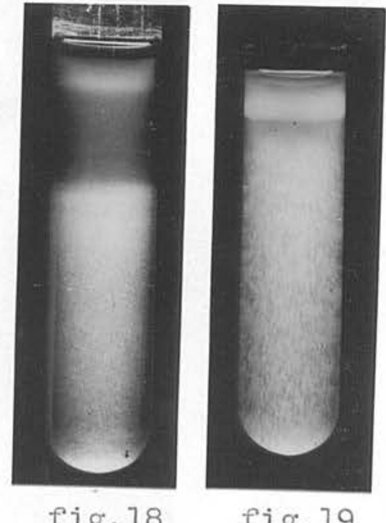

fig.18 fig.19

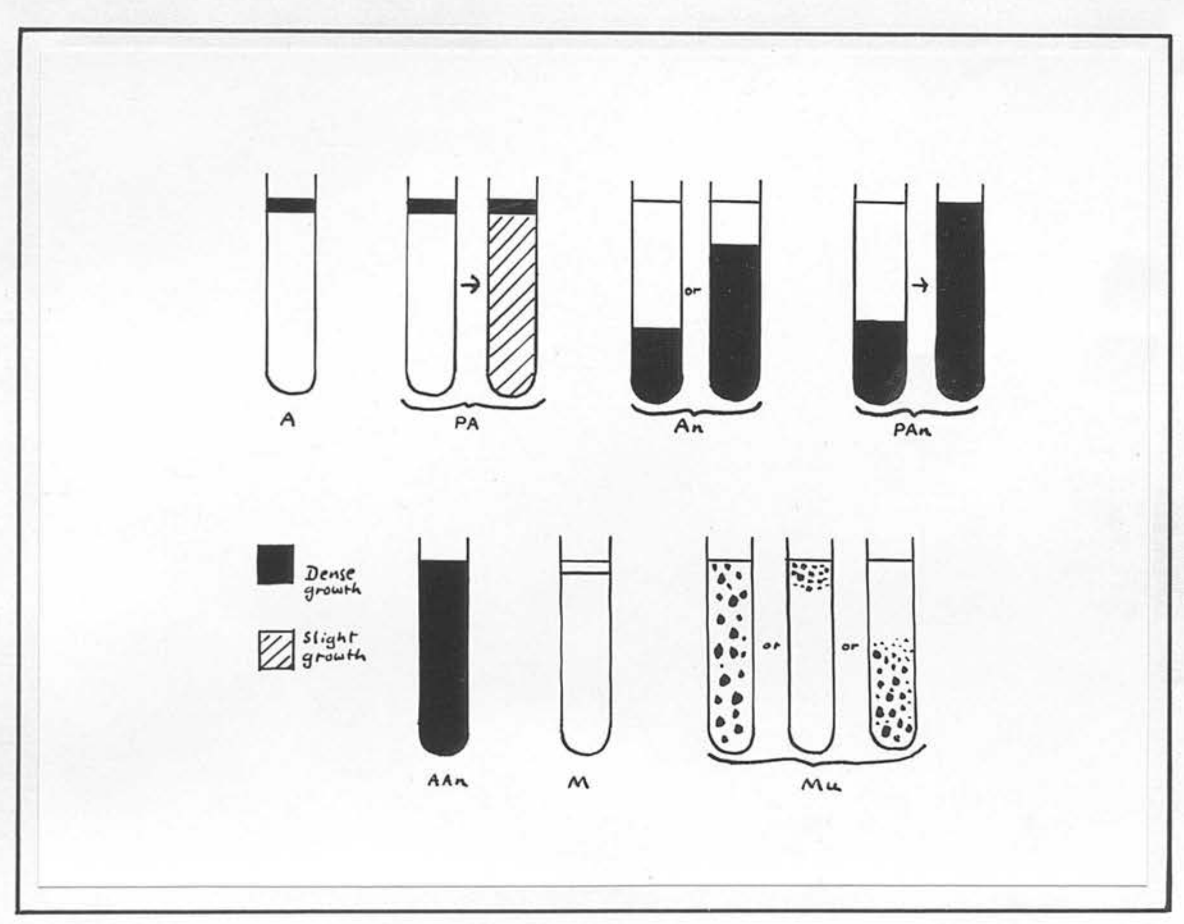

Fig. 20

Diagrammatic representation of growth variations described in Table 1.

Section 2.

A Study of some enterococci.

INTRODUCTION

It is generally recognized that the enterococci can be divided into two major types. One type includes Streptococcus faecalis, Str.zymogenes and Str.liquefaciens, and the other Str.faecium, Str.durans and certain unnamed organisms. The physiological tests separating these two types, however, are few and apparently do not always give satisfactory results in the hands of some investigators.

Therefore, the enterococci were examined for other differential properties. The results have suggested certain methods which, in conjunction with those in use at present, might assist the identification of the enterococci. The same methods have been found to be applicable to other types of lactic acid bacteria which are described in subsequent sections.

METHODS

The media and methods used are described, or referred to, in the appendix.

RESULTS AND DISCUSSION

A small selection of enterococci was obtained from a variety of sources (Table 1). Strains thought to be typical of recognized species and strains which possessed unusual characteristics, were selected. The object was to cover as far as possible the range of variation.

All were Gram-positive cocci, occurring singly, in pairs, and frequently in short to medium length chains. None reduced nitrate to nitrite, produced carbon dioxide from glucose, formed dextran from

sucrose, split hydrogen peroxide on 1·0%(w/v) glucose agar, or hydrolysed starch. All fermented glucose, fructose, mannose, galactose, maltose, lactose, cellobiose, and split aesculin. All grew at 10° and 45°.

The organisms were grouped into two major types (Table 2), the _faecalis_ type and the _faecium_ type. This division agrees with the findings of Skadhauge (1950), Sharpe & Shattock (1952), Shattock (1955) and Lake, Deible & Niven (1957), while the recognition of _Str.faecalis_ - or _Str.glycerinaceus_ - and _Str.faecium_ as separate species agrees with the findings of Orla-Jensen (1919 & 1943), Olsen (1949), Skadhauge (1950), Shattock (1955), Barnes (1956) and Lake _et al_ (1957).

In this investigation no clear-cut distinctions could be made between the two types in the carbohydrates utilized. Some strains of the _faecalis_ type fermented melezitose aerobically and anaerobically, some mutantly, and some not at all. Some strains of the _faecium_ type behaved in a similar way with melibiose. A clear-cut distinction was achieved, however, when the ability to utilize glycerol, sorbitol, and mannitol, and their manner of doing so, were considered together.

The strains of the _faecium_ type which utilized glycerol, only did so aerobically, whereas all but one of the _faecalis_ type utilized glycerol aerobically and anaerobically. The one exception, _Str.faecalis_ NCIB 370, only utilized glycerol aerobically. Under anaerobic conditions, three of the strains of the _faecalis_ type utilized glycerol mutantly. The mutants were otherwise physiologically identical with their parent cultures. On glycerol HBD agar, all the glycerol utilizing _faecium_ types formed peroxide. None of the

faecalis type did so provided an aerobically-grown inoculum from a glucose agar slope was used. If the inoculum was taken from glucose liquid medium, slight peroxide reactions occasionally appeared. This suggests that a proportion of the cells in liquid culture had grown under anaerobic conditions and that they had to adapt to peroxidative metabolism. When sodium fumarate, 0·5%(w/v), was added to glycerol soft agar as an additional hydrogen acceptor, all strains of the *faecalis* type, including the mutant and aerobic strains, produced dense anaerobic growth, whereas the *faecium* type strains still continued to utilize glycerol aerobically only.

To sum up, the two groups can be distinguished by their growth in glycerol soft agar containing fumarate and by their peroxide forming ability on glycerol HBD agar. These methods are based on the findings of Gunsalus (1947) who suggested the value of differences in glycerol utilization as tests for distinguishing *Str.faecium* from *Str.glycerinaceus* (*Str.faecalis*).

Mannitol was utilized aerobically and anaerobically by all organisms of the *faecalis* type and by many of the *faecium* type. In cultures of the *faecium* type, however, a narrow zone containing no growth frequently separated aerobic and anaerobic growth. This zone was attributed to the downward diffusion of peroxide from the more rapidly occurring aerobic growth. The inclusion of pyrolusite in the medium confirmed this suggestion as the zone now failed to appear and pyrolusite was dissolved in that area. On mannitol HBD agar all the mannitol utilizing strains of the *faecium* type, but none of the *faecalis* type, produced a peroxide reaction. This behaviour, then, was a useful test for distinguishing the mannitol utilizing strains of the two types, the non-mannitol utilizing organisms being of the

faecium type only.

Sorbitol was utilized aerobically and anaerobically by all strains of the *faecalis* type but by only one of the *faecium* type. This strain, however, only utilized sorbitol mutantly. The mutant, which was otherwise similar to the parent culture, formed peroxide on sorbitol HBD agar. None of the *faecalis* type strains did so. The two groups can be distinguished on sorbitol, therefore, as on mannitol.

Inositol utilization was of limited use as a test, but the manner in which it was utilized by strains of the *faecalis* type capable of doing so is worth noting. In inositol soft agar, growth was aerobic only but if fumarate was added to the medium anaerobic growth also occurred. Peroxide was not formed on inositol HBD agar.

As indicated above, peroxide formation on various media serves to distinguish the two groups. Another example was the production of peroxide by the *faecium* type strains on sugar-and sugar alcohol-free HBD agar. If glucose, 0·5%(w/v), was included in the medium, only three strains still formed peroxide. The reaction, however, was stronger than that occurring in the absence of added glucose.

Growth and peroxide production on HBD and MDO agar containing the alcohols - provided they were utilized - was much more pronounced than it was in the absence of these substances. This distinction was most evident on MDO agar because of the dependence of the peroxide reaction in this medium on the lactic acid formed. If the alcohols were not utilized, the peroxide reaction was similar to that occurring on the substrate-free medium.

A few strains of both types produced β haemolysis. The remainder of the *faecium* type organisms produced varying degrees of α haemolysis.

If glucose was added to the medium, only the strains known to produce peroxide from glucose continued to produce a greening reaction. Therefore, as α haemolysis and peroxide formation appear to be similarly affected by the presence of glucose, it seems reasonable to assume that α haemolysis in this instance is caused by peroxide.

The methods employed in the examination of malate and citrate dissimilation were those used by Whittenbury (1956) in distinguishing between a strain of Str. faecalis and strains of Str. faecium.

All the strains of the faecalis type, except two, utilized malate as an energy source. The use of malate as an energy source has been shown previously for Str. faecalis by Campbell, Bellamy & Gunsalus (1943). Occasionally the tubes had to be tapped sharply to initiate effervescence of carbon dioxide. The pH rose to a value above 7·0. BCP, however, was rapidly decolourized in the body of the medium. Where the culture was in contact with the atmosphere - the top portion of the agar seal and the surface of the medium when the agar seal had dried out and permitted the free entry of oxygen - the indicator remained coloured.

All organisms of the faecium type dissimilated malate, but did not appear to use it as an energy source. The major change noted was the rise in pH. Gas was rarely released into the medium unless the cultures were heated.

If glucose 1·0% (w/v), or the same concentration of another fermentable sugar, was added to the medium, a sharp contrast in the reactions of the two types resulted. All the faecalis type strains produced acid but no gas, indicating that malate was not dissimilated. All the faecium type strains, on the other hand, dissimilated malate vigorously. After 3 or 4 days the pH had

usually risen to 7·0 or higher. This medium, therefore, was useful in distinguishing between the two groups.

Citrate was dissimilated in the absence but not in the presence of 1·0%(w/v) fermentable sugar. It was used as an energy source by all the organisms of the faecalis type and by some of the faecium type. Campbell et al.(1943) have reported the use of citrate as an energy source by Str.faecalis. The faecalis type organisms utilized citrate equally well in media initially at pH 6·0, 7·0 and 8·0, though at pH 8·0 the tubes needed a sharp tap to initiate effervescence. On the other hand, the faecium type strains utilized citrate well in a medium at pH 6·0, slightly or not at all at pH 7·0, and not at all at pH 8·0. Therefore, the ability to dissimilate citrate at pH 8·0 was useful in distinguishing between the two types.

Malate and citrate dissimilation by faecalis type organisms and citrate dissimilation by faecium type organisms, though inhibited in each case by 1·0%(w/v) fermentable sugar, were not inhibited when the sugar concentration was reduced to 0·25%(w/v). Lake et al (1957) separated Str.faecalis from Str.faecium on citrate metabolism but, as no details were given, it is not clear whether they failed to encounter citrate-utilizing strains of Str.faecium or adjusted the conditions as was done here.

In a medium containing malate and a fermentable carbohydrate some strains of the faecium type gave rise to viscous cultures and ropy growth which could be drawn out to a length of 3-4 in. In a citrate medium, many faecalis type strains behaved similarly. The capsules formed by the faecalis type strains, examined by Duguid's (1948) wet India ink method, were unequal in size, though the largest were twice the size of those formed by the faecium type strains, which produced

capsules of uniform size. Other lactic acid bacteria, including the pediococci, were found to behave in a similar manner in citrate-and malate-containing media. Gunsalus & Niven(1942) reported on slime production by Str.liquefaciens grown in a glucose medium buffered at an alkaline pH. The material was isolated but not identified, though it was observed that some of the glucose disappearing from the medium could only be accounted for by the slime produced. In the present investigation conditions promoting slime formation were not examined in detail, though preliminary results indicate that if the pH is maintained at or above 6·0, slime is formed regardless of the type of energy source available.

All organisms initiated growth at pH 9·6, though, from the soft agar tests, it was clear that only a minute proportion of the inoculum of Str.durans and some intermediate organisms in the faecium type were able to grow at that pH.

Apo-catalase activity, restricted to strains of the faecalis type, was weak and best detected by heaping the growth before the addition of peroxide.

Classification of the enterococci.

The faecalis type comprises organisms recognizable as Str.faecalis, Str.liquefaciens, and Str.zymogenes. These organisms were considered to be sufficiently similar to justify their classification in one species, Str.faecalis, a conclusion previously reached by other investigators.

It was difficult to separate into species the organisms which have been referred to as the faecium type. Some were regarded as typical Str.faecium - those fermenting melibiose, arabinose and mannitol and utilizing glycerol aerobically - some as typical

Str.durans - those utilizing glycerol aerobically and producing β haemolysis but failing to ferment arabinose, melibiose and mannitol - but there were others with varying ranges of activity. Some appeared to be closely related to Str.faecium and some to Str.durans, whilst others occupied an intermediate position. The motile strain appears to be very similar to the yellow pigmented motile organisms described by Graudal(1952), to the motile strains described by Langston, Gutierrez & Bouma(1960), possibly to those described by, or referred to, by Hugh(1959) and to the motile Group D streptococcus mentioned by Sherman(1938). Over a period of 4 years this strain has lost its ability to produce ammonia from arginine, but, apart from this variation and the ability to ferment inulin, it is very similar to Str.faecium. As the differences among the strains were mainly restricted to carbohydrate and sugar-alcohol utilization - a gradual loss of such ability occurring from Str.faecium to Str.durans - the organisms in this group were regarded as the one species, Str.faecium.

CONCLUSIONS

Apart from the tests already in common use, a number of others described in this investigation were useful in separating the enterococci into two major types classified as Str.faecalis and Str.faecium. The tests are summarized in Table 3.

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Table 1.

Source and identification of cultures.

	Identification.	Designation when received.	Source.
faecalis type	Str. faecalis	Str. faecalis 581	NCDO
		Str. faecalis 775, 2707 and 370	NCIB
		Str. faecalis B8, H69D5, N83 and S161.	Dr. P.M.F. Shattock.
	Str. liquefaciens	Str. liquefaciens 588 and 598	NCDO
		213 and BLACK	Dr. T. Gibson
		Str. liquefaciens N97, E1V, and GB 122	Dr. P.M.F. Shattock
		Str. liquefaciens S41	Own isolate from silage.
	Str. zymogenes	Str. zymogenes 585 and 587	NCDO
faecium type	Str. faecium	Str. faecium 944 and 945	NCDO
		Str. faecalis 582	NCDO
		Str. faecium HGH511, IMEC and P5	Dr. P.M.F. Shattock
		S1, S2, S3, S4, S5, S6, 1, 60 and 73	Own isolates from silage.
		JMA 8	Dr. T. Gibson
	Intermediate 1.	Str. faecium 942 and 943	NCDO
		Str. faecalis 580	NCDO
		Str. faecalis 8274	NCIB
		Str. faecium Hobbs 2678	Dr. P.M.F. Shattock
		Enterococcus 2703	Dr. T. Gibson
	Intermediate 2.	32	Own isolate from silage.
	Intermediate 3.	SD 20, SD1 and JMA 3	Dr. T. Gibson
		Str. durans 498	Dr. P.M.F. Shattock
	Str. durans	98 D	Dr. T. Gibson
		Str. durans 596	Dr. P.M.F. Shattock
	Motile strain	43	Own isolate from silage.

NCDO - National Collection of Dairy organisms.
NCIB - National Collection of Industrial Bacteria.

Table 2.

Classification of the enterococci

Test	faecalis type			faecium type					
	Str. faecalis.	Str. zymogenes.	Str. liquefaciens.	Str. faecium.	Intermediate 1	Intermediate 2	Intermediate 3	Str. durans	Motile strain
Arabinose	−	±	−	+	+	−	−	−	+
Rhamnose	+	+	+	±	±	−	−	−	+
Melibiose	−	−	−	+Mu	−	+	−	−	+
Sucrose	+	+	+	±	±	+	−	−	+
Trehalose	+	+	+	+	+	+	±	−	+
Melezitose	+Mu	−	+	−	−	−	−	−	−
Raffinose	−	−	−	±	±	+	−	−	+
Glycerol	*+Mu	+	+	A̲	A	A	A	A	A
Mannitol	+	+	+	±	±	−	−	−	+
Sorbitol	+	+	+	M̲u̲	−	−	−	−	−
Inositol	±	±	±	−	−	−	−	−	−
Inulin	−	−	−	−	−	−	−	−	+
Hippurate	±	+	±	±	±	−	−	−	−
Growth and acid at 50°	−	−	−	±	±	−	−	−	−
Motility	−	−	−	−	−	−	−	−	+
Gelatin liquefied	−	−	+	−	−	−	−	−	−
β Haemolysis	−	+	−	−	−	−	−	+	−
α Haemolysis	−	−	−	+	+	+	+	−	+
Survival 63°/30 min.	±	+	+	±	+	+	+	+	+
Tellurite	+	+	+	−	−	−	−	−	−
Tetrazolium	+	+	+	−	−	−	−	−	−

continued.

Table 2 - continued.

Test	Str. faecalis.	Str. zymogenes.	Str. liquefaciens.	Str. faecium.	Intermediate 1	Intermediate 2	Intermediate 3	Str. durans	Motile strain
	faecalis type			faecium type					
Growth at pH 9·6	+	+	+	+	+	F	F	F	+
6·5% salt	+	+	+	+	+	+	±	±	(+)
Peroxide formed on:-									
HBD agar	–	–	–	+	+	+	+	+	+
HBD agar plus glucose	–	–	–	±	–	–	–	–	+
" glycerol⨍	–	–	–	+	+	+	+	+	+
" mannitol⨍	–	–	–	+	+	+	+	+	+
" sorbitol⨍	–	–	–	+	+	+	+	+	+
" inositol⨍	–	–	–	+	+	+	+	+	+
Apo-catalase	±	+	±	–	–	–	–	–	–
Organic acids dissimilated									
Malate	++	++	++ –	+	+	+	+	+	+
Malate plus glucose	–	–	–	++	++	++	++	++	++
Citrate pH 6·0	+	+	+	±	±	–	±	±	+
Citrate pH 8·0	+	+	+	–	–	–	–	–	–
Reduction of litmus milk (8 hr.)	+	+	+	–	–	–	–	–	–
Litmus milk (2 days)	ARC	ARC ARCL	ARCL	R ARC	R ARC	ARC	r –	–	ARC
0·1% MB milk (2 days)	RC	RC RCL	RCL	RC R	RC R	RC	r –	–	RC
Reduction of 0·1% MB milk (8 hr.)	+	+	+	–	–	–	–	–	–

⨍ Peroxide reaction by faecium types was more pronounced than that on substrate-free HBD agar if the substrate was utilized.

continued.

