SILAGE BACTERIOLOGY

I. WATER ACTIVITY AND TEMPERATURE RELATIONSHIPS OF SILAGE STRAINS OF LACTOBACILLUS PLANTARUM, LACTOBACILLUS BREVIS, AND PEDIOCOCCUS CEREVISIAE

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[*Manuscript received January* 30, 1963]

Summary

Logarithmic growth rates and apparent lag periods of *Lb. plantarum*, *Lb. brevis*, and *P. cerevisiae* are relatively resistant to lowering of the water activity of the growth medium. At 30°C, limiting water activity values for growth of these species have been found to be 0·920–0·930, 0·945–0·950, and 0·930–0·940, respectively. No appreciable interaction of water activity and temperature, in relation to growth of these bacteria, could be demonstrated.

Lag period and logarithmic growth rate data obtained here indicate higher temperature optima than previously recorded for *Lb. plantarum* and *P. cerevisiae*.

It has been shown that, within the range likely to be encountered in juice expressed from herbage when ensiled, any direct effects of decreased water activity on multiplication of these lactic acid bacteria would be small and seem unlikely to have a substantial bearing on the outcome. On the other hand, the possibility of indirect effects remains to be investigated.

I. INTRODUCTION

It has long been recognized that plant moisture content and the temperature level in the silo are important variables in relation to ensilage. Indeed, Hayden *et al.* (1945) concluded that the proportion of dry matter in the crop was a more important determinant factor than the method employed. There have been numerous reports in the literature which illustrate adverse effects of high moisture content on fermentative changes during ensilage and it is now evident that wilting prior to ensiling may have significant effects other than reduction of effluent flow. Many of these reports provide presumptive evidence, at least, that the composition of the silage microflora is markedly influenced by differences in moisture content. De Man (1952) found that silage pH varied inversely with dry matter content within the range 14·6–19·3%. Archibald and Kuzmeski (1954) concluded that a high butyric acid content is associated with high moisture content, and Nordfeldt (1957), in an examination of over 700 silages, found a significant positive correlation between dry matter and lactic acid contents and significant inverse relationships between dry matter content, on the one hand, and pH, crude protein loss, and total dry matter loss on the other. Stirling (1954) stated that excess moisture, particularly surface moisture, favoured the development of *Clostridium butyricum*.

Changes in moisture content are accompanied, inevitably, by changes in the solute content of juice which can be expressed from the plant material following rupture of cell membranes. The attendant alteration in water activity (a_w) of the

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substrate might be expected to have a significant bearing on bacterial development. Scott (1956) has reviewed in detail the subject of water relations of food spoilage microorganisms and the data given there have since been supplemented by a series of papers by Wodzinski and Frazier (1960, 1961*a*, 1961*b*, 1961*c*, 1961*d*). Apart from data given by the latter authors (1961*b*) for *Lactobacillus viridescens*, a species associated with ham spoilage, no detailed account has yet been found of a_w relationships of lactic acid bacteria. The present work describes some investigations of this nature with the three most common lactic acid-producing species found in silage. The possibility of interrelated effects of incubation temperature and a_w differences has also been considered.

II. Materials and Methods

(*a*) *Bacteria Studied*

Lactobacillus plantarum, *Pediococcus cerevisiae*, and *Lactobacillus brevis*, less commonly, have proved to be the predominant lactic acid bacteria in an extensive range of silages made in this Laboratory. Locally isolated strains of these species were used in the investigation described here. Stock cultures were preserved under vacuum, after lyophilization, and working stocks were stored in the refrigerator between monthly transfers on a glucose–tryptone–yeast extract agar (De Man, Rogosa, and Sharpe 1960). Growth studies were made on cultures incubated in water-baths which were controlled to within $\pm 0 \cdot 1$ degC of nominal temperatures.

