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A STUDY OF THE LACTOBACILLI ASSOCIATED WITH GRASS SILAGE.

by

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C O N T E N T S.

INTRODUCTION.

Introduction.

In making silage, green fodder is placed in a silo, thoroughly compressed to exclude oxygen as far as possible, and allowed to ferment. The aim is to produce material of sufficiently low pH (4 - 4.2) to prevent undesirable fermentations and proteolysis.

Many inter-related factors govern the pH level of silage at any stage in the process, but the attainment of a low pH depends principally on the fermentation of plant carbohydrates to acids, in particular, lactic acid. Thus bacteria capable of forming lactic acid as a major end-product in their fermentation of 'sugars', are of primary importance in the production of good silage. Of special importance are the lactobacilli, since they are the only organisms which can reduce the pH in silage to 4 or less.

In the past, lack of a suitable selective medium for lactobacilli has often made it impossible to assess accurately their numbers on fresh fodder and in silage in the early stages of the fermentation. The first part of this work therefore, deals with the evolution of a selective medium suitable for the enumeration of lactobacilli on grass and in silage. This is followed by a study of the development of lactobacilli and the other lactic acid bacteria in laboratory silage made from grass subjected to different treatments under controlled conditions.

Part III is an account of the properties and the identification of representative strains of grass and silage lactobacilli, while part IV concerns experiments designed to assess the/

the value of inoculation of grass before ensilage, with selected strains of lactobacilli.

PART I.

The evolution of a selective medium for the enumeration of lactobacilli on grass and in silage.

REVIEW OF LITERATURE.

The enumeration of lactobacilli in mixed microbial populations has always presented considerable difficulty, largely because of their exacting nutrient requirements. In order to satisfy these requirements, the artificial media used for their cultivation must necessarily be complex.

Growth does not occur in the absence of a fermentable substrate (Orla-Jensen, 1919; Davis, 1935-6), and so glucose which is almost always readily fermented by these organisms has been commonly incorporated in media. However, some authors, for example McLaughlin (1916), have used a mixture of sugars to provide suitable energy sources for those types that do not ferment glucose readily.

The form of nitrogen supplied is of great importance. Even the less fastidious of these organisms appear to require eight to ten amino-acids where excess of all accessory growth factors is present (Tittsler et al., 1952). Probably for this reason, casein hydrolysate has often been found to be a particularly suitable source of nitrogen (Orla-Jensen, 1919; Cunningham & Smith, 1939; McLaughlin, 1946), although ordinary commercial peptones have frequently been used.

In addition to their basic requirements for carbon and nitrogen, a wide range of accessory growth factors is essential. Yeast extract has frequently been employed to satisfy these requirements (Orla-Jensen, 1919; Pederson, 1930b; Allen & Harrison, 1936), but some authors have preferred to use tomato juice/

juice (Mickle & Breed, 1925; Kulp, 1927; Charleton, Nelson & Werkman, 1934; Valley & Herter, 1935; Harrison & Hansen, 1950a)

Various other substances have been used at different times, for example, bean extract, (Sherwood, 1939-40; Allen & Harrison 1936), grass and silage extract (Cunningham & Smith, 1939), kraut juice (Pederson, 1930b), whey (Sherwood, 1939-40), and malt sprouts (Olsen, 1948).

With regard to mineral nutrients, probably only manganese deserves special mention. It appears that certain lactobacilli have rather a high requirement for that element and that it may be deficient even in complex media. (Orla-Jensen, 1919; Demeter, 1949).

Owing to the complex nature of the media required for their cultivation, the only method of isolating lactobacilli from mixed microbial populations where they may be greatly outnumbered by other organisms, would appear to be the employment of selective inhibitors.

The aciduric nature of this group has long been recognised, and this property has often been exploited in devising media for their isolation. Solid or liquid media are adjusted to low pH, in some cases as low as pH 4.2 (Allen & Harrison, 1936). Kendall (1910) states that this method of isolation was first used by Johanneson in 1897. Various acids have been used to acidify the media, including lactic (Hadley, 1933), citric (Allen & Harrison, 1936), and a mixture of citric, tartaric and malic acids (Olsen, 1948). Acetic acid seems to have been particularly/preferred by several authors, (Salge, 1904; Kendall, 1910; Hunter/

Hunter, 1921; Weinstein & Rettger, 1932; Winblad, 1941). According to Kendall (1910), it was first used for this purpose by Heymann in 1898. Other workers have not specified which acid they used, but presumably they used a mineral acid.

According to Allen & Harrison (1936), the disadvantage of using a low pH for isolation is that the count of lactobacilli obtained may be only a fraction of the actual numbers present in the material examined. These authors used a citrate buffer at pH 4.2 (Davis, 1931), and so the possibility exists that the presence of citrate and citric acid contributed to the toxic effect of the low pH.

Davis (1935-36) has tentatively suggested that a concentration of sodium lactate in the region of 6% might be of use in differentiating lactobacilli from streptococci in cheese.

It may also be mentioned here that some lactobacilli have an unusually high tolerance of ethyl alcohol, sometimes being able to grow in concentrations as high as 20% (Olsen, 1948; Fornachon, Douglas & Vaughn, 1949).

L. casei has been shown to be capable of growth in high concentrations of glucose (Muedking, 1947).

From the available data, it would appear that the most useful substances to examine for their value as selective inhibitors would be lactate, citrate, acetate, ethyl alcohol and possibly glucose in high concentrations.

EXPERIMENTAL AND RESULTS.

Experimental and Results.

Since the selective media previously employed were of rather doubtful value, a non-selective medium was used for the isolation of lactobacilli at the outset of this work. This medium, tomato agar, was similar to that used by Kulp (1927), and had the following composition:- tomato extract, 40 ml. (Appendix p.118); peptone, 1 g.; agar 1.5 g.; water to 100 ml., pH 6 - 6.2.

However, certain types were isolated from silage which although capable of growth on crowded plates, did not grow satisfactorily in pure culture. It was therefore obvious that work involving the use of selective inhibitors could not be embarked upon until a satisfactory agar medium had been devised.

Development of an improved agar medium.

In the past decade, much attention has been given to oleic acid as a growth factor for lactobacilli (Kodicek & Worden, 1945; Williams & Fieger, 1946; Hoff-Jørgensen, Williams, & Snell, 1947; Whitehill, Oleson, & SubbaRow, 1947; Hutchings & Boggiano, 1947). It was probably first used for this purpose by Salge (1904), and has occasionally been included in media by various earlier workers, (Kendall, 1910; Hunter, 1924). The stimulatory effect of butterfat noted by Sherman & Albus (1922), may have been due to its oleate content.

A disadvantage of oleic acid appears to be its toxicity in concentrations above the optimum level (Kodicek & Worden, 1945; Williams & Fieger, 1946; Hutchings & Boggiano, 1947; Williams, Broquist/

Table 1.

Effect of various additions on the growth of a strain growing feebly on tomato agar and glucose agar.

Incubation 3 days at 30°C.

	Medium	Amount of growth
1.	G.A.	±
2.	" + 10% Y	++
3.	" + 10% T	+
4.	" + O	+++
5.	" + 5% Y + 5% T	++
6.	" + 5% Y + 5% T + O	+++++
7.	T.A.	++
8.	" + 10% Y	+++
9.	" + O	+++
10.	" + 10% Y + O	+++++

G.A. = glucose agar

T.A. = tomato agar

Y = yeast autolysate

T = tomato extract

O = oleic acid (20 μ g./ml.)

Broquist & Snell, 1947), but this effect may be overcome by including in the medium such substances as bovine serum albumen (Williams & Fieger, 1949; Tomarelli, Norris, Rose & György, 1950), tween 40 (Kitay & Snell, 1950), acacia suspension, dialysed whey, or tween 80 (Tomarelli et al., 1950).

Some work was therefore carried out to examine the possibility that oleic acid would stimulate the growth of the poor-growing strains mentioned above. The effect of yeast autolysate and tomato extract was also determined in these experiments.

The effect of yeast autolysate, tomato extract, and oleic acid.

The strain used was one that gave on tomato agar, the poorest growth of all those isolated. The basal medium contained 1 g. each of peptone (Evans), lemco and glucose, and 1.5 g. agar in 100 ml. water; pH 6.0. Yeast autolysate and tomato extract were prepared as described in the Appendix (p.118), and adjusted to pH 6 - 6.2 before sterilization at 15 lb./sq.in. for 15 min. Oleic acid was added as an aqueous sol[illegible] prepared by the method of Williams & Fieger (1946), and used at the same concentration, 20 μg./ml., throughout.

Tomato agar was used as a control and the effect of adding yeast autolysate and oleic acid to it was also determined. The optimum rate and the final amount of growth were assessed in stab cultures (using a loop) in the various media. The results are shown in table 1.

It can be seen that oleic acid stimulated growth in all media to which it was added, and that the best growth occurred where/

where all three constituents were used. A fairly high concentration of tomato extract, i.e. greater than 10%, appears to be necessary for maximum stimulation.

The effect of manganese. For this series of tests, the basal medium used was as follows; 1 g. each of glucose, peptone and lemco; tomato extract, 20 ml.; yeast autolysate, 5 ml.; oleic acid, 2 mg.; agar, 1.5 g.; water to 100 ml.; pH 6 - 6.2. In addition to the poor-growing strain previously used, a strong-growing strain was also tested to ensure that its growth was not reduced in the oleate medium. Optimum growth was now assessed by the number and size of colonies growing on poured plates inoculated with loop dilutions of the test organisms.

A series of plates with oleate agar containing 14.2 mg. manganous sulphate per litre was inoculated with the two strains in addition to a control series. There appeared to be no difference in the number and size of colonies growing on the two sets of plates. Both strains produced large colonies in oleate agar with and without added manganese.

It was concluded that manganese would not have to be added to the medium.

The effect of pH. Oleate agar was adjusted with dilute hydrochloric acid to pH values of 4.5 to 7.0 in 0.5 unit steps. The pH of each batch was taken after sterilization, and the different batches were inoculated with the same two strains used in testing the effect of manganese. Best growth appeared to occur in the range pH 5.0 - 6.0 approximately, and it was therefore decided to adjust the final medium to pH 5.4 - 5.6.

This/

This medium, referred to hereafter as oleate agar, has the following composition: 1 g. each of peptone, lemco, and glucose; tomato extract, 20 ml.; yeast autolysate, 5 ml.; agar, 1.5 g.; oleic acid, 2 mg.; water to 100 ml.; pH 5.4 - 5.6.

The effect of lemco. In a medium containing so many complex constituents, it was thought possible that lemco was not necessary. However, when oleate agar containing no lemco was tested, growth was considerably reduced. Lemco was therefore retained in the medium.

Some workers have used tween 80 in preference to oleic acid. It is a sorbitan mono-oleate polyoxyalkylene derivative (Glassman, 1948), and supplies oleic acid in a water-soluble and non-toxic form, (Williams, et al., 1947; Tomarelli, Norris & György, 1949). Some work was carried out to show if it could be used to replace oleic acid.

The effect of tween 80. The basal medium used was the same as for oleate agar but contained no oleic acid. The 'T.B.' quality of tween 80 was used. The same two strains as were used before plus an additional weak-growing strain were employed in this experiment. Concentrations of tween varying from 0.005 - 0.4% were sterilized in the medium, and a control of the same medium containing no tween was used. Oleate and tomato agars were also inoculated as controls.

For the two weak-growing strains, 0.005% tween gave a very marked stimulation, and 0.4% did not appear to inhibit them to any great extent. The optimum concentration was in the range 0.01 - 0.1%. Tween media were slightly better than oleate agar/

Table 2.

Comparison of plate counts of different strains of lactobacilli on tween and tomato agars, and the approximate sizes of colonies

Strain	Diln.	Plate count	
		Tween agar	Tomato agar.
1. strong-growing	10^{-6}	39 (2 m.m.)	45 (2 m.m.)
2. weak-growing	10^{-5}	4 ($<\frac{1}{2}$m.m.)	7 (1-2 m.m.)
3. weak-growing	10^{-5}	11 (pin-point)	33 (2 m.m.)

Table 3.

Growth of different strains of lactobacilli on tween agar compared with that on tomato agar taken as +.

Homofermentative strains		Heterofermentative strains	
1	> +	12	+
2	+	13	++
3	> +	14	++
4	++	15	+
5	++++	16	++
6	+++	17	++
7	+++	18	+++
8	+++	19	+++
9	++	20	+++
10	++		
11	+++		

agar and markedly superior to tomato agar. Even the strain which grew strongly in tomato agar produced slightly larger colonies in the media containing tween.

It was therefore decided to substitute 0.05% tween 80 for oleic acid in the oleate agar. This medium is referred to hereafter as tween agar.

Plate counts of the three test organisms were compared on tween and tomato agars. Week-old cultures in glucose yeast autolysate semi-solid agar were used. The results are given in table 2.

From these results it can be concluded that tween agar is much superior to tomato agar for the weak-growing strains, and is at least as good for the strong-growing strain.

Stab cultures of a selection of silage lactobacilli in tween and tomato agars were compared. The results are given in table 3.

It can be seen that the majority of strains tested grew more profusely in tween agar than in tomato agar. Those which did not give heavier growth in tween agar, grew profusely in both media. No strain grew more poorly in the new medium.

It was concluded from these results that tween agar was a suitable general medium and that it could be used as a basal medium to test the effect of various selective inhibitors.

The/

The use of inhibitors.

The various substances tried, were tested in tween agar except where high concentrations of glucose were used. The trials of glucose were carried out before the experimental work on tween agar was completed.

The organisms used in these experiments were nine heterofermentative and eleven homofermentative lactobacilli and one Gram-negative rod, all of which were isolated from silage. The Gram-negative rod was included to determine roughly the differential effect of the various inhibitors. Stab cultures (using a loop) were used to assess the degree of inhibition. Tween agar without any addition was used as a control.

The effect of glucose in high concentrations. 5% yeast autolysate, nutrient agar was the basal medium used here, and concentrations of glucose varying from 8% to 16% were sterilized in the medium. It was found that while the Gram-negative rod still grew vigorously in 16% glucose, a few of the lactobacilli showed signs of inhibition. Work on glucose was therefore discontinued.

The effect of ethyl alcohol. Absolute alcohol was sterilized by Seitz filtration and concentrations of 4% to 16% in 2% steps were added aseptically to molten tween agar in bottles. After thorough mixing, the medium was distributed in $\frac{1}{4}$ oz. screw-capped vials which prevented loss of alcohol by volatilisation during incubation. Stab inoculations were again made.

The Gram-negative rod was almost completely inhibited by

4%/

4% alcohol but so also were three of the lactobacilli. At a concentration of 6%, more lactobacilli were showing signs of inhibition.

It appeared that alcohol might be of some limited use. The effect of citrate, lactate and acetate. In this case the sodium salts of the three acids were autoclaved separately in concentrated aqueous solution and added aseptically to tween agar. Being salts of a weak acid and strong base, their solutions tended to raise the pH of the tween agar, in some cases to a little above pH 6. This, however, was not considered to be a serious drawback in a rough screening test.

On the basis of Davis's work (Davis, 1935-36), 2%, 4% and 6% lactate were tested. One strain was completely inhibited by 2%, all strains showed slight inhibition at 4%, which became more marked at 6%. With citrate, there was slight inhibition of all strains at 2% and very marked inhibition at 4%. The inhibitory effect of citrate for lactobacilli appears to operate through an induced deficiency of certain mineral nutrients, including manganese (McLeod & Snell, 1947).

However, with acetate, rather striking results were obtained. Virtually all the strains of lactobacilli tested seemed to grow as well with 4% acetate in the medium as in tween agar alone. On the other hand, the Gram-negative rod was completely inhibited by 0.5% acetate. Koser (1923) found that members of the coli-aerogenes group were inhibited by low concentrations of acetate, while Owen (1946) also states that acetic acid is of use in suppressing Gram-negative rods. It

appeared/

appeared that further exploration of the effect of acetate might prove fruitful.

Further experiments with acetate. The acetate was now used in the form of an acetic acid/sodium acetate buffer at pH 5.4 (Clark, 1922). As a preliminary, a 3 M. solution was autoclaved separately, and added aseptically to tween agar to give a final concentration of 0.3 M. acetate in the medium. Stab cultures were made of the same series of lactobacilli as were used previously. Of the twenty strains tested, two were completely inhibited and growth of a few more was considerably reduced.

When 0.2 M. acetate was tried, the same two strains were again completely inhibited and growth of a further three strains was considerably reduced. However, of these five strains, the two which were completely inhibited, and two of the other three strains had been particularly selected as being of unusual type. The fifth strain belonged to a small group of which several representatives had been isolated. All the other silage lactobacilli grew strongly in 0.2 M. acetate tween agar.

In order to determine the selective effect of this concentration of acetate, various types of silage organisms were tested including 53 Gram-negative rods, 4 micrococci, 3 corynebacteria, 5 homofermentative and 1 heterofermentative streptococcus, 1 Bacillus subtilis and 1 Bac. licheniformis. Of these one micrococcus (pediococcus?) and the heterofermentative streptococcus grew strongly, while one homofermentative

streptococcus/

Table 4.

Comparison of plate counts of various lactobacilli on tween and acetate agars.

(For each strain the counts were obtained from equal inocula.)

Homofermentative lactobacilli	Tween agar	Acetate agar
1	67	72
2	58	50
3	287	334
4	35	46
5	67	60
6	46	37
7	213	22
8	213	157
Heterofermentative lactobacilli		
9	286	318
10	52	59
11	77	61
12	63	55

streptococcus, an enterococcus, gave slight growth. These results appeared to be rather promising, and so it was decided to put the medium to a more severe test.

A batch of experimental silage was made and examined daily from the fresh grass stage up to the seventh day. Tween agar containing 0.2 M. acetic/acetate buffer was used as a plating medium. This medium is referred to hereafter as acetate agar. Double layer plates were used.

During the experiment, the colony count on the acetate agar varied from less than 6/g. on the fresh grass (lowest dilution 1/6) to approximately sixty millions/g. silage at the peak count. Of 140 cultures isolated at various stages in the fermentation, 139 were lactobacilli and the other was a heterofermentative streptococcus. Occasional pin-point colonies appeared on the plates, but these, when picked into tween semi-solid agar often failed to grow. One such colony, however, proved to be an aerobic spore-forming rod.

From these results, it seemed that the medium was highly selective for lactobacilli (with the exception of hetero-fermentative streptococci and occasional micrococci (pediococci?)). It now remained to determine to what extent the numbers of lactobacilli were reduced on the acetate agar. To do this, plate counts of representative strains of silage lactobacilli on tween and acetate agars were compared. The results are presented in table 4. The two strains which were completely inhibited were not included in these tests.

It can be seen that only two strains (nos. 7 and 8) were significantly/

significantly inhibited on acetate agar. Both of these strains belong to the group previously mentioned as being inhibited to a significant extent. (These organisms belong to group II described in part III of this work.)

It was surprising to find in the silage experiment mentioned above, that no lactobacilli could be detected on the fresh grass by means of acetate agar, since Allen & Harrison (1936) reported that 5 out of 9 grass samples examined had lactobacillus counts in the range 10^6 - 10^7/g.. The method used by these authors was as follows:- Tubes of yeast extract glucose broth were inoculated with dilutions of silage and incubated anaerobically for 10 - 15 days at 30°C. The highest dilutions showing growth were then plated on a solid medium of similar composition, from which colonies were picked and identified.

A sample of grass was examined using acetate agar as well as the dilution method of Allen & Harrison described above. Silage made from this grass was also examined by the two methods after incubation at 30°C for 1, 2, 4 and 11 days.

A macerate of the grass ($^1/6$ dilution) had an acetate count of 13, while the highest dilution in broth showing growth was 10^{-5}. When all the dilutions in broth showing growth (undiluted macerate - 10^{-5}) were plated on yeast extract glucose agar, none of the colonies developing on the plates were those of lactobacilli. However, when these same broths were plated on acetate agar, lactobacilli were recovered from the/

the tube inoculated with 1 ml. of undiluted macerate, but not from any of the higher dilutions. Examination of the silage after different periods of incubation yielded similar results.

It can reasonaly be concluded therefore that acetate agar is selective for lactobacilli, and that it can yield a nearly maximal colony count when used for a mixture of strains. Only one type of lactobacillus has been found to be seriously inhibited on the medium. Of organisms other than lactobacilli which may grow on acetate agar the most important are probably heterofermentative streptococci.

In an attempt to inhibit this latter group of bacteria, other substances were examined. The procedure was the same as had been used previously except that in these experiments, the acids were sterilized in the medium.

The effect of glycollic acid (hydroxy-acetic acid). The concentrations used varied from 0.25 - 2%. At the highest concentration, 2%, the agar was considerably softened, and there was no differentiation between lactobacilli and heterofermentative streptococci.

The effect of propionic acid. Here the selective effect was very similar to that of acetate. The inhibitory effect on lactobacilli was just noticeable at 0.5%, and was proportionately greater at 1%. The heterofermentative streptococci and two strains of micrococci (pediococci?) tested, also grew at these concentrations. It was concluded that propionic acid was not quite so effective as acetate.

Acetate/

Acetate agar was therefore adopted as a routine plating medium for the isolation and enumeration of lactobacilli. The basal medium is tween agar which has the following composition:- peptone (Evans), 1 g.; lemco, 1 g.; glucose, 1 g.; agar, 1.5 g.; tomato extract, 20 ml.; yeast autolysate, 5 ml.; tween 80, 0.05 ml.; water to 100 ml.; pH 5.4. The medium is sterilized in the autoclave at 15 lb./sq.in. for 15 min. 2M acetate/acetic acid buffer at pH 5.4 is added to the molten agar just before plating to give a concentration of 0.2 M. acetate in the medium. The final pH of the medium is 5.4 and double layer plates are used.

New selective media for lactobacilli

After the work on acetate agar was completed, details of two new selective media were published.

Rogosa, Mitchell & Wiseman (1951a, 1951b) described a medium very similar to that detailed above, employing acetic acid/sodium acetate buffer at pH 5.4, as the selective inhibitor. Although it has not been compared with acetate agar, it seems likely to have a similar effect.

The other medium (Vaughn & Emard, 1951; Emard & Vaughn, 1952) is selective for lactic acid bacteria and clostridia. The selective inhibitor is sorbic acid which appears to have an effect similar to that of acetate.

Neither medium seems to be more highly selective for lactobacilli than acetate agar.

P A R T II

The development of lactobacilli and other lactic acid bacteria in silage.

The work to be described in this section is essentially one aspect of a research programme carried out by a team of bacteriologists and a chemist.

In order to regulate as far as possible the many variable factors which normally occur in field ensilage, the silage was made in small laboratory silos from single strains of grass, under controlled conditions.

The object of this work was to study the effect of different treatments, other factors being as constant as possible, on the microflora of silage at different stages, and on the quality of the material produced as judged by pH.

The treatments studied were those which might be encountered in normal farm practice, viz. temperature, moisture and laceration. Since preliminary work with acetate agar had indicated that lactobacilli might be scarce on grass, the effect of inoculation was also tried.

In all, five major team experiments were carried out, experiments 7 - 10 at different times during one season, and experiment 15 at the beginning of the next.

Since heterofermentative streptococci frequently formed colonies on acetate agar plates inoculated with dilutions of silage, the occurrence of these organisms in silage is also discussed.

REVIEW OF LITERATURE

The presence of lactobacilli in silage has long been recognised. Sherman (1916) reported the occurrence of aciduric bacteria in alfalfa silage which were similar to "the B. bulgaricus of milk and the B. acidophilus of the intestine" but differed in that they grew better than these organisms on ordinary laboratory media.

Hunter (1918) using glucose acetic acid agar as a selective medium, found that the "Bulgarian type" of organism was predominant in alfalfa silage.

Peterson & Fred (1920), Fred, Peterson & Anderson (1921a) and Peterson, Hastings & Fred (1925) have emphasised the importance of the pentose-fermenting group of lactobacilli in corn silage. Three species have been described, Lactobacillus pentosus, L. arabinosus and L. pentoaceticus (Fred, Peterson & Anderson, 1921b). They considered that L. pentoaceticus was the principal organism concerned in the fermentation, and found that it constituted about 50% of the total bacterial population of both inoculated and uninoculated corn silage after 8 - 10 weeks (Fred et al., 1921a)

In silage made from potato pulp from which starch had been extracted, Elema (1927) found only the rod-shaped lactic acid bacteria at 2 - 3 months. These, he identified as Streptobacterium plantarum, Sbm. casei and Betabacterium breve. In pulps which had been ensiled for about one year, a new species

of/

of streptococcus was found which he named, S. solanacearum.

Rikhter & Rybalkina (1927), from sorghum, corn and weed silage, isolated seven types of homofermentative lactic acid bacteria which were all morphologically similar. All fermented maltose and hexose sugar, and produced inactive lactic acid. None fermented lactose, and the temperature range was approximately 15 - 50°C with the optimum at 33°C.

Allen & Watson (1934) and Allen, Watson & Ferguson (1937) using various additions for making grass silage, concluded that lactobacilli were mainly responsible for acidification and that streptococci played a minor rôle. The species principally concerned in the fermentation was L. plantarum (Allen & Harrison, 1936; Allen, Harrison, Watson & Ferguson, 1937) but with the addition of whey, the types isolated were probably more closely related to L. casei (Allen & Watson, 1934; Allen, Watson & Ferguson, 1937). One strain isolated was probably related to L. pentoaceticus but this species was not thought to be important in grass silage. In general, the large majority of the strains isolated were homofermentative, fermented lactose and maltose, and either salicin, or arabinose, or both. About 50% of the strains isolated fermented sucrose, 30% fermented xylose, and usually inactive lactic acid was produced (Allen & Harrison, 1936).

Pette (1947) in determining the effect of sugar additions on the microflora of grass silage, considered that Betacoccus arabinosaceus was the most important organism in the first two weeks/

weeks, but thereafter only Sbm. plantarum, Sbm. casei and Bbm. breve were isolated.

Orla-Jensen (1947) states that in beet and in cabbage silage, the dominant organisms are Betacocci and Sbm. plantarum. In lucerne silage on the other hand, Betacocci are less numerous and Sbm. plantarum is the most important organism in the early stages. Bbm. caucasicum predominates in the later stages because of its more aciduric properties. (Orla-Jensen, 1947; Orla-Jensen, Orla-Jensen & Kjaer, 1947)

Olsen (1951) in a study of beet-pulp silage found Betacoccus arabinosaceus the dominant lactic acid organism in the early stages, but it was soon replaced by Sbm. plantarum and Sbm. casei. After 2 - 3 months only Bbm. breve was found.

Several workers have reported the presence of lactobacilli in A.I.V. silage where the pH has been reduced to less than 4 by means of mineral acids.

Virtanen (1937) found L. pentoaceticus in such material. Cunningham & Smith (1939, 1940) isolated different groups of bacteria from A.I.V. silage but recovered only lactobacilli from material more than 5 weeks old. These, they divided into 5 groups as follows:-

Group 1. L. plantarum

Group 2. Probably a degenerate form of (1).

Group 3. A motile type, possibly a new species.

Group 4. L. brevis

Group 5. Probably a degenerate form of (4).

The/

The fermentations involved in the making of sauerkraut, and of biologically-produced, pickled cucumbers, appear to be similar to the normal silage fermentation.

Pederson (1930a & b) found that cocci similar to Leuconostoc mesenteroides were active in the first stages of the sauerkraut fermentation, although a few were isolated which might have been L. dextranicum. These were followed by Lactobacillus pentoaceticus, which was probably the principal organism, L. buchneri, and a type related to L. wehmeri. Other groups isolated were L. plantarum and L. cucumeris.

With regard to cucumber fermentations, Etchells & Jones (1946) considered that L. plantarum was mainly responsible for acidification. Pederson & Ward (1949) and Pederson & Albury (1950), reached this same conclusion, but considered that a sequence of organisms usually occurred. The organisms, in order of appearance, were as follows:-

S. faecalis, Leuconostoc mesenteroides, Pediococcus cerevisiae, Lactobacillus brevis and L. plantarum.

They found that higher temperatures (86 - 97°F) tended to suppress Leuconostoc types, while at lower temperatures (45 - 65° F) a low acid producing rod, otherwise similar to L. plantarum was dominant. This organism, along with Leuconostoc mesenteroides was chiefly responsible for acid production at low temperatures. They also noted that there were frequently marked differences in the numbers of a particular group occurring in the material from year to year.

Although/

Although there is an abundance of literature concerning silage, and in it, the lactic acid bacteria are frequently mentioned, information on the particular types occurring, is scanty. However, from the available data, certain general conclusions may be drawn concerning the different yet related ensilage processes mentioned:-

L. plantarum is the principal agent in the fermentation of grass silage, and in most other silage fermentations. Its differentiation from L. casei is not clear-cut and this latter species may also be present. Streptococci may take part in the early stages of the process before lactobacilli become dominant. The streptococci participating in the fermentations of plant material are generally heterofermentative. L. brevis may occur in grass silage, but only after the period of active fermentation has ceased. It appears to be the principal organism concerned in the acidification of corn (maize) silage, although types related to L. plantarum also occur.

EXPERIMENTAL.

Methods.

Collection of samples.

Single strains of ryegrass, either S23 or S24 were used in each experiment. The grass was grown in plots kept only for this purpose at Boghall Experimental Farm.

Harvesting of the grass was done as aseptically as possible, by means of sterile sheep shears, the operator wearing sterile rubber gloves. The material was immediately transported back to the laboratory in sterile cotton bags which had an inner lining of grease-proof paper.

If on arrival at the laboratory the grass was wet with dew, this surface moisture was dried off by means of an electric hair-drier. The material was then thoroughly mixed and any weeds and pieces of withered grass were removed.

Strict aseptic precautions were used throughout the manipulation of the material, and all workers handling it wore sterile rubber gloves.

Treatments.

Except where the effect of laceration was being studied the material used was chopped into approximately 1" lengths.

In all, nine series of silage were made in each experiment and the treatments used were as follows:-

A. Incubation at 22°C.

B. " " 30°C. This was the control series.

C. " " 40°C.

D. Unchopped. This gave the opposite extreme from laceration.

E. Minced. The grass was finely minced by passing through a sterile meat mincer. This treatment gave the effect of extreme laceration.

F. Inoculated. In experiment 7 a Swedish commercial Siloferm inoculum was tried but it had died out, and so this data will be omitted. In experiments 8, 9 and 10, a mixed inoculum of a homofermentative (L1), and a heterofermentative lactobacillus (L16) was used. Young glucose, yeast autolysate, broth cultures were employed and no attempt was made to use a standard inoculum. The cultures were mixed and diluted with sterile water before being sprinkled on the grass with thorough mixing. In experiment 10 the inoculum was rather old. In experiment 15 a special 8-strain inoculum was used, the evolution of which is described in detail in part IV of this work. An inoculum of 2 million organisms/g. grass was employed.

