Acta vet. scand. 1961, 2, 357—374.

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

STUDIES ON ALTERATIONS IN THE RUMEN FLUID OF SHEEP, ESPECIALLY CONCERNING THE MICROBIAL COMPOSITION, WHEN READILY AVAILABLE CARBOHYDRATES ARE ADDED TO THE FOOD

IV. IDENTIFICATION OF THE GRAM-POSITIVE FLORA DEVELOPING DURING THE FEEDING EXPERIMENTS

By

Nils Krogh

As described in previous papers (*Krogh* 1959, 1960, 1961), the addition of readily fermentable carbohydrates in excess to hay-fed sheep invariably led to the development of a predominantly Gram-positive ruminal flora. When cane sugar or lactose was being fed a transient rise in the streptococcal flora preceded the establishment of a vast population of Gram-positive rods, whereas the feeding of starch did not always result in any increase in the streptococcal counts. Further, in some of the sucrose experiments a huge flora of yeasts turned up at the end of the feeding period. Several representatives of the various organisms mentioned have been subjected to further studies for identification, the results of which are given in the present paper.

METHODS

Details of the media and procedure used for the primary culture series were given in the previous papers. From tubes of highest dilution showing growth, usually corresponding to a dilution of the rumen fluid in the range 10^{-7}—10^{-9}, well-separated colonies of streptococci and Gram-positive rods were subcultured

and purified on suitable media. The yeast isolates were obtained from streak cultures of rumen fluid on glucose agar and purified by repeated plating. After being obtained in pure culture all rod strains were examined for motility, spore formation, catalase activity and nitrate reduction, and as these tests were always found to be negative, the rods were assumed to be lactobacilli and submitted to appropriate tests for further classification.

The following media and methods were used for further studies of the three groups of organisms in question:

1. Streptococci. The 28 strains first isolated were submitted to more comprehensive examination including fermentation tests on 16 different "sugars", hydrolysis of sodium hippurate and aesculin, growth in litmus milk, in milk with 0.1 and 0.01 % methylene blue and in fluid medium with 2.0, 4.0 and 6.5 % sodium chloride. Tests for reduction of nitrate, liquefaction of gelatin, gas and catalase production, heat resistance and growth at 45°C. were also carried out.

The physiological tests for 65 strains isolated later were restricted to fermentation of glucose, lactose, inulin, starch and mannitol, growth in the presence of 6.5 % sodium chloride and in fluid medium with pH 9.6, as well as a test for haemolysis.

All fermentation reactions were carried out in peptone water containing 1 % of the test substance and with bromothymol blue as indicator. Sodium hippurate and aesculin were added to final concentrations of 1.0 and 0.5 %, respectively, and the full-grown cultures examined for hydrolysis with sulphuric acid (50 % sol.) and ferric citrate (1 % sol.).

Peptone water containing 0.02 % nitrite-free potassium nitrate was used for the nitrate reduction test, the cultures being analysed for the presence of nitrite and ammonia as described by *Thjötta* (1946) after 3 and 7 days of incubation. When these tests were found to be negative, nitrate was tested for by diphenylamine (0.01 % in concentrated sulphuric acid).

Two per cent glucose broth which after heavy inoculation was sealed by a deep layer of sterile vaseline, was used for testing gas production. Heat resistance was tested for by heating inoculated nutrient broth for 30 minutes in a waterbath at 60°C. followed by incubation at the usual temperature together with unheated controls. Finally, test for growth at pH 9.6 was performed as described by *Shattock* and *Hirsch* (1947) and haemolysis was examined for on cow blood agar.

Of the "sugars" used for studying biochemical reactions of streptococci as well as of lactobacilli, the following were sterilized in 10 % solution by Seitz filtration: Arabinose, xylose, rhamnose, galactose, laevulose, mannose, maltose and inulin, whereas the other test substances in similar concentrations were autoclaved at 115°C. for 15 minutes.

The test media were adjusted to pH 6.8 and after being dispensed the tubes were incubated for 2—3 days to ensure sterility. The test series were inoculated directly from 18—20 hrs old broth cultures, incubated at 38°C. and finally examined after 7 days. The final pH of all cultures was determined electrometrically.

2. *Lactobacilli.* The basal medium used for studying the lactobacilli was the peptone yeast-autolysate medium recommended by *Orla-Jensen* (1943). For the fermentation reactions the test substances were added to a final concentration of two per cent and all strains were tested against 28 different substrates including litmus milk, aesculin and sodium hippurate, the two latter media with and without addition of glucose. When strains under examination proved to be strictly anaerobic, 0.04 % l-cysteine hydrochloride was included in the media.

On the initial examination of the lactobacilli the test media were inoculated directly from young fluid cultures showing good growth. However, most of the strains have been re-tested after a lapse of one to two years, using centrifuged cultures re-suspended in sterile physiological saline as inoculum. The test series were examined at appropriate intervals and finally read after 14 days of incubation.

