THE EFFECT OF MUCINOLYTIC BACTERIA OF THE BOVINE RUMEN UPON SALIVA AND THEIR POSSIBLE ROLE IN BLOAT

by

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B.S., Kansas State University, 1959

A THESIS

submitted in partial fulfillment of the

requirements for the degree

MASTER OF SCIENCE

Department of Bacteriology

KANSAS STATE UNIVERSITY
Manhattan, Kansas

1961

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INTRODUCTION

Attention in recent years has become increasingly focused upon the microbial flora and physiology of the rumen. The microflora is responsible for many reactions beneficial to the host such as enzymatic breakdown of carbohydrates, fats and proteins and the synthesis of compounds utilized by the host. Whether or not these same organisms, or others in association, are also responsible for undesirable reactions, e.g. bloat, is a question yet unanswered.

Of the undesirable rumen conditions bloat is probably the most important economically. It is estimated to be the cause of an annual loss of 40 to 45 million dollars in the United States alone (Dougherty, 1953). Bloat is the result of gas forming in the rumen faster than it can be eliminated thus causing the rumen to distend. Normally gas is expelled from the rumen by eructation or by absorption into the blood and subsequent elimination with the expired air. Eructation is the more common.

Four main theories have been proposed to explain the etiology of bloat (Annison and Lewis, 1959): (1) the production of foam due to saponins in the diet; (2) the failure of the belching reflex due to lack of stimulation by fibrous materials upon the rumen wall; (3) the inhibition of the belching reflex by a specific substance; (4) the existence of an allergic condition. The evidence seems clear for each in specific instances.

Saponins are, according to Thompson et al. (1957), naturally occurring plant glucosides which have soaplike properties. Lindahl et al. (1954) produced bloat symptoms in sheep and cattle with alfalfa

saponin administered orally. Gutierrez et al. (1958) reported isolation of bacteria from the rumen which could produce slime from alfalfa saponin in vitro. This inferred that bacteria by producing slime cause bloat. However, saponin itself is a foaming agent. Van Horn (1959) found that bovine saliva added to a saponin solution reduced its ability to foam in vitro. Van Horn further showed that bovine saliva, in amounts of two to six liters, given daily to cattle on alfalfa pasture controlled bloat for two to four hours. The addition of 40 gm animal mucin to the rumen controlled bloat for two hours.

Since additional saliva and/or mucin controlled bloat for only relatively short periods, the possibility of an enzymatic degradation of the saliva, or some part of it, was considered likely. Work was accordingly initiated to determine if mucinolytic bacteria inhabited the rumen, and if so, whether or not the rate of degradation was a factor in the production of frothy bloat.

REVIEW OF LITERATURE

Saliva, Its Function and Effect Upon Bloat

Nichols (1959) explains that ruminants salivate about fifteen gallons of saliva per day depending upon the type of feed being given. Insalivation of feed begins in the mouth and is augmented by mastication. Much saliva is swallowed between periods of mastication. Coarse feeds and acids stimulate salivation.

Ruminant saliva is chiefly aqueous (99.12 per cent water), alkaline (pH 8.53), and has a lower surface tension than water (47.1 dynes/cm as compared to 71.3 dynes/cm for water). It is slightly

more dense and much more viscid than water. Besides water it contains salts in solution, soluble organic substances and mucinous substances. It contains about 250 mg per cent protein. Of the total nitrogen 86-89 per cent is mucoprotein (Reid and Huffman, 1949). Ruminant saliva is semi-colloidal and supports a stable froth.

Johns (1958) considered saliva a factor in bloat because of several of its properties. A large secretion, depending on its composition, could assist in preventing bloat by buffering a fall in pH, increase its severity by adding to the carbon dioxide evolved, or assist in foam formation. Johns also believes that mucoprotein present in saliva forms a stable viscous type foam which involves a great change in surface tension.

There is no doubt that saliva acts as a buffer in the rumen. McManus (1959) diverted saliva from the actively fermenting rumen of sheep and found a corresponding increase in volatile fatty acid and a drop in pH of the rumen contents.