(*b*) *Culture Media*

Liquid media commonly used for culture of lactic acid bacteria are high in solute content and therefore of limited use in a_w studies. For example, Briggs' (1953*a*) broth has an a_w level of approximately 0·990. The following basal medium (subsequently referred to as GTY medium) was found to give more than adequate growth of the bacteria used and had an a_w level of 0·9975 at 25°C:

Glucose	3·0 g	$MgSO_4.7H_2O$	0·23 g
$CH_3COONa.3H_2O$	2·27 g	$MnSO_4.2H_2O$	0·05 g
KH_2PO_4	1·36 g	$FeSO_4.(NH_4)_2SO_4.6H_2O$	0·02 g
Tryptone (Oxoid)	7·5 g	pH	6·5
Yeast extract (Difco)	2·5 g	Distilled water	to 1000 ml
"Tween 80"	1·0 ml		

The a_w of the basal medium was adjusted to required levels by appropriate additions of a mixture of NaCl, KCl, and Na_2SO_4 in molar proportions of 5 : 2 : 1 (Scott 1953). These additions caused appreciable shifts in pH of the medium, which were corrected before sterilization. All media were sterilized by autoclaving in screw-top bottles at 121°C for 15 min. Weight lost during sterilization was made up by addition of sterile distilled water and the screw-tops were then closed tightly.

Water activities were determined by the thermo-electric method of Baldes and Johnson (Van Andel 1952).

(c) *Procedures*

In order to reduce dilution effects, inocula were concentrated by centrifuging and resuspending in a small volume of basal medium such that one drop (*c.* 0·03 ml)

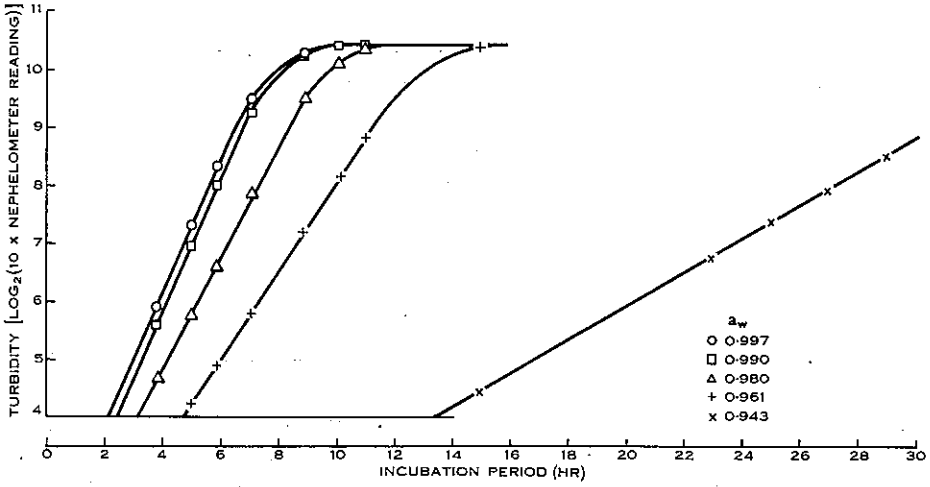

Fig. 1.—Growth curves of *Lactobacillus plantarum* (strain LP6) at 30 °C in GTY medium at various a_w levels.

produced a measurable turbidity when added to 10 ml of test medium. All treatments were examined in triplicate. Initially, culture tubes were sealed in the flame

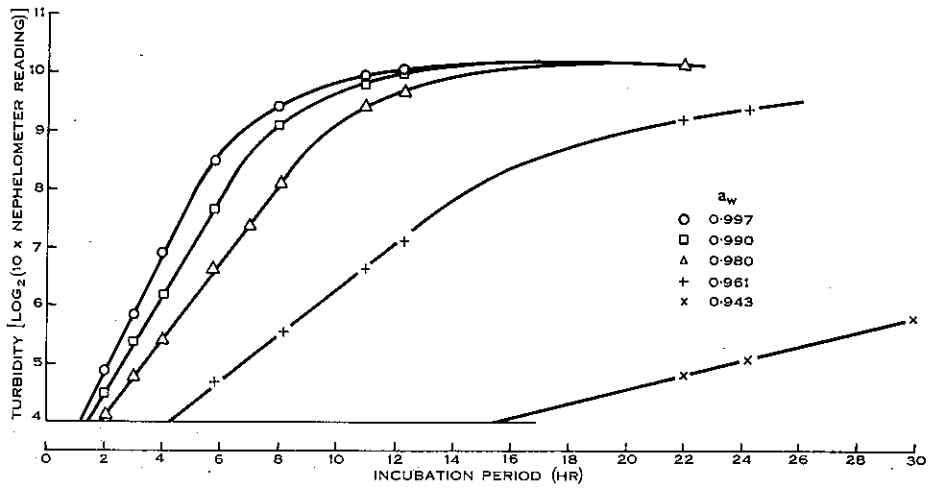

Fig. 2.—Growth curves of *Pediococcus cerevisiae* (strain PC4) at 30 °C in GTY medium at various a_w levels.