Table 2 - continued.

Mu = One or more strains mutant.

* = One strain aerobic only in absence of fumarate.

A = Aerobic only.

F = Few colonies.

ARC = Acid, reduced, clotted.

RC = Reduced, clotted.

RCL = Reduced, clotted, liquefied.

ARCL = Acid, reduced, clotted, liquefied.

R = Reduced

r = Slight reduction.

+ = All strains positive.

\- = All strains negative.

± = Some strains positive and some negative.

(+) = Weak reaction.

++ = Vigorous gas production.

Table 3.

The differentiation of the enterococci

	faecalis type	*faecium* type
Tolerance of:		
Tellurite	+	− (some strains produced slight grey growth)
Reduction of:		
Litmus milk (8 hr.)	+	−
Tetrazolium (8 hr.)	+	−
Fermentation (in soft agar)		
Glycerol + fumarate	+	− (Many utilize glycerol in presence of oxygen)
Melezitose	±	−
Melibiose	−	±
Peroxide formed on:*		
HBD agar	−	+
HBD agar containing sugar-alcohols	−	+
Organic acids:		
Malate as energy source	±	−
Gas from malate + glucose	−	+
Gas from citrate pH 8·0	+	−
Apo-catalase	±	−

*Inoculum must be from aerobically incubated slope culture.

Section 3.

A Study of the genus Pediococcus

INTRODUCTION

The pediococci have received only spasmodic attention and are as yet inadequately characterized. They are similar to the streptococci in their homofermentative action on glucose and their coccal form, but they differ in that they divide in two planes, forming tetrads. The pediococci have been encountered most frequently in plant materials, various foods and alcoholic beverages such as beer. Rarely, however, have they been the dominant fermentative organism wherever they were found and, in consequence, they have not attracted much attention. The following review is, therefore, devoted to an assessment of the present status of the genus *Pediococcus* and related organisms.

In Shimwell's(1948) opinion there is insufficient evidence to separate the pediococci from the streptococci, even though the pediococci divide in two planes and produce inactive lactic acid, whereas the streptococci divide in one plane only and form L(+) lactic acid. This opinion is supported by the work of Nakagawa & Kitahara(1959) and Deible & Niven(1960) who found that tetrad-forming organisms, classified as pediococci, produced L(+) lactic acid instead of the expected inactive acid. Orla-Jensen(1943) also recognized an L(+) lactic acid forming organism as a pediococcus. Originally he called the organism *Tetracoccus*, no.1 (Orla-Jensen 1919) but subsequently (Orla-Jensen, 1943) referred to it as *Pediococcus halophilus*, the name given to this organism by Mees(1934). Kitahara & Obayashi(1955) observed that, when an inactive lactic acid forming strain of *P. hennebergi* was cultured in a medium deficient in

nicotinic acid, a precursor of co-racemase, the resultant lactic acid was almost exclusively of the L(+) form. This work appears, therefore, to confirm Kitahara's opinion (1940) that pediococci are naturally L(+) lactic acid formers producing, in relation to their racemase activity which varies in different strains and cultural conditions, L(+), or mostly L(+) and some D(-), or inactive lactic acid.

Should this be a general finding, the use of optical rotation of lactic acid as a generic characteristic may be of limited value. Langston & Bouma(1959a) noted the production of optically inactive lactic acid instead of the expected L(+) lactic acid by two strains of Streptococcus liquefaciens (Str. faecalis). This illustrates the limited value of the optical rotation of lactic acid in separating pediococci and streptococci.

Felton, Evans & Niven(1953) have suggested that the pediococci should be removed from the Lactobacteriaceae. They based their opinion on the fact that some pediococci possess a weak peroxide-splitting ability when grown on a medium containing a low concentration of sugar. They presumed this activity to be due to catalase.

Jensen & Seeley(1954) supported this view by finding that similar strains oxidized p - phenylene diamine. This suggests that pediococci possess both a cytochrome c and a cytochrome oxidase. The observation, however, needs to be confirmed by the demonstration of the cytochromes themselves. As described in Section 1, the oxidation of p - phenylene diamine by similar types of pediococci was not observed.

The property of hydrogen peroxide-splitting ability as evidence for removing the pediococci from the Lactobacteriaceae is now questionable for two reasons. First, other types of lactic acid bacteria have been found to have a similar activity, namely strains of

Lactobacillus plantarum (Dacre & Sharpe,1956, Langston & Bouma,1959b), Leuconostoc mesenteroides (Langston & Bouma,1959a, Whittenbury,1960) and Str.liquefaciens (Langston, Gutierrez & Bouma,1960).

Second, it now seems clear that the enzyme concerned is not catalase. Delwiche(1961), examining the partially purified enzyme obtained from a strain of pediococcus, was unable to detect haemin in the extract or to inhibit peroxide-splitting activity with 1×10^{-2} M azide or cyanide. His observations have been confirmed (Section 1) using whole and broken cell-suspensions of pediococci.

The finding of a catalase-positive, cytochrome-possessing, motile strain of Lb.delbruekii (Vankova 1957) requires confirmation. The evidence presented is insufficient to distinguish this organism from Bacillus strains which can produce lactic acid but do not readily sporulate on media used for lactobacilli.

A further type of catalase-like activity in the lactic acid bacteria has since been demonstrated (Whittenbury, 1960) and described in Section 1. Some pediococci, not possessing the formerly described peroxide-splitting ability, contain what appears to be a constitutively formed apo-catalase as they develop a very active peroxide-destroying ability when haematin is made available to them. As strains of Lb.plantarum, Lb.casei, Lb.brevis, Lb.viridescens, Lb.fructivorans, non-dextran forming leuconostocs and Str.faecalis also possess this property, it seems that the pediococci are more closely related to the typical lactic acid bacteria than to the haemin-synthesizing, cytochrome-possessing micrococci.

The present evidence indicates, therefore, that the pediococci should be retained in the Lactobacteriaceae and that they are only completely separated from the streptococci by their mode of cell division.

A satisfactory separation of the genus Pediococcus into species has still to be achieved. Pederson(1949) recognized only two species, P. cerevisiae and P. acidilactici. They were separated by the hop tolerance of the former and the higher optimum growth temperature of the latter. Recently, however, Nakagawa & Kitahara(1959) described five species in some detail. Separation was founded on pH sensitivity, growth temperatures, catalase activity, carbon dioxide requirement, salt tolerance, hop tolerance and range of fermentation. Two of the species accommodate the two mentioned by Pederson(1949) and are similarly named. A third species, P. pentosaceous, appears similar to P. acidilactici as described by Pederson(1949), but has a lower maximum growth temperature than P. acidilactici described by Nakagawa & Kitahara. The two remaining species, P. urinae equi and P. halophilus, differ considerably from the others. Both are sensitive to acidity, tolerant of strongly alkaline conditions and produce L(+) lactic acid. The outstanding feature of P. halophilus, separating it from all other species, is its ability to proliferate in the presence of 18-20% salt. The work of these investigators appears to form a basis for a classification of the pediococci.

Langston & Bouma(1959a), whose work appeared at the end of this investigation, described five groups of pediococci, isolated from grass silage, which were distinguished by the substances fermented, arginine hydrolysis, and growth temperatures. If these groups are compared with the species described by Nakagawa & Kitahara(1959), Groups 1, 2 and 3 appear similar to P. pentosaceous, Group 5 to P. acidilactici and Group 4 to P. cerevisiae. Langston & Bouma's results help to confirm the basis of the classification proposed by Kitahara & Nagagawa(1959).

Deible & Niven(1960) have proposed the addition of a further species P. homari, which includes Aerococcus viridans, Williams, Hirsch & Cowan(1953), Gaffyka homari, Aaronson(1956) and similar organisms isolated from meat curing brines. These organisms produced L(+) lactic acid and were thought to be very similar to P. soyae as described by Sakaguchi(1958). From the available evidence, however, P. homari appears to be more similar to P. urinae equi described by Nakagawa & Kitahara(1959) than to P. soyae, an organism tolerant of high concentrations of salt and regarded by Nakagawa & Kitahara as synonomous with P. halophilus.

There appear, therefore, to be grounds for separating the pediococci in to five species at least. Representatives of all these species, either freshly isolated from silage and herbage or obtained from elsewhere, were examined in this investigation. Certain types of pediococci appeared similar to the enterococci. This is discussed.

METHODS

Media and Tests

Isolation medium. The pediococci isolated during this investigation were obtained from herbage embedded in a medium which was a modification of the acetate agar proposed by Keddie(1951) as a selective medium for lactobacilli. Tomato juice was omitted and the concentration of meat extract (Lab Lemco) and peptone (Evans) were reduced by half.

Tests and other media. These are referred to or described in the appendix.

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RESULTS AND DISCUSSION.

The tetrad-forming organisms (Table 1) were divided into six groups. None reduced nitrate to nitrite, liquefied gelatin, produced carbon dioxide from glucose, formed dextran from sucrose or split hydrogen peroxide on 1·0%(w/v) glucose agar. None reduced 2:3:5 triphenyl-tetrazolium-chloride significantly, tolerated potassium tellurite, hydrolysed starch, or dissimilated citrate. All, except Aerococcus viridans NCTC 8251, dissimilated malate in the presence of glucose and many did so slightly in the absence of glucose. Results of the test for survival after heating at 63°/30 min. were varied. Some organisms produced no growth, some a few colonies and a few 100-200 colonies per ml. of milk suspension plated on glucose agar. Slight acidity or no change was produced in litmus milk. No organism produced any change in 0·1%(w/v) methylene blue milk. All grew at 10° and 37°. All fermented glucose, fructose, galactose and mannose and none inositol or inulin. None dissimilated citrate in the presence or absence of glucose.

The identification of Groups 1, 2, 2a, 3 and 4. (Table 2)

The organisms of these groups could be considered to be typical pediococci.

Carbohydrate utilization was of little value as a means of separation, but the ability to utilize glycerol and the manner of its utilization was a useful differential characteristic.

All of the organisms except those in Group 3 utilized glucose equally well aerobically and anaerobically. One of the Group 3 strains, Pediococcus NCDO 1250, initiated growth in the anaerobic part of glucose medium adjusted to pH 6·8 (fig.4, Section 1). As the acid

diffused towards the surface, however, growth appeared in its wake suggesting that the lowering of pH was overcoming the organism's requirement for anaerobic conditions. This appeared to be confirmed by the following observations. Acid-producing growth appeared simultaneously, aerobically and anaerobically, in a glucose medium adjusted to pH 5·0. Growth appeared fairly rapidly on aerobically incubated glucose agar initially at pH 5·0, but very late and slightly, or not at all, on glucose agar initially at pH 6·8. With all sugars utilized the growth behaviour of this strain was the same as with glucose.

The remaining two strains in Group 3 showed a slight initial preference for anaerobic conditions but only in media at the higher pH. One of these strains, S 13, showed a marked initial preference for anaerobic conditions when fermenting lactose at the higher pH.

The ability to produce hydrogen peroxide on sugar-free HBD agar but not on glucose HBD agar separated Groups 1, 2 and 2a from all the other organisms. One strain in Group 3, Pediococcus NCDO 1250, produced peroxide both in the presence and absence of added energy sources. Growth and peroxide production by Groups 1, 2 and 2a was greater on glycerol HBD agar than on sugar-free HBD agar. This observation confirmed that the organisms required oxygen when utilizing glycerol in soft agar.

Ammonia production from arginine aided the separation of Groups 1, 2 and 2a from Groups 3 and 4. Varying the amount of glucose in the medium from 0·5 to 2·0%(w/v) did not affect the result.

Salt tolerance distinguished Group 4 from all other groups. The presence of 5·0% salt in the medium hastened the appearance of

growth of this organism but did not otherwise affect the organism's behaviour as observed in media lacking salt. Although 18·0% was tolerated by this organism, comparatively few cells in the inoculum gave rise to growth (fig.16C, Section 1). Organisms in Groups 1, 2 and 2a tolerated 8-10%(w/v) salt but growth at these concentrations was restricted to a few scattered acid-producing colonies which were sometimes present only in the middle zone of the medium (fig.17, Section 1).

The groups were compared with the amended species descriptions given by Nakagawa & Kitahara (1959), taking into account their different methods and cultural conditions. Group 1 is identifiable as *P.pentosaceous*, Group 2 and 2a as *P.acidilactici*, Group 3 as *P.cerevisiae*, and Group 4 as *P.halophilus*.

One of the organisms examined by Nakagawa & Kitahara(1959) and identified by them as *P.halophilus* was Orla-Jensen's(1919) *Tetracoccus* no.1 obtained from R.H.Mees. Orla-Jensen isolated it from anchovy pickle brine, containing 15·0%(w/v) salt, and found that it tolerated 15·5%(w/v) salt, and required 5·0%(w/v) for good growth. He also noted its lack of surface growth in stab culture, its inability to liquefy gelatin or to reduce nitrate to nitrite, its ability to form L(+) lactic acid, its maximum growth temperature (37·5° - 40°), and its ability to ferment, amongst other substrates, arabinose, mannitol and salicin. Mees(1934), when he proposed the species *P.halophilus*, noted that the organism was catalase negative. This organism, therefore, appears to have been well described as long ago as 1919.

Table 3 summarizes the differentiation of the groups.

The identification and description of Group 5.

The results (Table 4) indicate that the two strains examined are very similar. The question is to which genus do they belong.

One of the two strains in this group, A.viridans 8251, is the type culture of the genus Aerococcus. Deible & Niven(1960) considered A.viridans similar to the pediococci and included it in the species P.homari.

The other strain in Group 5 is P.urinae equi. It originates from Mees, who first described this organism and proposed the species name (Mees,1934). Nakagawa & Kitahara(1959) examined the same organism, expanded its description and retained it in the genus Pediococcus.

Although these two organisms possess a broad similarity to the pediococci, being similar morphologically and fermenting glucose homofermentatively, they possess other characteristics not typical of previously described pediococci.

An unusual feature was their preference for aerobic conditions when utilizing sugars, in spite of the fact that peroxide was produced and they were unable to form a peroxide-splitting enzyme. They also produced dense aerobic, peroxide-forming, growth in sugar-free soft agar. Colonies reached their largest size, 2mm. in diameter, on sugar-free, lysed-blood agar. Large areas of greening surrounded the colonies - a feature of A.viridans on blood agar noted by Williams et al.(1953) - corresponding to peroxide zones formed on lysed-blood media containing benzidine or o-dianisidine. Growth also occurred on meat extract, peptone agar, colonies up to 0·5 mm. being formed. These features, along with ability to initiate growth at pH 9·8, acid-sensitivity, slight acid producing

powers, and strict requirement for aerobic conditions when utilizing glycerol, mannitol and sorbitol, might be considered sufficient to separate these organisms from the genus Pediococcus.

Williams et al.(1953) excluded A.viridans from the genus Pediococcus mainly on the grounds that it was not microaerophilic, did not form much acid from glucose, fermented mannitol frequently and raffinose rarely. On the basis of tolerance tests they suggested that A.viridans was similar to the enterococci. However, the ability of A.viridans to utilize mannitol and sorbitol only aerobically, its preference for aerobic conditions, its inability to form ammonia from arginine, to grow at 45°, or to produce much acid from glucose, and its acid sensitivity and morphology, are not a combination of features remotely characteristic of any enterococcus species.

Certain characteristics of the organisms of Group 5 suggest a closer relationship with the staphylococci than with either the pediococci or the enterococci. In particular they appear very similar to some of the D type staphylococci described by Abd-el-Malek & Gibson(1948). Both organisms and some D type staphylococci fermented sugars, grew at 37° but not at 45°, were unable to produce ammonia from arginine, or liquefy gelatin, or reduce nitrate to nitrite, and only produce a final pH of 5·2 in glucose liquid medium. Acid production from mannitol and glycerol - sorbitol was not examined - by the D type staphylococci was recorded as variable, and Gibson (priv.comm.) states that this implies late and slight acid production. Similar behaviour, in the writer's experience, has often been found with lactic acid bacteria and the Group 5 organisms in liquid culture if they were only able to utilize these substances aerobically.

Staph.aureus has been recorded as utilizing both glycerol (Collwell,1939 and Gunsalus & Sherman,1943) and mannitol (Collwell,1939) aerobically only. It is possible, therefore, that the D type staphylococci also utilize these substances aerobically only as do the two strains in Group 5.

A major difference between the staphylococci and the Group 5 organisms lies in the possession of haemin-enzymes by the staphylococci. The ability to produce peroxide, the inability to split peroxide and the inability of cell pastes to produce a blue colour in the presence of benzidine and peroxide, indicate that the Group 5 strains are both catalase and cytochrome negative. Jensen(1957), however, has described mutants of Micrococcus pyogenes var. aureus (Staph.aureus) which were unable to synthesize haemin and consequently produced small colonies on aerobically incubated media. Although these mutants possessed the apo-enzymes of catalase and cytochromes, forming these enzymes when haemin was available, they would probably be difficult to distinguish from the Group 5 organisms in the absence of available haemin. Morphology would be unlikely to distinguish them as it was found that anaerobically grown cultures of Staph. aureus, Staph.albus, the Group 5 organisms and pediococci contained cocci of similar size occurring singly, in pairs and tetrads. Only aerobically-grown cultures were markedly different, the staphylococci forming large rafts and clumps of cells and the aerococci smaller clumps and tetrads, whilst the pediococci appeared as they did in anaerobic culture. This aerobic variation may reflect the different abilities of the organisms to utilize oxygen as, anaerobically, when forced to rely on fermentation for securing energy, they were very similar in morphology.

Their behaviour with respect to oxygen may indicate that the Group 5 organisms represent a further stage in retrogressive development of non-haemin synthesizing staphylococci. They were preferentially aerobic when utilizing sugars and strictly aerobic in utilizing glycerol, mannitol and sorbitol. Under aerobic conditions, however, peroxide accumulated. In view of the toxic nature of peroxide, such behaviour appears illogical. It may indicate that they have lost the ability to synthesize haemin and the ability to utilize haematin as a co-enzyme, or the ability to synthesize the apo-enzymes. Group 5 organisms might, therefore, be staphylococci with impaired respiratory systems.

A closer examination was made of the possibility that the Group 5 organisms may be related to the staphylococci. As no D type staphylococci were available at the time of the investigation, a preliminary comparison was made with a strain of *Staph.aureus* and a strain of *Staph.albus*. The object was to determine whether there were any broad similarities in the reactions of the Group 5 organisms and the staphylococci to various tests.

Of the two staphylococci, *Staph.albus* resembled the Group 5 organisms most closely (Table 4). Two points not indicated in the table are of interest. Although *Staph.albus* and the Group 5 organisms were unable to produce acid aerobically or anaerobically at pH 5·0, *Staph.albus* produced dense non-acid forming growth aerobically at this pH. This may reflect differences in their oxidative abilities. There was also a similarity in the behaviour of both types of organism in the presence of salt concentrations close to the maximum they could tolerate, acid producing growth becoming restricted to the surface layers. As described earlier,

the pediococci tended to grow in the middle zone of the medium if the salt concentration affected growth distribution.

On the basis of the results described above and indicated in Table 4, a more detailed comparison of the Group 5 organisms with the staphylococci, particularly with the D type staphylococci of Abd-el-Malek & Gibson(1948) and haemin-deficient staphylococcal mutants, is merited before conclusions can be drawn on the taxonomy of the Group 5 organisms. Meanwhile, it is suggested that the genus Aerococcus be maintained and that P.urinae equi, Mees is a synonym of the type species A.viridans. Apparently similar organisms, such as Gaffyka homari and organisms included in the species P.homari (Deible & Niven,1960), should also be placed in the genus Aerococcus.

The similarity of P.pentosaceous and P.acidilactici to Str.faecium.