Phillipson and Reed (1958) studied the effect of rumen pressure on salivary secretion. They found that pressures of five to twenty millimeters of mercury would cause an increase in the rate of secretion of the parotid and submaxillary glands. Once stimulation had occurred further increase in pressure caused inhibition of salivation. They found considerable variation in this effect. Some animals did not produce an increased saliva flow in response to rumen pressure.

Emery et al. (1960) investigated the changes in the physical and chemical composition of both the bovine rumen and saliva with different hay-grain ratios. They noted that the concentration of hexosamines in both the saliva and rumen increased with increasing amounts of grain

fed. This would mean a higher mucoprotein content of the saliva.

Van Horn and Bartley (1960) found that bovine saliva or a solution of linseed meal mucin would increase the rate of gas release if added to 200 gm samples of frothy rumen contents in vitro. The addition of 80 ml amounts of either solution was more effective in releasing gas than was the addition of 160 ml amounts. Van Horn (1959) found the addition of 40 gm of animal mucin directly to the rumen completely controlled bloat in every animal for the first two hour period after pasturing. After four hours on pasture foaming reoccurred. To control foaming two to six liters daily of additional saliva were required. In these treatments no change was observed in the pH or the total volatile fatty acid of the rumen ingesta. By bubbling air through a standard saponin solution to give a stable foam, Yadava (1960) was able to measure, to some degree, the antifoaming ability of saliva and a linseed mucin solution. Both markedly reduced the production and stability of foam in vitro. Solutions of animal mucins and silicone preparations prevented foaming entirely.

Bovine Submaxillary Mucoprotein

The classification of protein-carbohydrate complexes has been reviewed by Meyer (1945) and more recently by Bettelheim-Jevons (1958). These complexes of protein and carbohydrate exist in a variety of configurations and in many different places. Bettelheim-Jevons used the word "aminopolysaccharide" for amino sugars free of protein and "mucoprotein" and "mucopolysaccharide" for complexes of protein or peptide with carbohydrates containing hexosamine, or neutral sugar, but no uronic acid or sulfate. The word "mucin" is now used, in a

physiological sense, to denote a slimy, viscous secretion.

Beyond the identification of the constituent amino acids and carbohydrate units only limited information is available about the structure of mucosubstances. Gottschalk (1957), however, succeeded in identifying the prosthetic group of bovine submaxillary mucoprotein (BSM) as being N-acetylneuraminic acid. BSM contains about 17 per cent N-acetylneuraminic acid and 17 per cent galactosamine (Gottschalk, 1958). The term "sialic acid" is now used as a collective one to mean the acylated derivatives of neuraminic acid ($C_9H_{17}NO_8$)(Bettelheim-Jevons, 1958).

Proof of the sialic structure of BSM was accomplished by Cornforth et al. (1958) when they synthesized N-acetylneuraminic acid from N-acetyl-D-glucosamine and oxaloacetic acid. Gottschalk and Graham (1959) also found that an influenza virus enzyme liberates a prosthetic group from BSM which was identified as 6-α-D-sialyl-N-acetylgalactosamine. The amount of hexosamine present was 10-17 per cent while the sialic acid content was 16-28 per cent. The variability depended upon preparation procedure.

Sialic acids are widely distributed in nature, especially in blood protein, mucus, milk and certain lipopolysaccharides (Whitehouse and Zilliken, 1960). More recently they have been found in bacteria. They are aminocarboxyoctuloses, and are regarded as both polyhydroxyamino acids and as ketoses. Theoretically they may combine with other sugars through the glucoside links and with other amino acids through the peptide bond, thus affording a chemical bridge between polypeptides and polysaccharides.

Methods of Detecting Mucinase Activity

Sialic acid can be enzymatically split from the protein moiety, thus assay of free sialic acid can be used to indicate mucoproteinase (mucinase) activity (Aminoff, 1959).

