A COMPARATIVE STUDY OF THE MORPHOLOGICAL AND PHYSIOLOGICAL CHARACTERISTICS OF NITRATE REDUCING AND NON-REDUCING STRAINS OF LACTOBACILLUS PLANTARUM

Thesis for the Degree of M. S.

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Donald Lewis Robach

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By

Donald Lewis Robach

AN ABSTRACT

Submitted to the College of Arts and Science
Michigan State University of Agriculture and
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the requirements for the degree of

MASTER OF SCIENCE

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Approved by Ralph N. Costilow

The purpose of this study was to determine if there were any taxonomic differences between strains of Lactobacillus plantarum which reduced nitrate in indole-nitrate medium (BBL) and those which did not. Five of the 10 cultures used in this investigation reduced nitrate.

All cultures had similar cellular and colonial morphology. They were all catalase negative, homofermentative, and microaerophilic. However, variations were noted in other physiological studies. One strain produced abnormally high titratable acidity in skimmed milk, formed dextro-rotary lactic acid and failed to ferment raffinose and melibiose. Therefore, it was classified as Lactobacillus casei. Differences between the other 9 strains were noted in the hydrolysis of esculin and sodium hippurate, nutritional requirements, and acid production from various carbohydrates. All 9 strains appeared to fit into the classification as given in Bergey's Manual (1948) for L. plantarum with the exception that 5 cultures reduced nitrate. However, the variations were such that none of these cultures could be placed in any one of the three groups proposed by Rogosa et al. (1953) for strains of L. plantarum. No other taxonomic characteristics were correlated with nitrate reduction. Therefore, it was concluded that nitrate reduction should be included as one of the variable characteristics of this species and that no further taxonomic division of the cultures should be made.

1. BREED, R. S., MURRAY, E. G. D., and HITCHENS, A. P. 1948 Bergey's Manual of Determinative Bacteriology. 6th. ed. Williams and Wilkins Co., Baltimore, Md.

2. ROGOSA, M. W., MITCHELL, J. A., WISEMAN, R. F., and DISRALLY, M. N., assisted by BEAMAN, A. J. 1953 Species differentiations of oral lactobacillus from man including descriptions of Lactobacillus salvarius nov. spec. and Lactobacillus cellobiosus nov. spec., J. Bacteriol., 65, 681-699.

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INTRODUCTION

Strains of lactic acid bacteria similar to Lactobacillus plantarum have been found to reduce nitrates in indole-nitrate medium (BBL) (Costilow and Humphreys, 1955; and Costilow et al., 1956). The authors observed that about one-half of the strains isolated had this ability. These isolates were found to be similar in many other respects to Lactobacillus plantarum but a complete comparative study was not made.

It is possible that nitrate reduction by this organism was not previously noted due to the test medium employed. Sacks and Barker (1949) as well as a number of other investigators have established that oxygen tension is a very important factor in the ability of bacteria to reduce nitrates. The composition of the medium may also influence the results obtained. Also, some investigators retained only strains which did not reduce nitrates, discarding all nitrate positive strains as a preliminary procedure (e.g., see Rogosa et al., 1953).

The present study is to determine whether those strains which have the ability to reduce nitrates should be included in the species Lactobacillus plantarum or be designated as a separate species from organisms which do not reduce nitrates.

REVIEW OF LITERATURE

Microbiological Reduction of Nitrate

According to Conn (1936), for a number of years one of the paramount features in the characterization of bacteria has been the ability to reduce nitrates. In most papers in which species are described and in Bergey's Manual (1948), a statement is given as to whether or not nitrates are reduced. Generally just a categorical statement, nitrates reduced or nitrates not reduced, is made as if the determination was so concise that no specifications of methods and conditions were necessary. This, however, is not the case for many factors enter into the determination. A fundamental prerequisite is to have a suitable medium for the nitrate test; i.e., the proper nutrients to support growth. Another basic factor is the Eh of the medium. It was demonstrated in the classic experiments of Gayon and Dupetit (1886) that oxygen inhibits the reduction of nitrate and the formation of nitrogen by denitrifying bacteria.

Weissenberg (1897) using three denitrifying bacteria found that complete denitrification occurred only in anerobic cultures, whereas the nitrate was reduced only as far as nitrite in aerobic cultures.

Strickland (1931) determined the influence of oxygen at various partial pressures on the reduction of nitrate to nitrite by cell suspensions of Escherichia coli. Under conditions of aeration

that should have maintained an equilibrium of oxygen distribution between the liquid and gas phases, he found as little as 0.36 per cent oxygen caused a 21 per cent inhibition of nitrate reduction, 1 per cent oxygen caused approximately 50 per cent inhibition, and 3.76 per cent oxygen caused 93 per cent inhibition. A tenfold increase in nitrate concentration did not modify these results; thus demonstrating that the inhibition was non-competitive.

Van Olden (1940) was first to apply modern manometric techniques to the study of denitrification. Using Micrococcus denitrificans, he found that the ability of washed bacteria to produce nitrogen from nitrate dependent upon their previous history. Bacteria that had grown anaerobically with nitrate were capable of causing rapid denitrification of nitrate under anaerobic conditions, whereas bacteria grown aerobically either with or without nitrate denitrified very slowly or not at all. Van Olden concluded that nitrate reductase is an adaptive enzyme in a sense. However, from his results it is impossible to decide which enzyme or enzymes failed to develop under conditions unsuitable for denitrification. Since nitrite was formed by some of the bacteria grown aerobically, it is possible that their inability to denitrify was at least partially due to the absence of nitrite reductase or some enzyme mediating a reaction between nitrite and nitrogen.

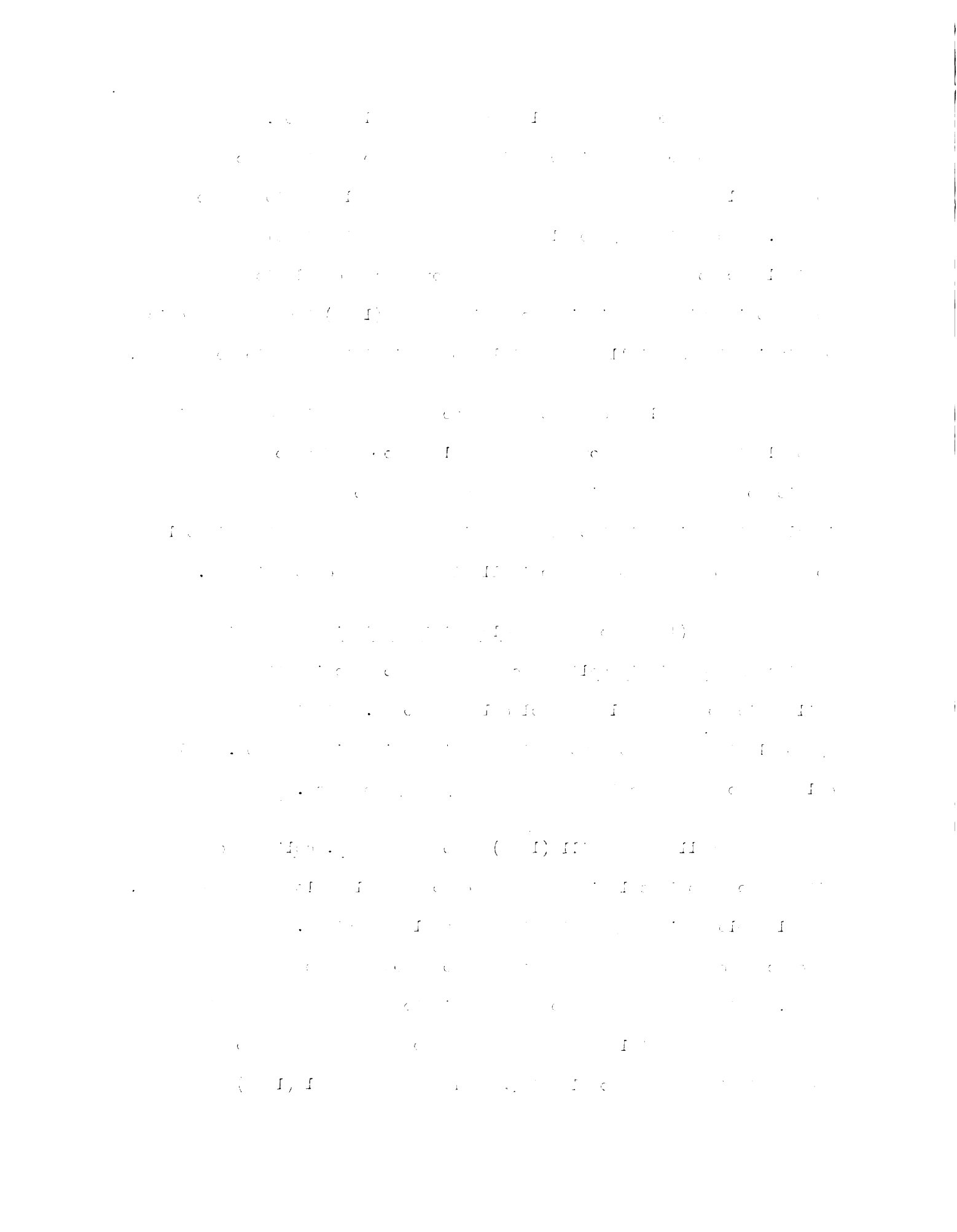
Lemoigne et al. (1946) found that when Bacillus megatherium, was grown in a medium containing nitrate as the sole source of nitrogen,

a pure oxygen atmosphere greatly increased the lag period. If there was a source of organic nitrogen in the medium or if the atmosphere contained less than 64 per cent oxygen, no great lag period was observed. From this they concluded that the mechanism involved in the assimilation of nitrate was arrested by oxygen; a conclusion that seems to harmonize with the findings of Weissenberg (1897) that the reduction of nitrite is especially susceptible to the inhibitory action of oxygen.