The cultures were maintained as stabs in yeast- extract glucose-agar (*Orla-Jensen* 1943), for short periods, otherwise they were freeze-dried in horse-serum glucose-suspensions (*Briggs et. al.* 1955).

Except for anaerobic incubation (CO_2-atmosphere), procedures similar to those described for the streptococci were employed for examination of the lactobacilli.

3. *Yeasts.* During feeding experiments with sucrose a large flora of yeasts turned up in the rumen of four different sheep and in each case a loopful of rumen fluid was streaked on glucose agar and incubated aerobically at 38°C. The yeast colonies developing in each culture appeared to be uniform in type and the same

seemed to be the case with the individual yeast cells. Moreover, three of the primary cultures were found to be much alike, whereas the fourth differed markedly from the others. From each culture one colony was subcultured and submitted to further studies.

The isolated strains have been classified according to the system of *Lodder and Kreger-van Rij* (1952), and their properties were studied by the following methods:

Beer wort (9° Bllg) and wort agar were used for general culture studies, 10 % gelatin in wort for studying giant colonies and mainly rice agar for examining the formation of pseudomycelium by the cover slip method.

Ascospore formation was searched for on the following media: Gypsum blocks in 0.1 % peptone water, Gorodkova's agar, carrot infusion agar (*McKelvey* 1926) and sodium acetate agar (Sodium acetate · $3H_2O$, 0.7 %, and agar, 1.5 %, in tap water; pH 6.5—7.0).

Fermentation reactions were determined in Einhorn tubes in a medium containing 1 % yeast extract, 1 % peptone and 2 % of the sugar to be tested.

Assimilation reactions (the auxanographic method), and the arbutin, ethanol and litmus milk tests were carried out according to *Lodder and Kreger-van Rij* (1952).

Although the yeast strains would grow vigorously at 38°C., the cultures were usually incubated at 25° C. However, for growth of giant colonies an incubation temperature of 18—20°C. was used and ascospore formation was tested at 20, 25 and 30°C.

RESULTS

1. *Properties of rumen streptococci.* Twenty-five out of the 28 strains first isolated originated from feeding experiments with sucrose and the remaining 3 isolates were obtained from a starch feeding experiment.

None of the strains liquefied gelatin, reduced nitrate, produced gas, hydrolysed hippurate, fermented glycerol or grew in 0.1 % methylene blue in milk.

All strains attacked glucose (final pH 4.2—4.6), laevulose, galactose, mannose, sucrose, maltose, lactose, raffinose, starch, salicin and aesculin.

Most of the strains (number in brackets) fermented trehalose (26), inulin (25), mannitol (21) and arabinose (16), whereas

18 isolates—including all mannitol negative strains—had no action on sorbitol.

Litmus milk was attacked by all isolates and was usually clotted as well as reduced, about one half of them grew in milk with 0.01 % methylene blue, and two strains—both of which were mannitol and inulin positive—grew in the presence of 6.5 % sodium chloride. Finally, all strains tested did grow at 45°C. but failed to survive 60°C. for 30 minutes.

The 65 strains tested later were obtained from feeding experiments with sucrose (10), lactose (43) and starch (12 isolates). Sixty-three of these strains proved to be identical in their response to the tests in question, i.e. fermented glucose, lactose, inulin and starch without attacking mannitol, showed α-haemolysis and did not grow in the presence of 6.5 % NaCl and at pH 9.6.

The remaining two strains were found to be non-haemolytic and in addition one differed from the pattern mentioned above by being negative to inulin and the other by fermenting mannitol and growing at pH 9.6.

2. *Properties of rumen lactobacilli.* One hundred and forty-nine isolates were submitted to further studies. Table 1 shows the number of strains tested from each of the three types of feeding experiments and the distribution of gas- and non-gas-forming bacilli.

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

Feeding experiment	Number of strains	
	Heterofermentative	Homofermentative
Sucrose	42	2
Lactoce	59	3
Starch	20	23

As will be seen the great majority of lactobacilli obtained from feeding experiments with sucrose and lactose proved to be heterofermentative, whereas more than half of the strains encountered when starch was being fed, were found to be homofermentative.

Characteristics of gasforming lactobacilli. The 121 strains included in this group were morphologically found to be much alike. Young fluid cultures showed distinct Gram-positive rods

of uniform shape, usually 0.6—0.8 × 2—4 microns, more rarely up to 6—8 microns in length. The rods were straight or slightly curved with rounded ends, usually occurring singly, although pairs and short chains could be observed. In young cultures the organisms grew with general turbidity, later the medium cleared and a deposit was formed which dispersed easily on shaking. In stab cultures incubated aerobically, no surface growth occurred, but all strains grew up to or nearly up to the surface of the medium.

