Bacterial Changes in the Rumen During the Onset of Feed-lot Bloat of Cattle and Characteristics of *Peptostreptococcus elsdenii* n. sp.

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Slime production by microorganisms in the rumen of cattle and sheep has been advanced as a possible factor in feed-lot bloat by Jacobson and Lindahl (1955), and for frothy legume bloat by Hungate *et al.* (1955). The slime serves to entrap the fermentation gases and the resulting frothy ingesta blocks the ruminant's gas eructation mechanism. Lindahl *et al.* (1957) have produced experimental bloat by oral administration of soluble alfalfa saponins to sheep. Rumen bacteria capable of degrading alfalfa saponins with the concomitant production of slime have been isolated from the rumen of cattle (Gutierrez *et al.*, 1958). Hungate *et al.* (1955) found rumen froth production was correlated with the severity of bloat symptoms in steers on legume pastures. The discovery by Smith *et al.* (1953) that certain high grain diets supplemented with protein could experimentally produce bloat in cattle offered an opportunity to study the microorganisms involved in the onset of feed-lot bloat. This investigation has been aimed at the enumeration, isolation, and identification of the predominant types of bacteria before and during bloat.

Experimental Methods

Five steers were fed a barley diet supplemented either with soybean oil meal, cottonseed oil meal, or linseed oil meal as a protein source. The rations are shown in table 1. The rumen samples were removed by stomach tube 6 to 20 days after the animals were put on the high starch diet and again as the steers bloated in order to detect any gross changes in the microbial population. Bloat symptoms did not occur until 2 to 3 weeks after the animals were on the full bloat diet. During bloat, the rumen contents were very frothy and a stable foam 60 to 100 mm in height was formed 5 hr after removal from the animals. The rumen samples were diluted serially as quickly as possible into tubes of starch-feed extract medium with the following composition in percentage: ground alfalfa, 2.0; soluble starch, 0.3; cottonseed oil meal, 0.5; dairy feed concentrate, 0.5 (Hamlin and Hungate, 1956); strained rumen fluid, 20.0; agar, 1.5; resazurin (redox indicator), 0.0001; NH_4Cl, 0.05; NaCl, 0.1; $MgSO_4$, 0.005; $CaCl_2$, 0.005; KH_2PO_4, 0.02; K_2HPO_4, 0.5; $NaHCO_3$, 0.5 (buffer used with 100 per cent CO_2). Cysteine hydrochloride, 0.04 per cent, was used as a reducing agent.

For the analysis of the fermentation products, the isolated strains were grown in 0.5 per cent peptone and 0.3 per cent yeast extract (Difco) with measured amounts of glucose or sodium lactate. Pure CO_2 was the gas phase and 0.5 per cent $NaHCO_3$ was used as buffer. Total volatile acids were determined by steam distillation of the culture fluid and the distillate titrated with 0.02 N NaOH using nitrogen as the gas phase. The acids were separated on a chromatographic cellulose column and identified from their Duclaux distillation constants and R_f values (Elsden *et al.*, 1956; Carroll and Hungate, 1954). After steam distillation, the culture fluid residue was extracted with diethyl ether for 24 hr, and lactic acid was determined by the method of Friedmann *et al.* (1927). Fermentation gases were identified with a semimicro-modification of the Newcomber-Haldane gas analysis apparatus, and the carbon dioxide dissolved in the medium was determined in an absorption train with 0.8 N NaOH.

Agar shake culture counts of the LC organisms (Elsden *et al.*, 1956) were made using 0.5 per cent peptone and 0.3 per cent yeast extract medium supplemented with 0.5 per cent sodium lactate. Utilization of various carbohydrates was tested with bromthymol blue indicator using the same concentration of peptone and yeast extract in a broth medium. Gram stain smears for direct microscopic examinations were made from rumen contents diluted 10 times with tap water. Obser-

TABLE 1

Experimental rations

Diet	Composition	Per Cent
A	Basal*	84
	Soybean oil meal	16
B	Basal	84
	Linseed oil meal	16
C	Basal	84
	Cottonseed oil meal	16

* Ingredients in basal portion of diet: 61 parts barley, 22 alfalfa, and 1 sodium chloride.

vations for capsule production by the isolated strains were made with India ink and a safranin counterstain. The shake culture and anaerobic techniques used were described by Hungate (1950).