In a comparison of the pediococci with the enterococci which are described in Section 2, it was seen that pediococci identified as P.pentosaceous and P.acidilactici were physiologically similar to Str.faecium. A closer physiological relationship appears to exist among these three species than between Str.faecalis and Str.faecium. These relationships are emphasised with examples in Table 5 where a strain of P.pentosaceous is compared with a similar strain of Str.faecium and with a strain of Str.faecalis.

Certain features, however, distinguish the two species of pediococci from these entrococci. The enterococci do not form tetrads or grow on acetate agar, but do grow at pH 9·6 and produce a final pH above 4·0 in glucose liquid medium. The pediococci form tetrads, grow well on acetate agar, do not grow at pH 9·6 and produce a final pH lower than 4·0 in glucose liquid medium.

The serological relationships have yet to be determined. Preliminary precipitin and gel diffusion tests (Norris & Whittenbury, unpublished data) revealed strong cross-reactions between Lancefield group D sera, prepared from Str.faecalis H69D5 and Str.faecium MEC1, and HCl extracts of P.pentosaceous and P.acidilactici. There was also a cross-reaction between a serum prepared from a strain of P.acidilactici and HCl extracts of strains of Str.faecalis and Str.faecium. A serum prepared from P.pentosaceous, however, only reacted with the homologous strain. These findings are being investigated in greater detail, no conclusions being drawn at this stage.

It is clear that if tetrad formation is missed during the morphological examination of the pediococci, many strains would be classified as Str.faecium or as variants of Str.durans, according to the tests commonly used in the identification of the enterococci. This observation particularly applies to investigations where very few tests are employed to differentiate between large numbers of enterococci.

CONCLUSIONS

The main conclusions drawn from this investigation are as follows. The organisms identified as pediococci are divisible into four species. P.pentosaceous, P.acidilactici, P.cerevisiae and P.halophilus. Two tetrad-forming organisms, P.urinae equi and A.viridans, appear to have properties in common with both the pediococci and the staphylococci. P.urinae equi is identified as an aerococcus and is considered to be a synonym of the type species A.viridans. Pediococci, identified as P.pentosaceous and P.acidilactici, appear to be physiologically similar to Str.faecium.

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Table 1.

Source and identification of tetrad-forming organisms.

Identification	Designation when received.	Source.
P.pentosaceous	W 1, W 2, W 3, W 4, W 5, W 50.	Own isolates from herbage and silage.
	M 1, M 11, M 12.	From silage by Cunningham & Smith (1940)
	P_2 & P_3	From silage by Dr. A.C. Stirling.
	Ln.citrovorum NCIB 7837	NCIB
	Ln.citrovorum NCIB 8106	NCIB
P.acidilactici	P 4, P 5, P 7	From silage by Dr. A.C. Stirling.
	Ln.mesenteroides NCIB 8018	NCIB
P.cerevisiae	S 13 & S 14.	From silage by Cunningham & Smith (1940).
	Pediococcus NCDO 1250	NCDO
P.halophilus	P.soyae IAM 1673	Dr. H.Iizuka.
A.viridans	A.viridans NCTC 8251	NCTC
	P.urinae equi, Mees IAM 1684	Dr. H.Iizuka.

Table 2.

Classification of the pediococci.

Classified as.	P. pento-saceous	P. acidi-lactici		P. cerev-isiae	P. halo-philus
Group	1	2	2a	3	4
Substrate utilized or dissimilated:-					
Arabinose	±	+	+	−	−
Xylose	±	+	+	−	−
Rhamnose	±	+	−	−	−
Melibiose	±	±	−	−	−
Sucrose	−	±	−	±	+
Lactose	±Mu	±	+	±	−
Trehalose	+	−	+	+	+
Maltose	+	−	−	+	+
Melezitose	−	±	−	−	−
Raffinose	−	−	−	−	−
Cellobiose	+	±	+	+	+
Glycerol	*A	A	A	−	+
Mannitol	−	−	−	−	−
Sorbitol	−	−	−	−	−
Salicin	+	+	+	+	+
Hippurate	−	−	−	−	−
Aesculin	+	+	+	+	+
Arginine	+	+	+	−	−
Growth in glucose soft agar	AAn	AAn	AAn	AAn/PAn	AAn
Growth and acid at.					
40°	+	+	+	−	−
45°	+	+	+	−	−
50°	−	+	+	−	−

continued.

Table 2 - continued.

Classified as	P. pentosaceous	P. acidilactici		P. cerevisiae	P. halophilus
Group	1	2	2a	3	4
Survival 63°/30 min.	±	±	-	-	NT
Final pH (glucose)	3·6	3·6	3·6	3·4 to 3·8	4·5
Growth on acetate agar	+	+	+	(±)(1)	-
Peroxide formation					
HBD agar	+	+	+	±(2)	-
" + glucose	-	-	-	±(2)	-
" + glycerol	+	+	+	±(2)	-
Growth and acid at:-					
pH 5·0	+	+	+	+	-
8·0	+	+	+	-	+
9·6	-	-	-	-	-
Pseudocatalase	±	-	-	-	-
Apo-catalase	-	+	-	-	-
Dissimilation of:-					
Malate	(±)	(±)	(+)	-	-
Gas from malate + glucose	++	++	++	++	++
Salt tolerance:-					
4, 8, 10, 12, 18%.	8-10	8-10	8	4	18

(1) *Pediococcus* NCDO 1250 was the only negative strain.
(2) *Pediococcus* NCDO 1250 was the only peroxide positive strain.

NT = not tested
± = only some strains positive
Mu = some mutant
A = aerobic only
* = 3 only produced slight acid
(+) = weak reaction

A = Aerobic.
PA = Preferentially aerobic.
AAn = Aerobic and anaerobic.
PAn = Preferentially anaerobic.
++ = vigorous gas production.

Table 3.

Differential characters of the pediococci examined in this investigation.

	P. pentosaceous (Group 1)	P. acidilactici (Group 2 & 2a)	P. cerevisiae (Group 3)	P. halophilus (Group 4)
Substrate utilized:-				
Trehalose	+	±	+	+
Maltose	+	−	+	+
Glycerol	*A	A	−	+
Growth and acid at:-				
45°	+	+	−	−
50°	−	+	−	−
Arginine Hydrolysis	+	+	−	−
Peroxide formation:-				
On HBD agar but not on Glucose HBD agar	+	+	−	−
Pseudocatalase	±	−	−	−
Apo-catalase	−	±	−	−
Salt tolerance %:				
8·0	+	+	−	+
18·0	−	−	−	+
Growth at:-				
pH 5·0	+	+	+	−
8·0	+	+	−	+

*Some strains give a very weak reaction.

Table 4.

Properties of Group 5 organisms and some properties of a strain of Staph. albus

Classified as	A. viridans NCTC 8251	P. urinae equi IAM 1684	Staph. albus
Group	5	5	
Substrate utilized or dissimilated			
Arabinose	-	-	-
Xylose	-	-	-
Rhamnose	-	-	-
Melibiose	-	(+)	-
Sucrose	+	+	+
Lactose	+	+	+
Trehalose	(+)	+	+
Maltose	+	+	+
Melezitose	-	-	-
Raffinose	-	+	-
Cellobiose	-	+	-
Glycerol	A	A	A
Mannitol	A	A	A
Sorbitol	A	A	A
Salicin	-	+	-
Hippurate	+	+	-
Aesculin	-	-	+
Arginine	-	-	+
Growth in glucose soft agar	PA	PA	AAn
Growth and acid at			
40°	-	-	+
45°	-	-	-
50°	-	-	-
Survival 63°/30 min.	NT	NT	NT
Final pH (glucose)	5·2	5·2	4·6
Growth on acetate agar	-	-	-
Peroxide formation			
HBD agar	+	+	-
" + glucose	+	+	-
" + glycerol	+	+	-
Growth and acid at:-			
pH 5·0	-	-	-
8·0	+	+	+
9·6	+	+	+
Pseudocatalase	-	-	-
Apo-catalase	-	-	catalase positive
Dissimilation of:-			
Malate	-	-	-
Gas from malate ± glucose	-	++	(+)
Salt tolerance:- 4, 8, 10, 12 18%	10	10	18

Legend as for Table 2.

Table 5.

Comparison of the characteristics of a strain of P.pentosaceous with Str.faecium - intermediate type SD20 - and Str.faecalis H69D5.

Characteristics common to Str.faecium Str.faecalis and to P.pentosaceous

	Str.faecalis Str.faecium P-pentosaceous
Substrate fermented or hydrolysed:	
Glucose	+
Fructose	+
Sucrose	-
Arabinose	-
Maltose	+
Cellobiose	+
Galactose	+
Raffinose	-
Melibiose	-
Mannose	+
Lactose	+
Trehalose	+
Inositol	-
Salicin	+
Aesulin	+
Arginine	+
Growth at:	
10°	+
45°	+
Tolerance of:	
6·5% NaCI	+
63°/30 min.	+

Characteristics common to Str.faecium and P.pentosaceous separating them from Str.faecalis

	Str.faecium P.pentosaceous	Str. faecalis
Substrate fermented or hydrolysed:		
Melezitose	-	+
Rhamnose	-	+
Sorbitol	-	+
Mannitol	-	+
Glycerol	A	+
Hippurate	-	+
Vigorous reduction of:		
Tetrazolium	-	+
Litmus milk	-	+
Tolerance of:		
MB milk	-	+
Tellurite	-	+
Organic acids		
Vigorous malate dissimilation	-	+
Gas, malate + glucose	+	-
Gas, citrate pH 8·0	-	+
Apo-catalase	-	+
Peroxide formed:		
Sugar-free HBD agar	+	-
Glycerol HBD agar	+	-
α haemolysis	+	-

Characteristics common to Str.faecalis and Str.faecium separating them from P.pentosaceous

	Str.faecalis Str.faecium	P.pentosaceous
Growth at pH 9·6	+	-
Final pH in glucose	4·1	3·6
Growth on acetate agar	-	+
Pseudo-catalase	-	+
Tetrads formed	-	+

Section 4.

A study of the genus Leuconostoc.

INTRODUCTION

The first clear definition of the heterofermentative streptococci was given by Orla-Jensen(1919), who separated them from the homofermentative streptococci and placed them in the new genus Betacoccus. He proposed two species within the genus but suspected that he was treading on treacherous ground.

Hucker & Pederson(1930 & 1931) recognised these organisms as belonging to the genus Leuconostoc (van Tieghem,1878) and proposed their classification into three species. The species were separated on their ability to ferment the pentoses and to form slime from and ferment sucrose.

The first species, Leuconostoc mesenteroides, (Cienkowski,1878) van Tieghem, fermented either or both arabinose and xylose and fermented and produced slime from sucrose. This description applied to many of the strains of both the species described by Orla-Jensen(1919), but did not take into account Orla-Jensen's observations on the ability of betacocci to acquire or lose the powers of pentose fermentation.

The second species, Ln.dextranicum, which fermented neither pentose but fermented and formed slime from sucrose, was based on Beijerinck's (1912) Lactococcus dextranicus. Beijerinck, however, though describing this organism's inability to ferment xylose, did not mention arabinose fermentation. Thus, Lactococcus dextranicus might have been similar to Ln.mesenteroides. A strain of Streptococcus [illegible] Evans(1916), isolated by Evans and deposited in the American Type Culture Collection by L.A. Rogers in 1925, was [illegible]

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The third species, which fermented neither the pentoses nor sucrose was identified as Streptococcus citrovorus, Hammer(1920) and designated Ln.citrovorus, the specific epithet appeared as citrovorum in the 4th edition of Bergey's Manual (Bergey,1934).

The most recent comprehensive study of the leuconostocs is by Garvie(1960) who divided the organisms into six groups.

Group I seems to have been identified as Ln.citrovorum. Nevertheless, the suggestion was made that the name of this species should be changed to Ln.cremoris. The chief reason given to justify this proposal was that several cultures, which are distinctly different from St.citrovorus as described by Hammer, have been in circulation with the designations 'St.citrovorus' or 'Ln.citrovorum'. The history of these cultures shows that they do not provide any acceptable evidence on the characters of the type culture, which appears to have been lost at an early date.

Group II comprised organisms identified as Streptococcus kefir by Abd-el-Malek & Gibson(1948), and others obtained elsewhere. Garvie rejects the epithet kefir because the amended description of Str.kefir(Migula), Evans(1918), does not apply to these organisms. Evans(1918) is reported as having stated that Str.kefir fermented raffinose, salicin and mannitol, whereas, in fact, she clearly indicated that raffinose was fermented by most strains, salicin by one out of twenty three and mannitol by none. These characters do apply to Garvie's Group II organisms and to Abd-el-Malek & Gibsons' organisms. Hucker & Pederson (1931 p.103) also report Evans' work incorrectly. The major characteristic of this group was its limited range of fermentation. None of the nine organisms in the group formed slime from sucrose and only one fermented a pentose.

Group IV organisms were identified as Ln.dextranicum though it is indicated (Table 3, Garvie) that out of five strains one does not ferment sucrose even though it forms slime from sucrose

Groups III, V and VI were identified as Ln.mesenteroides. Group III comprises sucrose-fermenting organisms which do not form slime from sucrose and two non-sucrose fermenting organisms (Table 3, Garvie).

McClesky, Faville & Barnett(1947) and Leiva-Quiros & McClesky(1947) attempted to subdivide strains of Ln.mesenteroides, isolated from cane juice, into homogenous subgroups, but were only able to recognize one such group and emphasized the heterogenous nature of the species.

From this brief review, it is concluded that there may exist three species, Ln.mesenteroides, Ln.dextranicum and Ln.citrovorum and probably a fourth Str.kefir. In the present investigation, a number of leuconostocs, isolated from grass and silage, are compared with named strains and these four species.

METHODS

The tests and media used are described in the appendix, apart from the following details.

Isolation medium. This was similar to that used in isolating pediococci.

Carbon dioxide production from glucose. The modification of Hayward's (1957) method was routinely used.

The addition of manganese as manganese sulphate and citrate as potassium citrate to the basal media. Manganese sulphate, 0·05%(w/v) was included in all media except those used in the initial experiments on the pattern of growth behaviour.

Potassium citrate, 0·5%(w/v) later reduced to 0·1%(w/v), was included in all media except those used in the examination of growth behaviour, those used in experiments on organic acid dissimilation, and those used in testing for gas production from glucose.

Citrate and malate dissimilation. Because of the heterofermentative nature of these organisms, glucose was replaced by a pentose when behaviour in the presence of a carbohydrate was being examined.

Organisms

Isolation of organisms.

Fresh, partially withered, withered and bruised grass, obtained from pastures, uncultivated land, and woodlands, was cut into small pieces and placed on the surface of setting acetate agar. When the agar had set, so trapping the grass, more agar was added until all the grass was covered. Incubation was at 22° or 27° for five days.

Various substrates - glucose, gluconate, sucrose, arabinose, and xylose - were used as the energy source. Sucrose was added to yield a final concentration of 5·0%(w/v) and the other substrates to yield a final concentration of 0·5%(w/v).

The number of colonies appearing were few, varying from none to fifteen or so per plate. The majority appeared on the surface of the partially withered and bruised grass and only very rarely on the fresh grass. The colonies, except those appearing on sucrose agar, were lens-shaped, of varying size, with their long axis generally lying along the length of the grass. Growth on sucrose agar varied, appearing as formless watery slime which split the agar, as slimy colonies, as tough gristly colonies and as non-dextran forming, lens shaped colonies.

The colonies were picked into liquid medium containing the substrates which had supported their growth. They were purified by streaking once on the substrate agar and then twice on glucose agar.

All were Gram-positive organisms and over 90% were cocci. A few of the cocci occurred as tetrads and proved to be pediococci whilst the remainder formed chains of varying length and eventually proved to be heterofermentative.

A number of leuconostocs were isolated on similar media from silage and some named strains were obtained from culture collections and individuals. A total of one hundred strains was examined.

RESULTS AND DISCUSSION

The classification of the organisms.

The organisms were often lanceolate on glucose agar but were coccal in liquid medium, occurring singly, in pairs, and in short to medium length chains. All strains produced small, greyish-white colonies on glucose agar containing hydrogen peroxide-destroying substances. None utilized sorbose, glycerol or sorbitol, produced ammonia from arginine, hydrolysed hippurate, reduced nitrate to nitrite, grew at 45° or split peroxide when grown on glucose 1·0%(w/v) agar slopes. All were able to utilize carbohydrates in media initially at pH 6·8 or 5·4 and grow at 15°. Although manganese and citrate were included in the media - the media being used in the study of other lactic acid bacteria - manganese did not appear to benefit growth and citrate only affected the manner of growth of one strain. Citrate was not dissimilated in the absence of sugar by any organism.

The organisms were divided into three sections on their ability to form peroxide and on their ability to form dextran from sucrose. These sections were subjected to a number of tests and subdivided into groups (Table 1.).

Some characteristics differentiating the groups are given in Table 2. Additional comments on the properties of the groups and on their classification are given below.

<u>Section A</u> - peroxide forming, non-dextran forming organisms,

<u>Group 1</u> - Eight organisms from fresh grass. Seven of the strains produced fair anaerobic growth in glucose medium but it was never as dense as the earlier formed aerobic growth. The aerobic growth in glucose medium was frequently separated from the anaerobic growth by a zone containing no growth (see Fig.19, Section 1). This was caused by the downward diffusion of peroxide from the surface growth, the peroxide inhibiting the initiation of anaerobic growth in that region. The remaining organism in this group, S70, only produced significant amounts of growth under aerobic conditions with any energy source. Attempts were made to improve its anaerobic growth. Diacetyl, 0·25%(w/v), had a stimulatory effect and citrate in a medium initially at pH 6·0 had a slight stimulatory effect. The slightly increased growth occurring in both cases gave rise to slight acidity.

In the absence of peroxide-destroying substances, growth on aerobically incubated media was slight and rapidly lost viability. An interesting feature of these organisms was their ability to form a peroxide-splitting enzyme in the presence of extracts of clover and grass leaves as well as on haematin-containing media. This behaviour has been referred to in Section 1.

Carbon dioxide production from glucose was slight in the case of seven strains and not evident with S70. This was thought to be due to their inability to grow well under anaerobic conditions which would rapidly occur under the agar seal. Their heterofermentative nature was confirmed by the method of Williams & Campbell(1951). Carbon dioxide produced by growth on 2·0%(w/v) glucose, pyrolusite agar slopes, was trapped as barium carbonate. Homofermentative streptococci,

used as controls, produced traces of carbon dioxide whereas the organisms in this group formed relatively large amounts.

These organisms could be identified as non-dextran forming variants of Ln.mesenteroides in the classification of Hucker & Pederson(1931). It is considered, however, that their collective characteristics suggest that they might constitute a new species for which the name Ln.gramineum is provisionally proposed. Their behaviour with resepct to oxygen, their apo-catalase activity and the ability of the one strain examined to form cytochromes (Section 1) when grown on heated-blood medium, suggests that they have an impaired respiratory system and that their natural environment might be aerobic.

Group 2 - Two organisms from fresh grass. These organisms were similar to the Group 1 organisms but failed to grow at 10° and were apo-catalase negative. They could be identified as Ln.mesenteroides as defined by Hucker & Pederson(1931) but they appear to be more closely related to Ln.gramineum.

Group 3 - Four organisms from milk identified as Str.kefir(Migula), Evans(1918) by Abd-el-Malek & Gibson(1948).

These organisms were slow growing and slow to initiate growth. A notable feature was the number of instances of weak, late and microaerophilic substrate utilization. Arabinose was only fermented by two strains and in an unusual manner. Acid-producing growth appeared in a sharply-defined zone stretching from the bottom of the tube to within 0·5 cm. of the surface. Growth was very slight, being only slightly better than that occurring in the sugar-free medium, and did not improve on further incubation. The pH, however, fell from 6·8 to 5·4. Growth and acid production was not improved by the variation of medium constituents, medium pH, or by serial subculture through

arabinose medium. Whilst action on arabinose was constant in soft agar, it was variable in liquid medium incubated aerobically. If the liquid cultures were incubated anaerobically, however, arabinose utilization was always observed. These observations may account for the variable and weak pentose fermentations noted by Orla-Jensen(1919) for some betacocci, and the acquiring of the ability to ferment arabinose noted for some leuconostocs by Pederson & Albury(1955).

