Physiological Characteristics and Vitamin Requirements of Lactobacilli Isolated from Milk and Cheese

By J. G. FRANKLIN AND M. ELISABETH SHARPE

*National Institute for Research in Dairying,
University of Reading*

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SUMMARY

The vitamin requirements and physiological characteristics of 179 strains of lactobacilli isolated from milk and cheese have been studied and their relationship to classification discussed. The vitamin requirements showed good correlation with the results of the physiological and biochemical tests with strains of the subgenus *Streptobacterium* but the correlation was poor among the beta bacteria. It was not possible to differentiate the physiologically similar *Lactobacillus brevis* and *L. buchneri* species by their vitamin requirements. The nutritional data indicated that the species *L. plantarum* probably consists of more than one variety, and confirmed the reliability of a thiamine requirement as a means of differentiating heterofermentative and homofermentative lactobacilli. The nutritional data also proved useful with some strains by verifying their unclassifiable status or by making possible their classification when they could not be identified with certainty on the basis of biochemical tests alone. The nitratase activities of strains of *L. casei*, *L. plantarum*, *L. brevis*, *L. buchneri* and pediococci were also studied. Four of the *L. plantarum* and 2 of the *L. casei* strains were able to reduce nitrate but none of the other species tested showed this characteristic.

INTRODUCTION

In a recent study of the bacterial flora of cheese milk and Cheddar cheese (Franklin & Sharpe, 1963), 1755 strains of lactobacilli were isolated and classified by using a variety of biochemical and physiological tests. Whilst most of these strains were satisfactorily assigned to appropriate species, a few strains could not be identified because their biochemical characteristics were intermediate between species. Rogosa and co-workers (1947, 1953, 1961) found that the biochemical and cultural characteristics of members of the genus *Lactobacillus* correlated well with their vitamin requirements. Accordingly, the vitamin requirements of representative strains of these milk and cheese isolates were investigated to determine whether a similar correlation occurred with these organisms and also to see whether knowledge of their vitamin requirements assisted in the identification of the unclassified strains.

Costilow & Humphreys (1955) observed that some strains of *Lactobacillus plantarum* reduced nitrates under certain conditions and this was later confirmed by Rogosa (1961). Because of the ready availability, in this investigation, of a large number of strains of lactobacilli and pediococci from different sources, the oppor-

tunity was taken of examining these organisms for nitrataase activity to determine the extent to which these observations were confirmed. The strains tested, however, were not always the same as those used in the nutritional study.

METHODS

Lactobacilli isolated from milk and cheese as described by Franklin & Sharpe (1963) were classified according to the schemes of Rogosa & Sharpe (1959) and Perry & Sharpe (1960), by using the following tests: microscopic appearance; catalase production; growth at 15° and 45°; production of gas from glucose; production of ammonia from arginine; splitting of aesculin; growth in 0.1 % Teepol (Shell Chemical Co. Ltd., Marlborough House, 15-17 Great Marlborough St, London W. 1.) and fermentation of sugars. Cultures were grown in MRS broth (de Man, Rogosa & Sharpe, 1960) and washed suspensions were used for inoculation of the test media.

In addition to the above tests, some strains were also tested for the reduction of nitrate using a medium of low carbohydrate content maintained at a relatively high pH within the nitrataase activity range (Rogosa, 1961). The composition of this medium was as follows (% w/v): Oxoid peptone, 1.0; Oxoid Lab-Lemco, 1.0; Oxoid yeast extract, 0.5; glucose, 0.2; Tween 80 (Polyoxyethylene sorbitan oleate, Honeywill and Stein Ltd., Devonshire House, Mayfair Place, Piccadilly, London W. 1) 0.1; K_2HPO_4 , 0.2; $CH_3COONa \cdot 3H_2O$, 1.0; tri-ammonium citrate, 0.2; $MgSO_4 \cdot 7H_2O$, 0.02; $MnSO_4 \cdot 4H_2O$, 0.005; KNO_3 , 0.1. The pH value was adjusted to 7.5-7.6 before sterilization. The method of testing for nitrate reduction was that used by Rogosa (1961). The vitamin requirements of the lactobacilli were determined by using the medium and methods described by Rogosa, Franklin & Perry (1961).

The taxonomy of the lactobacilli and many of the tests used were discussed recently by Rogosa & Sharpe (1959) and by Sharpe (1962).

RESULTS AND DISCUSSION

The milk and cheese lactobacilli isolates which could be satisfactorily classified on the basis of physiological tests belonged to the four species *L. casei*, *L. plantarum*, *L. brevis* and *L. buchmeri*. As it was not practicable to examine the vitamin requirements of all the 1755 strains, representative typical strains were studied from each of these species, together with those strains which could not be classified biochemically into named species or which, although resembling a particular species sufficiently to warrant inclusion in that group, nevertheless differed from the typical reactions of that species in one or more important characteristics. For reasons of simplicity in this publication, the description 'typical' is applied to named strains showing the differentiating physiological characteristics or nutritional requirements of species of lactobacilli as described by Rogosa & Sharpe (1959) and Rogosa *et al.* (1961).

