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The Characteristics of *Lactobacillus plantarum*, *L. helveticus* and *L. casei*

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SUMMARY: The characteristics of 152 strains of lactobacilli were examined, and strains divided into three groups. *Lactobacillus helveticus* fermented inositol, sorbose, glycerol and rhamnose but not melibiose or raffinose, failed to grow in 4% bile salt, but gave a positive Voges-Proskauer reaction and a rapid acid clot in Yeastrel glucose litmus milk (Y.G.L.M.). *L. casei* would not ferment these sugars, nor tolerate 4% bile salt, rarely gave a positive Voges-Proskauer reaction, but produced a rapid acid clot in Y.G.L.M. *L. plantarum* always fermented melibiose, usually raffinose and sometimes rhamnose, but did not ferment inositol, sorbose or glycerol. Strains of these species tolerated 4% bile salt but gave a negative Voges-Proskauer reaction and produced an acid clot in Y.G.L.M. only slowly.

The identification of *Lactobacillus helveticus*, *L. casei* and *L. plantarum* has in the past been difficult and uncertain. These organisms have been confused with each other and with other species in the genus *Lactobacillus*.

Bacillus casei α , γ , ζ , and ϵ were isolated from Emmenthal cheese and differentiated from each other by von Freudenreich & Thoni (1904) using production of acid and gas, carbohydrate fermentation, growth temperatures and colonial appearance. Later, Orla Jensen (1919) reclassified *Bacillus casei* α as *Streptobacterium casei*, and *Bacillus casei* ϵ as *Thermobacterium helveticum*; he also described *Streptobacterium plantarum*. The thermobacteria were distinguished from the streptobacteria mainly by their higher growth temperatures and with some species, by the optical activity of the lactic acid produced. This separation of *S. plantarum* from *S. casei* was based on the production of inactive lactic acid by the former, and dextro-lactic acid by the latter. He stated, however, that strains of *S. casei* might produce mainly inactive acid on first isolation and that some of his strains of *S. plantarum* produced mainly dextro-lactic acid. Further characteristics such as 'as a rule prefers maltose to saccharose and lactose' do not help in the definition of the two species. Pederson (1936) gave a clear description of *Lactobacillus plantarum* but did not compare it with *L. casei* or *L. helveticus*.

Sherwood (1939) isolated 594 strains of lactobacilli from New Zealand Cheddar cheese and examined a number of them in detail. He divided them into *Lactobacillus plantarum*, *L. casei* and a group of strains with intermediate properties. Both species and the intermediate group were further subdivided on the basis of sugar fermentations, and the effect of salt and heat on acid production. It seems possible that the second group of *L. casei*, which have a higher growth temperature and ferment rhamnose, are strains of *L. helveticus*.

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Curran, Rogers & Whittier (1933) studied *Lactobacillus acidophilus* and found that by growth temperatures, morphology and carbohydrate fermentation, this species could be separated from another group of organisms thought to contain strains of both *L. casei* and *L. bulgaricus*. Pederson (1947), however, found some strains of *L. acidophilus* which produced dextro-rotatory lactic acid; in this and other respects they were similar to strains of *L. casei* and he concluded that they were rough and smooth variants of a single type. The higher maximum growth temperature of *L. acidophilus* was used to separate this species from *L. casei* by Sherman & Stark (1927).

In a study of 500 lactobacilli from oral sources, Rogosa, Wiseman, Mitchell, Disraely & Beaman (1953) recognized three varieties of *Lactobacillus casei*, each variety being divided into three or more groups, in some cases by fermentation of a single sugar. They also described three subgroups of *L. plantarum*, but did not define *L. helveticus*. They found no difficulty in distinguishing *L. acidophilus* from *L. casei*.

Briggs (1953) was one of the few workers to attempt the study of the genus as a whole, and, using their wide range of growth temperatures (15–48°) and salt (NaCl) tolerance, she placed strains of *Lactobacillus helveticus* in one group (group IV). *L. plantarum* and *L. casei*, with a much lower maximum growth temperature but the same salt tolerance, were put in another group (group VI). By these tests *L. plantarum* could not be distinguished from *L. casei*. *Bergey's Manual* (1948) does not give distinguishing characteristics for these species, apart from the type of lactic acid produced. This test does not appear to be very reliable (Orla-Jensen, 1919; Pederson, 1947; Harrison & Hansen, 1950), and although possibly useful in the detailed examination of a few cultures, is laborious for the identification of large numbers of strains (Sherwood, 1939; Briggs, 1953).

