

From the Veterinary College of Norway, Department of
Internal Medicine, Oslo.

IDENTIFICATION OF THE GRAM-POSITIVE RUMEN FLORA OF CATTLE AND SHEEP IN CLINICAL CASES OF ACUTE INDIGESTION

By

Nils Krogh

A predominantly Gram-positive flora consisting of lactobacilli and/or streptococci was found in the rumen of cows and sheep suffering from acute indigestion due to overeating or overfeeding (*Krogh* 1962). A similar rumen lactobacillosis has later been encountered in cases of digestive disturbances in calves (unpublished data). Several isolates of the various predominating microorganisms from these cases have been subjected to further studies for classification. The results of these studies are given in the present paper.

MATERIALS AND METHODS

In addition to rumen studies in three cows and two sheep which are described in a previous paper (*Krogh* 1962), rumen flora studies have also been conducted on two calves aged 6 and 3—4 months, (Calf 1 and Calf 2, respectively). Direct microscopy of the rumen fluid from the two calves disclosed a bacterial population completely dominated by Gram-positive rods. In both cases the primary cultures gave colony counts of lactobacilli of the order 10^{10} — 10^{11} per ml. rumen fluid.

The media and procedures used in studying the streptococcal and lactobacillus isolates were similar to those described in an earlier paper (*Krogh* 1961). However, in some cases tomato juice prepared according to *Briggs* (1953), was added to the peptone yeast-autolysate medium (CYG medium) in a final concentration of 10 % (CYGT medium). The selective medium (SL medium)

presented by *Rogosa et al.* (1951) for culturing oral lactobacilli has also been employed. These two media were mainly used for comparing cell and colony morphology of some of the lactobacillus strains with that obtained on the CYG medium. The physiological tests employed for identification of the streptococcal isolates were similar to those described by *Mann et al.* (1954).

RESULTS

1. *Properties of the rumen streptococci.* Thirteen strains isolated from Cow 2, which was the only animal showing a significant rise in the streptococcal colony count, were studied. Twelve of these strains proved to be identical in their physiological reactions, *i. e.* fermented lactose, starch and inulin very readily with a final pH in the range of 4.0 to 4.5. They did not attack mannitol, failed to grow in the presence of 6.5 % sodium chloride and at pH 9.6, and were α -haemolytic. The remaining strain differed from the others by not fermenting starch and by being non-haemolytic.

2. *Properties of the rumen lactobacilli.* One hundred and seventeen strains originating from 6 animals were studied. Table 1 shows the number of strains isolated from the various animals and the distribution of homo- and hetero-fermentative isolates.

Table 1. Grouping of rumen lactobacilli according to origin and gas production.

Origin of strains	Number of strains	
	Heterofermentative	Homofermentative
Cow 1.	17	4
Cow 3.	14	0
Calf 1.	0	41
Calf 2.	20	0
Sheep 1.	9	0
Sheep 2.	2	10

Exclusively gasforming strains were encountered in three of the animals, non-gasforming in one, and a mixture of the two types in the remaining two animals. However, each of the latter showed a marked preponderance of either heterofermentative or homofermentative strains.

Characteristics of the gasforming lactobacilli. All sixty-two heterofermentative strains produced ample amounts of gas in fluid cultures and did not show surface growth in stab cultures incubated aerobically.