In general, except for a few workers, the evidence summarized strongly indicates that oxygen has a deleterious effect on the reduction of nitrate and nitrite and that one or more of the enzymes involved in denitrification is adapative in the sense that it is only formed by bacteria grown anerobically in the presence of nitrate.

Woods (1938) found that Clostridium welchi and certain strains of Escherichia coli reduced nitrate to ammonia with the utilization of four moles of molecular hydrogen. Nitrites and hydroxylamine were shown to be intermediates in this reaction. Molecular hydrogen was activated by the enzyme hydrogenase.

Lascelles and Still (1946) reported that E. coli reduced nitrate to ammonia only in the presence of benzyl viologen as a carrier. Benzyl viologen is a dye which is reversibly reducible. In the absence of a carrier, the reduction did not proceed beyond the nitrite stage. This and the action of some inhibitors suggested that different enzymes are responsible for the reduction of nitrate from those reducing nitrite and hydroxylamine. Colter and Quastil (1950) have



shown that hydroxylamine reduction to ammonia and molecular nitrogen can be catalyzed by hemoglobin.

Sacks and Barker (1949) found that oxygen affects nitrate reduction and denitrification in two ways; by supressing the formation of enzyme systems that catalyze these reactions, and by directly interfering with the action of enzyme systems when they are present in the bacteria. They concluded that the exposure of bacteria to oxygen during growth supresses the formation of enzyme systems responsible for nitrite reduction much more than those responsible for the reduction of nitrate to nitrite. This was based on the observation that nitrate could be reduced only as far as nitrite in oxygen tensions of about 5 per cent. At lower oxygen tensions there was an abnormally large accumulation of nitrite, but this was accompanied by denitrification. Both the accumulations of nitrite and the rate of denitrification were greatly affected by relatively small changes in oxygen level.

Krasna and Rittenberg (1954) have shown that both whole cells and extracts of Proteus vulgaris catalyze the reduction of nitrate to nitrite by molecular hydrogen. They, also, found that no nitrate could be reduced until all the oxygen was removed; therefore, the lenth of induction period was dependent on the oxygen content of the system.

Nason and Evans (1953) working with nitrate reductase of Neurospora found that it catalyzed the reduction of nitrates to nitrites according to the equation; $NO_3 + TPNH \cdot H \rightarrow NO_2 + TPN + H_2O$. The enzyme,

which had been concentrated approximately seventy fold, had a pH optimum at 7.0 and was observed to be a flavo protein with flavinadeninedinucleotide (F. A. D.) as the prosthetic group. It was suggested that sulfhydryl (-SH) groups were present on the enzyme as well as a heavy metal constituent. It was of an adaptive nature and not stimulated by molybdenum.

Nicholas et al. (1954) found that cell free extracts of molybdenum-deficient Neurospora crassa showed a striking decrease in nitrate reductase (from one-tenth to one-thirtieth of the controls). Individual deficiencies of other micronutrient elements (salts of iron, manganese, zinc, magnesium, boron or copper) did not result in a decrease of the enzyme. This is in agreement with Nason and Evans (1953). Nicholas et al., also, found that both Neurospora crassa and Aspergillus niger required molybdenum when nitrate, nitrite or ammonia was used as the sole source of nitrogen. When ammonia was the sole source of nitrogen, nitrate reductase was not formed and the molybdenum requirement was less. Therefore, this element is required for metabolic processes other than the reduction of nitrate.

Costilow and Humphreys (1955), found that about one half of the strains of Lactobacillus plantarum tested reduced nitrate to nitrite when grown in indole nitrate medium (BBL). These same cultures were unable to reduce nitrates in nitrate broth (Difco) or in indole nitrate medium with the 0.1 per cent agar omitted. The addition of yeast extract increased the rate of nitrate reduction but the oxygen tension of the test medium appeared to be the deciding factor as to whether nitrate was reduced by some strains or not.

Classification of Lactobacillus plantarum

Tittsler (1952) in giving the "Introduction" to the Symposium on the Lactic Acid Bacteria stated that this group of organisms attracted the attention of bacteriologists since the beginning of bacteriology due to their practical importance to the food and fermentation industries. Lactobacilli, particulary, have been subjected to much basic research yet there is a great need for satisfactory means of identifying, differentiating, and characterizing the genera and species in the family Lactobacteriaceae. L. plantarum (Orla-Jensen), typifies the confusion that has existed as illustrated by the nineteen probable synonyms listed in the sixth edition of Bergey's Manual. The probable synonyms are; Bacillus pabuliacidi, Lactobacillus pabuliacidi, Bacillus cucumeris fermentati, Lactobacillus cucumeris, Bacillus wortmannii, Bacillus listeri, Lactobacterium listeri, Lactobacillus listeri, Bacillus naercki, Bacillus leichmanni, Bacillus beijerinckii, Lactobacillus beijerinckii, Bacterium brusae asiaticae, Lactobacillus busaeasiaticus, Bacterium brassicae, Lactobacillus brassicae, Lactobacillus pentosus, Lactobacillus arabinosus and Lactobacillus wortmannii.

Pederson (1952) found that lactic acid bacteria obtained their energy by partial fermentation of sugars without the utilization of free oxygen. This necessitates the utilization of large quantities of sugar to obtain relatively small amounts of energy for growth, which results in comparatively large amounts of determinable fermentation end

products. Therefore, species within a group acting similarly could be partially identified by these end products of fermentation. Homofermentative species, of which L. plantarum is a member, produce lactic acid as a major end product with small amounts of acetic acid and carbon dioxide from hexose sugar fermentations. Equimolar amounts of lactic acid and acetic acid are produced from the fermentation of the pentose sugars.

Snell (1952) stated that L. plantarum will ferment up to 95 per cent of the utilized glucose to lactic acid and the remainder of the sugar is converted to carbon dioxide, traces of volatile acids, and cellular protoplasm. All of the most recent evidence indicates that lactic acid is formed by way of the Embden-Meyerhof scheme.

Pederson (1936) discovered the greatest variation between strains of Lactobacillus plantarum existed in their ability to ferment individual sugars, ranging from a definite failure to ferment to a definite positive fermentation. The majority of the strains formed acid from glucose, fructose, mannose, galactose, sucrose, maltose, lactose, raffinose, and salicin, and to a lesser extent sorbitol, mannitol, dextrin, and glycerol. The majority of the strains did not ferment rhamnose, starch, and inulin. A few strains had only slight or no action on arabinose and even less on xylose. The same range of variation occured even where cultures were obtained from identical material. This is in near agreement with what is now given in Bergey's Manual (1948). Similar results were also observed by

Rogosa et al. (1953) and Tittsler et al. (1947).

Snell (1952) points out that all living organisms require a utilizable form of energy, appropriate nitrogen and carbon containing compounds to permit synthesis of the various components of their protoplasm, and certain inorganic salts for growth. These nutritional requirements for the lactic acid bacteria are among the most complex so far studied. He further states that the most important compounds utilized by lactic acid bacteria as a source of energy are the soluble carbohydrates.

Vitamin and Amino Acid Requirements of L. plantarum

Due to the advent of microbiological assay techniques for various amino acids and vitamins much progress has been made in the study of the nutritional requirements of bacteria. The lactic acid bacteria in general, because of their fastidious nutritional requirements have been subjected to many investigations. L. plantarum is one of the lactic acid organisms which has been commonly used. This review will be limited to the requirements of this organism and its synonyms.

Vitamin requirements. Peterson and Peterson (1945) in preparing a comprehensive review of the vitamins and growth factors required by microorganisms noted that biotin, niacin, pantothenic acid and riboflavin were the most frequently reported as being essential. All of these vitamins have been established as essential for L. plantarum.