Streak cultures on agar plates presented two morphologically different kinds of colonies. The one appeared as round, convex and opaque colonies approximately 1 mm. in diameter with entire edge and smooth, glistening surface. The other type was smaller, usually not exceeding 0.5 mm., flat and dull with irregular edge and rough surface. Frequently both types were present in the same culture, the smooth colonies as a rule occurring scattered in the dense growth of the others. Gram-stained films usually revealed Gram-positive rods of somewhat different morphology, the smooth colonies presenting cells of similar shape and size to those found in fluid medium, whereas cells from rough colonies proved to be more pleomorphic, curved and curled with more tendency to form chains. Subcultures from single colonies gave, as a rule, growth of both colony types, although this was less pronounced for the smooth colonies when subcultured directly from plate to plate. Biochemical tests on cultures originating from smooth or rough colonies of the same strain gave identical results.

All strains produced ample amounts of gas and the results of the biochemical reactions for 117 of the isolates are summarized in Table 2.

On the whole, the fermentative pattern for all strains was found to be much the same. Thus when the few strains attacking hippurate, melezitose or cellobiose are excluded, the remaining 108 strains only differ in their behaviour to arabinose, lactose and salicin.

A characteristic feature of all but seven of the strains tested was that arabinose as well as xylose was strongly fermented and frequently more readily attacked than any of the other test substances. The seven arabinose negative but lactose positive strains were, together with eight other isolates, obtained from one of the lactose experiments and the latter strains behaved exactly

Table 2. Biochemical characteristics of the heterofermentative rumen lactobacilli.

Group	1	2	3	4	5	6	7	8
No. of strains	50	7	16	2	33	2	5	2
Arabinose	+	—	+	+	+	+	+	+
Lactose	—or+s	+	+	—	+	+	+	+
Salicin	—	—	—	+	+	+	+	+
Hippurate	—	—	—	—	—	+	—	—
Melezitose	—	—	—	—	—	—	+	—
Cellobiose	—	—	—	—	—	—	—	+

All strains fermented xylose, galactose, laevulose, glucose, sucrose, maltose, melibiose, raffinose and hydrolysed aesculin. None fermented rhamnose, mannose, trehalose, inulin, dextrin, starch, glycerol, adonitol, mannitol, sorbitol, dulcitol and inositol.

+s: Slightly attacked. Four strains are not included in the table (see text).

oppositely in respect to arabinose and lactose, the fermentation reactions being very clear-cut, but for the rest all 15 strains gave identical results.

The reaction to lactose varied. About one half of all strains showed strong fermentation of this sugar, whereas most of the others were found negative or presented slow and weak growth with only slight fall in pH. Further, seven of the strains in Group 3 proved to be clearly positive to lactose when tested shortly after isolation but negative on retesting, and two other strains in the same group showed the opposite reactions. To a lesser extent similar questionable results were obtained for the salicin test, i.e. weak positive reactions changing to negative on retesting.

The results given for the aesculin test are based on the ferric citrate reaction which was found positive for all strains. However, without any addition of glucose to the aesculin medium the majority of the strains showed weak growth with no or slight drop in pH and no loss of fluorescence. On the other hand, heavy growth with low pH and, usually, loss of fluorescence was obtained when glucose was included, but the ferric citrate test was then frequently found to be negative. When the pH of such acid cultures was raised towards neutrality the fluorescence often reappeared and the ferric citrate test changed to positive. However, it did also happen that the latter test was never found positive for cultures with glucose, thus indicating that the addition of sugar

hampered the hydrolysis of aesculin. The result of the aesculin test in aesculin-glucose medium was, therefore, found less reliable than in medium without any addition of a fermentable substance.

Nearly all strains failed to hydrolyse hippurate and showed no or very weak growth in this medium whether glucose was added or not.

Regarding the action on litmus milk, as a rule, all lactose positive strains clotted and decolorized this substrate.

Of the 4 strains not included in Table 2 three only disagreed with the strains in Group 1 and 5 by fermenting inulin, whereas the remaining strain differed from Group 1 by attacking inulin and trehalose and by being negative to galactose, raffinose and melibiose.

Characteristics of non-gasforming lactobacilli. The biochemical properties of the homofermentative strains are listed in Table 3. All strains in Group 1 originated from starch feeding experiments, those in Group 2 from experiments with starch and lactose and the remaining two strains were isolated when sucrose was being fed.

Table 3. Biochemical characteristics of the homofermentative rumen lactobacilli.

No. of strains	Xylose	Arabinose	Rhamnose	Sorbitol	Mannitol	Adonitol	Mannose	Melibiose	Cellobiose	Melezitose	Raffinose	Inulin	Dextrin	Starch	Aesculin	Hippurate
Group 1: 20	—	—	—	—	—	—	—	+	+	15	+	+	+	+	17	—
Group 2: 6	—	—	—	+	+	v	+	—	+	+	—	—	—or+s		+	+
Group 3: 2	+	+	1	+	+	—	+	+	1	+	+	—	+	+	+	—

All strains fermented glucose, laevulose, galactose, sucrose, maltose, lactose, trehalose, salicin and clotted and decolorized litmus milk. None fermented glycerol, dulcitol and inositol.