Results

Microscopic observations and culture counts. Examination of 1:10 dilution smears before bloat, during slight to moderate bloat, and during moderate to severe bloat showed gross differences that could be distinguished visually. Before bloat, the microflora consisted mostly of small single or paired cocci and rods; in slight to moderate bloat, the smears showed an increase in the number of short chained, encapsulated streptococci and LC type organisms; and in the one case of moderate to severe bloat which occurred during the experiments, the predominating organisms were long-chained LC and small cocci. Figures 1 to 3 and 6 to 10 give a comparison of the changes that occurred in the microflora during the onset of feed-lot bloat.

Dilution series of the rumen samples in the starch feed extract medium showed many colonies at 24 hr. Morphology, gram reaction, and capsule formation were checked on 21 colonies picked at random from the highest dilution tube of each experiment. Before bloat occurred, smears of the colonies from animals 488, 490, and 491 showed approximately equal numbers of gram negative rods and gram positive cocci. Samples from steers 488 and 490 also showed LC type organisms. Dilution series of animals 79 and 61 developed mostly gram positive cocci, singly or in pairs similar to *Streptococcus bovis*, and a few rod-shaped bacteria. Growth in the dilution series made from samples removed from all the steers as they bloated gave an increase in the numbers of short chain encapsulated streptococci. The magnitude of the changes is shown in table 2.

Growth of a large streptococcus similar in morphology to the LC organism described by Elsden *et al.* (1956) occurred only sporadically in the starch-feed extract agar tubes and better growth resulted when the culture counts were made in yeast extract peptone medium supplemented with 0.5 per cent sodium lactate. The counts of the large, gram negative LC type streptococcus also increased during bloat and the numbers reported are probably lower than the organisms present in the samples because of the difficulty in breaking the extremely long chains while making the serial dilutions (table 2 and figures 3 and 8).

Isolation of cultures. Pure cultures were obtained of the small, encapsulated streptococcus which showed an increase during mild bloat and the cultural and physiological characteristics of the strains were studied in detail. Twenty-two strains were isolated at random from the higher dilution tubes of the different experiments. Usually 90 to 95 per cent of the strains possessed capsules which were variable in size. An interesting characteristic of the isolated strains was the ability to attach to potato and corn starch grains (figure 5). This property has been reported for another organism found in animals on high starch diets, *Bacteroides amylophilus*, and this characteristic probably gives the strains an advantage in competition with other starch-digesting bacteria (Hamlin and Hungate, 1956). Twenty-four strains of LC type organisms were also isolated from bloated steers and the fermentation products and cultural characteristics investigated.

Characteristics of the encapsulated, short-chain cocci. The cells were 0.8 to 1.0 μ in diameter, in pairs and short chains of 4 to 6 cells (figure 5). The cocci were gram positive in 4 to 6 hr cultures and later became gram variable. Surface colonies were 1 to 4 mm, round, white, and mucoid. Subsurface colonies were lens-shaped, sometimes spreading out in a complex arrangement. The strains grew in a starch-feed extract agar stab in the zone with oxidized resazurin. Slightly better growth occurred in the reduced portion of the tube. The results of carbohydrate and cultural tests were as follows: Salicin, mannose, inulin, maltose, galactose, fructose, sucrose, lactose, starch, raffinose, cellobiose, and glucose were fermented. D-Sorbitol, D-xylose, rhamnose, arabinose, esculin, trehalose, glycerol, and cellulose were not attacked. Five other strains of the small streptococci fermented the same carbohydrates with the exception that four strains were able to utilize trehalose.

Nitrate was reduced to nitrite; the Voges-Proskauer reaction was negative; gelatin was not liquified; and catalase and H_2S were not produced. The test for indole was negative. Growth in litmus milk formed acid with reduction and coagulation. The strains could grow at 45 C but did not survive heating at 60 C for 30 min. Growth occurred in 2.0 but not in 6.5 per cent NaCl. The strains were not iodophilic.