after evacuating and flushing several times with nitrogen. It was found, however, that none of the strains performed differently under nitrogen and air, so, in later experiments, tubes were simply closed with sterile rubber bungs to prevent evap-

oration. Growth was measured as increase in turbidity, either nephelometrically or by light transmission at a wavelength of 600 mμ. Preliminary investigation established straight-line relationships between optical measurements and total (microscopical) cell counts up to the equivalent of an optical density of 0·5. On the other hand, no satisfactory relationship could be established between optical properties and viable count for any of the three species.

Total acid titres in ryegrass juice cultures were determined by potentiometric titration with the necessary corrections for juice titres before incubation.

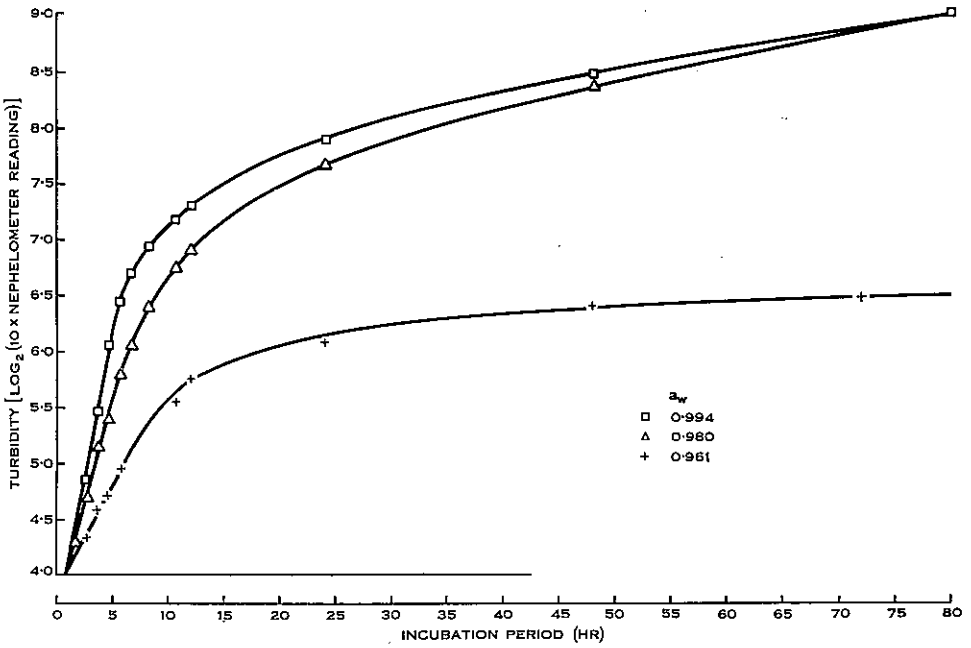

Fig. 3.—Growth curves of *Lactobacillus brevis* (strain LB2) at 30°C in GTY medium at various a_w levels.

III. Results

(*a*) a_w *Relationships at* 30°C

Growth curves for typical silage strains of *Lb. plantarum*, *Lb. brevis*, and *P. cerevisiae*, at various a_w levels, in cultures incubated at 30°C, are shown in Figures 1–3. *Lb. plantarum* and *P. cerevisiae* gave similar types of curve and comparable responses to decreasing a_w level, although the latter withstood such decreases less well than did the former. Growth curves of *Lb. brevis* differed notably in form from the others and this species was less resistant to lowered a_w. In media where a_w level was not a limiting factor, the curve given by *Lb. brevis* was characterized by a prolonged phase of deceleration, during which time as much as 90% of the total cell mass was produced, compared with approximately 50% in the cases of the other two species.

Six strains of *Lb. plantarum*, four of *P. cerevisiae*, and four of *Lb. brevis* were grown at 30°C in media adjusted to a_w levels within the range of 0·9975–0·920.

