

THE CHARACTERISTICS OF SOME RUMEN LACTOBACILLI¹

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During the past few years several investigators have reported the isolation of lactobacilli or lactobacilli-like organisms from the rumen liquor of cattle and sheep. Gall and Huhtanen (1951) isolated several gram positive, nonspore-forming rods from rumen fluid which produced lactic acid from glucose, but they were not further identified. Some lactobacilli found by Rodwell (1953) in a sheep's rumen were described as resembling *Lactobacillus leichmannii* or *Lactobacillus delbrueckii*. Huhtanen and Gall (1953) isolated two types of rods from rumen fluid which they stated were closely related to the genus *Lactobacillus*. Wasserman *et al.* (1953) characterized some isolates from a bovine rumen as variants of *Lactobacillus bifidus*. A rumen lactobacillus was classified into a group containing *Lactobacillus brevis*, *Lactobacillus buchneri*, and other heterofermenters by Briggs (1953b). *L. brevis*, an anaerobic variety of *Lactobacillus lactis* and an organism similar to the motile lactobacillus of Harrison and Hansen (1950) were discovered by Mann and Oxford (1954) in the rumens of young calves. Mann and Oxford (1955) also found *Lactobacillus fermenti* and *Lactobacillus acidophilus* in the rumens and abomasums of calves and kids. The number of lactobacilli in the rumen appears to vary with the diet, according to Briggs (1955), who reported that lactobacilli predominated in the rumen of animals on concentrate diets, but were less numerous in grass-fed animals.

This report deals with the isolation and identification of lactobacilli mainly from bovine rumen fluid. A sample of ovine rumen fluid yielded two cultures in some preliminary work which stimulated the investigation leading to this report.

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MATERIALS AND METHODS

One sample was taken from a fistulated sheep and eight from a fistulated cow, both of which received a high roughage diet for the duration of the study. Rumen fluid was drawn through a tube into a flask by a vacuum pump, shaken and strained through cheese cloth and used for inoculation.

Preliminary studies indicated that enrichments with plain skim milk and rumen fluid plus glucose or plating with SL agar (Rogosa *et al.*, 1951), incubated anaerobically, would not suffice as isolation media. Consequently, serial dilutions were made with rumen fluid to 10^{-12} in SL medium, Briggs medium (1953a), and fortified skim milk. The fortified skim milk contained yeast extract, 5 g; glucose, 10 g; "V-8 juice," 100 ml; and skim milk, 1,000 ml; final pH, 6.8. These media were dispensed into screw top tubes in 9 ml amounts, one ml of rumen fluid placed in the first tube and the series carried to 10^{-12} . All routine incubations were at 37 C and extended from 2 to 7 days. V-8 juice strained through 2 layers of cheese cloth was substituted for tomato juice in all media. Several unsuccessful attempts were made to isolate obligate anaerobes by adding 0.05 per cent cysteine to the Briggs' and SL media and flushing with nitrogen.

After incubation V-8 juice agar was used for plating and isolation. This medium contained strained V-8 juice, 200 ml; tryptone, 5 g; tryptose, 5 g; yeast extract, 5 g; agar, 15 g; and distilled water, 800 ml, with a final pH of 6.8. The composition was obtained from Dr. R. P. Tittsler in a personal communication. In many cases duplicate plates were prepared and one set incubated anaerobically. The plates were incubated for 48 hr and colonies were picked into tubes of fortified litmus milk. These cultures were replated for purification and to determine the colony type.

Preliminary procedures were performed as follows: gram stain, examination for spores and

TABLE 1
Characteristics of identified rumen lactobacilli

Identified as	No. of Strains	Milk				Growth								Gas from Glucose	Lactose	Fructose	Mannose	Salicin	Trehalose	Cellulose	Mannitol	Rhamnose	Mellibiose	Raffinose	Arabinose	Xylose	Melezitose	Esculin	Starch	Hippurate
		Plain		Y.G.L.M.*		48	45	16	2% Bile salts	4% Bile salts	2% NaCl	4% NaCl																		
		Co-agu-lated	%	Coagulated	2nd day								3rd day																	
<i>Lactobacillus acidophilus</i>	12	+	0.5	5†	11	-	+	-	1	+	-	+	+	+	+	+	+	11	+	-	-	-	-	-	-	-	-	1	3	
<i>Lactobacillus brevis</i>	5	-	0.1	-	-	+	+	-	-	+	4	-	+	+	+	+	+	-	-	-	-	-	-	-	-	-	-	-	+	
<i>Lactobacillus buchneri</i>	30	-	0.2	-	-	+	+	-	4	-	-	-	+	+	+	+	+	-	-	-	-	-	-	-	-	-	-	+	+	
<i>Lactobacillus casei</i>	4	+	1.0	+	+	+	+	-	-	+	+	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
<i>L. casei</i> (non-lactose fermenting)	46	-	0.1	36	45	+	+	-	-	+	+	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
<i>Lactobacillus fermenti</i>	6	4	0.5	-	-	-	+	+	+	+	3	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
<i>Lactobacillus plantarum</i>	52	+	0.6	43	46	+	+	-	+	+	+	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+

* Yeast-glucose-litmus milk. Glucose, maltose, galactose, and sucrose were fermented by all strains; none fermented glycerol or produced a positive Voges-Proskauer reaction.