Snell and his co-workers (1938, 1939, 1941) have demonstrated the requirements of L. arabinosus 17-5 for nicotinic acid, pantothenic acid, and biotin. Cheldelin et al. (1945) using one strain of lactic acid bacteria labeled as L. plantarum, two as L. arabinosus, and two as L. pentosus demonstrated a requirement for pantothenic acid. Rosen and Fabian (1953) working with ten isolates of L. plantarum from cucumber fermentations demonstrated that each required biotin, niacin, and pantothenic acid as did L. arabinosus 17-5. This was substiantiated by Costilow and Fabian, (1954) using four strains of L. plantarum isolated from cucumber fermentations. Kreuger and Peterson, (1948) reported similar results for L. pentosus 124-2. Rogosa et al. (1953) demonstrated that all strains of the L. plantarum group tested required niacin and pantothenic acid. Results as to biotin requirements were not given.

Snell and Mitchell (1942) using L. arabinosus 17-5 and L. pentosus noted that p - aminobenzoic acid was nonessential as did Snell (1948, 1950, 1951) using L. plantarum and L. arabinosus. Costilow and Fabian (1954) noted that three of four L. plantarum strains tested required p - aminobenzoic acid. Kreuger and Peterson (1948) found this vitamin to be stimulatory for L. plantarum and L. arabinosus while Isbell (1942) and Lewis (1942) observed it to be essential for L. arabinosus. In the concentrated media of Shankman et al. (1947) the omission of this vitamin had no apparent effect on L. arabinosus 17-5 or L. pentosus.

Snell and Strong (1938) noted riboflavin was not required by L. pentosus 124-2, L. arabinosus 17-5, nor by L. brassicae (8041). Campbell and Hucker (1944) obtained similar results with cultures labeled L. arabinosus F-17-5, Bacillus cucumeris fermentatae L-25, and two strains of L. plantarum. Rogosa et al. (1953) reported that some strains of L. plantarum required riboflavin which is in agreement with Rogosa et al. (1947) and Costilow and Fabian (1954).

Bohonos et al. (1941, 1942) reported that neither L. arabinosus or L. pentosus required pyridoxine but was synthesized by them. But, Shankman et al. (1943) found that both L. arabinosus 17-5 and L. brassicae (8041) were stimulated by pyridoxine when short incubation periods were used. No stimulation of L. pentosus was observed. Costilow and Fabian (1954) noted that only one of the four strains of L. plantarum tested required pyridoxine. Rogosa et al. (1953) observed pyridoxine to be neither required or stimulatory.

Shankman et al. (1943) and Baumgarten et al. (1944) noted neither thiamine or folic acid were essential for L. arabinosus 17-5. However, the latter workers have observed folic acid to be stimulatory. L. pentosus did not require these two vitamins, (Shankman et al., 1943). Rogosa et al. (1953) using strains of L. plantarum found neither thiamine or folic acid were essential or stimulatory.

Snell and Wright (1944) observed that pyridoxine, thiamine, and riboflavin stimulated the growth of L. arabinosus 17-5 during the first few hours of incubation.

Amino acid requirements: The findings of various workers on the amino acid requirements of L. plantarum are still more conflicting than on the essential vitamins. Snell (1952) contends that the number and the identity of amino acids required by lactic acid bacteria are highly dependent upon the different levels of vitamins that are supplied in the medium. He concluded that the conflicting data concerning amino acid requirements of lactic acid bacteria that appears in the literature is due primarily to this; and, also, to contamination of certain commercially available amino acids with other amino acids. To exemplify this statement he puts forth the following fact: in media that is low in biotin, Lactobacillus arabinosus requires aspartic acid but grows without it when large amounts of biotin is supplied. This suggests that biotin is in some way essential for synthesis of aspartic acid by the organism. This hypothesis has been confirmed by Lardy et al. (1949) and Snell (1951) using tracer experiments. Holden et al. (1951) and Snell (1948, 1951a) have shown that vitamin B_6 may also alter an organism's need for an amino acid.

Even using the same strain of L. plantarum (L. arabinosus 17-5) investigators have found considerable differences in amino acid requirements. The number of amino acids found to be required by this strain during six investigations varied from ten (Hegsted, 1944) to five (Dunn, 1947). In all six of the investigations (Shankman, 1943; Kuiken et al. 1943; Hegsted, 1944; Lyman et al., 1947; Dunn et al. 1947; Costilow and Fabian, 1954) five amino acids (viz., glutamic acid,

valine, leucine, isoleucine, and tryptophane) were noted to be essential. Cystine was found to be presently essential in all cases except by Dunn (1947), who found it to be stimulatory even in an enriched medium. Rissen et al. (1947) also noted that cystine was stimulatory for L. arabinosus 17-5. Threonine was observed to be required in three of the investigations (Shankman, 1943; Kuiken et al. 1943; and Costilow et al. 1954), and methionine was demonstrated to be essential in three (Shankman, 1943; Dunn et al. 1947; and Hegsted, 1944). Only Kuiken et al. 1943 found lysine to be required. Phenylalanine was found to be required in three investigations (Shankman, 1943; Kuiken et al. 1943; and Hegsted, 1944); arginine in two (Hegsted, 1944; Dunn et al. 1947); and tyrosine in two (Shankman, 1943; and Hegsted, 1944).

Dunn et al. (1947) using Leuconostoc mesenteroids noted considerable variations between strains as to amino acid requirements. Requirements varied from fifteen amino acids for one strain to two for another. They further noted that L. pentosus required glutamic acid, valine, isoleucine, leucine, and cysteine. L. arabinosus 17-5, however, did not require cysteine, but required tryptophane and in some instances methionine and arginine.

Valine, leucine, isoleucine, glutamic acid, and phenylalanine were reported by Kruger and Peterson (1948) as being essential for L. pentosus 124-2. In the same report it was noted cystine, threonine, and alanine stimulated growth but tryptophane showed no effect upon ommission.

L-tryptophane, L-leucine, DL-isoleucine, DL-valine, L-glutamic acid, L-cystine, DL-threonine, DL-alanine were found to be required for growth by four strains of L. plantarum isolated from cucumber fermentations by Costilow and Fabian (1954). They further found that three strains required DL-phenylalanine and L-tyrosine and one strain needed L-arginine for growth.

Lyman et al. (1947) demonstrated that L. arabinosus 17-5 required threonine, lysine, and alanine if pyridoxine was omitted but did not require them when pyridoxine was present. This shows conclusively that there exists a relationship between the composition of the media used for testing and the amino acids found to be essential. In this report, it was noted that if CO_2 was not available, arginine, phenylalanine and tyrosine were also required. However, pyridoxine was not required when all these amino acids were present.

Stokes and Gunness (1943) reported that pyridoxamine would eliminate the requirements of L. arabinosus 17-5 for lysine, threonine, and alanine but pyridoxine would not. However, if the basal medium containing pyridoxine was sterilized for thirty minutes at 15 pounds similar results were obtained as when pyridoxamine was used. These observations might account for many of the conflicting results obtained in the various investigations.

EXPERIMENTAL PROCEDURES AND METHODS

Source and method of handling cultures: Ten pure cultures were used for this study. Seven were isolated from cucumber fermentations and typed as Lactobacillus plantarum in our laboratory. One strain (B-227) was obtained from the Northern Utilization Research Branch (N.U.R.B.). Strain 246 was obtained from Dr. C. S. Pederson, Cornell, Agricultural Experimental Station, Geneva, New York. The tenth strain was L. arabinosus 17-5.

Each culture was streaked out on selective lactobacillus agar plates and isolated colonies picked. Again frequent transfers on trypticase sugar agar were made and microscopic examination was performed to insure purity of culture when good growth was observed. One inoculated tube of each strain was placed in the refrigerator for reference and a duplicate of each was used for further study.

Morphological studies: Colonial morphology was observed on both trypticase sugar agar and selective lactobacillus agar (Rogosa et al., 1951) streaked and poured plates. Cell morphology was observed by use of the microscope. Motility was determined by observation of growth in a semisolid media (cystine trypticase agar), and by microscopic examination of a hanging drop slide. The cultures were observed as to growth on nutrient agar slants, spore production, and gram staining reaction.

Physiological studies: Cultures were inoculated into indole nitrate medium (BBL) to test their ability to reduce nitrates. Observations were made after 3 and 6 days incubation. Testing procedures followed were as outlined in the Manual of Methods for Pure Culture Study of Bacteria (1946) using sulphanilic acid and alpha-napthylamine to test for the presence of nitrites.

The cultures were observed for catalase production by the addition of 3 per cent hydrogen peroxide to trypticase agar stab and trypticase broth cultures.

Fermentation studies were conducted on the following substrates at a concentration of 2 per cent; adonitol, arabinose, cellobiose, dulcitol, glucose, inositol, inulin, lactose, levulose, maltose, mannitol, mannose, melibiose, melezitose, raffinose, rhamnose, salicin, sorbitol, sorbose, sucrose, trehalose, xylose, alpha methyl-d-glucoside, and alpha methyl-d-mannoside. Galactose was used at a concentration of 1.4 per cent. Basal media used for the fermentation studies were cystine trypticase agar (BBL) and liquid medium No. 3 of Rogosa et al. (1953). The indicator was observed daily and a notation made on the day it changed color distinctly. After two weeks' incubation, the pH of the contents of each tube containing the liquid media was observed.