Growth in liquid yeast extract-peptone medium supplemented with different carbohydrates was variable. Slimy growth with a ropy, viscous characteristic was produced from salicin, galactose, fructose, sucrose, and cellobiose. Less slime was evident from maltose, inulin, and lactose. Quantitative experiments on the fermentation products of glucose by the small cocci showed that lactic acid was the major end product with small amounts of CO_2 being produced. From 300 mg of glucose 2.2 mEq of lactic acid and 0.4 mEq of CO_2 were found. Tests for succinic acid and ethanol were negative. Traces of volatile acids were encountered. Five additional strains of the cocci yielded 0.5 to 0.7 mEq of lactic acid from 80 mg of glucose in a yeast extract-peptone broth. The strains are similar to *S. bovis* except for the lack of high temperature tolerance and their gram variability. Since it had not been established whether *S. bovis* could agglutinate on the surface of starch grains, four known strains of *S. bovis* were tested for the capacity to adhere to corn starch. Smears made

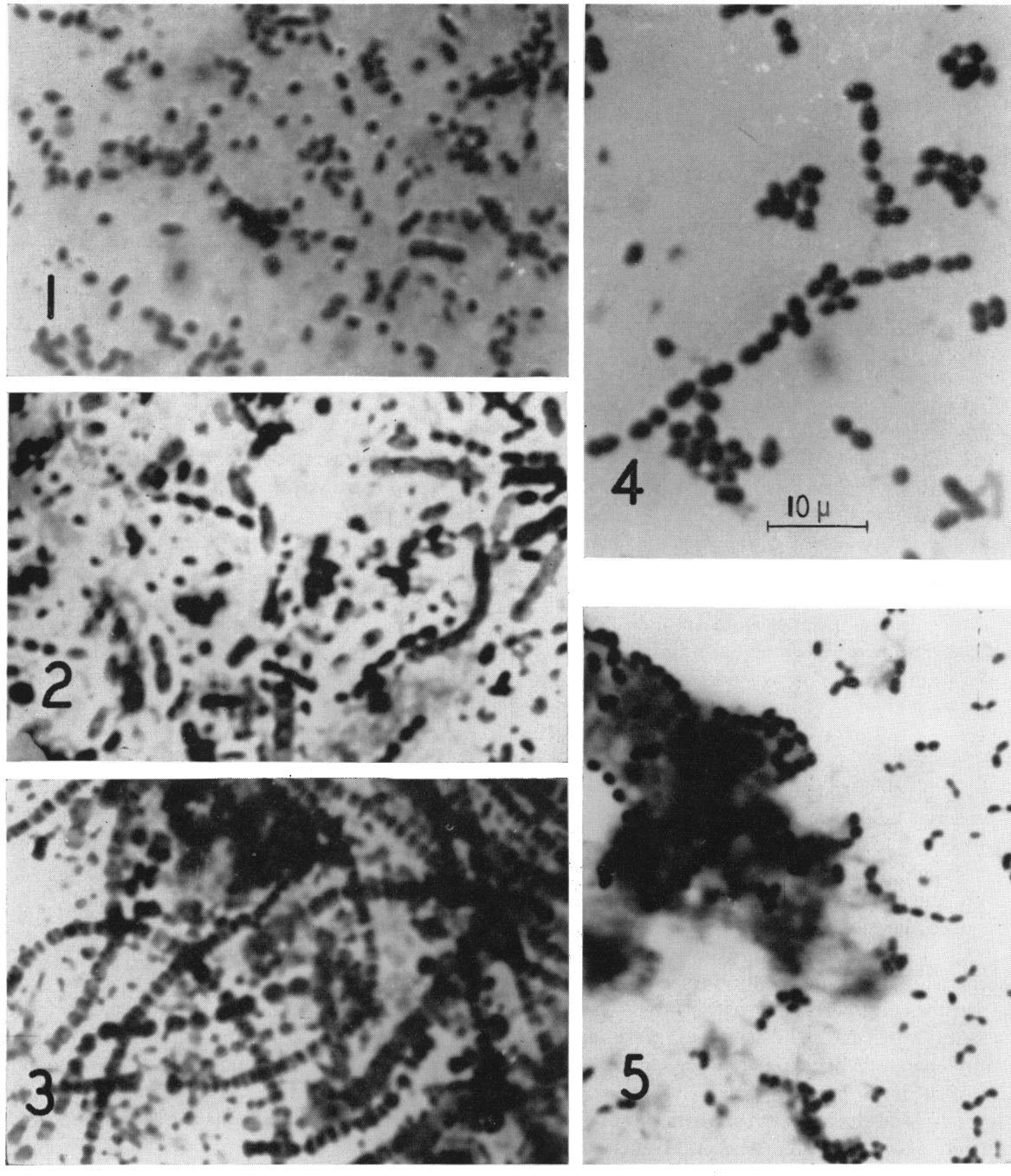

Figure 1. 1:10 dilution of rumen contents from animal no. 61 before bloat occurred, Gram stained.