The 42 strains originating from four of the animals (two cows and two sheep), were found to be very similar with regard to cell morphology and growth characteristics. The growth in fluid cultures as well as on solid media presented fairly uniform and distinctly stained Gram-positive rods, usually $0.7\text{--}0.9 \times 2.0\text{--}5.0$ microns in size. The cells were straight or slightly curved with rounded ends. They occurred most frequently singly or in pairs, more rarely in short chains. The organisms grew with uniform turbidity in young fluid cultures. Later a deposit was formed which, apart from the strains from Cow 1, dispersed easily on shaking. Streak cultures on CYG agar gave, as a rule, growth to two different colony types from each strain, *viz.* round, smooth, entire colonies up to 1 mm. in diameter, and much smaller, flat and dull colonies with irregular edge and rough surface. This variation in colony appearance was found very similar to that frequently observed for gasforming lactobacilli isolated from experimentally overfed sheep (Krogh 1961). However, this pleomorphism in colony morphology seemed to be related to the CYG medium since exclusively round, smooth and entire colonies were growing when the same strains were cultured on SL agar. In addition, the consistency of the growth seemed to be influenced by the medium used in some cases. Thus, the strains isolated from Cow 1 showed extremely mucoid growth in CYG broth and on CYG agar, whereas they presented pasty colonies on SL agar.

The 20 strains isolated from Calf 2 could be separated in two groups according to growth characteristics and arrangement of the microorganisms on CYGT media. Thus, twelve strains grew with fairly large and slightly fuzzy colonies on agar plates and in voluminous masses in fluid medium, the growth consisting of uniform small rods in long chains. The remaining strains presented smooth and somewhat smaller colonies, a deposit in broth which was easily dispersed, and the cells were usually occurring singly. These differences seemed to be consistent as they were invariably observed in serial subcultures.

The results of the biochemical reactions for all gasforming strains are summarized in Table 2.

Table 2. Biochemical characteristics of the heterofermentative rumen lactobacilli.

Origin of strains	Cow 1.		Cow 3.		Calf 2.	Sheep 1.	Sheep 2.	
Number of strains	17	12	2	2	20	9	1	1
Xylose	+	+	+	—	—	+	+	—
Arabinose	+	+	+	+	+	+	+	—
Laevulose	—or +s	+	+	+	+	+	+s	+
Lactose	+	—	+	+	+	+	+	+
Raffinose	+	+	+	19	+	+	+	+
Salicin	v	—	+	—	—	+	—	—
Aesculin	+	+	+	—	—	+	—	—

All strains fermented glucose, galactose, sucrose, maltose and melibiose. None fermented rhamnose, mannose, cellobiose, trehalose, melezitose, inulin, dextrin, starch, glycerol, adonitol, mannitol, sorbitol, dulcitol and inositol, or hydrolysed hippurate.

+s: Slightly attacked. v: Positive, weak or negative reactions. The figure indicates the number of strains giving a positive result.

The 40 strains originating from two cows and one sheep were identical in their response to nearly all substrates tested. The only differences were found in their behaviour to lactose, laevulose and salicin. With the exception of the two last-named substrates which might show questionable results, the other test substances gave clearcut reactions with the final pH in positive tests in the range 4.0—4.5. The rapid fermentation of arabinose and xylose, and the hydrolysis of aesculin as judged by the ferric citrate test, were characteristic features of all strains, whereas the ability to clot milk was confined to the lactose-positive isolates.

The remaining 22 gasforming strains isolated from another sheep and from one of the calves differed from the aforementioned group by not attacking aesculin and salicin and, with one exception, by being xylose negative. Two strains did not ferment arabinose or raffinose, and a third gave only a weak fermentation of laevulose. The other "sugars" tested gave uniform and clearcut results. The 20 strains originating from the calf showed a particularly strong fermentation of lactose, maltose and glucose with the final pH usually in the range 3.7—4.2. These strains also reduced, acidified and clotted litmus milk very readily, whereas this substrate was less strongly attacked by the two sheep strains. *Characteristics of the non-gasforming lactobacilli.* The 45 strains

isolated from the cow and the calf appeared to be very similar culturally and morphologically. In fluid cultures all strains showed heavy growth with uniform turbidity within 24 hrs. A liberal deposit was formed later which could easily be dispersed on shaking. The colonies developing on CYG and CYGT agar were from 0.5 to 1.0 mm. in diameter and appeared greyish, circular and smooth with entire edge and pasty consistency. These strains did not grow on SL agar in contrast to the gas-forming strains which were growing well on this medium. These strains also seemed to be more strictly anaerobic than the heterofermentative isolates and frequently did not grow in the upper layer of the stab cultures.