G. Wetted. 125g. sterile water was thoroughly mixed with 175g. of grass. This gave the opposite extreme to wilting.

H. Wilted. The grass was spread out in a thin layer on a piece of sterile muslin on a wire tray, and wilted by a stream of warm air from an electric hair-drier.

I. Wilted + water. Water was added to wilted material to restore its dry matter content as closely as possible to the original/

Table 5.

Dry matter and crude protein content of the grass samples used in experiments 7 - 10 and 15.

Expt. No.	Grass	C.P. (% D.M.)	D.M.%			
			Fresh	Wetted	Wilted	Wilted + water
7	S24, young first-cut	14	21.1	13.6	34.6	21.2
8	S24, pre-flowering first-cut	8.1	21.4	19.6	28.1	16.9
9	S24, stemmy second-cut	9	25.6	15.4	41.2	24.7
10	S23, young first-cut	10.8	21	20.2	30	18
15	S24, young first-cut	21.4	20.2	14.4	22.3	17.8

C.P. = crude protein content.

D.M. = dry matter content.

original level of the fresh material. The reason for this treatment was to find out if any differences which occurred in the wilted material could be attributed to its low moisture content, or were a result of some secondary effect of wilting.

Experiments 7 - 9 were carried out using grass from the same plot at different stages of growth, while in experiment 10 young grass sown in summer of the same year was used. Since none of this material had a high crude protein content, experiment 15 was done at the beginning of the following year using material with a crude protein content of 21% (on a dry matter basis).

Information on the material used is given in table 5.

It can be seen that in experiments 8, 10 and 15, too much water had been added in treatment I (wilted + water) and the dry matter content in each case was appreciably lower than that of the fresh material. In experiment 15, wilting (treatment H) was not very successful and the dry matter content was only slightly increased. Insufficient water was added in experiments 8 and 10 to give a significant reduction in dry matter content.

Preparation and filling of laboratory silos.

The containers used were 8 x 1¼" Pyrex tubes. These were plugged with cotton wool and sterilized in the autoclave. 50g. of material after appropriate treatment was packed into each tube by means of a sterile glass or wooden rod, the diameter of which was slightly less than that of the container.

The tubes were then sealed with rubber stoppers containing a sintered glass mercury value. This allowed egress of gases formed/

PLATE I

LABORATORY SILO WITH SINTERED GLASS
MERCURY VALVE

formed during the fermentation, while conditions inside the tube were kept anaerobic. The seals were sterilized before use by holding over formalin for several hours in a closed container.

As soon as all the tubes were filled and sealed, they were placed in thermostatically controlled water baths, and incubated in the dark. Except where the effect of temperature was being investigated, incubation was at 30°C.

Treatment for bacteriological examination.

With fresh or wilted material, 50g. quantities were weighed out after thorough mixing of the bulk to ensure representative sampling. In the case of silage, the entire contents of a tube (50g.) were removed by means of a sterile, stainless steel corkscrew. In this way sampling error was eliminated. The material was then finely chopped, by means of sterile shears, into a sterile glass macerator jar, and made up to 300g. with sterile water. Maceration was carried out in a top-drive macerator for two minutes. The macerate obtained was a 1/6 dilution of the original material. This macerate was used in all bacteriological and chemical determinations.

The macerator was washed thoroughly between examinations, and sterilized by swabbing with alcohol and flaming. A sponge rubber washer surrounding the macerator spindle at first proved difficult to sterilize. This was overcome by covering it before each maceration with a sterile disc of thin rubber sheeting, slotted in the centre so that it fitted closely round the spindle.

Sterility/

Sterility checks on the macerator were done frequently by running 'blanks' of 300 ml. sterile water and plating 1 ml. quantities of these. Contamination from this source was negligible.

Bacteriological examination.

Decimal dilutions of the macerate were plated on acetate agar, double layer plates being used. Plates were incubated at 30°C for 5 days when they were counted.

Four colonies were usually picked from suitable plates in each examination, into tween semi-solid (T.S.S.) medium. This medium has the same composition as tween agar (Part I) but contains only 0.1% agar.

A disadvantage of solid media containing tween 80, is that, in it, colonies of all lactic acid bacteria are of the smooth type and usually differ only in size. This has been emphasised by other workers, for example, Rogosa & Mitchell (1950). For this reason, different colony types could not be picked and a random selection was therefore made. Whenever tiny colonies appeared on the plates, at least one was picked to establish the identity of the organism which was producing it.

The isolates in T.S.S. medium were incubated at 30°C and, as soon as growth appeared, films were prepared for morphological examination. At first, these were stained by Gram's method, but after having examined several hundred isolates from acetate agar in this way, no Gram-negative organisms were ever obtained. Nigrosin films were therefore prepared thereafter. This method has the advantage of being quicker and it also permits better differentiation/

differentiation of rod and coccal forms, a separation which occasionally caused difficulty.

At this stage, any organisms which obviously were not lactic acid bacteria were discarded. For example, aerobic spore-formers occasionally produced tiny colonies on acetate agar plates poured with dilutions of fresh grass. These were generally recognised easily by type of growth in T.S.S. medium and morphology. Infrequently, yeasts were encountered.

In experiments 7 - 10 the isolates were first of all purified by plating on tween agar. Young cultures in buffered tween broth (B.T.B.) (or the same medium with 0.1% agar) were used to inoculate a series of media employed for primary differentiation. The composition of B.T.B. is given in the Appendix (p. 118).

In addition to morphology, the routine tests used for grouping the isolates were as follows:-

1. Catalase test. A few ml. of 5 vol. hydrogen peroxide were added to young glucose yeast autolysate broth (G.Y.B. Appendix, p. 118) cultures, which were then carefully examined for bubbles of oxygen.

Broths containing tween 80 are not very suitable for this test as cultures of heterofermentative lactic acid bacteria sometimes emit bubbles of CO_2 when the medium is disturbed.

2. Production of CO_2 from glucose. The method of Gibson and Abdel-Malek (1945) was used but 5% yeast autolysate was substituted for 0.25% Yeastrel. Milk was used as the basal medium/

medium.

3. Reactions in litmus milk. Incubation was at 30°C and cultures were examined at 1, 2, 3, 7 and 14 days approximately, for production of acidity, time of clotting, and reduction of litmus.

4. Growth at 45°C. T.S.S. medium was used and tubes were incubated in a waterbath at 45°C ± 0.1°C.

In experiment 15, 10 colonies per plate were picked in each examination and fewer tests were carried out. These were as follows:-

1. Morphology in T.S.S. medium (Nigrosin films)

2. Catalase test. The method was similar to that used previously but the G.Y.B. cultures were neutralized before addition of the hydrogen peroxide.

3. CO_2 from glucose. In this case a quick test was used. If a red-hot loop is plunged into young cultures in T.S.S. medium, violent evolution of gas results if the organism is heterofermentative. This method suffers from the disadvantage that the cultures must be tested at the right stage if reliable results are to be obtained. In general, incubation for 2 days at 30°C was found to give best results.

In experiments 7 - 10, the isolates were divided into 7 groups on the basis of morphology, production of CO_2 from glucose, and growth at 45°C.

Lactobacilli/

Lactobacilli

1. LO-1 Homofermentative, 45-
2. LO-2 " 45+
3. LE-1 Heterofermentative 45-
4. LE-2 " 45+

Streptococci

5. SO Homofermentative
6. SE Heterofermentative

Tetrad-forming cocci

7. P Homofermentative. The tetracocci isolated were of two types, (a) fairly large cells, catalase-positive, and (b) small cells, apparently catalase-negative.

Both types produced relatively large colonies on acetate agar and all of those tested grew at 15 and 45°C, and produced at most only slight acidity in litmus milk. Type (b) probably belongs to the genus Pediococcus (Pederson, 1949) and, in view of the recent work by Felton, Evans & Niven (1953) on the production of catalase by pediococci, it is considered likely that type (a) also belongs to this group.

In experiment 15 the same system was used but division on the basis of growth at 45°C could not be done.

In these screening tests it was occasionally difficult to differentiate pediococci from homofermentative streptococci, and heterofermentative streptococci from heterofermentative lactobacilli.

Representative/

Representative cultures of lactobacilli were retained for full study and the results of this are discussed in part III.

In all experiments, examinations were made of the fresh material, and of the silage after incubation for 1, 2, 3 and 8 days (7 days in experiments 9 and 10). These examinations were expected to cover the period of active fermentation. A final examination was made after approximately 6 months incubation which gave silage of about the same age as that which would normally be used on the farm.

In order that bacterial counts could be compared in the different treatments, they have been calculated on a dry matter basis. Acetate agar counts are given in tables 33 - 40 in the Appendix, and 'total' counts on glucose yeast autolysate agar (G.Y.A.) are included for comparison. G.Y.A. has the same composition as G.Y.B. (Appendix, p.118) but contains 1.5g. agar/100 ml. These counts were done by Dr. A. C. Stirling.

The data for lactic and butyric acids were provided by Dr. L. A. Mabbitt. Volatile acids were determined by the Wiegner method and lactic acid by the method of Barnett (1951).

The pH values of the macerates were determined electrometrically and are given in tables 41 - 44 in the Appendix.

R E S U L T S.

Results of experiments 7 - 10 and 15.

Acetate agar counts of fresh grass.

The preliminary observation that lactobacilli could be very scarce on fresh grass was confirmed. In experiments 7 and 8, none were detected while in experiments 9 and 15 the acetate agar counts were 1/ml. macerate. Thus in four of five samples of grass examined, the theoretical acetate agar count was 30/g. dry matter or less. In experiment 10, however, the lactobacillus count of the fresh grass was approximately 5,000/g. dry matter but even here, comparison with the G.Y.A. count shows that they were outnumbered by other organisms in the approximate ratio of 7,000 : 1.

The occurrence of relatively large numbers of these organisms on one sample, and their comparative scarcity on the others, has given much useful information on their value in the ensilage process. On the other hand, their frequent scarcity on the fresh grass has resulted in considerable tube to tube variation with respect to the species developing, and it has therefore been difficult to detect any regular sequence in the development of the several types.

In experiment 8, lactobacilli were abnormally scarce throughout the whole fermentation and the dilutions examined were frequently too high. Many blanks therefore occur in the data for this experiment.

The effect of various treatments on counts on acetate agar.

THE EFFECT OF TEMPERATURE.

FIG. 1

EFFECT OF TEMPERATURE

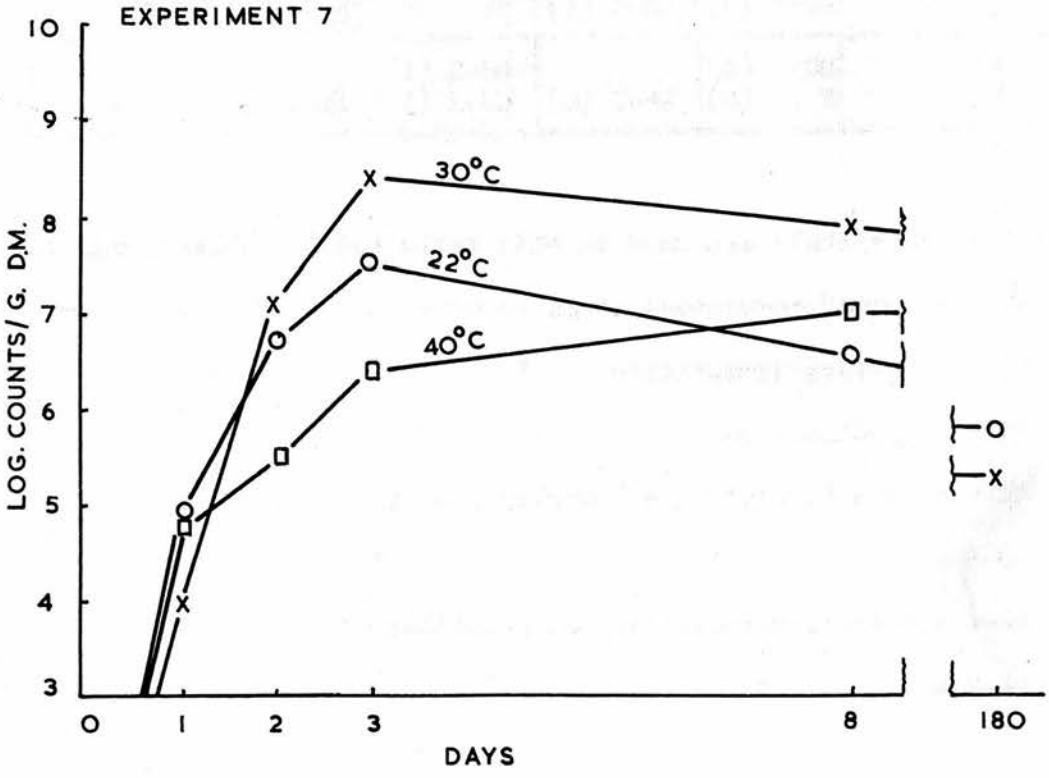


Table 6.

Types and numbers of organisms isolated from acetate agar plates.

Experiment 7.

Treatment	Fresh Grass	Incubation period				
		1 day	2 days	3 days	8 days	6 months
22°C		SE (4)	SE (4)	SE (1) LO-1 (3)	SE (3)	SO? (2)
30°C	-	LO-1 (2)	SE (3) LO-1 (1)	SE (4)	SE (5)	SO? (2)
40°C		SO (2) P (2)	LE-2 (4)	LO-2 (1) LE-2 (3)	LE-2 (5)	-

The following symbols are used in this table and in tables 7 and 8.

SO = homofermentative streptococcus

SE = heterofermentative "

P = pediococcus

LO-1 = homofermentative lactobacillus 45-

LO-2 = " " 45+

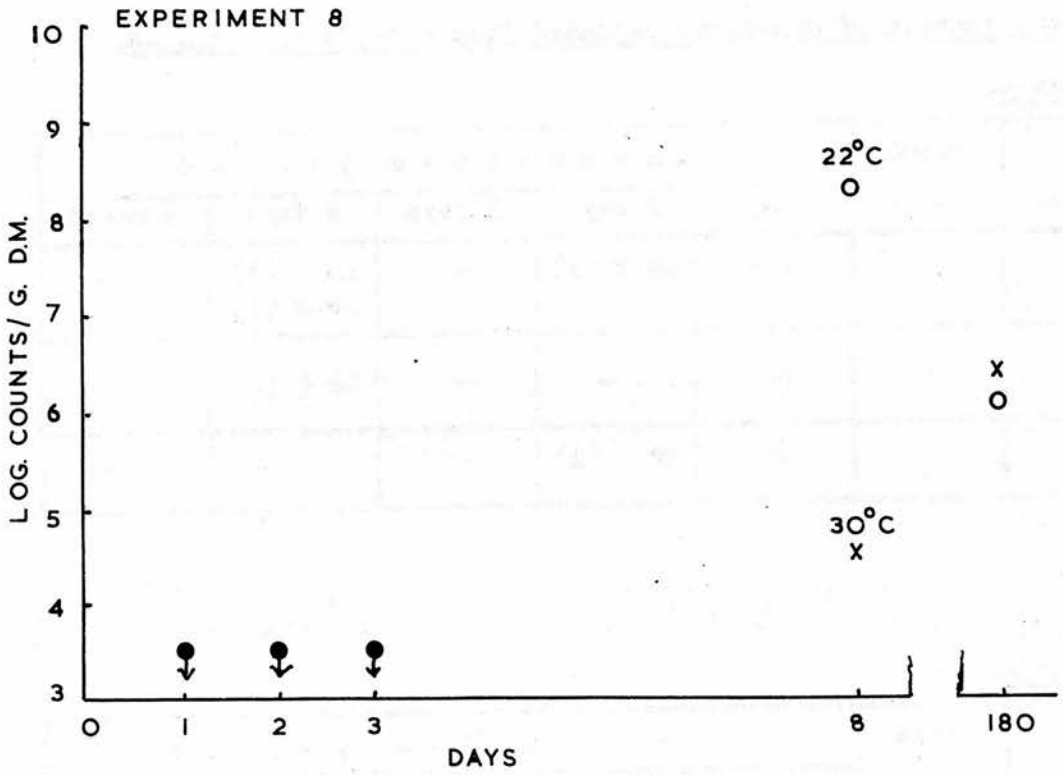
LE-1 = heterofermentative lactobacillus 45-

LE-2 = " " 45+

Note: Homofermentative streptococci (SO) were recovered from tiny colonies and were not included in counts.

FIG. 2

EFFECT OF TEMPERATURE



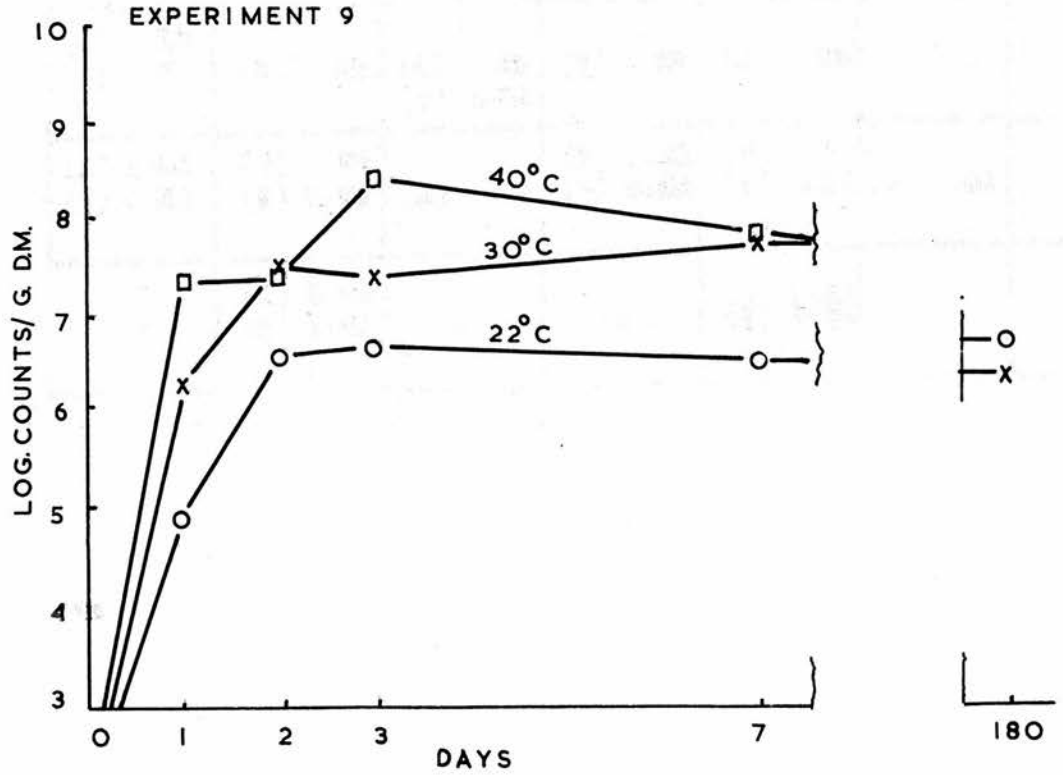


Table 7.

Types and numbers of organisms isolated from acetate agar plates.

Experiment 8.

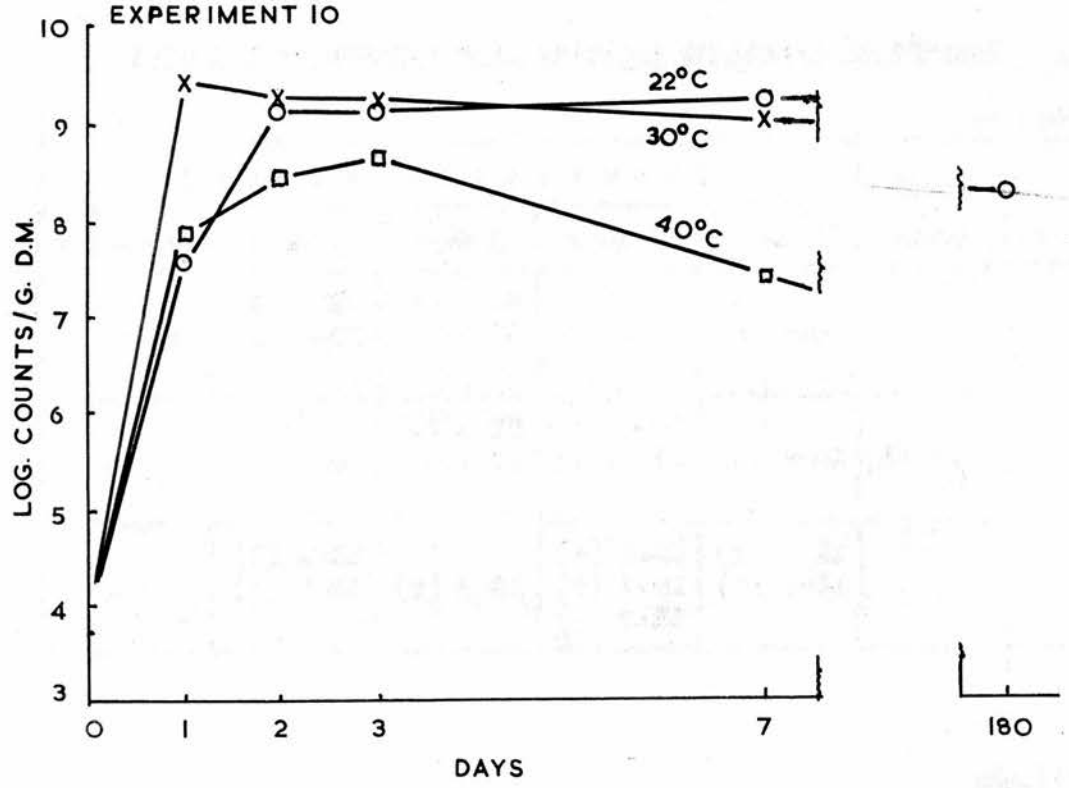
Treatment	Fresh Grass	Incubation period				
		1 day	2 days	3 days	8 days	6 months
22°C		-	LE-2 (3)	-	LO-1 (3) LE-2 (1)	-
30°C	-	-	-	-	LO-1 (4)	-
40°C		-	SO (2)	-	-	-

Experiment 9.

Treatment	Fresh Grass	Incubation period				
		1 day	2 days	3 days	7 days	6 months
22°C		SE (4)	SE (3)	SE (3) LO-1 (1)	SE (2)	SE (3) P (1)
30°C	LO-1 (1)	P (2) LO-1 (2)	LO-2 (2) LE-2 (1)	SE (2)	SO (1) LE-2 (2)	LO-2 (2) LE-2 (2)
40°C		LO-2 (1) LE-2 (3)	-	-	LO-2 (2) LE-2 (2)	-

FIG. 3

EFFECT OF TEMPERATURE



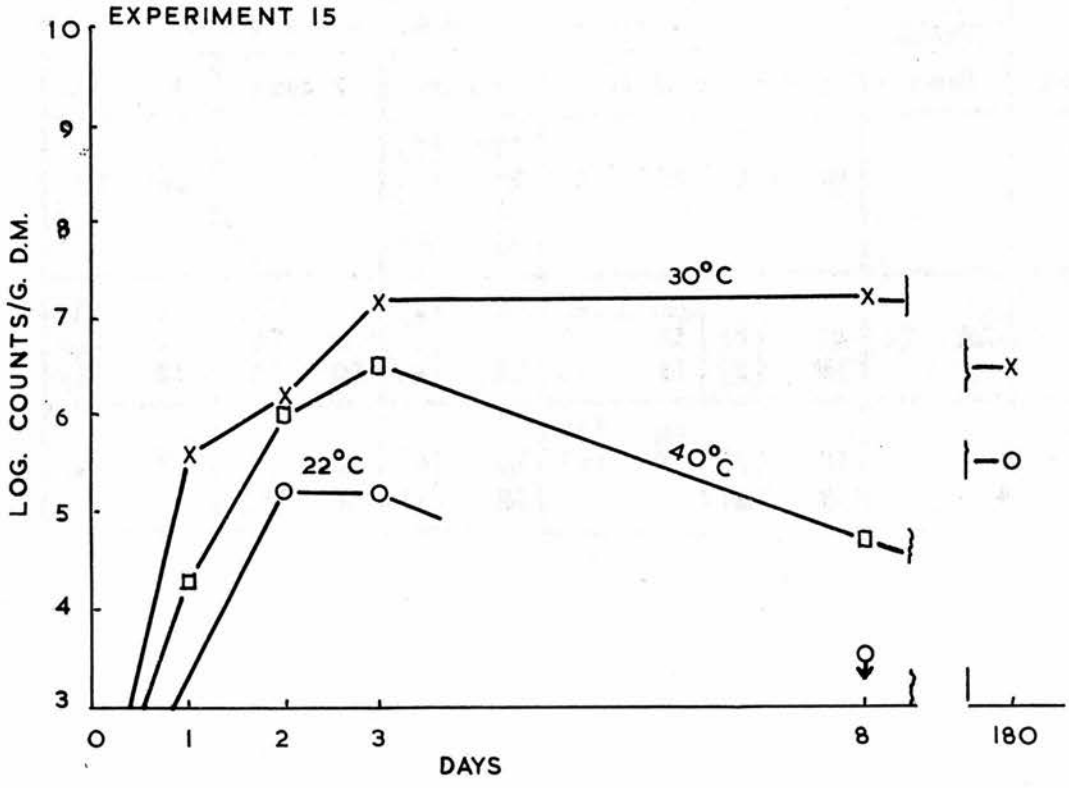


Table 8.

Types and numbers of organisms isolated from acetate agar plates.

Experiment 10.

Treatment	Fresh Grass	Incubation period				
		1 day	2 days	3 days	7 days	6 months
22°C		LO-1 (4)	LO-1 (3)	SE (1) LO-1 (3)	SE (2) LO-1 (2)	LO-1 (2)
30°C	LO-1 (4)	LO-1 (4)	LO-1 (2) LE-1? (2)	SE (1) LO-1 (3)	LO-1 (4)	-
40°C		SE (1) LO-1 (1)	LO-1 (1) LO-2 (1) LE-2 (1)	LE-2 (2)	LO-2 (1) LE-2 (1)	-

Experiment 15.

Treatment	Fresh Grass	Incubation period				
		1 day	2 days	3 days	8 days	6 months
22°C		SE (10)	SE? (10)	SE? (7) SE (1) LE (1) LO (1)	-	LO (10)
30°C	LE (1)	SE (8) LE (2)	SE (4) LO (3) LF (1)	SE (2) LO (1) LE (6)	P (5) LO (5)	P (5) LO (4) LE (1)
40°C		LO (7) LE (2)	LE (8) P (1)	LO (8) LE (2)	LO (9) LE (1)	P (2)

The effect of various treatments on counts on acetate agar.

The effect of temperature
(Treatments A, B, and C)

With the exception of experiment 9, the highest counts were obtained at 30°C. The reason for this exception is probably that the majority of lactobacilli isolated in experiment 9 were capable of growth at 45°C and could therefore compete more effectively with other bacteria at 40°C than at 30°C.

In experiment 15, where lactobacilli were scarce on the fresh grass, a fairly clear succession of types is seen in the control series (30°C). Even although no heterofermentative streptococci were isolated from the fresh material, they were the dominant organisms on the acetate agar plates at one day when the count was 380,000/g. (on a dry matter basis). They were gradually replaced by lactobacilli until at 3 days they were outnumbered in a ratio of 7 : 2. At 8 days they had disappeared and the count was made up of equal proportions of lactobacilli and pediococci. These two groups were still present in the same proportions at 6 months. There was haphazard occurrence of homofermentative and heterofermentative lactobacilli probably because of tube to tube variation.

Where lactobacilli were initially present in appreciable numbers (Experiment 10), they dominated the lactic acid flora throughout at 30°C, but appeared to be gradually ousted by heterofermentative streptococci at 22°C, up to 8 days.

In general, a temperature of 22°C seemed to favour heterofermentative streptococci at the expense of lactobacilli. At

30°/

30°C, lactobacilli usually formed a much greater proportion of the lactic acid flora than heterofermentative streptococci, while at 40°C, practically only lactobacilli were encountered.

The pH values obtained at the different temperatures seem to agree with these results. The lowest values always occurred with incubation at 40°C and the highest at 22°C. However, isolations from G.Y.A. plates indicated that at 40°C, homofermentative streptococci capable of growth at 45°C were frequently numerous. These organisms possibly have an influence on the fermentation at 40°C, at least in the early stages.

THE EFFECT OF LACERATION.

Table 9.

Types and numbers of organisms isolated from acetate agar plates.

Experiment 7.

Treatment	Fresh Grass	Incubation period				
		1 day	2 days	3 days	8 days	6 months
Unchopped		-	SE (4)	-	-	-
Control (1" lengths)	-	LO-1 (2)	SE (3) LO-1 (1)	SE (4)	SE (5)	SO? (2)
Minced		SE (4)	SE (3) LO-1[x] (4)	SE (2) LO-1[x] (3)	LE-2 (3)	LE-2 (3)

[x] Isolated from low dilution plate.

The following symbols are used in this table and in tables 10 and 11.