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

+s: Slightly attacked. v: Variable reaction (see text).

The strains in Group 1 proved to be more strictly anaerobic than any of the other isolates studied. This fact was already evident from the primary cultures as no growth of these bacteria occurred in the upper layer of the shake agar and they failed to grow within 1—2 cm. from the surface in stab cultures. To obtain

growth or satisfactory growth it was found necessary to add a reducing substance to the media.

The strains grew with uniform turbidity in young fluid cultures, later a deposit was formed. In deep agar medium the colonies were found to be lenticular and compact with entire edge and up to 1.0—1.5 mm. in diameter and surface colonies appeared round, convex, entire, opaque, smooth and glistening.

Young fluid cultures contained somewhat pleomorphic rods, usually 0.6—0.7 × 1.5—3.0 microns, occurring singly or often in small clumps without any tendency to form chains. Club-shaped cells were frequently observed and branched and cleft forms did also occur. After a few days on solid media the pleomorphism was still more pronounced and globular cells could also be seen.

The Gram-reaction varied from distinctly positive to uneven and granular, in the latter case the organisms were not unlike Gram-positive cocci.

Regarding the fermentation reactions most of the fermentable substances—starch included—were attacked very readily, usually showing heavy growth within one to two days and the litmus milk was, as a rule, clotted and decolorized within the same interval. However, some of the substrates—especially trehalose, cellobiose and melezitose and to a lesser extent inulin and salicin —underwent a more slow fermentation and five of the strains were judged to be negative to melezitose. The final pH in all positive tests was usually found in the range 3.8—4.5.

Seventeen of the strains hydrolysed aesculin and proved to attack this substance more easily than was the case with the heterofermentative strains as loss of fluorescence and marked fall in pH was regularly found in addition to a positive reaction to ferric citrate.

The six strains in Group 2 presented a very characteristic morphology by occurring as long bent chains consisting of uniform rods approximately 0.7—0.8 × 2—3 microns in size. The growth in liquid media differed from the strains previously described as a bulky deposit with clear medium was formed in young cultures. The colonies in shake agar were disc-like and compact, on agar plates circular, convex and smooth with entire edge, greyish and opaque and up to 1 mm. in diameter. No surface growth occurred in stab cultures.

These strains differed biochemically from those in Group 1

by attacking mannose, sorbitol, mannitol and hippurate, and by not fermenting melibiose, raffinose and inulin. Dextrin was slightly attacked by all strains, and four of them gave feeble growth in the starch media. With the exception of the two latter substrates and adonitol, the fermentation reactions were clear-cut with a very low final pH in positive tests, usually in the range 3.5—4.0, although values as low as 3.2 were found. Litmus milk was readily attacked and, as a rule, clotted and reduced within one to two days. The action on adonitol varied and was also found variable within the same strain. Thus, two strains gave positive reactions, whereas the other four were either found to be positive when first tested and negative on retesting, or vice versa.

The remaining two (Group 3) non-gasforming strains showed growth characteristics in liquid and solid media similar to the strains in Group 2. Microscopically they were found to form chains consisting of cells of variable size, from coccoid to short rods, sometimes rather like streptococci and thus being different from the chains presented by the former group. They were biochemically distinguishable from Group 2 by fermenting pentoses, melibiose, raffinose and starch, whereas adonitol and hippurate were not attacked. However, the two strains were not identical in their biochemical reactions as the one did not ferment rhamnose and cellobiose and the fermentation of dextrin and starch was less active than by the other strain.

3. *Properties of rumen yeasts.* Three of the isolates proved to be identical and showed the following morphological characters: After an incubation period of 2—3 days the wort cultures presented round or oval to long-oval cells measuring 4—6 $\times$ 4—9 microns, more rarely cylindrical cells up to 12—15 microns in length were found. Multipolar budding and small finger-branched clusters were observed. Young colonies on wort agar were circular, convex or conical, smooth and glistening with whitish or slightly yellowish colour. After one month on wort gelatin the giant colonies appeared greyish-white and dull with a radially folded surface surrounded by a narrow zone of pseudomycelium. Streak cultures on rice agar gave a well-developed tree-branched pseudomycelium below the surface of the medium with blastospores occurring singly, in pairs and in small wreaths.

The main physiological properties are summarized in Table 4. Of the sugars tested, glucose, galactose, sucrose and maltose were assimilated as well as fermented, whereas lactose and raf-

finose were not utilized. Among the nitrogen compounds used for assimilation tests, ammonium sulphate was assimilated but potassium nitrate was not. The arbutin test was found to be weakly positive and slight growth with formation of a thin smooth pellicle was obtained with ethanol as sole source of carbon. No coagulation occurred in litmus milk, but the pH rose from 6.5 to about 7.8. Finally, dry, smooth islets and a narrow ring were occasionally formed on wort.