In general, the cells were found to be very pleomorphic, varying from coccoids to slightly bent and club-shaped rods. Branched and globular forms were also frequently seen. The reaction to the Gram-stain varied from distinctly positive to uneven and granular.

The biochemical properties of these strains are presented in Table 3.

Table 3. Biochemical characteristics of the homofermentative rumen lactobacilli.

Origin and number of strains	Mannose	Galactose	Maltose	Lactose	Melibiose	Raffinose	Dextrin	Starch	Inulin	Salicin	Melezitose	Cellobiose	Trehalose
Cow 1 (4).	—	+	+	+	+	+	+	+	3	3	v	1	— or v
Calf 1 (41).	—	+	+	+	+	+	+	+	+	±	±	— or +s	
Sheep 2 (10).	+	—	4	—	—	—	—	—	—	—	— or v	—	—

All strains fermented glucose, laevulose and sucrose, and hydrolysed aesculin. None fermented arabinose, xylose, rhamnose, glycerol, adonitol, mannitol, sorbitol, dulcitol and inositol, or hydrolysed hippurate.

+s: Slightly attacked. v: Variable reactions. ±: Majority of strains did not ferment. The figures indicate the number of strains giving a positive result.

The strains were showing a fairly uniform fermentative pattern. The majority of the fermentable substances, dextrin and starch included, were very readily attacked and showed a final pH in the range of 3.6—4.3. These results were clearcut and

reproducible, and the litmus milk was usually reduced, acidified and clotted. However, a few of the substrates tested were not attacked by all strains, underwent slow fermentation or gave questionable results. This was particularly the case with melezitose, trehalose and cellobiose, and to a lesser extent also salicin and inulin. Thus, when melezitose was tested initially, it was fermented by three of the four cow strains. When retested one year later only one of the originally positive strains still possessed this ability, and the initially negative strain had gained some fermentative power. A similar behaviour to melezitose was also noticed in some of the calf strains. The majority of the 45 strains proved to be cellobiose and trehalose negative, a couple of strains gave positive or variable reactions, while the rest showed a slow and weak fermentation of these substances. Finally, salicin was fermented strongly by 11 strains, was weakly attacked by seven, while the remaining 27 strains lacked the ability to ferment this substrate.

The 10 sheep strains proved to be distinctly different, morphologically as well as biochemically, from the other non-gas-forming isolates. They were growing well in CYG broth and on the corresponding agar, although the growth showed some pleomorphism on the latter medium. After 24 hrs. of incubation the colonies were found fairly small (0.5—0.75 mm.), greyish, smooth, flat and crenated. After prolonged incubation (2—3 days), a slightly fuzzy appearance developed. However, when cultured on SL agar the growth appeared more uniform and produced somewhat larger and raised colonies with entire edge. In stab cultures the growth usually ceased before reaching the surface.

The microorganisms were, as a rule, of fairly uniform shape and size. They appeared as straight or slightly bent rods which usually occurred singly although short chains were also observed. The size was most commonly found to be in the range of 0.6—0.8 \times 1.5—3.0 microns, but sometimes up to 6—8 microns in length. However, tiny, irregular colonies which occasionally occurred on CYG agar usually consisted of very pleomorphic, curled rods.

As can be observed in Table 3, the present strains showed more limited fermentative powers than the other homofermentative strains tested. The only sugars which were regularly fermented by all strains proved to be glucose, laevulose, mannose and sucrose, whereas only four of the isolates were able to fer-

ment maltose. All strains were found to be negative to melezitose when first tested, but five of them fermented this sugar when retested one year later. The final pH in positive tests was in the range of 4.2—4.6 for nine strains and within 3.9—4.1 for the tenth. The ferric citrate test on aesculin was found positive for all strains, whereas none of them changed litmus milk.