The aim of the present work was to find simple tests which, by adding to the known characteristics, would clearly distinguish between *Lactobacillus helveticus*, *L. casei* and *L. plantarum*.

METHODS

Cultures and maintenance. The 152 cultures examined in this work are those comprising group IV (25) and group VI (127) in the classification of Briggs (1953) (Table 1). These groups consisted mainly of strains of *Lactobacillus casei*, *L. plantarum* and *L. helveticus*, but included named strains of many other species; amongst them were *L. acidophilus*, *L. bulgaricus*, *L. fermenti*, *L. delbrueckii*, *L. lactis*, *L. arabinosus*, *L. odontolyticus* and *L. pentosus*. Strains isolated from Stilton cheese, silage and fresh and sour milk had also been classified in groups IV and VI and were included in this work.

The cultures were maintained and stored in the same media and under the same conditions as those described by Briggs (1953). The carbohydrate fermentation tests, and those for the tolerance of bile salt, were described by Wheeler (1955).

Voges-Proskauer reaction. The medium, consisting of Neopeptone 1.5%, glucose 2%, NaCl 0.5%, Tween 80 0.1% and Yeastrel 0.3%, had a final

pH of 6.8 and was tubed in 5 ml. quantities. The tubes were inoculated with 0.05 ml. of an actively growing culture and incubated at 30° for 4 days. The cultures were then tested by the method of Barritt (1936).

Action on Yeastrel glucose litmus milk. The production of acid and the coagulation of milk by these strains was studied in separated milk containing 0.8% Yeastrel, 1% glucose and 1.5% of a 1% solution of litmus (Y.G.L.M.). Tubes of this medium were inoculated with a 4 mm. loopful of a 48 hr. culture in Y.G.L.M. and incubated at 30°. The tubes were examined for acid and coagulation after incubation for 48 and 72 hr.

Table 1. *Physiological classification of the lactobacilli* *

	Gas from glucose	NH ₃ from arginine	Survival at		Growth at			Tolerance 4% NaCl	No. of strains
			60° for 30 min.	65° for 30 min.	15°	45°	48°		
Group I	-	-	-	-	-	+	-	-	34
Group II	-	-	-	-	-	-	-	-	16
Group III	-	-	+	-	-	+	-	-	20
Group IV	-	-	-	-	+	+	+	+	25
Group V	-	-	+	+	-	+	+	-	82
Group VI	-	-	-	-	+	-	-	+	127
Group VII	+	+	-	-	+	-	-	+	55
Group VIII	+	+	-	-	-	+	-	-	31
Section 1	-	-	+	-	-	+	+	-	24
Section 2	-	-	-	-	-	+	-	+	11
Section 3	-	-	-	-	+	+	-	+	12
Section 4			Miscellaneous reactions						15

* After Briggs (1953).

RESULTS

Using these methods, 141 of the 152 strains could be identified; one of the remaining eleven was found to be *Lactobacillus brevis*, but the other ten could not be named. The 141 strains were distributed between three species. Twenty-three strains were *L. helveticus*, 35 *L. casei* and 83 *L. plantarum*; the properties of these species are shown in Table 2. All strains were homofermentative and were separated from other homofermentative strains by the tests described by Briggs (1953), i.e. *L. plantarum* and *L. casei* grew at 15° but were unable to grow at 45° or 48°, *L. helveticus* grew at all three temperatures, and with a few exceptions, strains of all three species tolerated 4% NaCl (Table 1).

It can be seen (Table 2) that, omitting the sixteen sugars which gave positive or variable results and the four which gave negative results with all three species (Table 2, footnote), the remaining sugars allow clear differentiation of the three species. Further confirmation was obtained by the tests for tolerance of 4% bile and the action of the organisms on Y.G.L.M.

LACTOBACILLUS HELVETICUS

Of the 23 strains in this group, 9 were received as *Lactobacillus helveticus*, 6 as *L. casei*, 3 as *L. acidophilus* and 3 as *L. delbrueckii*; 2 were oral strains and the remainder were isolated from yoghurt.

All strains of *Lactobacillus helveticus* fermented inositol, sorbose and rhamnose, the majority fermented glycerol, but none fermented melibiose, raffinose, arabinose, inulin, adonitol or xylose. No strains would grow in the presence of 4% bile salt, but all except one gave a positive Voges-Proskauer reaction. All strains gave an acid clot in Y.G.L.M. after 48 hr. incubation at 30°.