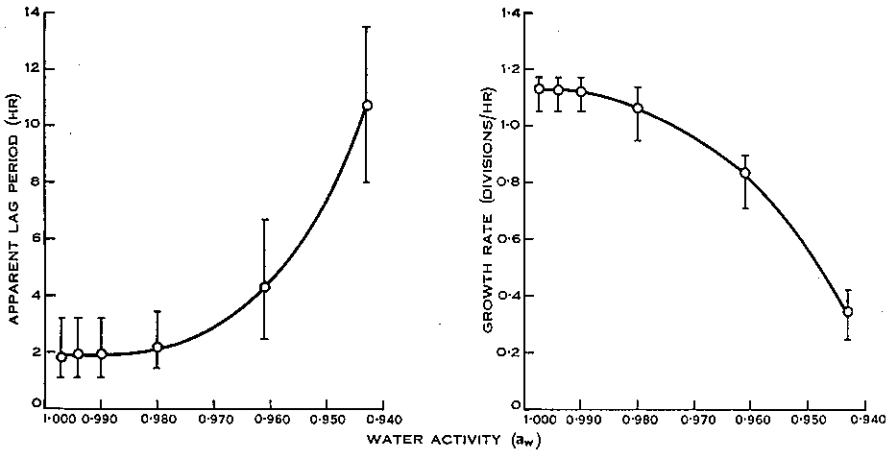

Fig. 4.—Effects of change in a_w of GTY medium on apparent lag period and growth rate in the logarithmic phase for *Lactobacillus plantarum* at 30°C. Range of values for six strains indicated.

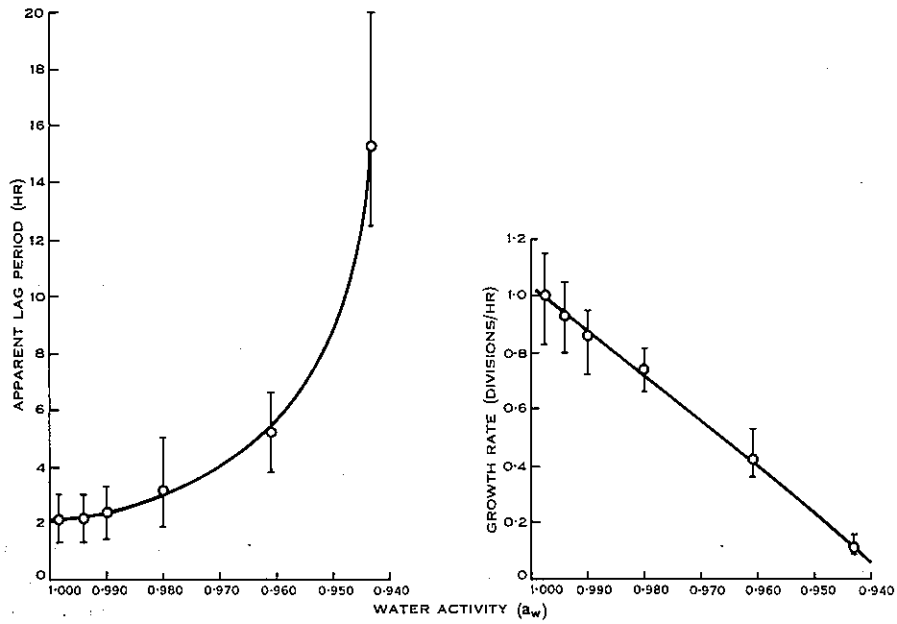

Fig. 5.—Effects of change in a_w of GTY medium on apparent lag period and growth rate in the logarithmic phase for *Pediococcus cerevisiae* at 30°C. Range of values for four strains indicated.

Mean growth rates and apparent lag periods for these organisms are shown in Figures 4–6. The order of resistance to decreasing a_w is *Lb. plantarum*, *P. cerevisiae*,

Lb. brevis. The growth rate : a_w curve of *P. cerevisiae* differs in shape from those of the other two species and shows no evidence of an optimal value within the range

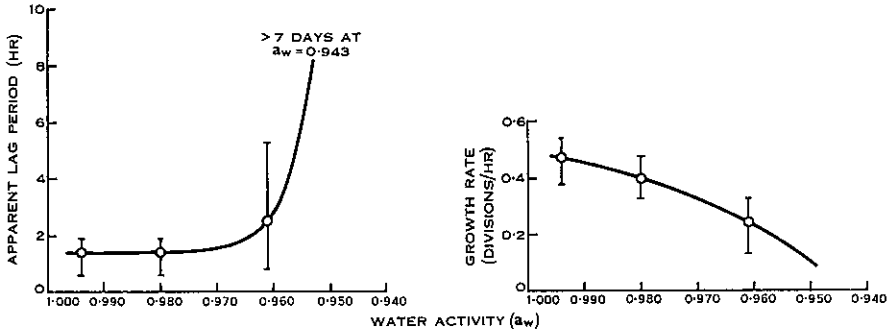

Fig. 6.—Effects of changes in a_w of GTY medium on apparent lag period and growth rate in the logarithmic phase for *Lactobacillus brevis* at 30 °C. Range of values for four strains indicated.