As all strains fermented sucrose but only two a pentose, two could be classified as non-dextran forming strains of <u>Ln.dextranicum</u>, (Hucker & Pederson,1931) and two as non-dextran forming variants of <u>Ln.mesenteroides</u> in the scheme of Hucker & Pederson(1931). It is considered, however, that weak pentose fermentation is not a significant activity on which to separate these otherwise very similar organisms into two species. No outstanding features separate these organisms from all others. They are, meanwhile, identified as <u>Str.kefir</u>(Migula), Evans(1918) and provisionally renamed <u>Ln.kefir</u>.

<u>Group 4</u> - Four organisms from herbage and four named strains known to be non-dextran forming; <u>Ln.mesenteroides</u> NCDO 803, 869, 870 and 871.

Sucrose was fermented vigorously by five strains and weakly by three strains. Occasionally within the first 18-24 hr. of incubation, a narrow zone of no growth caused by peroxide diffusing from the surface growth separated aerobic and anaerobic growth in glucose soft agar. The zone soon disappeared on further incubation. Six of the organisms dissimilated malate in the absence of sugar and the same six did so vigorously in the presence of arabinose.

These organisms are identified as non-dextran forming variants of <u>Ln.mesenteroides</u> as defined by Hucker & Pederson(1931). Apart from forming peroxide and failing to produce dextran, they appeared

to be very similar to dextran-forming organisms in groups identified as Ln.mesenteroides.

Section B - non-dextran forming, non-peroxide forming organisms.

Group 5 - Four named organisms, Ln.citrovorum NCDO 543, 705, 828 and 829.

These organisms were distinctive because they fermented fewer sugars than any of the other organisms examined. Three fermented sucrose mutantly within four to five days. The sucrose fermenting mutants were otherwise identical to their parent strains. They did not form dextran from sucrose and could not be induced to do so.

Apart from the ability of three organisms to ferment sucrose mutantly, the reactions of all four identify them as Ln.citrovorum (Hammer) Hucker & Pederson(1931). The action on sucrose may not invariably be detected if liquid media are used. This may explain why Garvie(1960) did not observe sucrose fermentation by the same strains.

Unfortunately, the ability to ferment sucrose is a characteristic used in separating Ln.mesenteroides and Ln.dextranicum from Ln.citrovorum. According to the presently used classification, then, strains of Ln.citrovorum may give rise to non-dextran forming variants of Ln.dextranicum.

Section C - dextran forming, non-peroxide forming organisms.

Group 6 - Two named strains, Ln.dextranicum NCDO 529 and NCIB 3355

Few sugars were fermented. Dextran production was slow. One strain produced colonies up to 3·5 mm in diameter, which were hemisphherical and composed of clear and opaque slime, the other strain produced flat watery colonies. One strain grew microaerophilically at 37°.

The organisms are identified as Ln.dextranicum as characterized by Hucker & Pederson(1931). Apart from their ability to form dextran, they appear to be very similar to the organisms in the previous group.

Group 7 - Eight organisms from herbage.

Group 8 - Two organisms from herbage.

Group 9 - Two organisms, one from herbage and Ln.mesenteroides NCDO 538.

The organisms in these three groups differed in the type of substrates they utilized, in their manner of growth in glucose medium and in growth temperature range. A feature common to all groups, however, was the manner in which the pentoses were fermented. Growth was slight and uniformly distributed but only slightly more dense than that occurring in the sugar-free medium. It was not improved by subculture through pentose media or by varying the pH of the medium or by varying the constituents of the medium. The final pH in each instance was 5·4.

The appearance of the dextran varied. Four strains in Group 7 and one strain in Group 8 formed tough, small cartilaginous colonies; two strains in Group 7 formed transluscent, hemispherical slimy colonies up to 3·5 mm. in diameter; and the remaining strains in all groups produced watery slime.

The organisms failing to ferment a pentose conform to Ln.dextranicum (Hucker & Pederson,1931) and the remaining strains to Ln.mesenteroides as defined by Hucker & Pederson(1931). The ability to ferment a pentose weakly, however, is considered to be an unsatisfactory characteristic with which to separate these otherwise very similar organisms into two species. The three groups are, therefore, classified as being intermediate between Ln.dextranicum and Ln.mesenteroides.

<u>Group 10</u> - Thirty four organisms from herbage.

All strains fermented at least one pentose vigorously. Dextran appeared as watery slime, as hemispherical slimy colonies with a diameter of 2-4 mm., or as cartilaginous colonies often surrounded by clear slime. Six strains were pseudocatalase positive. Thirteen strains dissimilated malate slightly in the absence of sugar and vigorously in the presence of arabinose or xylose.

These organisms are identified as <u>Ln.mesenteroides</u> according to Hucker & Pederson(1931).

<u>Group 11</u> - Fourteen organisms from herbage and silage.

These organisms are very similar to those in the previous group, differing mainly in their manner of growth in glucose medium. Dextran appeared as large clear or opaque hemispherical slimy colonies 3·5-4·0 mm. in diameter.

These organisms are identified as <u>Ln.mesenteroides</u> as defined by Hucker & Pederson(1931).

<u>Group 12</u> - Twelve organisms from herbage and silage.

Of all the organisms examined, this group possessed the widest range of positive properties. They formed large, domed, translucent colonies up to 6·0 mm. in diameter on sucrose agar.

These organisms are identified as <u>Ln.mesenteroides</u> in the Hucker & Pederson(1931) classification.

<u>Discussion of classification.</u>

A division of the organisms into species according to Hucker & Pederson(1931) was unsatisfactory. Sucrose and pentose utilization, as indicated in the last section, appear to be of questionable value as taxonomic characteristics.

An attempt at classification according to the utilization of any particular carbohydrate, or group of carbohydrates, is complicated by instances of weak, slow, microaerophilic or mutant utilization. However, the manner of growth in the glucose medium and whether a large or small number of substrates were utilized, proved useful in grouping the organisms.

Apo-catalase activity separated the preferentially aerobic Group 1 organisms from all others. Peroxide formation was of value in the initial grouping of the organisms. As representative strains from each group took up oxygen on glucose, it appears that the peroxide-positive organisms may possess flavoprotein oxidases and the peroxide-negative organisms flavoprotein peroxidases. These types of oxidases have been described in other lactic acid bacteria by Dolin(1957) and Strittmatter(1959).

The ability or inability of organisms to form dextran remained constant throughout the investigation and was used in the initial grouping of the organisms. The naked-eye morphological appearance of dextran (Figs. 1-9) fell broadly into five categories; watery slime spreading over the plate; small, domed, clear to opaque slimy colonies; large, domed, clear to opaque slimy colonies; soft, rugose colonies of varying size; and small, tough, opaque, cartilaginous colonies causing indentations in the agar. The latter two types of colony frequently became surrounded by a zone of clear slime.

Dextran formation was most pronounced at 22°. At 30° the cartilaginous and rugose colonies were frequently similar in size to glucose-grown colonies. The sucrose-grown colonies, however, were distinctive as they were tough, of irregular shape, and embedded in the agar, whereas the glucose-grown colonies were buttery in texture.

At 37° many dextran-formers produced colonies on sucrose agar which were indistinguishable from glucose-grown colonies.

The significance of dextran production as a taxonomic characteristic is open to question as Pederson & Albury(1955) and Langston & Bouma(1960) reported experiments in which they were able to change non-dextran forming leuconostocs into dextran-formers after a long series of subcultures through various media. Pederson & Albury also reported the accomplishment of the reverse change by subculturing organisms through media of increasing salt concentration. On the other hand, an attempt to confirm these observations with the non-dextran and dextran forming organisms examined in this investigation failed after two months continual subculture through similar media. Dr. C.W. Langston kindly provided three of his strains which had at one time been induced to form dextran but his results could not be repeated in this investigation. Other workers, Abd-el-Malek & Gibson(1948) and Niven, Castellani & Allanson(1949) also failed to induce non-dextran forming leuconostocs to form dextran. Further evidence on this question appears to be required.

The ability to dissimilate malate and citrate was restricted to the organisms with the greatest number of positive properties. Because of the heterofermentative nature of these organisms and the methods used in detecting organic acid breakdown, the ability to dissimilate organic acids in the presence of a sugar could only be tested with a pentose. The taxonomic value of this test was limited, therefore, as it could only be applied to the vigorous pentose fermenters. Malate, though dissimilated slightly in the absence of a sugar, did not enhance growth and therefore did not appear to be used as an energy source. Citrate was only dissimilated in the

presence of a sugar.

Growth temperature range correlated fairly well with the general activity of the groups. The organisms with few positive properties grew over the narrowest temperature range, whilst the organisms with many positive properties grew over the widest temperature range.

CONCLUSIONS

The organisms were divided into groups by various tests. The classification of the organisms according to Hucker & Pederson(1931) proved unsatisfactory. No alternative classification which would group the organisms into homogenous and distinctive species was evolved. A number of groups are clearly distinguishable but intermediate groups suggest a gradual transition from one distinctive type to the next. - E.g. Group 12 can be distinguished from Group 5 on the basis of carbohydrate utilization, dextran production, organic acid dissimilation and growth temperature range, Groups 6, 7, 8, 9, 10 and 11, however, appear to represent transitional stages between these two groups - .

Some of the characteristics of these organisms, especially of the Group 1 organisms, suggest that they may have an impaired respiratory system. The leuconostocs, therefore, may have developed from aerobically respiring organisms and the groups may be representative of various stages of retrogressive development. These suggestions require further investigation.

For reference purposes, and because some of the groups may be representative of the dominant type of leuconostoc in a particular environment, the organisms are provisionally classified as follows:-

Groups 1 and 2 as Ln.gramineum; Group 3 as Ln.kefir; Groups 5 and 6 as Ln.dextranicum; Groups 4, 10, 11 and 12 as Ln.mesenteroides; and Groups 7, 8 and 9 as being intermediate between Ln.dextranicum and Ln.mesenteroides.

It is suggested that the description of Ln.dextranicum given by Hucker & Pederson(1931) should be amended to include Ln.citrovorum as defined by the same authors. Of four organisms identified here as Ln.citrovorum, three gave rise to sucrose fermenting, non-slime forming mutants. As Ln.dextranicum and Ln.citrovorum are separated only on sucrose utilization and dextran formation, there appears to be no useful purpose served in separating these very similar organisms into two species on the basis of dextran production alone. The name dextranicum (Beijerink,1912) is retained in favour of citrovorum (citrovorus, Hammer, 1920) as it is the earlier epithet.

No amended descriptions of species are given, as further information is required on the significance of features used in separating the organisms into species, and on the interrelationships of the types.

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Table 1.

Properties of the Leuconostocs.

	Section A				Section B	Section C						
Groups:	1	2	3	4	5	6	7	8	9	10	11	12
Dextran from sucrose	−	−	−	−	−	+	+	+	+	+	+	+
Peroxide formation												
Glucose HBD agar	+	+	+	+	−	−	−	−	−	−	−	−
Glucose MDO agar	+	+	+	+	−	−	−	−	−	−	−	−
Growth in glucose soft agar	PA	PA	AAn	AAn	AAn A/An	A/An	AAn	A/An	AAn	A/An	AAn	AAn
Substrate utilized:-												
Sucrose	+	+V	+	+V	V	+	+	+	+	+	+	+
Arabinose	+	+	$\bar{S}$	+	−	−	$\bar{S}$	−	S	±	+	+
Xylose	−	±	−	±	−	−	$\bar{S}$	S	S	+V	+	+
Fructose	+	+	+V	+	−	+	+	+	+	+	+	+
Maltose	+	+	+V	+	−	±	±V	+	+	+	+	+
Lactose	±V	±	+V	±V	±	±V	−	+	+	+V	−	+
Cellobiose	−	−	−	±V	−	−	+	−	+	±	+	+
Melibiose	+	+	+V	±V	−	−	−	+	+	+	−	+
Mannose	±	+	+V	+	−	+	±V	−	−	±	+	±
Galactose	+	+	+	+	+	±	−	−	−	+	±	+
Melezitose	±	−	−	−	−	−	−	−	−	−	−	−
Raffinose	+	±	+V	+	−	−	−	+	+	±	−.	+
Mannitol	±	−	±V	±V	−	−	±V	+V	+V	±	±	±
Salicin	−	−	−	±V	−	−	+	−	+	±V	+V	±
Pseudocatalase	−	−	−	−	−	−	−	−	−	±	−	−
Apo-catalase	+	−	−	−	−	−	−	−	−	−	−	−
Growth and acid production at:-												
5°	+	−	+	−	−	−	+	+	+	+	+	+
10°	+	−	+	+	+	+	+	+	+	+	+	+

continued.

Table 1 continued

Groups:	Section A 1	2	3	4	Section B 5	Section C 6	7	8	9	10	11	12
Growth and acid production at:-												
37°	±	+	+	+	-	±	-	+	+	+	+	+
40°	-	-	+	+	-	-	-	-	+	±	-	+
Organic acid dissimilation.												
Malate	-	-	-	±	-	-	-	-	-	±	+	+
Malate + Arabinose	-	-	NT	±	NT	NT	NT	NT	NT	±	+	+
Citrate + Arabinose	-	-	NT	-	NT	NT	NT	NT	NT	-	-	+

Growth in fructose medium - if fructose utilized - was AAn and in pentose medium - if pentose utilized - AAn, except for Group 3 which was An.

NT = Not tested.

V = Slow, late, micro-aerophilic or mutant utilization.

S = Acid, but very slight growth.

AAn = Aerobic and anaerobic growth.

A/An = Aerobic first but anaerobic growth follows rapidly and was dense as surface growth.

An = Anaerobic growth only.

PA = Preferentially aerobic.

\+ = All strains positive.

\- = All strains negative.

± = Some strains positive and some negative.

Table 2

Some characteristics differentiating the leuconostocs

	Section A.				Section B.	Section C.						
Groups:	1	2	3	4	5	6	7	8	9	10	11	12
Peroxide formation												
Glucose HBD agar	+	+	+	+	-	-	-	-	-	-	-	-
Apo-catalase	+	-	-	-	-	-	-	-	-	-	-	-
Dextran from sucrose	-	-	-	-	-	+	+	+	+	+	+	+
Growth in glucose preferentially aerobic	+	+	-	-	-	-	-	-	-	-	-	-
Slight(S) Dense(D) or no(-) acid producing growth with:												
Arabinose	D	D	S -	D	-	-	S -	-	S	D -	D	D
Xylose	-	D -	-	D -	-	-	S -	S	S	D - S	D	D
Growth at:												
5°	+	-	+	-	-	-	+	+	+	+	+	+
10°	+	-	+	+	+	+	+	+	+	+	+	+
37°	±	+	+	+	-	±	-	+	+	+	+	+
40°	-	-	+	+	-	-	-	-	+	±	-	+
Organic acid dissimilation. Gas from malate + Arabinose or Xylose	-	-	NT	±	NT	NT	NT	NT	NT	±	+	+
pH rise in malate	-	-	-	±	-	-	-	-	-	±	+	+
Gas from citrate + Arabinose	-	-	NT	-	NT	NT	NT	NT	NT	-	-	+

NT = Not tested as pentose weakly or not utilized.
\+ = All strains positive.
\- = All strains negative.
± = Some strains positive and some negative.

EXPLANATION OF PLATES 1, 2, and 3.

Colony formation at 2 weeks at 22°c on sucrose, 5·0%(w/v), agar. 1·4 x magnification.

PLATE 1

Fig. 1 Group 10 organism. Clear, domed colonies.

Fig. 2 Group 6 organism. *Ln.dextranicum* NCDO 529. Hemispherical colonies, clear and opaque.

Fig. 3 Group 7 organism. Translucent, hemispherical colonies of varying size.

PLATE 2

Fig. 4 Group 10 organism. Soft, opaque, slightly rugose colonies.

Fig. 5 Group 10 organism. Cartilaginous colonies surrounded by an inner ring of opaque slime and an outer ring of clear slime.

Fig. 6 Group 6 organism. *Ln.dextranicum* NCIB 3355. Flat, watery, opaque slime.

PLATE 3

Fig. 7 Group 10 organism. Soft, rugose colonies.

Fig. 8 Group 7 organism. Cartilaginous colonies.

Fig. 9. Group 10 organism. Cartilaginous colonies.