The neuraminic acids exhibit characteristic color reactions in acid media with p-dimethylaminobenzaldehyde (direct Ehrlich reaction), orcinol (Dials reagent), thiobarbituric acid following oxidation by sodium periodate, and tryptophan in perchloric acid (Whitehouse and Zilliken, 1960). The direct Ehrlich provides the most specific but least sensitive color reaction and indicates the entire neuraminic acid present.

Aminoff (1959) developed two methods of determining the amount of free sialic acid while in the presence of the bound compound. He was able to follow the effect of Receptor Destroying Enzyme (RDE) in releasing sialic acid from mucoid substances. Formal and Lowenthal (1956) developed a technique based upon the observation that synovial fluid, when dropped into acid-alcohol, forms a firm clot. After incubation with hyaluronidase the hyaluronic acid component of synovial fluid is depolymerized and the synovial fluid is no longer capable of forming a clot. Boas (1953) used a cation exchange resin to absorb the basic hexosamines (glucosamine and galactosamine) found in tissue and allow the interfering chromogens to pass through. The hexosamines were then eluted and determined by a modified Elson and Morgan method (Elson and Morgan, 1933).

Occurrence of Organisms Possessing Mucin Active Enzymes

Burnet and Stone (1947) first reported a mucin splitting enzyme and named it the Receptor Destroying Enzyme of Vibrio cholerae. Bergamini (1956) listed organisms having enzymatic activity upon epithelial mucin resulting in a decrease in viscosity of the mucin. Included were strains of Actinomyces albus, Staphylococcus aureus, Bacterium prodigiosum, Bacillus anthracis and a "coccobazillen" not identified. Gottschalk (1957) found neuraminic acid up to 53 per cent is enzymatically liberated from bovine submaxillary mucoprotein by influenza virus. Vibrio cholerae (RDE) also releases neuraminic acid. He named the enzyme common to both "Neuraminidase". Salton and Ghuysen (1959) found that lysozyme and an enzyme from Streptomyces F_1 liberated N-acetylmuramic acid and N-acetylglucosamine from the cell walls of Micrococcus lysodeikticus. Formal and Lowenthal (1956) studied the distributions of ovamucinase activity in strains of Shigella. They found that 24 of 27 strains of Shigella flexneri X and two of nine strains of Shigella flexneri Y demonstrated activity. Using the same method of determining ovamucinase activity (CTAB), Lowenthal and Berman (1959) found five of 17 Clostridia species tested were enzymatically active. Williams and Powlen (1959) used human parotid saliva as the sole source of nutrients and found that populations of strains of Staphylococcus aureus, Aerobacter cloacae, vegetative and spore cells of Bacillus subtilis and Bacillus cereus survived and grew suboptimally.

APPARATUS, METHODS, AND MATERIALS

Animals Used

Identical twin heifers were used throughout these experiments. They each had a permanent rumen fistula fitted with a plastic canula and cap. When pasturing alfalfa the animals were kept in a dry lot in the afternoon and night and pastured in the morning. Holstein twins 15 and 16, Guernsey twins 22 and 23, and Jersey twin 88 were used to test the contribution of isolated bacteria to bloat. Saliva was collected from cows 85, 31, and 32.

Saliva Collection

Two methods were used to collect saliva. Lentin* was injected subcutaneously in the neck to stimulate salivation, and the resulting excess of oral saliva was allowed to fall into a funnel above a gallon jug. In this manner 1000 to 2000 ml were collected in one hour. Brown Swiss twins 31 and 32 were used.

In the second method a plastic funnel (10 cm) was attached to rubber pressure tubing four feet in length. This piece of tubing was connected to another one by a cast aluminum Y-shaped tube, one end of which could be open to serve as a pressure release. The second tube, also four feet long, was connected to a 500 ml vacuum flask. A second flask was used as a trap between the collecting flask and the source of vacuum. When collections were made in the barn the milking machine pump provided the necessary vacuum.

*The trade mark of Merck and Co., Inc., Rahway, New Jersey for the drug carbachol.