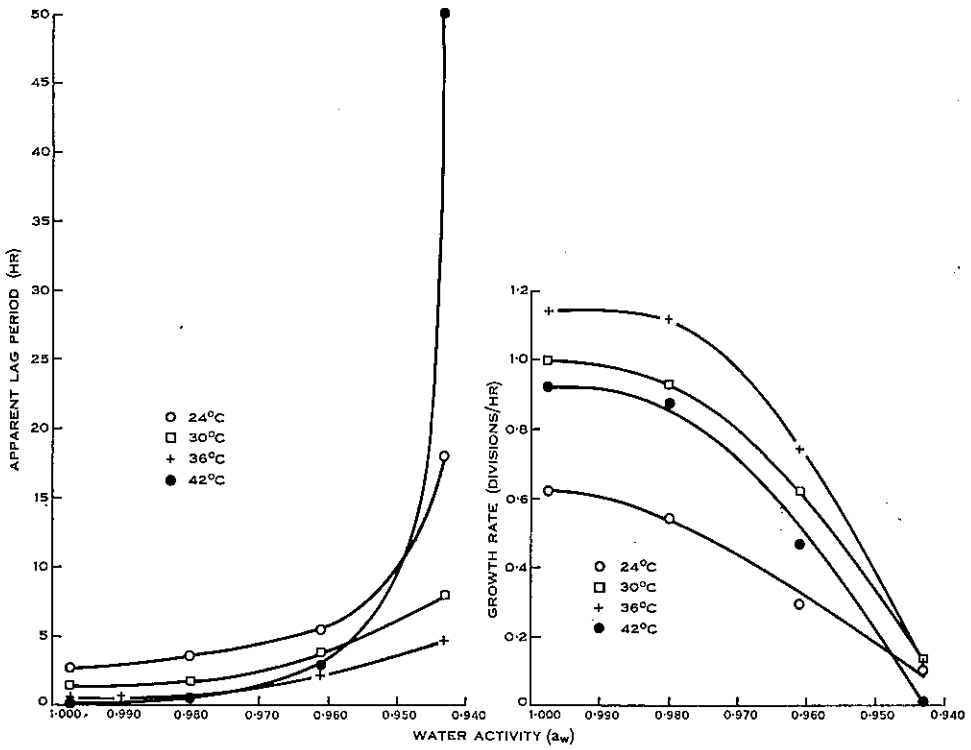

Fig. 7.—Effects of changes in temperature and a_w of GTY medium on apparent lag period and growth rate in the logarithmic phase for *Lactobacillus plantarum* (strain LP3).

studied. The growth rate of *Lb. plantarum* was only reduced by 6–7% when the a_w was lowered from 0·9975 to 0·980, whereas that of the pediococcus fell by 23%.

Limiting a_w levels for growth of the three species studied were found to be 0·920–0·930 for *Lb. plantarum*, 0·930–0·940 for *P. cerevisiae*, and 0·945–0·950 for *Lb. brevis*.

(*b*) *Temperature Relationships*

Growth of typical strains of *Lb. plantarum* and of *P. cerevisiae* was studied over a range of a_w levels and at four temperatures, namely 24, 30, 36, and 42°C. Plots of logarithmic growth rates and apparent lag periods for one strain of each are shown in Figures 7 and 8. The results of this experiment indicate a temperature optimum in the vicinity of 36°C for *Lb. plantarum* and some strains of *P. cerevisiae*.