In addition to observing the indicator of the cystine trypticase agar, notations were made as to motility and gas formation.

Tubed media No. 5 of Rogosa et al. (1953) was inoculated, plugged with sterile agar, and observed for gas formation.

Tests to determine if sodium hippurate and esculin could be hydrolyzed by the organisms were performed as outlined by Rogosa et al. (1953).

Sterile skimmed milk was inoculated and incubated for two weeks at which time it was observed for curd formation and the titratable acidity determined. Reaction of the cultures on litmus milk was also noted.

Medium No. 5 of Rogosa et al. (1953) was used as the production medium for determination of optical activity of lactic acid produced. Arabinose and xylose were not added for all strains were homofermentative. The medium was dispersed at the rate of 300ml. per 500ml. Erlenmeyer flask and 10 grams of calcium carbonate added. It was autoclaved at 15 pounds pressure for 15 minutes. Flasks were inoculated with 1ml. of culture which had been grown in media No. 5. After 2 weeks of incubation, volatile acids were distilled off and the lactic acid extracted with ether and finally zinc lactate formed. The preparation of zinc lactate and determination of optical activity were performed according to the methods of Currier et al. (1933) with modification from the procedure of Brinn et al. (1952).

Nutritional studies were performed using the synthetic medium of Sauberlich and Baumann (1946). Cultures were grown in micro-

inoculum broth, centrifuged, washed, resuspended, and diluted with sterile saline. One drop of this suspension was used to inoculate the media for nutritional studies. After an incubation of 3 days the pH of the contents of each tube was measured and the acid titrated with sodium hydroxide (about 0.1 N) to pH 7.0.

All cultures were incubated at 30°C. All tests were completed in duplicate except for that of the optical activity of lactic acid.

RESULTS

Five of the ten strains believed to be Lactobacillus plantarum used in this study had been found to reduce nitrates to nitrites on BBL indole nitrate medium (Costilow 1954, unpublished data). This was rechecked at the onset of this study and found to be true. The cultures were divided into 2 groups on this basis. The strains reducing nitrate (NO_3^-) were 17-5, L-10, A-6-4, A-231-3 and A-85-2. The non-nitrate reducing (NO_3^-) strains were B-227, 246, A-73-1, A-160-1 and A-242-1. Since this study was undertaken to determine any other differences between these two groups, the data are presented in a comparative form.

Morphology

Cell morphology: All ten cultures studied were gram positive rods measuring 3/4 to 1 μ in width and ranging from 3 to 5 μ in length. The cells had typical rounded ends and appeared singly, in short chains, and small groups. They were further characterized as being non-motile and without the formation of endospores.

Colonial morphology: Colonies appeared similar on both trypticase sugar agar and selective lactobacillus agar. All strains formed circular colonies having smooth, convex surfaces with entire edges and were opaque, with a slight off-white color. They differed only in the rate of growth. Growth on agar slants was very slight.

Physiology

All organisms were catalase negative and in no case was gas produced from carbohydrate fermentation. All strains grew on sterile skimmed milk forming acid and causing coagulation. However, there were differences in the amount of titratable acidity produced and the rate of curd formation (table 1). Similar results were observed from litmus milk.

Hydrolysis: Only strain 17-5 of the nitrate positive organisms had the ability to hydrolyze both sodium hippurate and esculin. Strains A-6-4 and A-85-2 caused hydrolysis of sodium hippurate and L-10 hydrolyzed esculin. Strain A-231-3 demonstrated no ability to hydrolyze either.

Nitrate negative strains which demonstrated hydrolysis of both sodium hippurate and esculin were A-73-1, A-160-1, and A-242-1. B-227 caused the hydrolysis of sodium hippurate and 246 hydrolyzed esculin (table 1).

Carbohydrate fermentations: The results of the studies of carbohydrate fermentations in cystine trypticase agar are presented in table 2, and those in Rogosa's medium No. 3 in table 3. The data from the two media were comparable in all but 5 instances. In medium No. 3, strains A-231-3 and A-85-2 demonstrated acid production from dulcitol, strains A-73-1 and A-160-1 produced acid from alpha-

TABLE 1

Hydrolysis of Sodium Hippurate and Esculin and Final Titratable Acidity in Skimmed Milk

NO_3^- strains					NO_3^- strains				
17-5	L-10	A-6-4	A-231-3	A-85-2	B-227	246	A-73-1	A-160-1	A-242-1
Sodium hippurate hydrolysis									
+ *	-	+	-	+	+	-	+	+	+
Esculin hydrolysis									
+	+	-	-	-	-	+	+	+	+
Final titratable acidity (as % lactic) in skimmed milk									
.79	.56	.61	.49	.39	1.37	.53	.61	.62	.61

* + = hydrolysis

\- = no hydrolysis

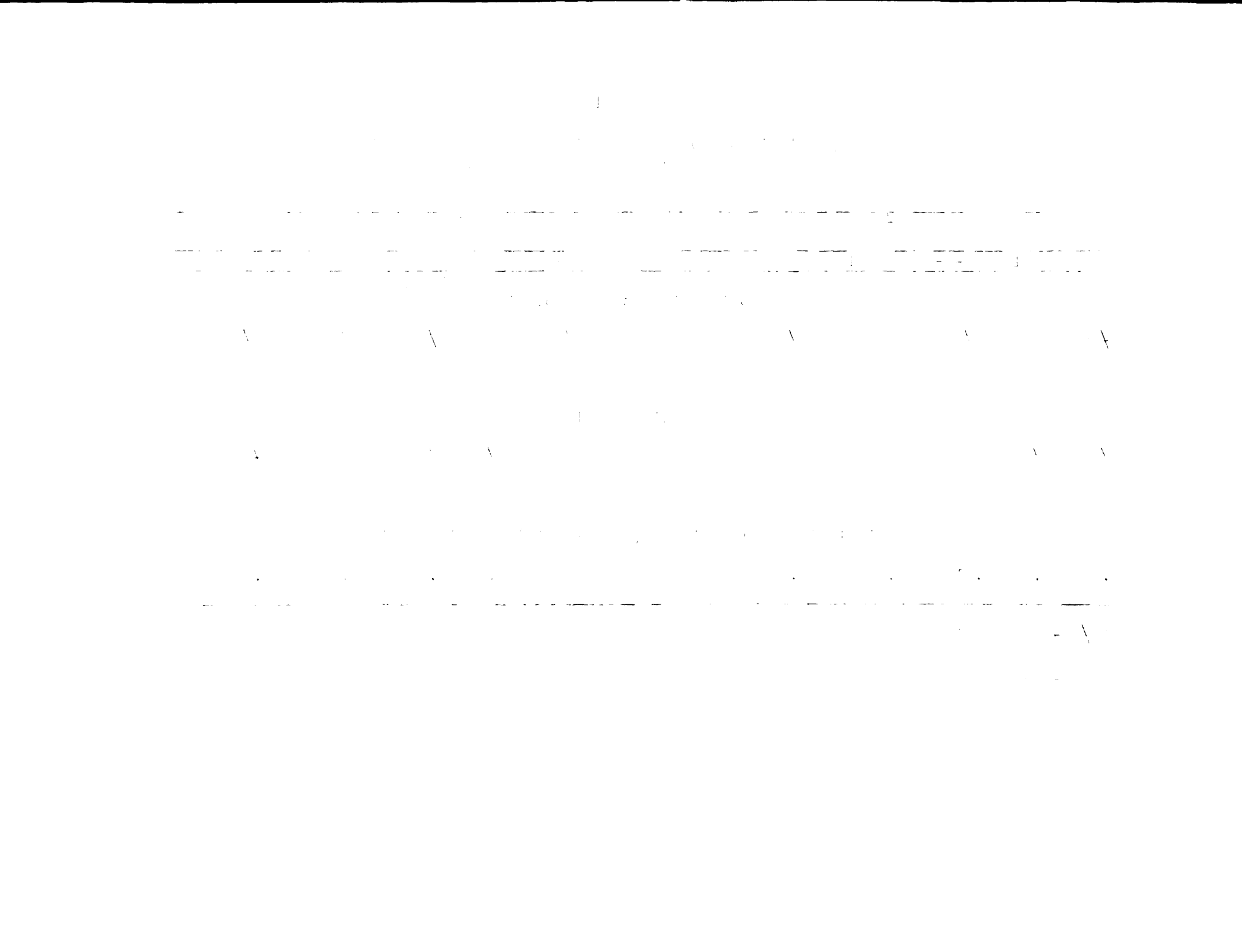


TABLE 2

The fermenting ability of various strains of L. plantarum on some carbohydrates in cystine trypticase agar*

	NO_3^- strains					NO_3^- strains				
Carbohydrate	17-5	L-10	A-6-4	A231-3	A-85-2	B-227	246	A-73-1	A-160-1	A-242-1
Arabinose	4+**	4+	4+	4+	-	4+	4+	-	4+	-
Dulcitol	-	-	4+	-	-	-	-	-	-	-
Melibiose	4+	4+	4+	4+	4+	-	4+	4+	4+	4+
Melezitose	4+	-	-	-	-	4+	4+	4+	4+	4+
Raffinose	4+	4+	4+	4+	4+	-	4+	4+	4+	4+
Rhamnose	4+	4+	4+	3+	-	4+	4+	-	-	-
Xylose	-	-	4+	4+	3+	-	-	-	-	-
Alpha CH_3-d-glucoside	4+	-	4+	4+	-	-	-	-	-	4+
Alpha CH_3-d-mannoside	4+	-	-	-	-	-	4+	-	-	4+

* All strains fermented celebiose, galactose, glucose, lactose, fructose, maltose, mannose, mannitol, salicin, sorbitol, sucrose, and trehalose. No strains fermented adonitol, inositol, innulin or sorbose.