Table 4. Physiological characteristics of rumen yeast isolates.

Strain number	Assimilation and fermentation of sugars						Assimilation of nitrogen compounds						
	Glucose	Galactose	Sucrose	Maltose	Lactose	Raffinose	Ammonium sulphate	Asparagine	Urea	Potassium nitrate	Histidine	Splitting of arbutin	Growth in ethanol
1—3	+	+	+	+	—	—	+	+	+	—	—	+s	+s
4	+	—	—	—	—	—	+	+	+	—	—	—	+

+s: Weakly positive or slight growth.

The morphological properties of the fourth yeast isolate were as follows: The cells in young wort cultures were short-oval to cylindrical, usually 2—5 × 3—8 microns, and showed multipolar budding. On wort agar long rod-shaped cells, often occurring in chains, were also observed, and the colonies appeared grey and dull and less raised than those of the former strains. The giant colonies were flat and smooth with a raised centre and a broad and voluminous peripherial zone of pseudomycelium. Streak cultures on rice agar presented a well-developed pseudomycelium at the surface of the medium with blastospores mainly occurring in branched verticils (type "Mycotoruloides") in young cultures, later on compact spherical clusters were dominating.

The physiological reactions revealed that of the sugars tested only glucose was assimilated and fermented, whereas the tests on assimilation of nitrogen compounds gave similar results to those obtained for the previous strains. Further, arbutin was not attacked and no change seemed to occur in litmus milk, but the strain grew well in ethanol, forming a dull, wrinkled pellicle. On wort a thin, smooth pellicle was found after one day of incubation, later it usually became wrinkled and a ring was formed.

Formation of ascospores could not with certainty be demonstrated in any of the strains under investigation. For further examination on this important point one strain of each type was sent to Mrs. *Kreger-van Rij,* Centraalbureau voor Schimmelcultures, Yeast Division, Delft, who later reported that she failed to find spores.

CONCLUSION AND DISCUSSION

Ninety-two out of 93 streptococcal strains isolated from the rumen of sheep receiving liberal amounts of readily fermentable carbohydrates proved to be amylolytic. The results of the wide-ranging tests on 28 of the isolates fit closely the descriptions given for *Str. bovis* (*Bergey's Manual* 1957), except that none of the strains survived 60°C. for 30 min.. However, there seems to be some controversy as to the heat resistance of *Str. bovis*. Thus, all strains examined by *Shattock* (1949), failed to pass this test and so did also the majority of the isolates tested by *MacPherson* (1953). These variable results are probably due to the technique used, i.e. whether the test, as in the present studies is carried out on freshly inoculated broth, or on fullgrown cultures.

Two of the strains were able to grow in the presence of 6.5 % sodium chloride but did otherwise show *Str. bovis* pattern and may therefore be regarded as a salt-resistant variety of this species.

The examination of the remaining 65 isolates was restricted to tests similar to those employed by *Mann et al.* (1954) for differentiating rumen streptococci. Sixty-three of the strains proved to be slightly α-haemolytic and fermented inulin in addition to starch, but did not attack mannitol and failed to grow at pH 9.6 and in the presence of 6.5 % NaCl. Thus, according to the terms used by the latter authors, these strains can be classified as "typical amylolytic rumen streptococci" which probably are identical with mannitol negative strains of *Str. bovis*.

Of the remaining two strains one only differed from the pattern mentioned above by being negative to inulin, a character also presented by a few of the *Str. bovis* strains first isolated. The other strain showed some properties characteristic of *Str. faecalis* (non-amylolytic, mannitol positive and growth at pH 9.6), but differed from the typical pattern of this species by its ability to ferment inulin and lack of salt resistance.

Some authors, among others *MacPherson* (1953) and *Higginbottom and Wheater* (1954), have found *Str. bovis* from the rumen of sheep and cattle to be mannitol negative. Further, *Mann and Oxford* (1955), studying the rumen streptococcal flora of calves at various ages, report that the proportion of mannitol-fermenting amylolytic streptococci decreased with the age of the animal and suggest that this variety of starch-fermenting rumen streptococci is possibly more tolerant of low pH. Although the number of streptococcal strains tested during the present investigations is not very great, it may be worth mentioning that mannitol-positive amylolytic streptococci were not encountered among the 43 isolates obtained from lactose feeding experiments, whereas the majority of strains isolated when sucrose was being fed, fermented mannitol and so did also some of the relatively few isolates from the starch experiments.