PLATE 1

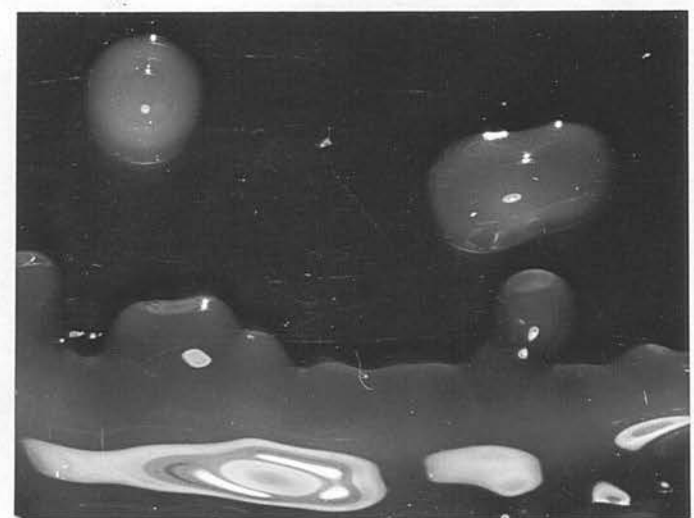

Fig. 1

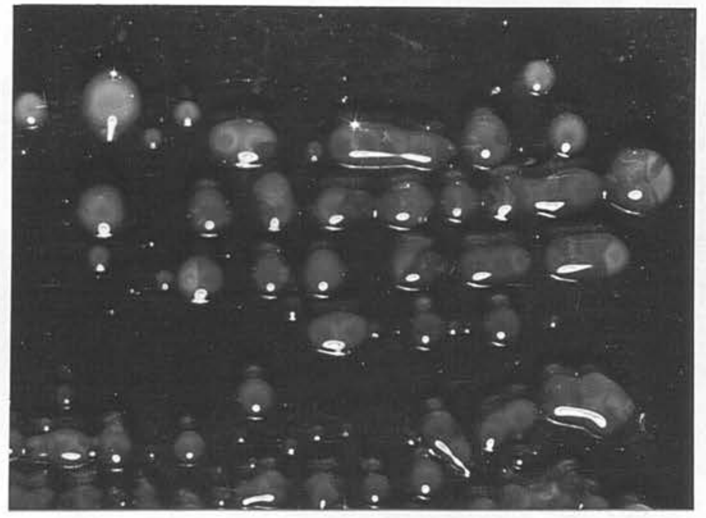

Fig. 2

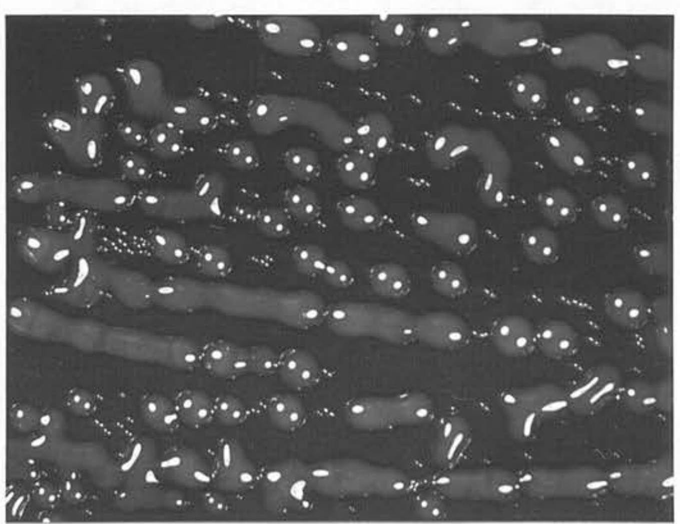

Fig. 3

PLATE 2

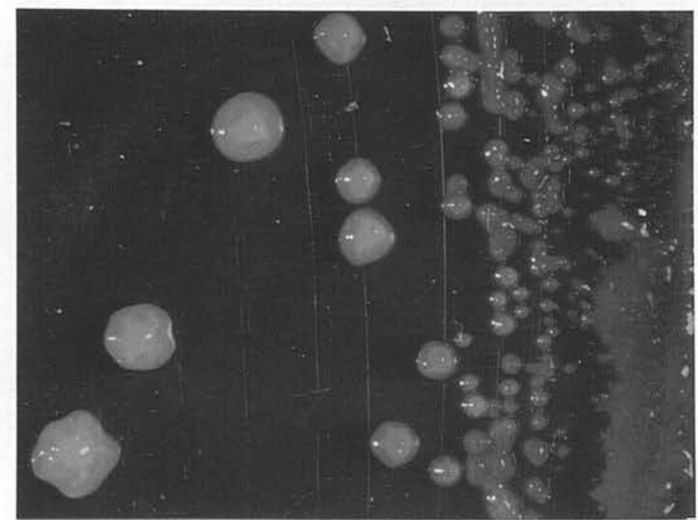

Fig. 4

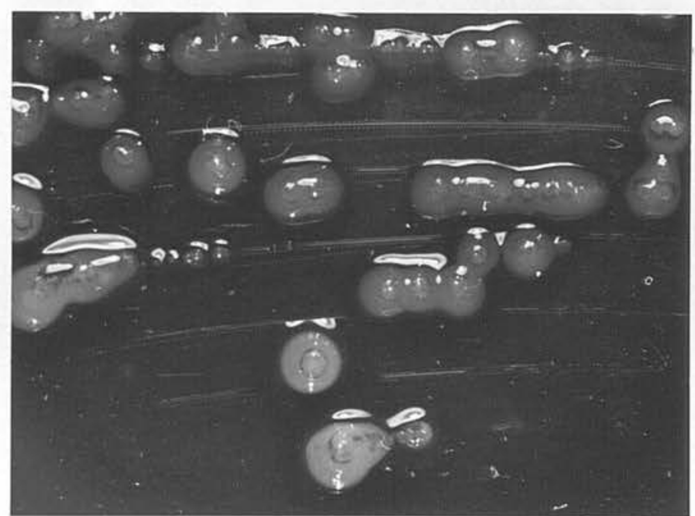

Fig. 5

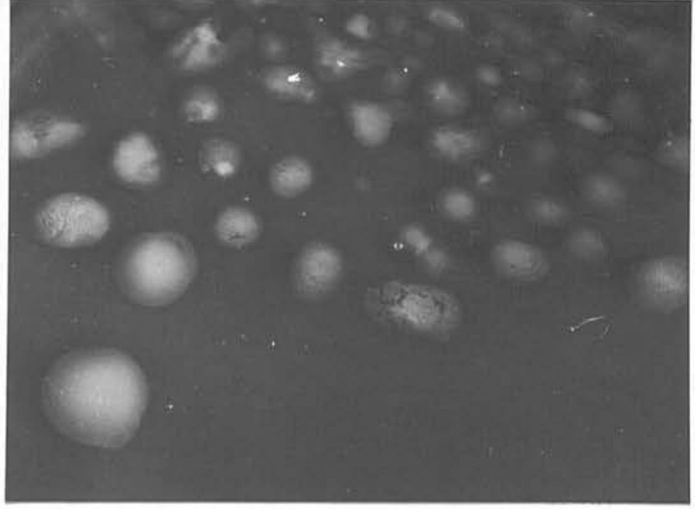

Fig. 6

PLATE 3

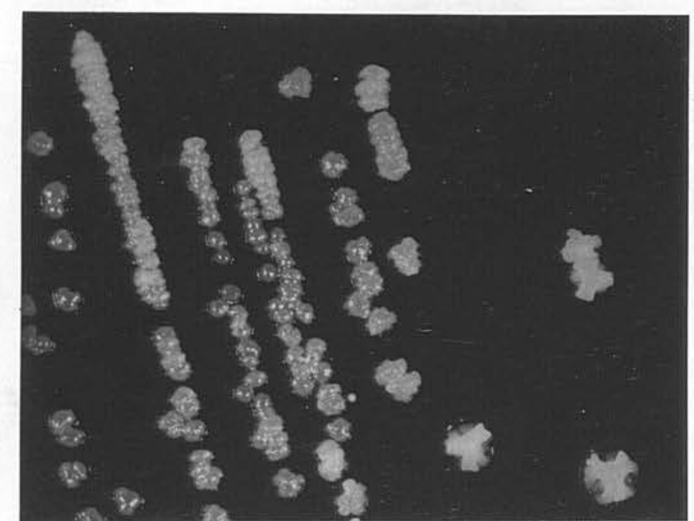

Fig. 7

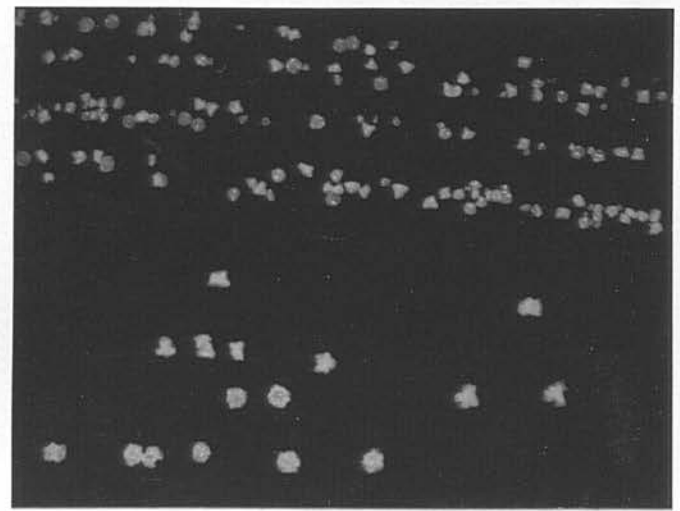

Fig. 8

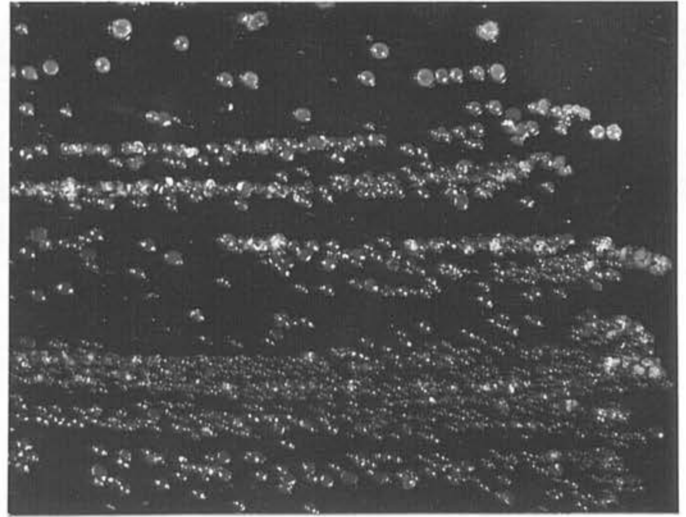

Fig. 9

Section 5.

A study of heterofermentative lactobacilli.

INTRODUCTION

These organisms have proved difficult to classify into clearly defined species. Orla-Jensen(1919) placed them in the new genus Betabacterium and proposed their classification into three species, Betabacterium breve, Bt.longum and Bt.caucasicum, which were differentiated on growth temperature ranges and sugar fermentations. Strain variation in sugar fermentation, however, led Orla-Jensen to remark that the line of demarcation between the first two species was somewhat vague.

Pederson(1938) recognized four species, Lactobacillus brevis (Bt.breve), Lb.fermentum (Bt.longum), Lb.buchneri, Lb.pastorianus and in addition an "inactive" group which included Bt.caucasicum. Their differentiation was based primarily on growth temperature ranges and secondarily on sugar fermentations. The "inactive" group was recognized by the few sugars fermented.

From Pederson's(1938) descriptions, however, there appears to be no clear-cut distinction between Lb.brevis and Lb.buchneri or between Lb.buchneri and Lb.fermentum. Lb.pastorianus was recognized only on the basis of earlier descriptions in the literature. These, however, do not satisfactorily distinguish Lb.pastorianus from Lb.brevis or Lb.buchneri.

The "inactive" group has been described most recently by Vaughn, Douglas & Fornachon(1949). Four species were recognized, Lb.trichoides Lb.hilgardii, Lb.fructivorans and Lb.desidiosus (Bt.caucasicum) (Table 1). All were relatively inactive in that they fermented few sugars and grew slowly in comparison with Lb.brevis. They had a marked preference

for acidic conditions.

Rogosa, Wiseman, Mitchell, Disraely & Beaman(1953) described Lb.brevis, Lb.buchneri and Lb.fermenti (Lb.fermentum) in greater detail, and proposed an additional species, Lb.cellobiosus. Nutritional requirements were introduced as differential characteristics.

Rogosa & Sharpe(1959) summarized the classification of the heterofermentative lactobacilli with the exception of those in the "inactive" group. Five species were recognized, Lb.brevis, Lb.buchneri, Lb.fermenti, Lb.cellobiosus and Lb.viridescens, a species proposed by Niven & Evans(1957). Their differential characteristics are abstracted from Rogosa & Sharpe(1959) and given in Table 2. Differences based on physiological activities are few but appear to be substantiated by nutritional and serological differences.

The main features used so far in the classification of the organisms have been differences in growth temperature range and sugar fermentations. Both, however, seem to be of limited value as differential characteristics.

Only two species can be clearly identified by sugar fermentation. They are Lb.buchneri, the only species fermenting melezitose (Rogosa et al. 1953), and Lb.cellobiosus, the only species fermenting cellobiose (Rogosa et al. 1953).

Keddie(1959) drew attention to the questionable validity of growth temperature range as a differential characteristic. A survey of the literature reveals varied and contradictory reports on the ranges given for a single species. Table 3 lists a number of such instances.

In the present investigation, a number of heterofermentative lactobacilli, isolated from fresh grass and silage, are compared

with named strains and with the species described in the literature. The results offer an explanation for some of the varied reports on growth temperature range and confirm the limited value of differences in sugar utilization as differential characteristics. Some additional tests are mentioned which may prove useful in the classification of these organisms.

METHODS

The media and methods used were similar to those used for the leuconostocs. They are described in the appendix and in Section 4. The ability to produce acid from different substrates was tested at pH 6·8-7·0 and pH 5·4.

RESULTS AND DISCUSSION

Organisms (Table 4)

A few were obtained from herbage in the manner described for the leuconostocs and a few were named strains obtained from individuals and culture collections. The majority, however, were obtained from field and laboratory silages using the selective medium for lactobacilli proposed by Keddie(1951).

Classification of the organisms

All were Gram-positive rods. None reduced nitrate to nitrite or split peroxide when grown on 1·0%(w/v) glucose agar. None utilized sorbose, sorbitol or glycerol, or grew and produced acid from glucose at 5°.

The organisms were initially divided into sections (Fig.1 and Table 5) according to their manner of growth in soft agar - pH 6·8-7·0 - containing different types of utilizable sugars. The sugars were

glucose - aldohexose, fructose - a ketohexose, and arabinose or xylose-pentoses. The few additional characteristics differentiating these sections are also given in Table 5. The sections, named after a species included, were subdivided into groups by various tests.

The brevis section.

The manner of growth of these organisms was the same as that described previously for Lb. brevis and Lb. buchneri (Section 1, p.12) with the following exceptions. One or two strains failed to utilize fructose. A few utilized xylose microaerophilically or initially anaerobically, and one utilized arabinose aerobically. All these organisms, however, utilized one of the pentoses aerobically and anaerobically.

Manganese stimulated surface growth but did not noticeably improve anaerobic growth. In the majority of cases citrate either had no effect on growth on soft agar containing sugars or stimulated anaerobic growth slightly. If citrate was dissimilated in the presence of a utilizable sugar, growth was markedly improved unless it was autoclaved with pentoses in the medium, when anaerobic growth was partially or completely inhibited.

Growth with other substrates as energy sources was similar to that observed with glucose except in the occasional instance of mutant or microaerophilic utilization.

Properties of the organisms are given in Table 6. Characteristics which serve to separate the five major groups from one another are given in Table 7. Other features of the organisms and comments on their classification are given below.

Group 1 - Although all strains utilized fructose and both pentoses, a few utilized fructose weakly and a few utilized xylose

microaerophilically or initially anaerobically. Some late, probably mutant, utilizations were observed with lactose and salicin. Malate was not dissimilated by the majority of the organisms. Some, however, produced a slight rise in pH in malate medium after a week. Slight gas production occurred in malate-pentose medium after three to four days incubation but the final pH was below 5·0.

These organisms were identified as Lb.buchneri according to the description of Rogosa & Sharpe(1959).

Group 2 - A few strains utilized fructose weakly. Sucrose and lactose were utilized vigorously by only a few strains; many either failed to utilize these substances or did so late and mutantly. Salicin also appeared to be utilized mutantly. A number of organisms produced peroxide on glucose and arabinose MDO agar but fewer did so on glucose and arabinose HBD agar. This observation appeared to be explained by the apo-catalase activity of these particular organisms, although apo-catalase positive organisms in many instances still produced peroxide. The anomalous behaviour of peroxide-splitting organisms releasing peroxide into the medium has been discussed previously (Section 1, p.27).

In so far as they are comparable, the description of these organisms classifies them as Lb.brevis as described by Pederson(1938) and Rogosa & Sharpe(1959).

Group 2a - The one strain in this group, Lb.brevis var rudensis NCIB 4617, only differed significantly from the organisms in the previous group in the following features. Arabinose was only utilized in the top 1·0 cm. of the medium. The organism produced a very strong peroxide reaction on both glucose and arabinose MDO and HBD agar and was apo-catalase negative.

Pigment production was not observed. This organism is considered to be related to the previous group.

Group 3 - Only one strain in this group dissimilated malate and did so vigorously in the presence of arabinose.

These organisms appear to form an intermediate group possessing similarities to Group 1, - vigorous raffinose and sucrose utilization - Group 2, - apo-catalase activity - and Group 4 and 5, - inability to produce ammonia from arginine. They could be classified as Lb.brevis as described by Pederson(1938) but not as described by Rogosa & Sharpe(1959).

Group 4 - These organisms utilized substrates in the manner described for the Group 2 organisms. All produced strong peroxide reactions on arabinose and glucose MDO and HBD agar despite the fact that they were apo-catalase positive.

These organisms could be classified as Bt.breve, Orla-Jensen(1919), and Lb.brevis as described by Pederson(1938) and Rogosa & Sharpe(1959).

Group 4a - These organisms are very similar to those in the previous group. They differed in being apo-catalase negative and in producing slight microaerophilic growth at 40°. They appear to be related to the Group 4 organisms.

Group 4b - The one organism in this group differed from Group 4 organisms in failing to produce ammonia from arginine and in being apo-catalase negative. The ability to utilize citrate suggests that this organism might be related to Group 4.

Group 5 - Individually, the strains in this group utilized fewer substrates than those in the previous groups. Late, probably mutant, and microaerophilic utilization was observed with all strains with one or more substrates. At 37° growth was aerobic or microaerophilic

whilst at 38-39° growth either did not occur or was very slight and microaerophilic.

Two of the organisms in this group were originally isolated from silage and described by Cunningham & Smith(1940). They were unable to obtain good growth from these organisms and did not, therefore, do more than to suggest that they might be degenerate strains of Lb.brevis or related to the "inactive" group of Pederson(1938). Slight growth was characteristic of the remaining strains in this group. Apart from the stimulation of surface growth by manganese, no alteration or variation in concentration of medium constituents significantly increased growth.

The description of these organisms classifies them as Bt.breve, Orla-Jensen(1919), which has a maximum growth temperature of or slightly above 37·5°, and as Lb.brevis, as described by Pederson(1938), which comprises organisms with various growth temperature ranges. The physiological description of these organisms, however, does not classify them as Lb.brevis as described by Rogosa & Sharpe(1959). As Pederson's(1938) description of Lb.brevis covers both Bt.breve (Orla-Jensen) and Lb.brevis (Rogosa & Sharpe) as well as the organisms in this group and in the preceeding groups it seems clear that Pederson might have included more than one now recognizably distinct type in Lb.brevis. This might explain some of the varying reports on growth temperature range given for Lb.brevis.

Group 5a - The one strain in this group is similar to the Group 5 organisms. It differs in producing slight growth at 40°, in utilizing melibiose and splitting aesculin, and in not forming peroxide. It is considered to be related to Group 5.

The taxonomic status of organisms in the brevis section.

The lactic acid bacteria in general are known to have complex nutritional requirements, which suggests that they might have evolved from more self-sufficient organisms. The characteristics of organisms in the brevis section provide additional evidence on the possible evolution of lactic acid bacteria.

The brevis section have a number of properties which suggest that the organisms might have evolved from an ancestor which possessed functional iron porphyrin-enzymes and respired aerobically. These properties are:

1. A representative strain from each group was shown to take up oxygen on glucose.
2. The preference for aerobic conditions when utilizing separately-sterilized hexoses.
3. The ability of some of the organisms to form peroxide. This suggests the loss of peroxidative ability by the peroxide formers.
4. The apo-catalase activity of many of the organisms.
5. The ability of the one organism of this section examined to form a cytochrome if provided with a source of haemin (Table 5 in Section 1). This and the previously mentioned property suggest a loss of ability by these organisms to synthesize iron porphyrins although the ability to synthesize the apo-enzymes of catalase and cytochrome is retained.

The survival of these organisms might be related to their powers of fermentation and acid tolerance. These properties allow them to compete effectively with more self-sufficient bacteria in oxygen-depleted environments, such as silage, which are rich in energy sources,

hydrogen acceptors other than oxygen, and growth factors.

It is possible, then, that the groups in the brevis section represent stages of an evolutionary development of an aerobically respiring organism. Fig.2 suggests a hypothetical evolutionary relationship of the different groups. It is based on positive properties of the organisms (see Table 6 and Table 7). From this diagram it can be seen that Group 5 organisms might have evolved from at least three of the major groups - Groups 2, 3 and 4 - as the most distinctive feature of Group 5 organisms is the lack of positive properties possessed by these groups.

If the groups are closely related and if they have evolved from one type of organism, as is suggested here, it is questionable as to whether the individual groups should be given species status. There appears to be no good reason for doing so at the present time. It is suggested, therefore, that all organisms in the brevis section should be classified as Lb.brevis (Orla-Jensen) Bergey et al. as this is the earliest well described species to which some of the organisms in this section appear to be similar. Lb.buchneri (Henneberg) Bergey et al. would be a synonym if this form of classification were accepted.

The fermenti section.

No preference was observed for either aerobic or anaerobic conditions. Neither citrate nor manganese affected the manner of growth. The organisms were separated into two groups, the properties of which are given in Table 6.

Group 6 - The substrate utilization pattern did not separate these organisms from all those in the brevis section. The pentoses if utilized were slowly or weakly fermented.

These organisms, apart from the ability of one strain to ferment mannitol, appear to be similar to Lb.fermenti as described by Rogosa & Sharpe(1959).

Group 7 - These organisms are considered to be very similar to those in the previous group. The main differences were: that one strain produced peroxide; that all grew and produced acid at 15°; and that two produced slight growth but no detectable acidity at 10°. It is interesting to note that their action on citrate was the reverse of that encountered with the citrate-dissimilating organisms in the brevis section. The reaction occurred in the presence but not in the absence of a pentose.

Keddie(1959) considered that these organisms occupied a position intermediate between Lb.buchneri and Lb.fermenti. They could be classified as Lb.buchneri according to Pederson(1938) but not according to Rogosa et al.(1953) or Rogosa & Sharpe(1959), who give melezitose fermentation as a positive characteristic of this species. These organisms are also quite distinct from those previously classified in the present investigation as Lb.buchneri. Their characteristics are considered to relate them closely to the strains in the previous group identified as Lb.fermenti. The only significant feature separating them was ability to grow at lower temperatures. They are, therefore, considered to be variants of Lb.fermenti.