** + to 4+ = varying degrees of acid production.

\- = no acid production.

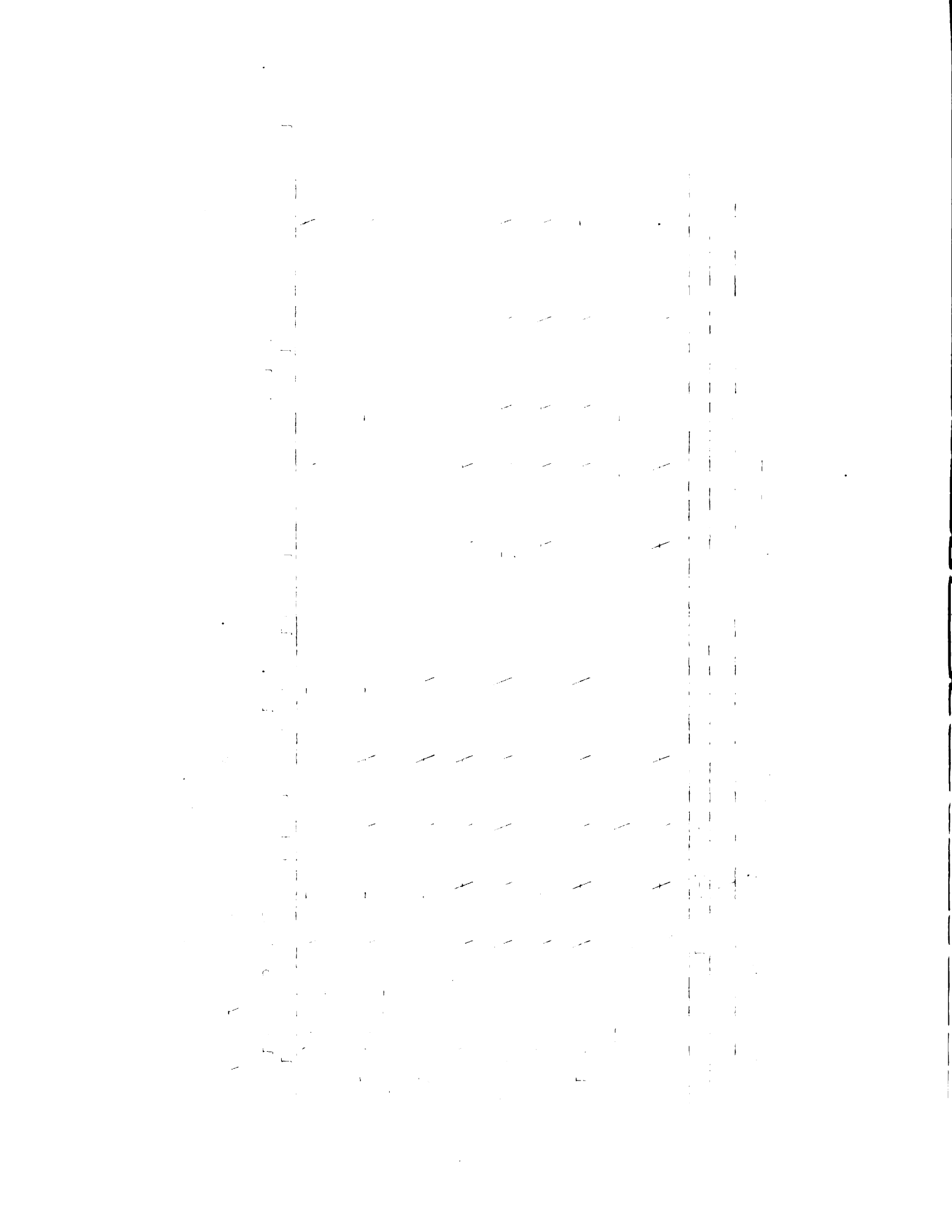


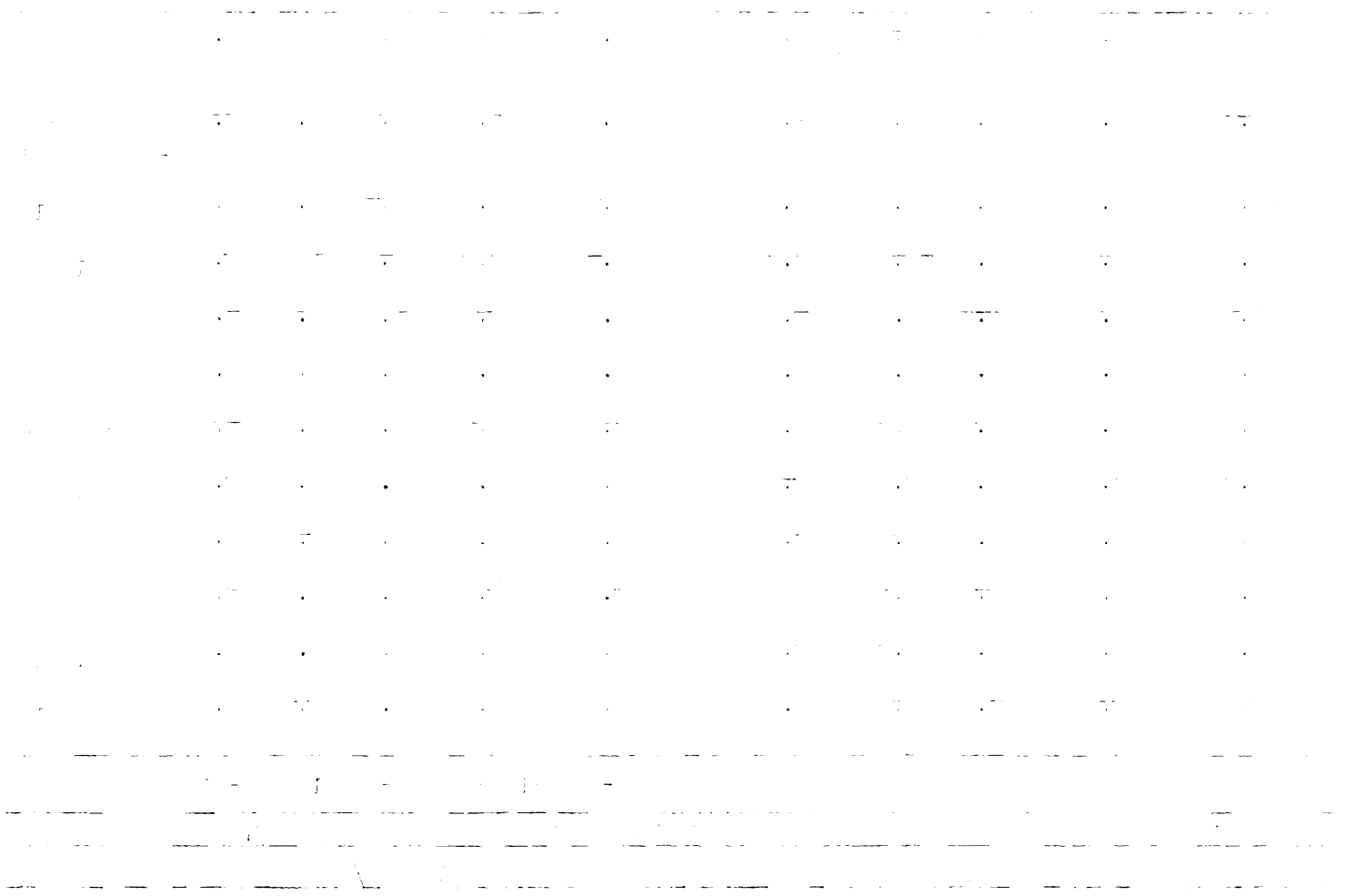
TABLE 3

The fermenting ability of various strains of L. plantarum on some carbohydrates in Rogosa's medium No. 3.