Collections in the pasture were made with the aid of the windshield wiper vacuum pump of an automobile (1950 Buick). In the actual procedure fistulated cow 85 was emptied of rumen contents and the rumen was flushed with water. The plastic funnel was placed over the cardiac orifice against the rumen wall and suction was applied. The free arm of the Y-shaped tube was used, by digital manipulation, to allow air to enter the line when necessary in order to prevent the vacuum from becoming strong enough to damage rumen tissue.

Mucoprotein Preparation

The collected saliva was handled in one of three ways. It was concentrated by evaporation in dialysis tubing suspended in a current of air coming from an air conditioner (15° C), extracted by precipitation with ethanol using the method given by Hawk, Oser and Summerson (1954), p. 356, or in the case of orally collected saliva, used unaltered. The precipitated and dry mucin was resuspended in phosphate buffer at pH 8-8.5. In some instances Seitz filtration was tried on orally collected saliva as well as that collected from the cardiac orifice. The latter was usually slightly green while the orally collected saliva was colorless and extremely viscid.

Measurement of Sialic Acid of Saliva and Other Solutions

Two color methods based on Ehrlich's reagent were used to follow the relative amount of sialic acid released and its disappearance from the growth medium. The "direct" Ehrlich method of Whitehouse and Zilliken (1960), p. 216, was used to determine the total neuraminic acid. The "pyrrole" test (Aminoff, 1959) was used to follow the amount

of free neuraminic acid in the growth medium. A Bausch and Lomb Spectrophotometer was used to make the determinations for filtered and non-filtered solutions of mucin and saliva collected both from the cardiac orifice and mouth.

Viscosity of Bovine Saliva and Other Solutions

An Ostwald type of viscometer was used at 23° C. Standard picnometers were used to determine the density of solutions tested. Viscosity was determined for orally collected saliva, concentrated (by evaporation) saliva and solutions of extracted mucoprotein. Viscosity was also determined before and after Seitz filtration of these different solutions.

Isolation of Bacteria Capable of Utilizing Bovine Saliva

One ml of frothy rumen fluid was inoculated into one of the following enrichment media: (1) 20 ml saliva collected from the cardiac orifice plus 30 ml Ashby's mineral salts solution (AMS), (2) 20 ml concentrated saliva (Seitz filtered and evaporated to 8/1 conc.) plus 30 ml AMS, (3) 10 ml concentrated (evaporated to 5-1) saliva. All were incubated at 37° C. Streak plate isolations were made on standard nutrient agar and/or Lord's (1959) carbon-free agar medium with added saliva or mucin (see Appendix table 1). Some preliminary characteristics of the isolated organisms were determined using standard techniques and tests according to Bergey's Manual of Determinative Bacteriology (Breed et al., 1957).

Measurement of the Effect of Mucinolytic Organisms Upon Bovine Saliva

To determine the ability of the isolated organisms to utilize saliva, freshly collected non-sterile oral saliva from Brown Swiss cow 31 was used as a substrate. The saliva was inoculated by several methods. These included standard wire loop inoculation, addition of 10 ml of active culture or 10 ml salt (AMS) washed cells resuspended in salts or water. Active cultures are saliva enrichments of isolated organisms carried on nutrient agar slants.

Into each of eight screw cap bottles (6 or 8 oz) was placed 90 or 100 ml of fresh oral saliva. In each group of eight bottles one was left uninoculated as a control, five were inoculated with isolated cultures, one was inoculated with Serratia marcescens (designated G) and the last inoculated with Escherichia coli B. All were incubated at 37° C for varying times. At intervals samples were withdrawn and determinations were made of total and free neuraminic acid content.

For experimental group SL-1, using 100 ml of saliva, one loop of the seven individual test organisms from nutrient agar slants was used as the inoculum. Total and free sialic acid was determined at 0, 4, 8, 12 and 28 hours of incubation. Group SL-2 (90 ml saliva) was inoculated with 10 ml of 72 hour active saliva cultures. Sialic acid determinations were made at 0, 12, 14, 16, 18 and 28 hours.

Experimental groups S-1 consisted of 10 ml of active culture washed three times in AMS and resuspended in these salts, and then inoculated into 100 ml saliva. Sialic acid was determined at 0, 12, 16, 20, 36 and 40 hours.