† The figures indicate the number of strains giving a positive result.

‡ Growth slight.

motility, and tests for catalase. Tests for nitrate reduction were made with both Briggs' medium and indole-nitrate broth (BBL), since Costilow and Humphreys (1955) noted that several strains of *Lactobacillus plantarum* reduced nitrate in the latter medium. Gas production was determined in Briggs' medium by the method of Rogosa *et al.* (1953).

Tests for the amount of acidity produced in skim milk, growth at 16, 45, and 48 C, type of lactic acid, carbohydrate fermentation, and hydrolysis of hippurate were performed as described by Rogosa *et al.* (1953) and Tittsler in a personal communication. The methods used to determine NaCl tolerance, bile salt tolerance, acetylmethylcarbinol production, action in yeast-glucose-litmus milk, and starch hydrolysis were described by Wheeler (1955*a, b*). Briggs' medium was used for the growth temperature tests and for the preparation of inocula.

The descriptions of various lactobacilli in the papers of Tittsler *et al.* (1947), Rogosa *et al.* (1953), Wheeler (1955*a, b*) and Sharpe (1955) were employed as guides for identification.

RESULTS

One hundred sixty-eight cultures were studied and 155 were identified. All of the strains were

gram positive, catalase negative, nonspore-forming, nonmotile rods. All of the isolates except four strains of *L. plantarum* failed to reduce nitrate. These four strains reduced nitrate to nitrite in indole-nitrate medium (BBL). In every other respect they resembled *L. plantarum*. No bifid forms were seen. The isolates grew on the surface of complex solid media containing reducing substances.

The characteristics of the identified isolates are summarized in table 1. In addition, volatile acid determinations helped to confirm the identifications. Cultures of *L. brevis*, *L. buchneri*, and *L. fermenti* all produced relatively large amounts of volatile acidity. Determinations were made of the optical rotation of the lactic acid produced by two strains of *L. acidophilus* and one strain of *Lactobacillus casei* (nonlactose fermenting). The former was inactive and with the latter, the D form was slightly predominant.

A group of 13 lactobacilli could not be identified on the species level. All were homofermenters that did not ferment lactose, but the fermentation patterns of the other carbohydrates were unique. At first growth failures were believed to be the cause, but the same results were obtained on repeated testing.

Because the isolation of lactobacilli resembling

TABLE 2
Cultures isolated from various media

	Plain Skim Enrich.	SL Agar	SL Broth	Briggs' Broth	Fortified Skim	Rumen Fluid Plus Glucose
<i>Lactobacillus acidophilus</i>	2* (2)†		4 (3)	1 (2)	3 (2)	2 (2)
<i>Lactobacillus brevis</i>			4 (5)	1 (6)		
<i>Lactobacillus buchneri</i>	2 (1)	1 (3)	15 (7)	4 (4)	8 (4)	
<i>Lactobacillus casei</i>			4 (4)			
<i>L. casei</i> (non lactose fermenting)....	2 (3)		30 (6)	8 (5)	6 (4)	
<i>Lactobacillus fermenti</i>			3 (4)	1 (7)		2 (2)
<i>Lactobacillus plantarum</i>	1 (3)	18 (3)	21 (6)	4 (6)	8 (6)	
Unidentified			7 (7)	6 (7)		

* These two cultures were isolated from ovine rumen fluid.

† Figures in parentheses are the logarithms of the highest dilution from which the cultures were isolated.

L. casei var. *alactosus* (Rogosa *et al.*, 1953) from rumen fluid was surprising, further testing was deemed necessary. A known culture (ATCC 11582) was obtained and studied. The ability of the isolates and ATCC 11582 to ferment inositol, dulcitol, adonitol, and sorbitol was determined. Dr. M. E. Sharpe kindly investigated the biochemical and serological characteristics of ATCC 11582 and three of the isolates. In the main, ATCC 11582 had the same characteristics as the isolated strains listed in table 1. All fermented adonitol, dulcitol, and sorbitol, but not inositol. Dr. Sharpe stated, in a personal communication, that she obtained almost the same results biochemically. She found that ATCC 11582 differed from the three isolates in fermenting inositol and dextrin but not dulcitol and all four hydrolyzed esculin. However, serologically the rumen strains differed from ATCC 11582. The three isolates reacted specifically, but weakly with a serum for the *L. casei* group. ATCC 11582 reacted specifically and strongly with a serum for the *L. casei-helveticus* group (Sharpe, 1955). The rumen isolates can apparently be classified as a nonlactose fermenting variety of *L. casei* not closely related serologically to ATCC 11582. The growth of these strains in a 45 C water bath was slight and in some cases questionable, but none of the strains fermented lactose or coagulated skim milk.