Regarding the classification of the lactobacilli, the great majority of the heterofermentative strains show biochemical reactions which, with one exception, are in closest agreement with those of *Lactobacillus brevis* (*Rogosa et al.* 1953). The discrepancy consists in that nearly all strains were found unable to hydrolyse hippurate, thus, in this respect being like *L. fermenti.* However, as aesculin was invariably hydrolysed, mannose and trehalose never attacked, and—except for the seven arabinose negative strains—xylose and arabinose always strongly fermented, it seems reasonable to identify all strains in Table 2, apart from those in Groups 2, 7 and 8, with *L. brevis.* It may be mentioned that aesculin-positive, hippurate-negative lactobacilli with fermentation reactions similar to those of the present strains have been isolated from the rumen of young calves by *Mann and Oxford* (1954).

The seven strains in Group 2 are apparently less closely related to *L. brevis* as they are lacking a distinctive character of this species, viz. the ability to ferment arabinose. In this respect they are more like *L. fermenti* and apart from the reaction to aesculin the biochemical properties of these strains agree with those listed by *Rogosa et al.* (1953), for a variant of the latter species. Thus, the strains in question may possibly be considered intermediates between *L. brevis* and *L. fermenti.*

The five strains in Group 7 are classified as *L. buchneri* since of all heterofermentative lactobacilli this species is the only one which ferments melezitose.

Finally, the two strains in Group 8 may presumably be identified with *L. cellobiosus nov. sp.* (*Rogosa et al.* 1953), the only discrepancy in biochemical reactions seems to be their failure to ferment trehalose.

Of the four heterofermentative isolates not included in Table 2., the three which only differed from the majority of strains by fermenting inulin may most likely be considered as a variant of *L. brevis,* whereas the remaining strain, showing a more unusual fermentative pattern, is regarded as unclassifiable.

As to the classification of the homofermentative isolates the 20 strains in Group 1, Table 3, may—according to biochemical reactions and colony and cell morphology—be identified with *L. bifidus.* Thus, the biochemical properties ascribed to this species by *Weiss and Rettger* (1934), agree with those of the present strains except that the majority of the latter attacked melezitose, a property also shown by strains of *L. bifidus* isolated from the cow rumen by *Gibbons and Doetsch* (1959).

Although *L. acidophilus* may show branched forms and a fermentative pattern much like that of *L. bifidus* (*Weiss and Rettger* 1934), it does not seem likely that the strains in question can be identified with this species as they never presented fuzzy colonies, were invariably found to be negative to mannose and showed, on the whole, wider fermentative powers than those usually ascribed to *L. acidophilus* (cp. *Rogosa et al.* 1953; *Wheater* 1955; *Jensen et al.* 1956).

The marked chain formation presented by the 6 strains in Group 2, Table 3, indicated they were belonging to the sub-genus *Streptobacterium* (*Orla-Jensen* 1919), and save for the hydrolysis of aesculin, their biochemical reactions fit those of *L. casei var. casei* (*Rogosa et al.* 1953). Regarding the action on aesculin the present strains agree with the description given for *L. casei* by *Sharpe and Mattick* (1957).

The remaining two strains which morphologically were found to be somewhat similar to the preceding group but differed biochemically by fermenting pentoses, melibiose and raffinose, are identified with *L. plantarum* as the fermentative reactions fit those given for this species, (*Rogosa et al.* 1953), the only discrepancy apparently being that one strain failed to act upon cellobiose.

The present studies of isolates obtained from the vast flora of lactobacilli developing in the rumen when various readily

available carbohydrates were being fed in excess, have revealed that the composition of the flora varied with the carbohydrate given. Thus, *L. brevis* and varieties of this species were found to be the overwhelmingly predominant organisms in feeding experiments with sucrose and lactose, whereas *L. bifidus* was never encountered under these conditions. On the other hand, the feeding of starch favoured the establishment of *L. bifidus,* which —at least at an early stage—seemed to be the dominating lactobacillus present in the rumen (*Krogh* 1961).

Regarding the occurrence of *L. bifidus* in the rumen it seems to appear from available literature that it has not frequently been encountered. *Clarke* (1959) isolated this organism from the rumen of two cows fed fresh clover and clover hay, and quotes that the only previous isolation of rumen organisms resembling *L. bifidus* was made by *Wasserman et al.* (1953). However, *Bauman and Foster* (1956), examining the rumen flora of cows fed high and low amounts of roughage in the form of alfalfa hay, report that the organisms found in greatest numbers from the high roughage samples were resembling *L. bifidus. Gibbons and Doetsch* (1959), have also found this organism in the cow rumen, but state that it is difficult to assess its significance in the physiology of the rumen. Finally, *Phillipson et al.* (1959), studying the rumen flora of sheep on various diets, isolated lactobacilli with morphological and physiological properties similar to those of *L. bifidus* and observed an increase in the colony counts when flaked maize was incorporated in the diet.