SO = homofermentative streptococcus

SE = heterofermentative "

P = pediococcus

LO-1 = homofermentative lactobacillus 45-

LO-2 = " " 45+

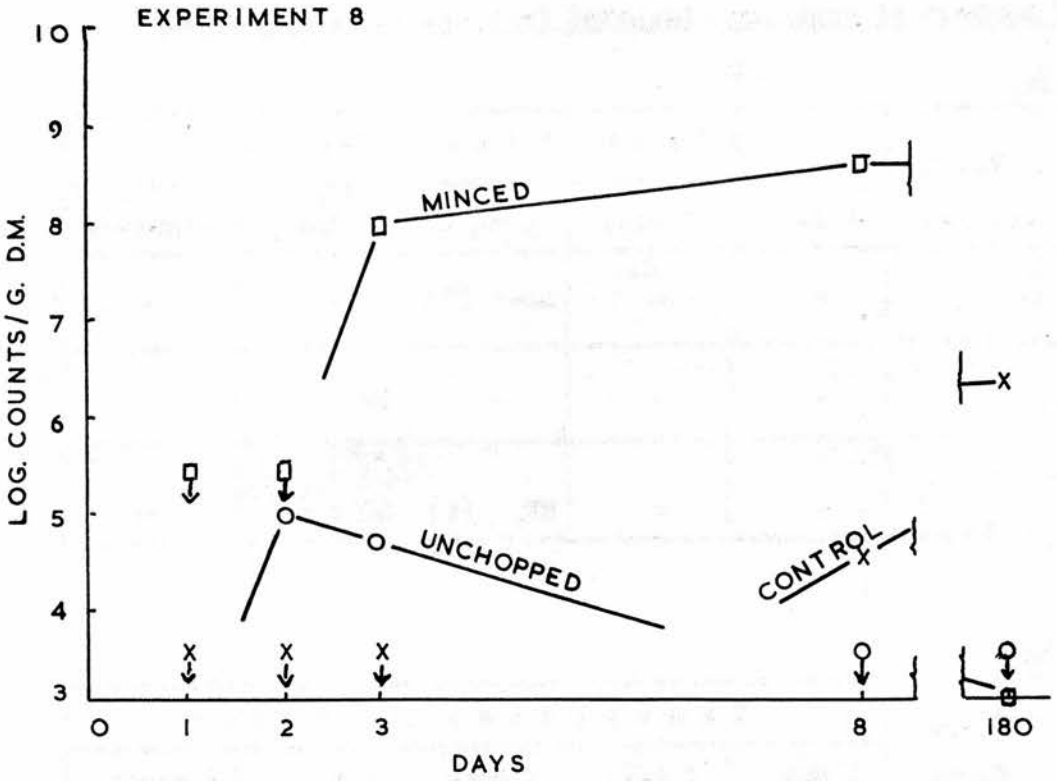
LE-1 = heterofermentative lactobacillus 45-

LE-2 = " " 45+

Note: Homofermentative streptococci (SO) were recovered from tiny colonies and were not included in counts.

FIG. 5

EFFECT OF LACERATION



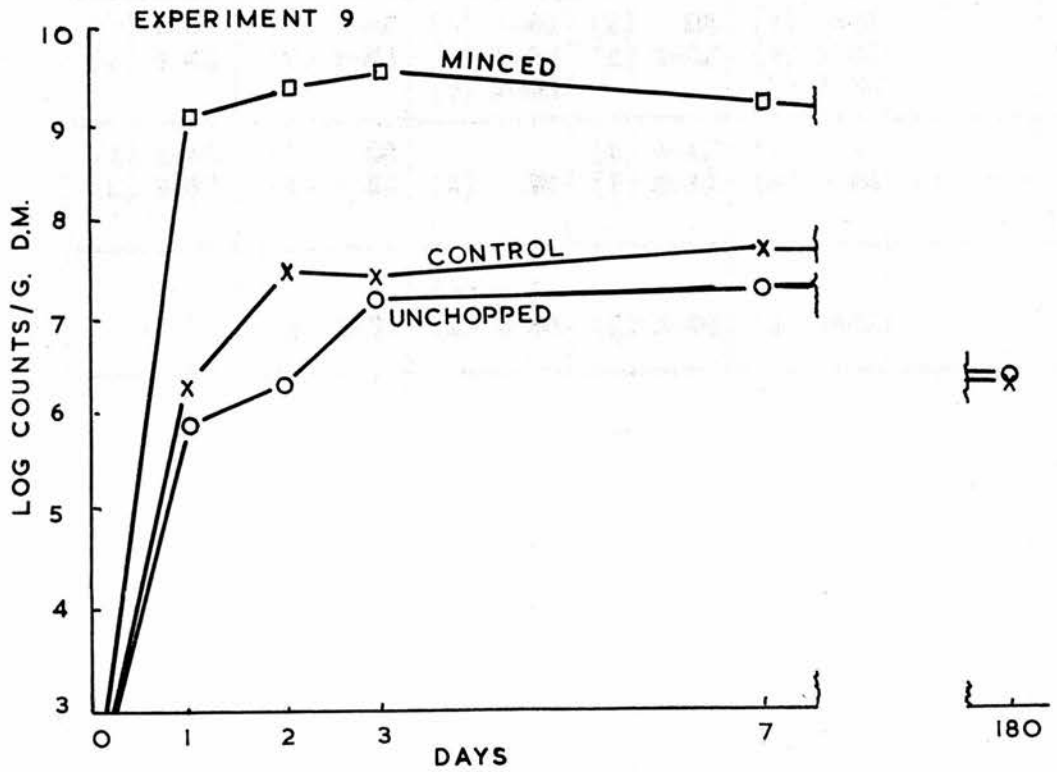


Table 10.

Types and numbers of organisms isolated from acetate agar plates.

Experiment 8.

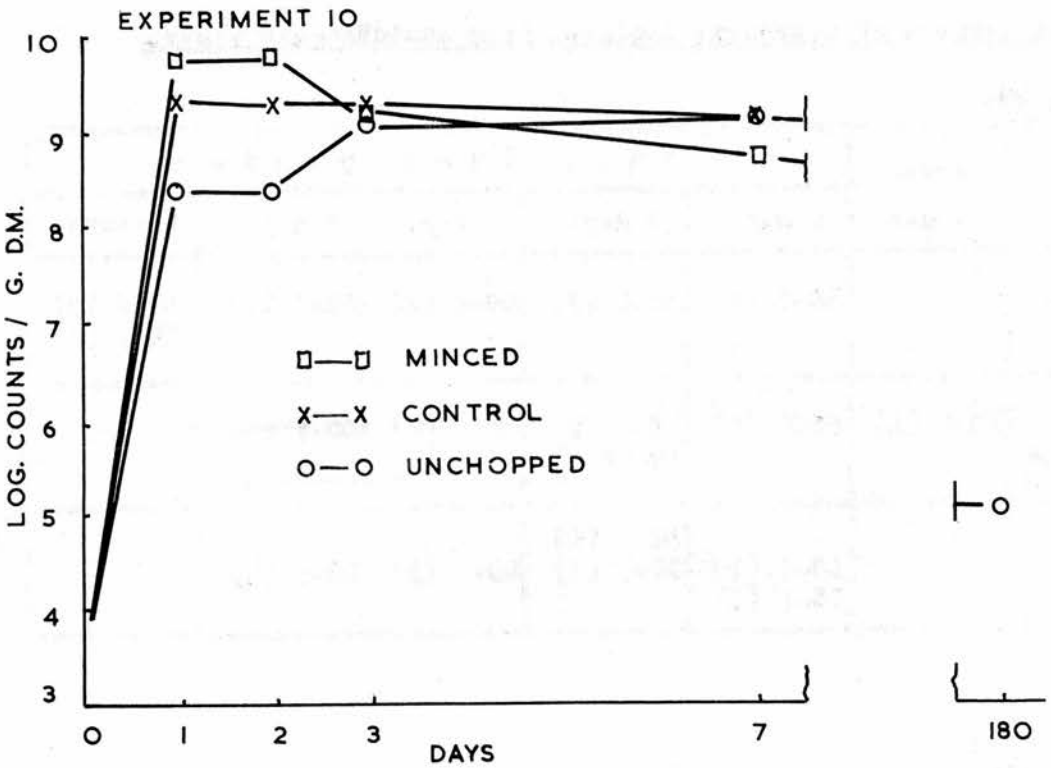
Treatment	Fresh Grass	Incubation period				
		1 day	2 days	3 days	8 days	6 months
Unchopped		-	-	LO-1 (3)	-	-
Control (1" lengths)	-	-	-	-	LO-1 (4)	-
Minced		-	-	SE (4)	LO-2 (3) LE-2 (1)	-

Experiment 9.

Treatment	Fresh Grass	Incubation period				
		1 day	2 days	3 days	7 days	6 months
Unchopped		LO-1 (1) LO-2 (1) LE-2 (2)	SE (2) LO-2 (2)	LO-1 (1) LO-2 (1) LE-2 (1)	LO-1 (1) LE-2 (2)	LO-2 (1) LE-2 (3)
Control (1" lengths)	LO-1 (1)	P (2) LO-1 (2)	LO-2 (2) LE-2 (1)	SE (2)	SO (1) LE-2 (2)	LO-2 (2) LE-2 (2)
Minced		LE-2 (4)	LE-2 (3)	LO-2 (1) LE-2 (2)	LE-2 (2)	-

FIG. 6

EFFECT OF LACERATION



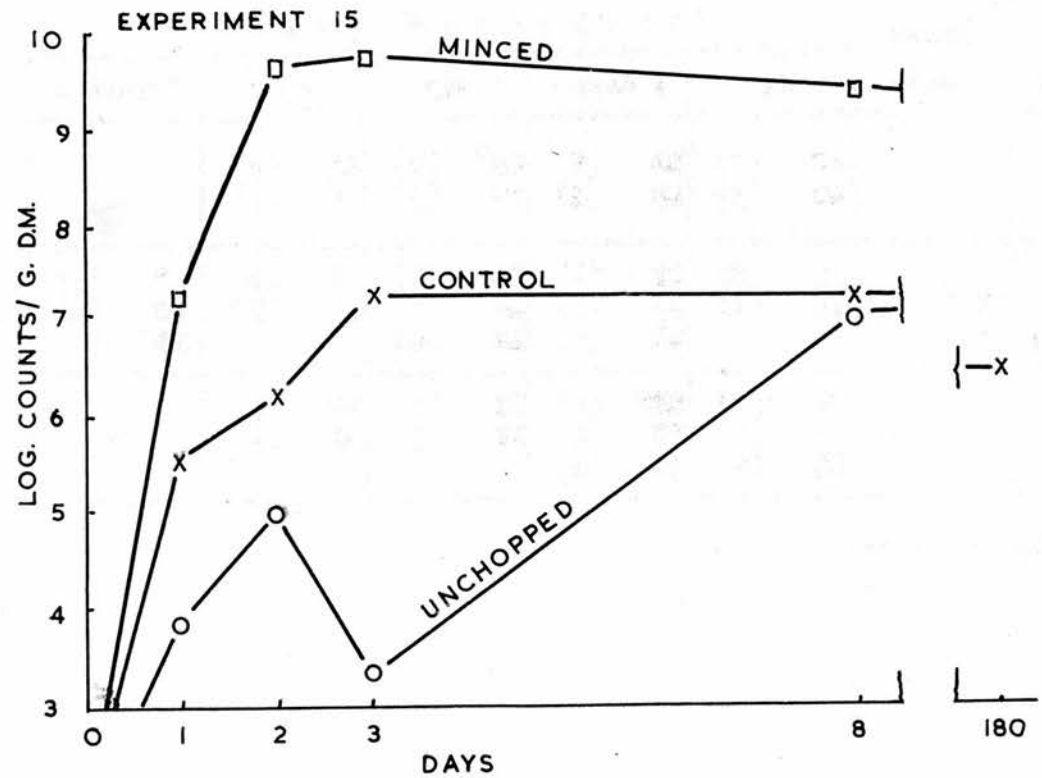


Table 11.

Types and numbers of organisms isolated from acetate agar plates.

Experiment 10.

Treatment	Fresh Grass	Incubation period				
		1 day	2 days	3 days	7 days	6 months
Unchopped		LO-1 (4)	LO-1 (2)	LO-1 (4)	LO-1 (2)	LO-2 (2) LE-2 (2)
Control (1" lengths)	LO-1 (4)	LO-1 (4)	LO-1 (2) LE-1? (2)	SE (1) LO-1 (3)	LO-1 (4)	-
Minced		LO-1 (2) LE-1 (2)	SE (1) LO-1 (3)	LO-1 (5)	LO-1 (4)	

Experiment 15.

Treatment	Fresh Grass	Incubation period				
		1 day	2 days	3 days	8 days	6 months
Unchopped		SE (7) LO (3)	SE (8) LE (2)	SO[x] (7) LO (3)	SE (9) P (1)	P (2)
Control (1" lengths)	LE-1 (1)	SE (8) LE (2)	SE (4) LO (3) LE (1)	SE (2) LO (5) LE (6)	P (5) LO (5)	P (5) LO (4) LE (1)
Minced		SE (1) LO (7) LE (2)	SE? (1) LO (6) LE (2)	LO (7) LE (3)	LO (6) LE (4)	-

[x] from tiny colonies.

The effect of laceration
(Treatments D, B and E)

Extreme laceration had the same pronounced effect in every case. Acetate agar counts were always considerably higher in the minced material, than in the control series. Unchopped material always gave slightly lower counts than in the control.

Mincing appeared to have a stimulatory effect on all microbial growth (anaerobes excepted) since these effects were also apparent in the G.Y.A. counts.

The stimulation of growth was least pronounced in experiment 10 where lactobacilli were fairly numerous on the fresh grass. Lactobacilli usually dominated the flora on acetate agar plates from minced material. It is possible that mincing, by release of nutrients and provision of a more suitable physical medium results in a general speed-up of microbial succession. However, the results indicate that it also tends to favour lactobacilli at the expense of other organisms. Better distribution of the lactobacilli in the minced material may have a contributory effect.

The pH values obtained with the different treatments are in agreement with these results. Minced material always had a very much lower pH than the control or unchopped material.

THE EFFECT OF MOISTURE

FIG. 7

EFFECT OF MOISTURE

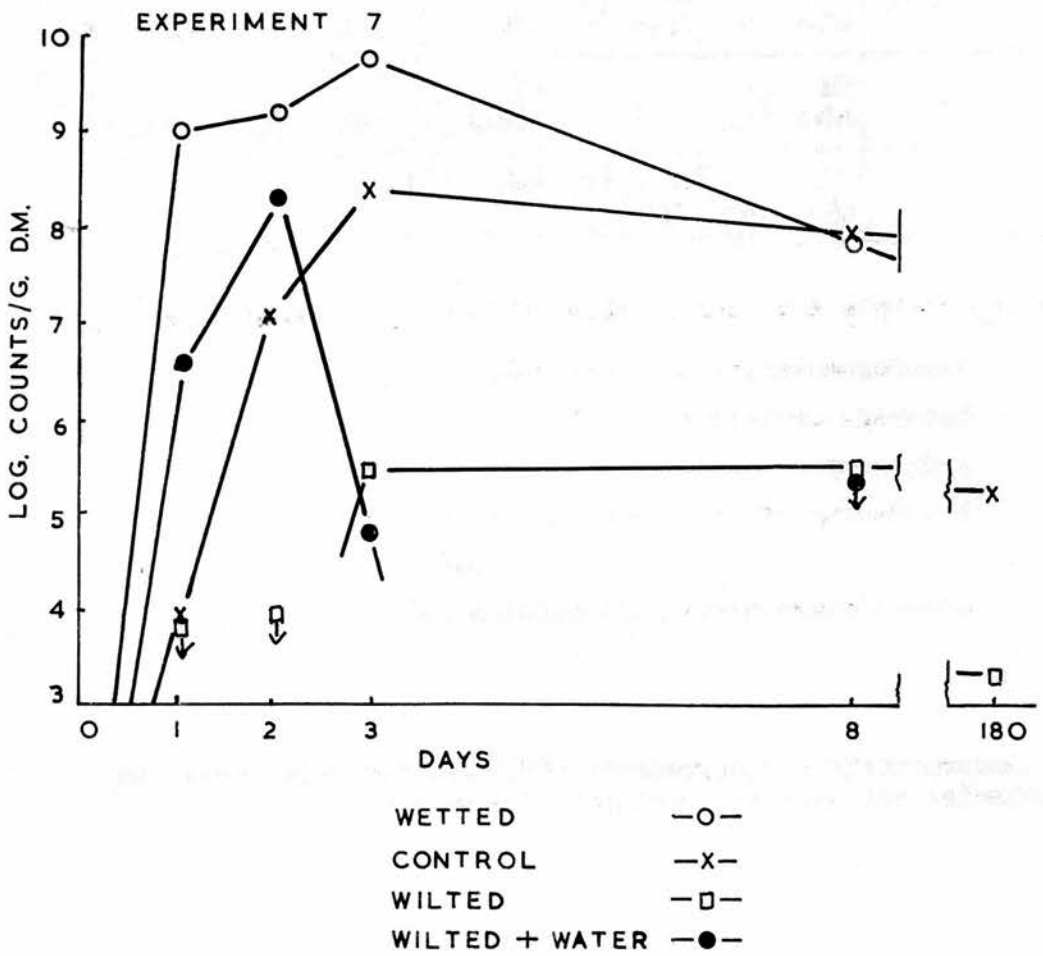


Table 12.

Types and numbers of organisms isolated from acetate agar plates.

Experiment 7.

Treatment	Grass	Incubation period				
		1 day	2 days	3 days	8 days	6 months
Wetted		SE (3) LO-1 (1)	SE (4)	-	SE (4)	-
Control	-	LO-1 (2)	SE (3) LO-1 (1)	SE (4)	SE (5)	SO? (2)
Wilted	-	SE (4) LO-1 (1)	-	P? (2) LE-2 (1)	SE (4)	LO-2 (2)
Wilted + water		SE (5)	SE (2) LO-1 (2)	SE (2)	-	-

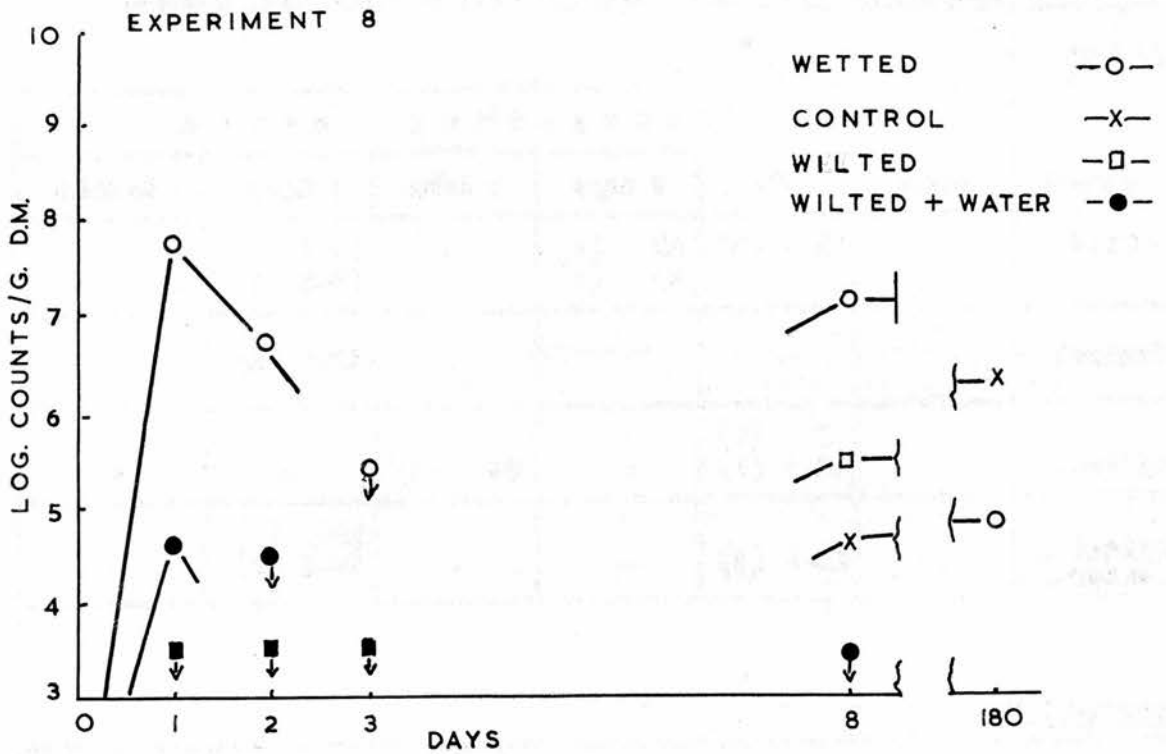
The following symbols are used in this table and in tables 13 and 14.

SO = homofermentative streptococcus
SE = heterofermentative "
P = pediococcus
LO-1 = homofermentative lactobacillus 45-
LO-2 = " " 45+
LE-1 = heterofermentative lactobacillus 45-
LE-2 = " " 45+

Note: Homofermentative streptococci (SO) were recovered from tiny colonies and were not included in counts.

FIG. 8

EFFECT OF MOISTURE



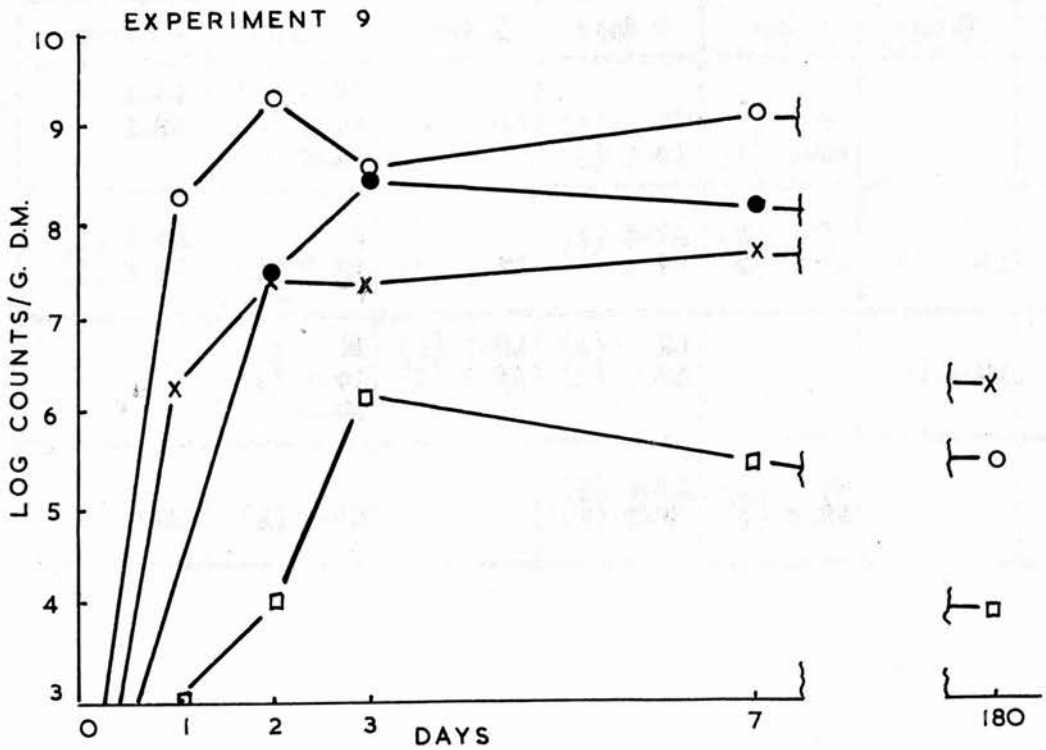


Table 13.

Types and numbers of organisms isolated from acetate agar plates.

Experiment 8.

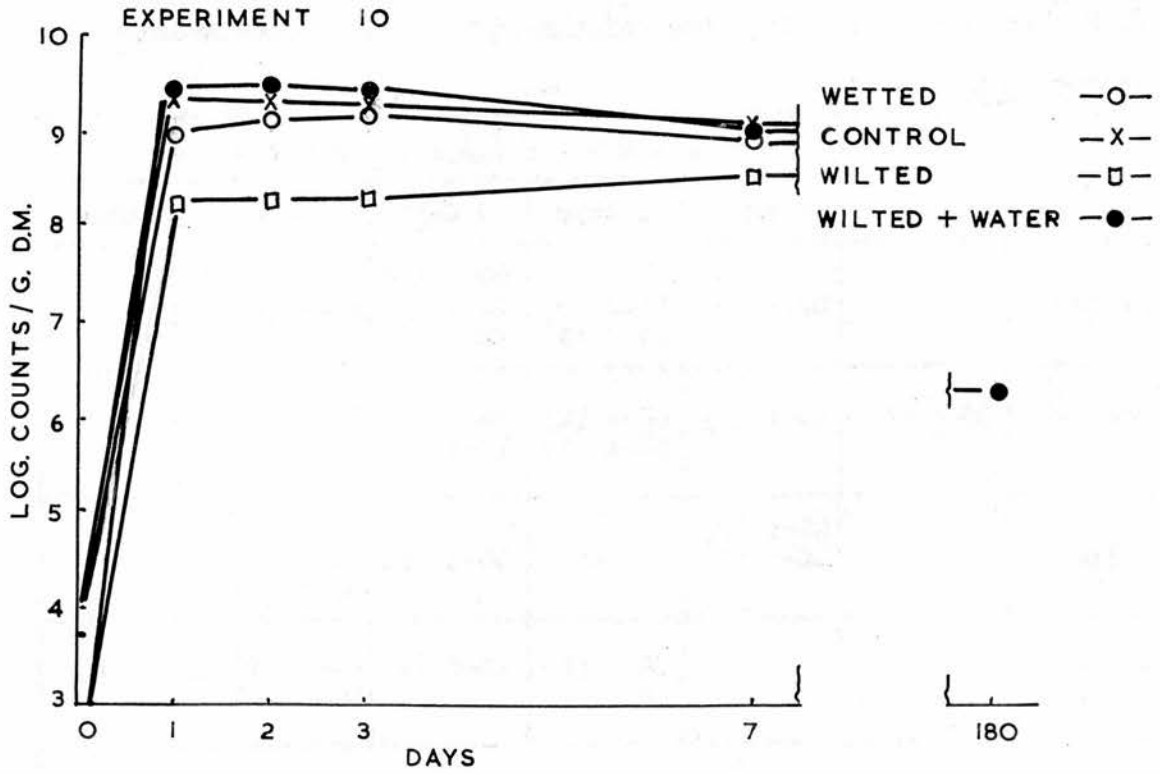
Treatment	Grass	Incubation period				
		1 day	2 days	3 days	8 days	6 months
Wetted		SE (4)	SE (1) SO (1)	-	LO-1 (4) LO-2 (3)	-
Control	-	-	-	-	LO-1 (4)	-
Wilted	-	P (1) LO-1 (1)	-	SO (2)	-	-
Wilted + water		LO-1 (3)	-	-	LO-1 (1) LO-2 (2)	-

Experiment 9.

Treatment	Grass	Incubation period				
		1 day	2 days	3 days	7 days	6 months
Wetted		P (1) LO-1 (3)	SE (1) LO-1 (3)	LO-1 (2)	LO-1 (1) LE-1? (1) LE-2 (1)	LO-2 (1) LE-2 (3)
Control	LO-1 (1)	P (2) LO-1 (2)	LO-2 (2) LE-2 (1)	SE (2)	SO (1) LE-2 (2)	LO-2 (2) LE-2 (2)
Wilted	LE-2 (1)	-	SE (2) LO-1 (2)	LO-1 (1) LE-2 (3)	SO (1) LO-1 (2) LE-2 (1)	-
Wilted + water		SE (1) LE-2 (3)	LO-1 (3) LE-2 (1)	-	LO-1 (4)	LO-1 (4)

FIG. 9

EFFECT OF MOISTURE



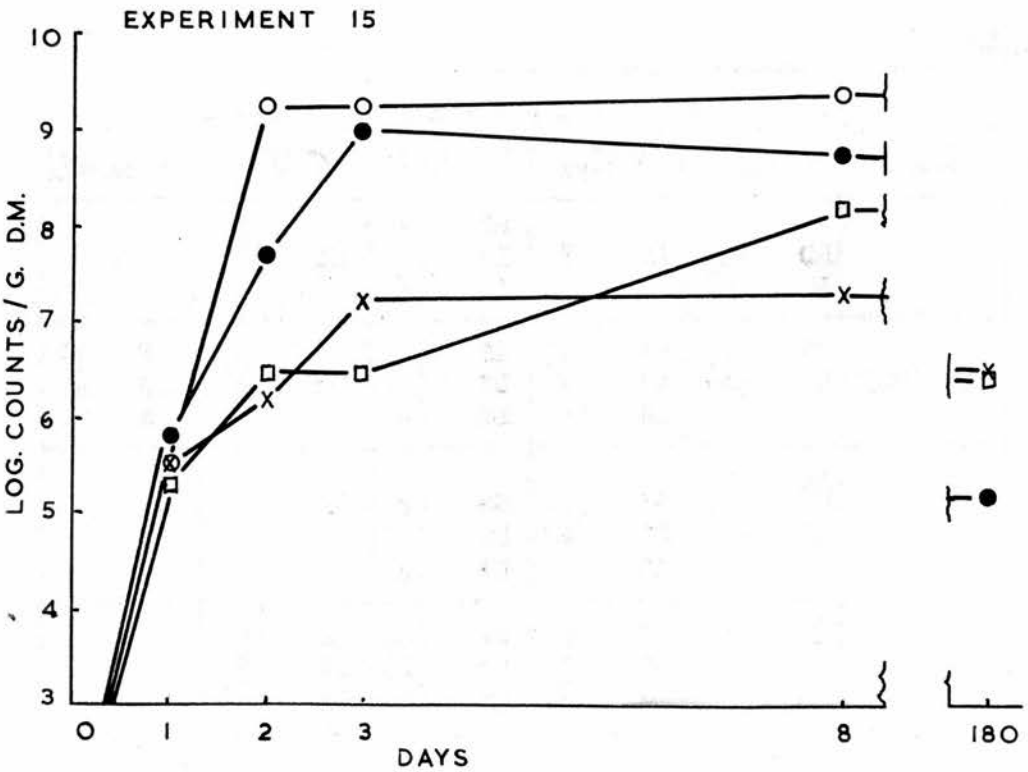


Table 14.

Types and numbers of organisms isolated from acetate agar plates.

Experiment 10.

Treatment	Grass	Incubation period				
		1 day	2 days	3 days	7 days	6 months
Wetted		LO-1 (3)	LO-1 (2) LE-2 (2)	SO (1) LO-1 (2) LE-2 (1)	LO-1 (2)	-
Control	LO-1 (4)	LO-1 (4)	LO-1 (2) LE-1 (2)	SE (1) LO-1 (3)	LO-1 (4)	-
Wilted	-	LO-1 (3) LE-1 (1)	-	LO-1 (2)	SE (1) LO-1 (1)	-
Wilted + water		-	SE (1) LO-1 (3)	LE-1 (2)	LO-1 (3) LO-2 (1)	LO-1 (4)

Experiment 15.

Treatment	Grass	Incubation period				
		1 day	2 days	3 days	8 days	6 months
Wetted		LO (9) LE (1)	LE (8)	SE (3) LO (1) LE (6)	LO (3) LE (7)	-
Control	LE (1)	SE (8) LE (2)	SE (4) LO (3) LE (1)	SE (2) LO (1) LE (6)	P (5) LO (5)	P (5) LO (4) LE (1)
Wilted	-	SO (2) SE (2) LO (2) LE (4)	SE (1) LO (4) LE (5)	SE (2) LO (1) LE (6)	LE (10)	-
Wilted + water		LO (2) LE (6)	P (1) LO (3) LE (6)	SE (4) LO (4) LE (2)	P (1) LO (3) LE (6)	-

The effect of moisture
(Treatments G, B, H and I)

Different degrees of moisture content resulted in marked differences in the acetate agar counts. Where sufficient water was added to the grass to make an appreciable difference in its moisture content, the counts were much higher than in the control series, with a resultant greater drop in pH. On the other hand, where wilting appreciably lowered the moisture content of the material, the counts were considerably lower than in the control and the pH values correspondingly higher.