	NO_3^- strains					NO_3^- strains				
	pH's are recorded and those which signify acid production are underlined.									
	17-5	L-10	A-6-4	A-231-3	A-85-2	B-227	246	A-73-1	A-160-1	A-242-1
Adonitol	6.0	6.0	6.0	5.9	5.9	6.0	5.9	6.0	5.9	5.9
Arabinose	4.6	4.8	4.8	4.7	5.9	5.1	4.5	5.9	4.8	5.9
Cellobiose	4.7	4.6	4.8	4.7	4.6	4.8	4.8	4.7	4.8	4.7
Dulcitol	6.0	5.9	4.4	4.5	4.4	6.0	6.0	6.0	6.0	5.9
Galactose	4.5	4.5	4.4	4.5	4.4	4.4	4.3	4.5	4.4	4.5
Glucose	4.4	4.3	4.2	4.3	4.2	4.3	4.3	4.1	4.3	4.4
Inositol	6.0	5.9	5.9	6.0	5.9	5.9	5.9	5.9	5.9	5.9
Inulin	5.9	5.9	5.9	5.9	5.9	5.9	5.9	5.9	5.9	5.9
Lactose	4.4	4.4	4.5	4.4	4.4	4.3	4.3	4.3	4.4	4.4
Levulose	4.4	4.5	4.4	4.3	4.2	4.2	4.3	4.2	4.4	4.2
Maltose	4.3	4.4	4.3	4.4	4.1	4.3	4.2	4.2	4.3	4.4
Mannitol	4.3	4.2	4.2	4.2	4.2	4.2	4.2	4.1	4.2	4.2
Mannose	4.2	4.4	4.3	4.3	4.2	4.2	4.2	4.2	4.4	4.3

TABLE 3 continued

	NO_3^- strains					NO_3^- strains				
	pH's are recorded and those which signify acid production are underlined.									
	17-5	L-10	A-6-4	A-231-3	A-85-2	B-227	246	A-73-1	A-160-1	A-242-1
Melibiose	4.4	4.2	4.1	4.3	4.2	6.0	4.3	4.0	4.3	4.3
Melezitose	4.4	6.0	6.0	6.0	6.0	4.4	4.4	4.5	4.5	4.4
Raffinose	4.5	4.6	4.5	4.6	4.6	6.0	4.5	4.6	4.6	4.5
Rhamnose	5.4	5.4	5.5	5.5	5.9	5.0	5.4	6.0	5.9	5.9
Salicin	4.9	4.9	4.9	4.9	4.8	4.9	4.9	4.9	4.9	4.9
Sorbitol	4.5	4.5	4.5	4.5	4.6	4.4	4.5	4.6	4.5	4.4
Sorbose	6.0	6.0	5.9	5.9	5.9	6.0	6.0	5.9	5.9	5.9
Sucrose	4.4	4.3	4.1	4.4	4.3	4.4	4.3	4.1	4.4	4.4
Trehalose	4.5	4.5	4.5	4.6	4.4	4.4	4.4	4.6	4.5	4.4
Xylose	6.0	5.9	4.7	4.6	4.9	5.9	5.9	5.9	5.9	5.9
Alpha CH_3-d-glucoside	4.5	5.9	4.5	4.4	6.0	5.9	6.0	5.0	5.3	4.4
Alpha CH_3-d-mannoside	4.5	6.0	5.9	6.0	6.0	6.0	4.5	4.6	6.0	4.4



methyl-d-glucoside and the former fermented alpha methyl-d-mannoside also. No acid production was observed with these carbohydrates in cystine trypticase agar.

All strains formed acid from cellobiose, trehalose, glucose, lactose, levulose, maltose, mannitol, mannose, salicin, sorbitol, and sucrose. No strain formed acid from adonitol, inositol, inulin or sorbose.

A-6-4 was the only strain which produced acid from dulcitol on both basal media. Alpha methyl-d-glucoside was fermented by strains 17-5, A-6-4, A-231-3 and A-242-1 and strains 17-5, 246, and A-242-1 fermented alpha methyl-d-mannoside on both basal media. Three strains, A-6-4, A-231-3 and A-85-2 fermented xylose. A-85-2, A-73-1, and A-242-1 produced no acid from arabinose, four strains L-10, A-6-4, A-231-3, and A-85-2 produced no acid from melezitose and 4 strains, A-85-2, A-73-1, A-160-1, and A-242-1 produced no acid from rhamnose. L. plantarum B-227 was the only strain which did not ferment melibiose or raffinose.

From the data observed there is no consistent difference in the fermenting ability of nitrate positive strains compared to nitrate negative strains.

Vitamin requirements: The medium used to test for vitamin requirements was the basal medium 1 proposed by Sauberlich and Baumann (1946) for microbiological assays using L. plantarum 17-5 as the assay

organism. The effect of the omission of individual vitamins from various lots of the medium on the acid production of the 10 cultures tested is given in table 4. All strains demonstrated a requirement for pantothenate, niacin, and biotin. One strain, L-10 was greatly stimulated by the presence of riboflavin and strain A-85-2 was affected to a slight extent. Thiamine, p-aminobenzoic acid and folic acid were not essential for any of the 10 strains. However, it should be noted that some organisms require either folic acid or p-aminobenzoic acid but not both together (Baker et al. 1955). Pyridoxine was not required by any of the organisms tested but was somewhat stimulatory for strain A-160-1.

Amino Acid Requirements: The same basal medium and procedure used for vitamin requirements were employed for amino acid requirements (tables 5 and 6). All strains tested required leucine, isoleucine, valine, cystine, tryptophane, and glutamic acid while none of the strains demonstrated a requirement for methionine, lysine, histidine, serine, glycine and proline. However, histidine and serine stimulated strains A-85-2 and B-227 and glycine stimulated strains A-73-1 and A-160-1. Seven strains, L-10, A-85-2, B-227, 246, A-73-1, A-160-1, and A-242-1 were noted to require tyrosine. Strains L-10, A-85-2, B-227 and A-242-1 required phenylalanine and strain A-73-1 was somewhat stimulated by its presence.

Threonine was either required or greatly stimulatory to all strains tested. Four strains, L-10, A-6-4, A-231-3 and B-227 demonstrated

TABLE 4

The effects of the omission of various vitamins from a synthetic medium on the acid production by various strains of L. plantarum.

	NO_3^- strains					NO_3^-- strains				
Vitamin omitted	17-5	L-10	A-6-4	A-231-3	A-85-2	B-227	246	A-73-1	A-160-1	A-242-1
	Ml. NaOH (0.1098N) to titrate acid production by various strains									
None	13.4	11.2	11.9	10.8	10.9	13.6	13.3	11.2	11.9	13.8
Thiamine	13.4	11.4	10.3	11.9	10.4	14.0	12.6	11.0	12.0	13.0
Pyridoxine	13.1	10.9	11.1	9.4	10.6	12.8	13.9	10.5	8.5	13.00
Pantothenic	3.3*	0.7	1.9	2.0	1.0	0.9	2.8	0.8	0.9	2.0
Riboflavin	13.5	5.1	11.6	11.3	8.0	11.6	13.8	11.2	12.0	14.0
Niacin	1.7	1.0	1.6	1.0	1.0	1.6	2.4	1.9	1.5	2.1
p-aminobenzoic acid	13.6	11.0	11.5	10.1	10.9	13.3	13.3	10.9	11.2	13.4
Biotin	4.0	1.9	2.0	1.8	1.2	4.7	1.7	1.1	1.0	1.5
Folic acid	14.1	11.3	11.7	10.1	11.1	13.6	12.9	11.6	11.9	14.6

* All results are underlined where the amount of NaOH necessary to titrate the acid production was less than one-half that of the control.

TABLE 5

The effects of the omission of various amino acids from a synthetic medium on the acid production by various strains of L. plantarum.

Amino acid omitted	NO_3^-/ strains					NO_3^-- strains				
	17-5	L-10	A-6-4	A-231-3	A-85-2	B-227	246	A-73-1	A-160-1	A-242-1
	Ml. NaOH (ca. 0.1098N) to titrate acid produced by various strains									
None	12.7	11.0	11.0	10.8	10.6	13.0	12.2	11.4	11.2	13.1
DL-leucine	1.7*	1.1	1.7	1.3	1.4	0.7	1.4	1.2	1.3	1.4
DL-isoleucine	1.6	1.6	1.7	1.4	1.7	1.6	1.9	1.5	1.7	1.7
L-cystine	2.0	1.0	0.9	1.6	1.0	1.3	3.2	0.9	0.9	2.1
DL-valine	1.4	0.8	1.3	0.4	1.0	1.4	1.1	0.4	0.3	0.8
DL-methionine	10.9	9.8	8.7	8.7	8.1	9.7	9.5	8.7	8.7	11.0
DL-tryptophane	0.5	0.5	0.7	0.5	0.6	0.8	0.5	0.4	0.4	0.5
L-tyrosine	12.2	1.0	11.1	9.5	0.6	2.1	0.7	0.6	0.7	0.6
DL-phenylalanine	11.6	2.5	10.3	8.5	0.6	0.5	13.0	6.7	8.4	0.7

* All results are underline where the amount of NaOH necessary to titrate the acid production was less than one-half that of the control.