DISCUSSION AND CONCLUSION

Thirteen streptococcal strains and 117 lactobacillus strains from the predominating Gram-positive rumen flora in seven clinical cases of acute indigestion in cattle and sheep have been investigated for identification. The streptococcal isolates were obtained from a mild case of indigestion in a cow and the lactobacilli from more severely affected cows, calves and sheep.

The 12 starch-fermenting streptococcal strains showed physiological properties similar to those described for the "typical amyolytic rumen streptococcus" (*Mann et al.* 1954), and which is most likely identical with the mannitol-negative variety of *Str. bovis*. The remaining strain lacked the ability to ferment starch and in this respect resembled *Str. inulinaceus*, the non-amyolytic variety of *Str. bovis* (*Bergey's Manual* 1957).

Out of the total number of 117 lactobacilli tested, 62 strains proved to be heterofermentative and 55 homofermentative. Forty of the gasforming strains were found to be aesculin-positive and showed fermentation reactions closely resembling those of *L. brevis* (*Rogosa et al.* 1953). However, the hippurate reaction of these strains did not correspond to that most commonly ascribed to *L. brevis*. In this respect they were like the aesculin-positive, hippurate-negative variety of this species isolated from the rumen of young calves (*Mann and Oxford* 1954), and from experimentally overfed sheep (*Krogh* 1961).

The remaining 22 gasforming isolates differed from the aforementioned strains by not hydrolysing aesculin and by being negative to xylose (21 strains) and arabinose (1 strain). These strains, therefore, showed a fermentative pattern characteristic of *L. fermenti* and are closely related to three varieties of this species listed by *Rogosa et al.* (1953, see group 3, 6 and 11 in Table 5). However, none of the present strains showed any action upon mannose.

With regard to the classification of the homofermentative isolates, the 45 strains obtained from a cow and a calf showed

cell morphology, growth characteristics and biochemical reactions resembling the majority of non-gasforming lactobacilli encountered in experimentally overfed sheep and which were identified as *L. bifidus* (Krogh 1961). The present strains as well as those from the feeding experiments gave clearcut and identical reactions to most of the substrates tested. However, five test substances, *viz.* trehalose, cellobiose, melezitose, salicin and inulin, gave more variable results. However, while most of the strains from the feeding experiments showed a slow fermentation of these substrates, negative reactions were frequently found among the present strains.

The remaining 10 homofermentative strains isolated from one of the sheep lacked the ability to ferment lactose, which in *Bergey's* classification would relate them to *L. delbrueckii* or to *L. leichmannii*. The other biochemical reactions, however, did not correspond to those described for any of the two species mentioned (Rogosa *et al.* 1953, *Bergey's Manual* 1957). Thus, the present strains differ from *L. delbrueckii* by not attacking galactose, in this respect being like *L. leichmannii* (Rogosa *et al.*), and by not fermenting dextrin (*Bergey's Manual*). However, since the strains in question otherwise are showing biochemical properties in agreement with *L. delbrueckii* and differ from *L. leichmannii* in their behaviour to cellobiose, trehalose and salicin (Rogosa *et al.*), they seem to be closer related to the former and may most likely be regarded as a variety of this species.

The distribution of the four classified species of lactobacilli in relation to the animal host and type of food consumed is given in Table 4.

Table 4. Relationships between species and age of animal, food consumed and classified rumen lactobacilli.

Animal	Food consumed	Species and number of lactobacilli			
		L. brev.	L. ferm.	L. bifid.	L. delbr.
Cow 1.	Concentrates + brewer's grains	17	—	4	—
Cow 3.	Concentrates	14	—	—	—
Calf 1.	Milk, hay + concentrates	—	—	41	—
Calf 2.	Unknown	—	20	—	—
Sheep 1.	Concentrates	9	—	—	—
Sheep 2.	Concentrates, roots + hay	—	2	—	10

The predominating rumen flora of lactobacilli appeared to be different qualitatively in the various cases. Thus, frequently only one single lactobacillus species occurred in each animal. However, the number of animals examined is small, and it is therefore difficult to say whether there is any definite relationship between species or age of animal, or type of food consumed, and the species of lactobacilli flourishing in the rumen.