That these effects can be mainly attributed to the moisture content is seen when treatment I (Wilted + water) is compared with the control series. Where successful readjustment of the moisture content was achieved, similar counts resulted. Where too much water was added the counts were higher. These effects are particularly obvious in experiment 9 where the material, being second-cut, had a high dry matter content.

These differences are not confined to the acetate agar counts but are also seen in the 'total' counts on G.Y.A.

The different treatments do not appear to have had any selective effect on any particular group.

THE EFFECT OF INOCULATION

FIG. 10

EFFECT OF INOCULATION

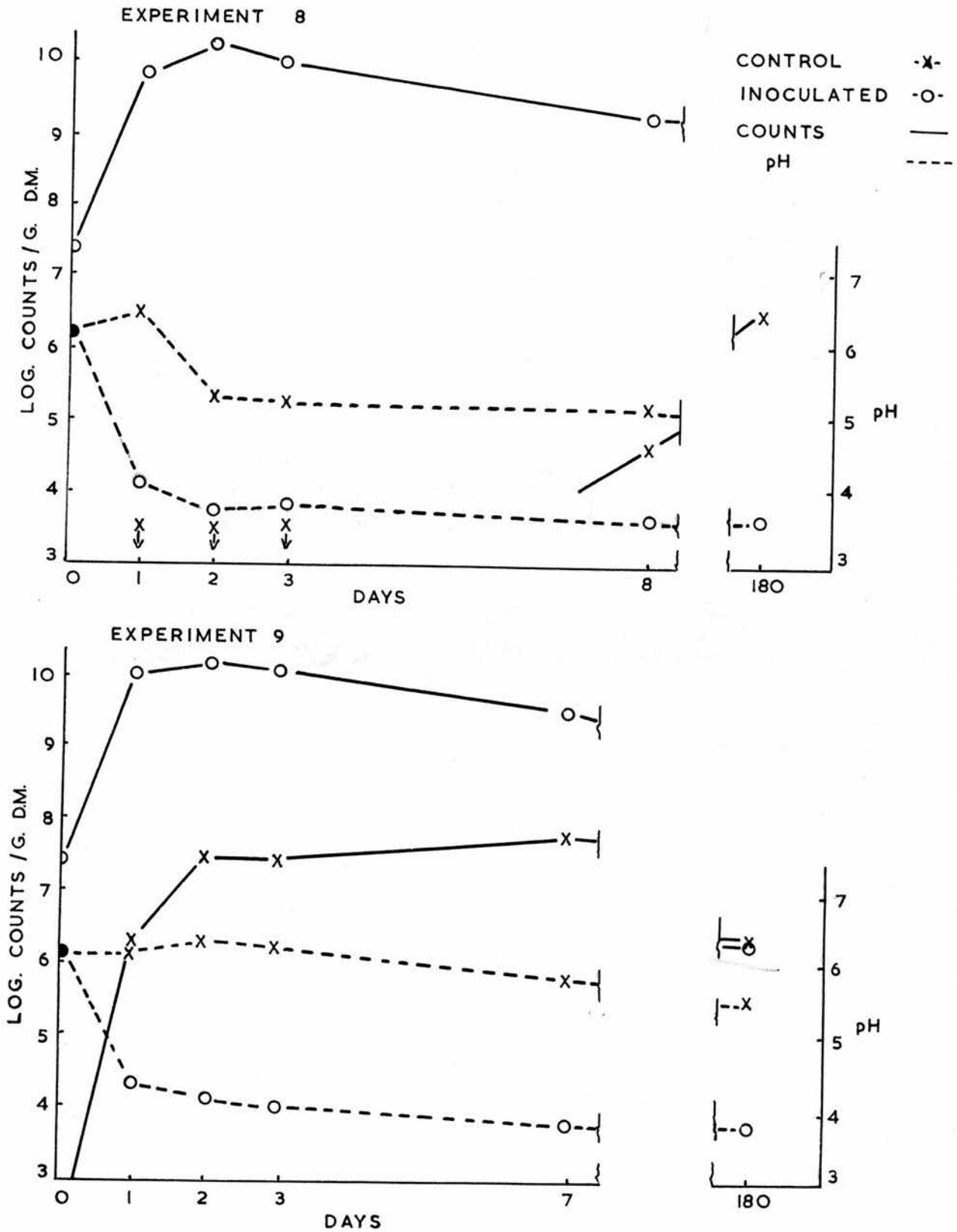
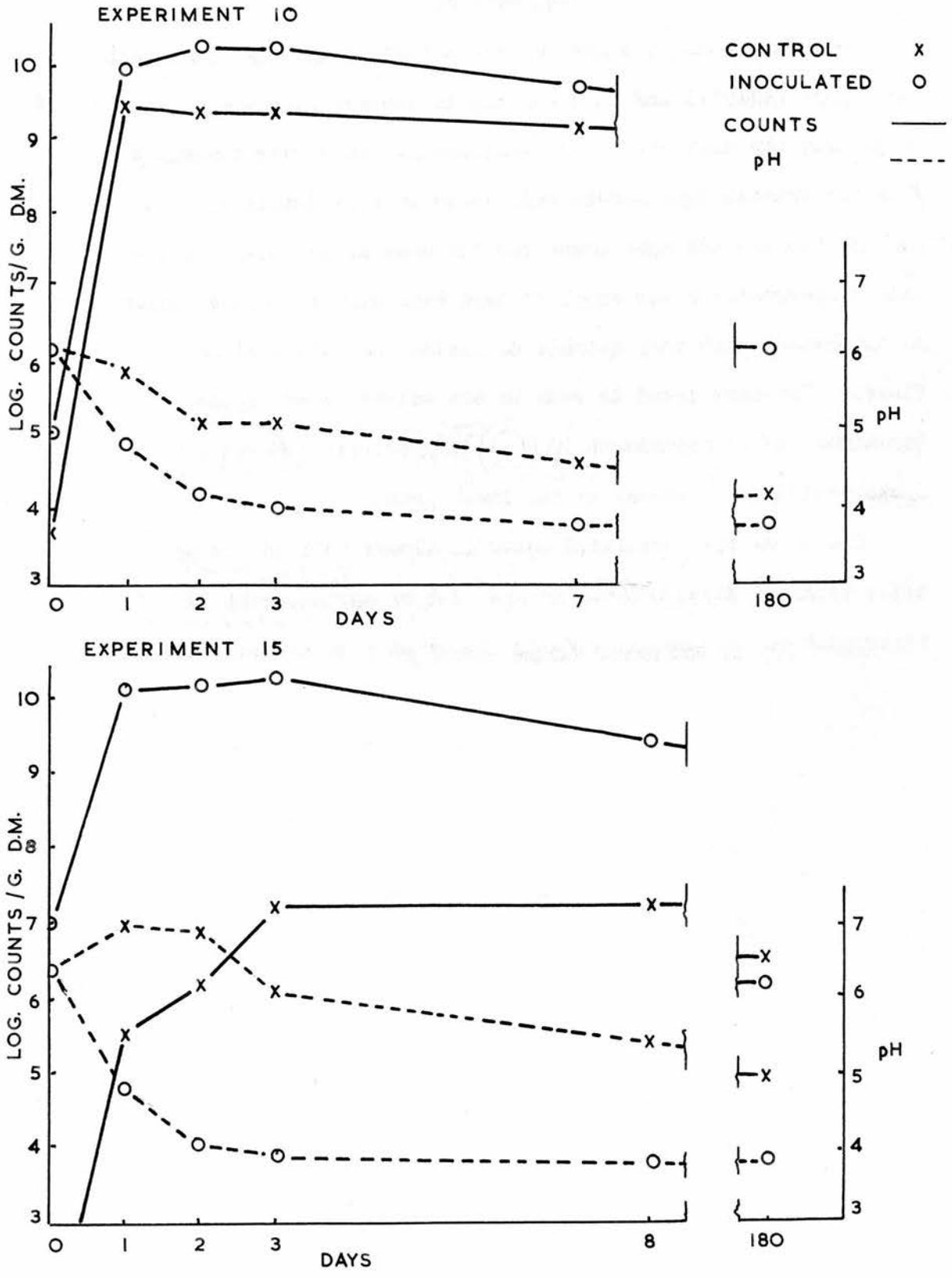


FIG. 11

EFFECT OF INOCULATION



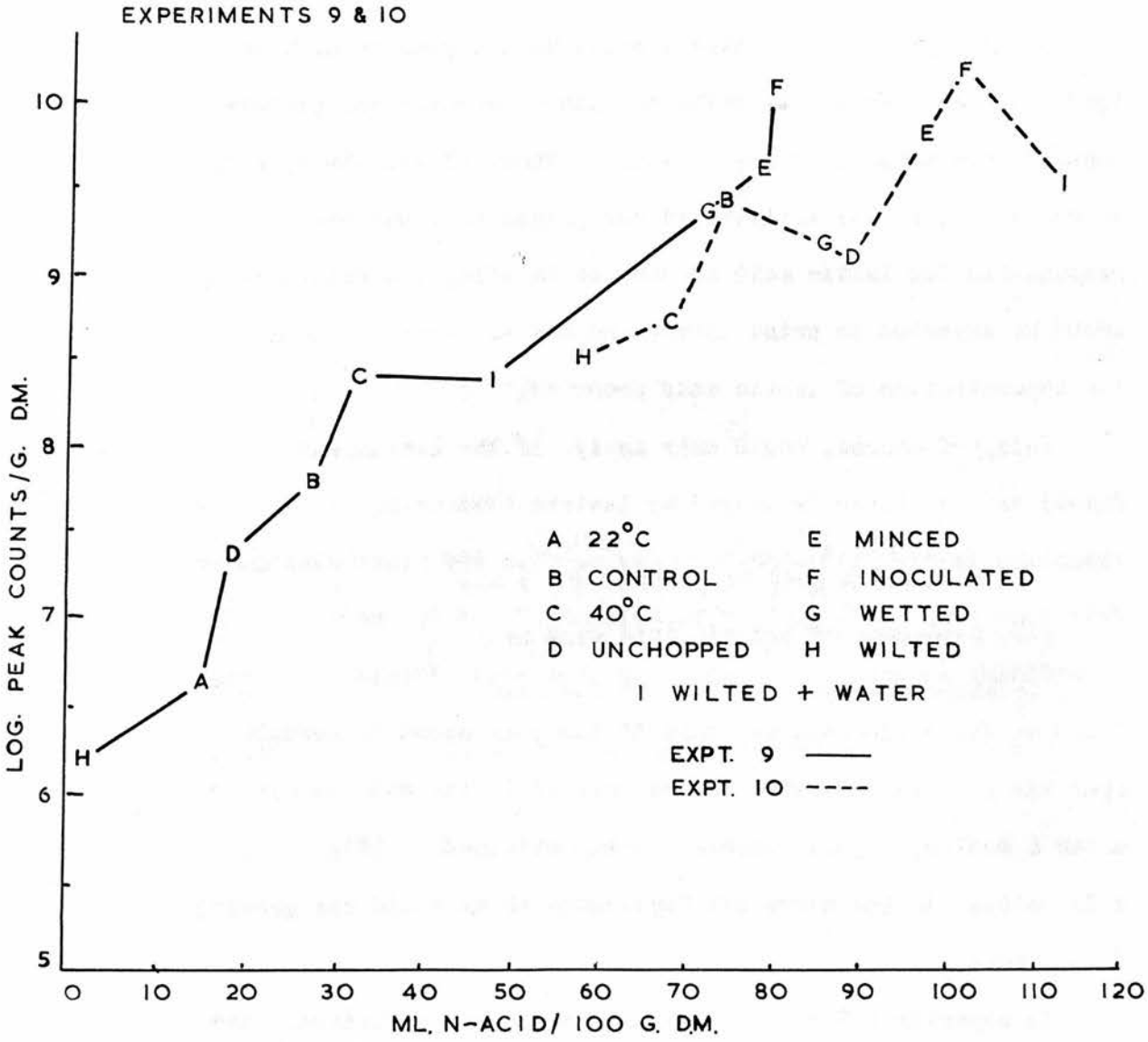
The effect of inoculation
(Treatment F)

Inoculation had the most marked effect of all the treatments. Inoculated material had always a higher acetate agar and G.Y.A. count than the control. Only lactobacilli were ever recovered from the acetate agar plates and, since in experiments 9, 10 and 15, the acetate agar count was the same as the G.Y.A. count, and in experiment 8 was equal to more than half the G.Y.A. count at one day, it is obvious that they quickly dominated the entire micro flora. The same trend is seen in the uninoculated series (treatment B) of experiment 10 where appreciable numbers of lactobacilli were present on the fresh grass.

The pH of the inoculated material always fell to 4.2 or below within 2 days, whereas in the control series, even in experiment 10, it had never fallen below pH 5 in the same time.

FIG. 12

CORRELATION OF PEAK COUNTS ON ACETATE AGAR WITH LACTIC ACID CONTENTS AT 6 MONTHS



Relationship between lactic acid production and counts on acetate agar.

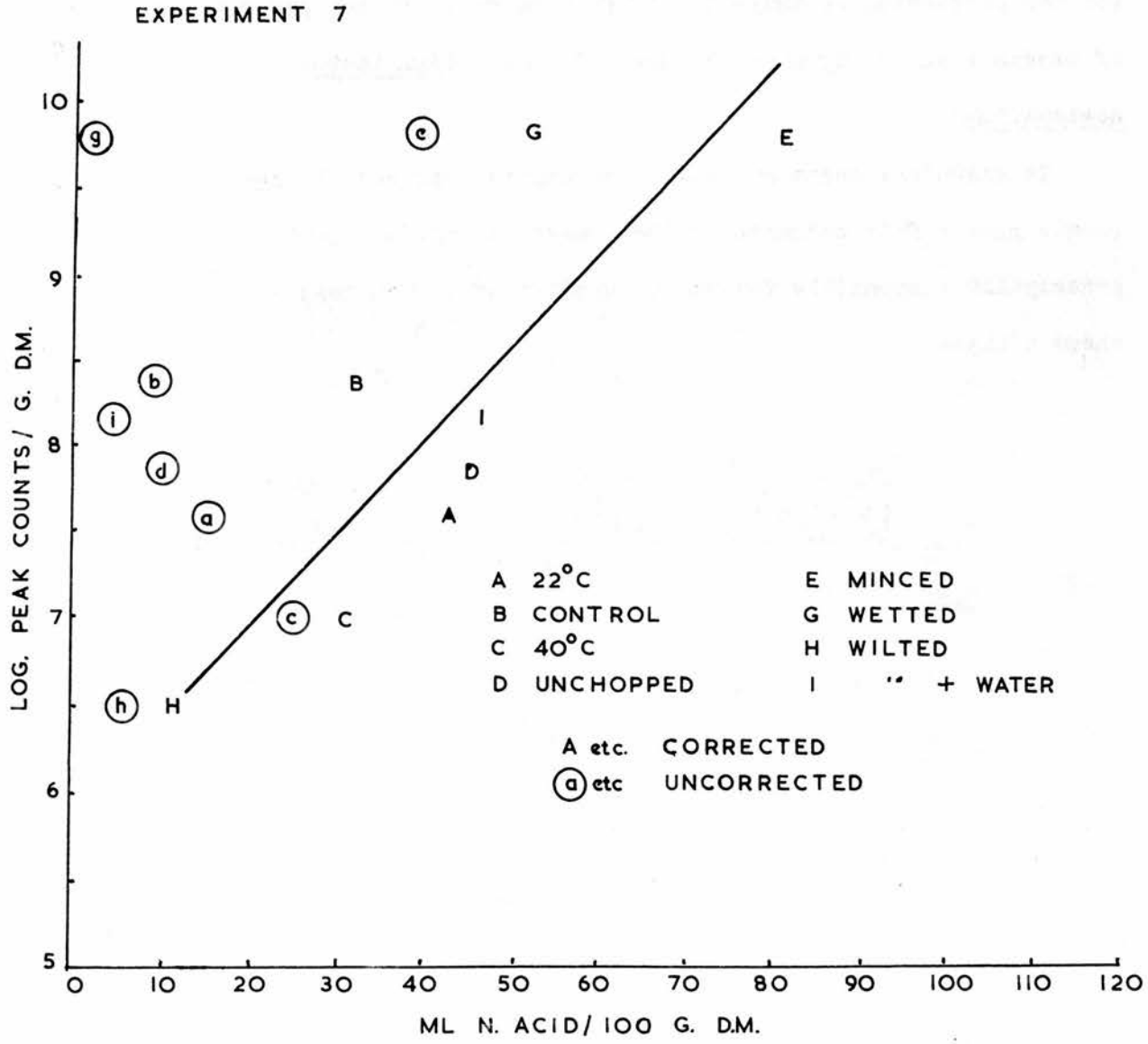
The possibility arises that some group of bacteria capable of producing a significant proportion of the lactic acid found in these silages, is not being detected on acetate agar. For example, homofermentative streptococci do not normally grow on acetate agar, and yet they might, under certain circumstances, produce considerable amounts of lactic acid. Thus, if acetate agar is indeed giving a true estimate of the principal organisms responsible for lactic acid production in silage, a relationship could be expected to exist between counts on acetate agar and the concentration of lactic acid produced.

This, of course, would only apply, if the lactic acid formed was not later destroyed by lactate-fermenting bacteria resulting in the production of butyric acid and other substances. This condition holds good in experiments 9 and 10 where significant amounts of butyric acid were never formed. It was found in these experiments, that if the peak count on acetate agar was plotted against final amounts of lactic acid occurring after 6 months, a good correlation was obtained. (Fig. 12, A is omitted in the curve for Experiment 10 as mould was growing in the tube.)

In experiment 7 where considerable amounts of butyric acid were produced in some silages in the later stages, this relationship did not occur. However, if one assumes that all the butyric acid produced after 8 days originates from lactic acid/

FIG. 13

CORRELATION OF PEAK COUNTS ON ACETATE AGAR WITH LACTIC ACID CONTENTS AFTER ALLOWANCE FOR A BUTYRIC FERMENTATION OF LACTATE

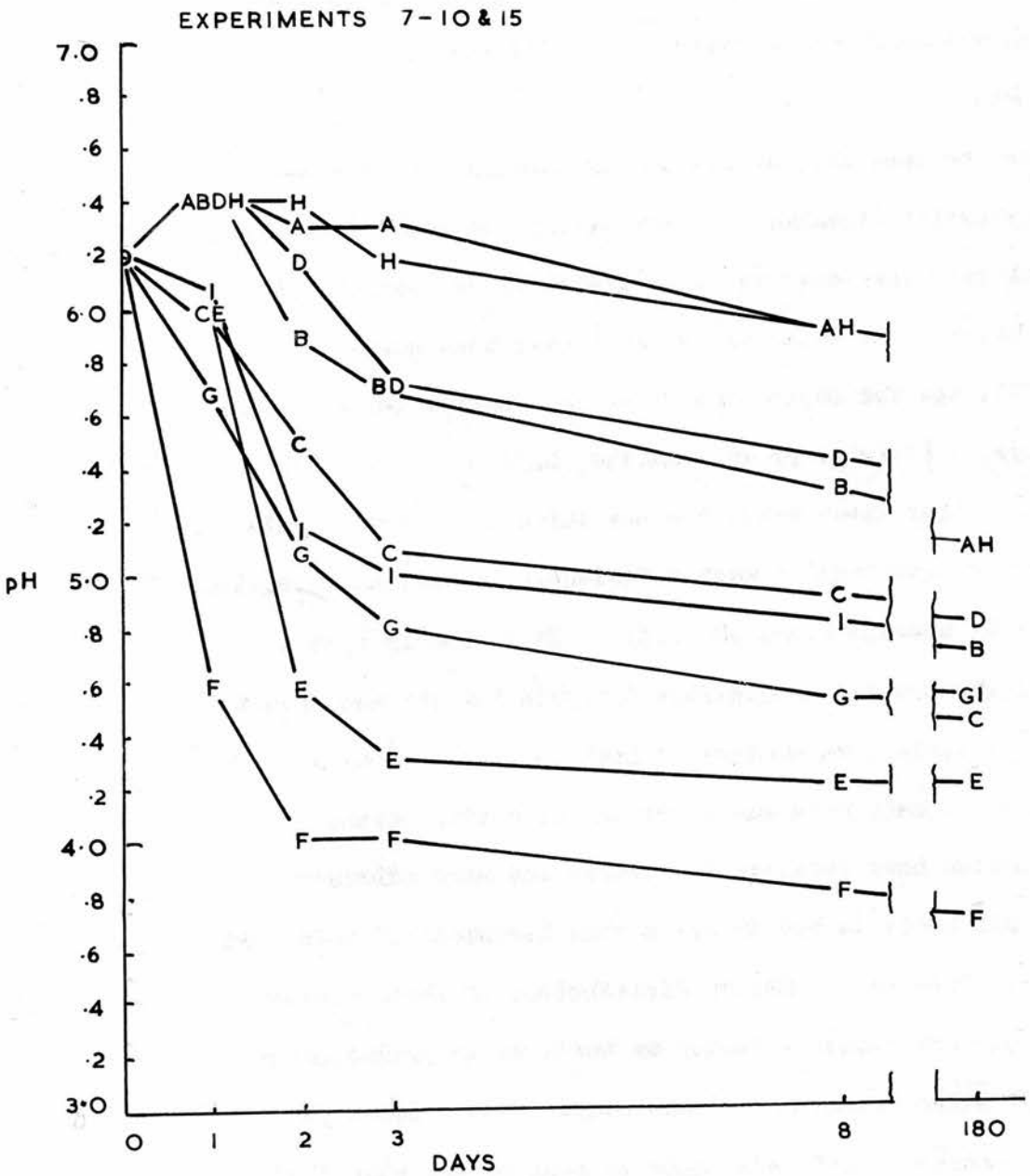


acid, and a correction is made for this, a good correlation is again obtained (Fig. 13). (Information concerning the appropriate correction to be made was provided by Dr. R. F. Rosenberger. It is based on the data of Bhat & Barker (1947) for the production of butyric acid from lactate (in the presence of excess acetate) by pure cultures of Clostridium lacto-acetophilum.)

It therefore seems reasonable to assume that acetate agar counts give a fair estimate of the numbers of those organisms principally responsible for the production of lactic acid in these silages.

FIG. 14

AVERAGE pH VALUES



A 22° C
B CONTROL
C 40° C
D UNCHOPPED
E MINCED
F INOCULATED
G WETTED
H WILTED
I WILTED + WATER

Discussion of experiments 7 - 10 and 15.

In order to compare the effects of the different treatments on the quality of the silages produced, as judged by pH, the average pH values for each treatment at different stages are shown in Fig. 14.

It can be seen that of all the treatments, inoculation with lactobacilli (treatment F) was easily the most effective. It was the only treatment which consistently reduced the pH to 4 and below. It would therefore appear that scarcity of lactobacilli was the major reason for the control series making poor silage. (Average pH at 6 months, 4.9)

Of the other treatments, the one which has overcome this deficiency of lactobacilli most efficiently is extreme laceration (treatment E, average final pH, 4.2). It is likely that a variety of factors are responsible for this but the net result has been the early predominance of lactobacilli. Greater availability of nutrients and provision of a more suitable physical medium have resulted in quicker and more extensive growth of all bacteria but it seems that lactobacilli have been particularly favoured. Better distribution of these bacteria is a possible contributory factor to their early predominance. Addition of water (treatment G) has resulted in a lower pH than in the control, and this tends to confirm the view that the beneficial effect of mincing is partly due to the medium being more moist through the release of plant juices.

Incubation at 40°C (treatment C) had a beneficial effect

where lactobacilli were scarce on the fresh grass. This effect was probably largely due to the inability of the majority of other bacteria to grow at this temperature rather than to a direct favourable effect on the lactobacilli themselves. The proliferation of homofermentative streptococci capable of growth at 45°C has probably contributed to the lowering of the pH, but the considerable drop which occurs after 8 days tends to suggest that their influence is mainly restricted to the early stages of the fermentation.

Wilting (treatment H) and incubation at 22°C (treatment A) have been least efficient in reducing the pH. With wilting, pH is a poor index of preservation since the preservative effect of this treatment is probably largely due to the limited growth of bacteria in general in the drier medium. That the much lower acetate agar counts obtained with this treatment is a result of the low moisture content of the medium is obvious when comparison is made with treatment G (wetted) and treatment I (wilted + water).

Low temperature incubation (treatment A) has resulted in poor silage largely because of the inability of the few lactobacilli present on the fresh grass, to compete favourably with other bacteria. It appears to favour heterofermentative streptococci at the expense of lactobacilli even when the former organisms are considerably outnumbered by the latter (Experiment 10).

Although tube to tube variation made it difficult to follow/

Table 15.

The relative numbers of homofermentative and heterofermentative lactobacilli isolated during the first 8 days of the fermentation.

Experiment	Homofermentative	Heterofermentative
7	53 %	47 %
8	84 "	16 "
9	51 "	49 "
10	85 "	15 "
15	49 "	51 "
Average	60 "	40 "

follow the sequence of types, there is considerable evidence that heterofermentative streptococci are usually much more numerous than lactobacilli in the early stages of the fermentation. At a later stage, lactobacilli become the dominant group but pediococci may also be present. There was no evidence that heterofermentative lactobacilli became dominant only in the later stages. Indeed it was rather surprising to find that a considerable number (40%) of the lactobacilli isolated were heterofermentative. The proportion of heterofermentative and homofermentative lactobacilli isolated during the first 8 days from the five experiments are given in table 15.

It might be suggested that acetate agar was perhaps more selective for heterofermentative lactobacilli, however, the average proportions of the two types isolated from tomato agar in early experiments were: homofermentative, 63%; heterofermentative, 37%.

It must therefore be concluded that heterofermentative lactobacilli occurred to a much greater extent in these experimental silages than would be expected from the findings of previous workers.

Inoculation of grass with different species of lactic acid bacteria.

Experiment K1

The object of this experiment was simply to add to grass, large inocula of representative strains of the major groups of lactic acid bacteria isolated from the experimental silages. It was thought that in this way, if sufficiently large inocula were used, the organisms introduced would be able to compete with the natural flora of the grass and the maximal effects of which they were capable could then be assessed.

Methods

The grass used was perennial ryegrass (strain S 24), which had been grown in a greenhouse during winter, without artificial illumination. The dry matter content was 17% and the crude protein content was 20% of the dry matter. Lactobacilli were not detectable on the fresh grass and the 'total' count on G.Y.A. was considerably lower than was normally found with field grass.

The methods employed were essentially the same as those described for the team experiments (p. 27) but, since the supply of grass was limited, 6" x $\frac{5}{8}$" tubes, holding 10g. of grass were used. At each examination this quantity of material was made up to 60g. with sterile water and disintegrated in a small capacity macerator. The macerate was therefore a $^1/_6$ dilution of the original material, as in the previous experiments.

Strains used for inoculation./

Strains used for inoculation.

The strains used, were, with one exception (L 94) selected because of their vigorous acid-producing capacity. As far as could be determined, they were representative of the types occurring most commonly in the experimental silages.

The homofermentative streptococcus (S 1) used, was identified as S. faecalis (Sherman, 1937). The heterofermentative streptococcus (S 53) agreed with the description of Leuconostoc mesenteroides (Hucker & Pederson, 1931). One homofermentative (L 1) and two heterofermentative (L 16 and L 94) lactobacilli were used. They are fully described in part III of this work.

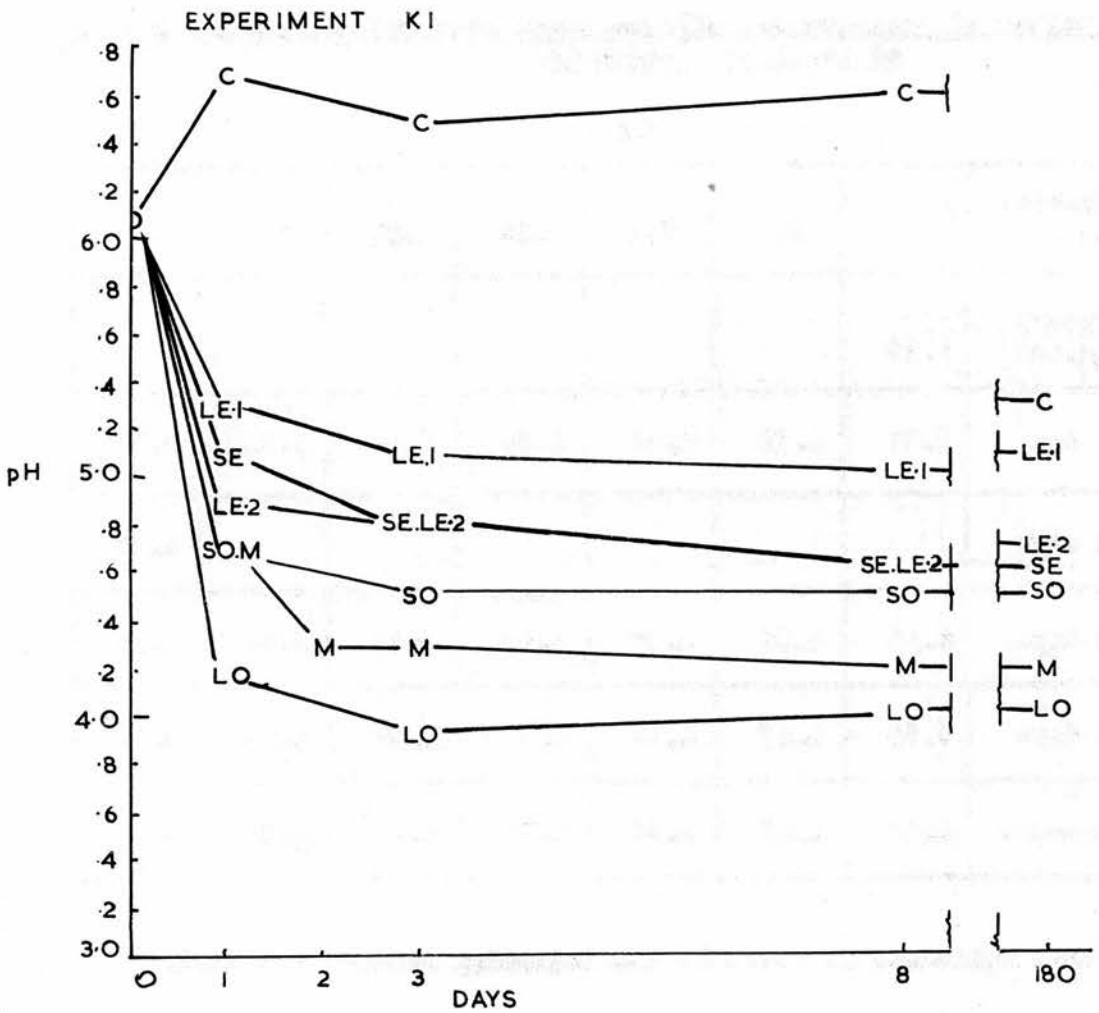
Different batches of the grass were inoculated with pure cultures in buffered malt sprouts extract molasses medium./ p.102 An inoculum of approximately 1 million cells per gram of grass was used. It had been intended to inoculate a series with a mixture of strains representative of the four major groups, and to trace their development in the silage. However, the method thought to be most suitable for tracing S. faecalis was that suggested by Reinbold, Swern & Hussong (1953) and a major constituent of their selective medium, ditetrazolium chloride was unobtainable. S. faecalis was therefore omitted from the mixed inoculum. The methods used for tracing the three strains used in the mixture were as follows:-

All strains grew quantitatively on acetate agar and therefore this medium gave a total count of the three organisms. The heterofermentative streptococcus (S 53) produced slime from/

FIG. 15

INOCULATION WITH VARIOUS LACTIC ACID BACTERIA

pH VALUES



C	CONTROL
SO	HOMOFERMENTATIVE STREPTOCOCCUS S1
SE	HETEROFERMENTATIVE " S53
LE·1	" LACTOBACILLUS L94
LE·2	" " L16
LO	HOMOFERMENTATIVE " L1
M	MIXTURE OF S53, L94 & L1

Table 16.