The cellobiosus section.

In a glucose soft agar initially at pH 6·8-7·0, growth was at first anaerobic. It was slight in some cases and delayed in others. The growth appeared in a zone stretching from the bottom of the tube to within 4-1·5 cm. of the surface, the distance varying with different

strains and sometimes with temperature. As incubation continued, growth developed towards the surface. In a medium initially at pH 5·4, no preference was noted for anaerobic conditions. Manganese did not affect the pattern of growth. Citrate, however, markedly improved the rate of growth and acid production of a number of the organisms including strains which did not dissimilate citrate. In the high pH medium containing citrate, growth rapidly developed in the surface regions.

When glucose was autoclaved in the medium, growth and acid production appeared rapidly and uniformly throughout in the majority of cases, and in every case if citrate was included and the pH of the medium adjusted to pH 5·4. Only at temperatures near the maximum for growth did preferences for anaerobic conditions become obvious again.

No preference for aerobic or anaerobic conditions were observed when separately sterilized pentoses were utilized. Other carbohydrates utilized gave rise to the type of growth observed with glucose.

The properties of these organisms are given in Table 6 and some characteristics differentiating the groups in Table 8.

Group 8 - The outstanding feature of these organisms was their preference for an acidic medium, At pH 6·8-7·0 only the pentoses were rapidly utilized - within 24-72 hr. Growth and acid production with other substrates was delayed and weak. The rate of growth and acid production increased as the pH of the medium was lowered, but the pentoses were still the most rapidly utilized substrates. Additional sugars were utilized at pH 5·4 but weakly and after ten days. After the pentoses, fructose was the most rapidly utilized sugar.

All grew at 10° though one strain did not form detectable acid. At 37° growth was either anaerobic or microaerophilic. At 40° only

the organisms producing anaerobic growth at 37° grew, but still anaerobically only.

Although these organisms did not dissimilate malate and citrate, they are classified as Lb.hilgardii as described by Vaughn et al.(1949). Their preference for pentoses, particularly arabinose if utilized, and their slow and often weak utilization of other energy sources suggests a close resemblance to Bt.caucasicum, Orla-Jensen(1919) - Lb.desidiosus, Vaughn et al.(1949). Bt.caucasicum was described as a pronounced arabinose form, utilizing arabinose under conditions when other sugars were not utilized. One of the organisms in this group, isolated from silage, examined in a citrate-less medium initially at pH 6·8-7·0, utilized arabinose vigorously at 24 hr., xylose at 48 hr., fructose slightly at ten days, and maltose and melibiose slightly at fourteen days. This organism appears to be very similar to Bt.caucasicum in its marked preference for a pentose. Lb.hilgardii might, therefore, be closely related to Bt.caucasicum.

Group 9 - These organisms grew vigorously and produced dense growth in the absence of citrate. A preference for anaerobic conditions in the high pH medium was most marked with lactose and cellobiose as energy sources. These organisms are identified as Lb.cellobiosus, Rogosa et al.(1953).

Group 10 - These organisms were slow to initiate growth but grew rapidly on continued subculture and in the presence of citrate. At 37° and 40° growth was restricted to the anaerobic regions of the medium.

These organisms do not appear to fit the description of previously described species.

Miscellaneous section.

Two groups were placed in this section as there was insufficient evidence to justify placing them in any of the three previous sections. Their properties are given in Table 6. Some characteristics differentiating them are given in Table 9.

Group 11 - In glucose soft agar - pH 6·8-7·0, or 5·4 - growth was initially aerobic. As incubation continued, anaerobic growth slowly developed but remained slight in comparison with the aerobic growth. Manganese stimulated the surface growth but not the anaerobic growth. Citrate had a marked effect, growth now occurring simultaneously and uniformly throughout the medium. Similar behaviour occurred in a citrate-less medium in which glucose had been autoclaved.

Similar growth behaviour was observed with fructose in the place of glucose with the exception that anaerobic growth in a citrate-less medium was slightly better with separately-sterilized fructose than with separately-sterilized glucose as an energy source.

Very few substrates were utilized. Sucrose was fermented late and mannitol late, probably mutantly. Both strains showed slight growth at 5° without producing detectable acidity. At 37°, one strain produced slight aerobic growth only and one strain slight microaerophilic growth. Both strains were apo-catalase positive when grown anaerobically on glucose HB agar but when grown aerobically only one strain was apo-catalase positive; the other strain produced only slight growth.

These organisms are identified as Lb.viridescens, Niven & Evans(1957).

Group 12 - In the first subculture from the stock culture, growth in glucose soft agar initially at pH 6·8-7·0 appeared as a thin disc well

down the medium. On continued subculture this effect was not observed and growth occurred uniformly throughout the medium. Manganese had no effect on growth and citrate a slight stimulatory effect. At pH 6·8–7·0 fructose was utilized vigorously and glucose comparatively weakly after three days, and maltose weakly after ten days. In a medium initially at pH 5·4, fructose was rapidly utilized and growth with glucose greatly improved. Autoclaving glucose in the medium improved growth at both the high and low pH. At the lower pH additional sugars were utilized, either weakly after nine to fourteen days or weakly and mutantly after nine days. At 37° growth was confined to a narrow disc, 2 mm. wide about 1·5 mm. below the surface. Growth did not develop outside this zone on further incubation (Fig.15B, Section 1).

These organisms are identified as *Lb.fructivorans*, Charlton, Nelson & Werkman(1934) and as described by Vaughn *et al*.(1949).

Discussion of classification.

Carbohydrate utilization was of limited value in the classification of the organisms. Apart from strain variation, there were instances of carbohydrates being utilized only at the higher pH or at the lower pH. Occasionally utilization was weak, slow, microaerophilic, or very delayed and mutant. Of greater value in classification, was the manner in which separately-sterilized sugars of different structure - glucose, fructose and arabinose or xylose - were utilized in citrate-less medium initially at pH 6·8–7·0. The organisms were initially grouped into four sections on this behaviour.

Hydrogen peroxide production and apo-catalase activity were useful in the separation of some groups.

Because of varied reports in the literature, the ability of organisms to grow at specific temperatures seemed to have only a limited value in the classification of the heterofermentative lactobacilli. The results of the present investigation, however, suggest that growth temperatures are useful in grouping the organisms and that reported differences in growth temperatures might have been due in some cases to the fact that the species embraced more than one now recognizable type of organism.

Temperatures close to the maximum for proliferation had a marked effect on the manner of growth of some organisms in glucose soft agar As citrate and glucose were autoclaved in the test medium, the majority of the organisms produced uniformly distributed growth at 30°. At 37-40°, however, many organisms in the _brevis_ section produced only aerobic growth and some strains in the _cellobiosus_ section produced only anaerobic growth. A third variation was noted with _Lb.fructivorans_, growth at 37° appearing as a disc 1-1·5 cm. below the surface (Fig.15B, Section 1). If glucose was replaced by a pentose and provided the pentose was vigorously utilized, the growth variations observed with glucose at the higher temperatures did not occur.

Ammonia production from arginine correlated with other reactions and was of value in separating the more closely related groups and groups with relatively few properties. The sugar concentration in the test medium, varied from 0·25%-2·0%(w/v), did not affect the reaction.

The ability to dissimilate malate and citrate in the presence and absence of a pentose had a limited value in the grouping of the organisms. A pentose was used because of the heterofermentative nature of these organisms, gas production being one of the criteria

used in detecting organic acid dissimilation. This particular test, therefore, was only applied to vigorous pentose fermenters.

Malate did not appear to be used as an energy source even though it was dissimilated by many organisms. Dissimilation was only vigorous when a pentose was included in the medium. Citrate, if dissimilated in the absence of a sugar, was used as an energy source. Different groups of organisms varied in their behaviour with citrate. Some organisms only dissimilated citrate in the absence of sugar, some only in the presence of a pentose, and some both in the presence and absence of a pentose.

Keddie(1959) has examined citrate and malate dissimilation by lactobacilli isolated from silage. It is not possible to compare his results with those obtained here, however, as a utilizable hexose was always included in the medium Keddie used in examining heterofermentative lactobacilli. The hexose was present in one of the medium constituents, tomato juice, and added in the form of small amounts of glucose.

CONCLUSIONS

The organisms were divided into four sections, the <u>brevis</u> section, the <u>fermenti</u> section, the <u>cellobiosus</u> section and a miscellaneous section.

The <u>brevis</u> section contained groups of organisms which appear to be closely related. Their close similarity was discussed and, although some were identified with previously described species, it was considered that they might all be variants of the one species, <u>Lb.brevis</u>, of which <u>Lb.buchneri</u> would be a synonym.

The fermenti section contained two groups, one identified as Lb.fermenti and the other as a low-temperature growing variant of this species.

The cellobiosus section contained three groups, one classified as Lb.cellobiosus, and one as Lb.hilgardii. The remaining group was not identified.

The miscellaneous section contained two groups, one classified as Lb.viridescens and one as Lb.fructivorans.

The characteristics of some organisms suggest that they might have an impaired respiratory system. Possible implications of these observations have been discussed with respect to organisms in the brevis section.

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Table 1.

Some differential characteristics of the "inactive" group.

Abstracted from Vaughn et al. (1949)

		Lb. hilgardii	Lb. fructivorans	Lb. trichodes	Lb. desidiosus
No. of cultures		(10)	(1)	(5)	(2)
Opt.cond. for glucose fermentation	Temp. range	30-35°	25-30°	25-30°	25-30°
	Initial pH range	4·5-5·5	4·5-5·5	4·5-5·5	5·0-7·0
Fermentation					
Xylose		++	-	-	-
Arabinose		-	-	-	++
Glucose		±	+	++	++
Fructose		++	++	++	++
Mannose		-	-	-	±
Galactose		+	-	-	++
Sucrose		+	-	±	-
Maltose		+	-	±	-
Lactose		±	-	-	-
Raffinose		±	-	-	-
l-malic acid		++	±	-	-
Citric acid		±	-	-	-
Alcohol tolerance %.		15-18	15	> 20	15

- = no activity

± = weak activity

\+ = moderate activity

++ = maximum activity

Table 2.

Differential characteristics of the heterofermentative lactobacilli Abstracted from Rogosa & Sharpe (1959).

	Lb. fermenti	Lb. buchneri	Lb. brevis	Lb. cellobiosus	Lb. viridescens
Growth at					
15°	−	+	+	+	+
45°	+	−	−	−	−
Growth in 0·1% Teepol	−	+	+	+	+
Serological gp.	F	E	E	−	−
Ammonia from Arginine	+	+	+	+	−
Aesculin split	−	±	±	+	−
Fermentation					
Arabinose	±	+	±	+	−
Cellobiose	−	−	−	+	−
Lactose	±	±	±	±	−
Mannitol	−	weak or −	weak or −	−	weak or −
Melezitose	−	+	−	−	−
Melibiose	±	+	+	+	−
∝-methyl D-Glucoside	±	+	±	−	−
Raffinose	±	+	±	+	−
Salicin	−	−	weak or −	+	−
Sucrose	±	+	±	+	±
Trehalose	±	−	−	+	±
Xylose	±	±	±	±	−
Mannose	±	−	−	weak ±	+
Requirement for					
Riboflavin	−	±	−	−	+
Pyridoxal	−	−	−	−	S
Folic acid	−	−	+	−	S

± = some strains + and some −; S = stimulatory

Table 3.

Some growth temperatures given in the literature for species of heterofermentative lactobacilli.

Species	Temperatures $^{\circ}$C	Author(s)
Lb.brevis (Bt.breve)	*c*. 15-37·5	Orla-Jensen (1919)
	10-15 to 40-45	Pederson (1938)
	questionable at 15; questionable or negative at 45	Rogosa *et al*. (1953)
	positive at 15; negative at 45	Rogosa & Sharpe (1959)
Lb.buchneri	10-15 to 44-48	Pederson (1938)
	negative at 16; positive or negative at 45	Rogosa *et al*. (1953)
	positive at 16; negative at 45	Rogosa & Sharpe (1959)
Lb.fermenti (Lb.fermentum and Bt.longum)	*c*. 18-45	Orla-Jensen (1919)
	15-18 to 48-50	Pederson (1938)
	negative at 16; positive or questionable at 45	Rogosa *et al*. (1953)
	negative at 16; positive at 45	Rogosa & Sharpe (1959)

Table 4.

Organisms examined.

Section	Group	No.	Strains and sources.
brevis	1	22	Lb.buchneri - NCIB 8037 and 8007; and 20 from silage.
	2	23	Lb.brevis NCIB 947; Lb.brevis X1 and X2 from Dr. M.E. Sharpe; and 20 from silage and fresh grass.
	2a	1	Lb.brevis var. rudensis NCIB 4617
	3	2	From silage.
	4	6	From silage.
	4a	4	From silage.
	4b	1	From silage.
	5	7	From silage. 2 isolated by Cunningham & Smith(1940).
	5a	1	From silage.
fermenti	6	4	Lb.fermenti NCDO 215, 218 and 305; and Lb.fermenti NCIB 5221.
	7	4	Isolated from silage by Dr. R.M. Keddie.
cellobiosus	8	7	Lb.hilgardii NCIB 8040; 4 strains received as Lb.hilgardii from Prof. R.H. Vaughn; and 2 from silage.
	9	2	Lb.cellobiosus 19LC and 5S7(33) from Dr. M. Rogosa.
	10	3	From silage.
Miscellaneous	11	2	Lb.viridescens S40A and S38A from Dr. M.E. Sharpe.
	12	2	Lb.fructovorans NCIB 8039 and 5223.

NCIB = National Collection of Industrial Bacteria.

NCDO = National Collection of Dairy Organisms.

Table 5.

Some characteristics differentiating the sections.

	brevis	fermenti	cellobiosus	Miscellaneous
Manner of growth in soft agar (pH 6·8-7·0)				
Glucose	PA (aerobic growth appears first)	AAn	PAn	PA or M
Fructose	PA (aerobic and anaerobic growth appear at same time)	AAn	PAn	PA or M
A pentose	AAn (with at least one pentose)	AAn (when a pentose utilized)	AAn (with pentose utilized)	not utilized
Growth and acid at:				
46°	-	+	-	-
10°	+	-	±	+
Apo-catalase	±	-	-	+

PA = Preferentially aerobic.
AAn = Aerobic and anaerobic.
PAn = Preferentially anaerobic.
M = Microaerophilic.

Table 6.

Properties of the heterofermentative lactobacilli.

Section:	brevis									fermenti		cellobiosus			Miscellaneous	
Groups:	1	2	2a	3	4	4a	4b	5	5a	6	7	8	9	10	11	12
Substrates utilized:-																
Glucose	+	(+)	+	+	+	+	+	+	+	+	+V	+	+	+	+	+
Fructose	+V	+V	+	+	+V	±	+	+	+	+	+V	+	+	+	+	+
Sucrose	+	±V	V	+	±V	–	–	±V	–	±	+	+V	+	+V	V	V
Arabinose	+V	+	+	+	+	+	+	+	+	$\underline{V}$	±	±	+	+	–	–
Xylose	+V	+	+	+	+	+	+	+	+	$\underline{V}$	–	+	±	+	–	–
Maltose	+	+	+	+	+	+	+	+	+	+	+	+V	+	+V	+	V
Lactose	±V	±V	V	+	±V	±V	+	–	+	+	+V	+V	+	+V	–	–
Cellobiose	–	–	–	–	–	–	–	–	–	–	–	–	+	±	–	–
Melibiose	+	±	+	+	+V	±	+	–	+	±	+V	+	+	+V	–	–
Raffinose	+	±	–	+	–	–	–	–	–	+V	+	+	+	+	–	–
Mannose	±	±V	–	+	±V	±V	–	±V	–	±	±V	–	+V	+	V	–
Melezitose	+	–	–	–	–	–	–	–	–	–	–	–	–	–	–	$\underline{V}$
Salicin	±V	+V	V	–	+V	+V	V	+V	+V	–	–	+V	+V	±	–	–
α-M-D glucoside	+	+	+	+	+	+	–	+	–	±V	–	–	–	±	–	–
Mannitol	±	±V	+	+	+V	+V	–	+V	+V	±	±V	–	–	+V	–	$\underline{V}$
Galactose	+	+	+	+	+	+	+	+	+	+	+V	–	+	+	–	–
Trehalose	NT	NT	NT	–	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT
Hydrolysis of:-																
Arginine	+	+	+	–	+	+	–	–	–	+	+	+	+	–	–	+
Aesculin	±	±	+	+	+	±	+	–	+	–	–	–	+	–	–	–
Hippurate	±	±	–	+	–	–	–	–	–	–	–	–	–	–	–	–

continued.

Table 6 continued.

Section:	brevis									fermenti			cellobiosus		Miscellaneous	
Groups:	1	2	2a	3	4	4a	4b	5	5a	6	7	8	9	10	11	12
Peroxide formed on:-																
glucose MDO agar	±	(±)	+	+	+	+	+	+	−	−	±	−	+	−	+	−
glucose HBD agar	±	(±)	+	−	+	+	+	+	−	−	±	−	+	−	+	−
Apo-catalase	−	+	−	+	+	−	−	−	−	−	−	−	−	−	+	+
Growth and acid at:-																
10°	+	+	+	(+)	(+)	(+)	(+)	(+)	+	−	−	(+)	−	−	(+)	(+)
15°	+	+	+	+	+	+	+	+	+	−	+	+	−	+	+	+
37°	+	+	+	+	(+)	+	(+)	(+)	+	+	+	+An or M	+	+An	(+)	(+)
40°	+	+	+	+	−	(+)	−	−	−	+	+	±An	+	+An	−	−
45°	−	−	−	−	−	−	−	−	−	+	+	−	−	−	−	−
46°	−	−	−	−	−	−	−	−	−	+	+	−	−	−	−	−
Organic acid dissimilation																
Malate	(±)	+	+	±	+	+	+	+	+	−	−	−	(+)	−	−	−
Malate + Arabinose or xylose	(+)	+	+	±	+	+	+	+	+	NT	±	−	+	+	NT	NT
Citrate	−	−	−	−	±	+	+	−	−	−	−	−	+	−	−	−
Citrate + Arabinose or xylose	−	−	−	−	(±)	(±)	(+)	−	−	NT	+*	−	+	+	NT	NT

A strain from each group took up oxygen on glucose.

*The one non-pentose utilizing strain not tested.

+ = All strains positive.
− = All strains negative.
± = Some strains positive and some strains negative.
(+) = Slight activity.
An = Anaerobic
M = Microaerophilic.
NT = Not tested.
V = Slow, late, micro-aerophilic or mutant utilization.

Table 7.

The differentiation of the five major groups in the brevis section.

Groups:	1	2	3	4	5
Sugar utilized.					
Raffinose	+	±	+	−	−
Melezitose	+	−	−	−	−
Sucrose	+	±	+	+	±
Gas from malate + arabinose	Slight	+	+	+	+
Citrate utilization (Gas and rise in pH)	−	−	−	+	−
Apo-catalase	−	+	+	+	−
Peroxide on glucose HBD agar	±	(±)	−	+	+
Ammonia from arginine	+	+	−	+	−
Growth and acid at 40°	+	+	+	−	−
Dense(D) or slight(S) growth	D	D	D	D	S

(+) = weak activity.

\+ = all strains positive.

− = all strains negative.

± = some strains positive and some strains negative.

Table 8.

Some differential characteristics of the cellobiosus section

Action on	Groups: 8	9	10
Cellobiose	−	+	±
Galactose	−	+	+
Aesculin	−	+	−
Arginine	+	+	−
Peroxide formation on Glucose HBD agar	−	+	−
Preference for acidic medium	+	−	−
Growth and acid at:			
10°	(+)	−	−
15°	+	−	+
37°	An or M	+	An
Organic acid dissimilation			
Rise in pH in malate sugar-free medium.	−	(+)	−
Gas from malate + pentose	−	+	+
Citrate utilized	−	+	−
Gas from citrate + pentose	−	+	+

(+) = slight activity
An = Anaerobic
M = Microaerophilic
\+ = all strains positive
− = all strains negative
± = some strains positive and some strains negative.