The findings made by the last-named authors and the results obtained in the present studies suggest that a starch-rich diet may favour the establishment of *L. bifidus* in the rumen. Further, the fact that the organism occurred in great numbers and frequently appeared in clusters about disintegrating starch grains in the rumen fluid (*Krogh* 1961), together with its vigorous fermentation of starch *in vitro,* indicates that it played an important part in the breakdown of starch in the rumen.

As to the classification of the yeast isolates, the three identical strains fit the descriptions given for *Candida tropicalis* (Castellani) Berkhout, and the fourth strain is identified with *Candida krusei* (Castellani) Berkhout; (*Lodder and Kreger-van Rij* 1952).

According to available literature yeasts do not frequently occur in the digestive tract of the sheep. *Uden et al.* (1958), examining the caecal contents of a large number of animals of

various species for the presence of yeasts, found only a small percentage of sheep to be positive and conclude that the suitability of these animals as hosts for yeasts of any species seems very limited.

Regarding the occurrence of yeasts in the sheep rumen, *Ingram and McGaughey* (1948), report to have isolated "candida-like" organisms, and *Rolle and Kolb* (1955), were occasionally, usually by use of enrichment methods, able to demonstrate the presence of yeasts in this part of the digestive tract. The latter authors fed large amounts of the isolated yeasts to sheep but failed to get the organisms established in the rumen and conclude they were not able to multiply there and may be regarded as occasional passengers introduced by the food. However, it appeared from the feeding experiments with sucrose (*Krogh* 1959), that yeasts can multiply tremendously in the rumen of sheep when the environmental conditions are suitable and the two species in question, *C. tropicalis* and *C. krusei,* may, therefore, be regarded as facultative saprophytes rather than merely passers-by.

ACKNOWLEDGEMENTS

The author wishes to express his gratitude to *The Agricultural Research Council of Norway* for financial aid in support of the present investigations. Thanks are also due to *Mrs. Kreger-van Rij,* Centraalbureau voor Schimmelcultures, Yeast Division, Delft, for confirming the cultural characters of the yeasts, and to *Mrs. Joy Gjoennes* and *Miss Anne-Marie Bakke* for technical assistance.

REFERENCES

1. *Bauman, H. E., and Foster, E. M.:* J. Bact. 1956, *71,* 333.
2. *Bergey's Manual of Determinative Bacteriology,* 7th ed. (1957). Ed. by Breed, R. S., Murray, E. G. D., and Smith, N. R.. London: Bailliére, Tindall and Cox, Ltd.
3. *Briggs, M., Tull, G., Newland, L. G. M. and Briggs, C. A. E.:* J. gen. Microbiol. 1955, *12,* 503.
4. *Clarke, R. T. J.:* J. gen. Microbiol. 1959, *20,* 549.
5. *Gibbons, R. J., and Doetsch, R. N.:* J. Bact. 1959, 77, 417.
6. *Higginbottom, C., and Wheater, D. W. F.:* J. Agric. Sci. 1954, *44,* 434.
7. *Ingram, M., and McGaughey, C. A.:* Nature 1948, *162,* 533.
8. *Jensen, R. G., Smith, K. L., Edmondson, J. E., and Merilan, C. P.:* J. Bact. 1956, *72,* 253.
9. *Krogh, N.:* Acta vet. scand. 1959, *1,* 74.

10. *Krogh, N.:* Acta vet. scand. 1960, *1,* 383.
11. *Krogh, N.:* Acta vet. scand. 1961, *2,* 103.
12. *Lodder, J., and Kreger-van Rij, N. J. W.:* The Yeasts. A Taxonomic Study. Amsterdam: North-Holland Publishing Company, (1952).
13. *MacPherson, M. J.:* J. Path. Bact. 1953, *66,* 95.
14. *Mann, S. O., Masson, F. M., and Oxford, A. E.:* J. gen. Microbiol. 1954, *10,* 142.
15. *Mann, S. O., and Oxford, A. E.:* J. gen. Microbiol. 1954, *11,* 83.
16. *Mann, S. O., and Oxford, A. E.:* J. gen. Microbiol. 1955, *12,* 140.
17. *McKelvey, C. E.:* J. Bact. 1926, *11,* 98.
18. *Orla-Jensen, S.:* The lactic acid bacteria. Det kgl. danske Vidensk. Selsk. Biol. Skrifter 1919, 8. rekke, *2,* 81—197.
19. *Orla-Jensen, S.:* The lactic acid bacteria. Ergänzungsband. Det kgl. danske Vidensk. Selsk. Biol. Skrifter 1943, *2,* Nr. 3, 1—145.
20. *Phillipson, A. T., Dobson, M. J., and Blackburn, T. H.:* Nature 1959, *183,* 402.
21. *Rogosa, M., Wiseman, R. F., Mitchell, J. A., Disraely, M. N., and Beaman, A. J.:* J. Bact. 1953, *65,* 681.
22. *Rolle, M., and Kolb, E.:* Zbl. Bakt. I (Orig.) 1955, *162,* 304.
23. *Sharpe, M. E., and Mattick, A. T. R.:* Milchwissenschaft 1957, *12,* 348.
24. *Shattock, P. M. F.:* J. gen. Microbiol. 1949, *3,* 80.
25. *Shattock, P. M. F., and Hirsch, A.:* J. Path. Bact. 1947, *59,* 495.
26. *Thjötta, Th.:* Generell Bakteriologi og Serologi Bd. I, 1946, 130.
27. *Uden, N. van, Carmo Sousa, L. Do, and Farinha, M.:* J. gen. Microbiol. 1958, *19,* 435.
28. *Wasserman, R. H., Seeley, H. W., and Loosli, J. K.:* J. Anim. Sci. 1953, *12,* 935.
29. *Weiss, J. E., and Rettger, L. F.:* J. Bact. 1934, *28,* 501.
30. *Wheater, D. M.:* J. gen. Microbiol. 1955, *12,* 123.