Experiment K1.

pH values of experimental silages inoculated with different species of lactic acid bacteria.

Incubation period	C	S1	S53	L16	L94	L1	M
fresh grass	6.10						
1 day	6.71	4.72	5.05	4.93	5.34	4.24	4.70
2 days							4.32
3 days	6.49	4.51	4.79	4.83	5.13	3.94	4.28
8 days	6.55	4.47	4.66	4.70	5.04	3.96	4.18
6 months	5.32	4.47	4.61	4.72	5.07	3.95	4.17

In this table and in table 17 the following symbols are used.

C = control

S1 = inoculated with homofermentative streptococcus (S1)

S53 = " " heterofermentative " (S53)

L16 = " " heterofermentative lactobacillus (L16)

L94 = " " " " (L94)

L1 = " " homofermentative " (L1)

M = inoculated with mixture of S53, L94 and L1

Table 17.

Colony counts of experimental silages inoculated with different species of lactic acid bacteria.

'Total' count on sucrose G.Y.A. (millions /g.).

Incubation period	C	S1	S53	L16	L94	L1
fresh grass	0.78	0.78 +1.7	0.78 +1.1	0.78 +1.8	0.78 +1.7	0.78 +1.7
1 day	89.	1,100.	1,900.	1,200.	580.	2,000.
3 days	290.	1,100.	570.	1,900.	540.	2,000.
8 days	15.	450.	51.	400.	160.	180.
6 months	0	0	0.21	0.0006	0.78	0.0006

Only slime-forming types were present in silages inoculated with S53, and no slime-formers could be detected in any of the other silages.

Acetate agar count (millions /g.).

Incubation period.	C	S1	S53	L16	L94	L1
fresh grass	0					
1 day	0	0	2,100.	1,100.	550.	2,500.
3 days	0.0001	0	640.	1,800.	500.	2,100.
8 days	0	0.016	68.	390.	lost	200.
6 months	0	0	0.34	0.0006	0.082	0.0006

Note: '0' denotes a count of less than 0.000006 millions /g.

from sucrose and so colonies of this organism could be detected on G.Y.A. containing 1% sucrose (Olsen, 1951). This medium also gave 'total' counts. The heterofermentative lactobacillus (L 94) was unusual in that it was chromogenic. Colonies on acetate agar were a characteristic deep orange-red colour, and so, on this medium it could be differentiated from the other two organisms in the mixture. Counts of the homofermentative lactobacillus (L 1) were obtained by subtraction. A control series of silage was run so that organisms from the grass did not interfere with the counting methods. A disadvantage was that L 94 was not a particularly vigorous type and so L 16 was included in the single strain inocula to compare the effects of the two strains.

The inoculum used was roughly 1 million S 53, and 100,000 each of L 1 and L 94 per gram of grass. Incubation was at 30°C and examinations were made at 1, 2 (mixed inoculum only), 3, and 8 days, and at approximately 6 months.

Results

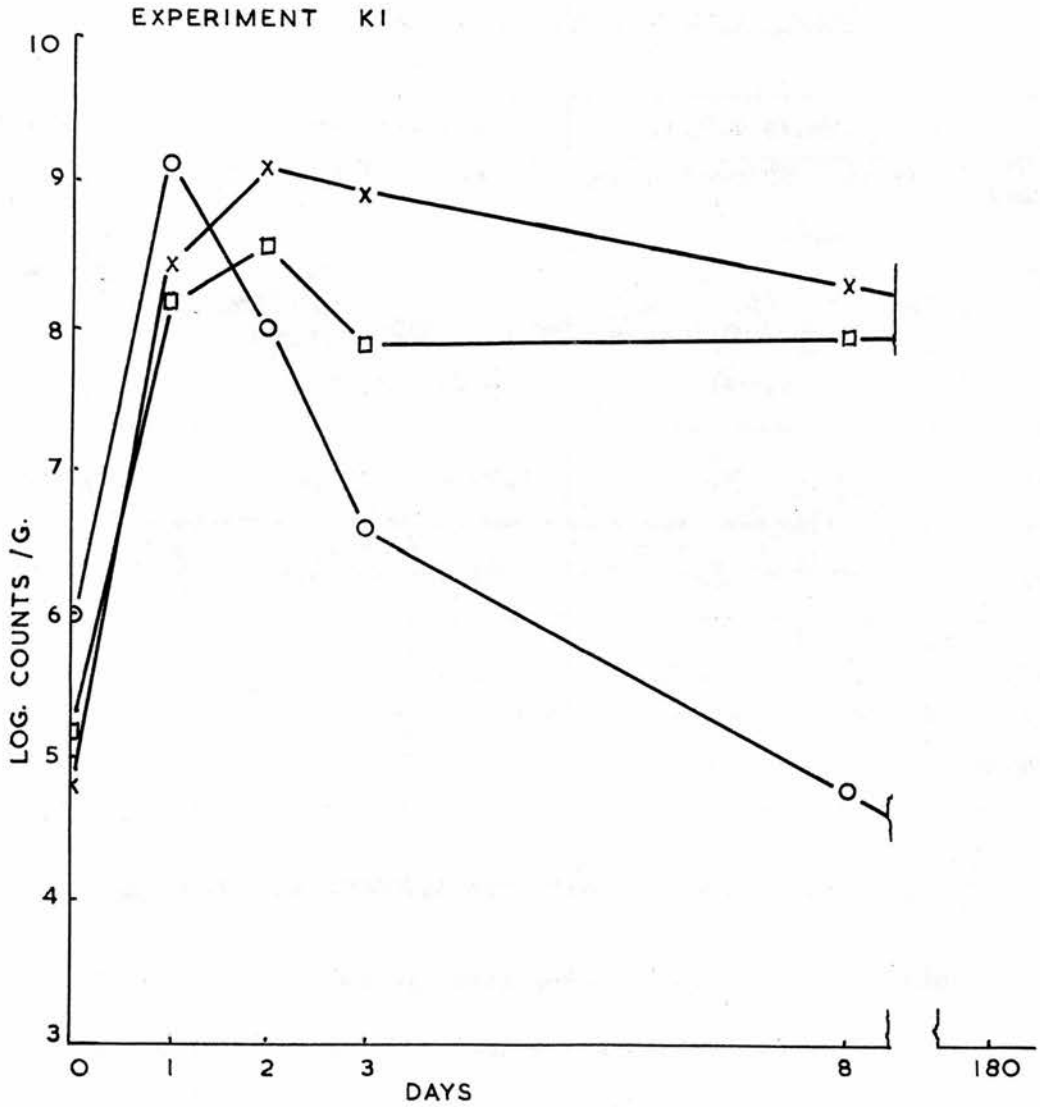
The counts obtained for the silages with single strain inocula are given in table 17. Comparison with the control series indicates that all strains multiplied profusely in the silage and the data suggest that the introduced strains dominated the flora from the first day onward. No slime-formers could be detected in the control and the acetate counts of this series were negative or extremely low throughout.

Reference to table 16 and figure 15 shows that all

FIG 16

INOCULATION WITH MIXED CULTURE OF LACTIC ACID BACTERIA

COUNTS



HETEROFERMENTATIVE STREPTOCOCCUS S53 —O—

" LACTOBACILLUS L94 —□—

HOMOFERMENTATIVE " L 1 —X—

Table 18.

Experiment K1.

Colony counts of experimental silage inoculated with a mixed culture of different species of lactic acid bacteria.

Colony counts in millions /g.

Incubation period	Sucrose G.Y.A.		Acetate agar		Estimated
	Total	Slime-formers S53	Total	Chromogenic L94	L1
fresh grass		1.1 (inoculum)		0.17 (inoculum)	0.07 (inoculum)
1 day		1,400.	1,800.	170.	230.
2 days	1,700.	96.	1,800.	360.	1,300.
3 days	1,100.	3.8	920.	84.	830.
8 days	340.	0.06	340.	110.	230.
6 months	0	0	0	0	0

Note: '0' denotes a count of less than 0.000006 millions /g.

S53 = heterofermentative streptococcus

L94 = heterofermentative lactobacillus (chromogenic)

L1 = homofermentative lactobacillus

inoculated silages had a lower pH than the control series. Considerable amounts of butyric acid were produced in the control and small amounts were formed where L 94 was used for inoculation. It seems likely that lactate-fermenting anaerobes were scarce on the grass and were only active where the pH remained above 5.

The homofermentative lactobacillus (L 1) was easily the most efficient organism in lowering the pH. Higher pH levels resulted when it was inoculated in mixed culture with hetero-fermentative organisms. The homofermentative streptococcus (S 1) was the most efficient of the remaining single strain inocula but at 6 months the heterofermentative organisms (L 94 excepted) had produced pH levels which were almost as low as with S 1. L 94 was appreciably less efficient than L 16 in producing a low pH.

The estimated counts of the different types in the series inoculated with the mixture of strains are shown in table 18 and figure 16.

The heterofermentative streptococcus (S53) originally present in higher numbers than the lactobacilli, dominated the microflora at 1 day and thereafter died off rapidly until at 8 days, the count was considerably lower than the initial inoculum. The peak count of lactobacilli occurred at 2 days when they dominated the microflora. The homofermentative organism (L 1) was predominant. From the third day onward the heterofermentative type gradually formed a greater

proportion/

proportion of the lactobacillus count until at 8 days the numbers of the homofermentative and heterofermentative strains were almost equal. It appeared that the latter type would become dominant some time after 8 days, however at the final examination the acetate agar count was nil and so there was no conclusive evidence of this trend.

Discussion of experiment K1

The grass used in this experiment having a high crude protein content would be considered difficult to ensile. Also the conditions of growth might lead one to expect a product low in fermentable substrates. However, inoculation with a homofermentative lactobacillus resulted in the production of good silage of pH 4. Inoculation with any other of the major groups of lactic acid bacteria resulted in a product of pH 4.5 - 4.8. It would therefore appear likely that only homofermentative lactobacilli are capable of producing silage of low pH from grass of high crude protein content.

This experiment has provided some additional evidence for a succession of types such as has been reported by some previous workers. However such a succession does not appear to be necessary for the production of good silage where adequate numbers of lactobacilli of the correct type are present initially. Indeed growth of heterofermentative organisms in this case has resulted in a product of higher pH (pH 4.2) than that in which there were only homofermentative types present (pH 4.0).

It/

It seems likely from this and from the previous experiments that initial proliferation of heterofermentative streptococci only occurs where lactobacilli are scarce on the fresh material. In such a case they might have a beneficial influence in providing a medium which would be more suitable for lactobacilli than for other organisms, through the formation of such substances as lactic acid, acetic acid and alcohol.

PART III

The properties and the identification of lactobacilli isolated from grass and silage.

The literature quoted in the preceding section of this thesis makes it evident that many previous workers have experienced difficulty in accurately identifying some of the silage lactobacilli. In addition, many obviously identical species are mentioned by various different names. Therefore, before describing the more detailed investigation of members of this group, it would appear relevant to survey briefy the present status of classification of the genus Lactobacillus Beijerinck, at least in so far as it concerns this present work.

In the sixth edition of Bergey's Manual (1948), the genus is divided into two more or less well-defined groups. The first comprises the homofermentative, and the second, the heterofermentative types.

Further sub-division of the homofermentative types is based largely on the work of Orla-Jensen (1919, 1943), and the main characteristics employed are, optimum temperature, optical rotation of lactic acid produced, and certain fermentation reactions. Two sub-genera are recognised, the first Thermobacterium Orla-Jensen, having an optimum temperature in the range 37 - 45°C, and the second, Streptobacterium Orla-Jensen, with an optimum temperature of 28 - 32°C. At first sight there would appear to be a distinct difference between these two groups, however, one finds, for example, that L. leichmannii included in the sub-genus Streptobacterium in the key, is described in the text as having an optimum temperature of 36°C.

Differentiation/

Differentiation of the heterofermentative types, comprising the sub-genus Betabacterum Orla-Jensen, is based largely on the work of Pederson (1938). Again the criteria employed are optimum temperature and the ability to ferment certain sugars. All the organisms in this sub-genus produce inactive lactic acid. Two groups are recognised on the basis of optimum temperature, but their separation is even less distinct than in the case of the homofermentative types, since the temperatures quoted are, 28 - 32°C and 35 - 40°C.

In all groups, the definitions of different species are based on original descriptions supplemented by data from subsequent work wherever possible.

Since the appearance of the sixth edition of Bergey's Manual (1948), three major works on the classification of this group have been published.

Shimwell (1948) has described methods for the differentiation of the brewery lactic acid bacteria employing criteria similar to those used by Orla-Jensen (1919; 1943), and Pederson (1938). His methods would appear to be of most value in the identification of those types of importance in the brewing industry.

Briggs (1953b) has divided the entire genus into eight groups by means of six physiological tests. These tests are similar to those used by several other workers in the classification of the streptococci (Sherman, 1937), and the lactobacilli. Perhaps the main value of this work lies in the fact that, as far as possible, a standard, nutritionally adequate/

adequate basal medium is used in the different tests (Briggs, 1953a). However, about 14% of the strains studied could not be classified, and further differentiation into species has not yet been attempted.

Recently, Rogosa et al.(1953) have published a classification of the lactobacilli with special reference to the oral types. This work can perhaps be regarded as confirmation and consolidation of the findings of Orla-Jensen (1919; 1943) and Pederson (1938). Much has been done to clear up the confusion which exists with regard to such criteria as colony type and fermentation characters. In addition, methods are described whereby the nutritional requirements of an organism may be used to help in its identification. However, the classification of the heterofermentative group is still obscure. For example, twelve variant types are described for L. fermenti, although a large majority of these had the same nutritional pattern.

Summing up, it would appear that Orla-Jensen (1919; 1943), Pederson (1938) and Rogosa et al. (1953) provide the most useful data for the identification of new isolates with previously described species.

E X P E R I M E N T A L .

Experimental

The pure cultures to be described, were for the most part selected as being representative of the dominant types of lactobacilli occurring in the differently treated silages during the period of active fermentation. A few of the strains were isolated from fresh grass. Details of the origin of the cultures are given in table 45 in the Appendix.

Media and Methods.

All cultures were purified by plating at least twice in tween agar.

Stock cultures were maintained in meat medium in $\frac{1}{4}$ oz. vials at room temperature. Transfers were made at approximately three month intervals to fresh meat medium with at least one preliminary sub-culture in T.S.S. medium.

Preparation of inocula.

Preliminary transfers were made from the meat cultures to T.S.S. medium, then at least two sub-cultures were made in B.T.B. (Appendix p.118) to ensure that the organisms were growing vigorously. The second transfer in B.T.B. was used to inoculate the various media. Occasionally, additional transfers of some poorly-growing strains were required.

Inoculation was by loop (one loopful) or by pasteur pipette (one drop except where otherwise stated) from 1 to 2 day-old cultures at 30°C.

Incubation of all cultures was at 30°C except where otherwise stated.

Differential/

Differential tests used.

1. Morphology. Nigrosin films of young tween broth cultures gave the most satisfactory differentiation of rod and coccal forms which was perhaps the main value of morphological studies. Gram films were prepared from young cultures in the same medium. Tween broth is similar to T.S.S. medium but contains no agar.

2. Production of catalase. Young G.Y.B. (Appendix p. 118) cultures and frequently also agar slope cultures of the same medium were tested with 5 vol. hydrogen peroxide and observed carefully for evolution of oxygen. In doubtful cases a modified Lind's apparatus (Cunningham, 1947, p.134) proved useful. Neutralised G.Y.B. cultures were used in this case.

3. Nitrate reduction. The medium used was tween broth (at pH 6.0) containing 0.1% potassium nitrate. Cultures were tested for the presence of nitrite after incubation for 7 days. All negative cultures were checked for the presence of nitrate (Cunningham, 1947, p.211).

4. Production of CO_2 from glucose. The same medium was used as in the initial screening tests (p.30). At least 2 drops from a pasteur pipette were used for inoculation. For types which grew at 45°C, incubation was at 37°C and for others, at 30°C. At least 7 day's incubation was used.

5. Colony type. Loop dilutions in poured plates of G.Y.A. containing 20% tomato extract were incubated for 5 days at 30°C. Colony size was determined roughly by naked eye, and structure by means of a low power stereoscopic microscope.

6./

6. Growth at 15 and 45°C. Originally, T.S.S. medium at pH 5.4 was used for growth at 45°C, however a few strains which will not grow at 45°C at this pH will do so if the pH of the medium is 6.5 (at room temperature). Again certain strains will grow at 45°C in unstoppered tubes but will not do so if the tubes are sealed with rubber stoppers. Rapid evaporation in unstoppered tubes appears to cause a lowering of the temperature below the critical level.

Stoppered tubes with T.S.S. medium at pH 6.5 were therefore incubated in a circulated water-bath at 45°C ± 0.1°C for 5 - 7 days. For the 15°C test, conditions are much less critical and T.S.S. cultures were incubated in a water-bath at 15°C ± 0.5°C for a minimum of 3 weeks.

The approximate limiting temperatures for growth of many of the strains were determined in T.S.S. medium at pH 5.4 in unstoppered tubes.

7. Fermentation reactions. Various basal media were used in a preliminary series of tests. The most satisfactory method resulting from these, involved the simultaneous use of two media which had the following composition:-

Basal medium I 0.5g. each of lemco and Evan's peptone; 5 ml. yeast autolysate; 0.05 ml. tween 80; water to 100 ml, indicator brom cresol purple. Adjusted to pH 7.0.

Basal medium II had the same composition as basal medium I but was adjusted to pH 5.4 and the indicator employed was brom cresol green.

Concentrated/

Concentrated solutions of the various substrates in distilled water were sterilized by Seitz-filtration and added aseptically to the basal medium. A minimal amount of heating was applied only where the substrate (e.g. inulin) did not dissolve readily in the cold. The final concentration in the medium was 0.5%. Fermentation was detected by colour change in the indicator.

Recently, Rogosa et al. (1953) have described a method using a basal medium adapted from their selective medium for lactobacilli (Rogosa et al., 1951a; 1951b). This method has since been adopted, being more reliable than those described above. Difco tryptose was substituted for trypticase which was not readily obtainable. The final pH of the medium after sterilization in the autoclave at 15 lb./sq.in. for 15 minutes was 6.1. The concentrations of different substrates employed were the same as those used by Rogosa and co-workers, i.e. 2% for all except galactose which was used at a concentration of 1.4%. All substrates were sterilized by filtration as in the first method described.

Control tubes containing Seitz-filtered distilled water instead of substrate were inoculated, and uninoculated controls containing each substrate were also incubated. After addition of the various substrates, all media were incubated for at least 2 days at 30°C to check for sterility before inoculation. Cultures were observed daily for 14 days and a note made of the day an obvious colour change occurred in the indicator (Alizarin red S). At the end of the incubation period, the pH/

pH of each culture was determined electrometrically.

The substrates used were as follows:-
D-glucose, D-laevulose, L-arabinose, D-lactose, D-mannitol, D-mannose, D-melezitose, D-melibiose, D-raffinose, D-sucrose, D-trehalose, D-xylose, D-cellobiose, D-galactose, D-maltose, L-rhamnose, D-sorbitol, L-sorbose, α-methyl-D-glucoside, α-methyl-D-mannoside, salicin, dulcitol, inositol, and inulin.

The fermentation characters mentioned later are those obtained by the method of Rogosa et al.(1953).

8. Acid production in milk. The milk used was from one animal which had had no penicillin injections during its current lactation. After separation, the milk was tubed in 9 ml. quantities and sterilized by steam at 100°C for 30 minutes on three successive days. Duplicate tubes were inoculated and incubated for 14 days at 30°C. Uninoculated controls were also incubated. After incubation, the cultures were titrated with N/10 sodium hydroxide using phenolphthalein as indicator. Acid was calculated as lactic acid, and the appropriate adjustment made for control acidity.

9. Hydrolysis of aesculin. The method used by Rogosa et al. (1953) was tried but did not give satisfactory results. The same medium used as a broth, containing no ferric citrate and with only 0.1% aesculin was finally employed. After 14 day's incubation, blackening on addition of ferric chloride indicated a positive result. Some rather indeterminate results were still obtained by this method. Throughout the incubation period/

period the cultures were examined by ultra-violet light to test for destruction of aesculin, but this method was only found useful in strongly positive reactions.

10. Hydrolysis of hippurate. Attempts to use the method described by Rogosa et al. (1953) proved unseccessful. No other method was tried.

11. Hydrolysis of arginine. The medium used was that of Niven, Smiley & Sherman (1942) for streptococci, but with the addition of 0.05% tween 80 and with the pH adjusted to 6.0. Some lactobacilli grew poorly in this medium but frequently cultures of such strains gave strongly positive reactions when tested with Nessler's reagent. The same medium without arginine was inoculated as a control. The cultures were examined at 5 and 8 days.

12. Production of CO_2 from citrate. The method of Gibson & Abdel-Malek (1945) was employed. Heavy inoculation with incubation at 35°C was used but only a tiny bubble of CO_2 was ever produced. Attempts to improve the medium by the addition of 0.05% tween 80, and the mineral supplement used by Rogosa et al. (1951a; 1951b) proved unsuccessful. Reduction of the citrate concentration from 3% to 1% had a similar result.

13. Production of acetoin from citrate. Liquid media similar to those described for the preceding test were used. Cultures were tested after 3 and 5 day's incubation (Cunningham, 1947, p.112) Strongly positive reactions were obtained with some strains but these were occasionally types which produced no

trace/

trace of CO_2 in the preceding test. From the data of Gunsalus & Campbell (1944) these results would appear to be contradictory. Obviously this, and the preceding test require further study before they can be of any value for purposes of differentiation.

14. Production of acetoin from glucose. Tween broth cultures were tested at 3 and 5 days as described above. No strain gave a definitely positive reaction.

15. Growth in litmus milk and yeast autolysate litmus milk. Litmus milk and the same medium with the addition of 5% yeast autolysate were inoculated and incubated at 30°C. The cultures were examined at 1, 2, 3, 7 and 14 days and the rates of acid production in the two media were compared.

16. Utilization of lactate. Sugar basal medium II (p. 56) made semi-solid by the addition of 0.1% agar was used, with the following additions sterilized in the medium: (a) 1% sodium lactate and (b) 1% sodium lactate + 0.25% sodium acetate. In addition to (a) and (b), a control series with no additions was inoculated and the cultures were incubated at 30°C for 28 days approximately. Utilization of lactate was assessed by comparing growth in (a) and (b) with that in the control series.

Series (b) was included since it is known that certain bacteria can utilize lactate only in the presence of acetate (Bhat & Barker, 1947).

17. Form of lactic acid produced. The medium used was similar to tween broth (at pH 6.5) but with 5% glucose instead of 1%. 300 ml. quantities were dispensed in 500 ml. conical flasks/

flasks and 10g. of chalk was added to each flask before sterilization. Incubation was at 30°C for 14 days. The lactic acid was separated as the zinc salt (Cunningham & Smith, 1940) and the optical rotation determined polarimetrically. No attempt was made to separate different fractions and therefore the designation 'inactive' (I), indicates that the lactic acid produced consisted either of the racemic form or of a mixture consisting of equal proportions of laevo- and dextro-forms.

18. Formation of slime from sucrose. The medium used was similar to tween agar but contained 5% sucrose and 12% Cox's gelatine instead of 1% glucose and 1.5% agar respectively. The pH was adjusted to 6.5. Stab cultures were employed and incubation was at 23.5°C for 14 days.

This temperature was selected as being sufficiently high to allow certain strains to grow without the gelatine melting.

19. Motility. Hanging drop preparations from young tween broth cultures were examined.

Results.

The organisms studied were catalase-negative, Gram-positive, rods. Coccoid and filamentous forms were frequently encountered. Nitrates were not reduced, and carbohydrates were fermented with the production of acid. Spores were never observed. A few strains were motile. The large majority of the strains were facultatively anaerobic and surface growth on solid media was generally slight. A few strains when freshly isolated produced colonies in G.Y.A. shake cultures only at depths greater than about 1 cm., but were much more tolerant of oxygen in richer media. True microaerophilic growth was exhibited by a few freshly isolated strains. In general, these deviations from the typical growth of a facultative anaerobe were most pronounced, and often only apparent under less favourable conditions of growth.

Utilization of lactate as the only source of carbon and energy was never detected.

The above characteristics are sufficient to identify the organisms as members of the genus Lactobacillus.

Primary separation into homofermentative and heterofermentative types was made on the ability to form CO_2 from glucose. In addition to the general characters of the group, the only single characteristic shared by all the strains in both groups was the ability to ferment glucose. Growth in litmus milk of all strains was stimulated to a greater or less extent by yeast autolysate.

Homofermentative/

Homofermentative lactobacilli

Thirty-seven of the strains tested failed to produce CO_2 from glucose. All fermented laevulose and mannose, while a large majority fermented trehalose, galactose, cellobiose and salicin. Sucrose, maltose, and lactose were less frequently attacked, but a majority of strains did so. All the substrates tested were attacked by at least our strain; that least commonly fermented was dulcitol.

Colony size and structure was found to be a very useful characteristic, providing solid media containing no tween were used. Colony forms were divided into three types as follows:

1) S. A large smooth lens-shaped colony always greater than 2 m.m. in diameter.

2) R. Small rhizoid type of colony with fine outgrowths in the medium.

3) I. This was used to designate two types of colony.

(a) Tiny lens-shaped types with outgrowths i.e. intermediate between 'S' and 'R' forms.

(b) Tiny irregular types.

Both (a) and (b) were less than one m.m. in diameter. Occasionally very tiny colonies were produced which were apparently quite smooth and lenticular in shape. These are easily differentiated from the typical 'S' form in size and have been designated 'I'.

All the strains could be divided into three groups on the basis of growth at 15 and 45°C, and colony type.

Group I/

Group I. (18 strains) Grew at 15 but not 45°C (except L96) Colony type 'S'.

Group II. (6 strains) Grew at 15 but not 45°C. Colony type 'R' (one strain 'I').

Group III. (13 strains) Grew at 45 (except L45) but not 15°C. Colony type 'R' or 'I'.

Group I is the most vigorous of the three groups, growing well on G.Y.A. without the addition of tween. Organisms of this group ferment a wider range of substrates than those of the other two.

Groups II and III generally grow poorly in media such as G.Y.A. without the addition of tween.

Table 19 Characteristics of homofermentative lactobacilli - Group I

	Strain	Acidity in milk % as lactic	Growth at 15°C	Growth at 45°C	Fermentation of: Arabinose	Melibiose	Sorbose	Inositol	Maltose	Mannitol	Raffinose	Sorbitol	Xylose	Melezitose	Rhamnose	Inulin	Dulcitol	α-methyl-mannoside	α-methyl glucoside	Aesculin hydrolysis	Arginine hydrolysis	Form of lactic acid
a	L19	1.23	+	-	-	-	+	+/-	+	+	-	+	-	+	-	+	-	+	+	-	-	D
a	"86	0.95	+	-	-	-	+	+/-	+	+	-	+	-	+	-	-	-	-	+	-	-	D
a	"96	1.20	+	+	-	-	-	+/-	-	+	-	+	-	+	+	-	+	-	-	-	-	D
b	L1	0.55	+	-	+	+	-	-	+	+	+	+	-	-	-	+	-	-	-	+	-	L
b	"62	0.45	+	-	+	+	-	-	+	+	+	+	-	-	-	+	-	-	+	+	-	D
b	"63	0.53	+	-	+	+	-	-	+	+	+	+	-	-	-	+	-	-	-	+	-	D
b	"78	0.43	+	-	+	+	-	-	+	+	+	+	-	+	-	-	-	+	+	+	-	D
b	"79	0.16	+	-	+	+	-	-	+	+	+	+	-	+	-	-	-	+	-	+	-	D
b	"106	0.17	+	-	-	+	-	-	+	+	+	+	-	+	-	-	-	+	-	+	+	D
b	"88	0.67	+	-	+	+	-	-	+	+	-	-	-	+	-	-	-	-	-	+	-	D
b	"114	0.05	+	-	+	+	-	-	+	-	-	-	-	-	+/-	-	-	-	-	-	+/-	D
b	"57	0.09	+	-	+	+	-	-	-	-	-	-	-	-	+/-	-	-	-	-	-	+	L
b	"77	0.05	+	-	+	+	-	-	-	-	-	-	-	-	+/-	+	-	-	-	-	+	D
b	"69	0.05	+	-	+	+	-	-	-	-	-	-	+	-	-	-	-	-	-	-	+	D
b	"71	0.05	+	-	+	+	-	-	-	-	-	-	+	-	+/-	-	-	-	-	-	+	D
b	"80	0.05	+	-	+	-	-	-	-	-	-	-	+	-	+/-	+	-	-	-	-	+	L
b	"84	0.05	+	-	+	-	-	-	-	-	-	-	+	-	+/-	-	-	-	-	-	+	D
	L26	0.08	+	-	-	-	-	-	+	-	-	-	-	+	-	-	-	-	-	-	+	-

In addition, all strains produce an 'S' type colony and (with the exception of L26) ferment glucose, laevulose, mannose, salicin (except L69), cellobiose, trehalose, galactose, sucrose, and lactose (sometimes weakly).