In order to get a more complete picture of the changes in saliva a final group (SL-3) was studied more closely than the others. This group consisted of 10 ml of a 56 hour active saliva culture of each test organism inoculated into 100 ml oral saliva. Tests for sialic acid were made at 0, 4, 8, 12, 14, 16, 18, 24 and 36 hours.

Effect of Isolated Organisms In Vivo

Twenty-four hour cultures of the isolated mucinolytic organisms grown in nutrient broth were poured into the rumen of fistulated cows then grazed on mature alfalfa to test their effect on animals on a non-bloat provoking diet. Five cows were used: Jersey 88, Holstein twins 15 and 16, and Guernsey twins 22 and 23. One of each twin was inoculated with the isolated bacteria just prior to entering the pasture. The other twin was left uninoculated to serve as a control. Either single pure cultures, or a mixture of the isolated organisms in volumes of 400, 800, 1200, 3000 ml and one gallon were used. The amount of bloat and foam in the rumen was estimated and rated on a 0-6 scale. The estimations were made from ½ to 3 hours after pasturing.

RESULTS

Isolation of Bacteria Capable of Utilizing Bovine Saliva

Five organisms were isolated in pure culture using bovine saliva as the sole source of nutrients. The organisms and the enrichment method used to isolate them are listed in table 1. Bergamini (1956) listed Serratia marcescens as a mycinolytic organism. It was therefore used, designated G, and treated in the same manner as the isolated

bacteria. Organisms A, B, and C were primary isolations and not pure cultures. D, E, F, H, and J were pure cultures. Compilation of the media used is found in Appendix table 1.

Characterization of the isolated organisms was incomplete and only touched upon. The findings are described in table 2. Organism F grew in liquid and solid media with a green diffusible pigment.

Measurement of the Effect of Mucinolytic Organisms Upon Bovine Saliva

Data showing the effects of organisms on undiluted saliva, are given in tables 3 through 6. The readings listed are in per cent transmission (color reaction between Ehrlich's reagent and sialic acid). Two wave lengths, 565 mμ for total and 560 mμ for free sialic acid, were used. As can be seen from table 7 the amount of sialic acid is less than may be determined, by the methods used, when saliva is Seitz filtered. For that reason all the experiments were carried out on unfiltered, unaltered saliva which was necessarily not sterile. The control readings, therefore, are very important in attempting to evaluate results. To facilitate interpretations graphs were also made.

In tables 3 through 6 values are given for total and free sialic acid in per cent transmission as determined by the methods indicated for experiments SL-1, S1, SL-2 and SL-3 respectively. Figure 1 shows the change of total and free neuraminic acid in saliva when inoculated with a loop of organisms. The values for the plots are found in table 3. To obtain some idea of the differences in activity, as a result of treatment of inoculum, the total and free sialic acid values (tables 4 and 5) for two experiments (S-1 and SL-2) are plotted in Appendix

figures 1 through 8. Since no determinations were made between 0 and 12 hours for experiments S-1 and SL-2 no connecting lines could be drawn between these points on these graphs. Appendix figures 1 through 8 are thus placed in the Appendix as data they represent are only explanatory and incomplete.

Readings in table 6 (Experiment SL-3) are plotted in figures 2 through 8 and are more complete. Data obtained for each organism inoculated is plotted with the control so that a direct comparison can be made. With organisms D, E, H, and J a marked difference appeared in the rate of sialic acid disappearance. Organisms F and G use sialic acid less rapidly but still faster than the control. With E. coli there is an apparent lack of utilization of sialic acid. It is possible that in this case the organism is synthesizing and then adding to the total amount of sialic acid present.

The values of sialic acid and viscosity for unaltered bovine saliva and other viscid solutions tested are given in table 7.