Figure 2. 1:10 dilution of rumen contents from animal no. 61, one week later, slight to moderate bloat symptoms, Gram stained. Several examples of the short chain encapsulated streptococci can be seen.

Figure 3. 1:10 dilution of rumen contents from animal no. 61, three weeks later, moderate to severe bloat symptoms, Gram stained. Long chains of LC type organisms and small cocci present.

Figure 4. Pure culture of LC type organism, crystal violet stain.

Figure 5. Pure culture of *Streptococcus bovis* type streptococcus attached to potato starch granules, stained with crystal violet.

Figures 1–5. Original magnification 1455 ×; 1091.25 × in reproduction here.

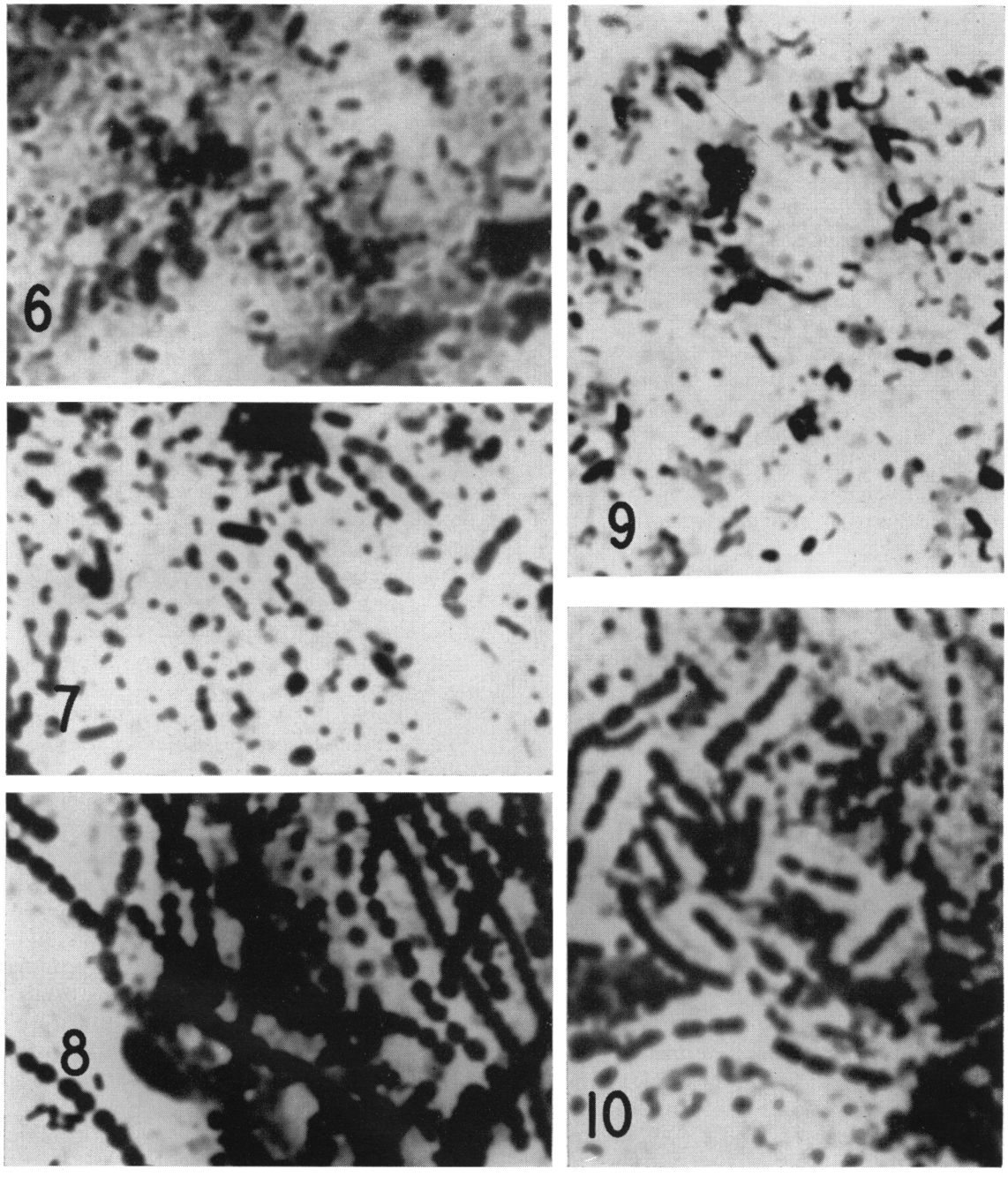

Figure 6. 1:10 dilution of rumen contents from animal no. 79 before bloat occurred, Gram stained.

Figure 7. 1:10 dilution of rumen contents from animal no. 79, three days later, slight to moderate bloat symptoms, Gram stained. LC and small cocci.

Figure 8. 1:10 dilution of rumen contents from animal no. 79, three months later, slight to moderate bloat, crystal violet stain. The LC type chains show the variation in flattening of adjacent cells.

Figure 9. 1:10 dilution of rumen contents from animal no. 491 before bloat occurred, Gram stained. Rods and cocci.

Figure 10. 1:10 dilution of rumen contents from animal no. 491, one week later, slight to moderate bloat symptoms, Gram stained. LC organisms and small streptococci.

Figures 6–10. Original magnification 1455 ×; 1091.25 × in reproduction here.

4 hr after the organisms had been inoculated into yeast extract peptone broth containing 0.1 per cent raw corn starch showed that *S. bovis* had the ability of attaching to the starch grains in the same manner as the lactic acid-producing streptococci isolates.

Characteristics of the lactate-utilizing organism. Strains of the large LC type streptococcus which showed an increase in the rumen samples during bloat were isolated in order to establish identity of the organism. Colonies on yeast extract peptone agar supplemented with 0.5 per cent sodium lactate were tan and lens-shaped in the agar depths. Surface colonies were round, slightly raised and had a glistening, mucoid appearance. At 48 hr the colonies were 0.2 to 1.0 mm in diameter. Older colonies attained 3 to 4 mm. Cells were 