Group I

(15+, 45-, colony type, 'S')

Of the 18 strains which comprise this group, one (L26) is atypical. The remainder ferment laevulose, mannose, salicin (except L69), cellobiose, trehalose, galactose, sucrose, and lactose (sometimes weakly). The atypical strain fermented all of these substrates except cellobiose, galactose and lactose. It also differed in that it never lowered the pH in sugar media below 4.9. In litmus milk, the litmus was completely reduced in 1 day but thereafter no further change occurred.

The strains in this group obviously belong to the sub-genus Streptobacterium Orla-Jensen (Bergey's Manual, 1948). With the exception of L26, the group can be further divided into two sub-groups on the basis of their fermentation of arabinose, melibiose, and inositol, degree of acidity in milk, and the form of lactic acid produced.

Group Ia (3 strains) These organisms produced at least 1% acid in milk calculated as lactic acid, failed to ferment arabinose and melibiose but produced a slight fermentation of inositol. Of the two strains tested, both produced dextro-rotatory lactic acid. In addition, they were morphologically distinct from the other members of Group I in that they produced in liquid media, long tangled chains of small square-ended rods.

These characters are in good agreement with those of L. casei (Orla-Jensen) Holland (Orla-Jensen, 1919; 1943: Rogosa/

Rogosa et al., 1953) although no preferential utilization of lactose to maltose and sucrose was detected. Strains L19 and L86 can be identified with the more commonly occurring type which Rogosa et al. (1953) have called L. casei var. casei, while L96, which grows at 45°C and ferments rhamnose, agrees with their description of L. casei var. rhamnosus. This latter variety has also been mentioned by Orla-Jensen (1919).

Group Ib The fourteen members of this group varied considerably in many of the characters studied. Reference to table 19 indicates that they form a gradual succession of types in the order in which they are given in the table. Obviously a dividing line could not be drawn at any point in the series.

This group differs from Group Ia in that usually less than 0.5% acid is formed in milk, 13/14 strains ferment arabinose and 12/14 ferment melibiose. Inositol is not attacked. In broth, the cells usually occur in pairs and short chains and are usually round-ended. All those strains examined produced inactive lactic acid.

The first five members of the group can easily be identified with L. plantarum (Orla-Jensen) Holland (Orla-Jensen, 1919, 1943; Pederson, 1936; Rogosa et al. 1953).

The remaining members of the group are obviously related to this species even although two strains, L80 and L84 failed to ferment melibiose, an important characteristic of L. plantarum (Orla-Jensen, 1943; Rogosa et al. 1953). They appear to be what previous workers have described as 'degenerate forms'/

forms' of L. plantarum, although the progressive loss of certain characters is accompanied by a corresponding gain of others. This trend is related to a general reduction in vigour of growth.

Table 20.

Characteristics of homofermentative lactobacilli - Group II

Strain	Acidity in milk, % as Lactic acid.	Colony type	Growth at 15°C	Growth at 45°C	Fermentation of: Salicin	Cellobiose	Trehalose	Galactose	Sucrose	Maltose	Lactose	Aesculin hydrolysis	Arginine hydrolysis	Motility	Form of lactic acid
L5	0.05	R	+	-	±	±	+	+	-	-	-	-	(slow +)	-	D
L50	0.12	I	+	-	+	+	+	+	-	+	±	-	-	-	D
L116	0.02	R	+	-	+	+	+	+	-	+	-	-	-	+	I
L107	0.04	R	+	-	+	+	-	+	-	+	-	-	-	+	.
L95	0.04	R	+	-	-	-	+	-	-	+	-	-	-	-	.
L118	0.09	R	+	-	-	+	-	+	-	+	+	-	-	-	.

In addition, all strains ferment glucose, laevulose, and mannose. No strain ferments mannitol, raffinose, sorbitol, xylose, arabinose, melibiose, sorbose, melezitose, rhamnose, inositol, dulcitol, inulin, α-methyl-mannoside or α-methyl-glucoside.

Group II

(15+, 45-, Colony type 'R')

Six strains were included in this group, and of these five produced an 'R' type colony, while the sixth produced a tiny irregular colony (I-type).

A characteristic of the group as a whole is a general lack of vigour both with regard to growth on media such as G.Y.A. and tomato agar, and also in fermentative capacity. Inability to ferment sucrose sharply differentiated them from all other homofermentative lactobacilli studied. Only one strain (L118) fermented lactose strongly. Morphologically the organisms were exceedingly tiny rods and coccoids occurring in pairs and short chains. Strain L107 differed from the others in being a rather long slender rod. Little acidity was produced in milk, and yeast autolysate had only a slight stimulatory effect.

The fact that these organisms are homofermentative and belong to the low temperature group indicates that they are related to the sub-genus Streptobacterium, yet they bear no ressemblance to any known species in this group. However, they appear to be related to the motile lacto-bacilli described by Cunningham & Smith (1940) both in temperature range for growth and in fermentation reactions. In addition two of the six strains (L116 and L107) were found to be actively motile. Motility in strain L107 was by a single flagellum attached close to one end of the cell. The type of flagellation in strain/

strain L116 could not be determined. Strain L116 appeared to produce inactive lactic acid but two other strains tested (L5 and L50) produced an excess of dextro-rotatory lactic acid. These organisms also differed from the Cunningham & Smith type in colony form and failure to hydrolyse aesculin.

Despite these differences, it is concluded that the organisms of this group are related to the motile lactobacilli described by Cunningham & Smith (1940), which, although apparently constituting a new species, were not named.

Table 21.

Characteristics of homofermentative lactobacilli - Group III

	Strain	Acidity in milk, % as lactic	Colony type	Growth at 15°C	Growth at 45°C	Fermentation of Cellobiose	Trehalose	Galactose	Maltose	Lactose	Mannitol	Raffinose	Melibiose	α-methyl glucoside	Aesculin hydrolysis	Arginine hydrolysis	Form of lactic acid
a	L13	0.62	R	-	+	+	+	+	+	+	-	-	-	+	+	-	I
a	"74	0.46	R	-	+	+	+	+	+	+	-	-	-	-	+	-	•
a	"52	0.50	I	-	+	+	-	+	+	+	-	-	-	-	+	-	I
a	"61	0.28	I	-	+	+	-	-	-	+	-	-	-	-	±	-	•
a	"82	0.09	I	-	+	+	+	+	+	-	-	-	-	-	+	-	I
a	"99	0.09	I	-	+	+	+	±	+	-	-	-	-	+	±	-	•
a	"98	0.04	R	-	+	+	-	±	+	-	-	-	-	-	+	-	I
a	"108	0.11	I	-	+	+	+	±	-	-	-	-	-	-	±	-	I
a	"30	•	R	-	+	+	-	-	+	-	-	-	-	-	+	-	•
a	"45	0.16	R	-	-	+	+	+	+	+	-	-	-	-	-	-	I
b	L3	0.28/0.59	I	-	+	+	+	+	+	+	±	+	+	-	+	-	•
b	"37	0.02	I	-	+	+	+	+	+	+	±	+	+	-	-	-	D
b	"33	0.25	I	-	+	-	+	+	+	+	±	+	+	-	-	-	•

In addition, all strains ferment glucose, laevulose, mannose, salicin (except L52), and sucrose.

No strain ferments sorbitol, xylose, arabinose, sorbose, melezitose, rhamnose, inositol, dulcitol, inulin or α-methyl-mannoside.

Group III

(15-, 45+, Colony type 'R' or 'I')

The 13 strains which comprise this group are readily identified with the high temperature group constituting the sub-genus Thermobacterium Orla-Jensen (Bergey's Manual, 1948). Although one strain failed to grow at 45°C (L45), the maximum temperature for the group as a whole was 46 - 47°C, while no growth occurred below about 21 - 23°C.

Five strains produced typical rough colonies while the remainder produced colonies which were classified as intermediate or irregular.

Morphologically, all types from short rods to filaments were produced, but generally the rods were larger and longer than those of the other two groups.

In addition to glucose, all strains fermented laevulose, mannose, sucrose and salicin (except L52), while no strain fermented xylose, arabinose, sorbose, melezitose, rhamnose inositol, dulcitol, sorbitol, inulin or α-methyl-mannoside.

The group can be further divided into two sub-groups on the basis of their fermentation of mannitol, raffinose and melibiose.

Group IIIa The ten strains which comprise this group do not ferment mannitol, raffinose or melibiose. Nine attack aesculin to some extent but their action is often weak and is never as strong as with some members of Group I.

Five of the strains attack lactose strongly and of these, the/

the three which were tested produced inactive lactic acid. About 0.2 - 0.6% acid was formed in milk.

These strains appear to correspond to the species L. acidophilus (Moro) Holland. Much confusion exists in the literature with regard to the taxonomy of this organism, and it is beyond the scope of this work to discuss its alleged relationship with L. casei (Pederson, 1947), or indeed the possibility that Moro's original isolate was in fact Microbacterium lacticum (Orla-Jensen, 1919). Suffice is to say that the strains isolated here agreed in all major characteristics with the modern concept of L. acidophilus, or Thermobacterium intestinale Orla-Jensen (Curran, Rogers & Whittier, 1933; Orla-Jensen, Orla-Jensen & Winther, 1936; Orla-Jensen, 1943; Rogosa et al., 1953).

The remaining organisms of this group failed to ferment lactose but three of these strains which were tested produced inactive lactic acid. In all other respects they were identical with the lactose-fermenting strains.

The inability to ferment lactose in addition to the other characteristics described, tends to suggest that these organisms belong to the species L. leichmannii Bergey et al. Although in the sixth edition of Bergey's Manual (1948), this species is included in the sub-genus Streptobacterium, its temperature range (inadequately defined in Bergey's Manual) would suggest its inclusion in the sub-genus Thermobacterium. From available descriptions of the species, it appears to

differ from L. acidophilus only in its inability to ferment lactose and in the formation of laevo-rotatory lactic acid. (Fred, Peterson & Stiles, 1925; Rogosa et al., 1953) One named strain of this organism (L. leichmannii A.T.C.C. 4797) was investigated and found to ferment lactose. This has also been noted for this strain by Tilden & Svec (1952). However, laevo-rotatory lactic acid was produced. Thus lactose fermentation is possibly a poor criterion for its differentiation from L. acidophilus.

The organisms isolated here produced inactive lactic acid and are therefore regarded as non-lactose-fermenting strains of L. acidophilus .

Group IIIb The three strains included in this group differed from those in Group IIIa in fermenting raffinose, melibiose and, to a less extent, mannitol. In addition, the only strain tested produced dextro-rotatory lactic acid. These characters differentiate them from L. acidophilus, although occasional slight fermentation of raffinose and melibiose has been reported for this species (Rogosa et al., 1953).

They appear to be very similar to the organisms isolated from the faeces of turkeys by Harrison & Hansen (1950), and identified by them as L. plantarum. However, these latter types grew at 45°C but not at 20°C and produced an excess of dextro-rotatory lactic acid. Although some strains of L. plantarum grow at 45°C (Orla-Jensen, 1919, 1943; Pederson, 1929), growth always occurs at 15°C and usually at 10°C. It is therefore considered that the organisms of Group IIIb

cannot/

cannot justifiably be identified with L. plantarum although their fermentation reactions are undoubtedly similar. They must therefore remain unclassified at present.

Heterofermentative lactobacilli

The remaining 24 strains investigated produced CO_2 from glucose and therefore belong to the sub-genus Betabacterium Orla-Jensen (Bergey's Manual, 1948). With the exception of a few atypical strains, all the heterofermentative types hydrolysed arginine. This is in agreement with previous workers (Briggs, 1953b; Rogosa et al., 1953). No strain hydrolysed aesculin.

With the exception of glucose, no substrate was fermented by all strains, but a large majority fermented maltose, sucrose, melibiose, arabinose, lactose and galactose. No strain fermented rhamnose, inulin, melezitose, sorbose, mannitol, dulcitol, sorbitol, inositol, or α-methyl-mannoside. Occasionally strains attacked salicin, trehalose and cellobiose but these were generally atypical in other respects also. A large majority grew at 45°C.

The relationship between colony type and other characters although not so clearly defined as in the homofermentative group, is still apparent.

Nineteen of the twenty-four strains could be placed in two more or less well-defined groups on the basis of growth at 15 and 45°C, fermentation of laevulose, and colony type. There was a good positive correlation between ability to ferment laevulose and growth at 15°C.

The remaining five strains did not form a compact group.

Group IV

Group IV (12 strains) Grew at 45 but not 15°C and did not ferment laevulose. One strain formed an 'S' type colony but the colony type of the remainder was 'R' or 'I'.

Group V (7 strains) Grew at 45 and 15°C, and all but one strain fermented laevulose. Colony type 'S'.

Group VI The remaining five strains have been included in this group although obviously of diverse types. They all failed to grow at 45°C but four strains grew at 15°C. Three produced 'S' type, and two 'I' type colonies.

Table 22.

Characteristics of heterofermentative lactobacilli - Group IV.

Strain	Acidity in milk, % aslactic	Colony type	Growth at 15°C	Growth at 45°C	Fermentation of Arabinose	Fermentation of Xylose	Fermentation of α-methyl-glucoside	Aesculin hydrolysis	Arginine hydrolysis
L103	0.02	R	-	+	-	-	+	-	+
"101	0.04	I	-	+	+	+	-	-	+
"110	0	R	-	+	±	-	+	-	+
"4	0.05	I	-	+	+	-	+	-	+
"8	•	S	-	+	+	+	+	-	+
"38	0.15	R	-	+	+	-	+	-	+
"58	0.09	R	-	+	+	-	+	-	+
"66	0.02	R	-	+	+	-	-	-	+
"68	0.09	R	-	+	+	-	+	-	+
"72	0.07	R	-	+	+	-	-	-	+
"94	0.15	I	-	+	+	-	+	-	+
"115	0.06	R	-	+	+	+	-	-	+

In addition, all strains ferment glucose, maltose, sucrose, lactose, galactose, melibiose and raffinose.

No strain ferments laevulose, salicin, trehalose, cellobiose, mannose, rhamnose, inulin, melezitose, sorbose, mannitol, dulcitol, sorbitol, inositol or α-methyl-mannoside.

Group IV

(45+, 15-, laevulose -, colony type 'I' or 'R')

Twelve strains (50%) belonged to this group. A surprising characteristic was the inability to ferment laevulose, a sugar which is frequently fermented more readily than glucose by many lactobacilli.

For the strains examined, the maximum temperature was approximately 46 - 47°C and the minimum about 23 - 24°C. On the basis of temperature limits for growth this species is therefore closely related to L. fermenti Beijerinck (Pederson, 1938), although the range is narrower than for that species. An important characteristic of L. fermenti (Betabacterium longum; Orla-Jensen, 1919) is the inability to ferment arabinose, although strains have been described which do so, in particular by Rogosa et al. (1953). The majority of the strains in this group fermented arabinose and occasionally xylose. However, it is perhaps significant that frequently the pH produced in arabinose was not so low as in glucose. Typical pentose-fermenters on the other hand e.g. L. brevis and L. buchneri, ferment arabinose more strongly than glucose. In addition, all the strains fermented sucrose, lactose and in particular raffinose, which is in good agreement with the characters of L. fermenti. It is therefore concluded that the strains in this group can be identified with L. fermenti Beijerinck although the majority are atypical with respect to laevulose and arabinose fermentation.

One/

One strain in this group (L94) was unusual in that it produced an orange-red pigment in both tween agar and G.Y.A. Chromogenesis in the lactobacilli is fairly uncommon, but it has been reported for strains of L. brevis and L. plantarum which cause 'rusty spot' in certain cheeses (Davis & Mattick, 1929; 1930: Breed & Pederson, 1938; Pederson & Breed, 1941).

Table 23.

Characteristics of heterofermentative lactobacilli - Group V.

Strain	Acidity in milk, % as lactic	Colony type	Growth at 15°C	Growth at 45°C	Fermentation of Arabinose	Fermentation of Xylose	Fermentation of Mannose	Fermentation of α-methyl-glucoside	Aesculin hydrolysis	Arginine hydrolysis
L7	0.07	S	+	+	-	-	±	-	-	+
"15	0.05	S	+	+	-	-	-	-	-	•
"16	0.04	S	+	+	+	-	+	+	-	+
"56	0.17	S	+	+	+	-	+	+	-	+
"65	0.10	S	+	+	+	-	+	-	-	+
"100	0.09	S	+	+	+	-	+	+	-	+
"111	0.04	S	+	+	+	±	-	+	-	+

In addition, all strains ferment laevulose (except L111), maltose, sucrose, lactose, galactose, melibiose and raffinose.

No strain ferments salicin, trehalose, cellobiose, rhamnose, inulin, melezitose, sorbose, mannitol, dulcitol, sorbitol, inositol, or α-methyl-mannoside.

Group V

(45+, 15+, laevulose +, colony type S)

The seven strains in this group had a maximum temperature of about 46 - 47°C, while the minimum was 15°C or less, but growth did not occur at 10°C. These organisms would therefore appear to belong to the 'intermediate' group of Pederson (1938) constituting the species L. buchneri (Henneberg) Bergey et al.. This species generally ferments mannose, sucrose, galactose, lactose and raffinose, in addition to glucose, laevulose and the pentoses. (Pederson, 1938).

The organisms isolated here agree well with this description except perhaps in the case of the pentoses. Xylose is usually not fermented, while only five of the seven strains ferment arabinose. In this case however, the pH produced in arabinose is lower than in glucose.

Rogosa et al. (1953) found that none of the strains which they identified as L. buchneri grew at 16°C while growth at 45°C was poor or absent. They classified the colony type as intermediate to rough. However, they claimed that an outstanding characteristic of L. buchneri is the ability to ferment melezitose, a property which is unique among the heterofermentative lactobacilli. Pederson (1929) also found this characteristic in strains (syn. L. mannitopoeus) isolated from spoiled tomato products. In this case, however, all the strains were isolated from one limited source. Nevertheless, he did not note melezitose fermentation as a special characteristic of the species/

species (Pederson, 1938), nor is it mentioned in Bergey's Manual (1948).

None of the strains of Group V fermented melezitose. However, although they differ in several respects from oral strains of the species (Rogosa et al., 1953) it is considered that they agree with the description of L. buchneri put forward by Pederson (1938).

Group VI
(45-, 15+)

As already mentioned, the five strains which grew at 15 but not 45°C, did not form a compact unit. There are three distinct types.

Type (i) Strains L112 and L117 formed small, irregular to rough colonies, fermented arabinose, xylose and glucose, and to a slight extent mannose. A lower pH was produced in media with arabinose or xylose than with glucose. Arginine was not hydrolysed. Morphologically, small coccoid elements were seen. These were similar to those produced by the propionibacteria under certain conditions of growth (Van Niel, 1928). However, lactate was not fermented and by means of paper chromatography, it was found that no propionic acid was present in tween broth cultures, although strong spots appeared in the acetic acid position. Propionibacteria normally produced at least twice as much propionic acid as acetic acid in their fermentation of glucose (Van Niel, 1928). It would therefore appear that strains L112 and L117 are indeed lactobacilli. They are probably degenerate forms of L. brevis (Orla-Jensen) Bergey et al. (Rogosa et al., 1953) and are fairly similar to members of Pederson's (1938) 'inactive' group.

Type (ii) Strains L102 and L109 formed smooth colonies and grew rather well on media such as G.Y.A. They fermented only glucose, laevulose, maltose, sucrose, trehalose and mannose (slightly). Arginine was not hydrolysed. Definite

rods/

rods were formed, and propionic acid was not produced in their fermentation of glucose. Lactate was not fermented.

Although the fermentation of trehalose is unusual, these organisms appear to be heterofermentative lactobacilli but must be regarded as being unidentifiable.

Type (iii) L81 formed a typical smooth colony and grew well in G.Y.A. Glucose, laevulose, maltose, cellobiose, mannose, galactose, salicin, and α-methyl-glucoside were fermented, and to a less extent, lactose, arabinose and xylose. Arginine was hydrolysed. Although fermentation of sucrose could not be detected, slime was formed in sucrose-gelatine. With such a combination of characteristics, this strain is obviously closely related to the genus Leuconostoc. However, definite rods were formed in both solid and liquid media, and on this basis it is regarded as a lactobacillus.

Slime-formation has been observed for both a heterofermentative (Meyer, 1939), and a homofermentative (Perquin, 1940) lactobacillus. Strain L81 does not conform to the characteristics of the heterofermentative type Betabacterium vermiforme quoted by Orla-Jensen (1943), and must be regarded as being unidentifiable.

Table 24.

Differential characteristics of silage lactobacilli.

Group	CO_2 from glucose	Colony type	Growth at 15°C	Growth at 45°C	Fermentation of Sucrose	Fermentation of Laevulose	Fermentation of Arabinose	Fermentation of Melibiose	Fermentation of Raffinose	Form of lactic acid	Identity
I a	-	S	+	∓	+	+	-	-	-	D	*L. casei*
b	-	S	+	-	+	+	(+)	(+)	±	I	*L. plantarum*
II	-	R or I	+	-	-	+	-	-	-	D or I	*Streptobacterium sp.*
III a	-	R or I	-	(+)	+	+	-	-	-	I	*L. acidophilus*
b	-	I	-	+	+	+	-	+	+	D	*Thermobacterium sp.*
IV	+	R or I	-	+	+	-	(+)	+	+	•	*L. fermenti* ?
V	+	S	+	+	+	(+)	(+)	+	+	•	*L. buchneri* ?
VI	+	S or I	(+)	-	∓	±	±	-	-	•	*Betabacterium spp.*

With regard to colony type R = rough; S = smooth; I = intermediate or irregular.

(+) indicates that a large majority of strains are positive.

± and ∓ indicate variable reactions.

Discussion

Identification of the isolates has not proved to be easy, and has required the examination of a large number of characteristics for each strain. Even then, proper identification has been found to be impossible in some cases.

However, the characteristics which have proved to be most useful in this study are, formation of CO_2 from glucose, growth at 15 and 45°C, and colony type (Table 24). This last-named character has frequently been described as being unreliable since it is subject to great variation, particularly in the case of L. acidophilus. Although Rogosa et al. (1953) have done much to clear up the misunderstanding which has occurred in the past over the so-called rough → smooth variation, such a change undoubtedly occurs (Rogosa et al.1953; Olsen, 1949). However it is not as widespread as was previously believed, and there is always a tendency to revert to the original colony type (Rogosa et al. 1953). It does not appear to involve a corresponding change in other characters (Rogosa et al. 1953). In the present study, colony type, despite its limitations, has proved to be useful, and has shown a definite correlation with other characters (Table 24).

Sugar fermentations are frequently referred to as being subject to great variation, yet they feature to a large extent in the classification of this group (Orla-Jensen 1919, 1943; Pederson, 1936, 1938; Tittsler, Geib & Rogosa, 1947; Rogosa et al. 1953). In this work, it was found that if the results obtained/

obtained with sugar basal media I and II were taken together, they agreed in a very large majority of cases with the results obtained using the basal medium of Rogosa et al. (1953) (The fermentation of thirteen substrates were compared on the three different media.) Differences which did occur were in the case of substrates which were weakly fermented. With sugar basal media I and II, it was frequently noted that some substances were readily attacked at pH 5.4 but only slightly or not at all at pH 7.0. On the other hand, indicator changes have been observed in the medium at pH 5.4 where no fermentation had taken place (as judged by electrometric pH determination), and also in inoculated controls where no fermentable substrate was present. These examples illustrate how many so-called 'variations' in sugar fermentations may have occurred in the past. In addition, the basal media used by many previous workers appear to have been nutritionally inadequate. The use of a nutritionally adequate basal medium for the determination of fermentation reactions was amply stressed by Orla-Jensen (1919). Rogosa et al.(1953) have also pointed out how many of the past variations in fermentation reactions may be attributed to the use of unsuitable media and methods for the detection of fermentation.

In short, it may be stated that fermentation reactions will only be reliable provided that (1) the cultures used are in a vigorous state, (2) the basal medium used is adequate, both nutritionally and with respect to buffering capacity, and

(3)/

Table 25.

Distribution of various groups of lactobacilli in experimental silages.

Expt. No.	Treatment									
	Grass	22°C (A)	30°C (control B)	40°C (C)	unchopped (D)	minced (E)	wetted (G)	wilted (H)	wilted + water (I)	Inoculated with 'siloferm' (Expt. 7 only)
7						Gp.Ib (1)				Gp. Ia (1)
8					Gp.Ib (1)	Gp.IV (1)			Gp.IIIa (1)	
9	Gp.Ib (1) (fresh) Gp.V (1) (wilted)		Gp.Ib (1)	Gp.IV (1)		Gp.IIIa (1) Gp.IV (2)		Gp.Ib^{x}(1)	Gp.Ib^{x} (1)	
10	Gp.Ib (1)	Gp.Ib (1)				Gp.Ib^{x} (2) Gp.VI (1)			Gp.IIIa (1) Gp.Ia (1)	
15	Gp.VI (1)				Gp.II (1)	Gp.Ia (1) Gp.IIIa (2) Gp.II (1)	Gp.IIIb (1) Gp.IIIa (1) Gp.VI (1)	Gp.V (1) Gp.IV (2)	Gp.IV (1) Gp.Ib (1) Gp.V (1)	

^{x} typical L. plantarum.

(3) a reliable method is used for the detection of fermentation.

In this work, nutritional studies have not so far been used as an aid to identification. However, there is an increasing amount of evidence in the literature that such studies may be of great value in the classification of this group. (Rogosa, Tittsler & Geib, 1947; Tittsler et al.,1952; Rogosa et al., 1953)

Serological studies have proved to be of considerable value in the classification of the streptococci, and they might also be expected to be of use in the taxonomy of the lactobacilli. Although some preliminary observations have been made (e.g. Orland, 1950; Sharpe, 1952), serological methods have not yet been developed to the point of being generally applicable to the whole lactobacillus group.

With regard to the actual species isolated, it was rather surprising to find that L. acidophilus, an intestinal type, was frequently recovered from the experimental silages. It is possible that these types were of intestinal, rather than of plant origin, and were present on the grass through contamination with bird-droppings. Such an explanation is however, largely a matter of conjecture, and whatever their original source, they were undoubtedly capable of multiplication in silage.

It has not been possible to demonstrate any selective effect of a particular treatment on the occurrence of any one species (table 25). However, L. plantarum was encountered much less frequently than would be expected from the previous literature.

Very/

Very few completely typical strains of this species were isolated, and these were only recovered from relatively low dilutions. L. plantarum was isolated only twice from freshly harvested grass (Experiments 9 and 10), and these strains were atypical (L71 and L77). These results tend to suggest that not only are lactobacilli in general scarce on growing crops of grass, but that L. plantarum itself is of very infrequent occurrence on grass.

In addition, a few strains were identified as L. casei. Although many previous workers have stated that its differentiation from L. plantarum is not clearly defined, the strains isolated in this work appear to be quite distinct . However the differential characters used have been based on more recent work which has considerably clarified the position of these two species (Orla-Jensen, 1943; Rogosa et al. 1953). Indeed, less clearly defined differences exist between many other species in this group. For example, from the data of Rogosa et al.(1953), it would appear that the only difference between L. bulgaricus and L. helveticus is that the latter species ferments maltose while the former does not.

The heterofermentative lactobacilli formed a greater proportion of the strains isolated than would be expected from the previous literature. Typical strains of L. brevis were never isolated, but the majority of the isolates appeared to agree most closely with the descriptions of L. fermenti and L. buchneri.

P A R T IV

Further inoculation studies

The observations that lactobacilli were usually scarce on the samples of grass examined, and that addition of lactobacilli to such material was the only treatment, of those tried, which invariably resulted in silage of low pH, suggested that further inoculation studies might prove fruitful.

The following work, therefore, deals with the selection of strains suitable for the inoculation of grass, and the evolution of a medium in which large quantities of the organisms could be produced relatively cheaply.

Preliminary experiments are described, which were designed in an attempt to assess the effect of inoculation on a semi-field scale.

REVIEW OF LITERATURE

Many previous authors have reached the conclusion that there are adequate numbers of lactic acid bacteria on the green crops normally ensiled (Völtz & Jantzon, 1916; Völtz, 1922; Allen, Harrison, Watson, & Ferguson, 1937; Stone et al. 1943; Watson, 1947). On the other hand Gorini (1915) states that although there are always lactic acid bacteria present on green crops, they are not always of such vigour, or present in sufficient numbers to predominate in silage.

Actual inoculation experiments have met with varied success. Malpeaux & Lefort (1911-1912) obtained palatable silage from such diverse materials as beet pulp, distillery pulp, sliced mangolds, beet-leaves, and maize after addition of a 'ferment' called 'lacto-pulp'.

Gorini (1901-1913; 1915; 1920; 1921; 1928) has repeatedly emphasized that to ensure a good lactic fermentation, lactic acid bacteria should be added to the fodder before ensiling. He states that the bacteria used should be of a type normally occurring on green plants, and capable of fermenting sugars other than lactose. In this way, a good lactic fermentation is assured, and butyric acid bacteria are inhibited. He also states that inoculation is of particular importance where leguminous crops are being ensiled.

Remn & Weiske (1914) found that inoculation of beet, and of clover, had a beneficial effect. On the other hand, Völtz & Jantzon (1916) could demonstrate no improvement in silage made/

made from beet leaves when pure cultures of B. cucumeris fermentatae and B. lactis acidi were added. Nevertheless Völtz (1922) later recommends inoculation although he states that there are adequate numbers of lactic acid bacteria on all green plants.

Peterson & Fred (1920) found that addition of pentose-fermenting lactobacilli to corn resulted in a silage which had a higher concentration of volatile and non-volatile acids than a control silage, whereas addition of a mixed culture of B. bulgaricus and B. lactis acidi gave a product similar to the uninoculated control. Inoculation of sterilized fodder with pentose-fermenters resulted in normal acidification. Later, in association with Anderson (1921a), using the same bacteria, they concluded that although inoculation speeded up the fermentation, at a later stage both inoculated and uninoculated material had the same chemical and bacteriological composition.

Frazier (1921), in similar experiments, reached this same conclusion.

Wyant (1920) obtained a palatable corn silage by addition of pure cultures of B. bulgaricus and B. lactis acidi. She could later recover only the latter organism from the material. Burri & Kürsteiner (1919) suggest that control of butyric acid bacteria in 'sweet' silage may be achieved by inoculation with cheese lactic acid bacteria.