Table 2. Differentiating characteristics of *Lactobacillus plantarum*, *L. helveticus* and *L. casei*

	<i>L. plantarum</i>	<i>L. helveticus</i>	<i>L. casei</i>
No. of strains ...	83	23	35
Growth at			
15°	+	+	+
45°	-	+	-
48°	-	+	-
Tolerance of 4% bile salt	+ (88%)*	-	-
Voges-Proskauer reaction	- (80%)	+	- (77%)
Acid clot in Y.G.L.M.			
2 days	-	+	+ (71%)
3 days	- (87%)	+	+
Carbohydrate fermentations†			
Inositol	-	+	- (88%)
Sorbose	-	+	- (83%)
Glycerol	- (90%)	+ (87%)	-
Rhamnose	- (59%)	+	-
Melibiose	+	-	-
Raffinose	+ (77%)	-	-
Arabinose	- (53%)	-	-
Xylose	- (87%)	-	-
Inulin	-	-	- (78%)
Adonitol	-	-	- (90%)

* The figures in parentheses indicate the percentage of strains giving the specified reaction.

† The following sugars were (a) fermented by all strains: amygdalin, cellobiose, glucose, galactose, lactose, fructose, maltose, mannitol, mannose, melezitose, salicin, sucrose, trehalose; (b) fermented by some strains of each species: dextrin, dulcitol, sorbitol; (c) fermented by none of the strains: aesculin, glycogen, erythritol, starch.

LACTOBACILLUS CASEI

The 35 strains found to belong to this species were 15 received as *Lactobacillus casei*, 3 as *L. plantarum*, 3 as *L. helveticus*, 1 as *L. lactis*, 1 as *L. brevis* and 1 as *L. delbrueckii*. The other strains were isolated from raw milk (2), sour milk preparations (4), cheese (2) and the mouth (3).

None of these strains fermented glycerol, rhamnose, melibiose, raffinose, arabinose or xylose, but the majority gave a positive Voges-Proskauer reaction. All strains produced acid and nearly all coagulated Y.G.L.M. in 48 hr. followed by reduction in 72 hr.

Strains of *Lactobacillus casei* were readily distinguished from *L. helveticus* by their inability to ferment rhamnose and, with few exceptions, their inability to ferment sorbose, inositol or glycerol or to produce acetylmethyl carbinol.

LACTOBACILLUS PLANTARUM

Eighty-three strains were identified; 22 of *Lactobacillus plantarum*, 5 of *L. arabinosus*, 2 of *L. casei*, 2 of *L. pentosus*, 1 of *L. fermenti*, 1 of *L. pentoaceticus* and 1 of *L. pastorianus*, and of the isolated strains, 26 were from Stilton cheese and 2 from Swedish cheese, 14 were from silage, 1 from fish silage, 2 from sour milk, 1 from raw milk and 3 from the mouth.

All strains fermented melibiose and the majority, raffinose; about half also fermented rhamnose and arabinose and few glycerol and xylose. Inositol, sorbose, inulin and adonitol were not fermented by any of the strains. With few exceptions, they tolerated 4% bile salt and gave a negative Voges-Proskauer reaction. Only a few of the cultures in Y.G.L.M. had produced acid in 48 hr., and although after 72 hr. all were showing acid, only 13% had clotted the milk.

Lactobacillus plantarum is distinguished from *L. helveticus* and *L. casei* by its ability to ferment melibiose and generally raffinose, and from *L. helveticus* by its failure to produce acid from inositol, sorbose, glycerol and often from rhamnose. It also differs from both species in its tolerance of 4% bile salt, and its slow growth and production of acid clot in Y.G.L.M.

DISCUSSION

The tests described in this work, supplemented by the growth temperature and gas production tests of Briggs (1953), show that the three species *Lactobacillus plantarum*, *L. helveticus* and *L. casei* are distinct and can be differentiated from each other and from all other species in the genus *Lactobacillus*. Other tests, including hydrolysis of starch in an agar medium, tolerance of 6% NaCl and production of amino acid decarboxylases, were discarded either because the results were variable or because they did not differentiate the organisms. The rapidity of acid production in milk was chosen in preference to the total percentage acid formed because it was found that a number of strains of *L. plantarum*, which produced acid only slowly, nevertheless gave a final acidity equal to that of *L. casei*.

It will be seen in Table 2 that in certain tests not all the strains in a species gave the specified reaction of that species. In general, however, only one atypical reaction was given by one strain; in other respects its characteristics agreed with those of the species. Since no two strains gave a number of similar atypical reactions, e.g. a strain of *Lactobacillus casei* differing from the majority in fermenting inositol, did not necessarily also differ in fermenting sorbose, no sub-division of any of the three species into varieties was made.