2.0 by 2.4 μ in diameter, in pairs and short chains of 4 to 8 cells, with an occasional chain of 16 to 20 cells (figure 4). In the rumen the large cocci grow in much longer filaments and are flattened on adjacent sides (figures 3 and 8). Chains with 65 cells or longer were common. When grown on yeast extract-peptone medium, 18 strains were predominantly gram negative, although 6 cultures showed cells which had a slight tendency toward being gram positive. Long chains of LC in direct smears of rumen contents very frequently showed many gram positive granules at the periphery of the cells with the main areas of the cells being gram negative. On a few occasions, short chains of LC in direct smears of rumen contents were entirely gram positive. Fermentation products from 270 mg of lactic acid by a pure culture of the large coccus yielded in mmoles: CO_2, 1.68; H_2, 0.04; acetic acid, 0.43; propionic acid, 0.36; butyric acid, 0.36; and valeric acid, 0.46. In yeast extract peptone broth containing 0.27 per cent glucose, 0.38 mmole of caproic acid was produced by the organism.

The isolation of large numbers of LC type organisms from diets with a high content of starch and their end products of metabolism suggests the strains can be of importance in the rumen fermentation. Elsden *et al.* (1956) have discussed the taxonomic problem associated with the LC organism. Chain formation and the rapid fermentation of carbohydrates excludes the bacterium from the genus *Neisseria*. Although the strains are predominantly gram negative, the fermentation products, the ability to attack organic acids, and the morphology favor the genus *Peptostreptococcus* (Kluyver and Van Niel) Smith (Breed *et al.*, 1957). The species designation *Peptostreptococcus elsdenii* n. sp. is proposed for the isolated strains with the following description.

Living cells, spherical, 2.0 to 2.4 μ by 2.6 μ in pairs and chains of 16 to 20 cells. Stained smears show cells 1.8 by 1.6 μ in diameter. The adjacent sides of pairs of cells are flattened. Occasionally much larger cells occur. Extremely long chains are formed in the rumen. Granular material is usually present in stained smears. Capsules not observed. Nonmotile. Gram negative, although in a few strains gram positive cells are evident. Pleomorphic in old cultures.