Effect of Isolated Organisms Tested In Vivo

The results of inoculating fistulated cows with the isolated mucinolytic bacteria are shown in table 8. Of the 16 trials in which one of each pair of twins tested acted as a control, ten resulted in a more foam-bloat rumen content in the test animal. Three trials showed no difference, and three resulted in the control producing more foam-bloat than the test animal. Cow 88 had no control, but upon being inoculated she bloated and with no further inoculation, presumably because of carry over, continued to bloat daily for one week. She also

Table 1. Source of organisms isolated and tested.

Organism Designation	Saliva(1) as Isolation Medium	With added Supplements	Inoculum
A	Unaltered (200 ml)	20 ml AMS(2) 1 gm dry mucin	1 ml RF(3)
B	Unaltered Aerated 24 hours	20 ml AMS 1 gm dry mucin	1 ml RF
C	Previously incubated at 37°C	-	-
D	8/1 conc (20 ml)	30 ml AMS	1 ml RF
E	8/1 conc (20 ml)	30 ml AMS	1 ml RF
F	5/1 conc (10 ml)	20 ml AMS	1 ml RF
G	5/1 conc (15 ml)	-	1 loopful *S. marcescens*
H	5/1 conc (15 ml)	-	1 ml "C"
J	5/1 conc (15 ml)	-	1 ml "B"

(1) All saliva used here was collected from the cardiac orifice of cow No. 31.
(2) AMS is Ashby's mineral salts solution.
(3) RF stands for rumen fluid. Except for clearing by filtering through four layers of gauze it was unaltered.

Table 2. Preliminary characterization of isolated organisms.

Organism	Motility	Appearance on Nutrient Agar Slant	Action on Litmus Milk	Indole	H_2S	Gelatinase	Glucose	Sucrose
D	Neg	Yellowish white, viscid translucent colonies.	Proteolysis in 8 days	Neg	Neg	Neg	NC	NC
E	Pos	Slight yellow tint to colonies.	Proteolysis in 6 days	Neg	Neg	Neg	NC	A
F	Pos	Greenish white mucoid colonies. Medium became green.	Proteolysis in 2 days	Neg	Neg	Pos	AG	AG
H	Pos	Grayish white translucent colonies.	Acid coagulation	Neg	Pos	Pos	AG	AG
J	Pos	White, opaque, viscid colonies.	Acid curd. Proteolysis slight in 6 days	Neg	Neg	Neg	AG	AG

NC indicates no change in media
A indicates acid production
G indicates gas production

Table 3. Effect of isolated organisms upon orally collected saliva.(1)

Time in hours	Organism inoculated: D	E	F	G	H	J	E. coli	Control
	Total Sialic Acid As per cent transmission at wave length 565 mμ							
0	31	28	30	30	31	27	25	27
4	29	29	27	28	28	28	29	29
8	26	24	29	27	25	24	27	(2)
12	28	26	28	27	27	24	22	(2)
28	84	84	88	81	89	89	89	76
	Free Sialic Acid As per cent transmission at wave length 560 mμ							
0	78	78	89	78	78	86	85	88
4	96	91	89	96	96	94	85	92
8	90	95	92	97	90	94	95	(2)
12	100	98	90	96	92	92	98	(2)
28	100	100	100	100	100	100	100	100

(1) Experimental group SL-1
(2) No determination made

Table 4. Effect of isolated organisms upon orally collected saliva.(1)

Time in hours	Organism inoculated: D	E	F	G	H	J	E. coli	Control
	Total Sialic Acid As per cent transmission at wave length 565 mμ.							
0	32	31	33	37	32	31	30	32
12	42	45	36	35	31	62	29	32
16	63	73	38	41	40	75	32	31
20	67	81	74	72	67	80	77	77
24	75	88	80	78	73	88	88	88
36	73	82	80	76	72	80	84	85
40	73	83	77	77	76	81	80	86
	Free Sialic Acid As per cent transmission at wave length 560 mμ							
0	100	100	100	100	100	100	100	100
12	65	74	80	55	62	77	84	84
16	95	90	72	71	73	88	80	82
20	86	94	89	88	90	90	94	87
24	89	92	94	86	99	90	98	94
36	78	88	93	88	85	89	93	86
40	80	87	86	88	93	87	82	89