The strains included in the species *Lactobacillus helveticus* are, with two exceptions which could not be identified by the tests described here, those included by Briggs (1953) in her group IV, confirming that the wide range of temperatures at which strains of *L. helveticus* will grow is a valuable aid to their identification. The strains classified as *L. plantarum* or *L. casei* were those in Briggs group VI; nine strains of this group could not, however, be identified. Serological examination of the same strains by Sharpe (1955) also

confirms the identification of some of these organisms. All strains of *L. plantarum* belonged to the serological group *L. plantarum*, and 20 of the 23 strains of *L. helveticus* all possessed the same type antigen. Although no distinction could be made physiologically, the species *L. casei* was divided into two serological groups, and one of these groups, though possessing a different type antigen, had the same group antigen as strains of *L. helveticus*.

The results of the carbohydrate fermentation tests were highly reproducible and confirm that fermentation of melibiose is a characteristic distinguishing *Lactobacillus plantarum* from *L. casei* as was suggested by Orla-Jensen (1943). The results also show that *L. casei* can be distinguished from *L. acidophilus* by its ability to ferment mannitol and melezitose. Rhamnose, sorbose and inositol were consistently fermented only by strains of *L. helveticus*; *L. plantarum* strains were variable in their action on rhamnose, and a few strains of *L. casei* fermented sorbose and inositol. Orland (1950), in a study of the antigenic characteristics of a number of lactobacilli, found that one group possessing the same major antigen had similar fermentation reactions, in particular their ability to ferment rhamnose and sorbose. He thought all these strains were possibly of the same species although differently named, and this is supported by the work reported here. Some of his strains were included in the present study and the rhamnose-sorbose fermenters classified as *L. helveticus*. Although in general the carbohydrate fermentation tests confirm those of Rogosa *et al.* (1953) for *L. casei* and *L. plantarum*, it seems probable that the *L. casei* var. *rhamnosus*, which they describe, is really in fact *L. helveticus*. Its higher growth temperature and fermentation of rhamnose, sorbose and inositol identify it as a member of this species. Rogosa *et al.* (1953) did not describe a species *L. helveticus*.

Table 3.

LACTOBACILLUS PLANTARUM	LACTOBACILLUS CASEI	LACTOBACILLUS HELVETICUS
Growth at 15° but not at 45° or 48°.	Growth at 15° but not at 45° or 48°.	Growth at 15°, 45° and 48°.
Yeast glucose litmus milk: production of acid but rarely coagulation in 3 days.	Yeast glucose litmus milk: production of acid and generally coagulation in 2 days.	Yeast glucose litmus milk: production of acid and coagulation in 2 days.
<i>Distinguishing characters:</i> homofermentative. Ferments amygdalin, cellobiose, salicin, sucrose, melezitose and mannitol, also melibiose and raffinose and sometimes rhamnose. Does not ferment inositol, sorbose or glycerol.	<i>Distinguishing characters:</i> homofermentative. Ferments amygdalin, cellobiose, salicin, sucrose, melezitose and mannitol. Does not ferment melibiose, raffinose, rhamnose or glycerol, and rarely ferments inositol or sorbose.	<i>Distinguishing characters:</i> homofermentative. Ferments amygdalin, cellobiose, salicin, sucrose, melezitose and mannitol, also inositol, sorbose, rhamnose and glycerol. Does not ferment melibiose or raffinose.
Growth at 15° but not at 48°.	Growth at 15° but not at 48°.	Growth at 15° and at 48°. Growth in 2% but not 4% bile salt, usually grows in 4% NaCl.
Growth in 4% bile salt and generally in 4% NaCl.	Growth in 2% but not 4% bile salt, usually grows in 4% NaCl.	Rapid production of acid and clot in Y.G.L.M.
Slow production of acid and clot in Y.G.L.M.	Rapid production of acid and clot in Y.G.L.M.	

It is of interest to note that the few oral strains in our collection are distributed over a number of species. One was found to be a strain of *Lactobacillus acidophilus* (Wheater, 1955), 2 were *L. helveticus*, 3 were *L. casei* and 3 were *L. plantarum*, confirming the findings of Rogosa *et al.* (1953). Pederson (1936) suggested that *L. arabinosus* and *L. pentosus* were synonyms of *L. plantarum* and in the present work all strains of these two species were in fact identified as *L. plantarum*.

Since these tests have shown that *Lactobacillus plantarum*, *L. helveticus* and *L. casei* can be clearly identified, it is suggested that the descriptions in *Bergey's Manual* (1948) may now be supplemented as shown in the lists of distinguishing characters (Table 3).

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