Subsurface colonies in yeast extract peptone agar supplemented with sodium lactate, lenticular, 1 to 4 mm in diameter, tan, soft butyrous texture. Surface colonies round, smooth, raised, with glistening appearance. Growth in yeast extract peptone broth, a fine sediment in 24 hr.

Nitrate not reduced to nitrite. Indole negative. H_2S produced. Gelatin not liquefied. Voges-Proskauer test negative. Litmus milk reduced without coagulation. Catalase negative. Growth at 25, 30, 39, 45, but not at 50 C. Optimum, 39 C.

Substrates utilized: lactate, glucose, fructose, and maltose. Glycerol and sucrose fermented by some strains.

Not metabolized: D-xylose, rhamnose, salicin, mannose, arabinose, esculin, raffinose, lactose, trehalose, cellobiose, and sorbitol.

Fermentation products from sodium lactate: CO_2, H_2, acetic, propionic, butyric, and valeric acids. Additional product from glucose fermentation is caproic acid.

Obligate anaerobe. Habitat is the rumen of cattle and sheep.

Six strains of LC type organisms fermented the same carbohydrates except that three strains attacked glycerol and one strain failed to utilize sucrose. Our strains fit or are closely related to the original description for the LC organism from the rumen of sheep given by Elsden *et al.* (1956)

Observations on the rumen protozoa. Phase microscopic

TABLE 2

Cultural counts before and during bloat

Steer No.	Diet	Before Bloat No. per ml			Slight to Moderate Bloat No. per ml		
		Total count	Count of short-chain, encapsulated streptococci	Count of LC	Total count	Count of short-chain, encapsulated streptococci	Count of LC
61	B	9×10^8	7×10^7	$<1 \times 10^4$	2×10^9	2×10^8	2×10^8
488	C	6×10^9	6×10^6	2×10^7	8×10^8	4×10^8	1×10^8
490	A	1×10^{10}	3×10^7	1×10^6	1×10^{10}	5×10^9	2×10^6
79	B	3×10^8	8×10^7	$<1 \times 10^4$	2×10^9	1×10^9	8×10^7
491	A	2×10^8	1×10^7	$<1 \times 10^4$	6×10^9	5×10^8	8×10^6

examination of the living ciliates from 1:10 dilutions of both bloated and nonbloated rumen samples showed *Entodinium* was the predominant genus, whereas lesser numbers of *Diplodinium* species were also present. High starch diets have been reported to support large populations of *Entodinium* (Van der Wath and Myburgh, 1941). Ciliates of the genus *Dasytricha* and *Isotricha* were either entirely absent or present in numbers less than 2700 per ml. The latter count has been established as a minimum population figure for *Dasytricha* (Gutierrez, 1955). Since the holotrichs have been shown to selectively ingest certain rumen bacteria, the low numbers of these genera may be due to the lack of favorable bacteria (Gutierrez and Hungate, 1957). Although pH measurements were not made on the rumen samples, the presence of *Entodinium* in all of the samples indicates that the pH was not excessively low. At a pH below 6 all of the rumen ciliates disappear (Oxford, 1955).

Discussion

Rations which have a high content of carbohydrate have been shown to give rise to large populations of lactic acid producing cocci in ruminants (Hungate *et al.*, 1952; Bauman and Foster, 1956). The significant amounts of lactic acid produced in this type of diet favors the development of lactate-utilizing organisms such as LC. In the early stages of bloat engendered by feeding rations with a high content of starch supplemented with an ample protein source, the lactic acid starch-fermenting cocci and LC were among the predominant organisms. Microscopic observations made on rumen samples from bloating animals during the third month of the bloat-producing experiments showed LC, cocci, and rod-shaped bacteria. The increase in the numbers of encapsulated lactic acid streptococci during the onset of feed-lot bloat contributes to the theory that one of the factors leading to frothiness of the rumen contents is the slime produced by the rumen microorganisms. It should be emphasized that slime production is the property of various genera of rumen bacteria, and that other types of organisms may contribute to the frothing of rumen contents with different dietary conditions. Laboratory cultures of LC did not produce large amounts of slime, but did evolve much gas from lactic acid and carbohydrates. This suggests that LC augments the bloat symptoms by its breakdown of the lactic acid produced by the amylolytic cocci. An additional contributory factor may be the extremely heavy cellular proliferation of the LC type organisms which formed a filamentous mat of growth during bloat (figures 3 and 8). Besides lactic acid and carbohydrates, L-threonine, L-serine, and L-cysteine can also be attacked by LC (Lewis and Elsden, 1955). In an earlier study on the amounts and kinds of fatty acids produced by the rumen bacteria during feed-lot bloat in cattle, Jacobson and Lindahl (1955) presented evidence that valeric acid increased as the severity of bloat increased. The predominant bacterial populations were not identified, but the present findings indicate that the valeric acid was due to LC type organisms since this is the only organism known to produce valeric acid in the rumen at the present time. In this study, direct smears and culture counts provided conclusive evidence that as the severity of the bloat symptoms increased the number of LC organisms also increased.