Table 9.

Some characteristics differentiating groups in the miscellaneous section.

Groups:	11	12
Ammonia from arginine	-	+
Peroxide formation Glucose HBD agar	+	-
Manner of growth in glucose soft agar	PA	M (first subculture)
Marked preference for acidic medium	-	+

PA = Preferentially aerobic.

M = Microaerophilic.

+ = All strains positive.

- = All strains negative.

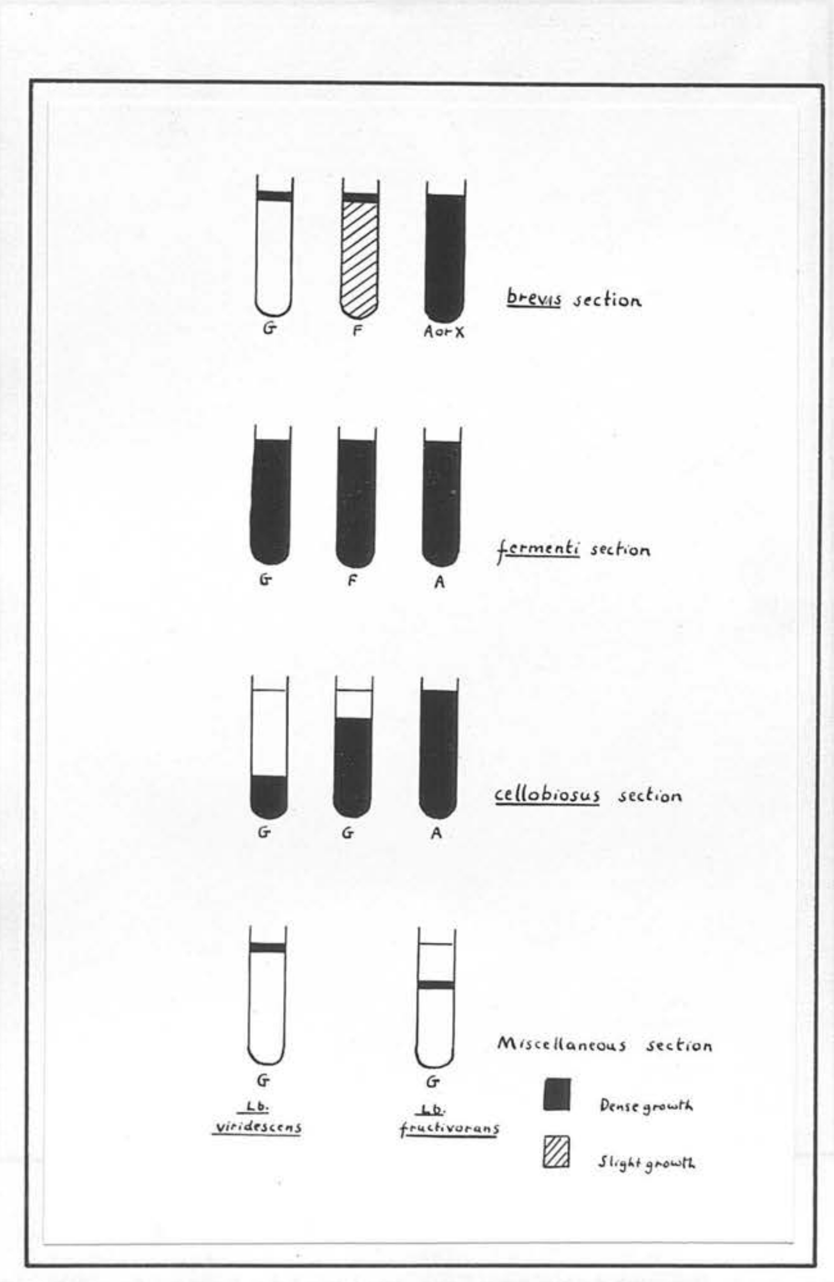

Fig. 1

Diagrammatic representation of the initial growth of the heterofermentative lactobacilli in soft agar containing various sugars.

G = Glucose; F = Fructose; A = Arabinose; X = Xylose.

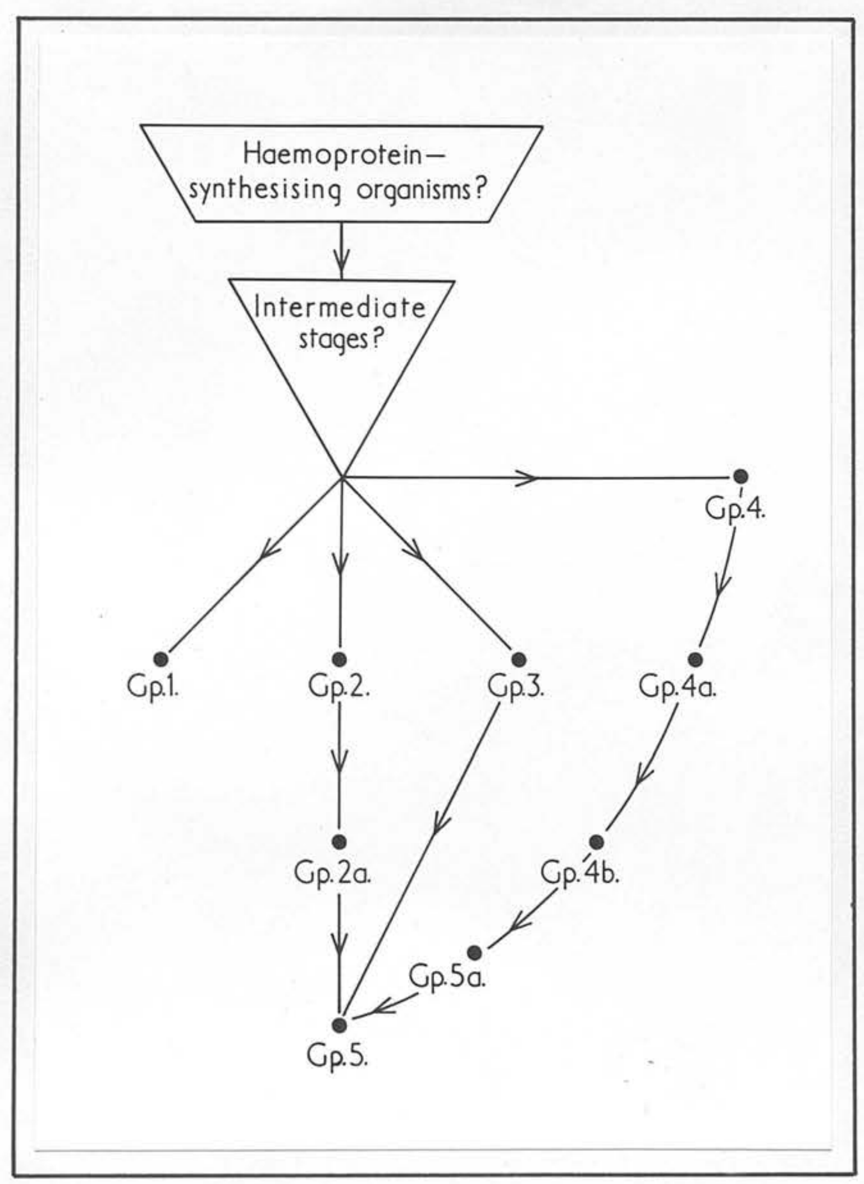

Fig. 2

A hypothetical evolutionary relationship of the groups in the brevis section.

Section 6.

Miscellaneous aspects concerned with the production of silage.

INTRODUCTION.

When fresh grass is ensiled and the subsequent changes observed at intervals, a regular sequence of events can be demonstrated.

Atmospheric oxygen trapped in the herbage is rapidly consumed by the plant enzymes. The aerobic bacteria generally die and organisms capable of anaerobic growth proceed to multiply if they are present. The period of active multiplication lasts for a few days only during which time sufficient acid, mainly lactic acid, is normally produced from the plant sugars by the lactic acid bacteria to ensure the preservation of the ensiled material. A phase of decreasing viable count sets in. Lactobacilli become the dominant organisms of those capable of multiplying in the now acidic material and continue to produce acid from the plant sugars.

The following discussion considers three topics relating to the production of silage. The first is concerned with preliminary studies on the occurrence of pediococci, lactobacilli, and leuconostocs on plant material. The second is a discussion on the reactions of the different types of lactic acid bacteria on the sugars and certain organic acids likely to occur in grasses and other crops, and how these reactions might affect the preservation of silage. The third describes the properties required of an organism as a silage inoculum and two organisms which might be of use.

The occurrence of lactic acid bacteria on plant materials.

Lactic acid bacteria have proved to be scarce on growing crops, as indicated in the review of Gibson & Stirling(1959). There have been occasional reports of crops with high counts of lactobacilli but it has

been suggested (Gibson & Stirling,1959) that such material may not have been fresh. Bacterial multiplication may take place during the wilting and harvesting of the material.

Counts of the organisms are usually made by plating macerated samples of the crop. Limitations of this method are that, usually, only the dominant organisms can be detected and that little or no information can be obtained as to the actual site of these organisms on the plant. The methods described below provided information both as to the actual site of the organisms and the ratio in which the various types occurred.

The medium used was a modified version of the selective medium for lactobacilli proposed by Keddie(1951) and is described in the section on pediococci. This medium permits the growth of leuconostocs and at least two species of pediococci which occur commonly in silage. Plants of various types were obtained as aseptically as possible and cut into sections which would fit into a petri dish. About 15 ml. of agar was poured into the dish and the plant sections placed on the liquid agar. Air bubbles were teased out from underneath the sections and the agar allowed to set, so trapping the plant section. A further layer of agar was poured, again about 15 ml., ensuring that all the plant section was covered with agar. The amount of plant material that could be placed in one dish depended on the shape of the material; - i.e. six or seven grass blade sections, or two stem sections with grass blades attached, could be placed in one dish. The colonies developing after three to five days incubation - at 22 or 27° - were, in the great majority of instances, on the plant tissue surface. This seemed to indicate that the organisms were firmly attached to the plant and not easily washed off by the addition of the agar.

A great variety of plant materials were examined. They included:- leaves of trees; - such as sycamore and elm; - leaves of kale, sugar beet, mangolds, and cabbage; leaves, stem sections, flowering heads and seeds of grasses and cereals including ryegrass, cocksfoot, various wild grasses, oats, barley and wheat; and sections of wild flowers including willow herb, dandelion, daisies, fumitory, gorse and broom.

Five separate examinations, consisting of about one hundred platings, were carried out over a period of thirty months during summer and autumn periods.

The results indicated that pediococci, leuconostocs, and lactobacilli were present in very small numbers and occurred only on certain portions of the plant. Fresh, undamaged plant material rarely yielded a colony; - i.e. of about four hundred grass blades and stem sections examined, less than ten gave rise to colonies and then to only one colony each. No colonies developed on the flower heads, seed pods, partially ripened heads of grain or seeds of the plants examined. Dead and partially wilted sections of kale, beet, mangold, and cabbage leaves, rarely yielded a colony. Of the colonies which did appear on this material, most proved to be yeasts, though two on kale were found to be pediococci. The great majority of colonies were found to occur on damaged, partially withered, and withered grass blades, on sheath material and on damaged plant leaves. In the latter case the damage appeared to have been caused either by insects or birds. Colonies appeared most frequently (Fig.1) when such leaves were also infested by aphids. One or two colonies often developed around the legs of aphids which were inadvertantly trapped in the agar plates of plant sections.

Generally, the colonies appearing on one section of plant material were all found to consist of organisms similar in morphology. Therefore, only two or three colonies per section were examined in detail. Of approximately four hundred colonies picked and purified, about 80% proved to be leuconostocs, 10% pediococci, and the remaining 10% heterofermentative and homofermentative lactobacilli. The leuconostocs appeared to be the most widely distributed of the types, occurring on grasses, flowering plants, and tree leaves. Pediococci were twice isolated from kale leaves and a number of times from grasses and cereals. The lactobacilli appeared to be the least widespread of all, as they were only isolated from grasses and, in the majority of instances, only from the base of the plant (Fig. 2 and 3).

No definite conclusions can be drawn from these preliminary studies though the results suggest three findings. First, that lactic acid bacteria are sparsely distributed on plant material, as has been shown previously. Secondly, that lactic acid bacteria do not normally occur or proliferate on undamaged aerial foliage, and thirdly, that the lactic acid bacteria encountered exist and probably proliferate on plant material which is damaged or beginning to die and decay.

<u>The action of lactic acid bacteria on sugars and non-volatile organic acids</u>.

Lactic acid, produced from plant sugars by lactic acid bacteria, is responsible for the natural acidic preservation of most ensiled plant materials. Different types of lactic acid bacteria contribute to this process. Leuconostocs and streptococci generally develop first but they are rapidly followed by acid-tolerant lactobacilli which dominate the microflora of a well preserved silage.

The following discussion is concerned with actions the different

types of lactic acid bacteria have on sugars and how this might affect the preservation of silage. Attention is also given to the action these organisms have on non-volatile organic acids of herbage and how this might affect the silage.

Action on sugars present in grass.

The rapid production of acid is obviously dependent on the availability of sugars in plants to the organisms. In rye-grass, for instance (Harwood,1954), the water-soluble sugars which would be immediately available are glucose, fructose, sucrose and perhaps fructosan. The latter substance, however, might not be utilized or hydrolysed rapidly enough to be of significance as an acid source in the early and critical period of preservation. It is considered, therefore, that glucose, fructose and sucrose are the significant sources of lactic acid. Other sugars, i.e. pentoses, hexoses, di- and tri-saccharides, might become available at later stages as xylans and oligosaccharides undergo enzymic or acidic hydrolysis.

Homofermentative cocci and rods-streptococci, pediococci and lactobacilli.

These organisms metabolize hexoses chiefly by the Embden-Meyerhof pathway. Theoretically, therefore, they form approximately 2 mol. of lactic acid per mol. of glucose or fructose utilized under anaerobic conditions. In practice, it is found that these organisms convert approximately 90-95% of the hexose to lactic acid. Sucrose may not be utilized by all the types occurring in silage but it appears from Harwood's(1954) results that sucrose is rapidly hydrolysed in silage to fructose and glucose which are both available to the homofermentative organisms so far encountered in silage.

It is clear, then, that the action of these organisms in silage is a desirable one, as they convert most of the readily available hexoses to lactic acid.

Heterofermentative cocci and rods-leuconostocs and lactobacilli.

These organisms metabolize hexoses via the hexose-monophosphate pathway which involves, theoretically, the formation of 1 mol. of carbon dioxide, 1 mol. of ethanol and 1 mol. of lactic acid per mol. of glucose or fructose utilized under anaerobic conditions. Sucrose, if utilized, will in the majority of cases give rise to similar products.

More complex reactions might also occur. A proportion of the fructose will be reduced to mannitol as the heterofermentative lactic acid bacteria are able to use fructose as an alternative hydrogen acceptor to those produced in energy yielding reactions. The amount of fructose reduced to mannitol will depend on the ability of the organisms present to utilize hexoses anaerobically without the aid of additional hydrogen acceptors. In the most extreme case, all of the fructose present might be reduced to mannitol in the utilization of glucose - i.e. by organisms of the Lactobacillus brevis and Leuconostoc gramineum type which, in laboratory media, will at first only utilize separately-sterilized glucose anaerobically if provided with hydrogen acceptors. If fructose is utilized as a hydrogen acceptor, the loss of fructose as mannitol will be partially compensated for by the formation of acetic acid in the place of ethanol.

Should dextran-forming leuconostocs dominate the microflora at an early stage before sucrose is hydrolysed, much of the glucose fraction of sucrose might be converted to dextran which is not utilized by lactic acid bacteria as an energy source.

A number of products which do not assist in the production of a low pH in silage might, therefore, be formed at the expense of potential lactic acid. Of these products, carbon dioxide constitutes a loss in dry matter, and ethanol and dextran constitute a wastage of

sugars in that they do not contribute to the fall in pH. Mannitol might also constitute a wastage, as few lactic acid bacteria can utilize it rapidly enough for it to be of use as an acid source in the early stages of preservation.

Heterofermentative lactic acid bacteria, therefore, appear to be less economical than the homofermentative lactic acid bacteria, in that they probably form only half as much acid, if not less, from the sugars.

The practical effect that these two types of organisms might have on silage preservation.

MacKenzie & Wylam (1957) have analysed the sugar content of rye-grass at various times during the growing season. On the basis of their results and on the assumption that sucrose, but not fructosan, is rapidly hydrolysed during ensilage, it appears that the ratio of fructose to glucose varies approximately from 1:1 to 2:1. Should the fructosan be utilized or rapidly hydrolysed, the ratio will increase in favour of fructose. In these circumstances, the heterofermentative lactic acid bacteria might produce considerably less than half the amount of acid which would be produced from these sugars by homofermentative lactic acid bacteria. If the water-soluble sugar concentration, including fructosan, in the grass were high, i.e. 20% of the dry matter, it is unlikely to matter which type of organism, heterofermentative or homofermentative, develops, as sufficient acid will probably be produced by either type to ensure preservation of the silage. If the sugar concentration were low, however, a situation might arise whereby a heterofermentative, but not a homofermentative, population would exhaust the available sugar without producing sufficient acid to preserve the silage. At the present time it is difficult to

estimate what this critically low sugar value would be, as much might depend on the buffering capacity of the silage material and to some extent on the action that lactic acid bacteria have on non-volatile organic acids present in plant material.

The action of lactic acid bacteria on non-volatile organic acids and the effect this action might have on silage preservation.

Of the non-volatile organic acids present in rye-grass, quinic, malic, citric and succinic constitute the major fraction. Of these, according to Hirst & Ramstad(1959), malic and citric disappear rapidly during the early stages of ensilage, whilst the quinic fraction remains fairly constant and the succinic fraction, if changing, generally increases. These acids will have a buffering effect and work against the production of an acid pH. Such behaviour can be estimated in the case of acids which undergo no further change. The possible effect of the destruction of citric and malic acids on the pH of silage material does not, however, appear to have been considered.

The action of lactic acid bacteria obtained from silage on these particular acids, described in previous sections, has been observed in media adjusted to pH 6·0, a pH closely similar to that of fresh rye-grass and cocksfoot. It is considered, therefore, that the results obtained have some bearing on what might occur during ensilage.

The lactic acid bacteria examined can be divided into three categories according to their action on malate; (1) those that have no action on malate; (2) those that dissimilate malate vigorously only in the presence of a fermentable sugar; and (3) those that dissimilate malate vigorously in the absence but not in the presence of 1·0%(w/v) fermentable sugar. With respect to the action on citrate they can be divided into four categories; (1) those that have no action on citrate;

(2) those that dissimilate citrate only in the presence of a fermentable sugar; (3) those that dissimilate citrate vigorously both in the presence and absence of a fermentable sugar; and (4) those that dissimilate citrate vigorously only in the absence of 1·0%(w/v) of a fermentable sugar. The action of lactic acid bacteria on these substances will, therefore, depend to some extent on the amount of sugar available.

The dissimilation of malic and citric acids was detected by carbon dioxide production and a rise in pH. These changes indicate that decarboxylation and base formation have taken place. The formation of the base is explained by the fact that these acids were present mainly as potassium salts; the removal of a carboxylgroup releases potassium which forms a base. As cultures containing sugars frequently became alkaline, it was clear that insufficient sugar was present to provide enough acid to neutralize all the base formed. The cultures also gave a strongly positive Voges-Proskauer reaction; this indicates the formation of neutral substances such as acetoin, probably at the expense of pyruvate which would be an intermediate compound formed during citrate and malate dissimilation. More base would, therefore, be formed. From evidence in the literature, it can be concluded that acetic and lactic acids will also be products of citric and malic acid breakdown. These acids, however, being derived from organic acid salts, will not contribute to the fall in pH, as they themselves will also be present mainly as salts.