(1) Experimental group S-1

Table 5. Effect of isolated organisms upon orally collected saliva.(1)

Time in: hours	Organism inoculated: D	E	F	G	H	J	E. coli	Control
	Total Sialic Acid As per cent transmission at wave length 565 mμ.							
0	28	28	27	29	29	27	26	30
12	74	27	35	56	73	70	25	27
14	78	50	46	79	84	76	26	28
16	81	86	67	84	88	79	29	31
18	78	89	77	81	83	76	27	36
28	82	91	84	80	85	85	33	80
	Free Sialic Acid As per cent transmission at wave length 560 mμ.							
0	100	100	100	100	100	99	100	100
12	100	100	95	100	100	100	100	100
14	100	100	100	100	100	100	100	100
16	100	100	100	100	100	100	100	100
18	100	100	100	100	100	100	100	95
28	100	100	100	100	100	100	100	100

(1) Experimental group SL-2.

Table 6. Effect of isolated organisms upon orally collected saliva.[1]

Time in: hours	Organism inoculated: D	E	F	G	H	J	E. coli	Control
Total Sialic Acid As per cent transmission at wave length 565 mμ								
0	23	21	21	22	22	21	22	21
4	25	22	21	23	23	22	20	24
8	25	26	24	24	24	29	23	21
12	78	59	23	25	36	78	20	21
14	82	78	28	34	74	86	23	27
16	76	80	45	43	73	84	21	26
18	92	91	77	72	82	86	25	37
24	86	87	78	72	84	89	23	81
36	89	92	76	77	85	91	26	86
Free Sialic Acid As per cent transmission at wave length 560 mμ								
0	80	94	87	87	84	86	88	90
4	91	84	96	97	89	88	90	99
8	80	86	93	96	81	75	91	90
12	100	93	80	72	67	87	93	93
14	93	98	58	67	99	90	84	88
16[2]	-	-	-	-	-	-	-	-
18	96	94	98	92	90	91	92	78
24	98	97	97	99	96	89	88	94
36	93	92	89	93	90	97	91	94

(1) Experimental group SL-3
(2) No determinations made

Table 7. Total and Free Sialic Acid content and viscosity of concentrated saliva, mucin and unaltered saliva.

Solution Tested	Sialic Acid in % T		Relative Viscosity(1) in Millipoises
	Total(565 mμ)	Free(560 mμ)	
Orally collected saliva used in experiment SL-3 (cow 31).	21	90	10.3381
Alcohol precipitated mucin in Ashby's salts (1X).	100	100	9.3710
Saliva collected from the cardiac orifice, evaporated to 5/1 concentration and Seitz filtered (cow 85).	42	86	10.6450
Saliva collected from the cardiac orifice, Seitz filtered, evaporated to 8/1 concentration and dialysed in tap water (cow 85).	65	92	11.3278
Saliva collected from the cardiac orifice and Seitz filtered (cow 85).	94	96	9.5456
Orally collected saliva Seitz filtered (cow 31).	100	100	9.3710

(1) Relative viscosity determined at 23° C by comparison with water in an Ostwald type viscosometer. Water has a viscosity of 9.358 millipoises at 23° C.