Allen & Watson (1934) and Allen, Watson & Ferguson (1937) found that addition to grass of mixed whey cultures of

L./

L. acidophilus, L. bulgaricus and L. casei did not produce any better silage than addition of whey alone. However, they suggest that inoculation may have a beneficial effect.

Malzahn, Bechdel & Stone (1942) and Stone et al. (1943) found no demonstrable difference in alfalfa silage due to inoculation. The last-named authors used a commercial culture 'Silogerm'.

Orla-Jensen (1947); Orla-Jensen, Orla-Jensen & Kjaer (1947); and Pette (1947), recommend the addition of whey cultures of L. casei. Orla-Jensen et al. (1947) also suggest that L. plantarum may be of use provided molasses is added to the whey to supply fermentable sugars.

Watson (1947); Shepherd, Hodgson, Ellis & McCalmont (1948); and Musgrave & Kennedy (1950), all conclude from a study of the literature that no benefits are obtained by inoculation, although Musgrave & Kennedy (1950) also state that inoculation may have a place in silage-making.

From time to time, it has been suggested that yeasts in addition to lactic acid bacteria, should be used for inoculation. Ruschmann & Gräf (1932) used various species of yeasts alone, and in combination with B. casei for the inoculation of grass from sewage meadows, in laboratory-scale silos. They concluded that provided sufficient sugar was added to the material, certain yeasts had a beneficial effect on the aroma of the product. If there was not enough sugar, the yeasts quickly used up all available supplies leaving none for the lactobacilli to convert

to/

to lactic acid, and the resultant product had a high pH. Again, if the sugar was not in excess, lactate was decomposed by the yeasts with a resultant rise in pH. Gerasimowa (1937) found that with soya bean silage, addition of yeasts promoted lactic acid production by suppressing butyric acid bacteria. Bacterial inoculation improved silage quality and reduced losses.

Inoculation has also been used in the production of sauerkraut. Henneberg (1917) suggests that it is useful for preparing a type of sauerkraut from various kinds of beet. Pederson (1930a, 1930b) found that inoculation with S. lactis and Leuconostoc mesenteroides produced good quality kraut, but thought it possible that cabbages already contained sufficient numbers of these bacteria. L. cucumeris and L. pentoaceticus had a detrimental effect probably largely due to their suppression of the lactic acid cocci in the early stages of the fermentation. It would appear that initial development of these cocci is essential for optimal flavour and aroma production in sauerkraut.

As regards the making of silage from steamed potatoes, it seems that all the naturally occurring lactic acid bacteria are killed off in the steaming process (Cunningham & Smith, 1943). Addition of pure cultures of lactic acid bacteria produces a very palatable product (Foth, 1914; Völtz, 1914; Cunningham & Smith, 1943). Round & Gore (1916) prepared good silage from steamed or raw potatoes by addition of ground corn of a 'ferment' prepared from it. They state that ground corn contains/

contains large numbers of lactic acid bacteria.

Thus, from a study of the available data, it would seem that adequate numbers of lactic acid bacteria are present on green crops. The effects of inoculation are doubtful. Where beneficial results have, apparently, been obtained, there is often no evidence that this is a result of inoculation. Again, where addition of cultures has had no detectable effect, the organisms used frequently appear to be particularly unsuitable for this purpose.

EXPERIMENTAL AND RESULTS.

The selection of strains suitable for inoculation.

In the preliminary studies of the effect of inoculation carried out in experiments 8 - 10, (p. 39) a mixture consisting of one homofermentative and one heterofermentative strain was used. Although the inoculated silages had pH levels considerably lower than those attained using any other treatment, the test could not be considered to be very severe, since none of the materials used had a high crude protein content. Before proceeding to more extensive trials of the effect of inoculation on material of high crude protein content, it was decided to attempt to evolve an inoculum composed of strains which would satisfy the following basic requirements:-

They should be able to ferment the 'sugars' available in grass to lactic acid, with the formation of minimal amounts of other products. They should be capable of reducing the pH in silage to 4 or less as quickly as possible, and also of doing so at the temperatures normally encountered in farm silage. Extensive growth, (and survival) in relatively simple and inexpensive media would be essential. In addition, the different strains in a multiple inoculum should not be mutually antagonistic.

The efficiency of lactic acid production. The results of experiment K1 (p. 47) indicate that, of the lactic acid bacteria, homofermentative lactobacilli ferment sugars most efficiently to lactic acid, and therefore only these strains have been considered.

Growth/

Growth in relatively simple media. As a preliminary, 34 representative strains of homofermentative lactobacilli were grown in stab culture in tomato agar. Of these, 16 strains grew well in this medium. The remainder multiplied less actively, and were not studied further.

Ability to produce low pH in silage. Since this part of the work was carried out during winter, and no fresh grass was available, several different media were tried in screening tests for effective strains. The media used were as follows:-

Glucose, yeast water medium. Glucose, 1 g.; yeast water, 100 ml. The yeast water was prepared by autoclaving 5 g. brewer's yeast in 100 ml. water at 15 lb./sq. in. for 15 minutes.

Malt sprouts, molasses medium. Milled malt sprouts, 4 g.; molasses, 5 g.; water, 100 ml.

Dried grass medium. Milled dried grass, 2 g.; water, 100 ml.

Dried grass, molasses medium. Milled dried grass, 2 g.; molasses, 5 g.; water 100 ml.

None of the media were filtered. All were adjusted to pH 6 - 6.2, before sterilization at 15 lb./sq. in. for 15 minutes. This pH level approximates to that of fresh grass. Duplicate tubes of each medium were inoculated from young cultures and incubated for 5 days at 30°C when the pH was determined electrometrically.

It was noted that in the dried grass medium, a few strains reduced the pH to about 3.7 - 3.8, while the remainder did not reduce it below 4.2, and in some cases not below 5. No strain produced/

Table 26.

pH values of cultures of different strains of lactobacilli in grass extract medium after incubation for 1, 2, and 3 days at 30°C.

Strain	Period of incubation		
	1 day	2 days	3 days
F1	4.8	4.5	4.5
F2	4.8	4.5	4.5
L1	4.0	3.7	3.6
L19	4.5	4.0	3.8
L50	5.9	4.6	4.5
L57	4.4	4.4	4.4
L62	4.1	3.8	3.7
L63	4.2	3.8	3.7
L70	4.3	4.2	4.1
L71	4.4	4.4	4.4
L77	4.4	4.3	4.3
L78	3.9	3.8	3.8
L79	3.9	3.9	3.8
L80	4.6	4.6	4.6
L86	4.8	4.6	4.5
L88	4.6	4.6	4.6

produced a low pH in glucose yeast water medium, while practically all reduced the pH in the remaining two media to 4.2 or less. A medium containing dried grass without added sugar therefore appeared to provide a useful screening test for the selection of suitable strains. Consequently, the following medium, grass extract medium, was prepared. 20 g. of milled dried grass was thoroughly macerated in 100 ml. of tap water and allowed to stand for about 30 minutes. The liquid was then squeezed out of the 'pulp' by means of a fruit juice extractor, filtered, tubed, and autoclaved at 15 lb./ sq. in. for 15 minutes. The pH of the medium after sterilization was 5.5. Six tubes of this medium for each strain tested, were inoculated by loop from young cultures, and incubated at 30°C. After incubation for 1, 2 and 3 days, duplicate cultures were removed from the incubator and the pH of each was determined electrometrically. The average pH values of the duplicate cultures are given in table 26.

From these results it is obvious that certain strains produced a considerably lower pH in grass extract medium than others, viz. L1, L19, L62, L63, L78 and L79, and for this reason they were selected for use in the inoculum. It is interesting to note that of these strains, L19, was identified as L. casei while the remainder were regarded as typical strains of L. plantarum. The majority of the remaining strains which did not produce a very low pH in this medium were atypical forms of L. plantarum.

Ability/

Table 27.

Fermentation of substrates occurring in grass and silage by inoculum strains.

Substrate	Strain							
	L1	L19	L62	L63	L78	L79	L71	L80
Glucose	+	+	+	+	+	+	+	+
Fructose	+	+	+	+	+	+	+	+
Sucrose	+	+	+	+	+	+	+	+
Fructosan	+	+	+	+	-	-	•	•
Arabinose	+	-	+	+	+	+	+	+
Xylose	-	-	-	-	-	-	+	+

Ability to ferment the 'sugars' present in grass and silage.

Since the amount of 'sugar' available in grass may be a limiting factor in attaining a low pH in silage, it is obviously desirable that strains used for inoculation should be capable of fermenting all the principal 'sugars' which may occur.

The free sugars in ryegrass are mainly glucose, fructose and sucrose, while the 'storage' carbohydrate is a fructosan (Laidlaw & Reid, 1952). The hemicellulose fraction is mainly composed of araban and xylan (Harwood, 1953) from which xylose and arabinose might be liberated in silage.

Of the sugars mentioned, only xylose could not be fermented by any of the strains so far selected. For this reason, the xylose-fermenting strains L71 and L80 were also included with the strains to be used for inoculation.

The fermentation reactions of the eight strains on the carbohydrates mentioned above are shown in table 27. The ryegrass fructosan was kindly supplied by Dr. C. Wylam of the Chemistry Department, University of Edinburgh.

Ability to produce low pH in silage at different temperatures.

All eight strains could grow at 10°C or below. The maximum growth temperature for L1, L62 and L63 was approximately 40°C, for L78, L79, L70 and L81, approximately 42°C, and for L19, just under 45°C. Thus a temperature range of approximately 10 - 45°C was covered by the eight strains. In order to determine the behaviour of these strains in silage at different temperatures, the following inoculation experiment was carried out.

FIG. 17

GROWTH OF LACTOBACILLI IN SILAGE AT DIFFERENT TEMPERATURES

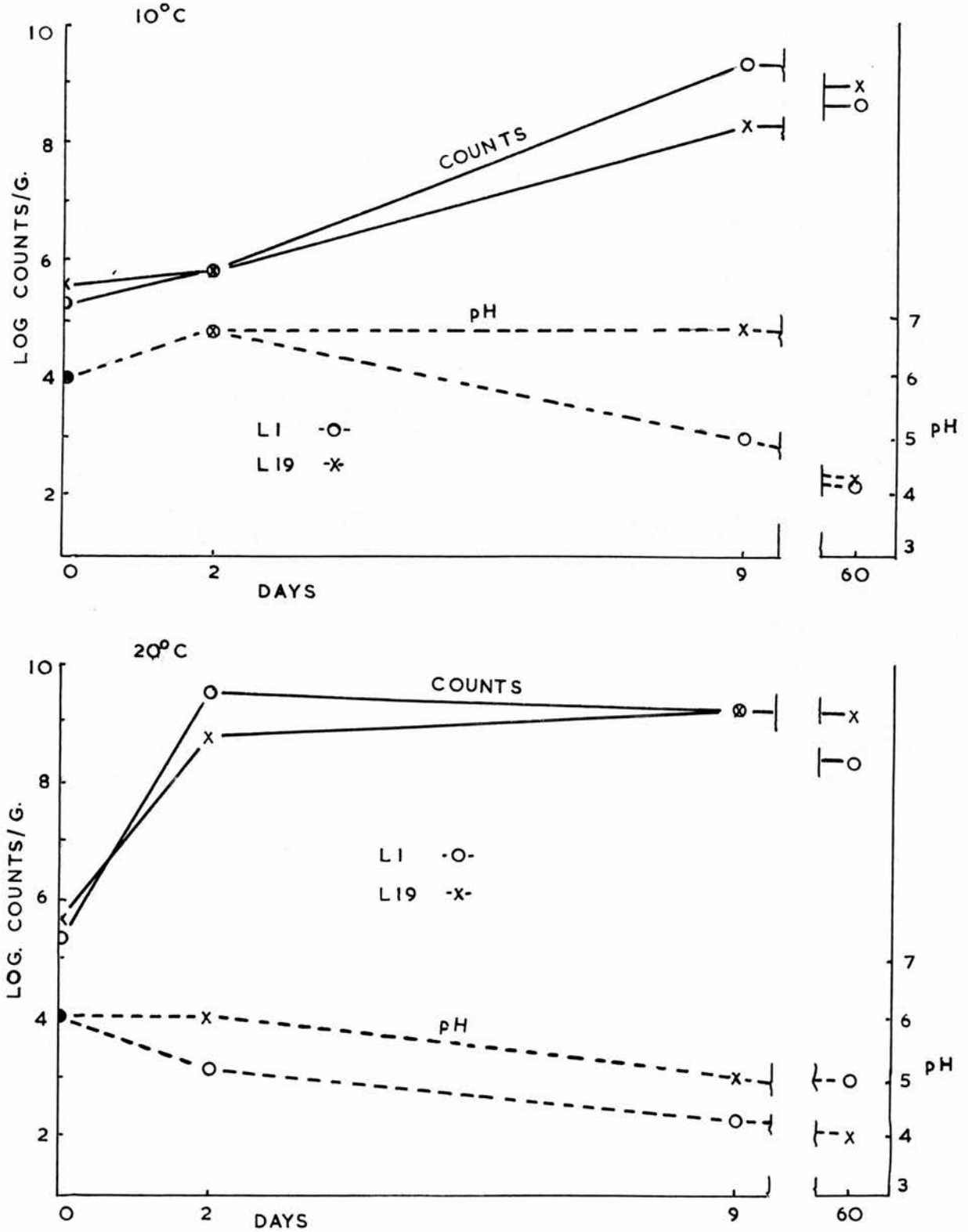
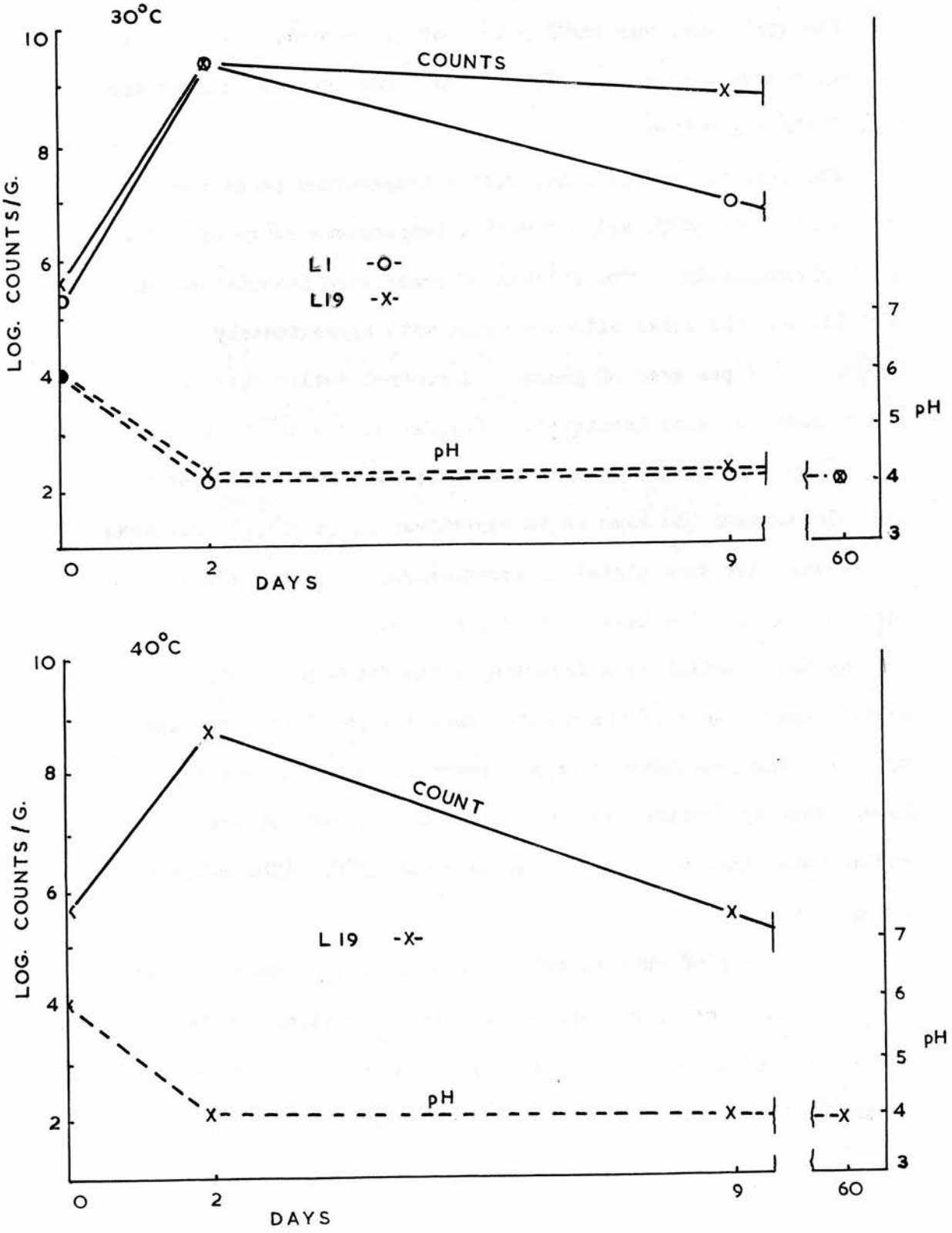


FIG. 18

GROWTH OF LACTOBACILLI IN SILAGE AT DIFFERENT TEMPERATURES



Growth of lactobacilli in silage at different temperatures.

Experiment K2.

The grass used was similar to that in experiment K1 (p.45). Its dry matter content was 20% and the crude protein content was 19% of the dry matter.

The strains used were L1, with a temperature range for growth of <10 - 40°C, and L19 with a temperature range of <10 - 45°C approximately. Two batches of grass were inoculated, one with L1, and the other with L19, each with approximately 200,000 cells per gram of grass. A control series with no inoculation was also incubated. Incubation was at 10, 20, 30 and 40°C. The methods used for inoculation and examination of the samples were the same as in experiment K1 (p. 45). Dilutions of the macerate were plated on acetate agar only, and examinations were made at 2 and 9 days, and at 2 months.

No lactobacilli were detected on the fresh grass and acetate agar counts of the control series were always very low or nil. The inoculated silages always had markedly lower pH levels than the control series, except in the case of the series inoculated with L1 and incubated at 40°C. (The maximum temperature for L1.)

The results of this experiment are shown in figures 17 and 18. At 10°C growth of both strains was very slow, but L1 reduced the pH more rapidly than L19. However, at the end of 2 months, the pH of both silages was 4 - 4.2.

At/

At 20°C growth was more rapid, but L19 still lagged considerably behind L1. At the final examination, the pH of the silage inoculated with L1 was approximately 5. The only explanation which can be suggested for this failure to reach a low pH is that there may have been a slow air leak into that tube, through a faulty mercury valve. However, the silage inoculated with L19 had reached pH 4 at 2 months.

Both strains behaved similarly at 30°C and the result was rapid multiplication with a correspondingly rapid drop in pH to 4.

With an incubation temperature of 40°C, L1 did not grow, but L19 rapidly lowered the pH of the material to 4.

These results show that these two strains are capable of producing a low pH in silage at temperatures from 10 - 40°C. However, the optimum temperature for producing a low pH quickly, appears to be about 30°C for L1, which has approximately the same temperature range as 6 of the 8 strains selected. The optimum temperature for L19 may be slightly higher.

Mutual antagonism. In order to ensure that none of the 8 strains had an antagonistic effect on any of the others, G.Y.A. plates were heavily seeded each with a single strain, and then spot inoculations of the other 7 strains were made on the surface of the agar. The plates were examined after incubation for 5 days at 30°C and no signs of inhibition were seen.

Thus, by means of various screening tests, 8 strains of/

of homofermentative lactobacilli were selected which, collectively, satisfied the requirements regarded as essential for an efficient inoculum. Examination of the characteristics of these 8 strains shows that some are probably identical, e.g. L1, L62 and L63. However it was thought that it might be advantageous to include all 8 strains in the inoculum to allow for any variations in growth rates at different temperatures, and also for possible variations in rates of fermenting different substrates. A relatively large number of strains would also be an advantage if 'phage infection ever occurred.

Having secured an apparently suitable multiple strain inoculum, it remained to design a simple but adequate medium for its propagation in large quantities.

Table 28.

Peak counts of inoculum strains in original malt sprouts molasses medium at 30°C.

Strain	Peak count (millions /ml.)
L1	690
"19	310
"62	500
"63	590
"78	990
"79	620
"71	270
"80	370

Average count /ml. = 500 millions (rounded figure).

Evolution of a medium for the propagation of inoculum strains.

From preliminary work it seemed that media containing malt sprouts, a by-product of the brewing industry, and molasses, a by-product of the sugar refining industry, might prove useful. Consequently, the first medium tried, had the following composition: milled malt sprouts, 4 g.; molasses, 5 g.; water, 100 ml. The pH was adjusted to 6.0 and 100 ml. quantities were dispensed in 6 oz. screw-capped bottles. This medium is referred to hereafter as the original malt sprouts molasses medium. Sterilization of this medium and of subsequent modifications of it was at 22½ lb./sq. in. momentarily. The bottles were inoculated with 1 ml. quantities of young cultures in the same medium. Incubation was at 30°C and plate counts were made daily for 3 days. The peak counts for each strain were usually reached at one day and are given in table 28.

The cell yields obtained were promising but unfortunately, the organisms died off very rapidly, and the counts after incubation for 3 days at 30°C were usually about 1 - 2 millions/ml. It was thought that this was possibly due to the poor buffering capacity of the medium, and that addition of a suitable buffer might prove beneficial. Since cultures were to be added to the grass by means of a sprayer in large-scale experiments, an extract of malt sprouts was used in all subsequent modifications of the medium. Malt sprouts extract was prepared as follows:- The malt sprouts were milled and the appropriate amount was steamed/

steamed in tap water for 30 minutes. The 'mash' obtained was then squeezed through muslin and made up to the original volume with water.

Modifications of the medium were tested with the object of improving the yield of cells. The modified media were dispensed in 100 ml. quantities in 6 oz. screw-capped bottles and were inoculated with 1 ml. of a young culture in the original malt sprouts molasses medium. L1 and L78 were used for these tests. Incubation was generally for 3 days, and plate counts were made daily.

The effect of added buffer. Addition of 1% K_2HPO_4 to the medium effectively countered the rapid death-rate which occurred in the medium with no added buffer. The control medium used in subsequent tests had therefore the following composition:- Extract from 4g. malt sprouts, 100 mL.; molasses, 5 g.; K_2HPO_4, 1 g.; water to 100 ml. The pH was adjusted to 6.0.

This medium gave lower peak counts than the original malt sprouts molasses medium probably because the malt sprouts solids were eliminated.

The effect of temperature. The temperatures used were, 20, 25, 30 and 35°C. Greater yields of viable cells were obtained at 25°C than at higher temperatures, and the organisms did not die as quickly at the lower temperature. At 20°C the peak counts were as high as those obtained at 25°C but multiplication was slower. For all subsequent experiments, the cultures were incubated at 25°C.

The/

The effect of pH. Different batches of medium were adjusted to pH levels in the range 5 - 7 in 0.5 unit steps. Similar results were obtained in the range pH 5 - 6 but a pH of 7 resulted in slower initial development although the peak counts reached were the same as for the lower pH levels. It appeared therefore that a pH level of 6, which had been originally used, was suitable.

The effect of different concentrations of molasses. The concentrations tried were 0, 0.1%, 0.25%, 0.5%, 1%, 2% and 10%. There was little difference in count with the different concentrations of molasses, which suggested that malt sprouts extract contained an adequate supply of fermentable substrate. However, as a safety measure, 1% molasses was added to all subsequent modifications.

The effect of added manganese. In view of the findings of Orla-Jensen (1919) and Demeter (1949), it was thought that manganese might be deficient in malt sprouts extract molasses media. Addition of 14.2 mg. manganous sulphate/litre resulted in a small increase in the yield of viable cells. However, this increase was not considered to be sufficiently large to justify the routine addition of manganese to the medium.

The effect of different concentrations of malt sprouts extract. Only two concentrations were compared, 4% and 20%. The higher concentration always gave markedly higher counts than the lower concentration and so 20% malt sprouts extract was used thereafter.

The/

Table 29.

Counts of inoculum strains in B.M.M. medium after incubation for 2 days at 25°C.

Strain	Count (millions /ml., rounded figures)
L1	1,500
"19	2,400
"62	1,000
"63	1,200
"78	1,400
"79	700
"71	200
"80	200

Average count = 1,000 millions /ml.

The final medium evolved, buffered, malt sprouts extract, molasses medium, referred to hereafter as B.M.M. medium had the following composition:- K_2HPO_4, 1 g.; molasses, 1 g.; 100 ml. extract from 20 g. malt sprouts (see p. 99). Adjusted to pH 6.

Inoculation was from young cultures in the same medium and an inoculum of 2% was found to give maximum numbers of viable cells after 1 - 2 day's incubation at 25°C. The 8 strains were grown separately and equal volumes of the different cultures were bulked and mixed before inoculation of the grass.

The maximum yields of viable cells for the different strains grown in this way are given in table 29. It can be seen that the final medium evolved gives an average yield of approximately 1,000 millions/ml. which is double that of the original malt sprouts molasses medium (table 28).

Survival of inoculum strains in B.M.M. medium. When cultures in B.M.M. medium were incubated for 2 days at 25°C and then stored at 10 - 12°C, it was found that the count of viable cells was not significantly reduced up to 7 days. At 14 days, a slight reduction in numbers was noted for some of the strains, while L71 and L80 were considerably reduced in numbers. One strain (L19) was stored for 7 weeks without a significant reduction in numbers.

It was considered that the ability of the inoculum strains to survive in B.M.M. medium was sufficiently good for the present work.

Inoculation experiments with the multiple-strain inoculum.

The inoculum was tested on a laboratory scale in experiment 15 (treatment F). This work has already been described (p. 39) but the results will be briefly summarized here. The grass used had a crude protein content of 21% (on a dry matter basis) and would therefore be regarded as being difficult to ensile. However, the pH of the inoculated material had fallen to 4.0 in 2 days, and was 3.7 at 8 days, while that of the control series never fell below 5 at any stage. It is obvious from these results that the inoculum was efficient in producing silage of low pH.

Brief mention may be made of the results of a silage experiment conducted in cooperation with the Chemistry Department of the University of Edinburgh. (Experiment 16)

Grass was taken from the same plot as that used in experiment 15, but one week later. It had a crude protein content of 27% (on a dry matter basis). The methods of ensilage and examination were essentially the same as used previously (p. 27). One series was inoculated with the multiple inoculum (300,000/g. grass), and a control series with no treatment was also set up. Incubation was at 30°C.

The pH of the inoculated silage fell to 4.4 in 1 day, but remained at this level up to 3 days, and rose to 5.0 at 8 days. Information on the 'sugar' content of the material (kindly supplied by Dr. C. Wylam and Dr. V. Harwood), showed that the glucose, fructose, sucrose and fructosan were initially present only/

only in low concentrations, and that little sugar remained at 24 hr. and none at 3 days. Thus, inability of the inoculum to reduce the pH of the silage to 4 was obviously due to lack of fermentable substrate.

Inoculation experiments in small concrete silos.

The work to be described was a series of 'team' experiments carried out on a semi-field scale. Only one aspect of this work will be considered here, namely the effect of inoculation as judged by pH.

Methods. The 'silos' used in these experiments were concrete cylinders and were of two sizes. The small silos were 3 ft. high and 3 ft. in diameter, while the large silos were 6 ft. high and 4 ft. in diameter. Provision was made for drainage, and during filling the material was thoroughly consolidated by tramping. 4 cwt. of material was put in the small silos, and 15 cwt. in the large ones. Sealing was effected by means of 'Sisalkraft' paper covered by a layer of soil. (6 cwt. for the large, and 4 cwt. for the small silos.) The silos were finally covered with tarpaulins or galvanized iron 'lids'. In addition, tube silos were filled with similar material to that used in the concrete silos and incubated at 30°C in the laboratory.

The concrete silos were thoroughly disinfected with lysol a few days before filling, and uninoculated material was always filled, and the silos sealed, before inoculation was started. The pH figures given in the tables are the average results of a relatively large number of samples taken at different points throughout each silo.

In all experiments, the silos were opened and sampled about 3 - 4 months after filling.

Experiment 11.

Material Spring-cut grass-clover mixture, dry matter 21%, and crude protein 12.8% of the dry matter.

Treatments

1. No treatment.
2. Uncut + molasses.
3. Uncut + inoculum.
4. Uncut + inoculum + molasses.
5. Lacerated.
6. Lacerated + molasses.
7. Lacerated + inoculum.
8. Lacerated + inoculum + molasses.

Additions

Molasses 2 gal. diluted with water, or water + culture, to 4 gal., added per ton. Applied by spray.

Inoculum 3 litres multiple inoculum in B.M.M. medium, diluted with water, or water + molasses, to 4 gal., added per ton. Applied by spray.

Laceration The fodder was passed through a Robust chopper.

As far as possible the different treatments were replicated in (a) large silos (b) small silos and (c) laboratory silos.

The acetate agar count of the fresh grass was 30/g.. 4 cultures were isolated but unfortunately three died before they could be examined. The one remaining was a heterofermentative streptococcus but it is considered likely that the other three were lactobacilli.

Table 30.

Experiment 11.

Average pH values.
(at 3 months)

Large silos.	Control.	Inoculated.
Uncut	4.0	3.6
" + molasses	3.9	3.6
Lacerated	4.0	3.6
" + molasses	4.0	3.6
Small silos.		
Uncut	•	•
" + molasses	3.9	3.6
Lacerated	3.9	3.7
" + molasses	4.1	3.7
Laboratory silos.		
Uncut	4.6	3.5
" + molasses	4.1	3.5
Lacerated	4.0	3.5
" + molasses	4.1	3.5

Results

All the silages made in the concrete containers were well preserved having pH levels of 4 or below. However, the pH levels of the inoculated silages were always 0.3 - 0.4 units below those of the uninoculated silages, and this is considered to be a significant difference.

There was no appreciable difference between the silages made in the large concrete silos, and those made in the small ones, in corresponding treatments.

The laboratory silos gave results similar to the concrete ones, but the differences between different treatments were more pronounced. This was possibly a result of incubation at 30°C, since the temperature in the concrete silos probably did not rise above 18°C. (The temperatures were not recorded in this experiment, but in a later one were found to be about 16°C, and 18°C was the highest recorded.)

That the material used in this experiment had a relatively low buffering capacity combined with a high content of fermentable carbohydrates, is indicated by the very low pH levels reached in some of the silages (pH 3.5 - 3.6). The good results obtained in the concrete silos can therefore be attributed to the favourable sugar/protein ratio of the initial material.

Experiment 12.

Material Autumn-cut grass-clover mixture, dry matter 18.3% and crude protein 16.4% of the dry matter.