SUMMARY

Streptococci, lactobacilli and yeasts have been isolated from the dominating Gram-positive flora developing in the rumen of sheep during feeding experiments with sucrose, lactose and starch.

Ninety-two out of 93 streptococcal isolates proved to be amylolytic and detailed studies on 28 of the strains revealed properties characteristic of *Str. bovis.*

One hundred and forty-one out of 149 isolates of lactobacilli were identified as follows (number in brackets): *Lactobacillus brevis* and variants (106), *L. buchneri* (5), *L. cellobiosus nov. sp.* (2), *L. bifidus* (20), *L. casei var. casei* (6), *L. plantarum* (2). Seven isolates were regarded as intermediates between *L. brevis* and *L. fermenti,* and one strain was found unclassifiable.

L. bifidus was encountered in starch feeding experiments only.

Of the 4 yeast isolates 3 strains were identified with *Candida tropicalis* and one strain with *C. krusei.*

ZUSAMMENFASSUNG

Untersuchungen über Änderungen der mikrobiellen Zusammensetzung des Pansensaftes beim Schaf bei Zuschuss von leichtverdaulichen Kohlehydraten zum Futter. IV. Identifizierung der während der Fütterungsexperimente zur Entwicklung kommenden Gram-positiven Flora.

Aus der dominierenden Gram-positiven Flora, die sich im Pansen bei Schafen während der Fütterungsversuche mit Rohrzucker, Milchzucker und Stärke entwickelt hatte, wurden Streptokokken, Lactobazillen und Hefepilze isoliert.

Von 93 isolierten Streptokokkenstämmen waren 92 amylolytisch. Eine eingehendere Untersuchung von 28 der Stämme zeigten die für *Str. bovis* characteristischen Eigenschaften.

Von 149 isolierten Lactobazillen-Stämmen wurden 141 folgendermassen klassifiziert (Anzahl in Klammern angegeben): *Lactobacillus brevis* und Varianten desselben (106), *L. buchneri* (5), *L. cellobiosus nov. sp.* (2), *L. bifidus* (20), *L. casei var. casei* (6), *L. plantarum* (2). Sieben Stämme wurden für einen intermediären Typ von *L. brevis* und *L. fermenti* gehalten, und ein Stamm liess sich nicht klassifizieren.

L. bifidus wurde nur in Fütterungsversuchen mit Stärke gefunden.

Von 4 isolierten Hefestämmen wurden 3 als *Candida tropicalis* und einer als *C. krusei* identifiziert.

SAMMENDRAG

Undersökelser over endringer i vomsaftens mikrobielle sammensetning hos sau ved tilskudd av lettfordöyelige kullhydrater til foret. IV. Identifisering av den Gram-positive flora som utviklet seg under foringsforsökene.

Streptokokker, lactobaciller og gjærsopp er blitt isolert fra den dominerende Gram-positive flora som utviklet seg i vommen på sauer under foringsforsøk med rørsukker, melkesukker og stivelse.

Av 93 isolerte streptokokk-stammer ble 92 funnet amylolytiske. Detaljert undersøkelse av 28 av stammene viste egenskaper karakteristiske for *Str. bovis.*

Av 149 isolerte lactobacill-stammer ble 141 klassifisert som følger (antall i parentes): *Lactobacillus brevis* og varianter av denne (106), *L. buchneri* (5), *L. cellobiosus nov. sp.* (2), *L. bifidus* (20), *L. casei var. casei* (6), *L. plantarum* (2). Syv stammer ble antatt å være en intermediær type av *L. brevis* og *L. fermenti,* og en stamme lot seg ikke klassifisere.

L. bifidus ble bare funnet i foringsforsøk med stivelse.

Av 4 isolerte gjærsopp-stammer ble 3 identifisert med *Candida tropicalis* og en med *C. krusei.*

(Received September 28. 1961).