Table 2 presents data concerning the media with which the cultures were isolated. Lactobacilli were not isolated from SL agar at rumen fluid dilutions greater than 10^{-3} even when cysteine was added and the plates were incubated anaerobically.

The first 2 of 9 samples, including the ovine, were used for preliminary tests to develop the method of isolation. Strains of *L. casei*, *L. plantarum*, and *L. buchneri* were found in all of the next 7 samples; *L. acidophilus* in 4, *L. brevis* in 2, and *L. fermenti* in 2. The 7 samples were taken over a period of 2 months. Two cultures of *L. acidophilus* were isolated by an enrichment in skim milk from the only sample of ovine rumen fluid studied.

DISCUSSION

It has been demonstrated in this study that several species of lactobacilli were regularly found in the rumen fluid of 1 cow. The identified species do not appear to be indigenous to the

rumen as all of them have been isolated from other sources. Although no total cultural counts were made, it is evident that the lactobacilli represented a numerically important part of the total rumen population, since some of the isolated species were present in numbers greater than 10^7 (table 2). In this connection, Wilson and Briggs (1954) obtained total counts ranging from 10^8 to 10^{10} per g. Briggs (1955) noted that lactobacilli predominated in the rumens of animals fed high concentrate diets, whereas the cow used in the present study was fed a high roughage diet.

Table 2 indicates that *L. acidophilus* and *L. casei* were probably passengers because they were not found in the higher dilutions nor in all of the samples. Only one culture, a strain of *L. acidophilus*, had amyolytic powers, indicating that the lactobacilli isolated from the roughage-fed animal used in this study were not important in the fermentation of starch. However, Mann and Oxford (1955) isolated several amyolytic strains of *L. acidophilus* from kids.

L. brevis and *L. fermenti* have both been found before in calves and kids. (Mann and Oxford, 1954, 1955).

Although the idea of a nonlactose fermenting variety of *L. casei* seems somewhat surprising, a number of such cultures have been isolated. Orla-Jensen (1942) listed two nonlactose fermenters. Rogosa *et al.*, (1953) isolated 30 oral strains which they classified as *L. casei* var. *alactosus*. Rodwell (1953) suggested that one of the strains he isolated from a sheep's rumen was similar to *L. delbrueckii* or *L. leichmannii* because it did not ferment lactose. It was a homofermenter which grew at 40 C and fermented glucose, fructose, mannose, sucrose, and trehalose. It is certainly possible that Rodwell's isolate may have been similar to the nonlactose fermenting strains of *L. casei* isolated in the present study. Sharpe, in a personal communication, stated that her group had come across several strains of *L. casei* which did not ferment lactose. Hunt and Rettger (1930) found a number of nonlactose fermenting lactobacilli in soil and grain. The descriptions of some of their strains indicate that they were similar in many ways to the rumen isolates.

The presence of *L. plantarum* in the rumen was expected because this organism has been isolated many times from many types of vegetable material.

Several attempts were made to isolate obligately anaerobic lactobacilli, but none were found. This, of course, does not mean that they were not present in the rumen. Mann and Oxford (1954) found an anaerobic variety of *L. lactis* in a calf's rumen.

Several characteristics of the lactobacilli seem to be important in the over-all rumen fermentation. They are active fermenters of many carbohydrates, producing lactic acid, or lactic acid, CO₂, and acetic acid. Their requirements for vitamins and amino acids are well known. Certain strains decarboxylate histidine to histamine which can cause rumen paralysis (Rodwell, 1953). Heavy grain feeding caused the lactobacilli to predominate in the rumen (Briggs, 1955) and increased the amount of lactic acid (Balch *et al.*, 1955). With these characteristics in mind the need for further research upon the numbers and species of lactobacilli in the rumen with different conditions of feeding and in different geographic locations certainly seems evident.

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SUMMARY

One hundred sixty-eight cultures of lactobacilli were isolated, 2 from a sample of ovine rumen fluid in a preliminary trial and the rest from 8 samples of bovine rumen fluid. The methods of isolation are described. One hundred fifty-five were identified as follows: 12 were *Lactobacillus acidophilus*, 5 were *Lactobacillus brevis*, 30 were *Lactobacillus buchneri*, 4 were *Lactobacillus casei*, 46 were *Lactobacillus casei* (nonlactose fermenting), 6 were *Lactobacillus fermenti*, and 52 were *Lactobacillus plantarum*. Two cultures of *L. acidophilus* were found in the ovine sample. All, except *L. acidophilus* and *L. casei*, were found in rumen fluid dilutions of 10⁻⁶ or 10⁻⁷. The classification of nonlactose fermenting variety of *L. casei* is discussed.

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