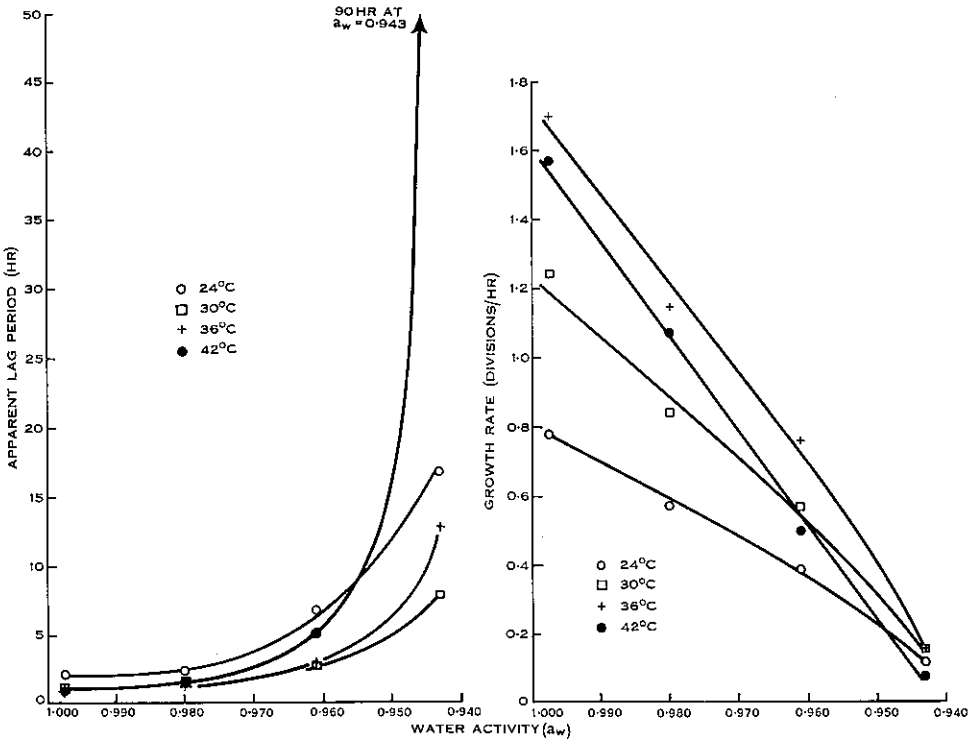

Fig. 8.—Effects of changes in temperature and a_w of GTY medium on apparent lag period and growth rate in the logarithmic phase for *Pediococcus cerevisiae* (strain PC4).

Other strains of the latter species appeared to have even higher optimal temperatures, since logarithmic growth rates were higher, and lag periods shorter, at 42°C than at lower temperatures. These strains are further characterized by ability to grow at 45°C and to withstand 60°C for 30–90 min.

The general shapes of a_w–growth curves and a_w–apparent lag curves do not appear to vary with temperature and it may therefore be concluded that, within

the temperature range studied, there is no significant interaction of a_w–temperature relationships for the species under consideration. Quantitative effects of temperature differences on response to decrease in a_w are, however, apparent in some instances.

(c) *Growth in Ryegrass Juice*

In order to gain some insight into the a_w levels initially met by bacteria on ensiled herbage, juice expressed from freshly harvested ryegrass was concentrated to various degrees by freeze-drying, clarified in the centrifuge, and sterilized by Seitz filtration. a_w levels were then determined and representative strains of *Lb. plantarum*, *Lb. brevis*, and *P. cerevisiae* were grown in samples of the juices at 30°C. Since growth could not be measured in terms of turbidity, acid formation was followed by titration of samples taken at intervals over a 4-day period. The results are summarized in Table 1. It is noteworthy that the a_w of the most concentrated

TABLE 1

ACID FORMATION BY LACTOBACILLUS PLANTARUM, LACTOBACILLUS BREVIS, AND PEDIOCOCCUS CEREVISIAE AT 30°C IN EXPRESSED RYEGRASS* JUICE ADJUSTED TO FOUR a_w LEVELS BY VACUUM CONCENTRATION FROM THE FROZEN STATE

Equivalent Moisture Content in Fresh Grass (% of dry matter)	a_w of Juice	Titratable Acidity (m-equiv/100 ml culture medium)								
		Lb. plantarum (strain LP3)			*Lb. brevis* (strain LB2)			*P. cerevisiae* (strain PC1)		
		16 hr	40 hr	90 hr	16 hr	40 hr	90 hr	16 hr	40 hr	90 hr
500	0·992	8	15	17	2	10	12	6	10	12
350	0·989	8	14	19	2	13	17	6	11	15
220	0·984	8	19	21	2	17	20	6	15	17
150	0·977	6	18	22	0·2	15	20	4	14	17

* New Zealand H1 variety.