Lactobacillus casei. The vitamin requirements were determined for 74 strains which were classified physiologically as *L. casei* or which resembled this species more than any other. Twenty-two per cent of these, giving the typical reactions of this species listed in Table 1, were able to grow at 45°, whilst 14 % of those giving the typical reactions did not grow at this temperature. Thirty-four per cent differed

Table 1. The differentiating biochemical and physiological characteristics of species used to classify *Lactobacilli* isolated from milk and Cheddar cheese

Species of <i>Lactobacillus</i>	Fermentation of			
	<i>L. casei</i>	<i>L. plantarum</i>	<i>L. brevis</i>	<i>L. buchneri</i>
Growth at 45°	maj	+	+	+
Gas from glucose	-	-	+	+
NH ₃ from arginine	-	-	+	+
Growth in 0.1 % Teepol	-	+	+	+
Arabinose	-	maj	+	+
Cellobiose	+	+	-	-
Lactose	+	+	maj	maj
Mannitol	+	+	weak	weak
Melibiose	-	+	+	+
Raffinose	-	+	maj	+
Salicin	+	+	-	-
Sucrose	+	+	maj	+
Trehalose	+	+	-	-
Xylose	-	maj	+	maj
Rhamnose	maj	maj	-	-
Melezitose	+	+	-	+
Aesculin cleavage	+	+	maj	maj

All species are catalase negative and grow at 15°. maj + = majority of strains positive; maj - = majority of strains negative.

from the typical in one character, 26 % in two characters, 3 % in three characters and 1 % in four characters. The extent to which the different tests gave atypical results with the 74 strains was as follows: sucrose negative, 33 %; trehalose negative, 23 %; cellobiose negative, 10 %; salicin negative 8 %; mannitol negative, 7 %; Teepol positive, 5 %; aesculin negative, 3 %; lactose negative, 3 %; melezitose negative, 3 %; rhamnose positive, 1 %; arabinose positive, 1 %.

The vitamin requirements for the 74 strains of *Lactobacillus casei* tested are given in Table 2. Most of the strains (91 %) had the typical vitamin requirements for the species, and no strain required thiamine. Only one strain had no requirement for riboflavin and this strain differed from the typical physiological and biochemical characteristics of the species only in its failure to ferment sucrose. The remaining strains having atypical vitamin requirements had no other atypical characteristics in common.

Table 2. *The vitamin requirements of 121 strains of the sub-genus Streptobacterium Orla-Jensen*

Nicotinic acid and pantothenic acid were required by all strains.

Species	No. of strains	Thiamine	Riboflavin	Pyridoxal	Folic acid
<i>L. casei</i>	62	—	+	+	+*
(74 strains)	5	—	+	S	+*
	3	—	+	S	—
	1	—	+	+	—
	1	—	+	—	+
	1	—	—	+	S
	1	—	+	—	—
<i>L. plantarum</i>	30	—	+	—	—†
(47 strains)	14	—	—	—	—†
	2	—	—	S	—
	1	—	+	S	—

* Typical vitamin requirement pattern for *L. casei* strains (Rogosa *et al.* 1961).

† Typical vitamin requirement pattern for *L. plantarum* strains (Rogosa *et al.* 1961).

+ = requirement; — = no requirement; S = stimulatory.

Lactobacillus plantarum. The vitamin requirements were determined for 47 strains classified physiologically as, or closely related to, *L. plantarum*. Twenty-four per cent of these gave the typical physiological reactions listed in Table 1, 24 % differed from the typical physiological pattern by one character, 9 % by two characters, 4 % by three characters and 39 % by four characters. The proportion of the 47 strains differing in particular characters from the typical were as follows: Teepol negative, 63 %; trehalose negative, 54 %; sucrose negative, 52 %; raffinose negative, 41 %; melibiose negative, 2 % (1 strain). Unlike the *L. casei* group, no strains of *L. plantarum* were able to grow at 45°.

The vitamin requirements of the 47 strains of *Lactobacillus plantarum* tested are given in Table 2. Nearly all the strains (94 %) had the typical vitamin requirements for the species although it is evident that there are two varieties of *L. plantarum*, one requiring riboflavin and one having no requirement for this vitamin. This confirms the findings of Rogosa *et al.* (1953). Three strains only differed from the typical patterns in showing a stimulatory action of pyridoxal.

The strains which had no riboflavin requirement had the typical physiological characteristics of this species or differed only in their failure to grow in 0.1 % Teepol, whereas with the exception of two typical strains and one strain which did not grow in 0.1 % Teepol, the remaining 27 strains which exhibited a requirement for riboflavin also differed from the typical physiological pattern in their failure to ferment one or more of sucrose, trehalose, raffinose and melibiose.