Since the small silos gave the same results as the large silos in experiment 11, only the former were used in this and the next experiment. The material was not lacerated.

Treatments

1. No treatment.
2. + water.
3. + inoculum.
4. + water + inoculum.
5. Untreated, uncovered.
6. + inoculum, uncovered.

Addition of water at the rate of 1 gal./cwt. was made to the material in the silo as it was being filled.

Addition of culture was the same as in Experiment 11. The two silos which were 'uncovered' were sealed in the usual way with Sisalkraft paper and soil, but were not covered by a tarpaulin. Consequently, rain water entered the silos, percolated through the silage and escaped via the effluent pipes. The effluent figures are included in table 31 to show the extent of this leeching action.

Acetate agar counts of the fresh material were not done.

Table 31.

Experiment 12.

Average pH values and amounts of effluent.
(at 3 months)

Small silos	pH.		Effluent (pints).	
	Control	Inoculated	Control	Inoculated
Untreated	4.9	4.4	13	14
Wetted	4.9	4.3	37	28
Uncovered	5.0	4.3	193	184

Laboratory silos	pH	
	Control	Inoculated
Untreated	5.7	•
Wetted	4.2	4.0

Results

The only silage which reached pH 4 in this experiment was the wetted, inoculated material in the laboratory silo. This suggests that the 'sugar' content of the fresh material was just enough to allow of good acidification under the near-ideal conditions of the laboratory silo. The pH levels of the silages in the concrete containers were never quite as low as 4, but those of the inoculated material were always 0.4 - 0.7 units below those of the uninoculated material.

Unfortunately, analysis of the 'sugar' content was not done, but it seems likely that failure to reach a pH level of 4 or below was due to lack of sufficient fermentable substrate.

Experiment 14

Material Summer-cut, grass-clover mixture, dry matter 13%, and crude protein 22.6% of the dry matter.

Treatments

1. Untreated.
2. + molasses.
3. + inoculum.
4. + inoculum + molasses.
5. Untreated, weighted.
6. + inoculum, weighted.
7. Untreated, not drained.
8. + inoculum, not drained.

None of the material was lacerated.

'Weighted' silos had a thick wooden platform inside the silo resting on top of the silage (+ earth). Approximately one ton of granite 'sets' were built on top of the wooden platforms, and the whole structure was covered with a tarpaulin.

The effluent outlets of two silos were sealed so that no drainage occurred. Other treatments were the same as in Experiment 11.

The acetate agar count of the fresh material was 101/g.. 4 cultures were isolated and identified as lactobacilli. Dilutions of the molasses were also plated on acetate agar, and the count obtained was 380/g. 4 colonies were picked and identified as lactobacilli.

Table 32.

Experiment 14.

Average pH values.
(at 3 months)

Small silos.	Control.	Inoculated.
Untreated	4.9	4.9
Molassed	4.3	4.7
Weighted	4.6	4.2
Undrained	5.0	4.9
Laboratory silos.		
Untreated	5.5	4.5
Molassed	4.7	5.1

Results

In this experiment, only the weighted inoculated material had a pH at about the critical level for good preservation. In all other treatments inoculation had no noticeable effect, even where molasses was also added. However, analysis of the molassed inoculated material (kindly carried out by Dr. C. Wylam) revealed that sugars and fructosan were completely exhausted.

Thus it would appear that the material used (C.P. 23%) was initially very low in sugars, and that addition of 20 lb. molasses per ton was insufficient for adequate acidification.

Discussion

It has been emphasized repeatedly in this work that lactobacilli were frequently scarce on the samples of fresh grass examined. This observation has been confirmed by Stirling (1953), who investigated 155 samples of various green crops using the same methods as those employed in this work (p. 25 *et seq.*).

There is also some evidence that the few lactobacilli which have been isolated from grass were of unsuitable type, i.e. were heterofermentative, or were unable to produce a low pH in grass extract medium. A few typical strains of *L. plantarum* and one of *L. casei* isolated from silage, were the only organisms which produced a pH of less than 4 in this medium. Their behaviour in grass extract medium is considered to be indicative of their value in silage. Thus efficient strains of lactobacilli would appear to be of very infrequent occurrence on fresh grass.

This evidence in itself would seem to be sufficient justification for the inoculation of grass with efficient strains of lactobacilli before ensilage. However, the grass samples examined in this work, and also those investigated by Stirling (1953), were taken aseptically from the growing crop. When silage is made on the farm, the material is normally subjected to considerable mechanical manipulation before actual ensilage, and such treatment might further contaminate the fodder with lactobacilli. Also, it has been shown by

Kroulik/

Kroulik (1953), that considerable increases (5 - 8 fold) in bacterial numbers may occur in the short period between cutting and ensiling a crop. In addition, there is some evidence that molasses, a frequent addition to silage, may be a source of lactobacilli, although the efficiency of such strains has not been studied.

Nevertheless, despite these additional considerations, it seems likely that low incidence of efficient lactobacilli on the crop as it is ensiled, may sometimes be a limiting factor in the production of silage of low pH particularly from material of high crude protein content.

On a laboratory scale, inoculation resulted in the rapid acidification of the material when adequate supplies of fermentable substrates were present.

Semi-field-scale experiments have yielded some evidence that inoculation is beneficial but the results are not completely conclusive. The main reason for this seems to be that where material of high crude protein content has been used, the supply of fermentable substrates was not sufficient for adequate acidification. Even when molasses was added at the rate of 20 lb./ton, a normal application in farm practice, silage of low pH was not produced, apparently through exhaustion of the 'sugars' before the critical pH level was reached.

SUMMARY.

Summary

The objects of the work reported in this thesis were (a) to develop a medium for the enumeration and isolation of lactobacilli, (b) to study the growth of these organisms in silage made under controlled conditions in the laboratory, (c) to characterize the lactobacilli isolated from this silage, and (d) to assess the value of inoculating grass with lactobacilli before ensilage.

(a) The first step was the development of a medium which was considered to be nutritionally adequate for all silage lactobacilli. This medium, tween agar, contained lemco, peptone, glucose, yeast autolysate, tomato extract, tween 80 and agar, and was adjusted to pH 5.4. Various substances were added to tween agar and examined for their value as selective inhibitors. They included citrate, lactate, acetate, propionate and glycollate, ethyl alcohol, and glucose in high concentrations. Of these the most efficient was found to be acetate in a concentration of 0.2 M. It allowed a large majority of silage lactobacilli to grow more or less quantitatively, while inhibiting the majority of other silage bacteria. The final medium, acetate agar, was prepared by adding the requisite amount of 2 M. sodium acetate/acetic acid buffer at pH 5.4 to molten tween agar just before plating. Double layer plates were used. The only organisms which in this medium could produce colonies comparable in size to those of lactobacilli, were heterofermentative streptococci/

streptococci and a few micrococci (pediococci ?).

(b) Acetate agar was used to trace the development of lactobacilli (and heterofermentative streptococci) in silage made from grass subjected to different treatments. It was noted that lactobacilli were frequently scarce on freshly harvested grass. In the silage there was frequently an initial proliferation of heterofermentative streptocci; these organisms were then gradually replaced by lactobacilli. During the period of active fermentation, heterofermentative lactobacilli occurred with greater frequency than is indicated by the previous literature.

In general, a temperature of 22°C seemed to favour heterofermentative streptococci at the expense of lactobacilli, while at 40°C only the latter types were recovered. The highest acetate agar counts were obtained with material incubated at 30°C, but usually the lowest pH values resulted when the silage was incubated at 40°C.

Laceration (mincing) promoted much more rapid and extensive bacterial multiplication, but appeared to have a particularly favourable effect on lactobacilli. This resulted in silage with a lower pH level than with any other treatment except inoculation.

Addition of water had a general stimulatory effect on bacterial growth, while wilting had the opposite effect.

Inoculation with lactobacilli was the only treatment which invariably resulted in silage with a pH of less than 4. When

grass/

grass rich in crude protein was inoculated with single cultures representative of four major groups of lactic acid bacteria, only a homofermentative lactobacillus reduced the pH of the material to 4. A higher pH resulted when this organism was used in mixed culture with heterofermentative lactic acid bacteria.

(c) The characters of 61 strains of lactobacilli isolated from experimental silages were examined in an attempt to identify the organisms. The criteria found to be of most value were, production of CO_2 from glucose, growth at 15 and 45°C, and colony type. Sugar fermentation reactions were also of use.

The principal homofermentative types isolated were identified as L. plantarum and L. acidophilus, but a few strains of L. casei were also isolated. Typical strains of L. plantarum occurred rather infrequently in the experimental silages. Some of the remaining organisms studied were similar to motile lactobacilli which have been reported to occur occasionally in A.I.V. silage. The others were similar to a type reported to occur in the caecal contents of turkeys.

A large majority of the heterofermentative strains could be placed in two groups. The organisms of the first group, comprising 50% of the heterofermentative types isolated, were closely related to L. fermenti. Those of the second group corresponded most closely with L. buchneri. The remaining few strains were unusual in several respects but two of them appeared/

appeared to be degenerate forms of L. brevis.

(d) Preliminary trials of the addition of lactobacilli to grass before ensilage having yielded promising results, further work on inoculation was undertaken. A n 8-strain inoculum was developed, in which the component strains were selected for their ability to produce low pH in grass extract medium, and to ferment the predominant 'sugars' occurring in grass and silage. These strains collectively were capable of growth in the range 10 - 45°C.

A medium was developed in which the inoculum strains could be propagated relatively cheaply.

On a laboratory scale, inoculation of grass rich in protein with the 8-strain inoculum yielded silage of pH less than 4 provided that the grass contained an excess of fermentable substrates.

Three semi-field scale trials in small concrete silos yielded rather inconclusive results. One batch of protein rich grass, when inoculated, yielded silage of lower pH than similar material without inoculation, but a pH of 4 was not reached. The reason for this failure was probably the low 'sugar' content of the grass-clover mixture ensiled. Even when molasses (+ inoculum) was added at the rate of 20 lb./ton the resultant silage had not a low pH. Analysis of this silage showed that 'sugars' had been completely exhausted.

APPENDIX.

Appendix.

Tomato extract

1½ lb. tomatoes were steamed in 300 ml. tap water for 30 min. The resultant pulp was then filtered. Frequently, bottled tomatoes (1½ lb. in 300 ml. water) were used in place of fresh fruit.

Yeast autolysate

1 lb. brewer's yeast was suspended in 500 ml. tap water and incubated at 50°C for 24 hr. The resultant autolysate was filtered, bottled, and sterilized in the autoclave at 22½ lb./sq. in. momentarily.

Buffered tween broth (B.T.B.)

Peptone (Evan's), 1 g.; lemco, 1 g.; glucose, 0.25 g.; K_2HPO_4, 0.5 g.; yeast autolysate, 5 ml.; tomato extract, 10 ml.; tween 80, 0.05 ml.; pH 6.0.

Glucose yeast autolysate broth (G.Y.B.)

Peptone (Evan's), 1 g.; lemco, 1 g.; glucose, 0.5 g.; yeast autolysate, 5 ml.; pH 6.0.

The above two media were sterilized in the autoclave at 15 lb./sq. in. for 15 min.

Table 33.

The effect of temperature. (see p.35).

Acetate agar counts $\times 10^6$/g. dry matter.

Incubation period	Treatment	Experiment. 7	8	9	10	15
Fresh grass		<0.001	<0.001	<0.001	0.005	<0.001
1 day	22°C	0.09	<0.003	0.08	44.0	<0.001
	30°C	0.01	<0.003	2.1	2,800.0	0.38
	40°C	0.07	<0.003	25.0	85.0	0.02
2 days	22°C	6.6	<0.003	3.7	1,400.0	0.14
	30°C	12.0	<0.003	29.0	2.100.0	8.7
	40°C	0.32	<0.001	24.0	320.0	1.0
3 days	22°C	38.2	<0.003	5.4	1,400.0	0.4
	30°C	230.0	<0.003	22.0	2,000.0	16.6
	40°C	2.1	<0.003	220.0	490.0	3.0
7 or 8 days	22°C	3.9	270.0	3.6	2,100.0	<0.003
	30°C	72.0	0.038	69.0	1,200.0	17.0
	40°C	9.0	<0.001	76.0	28.0	0.04
6 months	22°C	0.61	1.2	4.7	220.0	0.32
	30°C	0.22	270.0	2.1	<0.002	3.0
	40°C	<0.001	<0.001	<0.001	<0.001	<0.001

Table 34.

The effect of temperature. (see p.35).

G.Y.A. counts x 10^6/g. dry matter.

Incubation period	Treatment	Experiment				
		7	8	9	10	15
Fresh grass		9.1	61.0	220.0	35.0	2.6
1 day	22°C	3.0	4.0	97.0	480.0	<2.9
	30°C	49.0	230.0	160.0	3,800.0	<2.9
	40°C	4.9	13.0	28.0	430.0	17.4
2 days	22°C	190.0	1,600.0	1,500.0	4,800.0	<2.9
	30°C	1,400.0	1,100.0	2,100.0	23,000.0	87.0
	40°C	43.0	230.0	140.0	3,800.0	9.0
3 days	22°C	2,900.0	8,700.0	3,700.0	4,000.0	23.0
	30°C	820.0	180.0	1,200.0	2,300.0	260.0
	40°C	63.0	5.3	360.0	1,000.0	17.0
7 or 8 days	22°C	1,000.0	1,100.0	1,600.0	2,500.0	960.0
	30°C	140.0	5.5	140.0	1,300.0	81.0
	40°C	15.0	270.0	670.0	66.0	2.0
6 months	22°C	1.1	1.5	1,000.0	1,600.0	15.0
	30°C	2.5	2,700.0	2.4	2.1	2.0
	40°C	0.027	0.01	<0.001	0.005	<0.001

Table 35.

The effect of laceration. (see p.37).

Acetate agar counts x 10^6/g. dry matter.

Incubation period	Treatment	Experiment				
		7	8	9	10	15
Fresh grass		<0.001	<0.001	<0.001	0.005	<0.001
1 day	unchopped	<0.001	<0.001	0.76	280.0	0.008
	control	0.01	<0.003	2.1	2,800.0	0.38
	minced	2,100.0	<0.27	1,200.0	6,200.0	16.0
2 days	unchopped	77.0	0.11	2.1	280.0	0.1
	control	12.0	<0.003	29.00	2,100.0	8.7
	minced	5,900.0	<0.27	2,200.0	6,900.0	4,500.0
3 days	unchopped	0.027	0.05	16.0	1,400.0	0.002
	control	230.0	<0.003	22.0	2,000.0	17.0
	minced	2,300.0	75.00	4,200.0	1,400.0	6,900.0
7 or 8 days	unchopped	<0.027	<0.001	24.0	1,200.0	96.0
	control	72.0	0.038	69.0	1,200.0	17.0
	minced	880.0	540.0	2,000.0	4.9	2,500.0
6 months	unchopped	<0.002	<0.003	2.6	0.1	<0.001
	control	0.22	270.0	2.1	<0.002	3.0
	minced	1.1	<0.001	<0.001	<0.001	<0.001

Note: The material in the control series was chopped in 1" lengths.

Table 36.

The effect of laceration. (see p.37).

G.Y.A. counts x 10^6/g. dry matter.

Incubation period	Treatment	Experiment				
		7	8	9	10	15
Fresh grass		9.1	61.0	220.0	35.0	2.6
1 day	unchopped	20.0	210.0	100.0	2,600.0	<2.9
	control	49.0	230.0	160.0	3,800.0	<2.9
	minced	4,600.0	1,800.0	3,900.0	9,100.0	270.0
2 days	unchopped	200.0	610.0	2,900.0	4,300.0	2.9
	control	1,400.0	1,100.0	2,100.0	23,000.0	87.0
	minced	7,400.0	280.0	5,200.0	20,000,0	5,100.0
3 days	unchopped	45.0	480.0	1,400.0	2,300.0	46.0
	control	820.0	180.0	1,200.0	2,300.0	260.0
	minced	2,900.0	73.0	4,900.0	2,000.0	5,900.0
7 or 8 days	unchopped	45.0	0.40	220.0	780.0	110.0
	control	140.0	5.5	140.0	1,300.0	81.0
	minced	1,100.0	2,700.0	1,600.0	5.5	1,800.0
6 months	unchopped	0.44	0.007	3.1	2.2	15.0
	control	2.5	2,700.0	2.4	2.1	15.0
	minced	0.03	0.27	<0.002	0.005	0.003

Note: The material in the control series was chopped in 1" lengths.

Table 37.

The effect of moisture. (see p.38).

Acetate agar counts x 10^6/g. dry matter.

Incubation period	Treatment	Experiment 7	8	9	10	15
Grass	fresh	<0.001	<0.001	<0.001	0.005	<0.001
	wilted	<0.001	<0.001	<0.001	<0.001	<0.001
1 day	wetted	950.0	57.0	270.0	890.0	0.38
	control	0.01	<0.003	2.1	2,800.0	0.38
	wilted	<0.01	<0.001	0.001	190.0	0.19
	wilted + water	4.2	0.055	<0.001	3,200.0	0.60
2 days	wetted	1,600.0	0.59	2,600.0	1,200.0	1,500.0
	control	12.0	<0.003	39.0	2,100.0	8.7
	wilted	<0.01	<0.001	0.009	190.0	3.5
	wilted + water	210.0	<0.035	37.0	3,200.0	46.0
3 days	wetted	5,700.0	<0.3	380.0	1,600.0	1,500.0
	control	230.0	<0.003	22.0	2,000.0	17.0
	wilted	0.29	<0.001	1.5	190.0	3.5
	wilted + water	0.08	<0.001	230.0	2,300.0	1,000.0
7 or 8 days	wetted	58.0	14.0	1,100.0	790.0	2,100.0
	control	72.0	0.038	69.0	1,200.0	17.0
	wilted	0.31	0.35	0.33	310.0	130.0
	wilted + water	<0.27	<0.001	170.0	1,000.0	500.0
6 months	wetted	<0.004	0.063	0.3	<0.001	<0.001
	control	0.22	270.0	2.1	<0.002	2.1
	wilted	0,002	<0.001	0.008	<0.001	2.2
	wilted + water	<0.002	<0.001	<0.001	2.1	0.1

Table 38.

The effect of moisture. (see p.38).

G.Y.A. counts x 10^6/g. dry matter.

Incubation period	Treatment	Experiment				
		7	8	9	10	15
Grass	fresh	9.1	61.0	220.0	35.0	2.6
	wilted	4.4	0.038	180.0	310.0	1.0
1 day	wetted	1,600.0	1,000.0	4,500.0	5,800.0	830.0
	control	49.0	230.0	160.0	3,800.0	<2.9
	wilted	0.08	12.0	12.0	460.0	<2.6
	wilted + water	31.0	630.0	1,500.0	4,800.0	9.8
2 days	wetted	1,900.0	150.0	4,000.0	3,500.0	2,000.0
	control	1,400.0	1,100.0	2,100.0	23,000.0	87.0
	wilted	2.1	210.0	7.4	2,800.0	<2.0
	wilted + water	290.0	220.0	3,200.0	6,500.0	780.0
3 days	wetted	7,900.0	19.0	1,400.0	1,300.0	2,400.0
	control	820.0	180.0	1,200.0	2,300.0	260.0
	wilted	1.1	20.0	11.0	2,800.0	130.0
	wilted + water	0.44	45.0	2,800.0	2,900.0	1,200.0
7 or 8 days	wetted	63.0	26.0	3,600.0	510.0	1,700.0
	control	140.0	5.5	140.0	1,300.0	81.0
	wilted	54.0	120.0	8.0	460.0	51.0
	wilted + water	<0.27	0.77	230.0	810.0	360.0
6 months	wetted	0.09	0.054	0.02	<0.001	6.2
	control	2.5	2,700.0	2.4	2.1	2.1
	wilted	0.2	0.98	1.8	0.02	1.1
	wilted + water	<0.027	0.01	0.85	2.2	0.17

Table 39.

The effect of inoculation. (see p.39).

Acetate agar counts x 10^6/g. dry matter.

Incubation period	Treatment	Experiment			
		8	9	10	15
Grass	fresh	<0.001	<0.001	0.005	<0.001
	inoculated	26.0	31.0	0.095	11.0
1 day	control	<0.003	2.1	2,800.0	0.38
	inoculated	8,400.0	9,900.0	7,400.0	13,000.0
2 days	control	<0.003	29.0	2,100.0	8.7
	inoculated	14,000.0	13.000.0	14,000.0	15,000.0
3 days	control	<0.003	22.0	2,000.0	17.0
	inoculated	11,000.0	10,000.0	14,000.0	19,000.0
7 or 8 days	control	0.038	69.0	1,200.0	17.0
	inoculated	1,600.0	3,100.0	4,800.0	2,500.0
6 months	control	270.0	2.1	<0.002	2.1
	inoculated	<0.003	1.5	1.2	1.5

Table 40.

The effect of inoculation. (see p.39).

G.Y.A. counts x 10^6/g. dry matter.

Incubation period	Treatment	Experiment			
		8	9	10	15
Grass	fresh	61.0	220.0	35.0	2.6
	inoculated	514.0	160.0	39.0	.
1 day	control	230.0	160.0	3,800.0	<2.9
	inoculated	13,000.0	9,000.0	8,500.0	13,000.0
2 days	control	1,100.0	2,100.0	23,000.0	87.0
	inoculated	15,000.0	11,000.0	11,000.0	21,000.0
3 days	control	180.0	1,200.0	2,300.0	260.0
	inoculated	13,000.0	6,700.0	9,600.0	13,000.0
7 or 8 days	control	5.5	140.0	1,300.0	81.0
	inoculated	1,800.0	3,300.0	2,500.0	2,900.0
6 months	control	2,700.0	2.4	2.1	2.1
	inoculated	0.16	1.7	1.6	1.7

Table 41.

The effect of temperature. (see p.35).

pH values.

Incubation period	Treatment	Experiment				
		7	8	9	10	15
Fresh grass		6.2	6.2	6.2	6.2	6.4
1 day	22°C	6.5	6.1	6.2	6.3	6.9
	30°C	6.4	6.5	6.1	5.9	6.9
	40°C	6.0	5.4	6.1	5.9	6.4
2 days	22°C	6.5	6.2	6.0	5.8	6.9
	30°C	6.3	5.3	6.2	5.1	6.8
	40°C	5.7	5.0	5.8	5.5	5.6
3 days	22°C	6.2	6.2	6.3	5.8	6.9
	30°C	5.9	5.2	6.1	5.1	6.0
	40°C	5.0	4.9	5.5	5.0	5.3
7 or 8 days	22°C	6.1	5.7	6.1	4.9	6.6
	30°C	5.7	5.1	5.8	4.6	5.3
	40°C	4.7	4.8	5.4	4.5	4.9
6 months	22°C	5.2	5.0[x]	5.1	5.3	5.1
	30°C	5.3	7.0[x]	4.5	4.2	4.9
	40°C	4.6	4.3	4.3	4.2	4.8

[x] mould growth in tube.

Table 42.

The effect of laceration. (see p.37).

pH values.

Incubation period	Treatment	Experiment				
		7	8	9	10	15
Fresh grass		6.2	6.2	6.2	6.2	6.4
1 day	unchopped	6.5	6.5	6.1	6.0	7.0
	control	6.4	6.5	6.1	5.9	6.9
	minced	5.6	6.0	5.2	4.7	6.6
2 days	unchopped	6.5	5.7	6.2	5.5	6.9
	control	6.3	5.3	6.2	5.1	6.8
	minced	4.5	4.8	4.4	4.3	4.8
3 days	unchopped	5.1	5.2	6.1	5.2	6.8
	control	5.9	5.2	6.1	5.1	6.0
	minced	4.3	4.6	4.1	4.2	4.3
7 or 8 days	unchopped	5.4	4.9	5.7	4.9	6.2
	control	5.7	5.1	5.8	4.6	5.3
	minced	4.4	4.3	3.8	4.2	4.2
6 months	unchopped	5.0	4.9[x]	4.6	4.2	5.1
	control	5.3	7.0[x]	4.5	4.2	4.9
	minced	4.6	4.3	3.8	4.0	4.3

[x] mould growth in tube.

Note: The material in the control series was chopped in 1" lengths.

Table 43.

The effect of moisture. (see p.38).

pH values.

Incubation period	Treatment	Experiment				
		7	8	9	10	15
Grass	fresh	6.2	6.2	6.2	6.2	6.4
	wilted	6.3	6.2	6.1	•	6.4
1 day	wetted	5.4	5.0	5.9	5.4	6.6
	control	6.4	6.5	6.1	5.9	6.9
	wilted	6.3	6.4	6.0	6.3	6.9
	wilted + water	6.1	6.1	6.2	5.3	6.9
2 days	wetted	4.7	5.3	5.0	4.8	5.5
	control	6.3	5.3	6.2	5.1	6.8
	wilted	6.4	6.4	6.1	6.3	6.9
	wilted + water	4.8	5.5	5.7	4.5	5.5
3 days	wetted	4.5	5.3	4.1	4.7	5.2
	control	5.9	5.2	6.1	5.1	6.0
	wilted	6.2	6.3	5.9	6.1	6.6
	wilted + water	4.8	5.0	4.9	4.8	5.5
7 or 8 days	wetted	4.7	4.6	4.3	4.4	4.7
	control	5.7	5.1	5.8	4.6	5.3
	wilted	6.3	6.2	5.8	5.5	5.7
	wilted + water	4.8	4.8	5.1	4.4	5.0
6 months	wetted	4.7	5.2	4.0	4.0	4.6
	control	5.3	7.0[x]	4.5	4.2	4.9
	wilted	5.5	5.2	5.3	4.4	4.9
	wilted + water	4.7	4.7	4.2	4.0	4.9

[x] mould growth in tube.

Table 44.

The effect of inoculation. (see p.39).

pH values.

Incubation period	Treatment	Experiment			
		8	9	10	15
Fresh grass		6.2	6.2	6.2	6.4
1 day	control	6.5	6.1	5.9	6.9
	inoculated	4.1	4.3	4.9	4.7
2 days	control	5.3	6.2	5.1	6.8
	inoculated	3.8	4.1	4.2	4.0
3 days	control	5.2	6.1	5.1	6.0
	inoculated	3.9	4.0	4.0	3.9
7 or 8 days	control	5.1	5.8	4.6	5.3
	inoculated	3.7	3.8	3.8	3.7
6 months	control	7.0[x]	4.5	4.2	4.9
	inoculated	3.7	3.7	3.8	3.7

[x] mould growth in tube.

Table 45.

Origin of lactobacillus strains studied.

Strain	Expt.	Treatment	Age of silage (days)	Dilution	Medium from which isolated
L1	1	control	7	1/6 x 10^{-2}	Waksman's acid agar
"3	2	"	2	" x 10^{-6}	Tomato agar
"4	2	"	2	" x 10^{-4}	" " x
"5	2	"	3	" x 10^{-6}	" "
"7	2	"	5	" x 10^{-3}	" " x
"8	2	"	2	" x 10^{-2}	" " x
"13	3	minced	8	" x 10^{-6}	" "
"15	3	"	4	" x 10^{-7}	" "
"16	3	"	8	" x 10^{-6}	" "
"19	3	"	4	" x 10^{-6}	" " (anaerobic)
"26	4	unchopped	8	" x 10^{-4}	" "
"30	6	control	14	" x 10^{-6}	" "
"33	B.Y.	molassed	1	" x 10^{-5}	" " (at 45°C)
"38	6	wilted	2	" x 10^{-6}	" "
"45	6	"	6	" x 10^{-6}	" "
"50	B.X.	lacerated	7	" x 10^{-6}	" "
"52	4	minced	8	" x 10^{-5}	" "
"56	5	40°C	5	" x 10^{-5}	" "
"57	8	unchopped	3	" x 10^{-1}	Acetate agar
"61	9	minced	3	" x 10^{-6}	" "
"62	9	wilted	2	" x 10^{-1}	" "
"63	9	wilted + water	2	" x 10^{-3}	" "
"65	9	grass (wilted)	•	1/6	" "
"66	8	minced	8	1/6 x 10^{-4}	" "
"68	9	"	2	" x 10^{-5}	" "
"69	9	control	1	" x 10^{-3}	" "
"71	9	grass (fresh)	•	1/6	" "

x after pasteurization in milk at 63°C for 30 min.

Table 45 (continued).

Strain	Expt.	Treatment	Age of silage (days)	Dilution	Medium from which isolated
L72	9	minced	1	$1/6 \times 10^{-6}$	Acetate agar
"74	8	wilted + water	8	" $\times 10^{-1}$	" "
"77	10	grass (fresh)	•	1/6	" "
"78	10	minced	8	$1/6 \times 10^{-3}$	" "
"79	10	"	3	" $\times 10^{-4}$	" "
"80	10	22°C	8	" $\times 10^{-6}$	" "
"81	10	minced	1	" $\times 10^{-6}$	" "
"82	10	wilted + water	2	" $\times 10^{-5}$	" "
"84	10	" "	2	" $\times 10^{-5}$	" "
"86	7	inoculated with 'Siloferm'	3	" $\times 10^{-5}$	" "
"88	7	minced	2	" $\times 10^{-5}$	" "
"94	G 8	grass (fresh)	•	1/6	" "
"95	15	unchopped	1	"	" "
"96	15	minced	3	$1/6 \times 10^{-4}$	" "
"97	15	wetted	1	" $\times 10^{-3}$	" "
"98	15	minced	1	" $\times 10^{-1}$	" "
"99	15	wetted	8	" $\times 10^{-6}$	" "
"100	15	wilted	1	" $\times 10^{-1}$	" "
"101	15	"	8	" $\times 10^{-5}$	" "
"102	15	grass (fresh)	•	1/6	" "
"103	15	wilted + water	8	$1/6 \times 10^{-5}$	" "
"106	15	" "	3	" $\times 10^{-6}$	G.Y.A.
"107	15	minced	3	" $\times 10^{-6}$	"
"108	15	"	8	" $\times 10^{-6}$	"
"109	15	wetted	2	" $\times 10^{-6}$	"
"110	15	wilted	8	" $\times 10^{-5}$	"

Table 45 (continued).

Strain	Expt.	Treatment	Age of silage (days)	Dilution	Medium from which isolated
L111	15	wilted + water	3	$1/6 \times 10^{-6}$	G.Y.A.
"112	16	grass (fresh)	•	1/6	Acetate agar
"114	16	control	1	$1/6 \times 10^{-5}$	" "
"115	16	"	2	" $\times 10^{-6}$	" "
"116	16	"	2	" $\times 10^{-6}$	" "
"117	16	"	3	" $\times 10^{-6}$	" "
"118	16	"	8	" $\times 10^{-6}$	" "

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