It may also be mentioned that the three animals harbouring *L. brevis* were those which showed symptoms of lameness, apparently due to laminitis (Krogh 1962). Supposing that the etiology of this disorder in ruminants is similar to that in horses, *i. e.* a histaminosis due to formation of histamine by bacterial decarboxylation of histidine (Akerblom 1934), and considering that abnormally high concentrations of histamine have been found in the rumen in cases of overfeeding (Rodwell 1953, Dain *et al.* 1955), it seems reasonable to assume that *L. brevis* could be the organism responsible for a possible histamine formation in the present cases.

In regard to the occurrence of lactobacilli in clinical as well as in experimental cases of acute indigestion in ruminants (Krogh 1961), eight species have been diagnosed. Thus, in addition to the four species described above, the following have been found: *L. buchneri*, *L. cellobiosus*, *L. plantarum* and *L. casei*. Out of a total of 265 identified strains, there were 146 strains of *L. brevis*, which is, therefore, the species most frequently encountered, although found only in cows and sheep. *L. bifidus* has been isolated from cow, sheep and calf in a total number of 65 strains, *L. fermenti* from calf and sheep, and the remaining five species were obtained from sheep.

Available literature indicates that *L. delbrueckii* and *L. cellobiosus* have previously not been isolated from the rumen. On the other hand, *L. acidophilus* which has been demonstrated in the rumen by other workers (Mann and Oxford 1955, Jensen *et al.* 1956, Perry and Briggs 1957), was not found in the rumen by this author.

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SUMMARY

Thirteen streptococcal strains and 117 lactobacillus strains have been isolated from the predominating Gram-positive rumen flora of cattle and sheep suffering from acute indigestion due to overeating.

Twelve of the streptococcal isolates proved to be amyolytic and showed physiological properties characteristic of *Str. bovis*. The remaining strain was found non-amyolytic and resembled *Str. inulinaceus*.

The 117 lactobacillus isolates were identified as follows (number in brackets): *L. brevis* (40), *L. fermenti* (22), *L. bifidus* (45), a variety of *L. delbrueckii* (10).

ZUSAMMENFASSUNG

Identifizierung der Gram-positiven Pansenflora beim Rind und Schaf in spontanen Fällen akuter Indigestion.

Dreizehn Streptokokkenstämme und 117 Laktobazillenstämme wurden aus der dominierenden Gram-positiven Pansenflora beim Rind und Schaf bei durch Ueberfütterung verursachter akuter Indigestion isoliert.

Zwölf Streptokokkenstämme waren amyolytisch und zeigten die für *Str. bovis* charakteristischen physiologischen Eigenschaften. Der dreizehnte Stamm war nicht-amyolytisch und glich dem *Str. inulinaceus*.

Die 117 Laktobazillenstämme liessen sich folgendermassen identifizieren (Anzahl in Klammern): *L. brevis* (40), *L. fermenti* (22), *L. bifidus* (45), eine Variante von *L. delbrueckii* (10).

SAMMENDRAG

Identifisering av den Gram-positive vomflora hos storfe og sau ved spontantilfeller av akutt indigestion.

Tretten streptokokk-stammer og 117 lactobacill-stammer er blitt isolert fra den dominerende Gram-positive vomflora hos storfe og sau ved akutt indigestion forårsaket av foretning.

Tolv av streptokokk-stammene var amylytiske og viste fysiologiske egenskaper karakteristiske for *Str. bovis*. Den trettende stammen var non-amylytisk og lignet *Str. inulinaceus*.

De 117 lactobacill-stammer ble identifisert som følger (antall i parentes): *L. brevis* (40), *L. fermenti* (22), *L. bifidus* (45), en variant av *L. delbrueckii* (10).

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