Table 3. *The vitamin requirements of 46 strains of the subgenus Betabacterium Orla-Jensen*

Nicotinic acid and pantothenic acid were required by all strains.

Species	No. of strains	Thiamine	Riboflavin	Pyridoxal	Folic acid
<i>L. brevis</i> (27 strains)	9	+	+	—	+
	6	+	S	+	+
	4	+	+	+	+
	3	+	—	—	+*
	3	+	S	+	+
	2	+	+	—	+
<i>L. buchneri</i> (19 strains)	7	+	—	—	—†
	6	+	—	S	+
	6	+	+	S	+

* Typical vitamin requirement pattern for *L. brevis* strains (Rogosa *et al.* 1961).

† Typical vitamin requirement pattern for *L. buchneri* strains (Rogosa *et al.* 1961).

+ = requirement; — = no requirement; S = stimulatory.

Lactobacillus brevis. Vitamin patterns were determined for 27 strains classified as *L. brevis*. Eight strains gave the typical physiological reactions for this species as listed in Table 1 and 8 strains differed by only one character (Teepol negative, 5 strains; melibiose negative, 6 strains; NH_3 from arginine negative, 1 strain).

The vitamin requirements for the 27 strains of *Lactobacillus brevis* are given in Table 3 which shows that only three strains gave the typical vitamin requirement pattern for this species. All the strains possessed the typical thiamine requirement of the heterofermentative lactobacilli and all required folic acid, but this latter property was also found in other betabacteria. Contrary to the previous findings, riboflavin was essential for growth of, or was stimulatory to, 24 strains, and pyridoxal was essential for the growth of 13 strains. The atypical vitamin-requirement patterns obtained in this group were different combinations of the various vitamin requirements exhibited by so-called typical strains of *L. brevis*, *L. buchneri* and *L. viridescens*. All these strains had the physiological characteristics of *L. brevis*, however. None fermented melezitose and all differed in some respect from typical *L. viridescens* strains.

Lactobacillus buchneri. Vitamin requirements were determined for 19 strains classified physiologically as *L. buchneri*. Four strains gave the typical physiological characteristics for this species as shown in Table 1, 13 strains differed in one character from the typical and 2 strains in two characters. The proportions of the 19 strains differing in particular characters from the typical were as follows: Teepol negative, 13 strains; raffinose negative, 1 strain; sucrose negative, 1 strain; melezitose negative, 1 strain; salicin positive, 1 strain.

Table 4. *The physiological and nutritional characteristics of twelve unclassified lactobacilli isolated from milk and Cheddar cheese*

Strain no.	Nicotinic acid and pantothenic acid were required by all strains.											
	Requirement for						Fermentation of					
	Folic acid					+			+	+		
	Pyridoxal	S		S	S		+			+	+	S
	Riboflavin	+	+			+	+	+	+	+	+	+
	Thiamine	+	+	+	+					+		
	Aesculin cleavage					+		+	+			+
	Melezitose							+	+		+	+
	Rhamnose											
	Xylose									+		
	Trehalose	+	+					+			+	
	Sucrose		+	+	+	+					+	
	Salicin					+		+	+			+
	Raffinose		+	+	+							
	Melibiose			+	+							
	Mannitol					+	+	+	+		+	+
	Lactose		+	+	+	+	+	+	+	+		+
	Cellobiose					+		+	+			+
	Arabinose				+				+	+		
	Growth in 0.1% Teepol	+							+			
	NH ₃ from arginine			+	+					+		
	Gas from glucose	+	+	+	+			+		+	+	+
	Growth at	15° 45°				+	+		+			
				+	+	+		+	+	+	+	+
	Catalase											

S = stimulatory, ± = sometimes positive.

The vitamin requirements for the 19 strains of *Lactobacillus buchneri* are given in Table 3. All the strains required thiamine but only 7 strains exhibited the typical vitamin requirement pattern for this species. Pyridoxal was stimulatory and folic acid essential for the growth of the remaining 12 strains which could be equally divided into those which required riboflavin and those which did not. Some strains of *L. buchneri* have been observed to show a requirement for riboflavin and this is confirmed here, although in the present work this character was always accompanied by a requirement for folic acid. The atypical vitamin requirement patterns obtained in the group again appear to be different combinations of the so-called typical patterns for *L. brevis*, *L. buchneri* and *L. viridescens*. However, all the atypical strains produced ammonia from arginine and fermented melezitose, arabinose and melibiose, and could not, therefore, be classified either as *L. brevis* or *L. viridescens* species.

Unclassified *Lactobacillus* strains

Twelve strains were unclassified physiologically and their physiological and nutritional characteristics are given in Table 4. Some of the implications of these results in relation to their classification are discussed below.