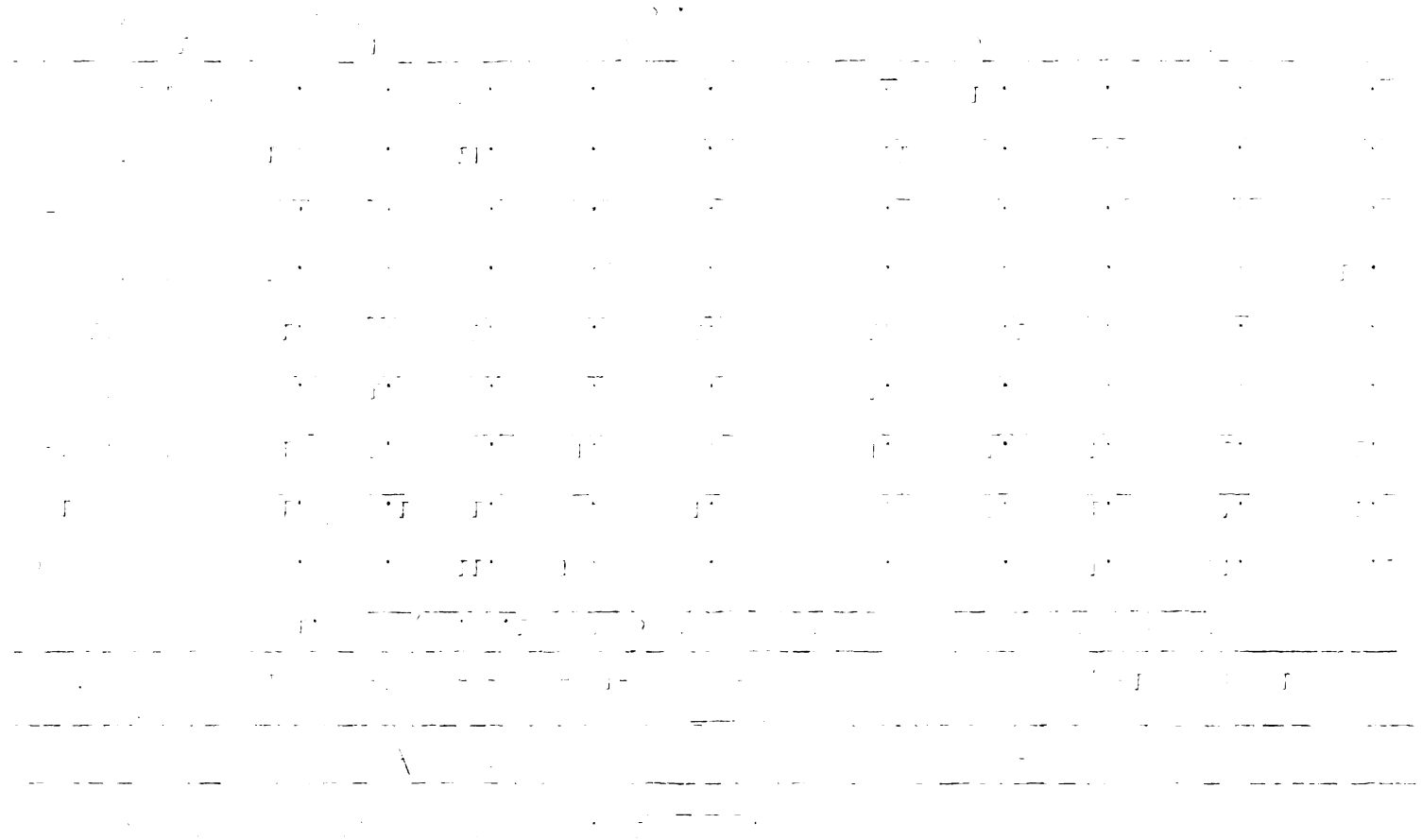
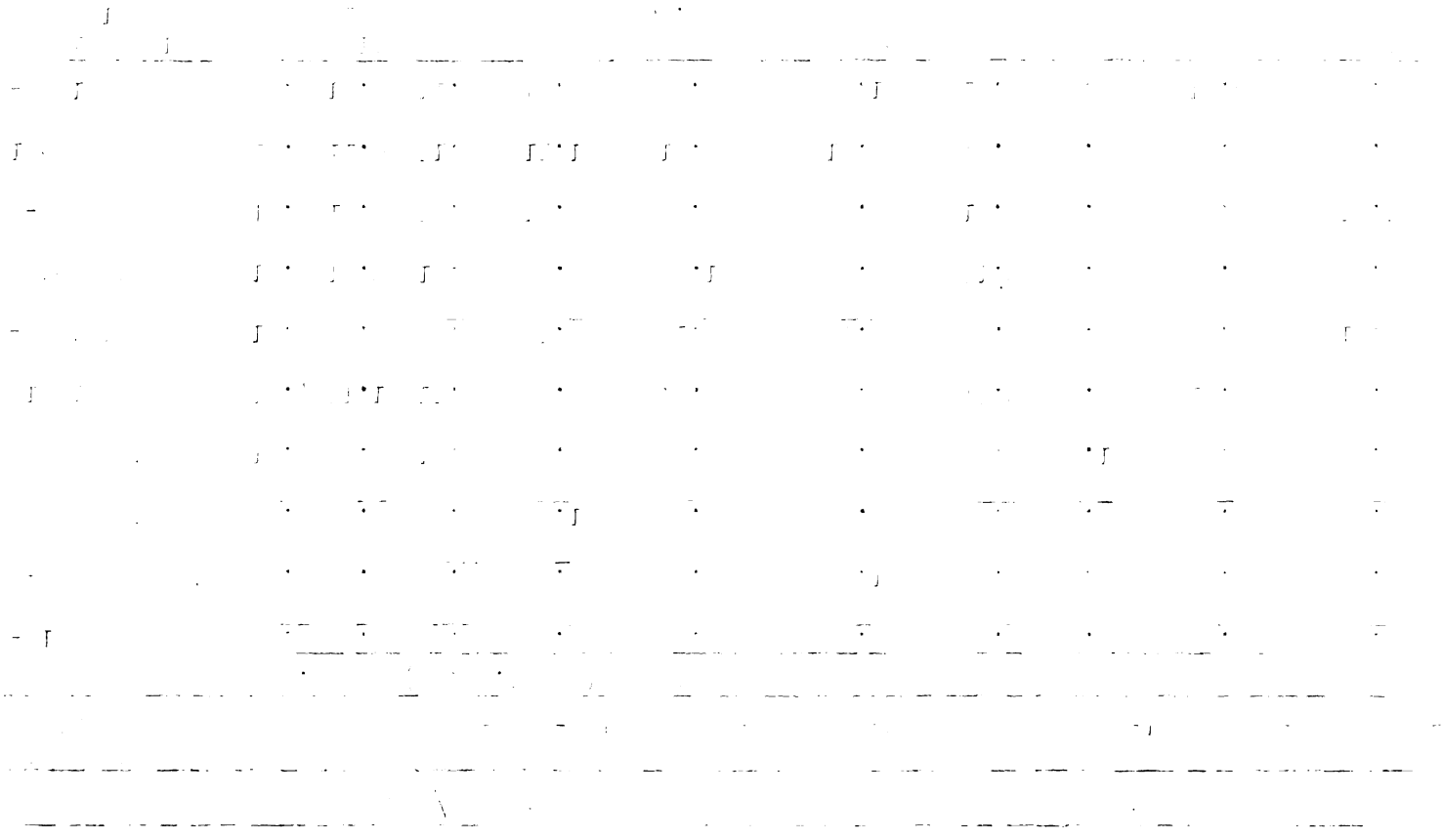


TABLE 6

The effects of the omission of various amino acids from a synthetic medium on the acid production by various strains of L. plantarum.

Amino acid omitted	NO_3^- strains					NO_3^- strains				
	17-5	L-10	A-6-4	A-231-3	A-85-2	B-227	246	A-73-1	A-160-1	A-242-1
	Ml. NaOH (ca. 0.1098N) to titrate acid produced by various strains									
L-glutamic acid	0.5*	0.5	0.6	0.6	0.6	0.4	0.6	0.5	0.5	0.6
DL-alpha alanine	7.7	2.2	4.9	3.5	7.0	6.1	6.3	7.3	7.7	9.6
DL-threonine	4.5	4.5	2.4	2.1	4.4	7.5	4.2	5.7	5.9	5.5
L-asparagine	10.3	9.6	10.6	6.6	6.0	5.5	5.2	7.1	7.4	9.5
L-lysine	12.7	11.1	11.4	8.0	10.5	9.9	11.2	9.5	10.2	13.0
L-arginine	12.8	8.5	2.0	1.3	1.2	1.2	12.2	8 .6	8.8	13.2
L-histidine	12.1	10.2	10.7	9.8	7.1	7.2	11.2	9.4	9.8	12.1
DL-serine	12.8	10.8	10.8	10.2	6.3	7.6	12.2	11.3	11.2	13.1
Glycine	12.8	11.5	11.5	11.1	10.9	11.8	12.3	6.8	7.4	13.2
L-proline	12.8	11.2	11.4	10.7	8.2	12.1	12.4	10.3	10.2	13.5

* All results are underlined where the amount of NaOH necessary to titrate the acid production was less than one-half that of the control.



a need for alanine while the other six strains (17-5, A-85-2, 246, A-73-1, and A-242-1) were somewhat stimulated when it was added to the media. Only two strains (B-227 and 246) required asparagine while five strains (A-231-3, A-85-2, A-73-1, A-160-1 and A-242-1) produced less acid when it was omitted. For arginine, it was noted that four strains required it for high acid production (A-6-4, A-231-3, A-85-2, and B-227).