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Summary

The bacterial changes that occurred in the rumen of five steers during the onset of feed-lot bloat are described. Twenty-two strains of lactic acid producing streptococci and 24 strains of LC type organisms which increased in numbers during bloat were isolated and the properties of representative types studied. The characteristics of the lactic acid cocci indicated the strains were similar to *Streptococcus bovis*.

The species designation *Peptostreptococcus elsdenii* n. sp. is proposed for the LC type organisms. *P. elsdenii* is a large, gram negative streptococcus which produces carbon dioxide, hydrogen, and acetic, propionic, butyric, and valeric acids from lactate. Caproic acid is an additional fermentation product from glucose. The increase in *S. bovis* type streptococci and *P. elsdenii* during the onset of feed-lot bloat suggests these organisms may play a role in the etiology.

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A Rapid Test for Detecting *Staphylococcus aureus* in Food[1, 2]

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Coagulase-positive staphylococci are responsible for approximately 75 per cent of the food-borne illnesses of established etiology that have been reported in the United States during recent years (Dauer and Sylvester, 1956, 1957). Ideally, to determine the relation of a suspected food to staphylococcus food poisoning, the presence of enterotoxin should be demonstrated. The methods for this purpose are cumbersome, require the use of laboratory animals or human volunteers (Dack, 1956; Matheson and Thatcher, 1955), and are not suitable for screening purposes. In lieu of demonstrating enterotoxin, a rapid method has been developed for detecting the bacteria that are known to produce enterotoxin, that is, the coagulase-positive staphylococci. The cultural methods currently in use for detection of these organisms have the disadvantage, for screening purposes, of requiring 2 to 3 days for completion (Chapman, 1946; Zebovitz *et al.*, 1955). The rapid method reported here indicates the presence of significant numbers of viable coagulase-positive staphylococci in 7 to 9 hr, thus providing a presumptive report on the food within the same working day in which it is examined.

EXPERIMENTAL DEVELOPMENT OF THE METHOD

Preliminary studies demonstrated that 10- to 20-fold more growth of staphylococci developed after 6 to 8 hr on a shaker[3, 4] at 35 C than was obtained by stationary incubation in air or water. Accordingly, shaking incubation at this temperature was used throughout the development of the method.

Development of selective growth medium. In order to select a readily available culture medium, the following common broth formulae were compared in terms of the amount of growth, as measured by turbidity,[4, 5] that could be obtained in 4 to 6 hr: (1) nutrient (Difco)[4]; (2) milk protein hydrolysate (BBL),[4] plus 0.1 per cent glucose; (3) brain heart infusion (BBL); (4) yeast extract and trypticase (BBL), in amounts used in staphylococcus medium no. 110 (BBL); (5) cooked meat medium (Difco) supernatant; and (6) trypticase soy (BBL). Growth was observed in the media in the presence and absence of 4 per cent added NaCl. More growth was obtained in brain heart infusion than any other broth except cooked meat supernatant. Since the difference between these two was insufficient to justify

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[2] This work was supported in part by the Federal Civil Defense Administration under Delegation no. 1 to the U. S. Department of Health, Education, and Welfare.

[3] Variable speed model, Eberbach Corporation, Ann Arbor, Michigan.

[4] The name of the manufacturer is given for some materials and equipment solely for the purpose of identification.

[5] Coleman Junior Spectrophotometer, Coleman Electric Company, Maywood, Illinois.