It is possible to suggest, from evidence in the literature, the stoichiometery of malic and citric acid dissimilation and thereby arrive at the comparative effect that these substances might have on the pH of silage. This, however, might not give a true picture of

what occurs under cultural or silage conditions.

Three points connected with silage production emerge from these observations. First, that the loss of carbon dioxide by decarboxylation will contribute to the loss in dry weight. Secondly, that so-called increases in the buffering capacity of silage might be due to the formation of bases from the non-volatile organic acid salts and not to the production of true buffers. Thirdly, that the acetic and lactic acid detected in silage should not, as is frequently done, be related directly to the original sugar content of the plant material.

The effect of organic acid dissimilation on silage preservation will depend on the amounts present in the plant material and the relative proportions of organic acids to water-soluble sugars. In rye-grass, organic acids appear to be present in small amounts and sugars in relatively high amounts. Organic acid dissimilation is, therefore, unlikely to effect the preservation of rye-grass silage. In other crops, however, such as cocksfoot and legumes, sugar values are lower and organic acid values apparently higher than they are in rye-grass. Citric and malic acid dissimilation, might, therefore, have a significant effect on cocksfoot and legume silage preservation.

<u>Concluding comments on the effect of sugar and non-volatile organic acid dissimilation on silage preservation.</u>

The various reactions that lactic acid bacteria might have on glucose, fructose and sucrose, and on citric and malic acids, have been outlined.

At certain levels of sugar content, it has been suggested that a heterofermentative population of lactic acid bacteria might exhaust the sugar supply in silage without producing sufficient acid to preserve the silage, whereas a homofermentative population would have

done so. As the dissimilation of citric and malic acids, presumed to be present mainly as metal salts, would counteract a fall in pH, a greater contrast would occur between an organic acid-dissimilating heterofermentative population and a homofermentative population which does not dissimilate these acids.

A feature which emerges from this discussion, is that silage materials with a critically low sugar content and materials with high organic acid content would probably benefit from an inoculum of a lactic acid bacterium with the characteristics described below.

<u>The characteristics required of a lactic acid bacterium for inoculation of silage</u>.

The features desirable in such an organism are as follows:-

1. It must be a vigorously growing organism able to compete with and preferably dominate other organisms likely to occur in silage.
2. It must possess a homofermentative pathway in order to produce the maximum amount of lactic acid from the immediately available hexose sugars.
3. It must be acid-tolerant and capable of producing a final pH of at least 4·0. Preferably, it should be able to produce this low pH as rapidly as possible in order to lessen the chance of losses in dry weight due to the production of ammonia, hydrogen and carbon dioxide by other microorganisms.
4. It must be able to ferment glucose, fructose, sucrose and preferably fructosan, and the pentoses which will appear during the silage process.
5. It must not produce dextran from sucrose or mannitol from fructose. In the event of the latter substance appearing due to action of heterofermentative organisms, it is desirable that this organism should ferment mannitol rapidly.

6. It should have no action on the organic acids.
7. It should possess a growth temperature range extending to 50° in order to combat any marked rise in temperature due to excess initial heating of the silage material. A feature of inoculation, however, would be the avoidance of the necessity for initial heating.
8. It should be able to grow in material of fairly low water content as might arise when silage material is wilted.

Choice of organisms from those available.

Immediately excluded from choice on grounds of metabolism, are the leuconostocs and heterofermentative lactobacilli, and on the grounds of acid tolerance, the streptococci. The choice would seem to lie, therefore, among the pediococci and the homofermentative lactobacilli.

Of the organisms available which have been isolated from silage, there are two strains which might be of use. One is a strain of *Pediococcus acidilactici* which grows and produces acid from glucose over a temperature range of 10°-50°, ferments glucose, sucrose, fructose arabinose, and xylose, does not form dextran from sucrose, is able to produce, in glucose broth, a final pH of 3·6, and dissimilates malate only in the presence of fermentable sugar and citrate not at all. The undesirable characteristics possessed by this organism is its ability to dissimilate malate and its inability to ferment mannitol.

The other organism is an unidentified strain of the *Lb. plantarum-casei* group which grows and produces acid over a temperature range of 10°-40°, ferments glucose, fructose, sucrose, mannitol and arabinose but not xylose, fails to form dextran from sucrose, and does not dissimilate either malate or citrate in the presence or absence of sugars. Other favourable features of this organism are that it is

motile and does not produce ammonia from arginine. Further work is necessary before it is possible to say how these two organisms comply with the other requirements that have been suggested.

In practice, it will be necessary to observe the behaviour of these organisms in plant material of known sugar and organic acid composition. Present analytical methods are laborious and time consuming and, with the possibility of day to day variation in sugar content, the results may be too delayed for practical purposes. Work presently going on in this laboratory is designed to overcome this problem. By using cell-suspensions of lactic acid bacteria and on the basis of the fermentation of sugars to lactic acid and the decarboxylation of citrate and malate, it is hoped that a total biological sugar, malate, and citrate, value of the plant material can be obtained within as short a period of time as 1-2 hr. In addition, it should also be possible to obtain individual sugar values by using organisms with different fermentative properties. If such a method should prove successful, it would be possible to follow the day to day variations of sugar and organic acid content in plant material and changes occurring during the day without involving a great amount of labour. It may also be possible to devise a system whereby plant material would be roughly classifiable into three categories with respect to its suitability for ensiling. Plant material would be placed in the first category when its sugar content is high and organic acid content low. This material could be ensiled without additions, as it would not matter which type of lactic acid bacterium developed.

Plant material would be placed in the second category when its content of sugars and organic acids are at such a level that a

heterofermentative fermentation might be detrimental. In this case it would be advisable to add an inoculum of a homofermentative organism which would guarantee preservation of the silage.

Plant material would be placed in the third category when its sugar and organic acid content are such that the addition of both an inoculum and a carbohydrate would need to be added to guarantee preservation. By using a homofermentative organism as the inoculum, it should be possible to economise on the use of carbohydrate additives.

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EXPLANATION OF PLATE 1.

Colonies appearing on plant material in a selective medium.

Fig. 1	A sycamore leaf.	A.	Leuconostoc colonies appearing around holes in the leaf.
Fig. 2	Cocksfoot.	B.	Lactobacillus colonies on the outside surface.
		C.	Lactobacillus colony on the inside surface.
Fig. 3	Cocksfoot.		Lactobacillus colonies on partially withered and withered blades.

PLATE 1.

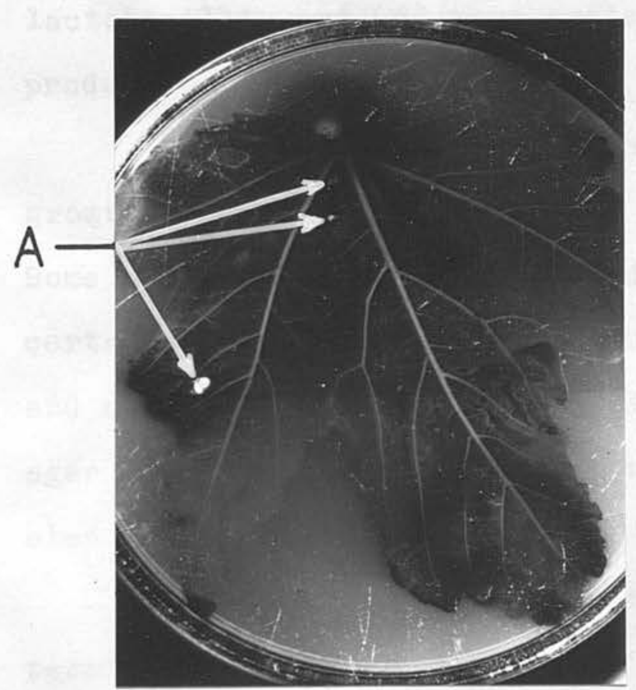

Fig. 1

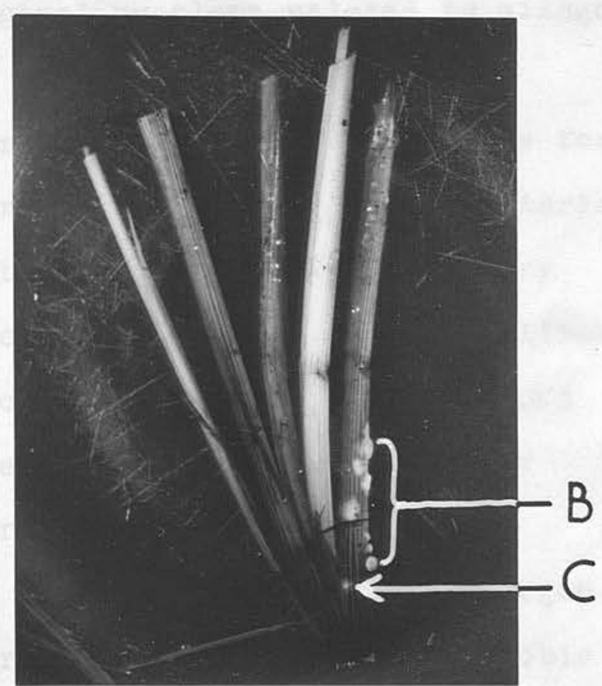

Fig. 2

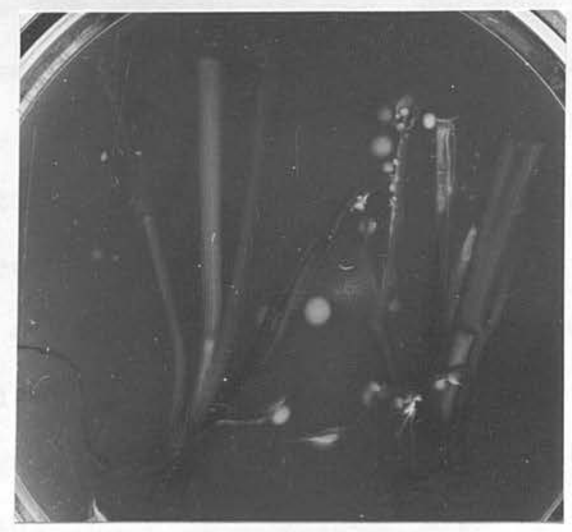

Fig. 3

Summary.

This thesis discusses (1) the oxygen relationships of lactic acid bacteria, (2) the differentiation of enterococci, (3) the genus *Pediococcus*, (4) the genus *Leuconostoc*, (5) the heterofermentative lactobacilli, and (6) some bacteriological problems related to silage production.

(1) A preference for anaerobic or microaerophilic conditions for growth was shown to be far from universal in the lactic acid bacteria. Some preferred and some required aerobic conditions when utilizing certain substrates. Others required or preferred anaerobic conditions and others did not exhibit a preference for either condition. Soft agar proved to be useful for demonstrating these requirements and also the occurrence of mutation in fermentation.

Lactic acid bacteria differed in their ability to form hydrogen peroxide independently of their preferences for aerobic or anaerobic conditions.

Two types of peroxide-splitting activity were detected though never in the same organism. One type was dependent on the presence in the medium of heated-blood or haematin. The other type was apparently due to a non-haem enzyme which was acid-sensitive. There was no correlation between peroxide-splitting activity and the ability to form peroxide.

In an examination of a few representative organisms, some appeared to be capable of forming cytochromes when grown on heated-blood agar. One showed traces of cytochrome when grown on glucose agar. It was suggested that lactic acid bacteria might have developed from haemin-synthesizing, aerobically respiring organisms.

Physiological tests based on the above findings and tests for action on citrate and malate were used in combination with previously described methods for the differentiation of lactic acid bacteria.

(2) Enterococci obtained from various sources were classified as _Streptococcus faecalis_ and _Str.faecium_.

(3) Organisms identified as pediococci were classified as _Pediococcus pentosaceous_, _P.acidilactici_ and _P.cerevisiae_. _P.acidilactici_ and _P.pentosaceous_ appeared to be physiologically similar to _Str.faecium_. Two other tetrad-forming organisms, _Aerococcus viridans_ and _P.urinae-equi_, had properties in common with both staphylococci and pediococci. _P.urinae equi_ was identified as _A.viridans_.

(4) The leuconostocs were provisionally classified in four species, _Leuconostoc gramineum_, prov.nov.spec., _Ln.kefir_, nov.comb., _Ln.dextranicum_ and _Ln.mesenteroides_.

(5) The heterofermentative lactobacilli were classified in six species, _Lactobacillus brevis_, _Lb.fermenti_, _Lb.cellobiosus_, _Lb.hilgardii_, _Lb.viridescens_ and _Lb.fructivorans_, and an unidentified group.

(6) Plant material was examined for lactobacilli, pediococci and leuconostocs by plating portions in a selective agar medium. These organisms were scarce, the leuconostocs occurring most frequently.

The heterofermentative and homofermentative lactic acid bacteria were discussed with respect to their efficiency in silage preservation. Activities considered were the production of lactic acid from sugars and dissimilation of citrate and malate.

A specification was given of an organism which could be accepted as suitable for the inoculation of silage, and two strains were described which might prove to be useful for this purpose.

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The author is grateful to the various investigators named in the text, who have kindly provided cultures.

APPENDIX

METHODS

The media and tests which have been used in the study of enterococci, pediococci, leuconostocs and heterofermentative lactobacilli are collected below.

Agar medium. This was as described in Section 1.

Liquid medium. This was the agar medium less agar.

Inoculum medium. This was as described in Section 1.

Acid production from carbohydrates. A soft agar medium, containing Seitz-filtered solutions of carbohydrates, was used as described in Section 1, and adjusted to pH 6·5 in the case of the enterococci and pediococci; 6·8-7·0 in the case of the leuconostocs; and 6·8-7·0 and 5·4 in the case of the heterofermentative lactobacilli. The concentration of bromocresol-purple (BCP), was doubled in this medium and in all other media used in tests with the enterococci.

Temperature tests. Soft agar containing BCP and glucose, 0·5%(w/v) - autoclaved in the medium - and adjusted to pH 6·8, was used. The ability of organisms to both grow and produce acid at various temperatures was determined in thermostatically controlled water baths. Baths running at 10° and 15° were kept in a cold room at 4°.

Heat resistance. The method of Abd-el-Malek & Gibson(1948) was followed using the agar medium containing glucose, 0·5%(w/v), adjusted to pH 6·5, for plating.

Motility. Hanging drop preparations of the condensation water of 12-15 hr. old slope cultures were examined. The medium was the agar medium containing glucose, 0·25%(w/v), adjusted to pH 6·5.

Gelatin liquefaction. The inoculum medium, from which glucose had been omitted and to which gelatin (Cox's), 12%(w/v) had been added, was used adjusted to pH 6·5. Incubation was at 22° for 2 weeks.

Ammonia production from arginine. The method and medium of Niven, Smiley & Sherman (1942) was used with the exception that yeast extract was replaced with yeast autolysate and Tween 80 included. After 4 days incubation the presence of ammonia was determined with Nessler's reagent.

Ability to initiate growth at pH 8·0 and above. The medium and method of Chesbro & Evans (1959) was used. Repeated experiments were carried out in a medium containing 0·15%(w/v) agar, inoculations being made before the agar set.

Diastase production. Plates of agar medium containing soluble starch, 0·2%(w/v), were inoculated and incubated for 1 week and then flooded with a solution of Gram's iodine.

Aesculin hydrolysis. Soft agar containing aesculin, 1·0%(w/v), glucose, 0·2%(w/v), and ferric citrate, 0·05%(w/v), and adjusted to pH 6·5, was inoculated and incubated for 1 week. Hydrolysis of aesculin was judged to have occurred when the loss of fluorescence was accompanied by blackening and the formation of crystalline aesculetin (Gemmell, M.priv.com.). Blackening did not necessarily indicate aesculin hydrolysis.

Hippurate hydrolysis. Liquid medium containing sodium hippurate, 1·0%(w/v), and glucose, 0·2%(w/v) and adjusted to pH 6·5, was used. After 2 weeks incubation the liberation of benzoic acid was detected by the addition of 50% sulphuric acid to c 1 ml. of clear decanted culture fluid.

Sodium chloride tolerance. Double strength liquid medium containing BCP and glucose, 1·0%(w/v), was adjusted to pH 6·5 and an equal volume of salt preparations added. The salt did not interfere with reactions of the pH indicator. In some additional experiments the liquid medium was replaced by a soft agar.

Potassium tellurite tolerance. The agar medium containing glucose, 0·5%(w/v), and tellurite, 1/2,500 p.p.m., as recommended by Skadhauge (1950), adjusted to pH 6·5, was used. Results were erratic at first but became constant when the meat extract concentration was doubled.

Reduction of 2:3:5 triphenyl-tetrazolium-chloride. This test was based on the method of Barnes' (1956). Liquid medium containing glucose 0·5%(w/v), and tetrazolium, 0·01%(w/v), adjusted to pH 6·0, was used. A heavy inoculum of 3 capilliary pipette drops was added and the cultures examined after 8 hr. with a final reading at 24 hr. Positive cultures were a deep magenta in colour whilst the cultures regarded as negative were either colourless or faintly tinged with a pink colouration.

Haemolysis. Brown's method (1919) was followed using nutrient agar containing salt, 0·5%(w/v), and defibrinated horse blood, 3·0%(v/v). Poured agar plates were incubated for 2 days at 30° and refrigerated overnight.

Hydrogen peroxide production, hydrogen peroxide-splitting activity and ability to take up oxygen on glucose. These tests were carried out as described in Section 1.

Malate dissimilation. The dissimilation of malate was determined in two media, one with and one without glucose. Liquid medium, containing 4·0%(w/v) DL malic acid, BCP, and, if required, glucose, 1·0%(w/v), was adjusted to pH 6·0 with potassium hydroxide and distributed in 4 ml. amounts to 5 x 1/2 in. test-tubes containing Durham tubes. The main function of the Durham tubes was to support the water-agar seals added after the tubes had been inoculated. Dissimilation of malate was judged by pH change, carbon dioxide production, and increase of growth.

Citrate dissimilation. The medium was similar to that used above. Citrate was added as potassium citrate, 3·0%(w/v), in the place of malate, and adjusted to the pH required, usually 6·0. Dissimilation of citrate was judged by pH change, carbon dioxide production, and increase of growth.

Tolerance of acetic acid-acetate buffer. The acetate medium of Keddie (1951), modified as described under Isolation medium, p. 51, was used. Agar plates were streaked and incubated aerobically for two days.

The reduction and tolerance of methylene blue in milk and the reduction of litmus milk. The ability of a large inoculum, 3 capilliary pipette drops, to reduce the dyes within 8 hr., with a final reading at 24 hr., was examined. A final reading on acidity, clotting and the onset of liquefaction was made at 2 days.

The production of nitrite from nitrate. Liquid medium containing glucose, 0·2%(w/v), and potassium nitrate, 0·5%(w/v), was adjusted to pH 7·0, inoculated, and incubated for 14 days. Tests for nitrite were made at 1, 3, and 14 days with Trommsdorf's reagent (Fred & Waksman, 1928).

Production of carbon dioxide from glucose. The method and medium of Gibson & Abd-el-Malek (1945), and a method similar to that of Hayward's (1957), were used. The medium used in the latter method was liquid medium to which glucose, 3·0%(w/v), was added and in which the concentration of meat extract (Lab Lemco) and peptone (Evans) had been doubled. The medium was adjusted to pH 7·0 and distributed to 5 x 1/2 in. test-tubes containing Durham tubes, inoculated, and sealed with water agar.

Dextran production from sucrose. The agar medium containing sucrose, 5·0%(w/v), was adjusted to pH 7·0. Plates were poured, streaked, and incubated at 22° for 7 days.

Miscellaneous details.

Unless otherwise stated; incubation was carried out at 30°; liquid media were distributed in 2·5-3 ml. amounts in 5 x 1/2 in. test-tubes; soft agar in 5 ml. amounts in 6 x 5/8 in. test-tubes; and all media were sterilized by autoclaving at 15 lb./sq.in. Milk preparations were sterilized by steaming for 30 min. on three consecutive days. Certain additives, such as tetrazolium and tellurite salts, were separately sterilized according to the methods suggested by the authors proposing their use.

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