Optical activity of lactic acid produced: The optical activity of the lactic acid produced by the various strains is given in table 7. There is a good correlation between the water of crystallization of the zinc lactate salt and the optical activity of the salts. Optically inactive salts theoretically should contain three moles of water of crystallization or 18.8%. Optically active salts contain 2 moles or 12.89% water of crystallization. Optically inactive lactate is derived from optically inactive lactic acid and active lactate from optically active lactic acid.

The lactate resulting from the lactic acid production by all cultures except B-227 was inactive. This is evidenced by both the water of crystallization and the specific rotation measurements. Therefore, these strains produced inactive lactic acid.

From the data observed the lactic acid formed by B-227 is a mixture of the d and l forms with the optically active d-lactic acid in excess. If the salt was pure d-lactate, the water of crystallization

TABLE 7

Water of crystallization in zinc lactate, and optical activity of lactic acid.

	NO_3^-/ strains					NO_3^-- strains				
	17-5	L-10	A-6-4	A-231-3	A-85-2	B-227	246	A-73-1	A-160-1	A-242-1
Water of crystallization of zinc lactate (per cent)	18.13	17.97	17.82	18.0	18.04	13.65	17.9	17.89	17.7	17.76
Specific rotation $[\alpha]_D^{20}$ (lactic acid)	0.0	0.0	0.0	0.0	0.0	4.20	0.0	0.0	0.0	0.0
Optical activity (lactic acid)	I	I	I	I	I	d	I	I	I	I

would be less (*i. e.*, 12.9% instead of 13.65%) and the specific optical rotation greater (8.8 instead of 4.2). It must be remembered that the specific optical rotation is given for the lactic acid *per se* rather than the salt. The rotation of zinc lactate is opposite of the lactic acid therefore, the salt demonstrated levo rotation on the polarimeter.

DISCUSSION

From the biochemical behavior of strain B-227 (i.e., its inability to ferment melibiose and raffinose, the formation of an extremely high titratable acidity in skimmed milk, and the formation of dextro-rotary lactic acid) it must be typed as Lactobacillus casei rather than Lactobacillus plantarum. This is according to the characteristics of these organisms as outlined by Rogosa et al. (1953), Tittsler et al. (1947) and Bergey's Manual (1948). Therefore, strain B-227 will not be considered for further discussion.

The fermenting ability of the other cultures tested was in close agreement with strain 17-5. Variations were observed on the following carbon compounds; arabinose, dulcitol, melezitose, rhamnose, xylose, alpha-methyl-d-glucoside, and alpha-methyl-d-mannoside. Similar variation between strains of L. plantarum were observed by Rogosa et al. (1953), Tittsler et al. (1947), Orla-Jensen (1943), and Harrison and Hansen (1950). Similar variations are reported in Bergey's Manual (1948). From the investigation of the fermenting ability of the various strains no significant difference between nitrate positive and nitrate negative strains was observed. Variations as to sodium hippurate hydrolysis and esculin hydrolysis were noted among the strains but they were not specific for nitrate positive or negative strains. Such variations were also noted by Rogosa et al. (1953).

This investigation demonstrated that the well known 17-5 strain

required the following vitamins; biotin, niacin and pantothenic acid. The amino acids required were cystine, glutamic acid, leucine, isoleucine, tryptophane and valine. This is in agreement with Hegsted (1944), Kuiken et al. (1943), Lyman et al. (1947), and Shankman (1943). Dunn et al. (1947) found that L. plantarum 17-5 did not require cystine but he was using a greatly enriched medium. The vitamin and amino acid requirements of the other 8 strains of L. plantarum tested were noted to be similar to strain 17-5 in respect to the above stated vitamins and amino acids. All 9 strains either required or were very dependent on threonine for rapid acid production. This is in agreement with Costilow and Fabian (1955) using the same basal medium. Lyman et al. (1947) and Stokes et al. (1943) using different media have shown threonine to be non-essential for L. plantarum.

Slight variations were noted among the isolates tested in respect to vitamin requirements. Riboflavin was greatly stimulatory to one strain and slightly stimulatory to a second strain. Pyridoxine was found to be slightly stimulatory for one strain. In testing for amino acid requirements a wider variation was noted among the strains in respect to tyrosine, phenylalanine, alanine, arginine, and asparagine. Histidine and serine were stimulatory for one strain. Previous reports have also been conflicting as to the requirements of this species for these nutrients. Since the basal medium was constant in these experiments the variations noted must be due to differences in strains rather than to the test medium. Using the same basal medium,

Costilow and Fabian (1955) found similar variations; and Dunn et al. (1947) and Shankman et al. (1947) obtained similar results with different media. From the observed nutritional requirements, there is no significant difference between nitrate positive and nitrate negative strains.

All the strains of L. plantarum investigated produced a racemic mixture of lactic acid. This is in agreement with several other investigators (Rogosa et al., 1953; Tittsler et al., 1947; Orla-Jensen, 1943; and Pederson, 1936).

The ability of microorganisms to reduce nitrates is a very important taxonomic tool. It is used extensively not only for bacterial taxonomy (Bergey's Manual of Determinative Bacteriology, 1948), but also for that of yeasts. In fact Lodder and Kreger-VanRij (1952) used nitrate assimilation not only to demonstrate species differences but, also, to differentiate genera. The genus Hansenula, in their taxonomic breakdown, is separated from the genus Pichia only by nitrate assimilation; Hansenula has the ability to reduce nitrate and Pichia does not. Since certain strains of Lactobacillus plantarum have been shown to reduce nitrates in indole nitrate medium (BBL), it would seem logical to use this physiological ability as a means of taxonomic separation. Rogosa et al. (1953) and Tittsler et al. (1947) have divided strains of L. plantarum into three species, L. plantarum, Lactobacillus pentosus, and Lactobacillus arabinosus, based primarily

on the organisms' ability to ferment various carbohydrates. However, the results obtained in this investigation failed to provide additional support for such a division. Thus, according to the scheme based on carbohydrate fermentation as outlined by Rogosa et al. (1953), strains A-231-3 and A-85-2 which did not ferment dulcitol in cystine trypticase agar could be placed in the species L. plantarum. Conversely, in medium No. 3 both strains fermented dulcitol; and, therefore, could not be placed in this species. Using either basal medium, 5 strains (L-10, 246, A-73-1, A-160-1 and A-242-1) could be classified as L. plantarum and one strain (17-5) as L. arabinosus. Strains L-10 and 17-5 reduce nitrates while the other 4 strains listed do not. No strain could be typed as L. pentosus. Strain A-6-4 could not be typed according to this scheme.

A more extensive study was performed by Rogosa et al. (1953) whereby they separated L. plantarum into 3 groups. The division was based on carbohydrate fermentations, the hydrolysis of sodium hippurate and esculin, and a nutritional pattern. From the results obtained in the author's work not a single strain would satisfy all of the requirements of any one of the three groups. However, this does not mean that these cultures are not L. plantarum. It does mean that there is grave doubt as to the advisability of dividing this group of organisms into three separate species.

From the foregoing information, it is apparent that there are no other common taxonomic characteristics that are correlated with

the ability of the strains tested to reduce nitrates. Therefore, it does not appear advisable to differentiate those cultures which reduce nitrates as a separate species. There is no more reason to expect all strains of a species to reduce nitrates than there is to expect them all to ferment a particular carbohydrate or require a specific vitamin. There are so many of these factors which vary within this group of organisms, that it would be necessary to create many species to satisfy all of the patterns possible. Therefore, it is the authors' opinion that such variations should be listed as strain differences rather than species differences for this particular group.

SUMMARY

Five of the ten strains typed as Lactobacillus plantarum used in this study were shown to reduce nitrates in indole nitrate medium (BBL). The purpose of this work was to determine if there were any other taxonomic differences between those strains which were capable of reducing nitrates and those strains which were devoid of this physiological ability.

The cultures were studied for morphological, cultural and physiological characteristics. All strains were uniform in that they were gram positive, non-spore forming, non-motile, rods and were catalase negative and homofermentative. Variations among the individual strains were noted in the hydrolysis of esculin and sodium hippurate, fermentation of various carbohydrates, and nutritional requirements. All but one of the ten cultures produced optically inactive lactic acid. The one culture producing the active form was proven to be Lactobacillus casei.

The variations noted among the nine strains classified as L. plantarum were in no way correlated with nitrate reduction. Therefore, it is concluded that nitrate reduction in such instances should be used only for strain differentiation.

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Thesis

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