Strain ML 68. Production of gas from glucose places this strain in the subgenus *Betabacterium*. Failure to produce ammonia from arginine, its inability to grow at 45° and its relative non-fermentative nature suggest that it might be classified as *L. viridescens* and, except that folic acid was not stimulatory, this is supported by the nutritional data.

Strain ML 78. This strain is also a member of the betabacteria but differs from ML 68 in its failure to grow in 0.1 % Teepol, its fermentation of lactose and raffinose and the lack of growth stimulation by pyridoxal. This strain does not, therefore, fit into any of the recognized species in this subgenus.

Strain ML 118. The physiological characters of this strain suggest that though capable of growth at 15° it belongs to the species *L. fermenti*. The vitamin requirements supported this classification although pyridoxal was stimulatory contrary to the usual pattern for this group.

Strain ML 161 gave identical results to ML 118 except for its failure to grow at 15°, and would, therefore, also appear to belong to the species *L. fermenti*.

Strain ML 568. The physiological results, with the exception of the failure to ferment trehalose, indicate that this strain is clearly a member of the species *L. casei*. This is not supported by the nutritional results, however, which were typical of *L. plantarum*.

Strain CL 833. This fermented few carbohydrates but the typical vitamin requirements of *L. casei* exhibited by this organism suggest that CL 833 should be assigned to this species.

Strain OL 1025. With the exception of the production of a small amount of gas from glucose and the failure to ferment sucrose, this strain had the typical physiological reactions of *L. casei*; its vitamin requirements, however, were those of *L. plantarum*. The absence of a thiamine requirement excluded it from the heterofermentative lactobacilli and the physiological characteristics, with the exception of gas production, were unlike those of any named *Betabacterium* species.

Strain CL 1261. This was very similar to CL 1025, having physiological characters closely related to *L. casei*, the vitamin requirements of *L. plantarum*, yet producing small quantities of gas from glucose.

Strain CL 1191. This seems to be intermediate between *L. casei* and *L. plantarum*, being unable to ferment melibiose and raffinose but able to grow in 0.1 % Teepol and fermenting arabinose. The vitamin requirements, however, were typical of *L. plantarum*.

Strain CL 1225. Although clearly a member of the subgenus *Betabacterium*, this organism cannot be placed into any of the named species in this group; this was confirmed by the nutritional data.

Strain CL 1226. This physiologically inactive strain showed the typical vitamin requirements of *L. casei* suggesting that it is most closely related to this species.

Strain CL 131. This strain resembles *L. casei* physiologically, although producing small volumes of gas from glucose, but this classification is not entirely supported by the nutritional pattern which was intermediate between those of *L. casei* and *L. plantarum*.

Table 5. Nitrate reduction by lactobacilli and pediococci isolated from milk and cheese

Species	No. strains tested	No. strains positive
<i>L. casei</i>	102	2
<i>L. plantarum</i>	12	4
<i>L. brevis</i>	18	0
<i>L. buchneri</i>	8	0
Pediococci	9	0

The results of this investigation confirm the consistency that exists between the physiological characters of strains of *Lactobacillus casei* and *L. plantarum* and their vitamin requirements. The nutritional patterns obtained with *L. plantarum* were mainly of two kinds and this supports the hypothesis (Rogosa *et al.* 1953) that *L. plantarum*, as at present defined, might be a heterogeneous group consisting of more than one variety.

In contrast to the results obtained with the streptobacteria, the correlation between the vitamin requirements and the physiological characteristics of *Lactobacillus brevis* and *L. buchneri* strains was poor. A wide variety of vitamin-requirement patterns were obtained for these species, although no pattern was found to be common to both species. The suggestion of Rogosa *et al.* (1961) that the separation of *L. brevis* and *L. buchneri*, dependent physiologically on the fermentation of melezitose only, could be achieved by use of nutritional data was not supported because, contrary to their observations, many strains of *L. brevis* were found to have a requirement for riboflavin, and folic acid was necessary for the growth of most of the *L. buchneri* strains examined.

A requirement for thiamine by heterofermentative species of lactobacilli and the lack of this requirement by homofermentative species was again confirmed as a very reliable differentiating character, and the usefulness of nutritional studies as an adjunct to the more traditional methods of classifying the lactobacilli was again demonstrated.

Nitrate reduction

Table 5 confirms the ability of certain strains of *Lactobacillus plantarum* to reduce nitrates. Two strains of *L. casei* also reduced nitrate but the proportion of positive strains was very much less than with *L. plantarum* species. None of the strains of the other species tested exhibited nitrataase activity. It is evident, therefore, that under conditions of low carbohydrate content and a relatively high pH value, some strains of lactobacilli are able to reduce nitrate; failure to do so cannot be regarded, therefore, as a typical property of the genus *Lactobacillus*.

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