juice was not particularly low. This degree of concentration would be equivalent to that of juice from grass wilted to a moisture content of 150% of the dry matter, a degree of wilting not usually exceeded with material intended for ensilage. It will be seen that retardation of acid formation by all three species was only evident in juice that had been concentrated to the equivalent of that in plant material with a moisture content below 220% of the dry matter; or a juice a_w of 0·984. The retardation observed in juice of $a_w = 0{\cdot}977$ was temporary in nature and largely overcome within 40 hr. It is doubtless a cumulative effect of increased lag period and slower growth rate, which have already been demonstrated for these organisms at this a_w level. However, the lag in the onset of acid formation in the case of *Lb. brevis* in this concentrated juice appears greater than can be accounted for by known a_w effects. Thus some specific inhibitory effect is indicated for this species.

IV. Discussion

Silage strains of *Lb. plantarum* and *P. cerevisiae* have proved to be among the non-halophilic bacterial species most tolerant of lowered a_w in the growth medium. Limiting a_w levels for growth of these two species have been shown to be closely similar to that reported for *Lb. viridescens* by Wodzinski and Frazier (1961*b*). *Lb. brevis* is rather less resistant to decrease in a_w than these other species but still more so than most bacteria. Briggs (1953*b*) found practical application for the ability of certain lactobacilli to grow at relatively low a_w levels when she used growth in 4% NaCl as a diagnostic feature of her physiological groups IV (*Lb. helveticus*), VI (*Lb. casei–Lb. plantarum*), and VII (*Lb. brevis*). Briggs' medium with 4% added NaCl has an $a_w = 0{\cdot}970$ and it is noteworthy that little more than half of the strains allotted to her group VII grew in this medium. Another known application is the long-established practice of adding some salt to various vegetable foodstuffs required to undergo a lactic fermentation in order to preserve them and to improve their flavour. *Lb. plantarum* and *P. cerevisiae* are known to figure largely, if not to predominate, in the bacterial flora of pickled olives and sauerkraut, for example. In silage making, too, salt has been advocated as a desirable additive.

Experience has indicated that the best moisture content for ensilage of most crops lies between 300 and 400% of the dry matter. Above this range, undesirable bacterial activity is likely to be encouraged and below it the author has observed that lactic acid formation and pH fall are commonly retarded, thus giving greater opportunity for continued post-harvest changes within the plant cells; in particular, respiration wastes much greater amounts of carbohydrates and the high temperatures which frequently result have adverse effects on protein digestibility (Watson and Nash 1960). Within the range of 300–400% moisture, herbage juice may be expected to have an a_w between $0{\cdot}987$ and $0{\cdot}990$, levels at which all three of the silage species studied have been shown to grow well, even if not optimally. The observed differences in tolerance to decrease in a_w, for the three species, although relatively small, indicate that *Lb. plantarum* would tend to outgrow *Lb. brevis*, especially, and even *P. cerevisiae*, in herbage of much reduced moisture content. The retarded rate of lactic acid accumulation, observed when the moisture content is reduced below 300% (a_w $0{\cdot}987$ in ryegrass juice) and sugars are not limiting, cannot be attributed to an adverse ionic environment in the light of the present study. Rather must we think in terms of reduced availability of nutrients for bacterial growth in such material. An investigation of factors controlling the release of cell contents in ensiled plant material may well prove fruitful. Although the a_w range likely to be encountered in ensiled herbage is not such that we may expect marked effects on the lactic acid bacteria, development of other types initially present may well be influenced by variations within this range. Work on this aspect of ensilage bacteriology therefore appears warranted.

The temperature optima indicated by growth rate and lag period data are appreciably higher than those recorded for *Lb. plantarum* and *P. cerevisiae* in "Bergey's Manual" (Breed, Murray, and Smith 1957) and by Gunther and White (1960). However, in view of the different methods employed, it is not certain that silage strains of these organisms differ in this respect from those studied elsewhere.

In a paper which has recently come to the author's notice (Nakagawa and Kitahara 1959), *P. pentosaceus* is differentiated from *P. cerevisiae* on the basis *inter alia* of the former's higher optimal temperature for growth and greater aero-tolerance. It may be therefore that some, at least, of the silage strains of *Pediococcus* would be more correctly identified with this newly defined *P. pentosaceus*. Further study will be needed to resolve this problem.

V. Acknowledgment

The author is indebted to Mr. W. L. Greenhill for the a_w measurements.

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