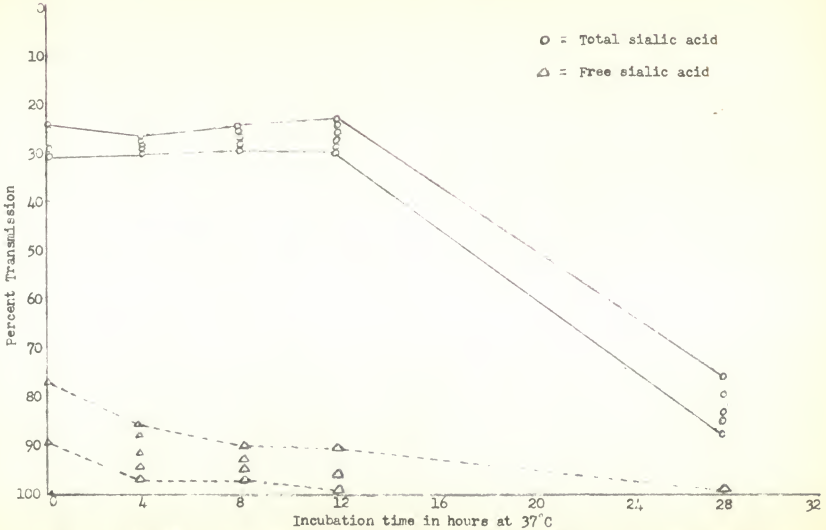

Fig. 1. Decrease in total and free sialic acid of orally collected bovine saliva resulting from inoculation with mucinolytic organisms. Points represent values given in table 3 of experimental group SL-1. The lines indicate the range using all seven test organisms and one control.

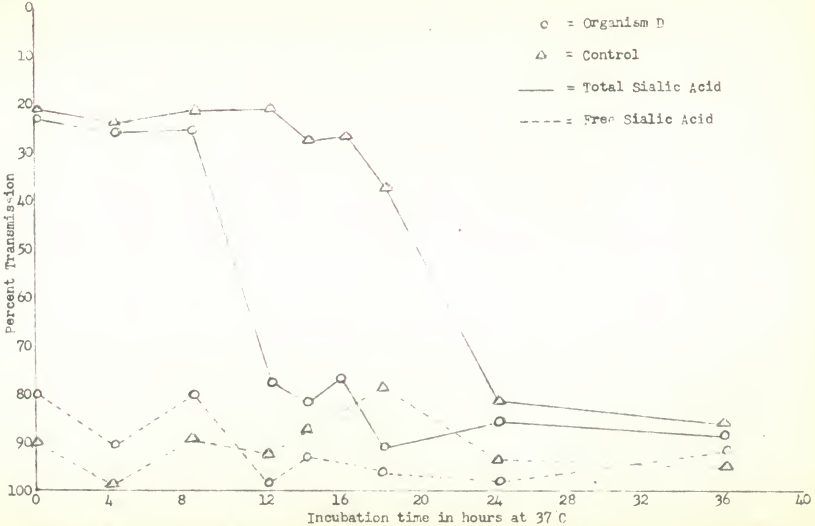

Fig. 2. Effect of isolated bacterium "D" on orally collected bovine saliva. This group was designated SL-3 (see table 6). Uninoculated saliva served as the control.

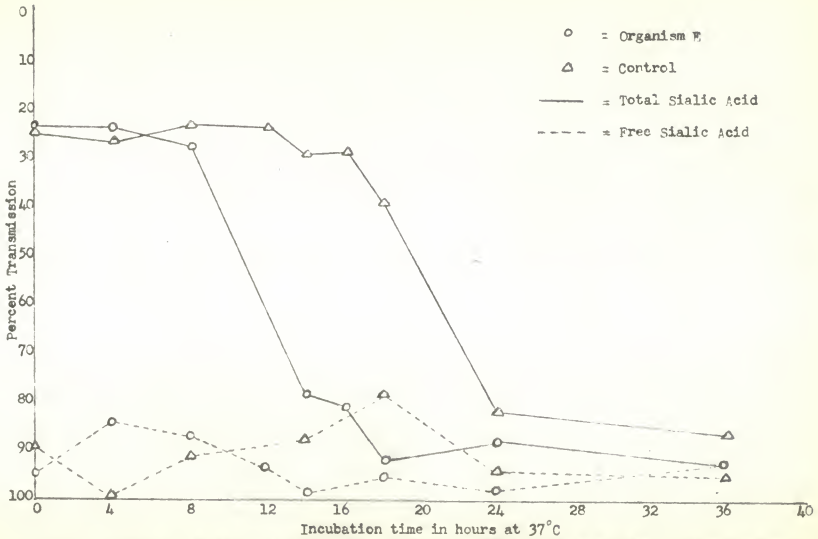

Fig. 3. Effect of isolated bacterium "E" on orally collected bovine saliva. This group was designated SL-3 (see table 6). Uninoculated saliva served as the control.

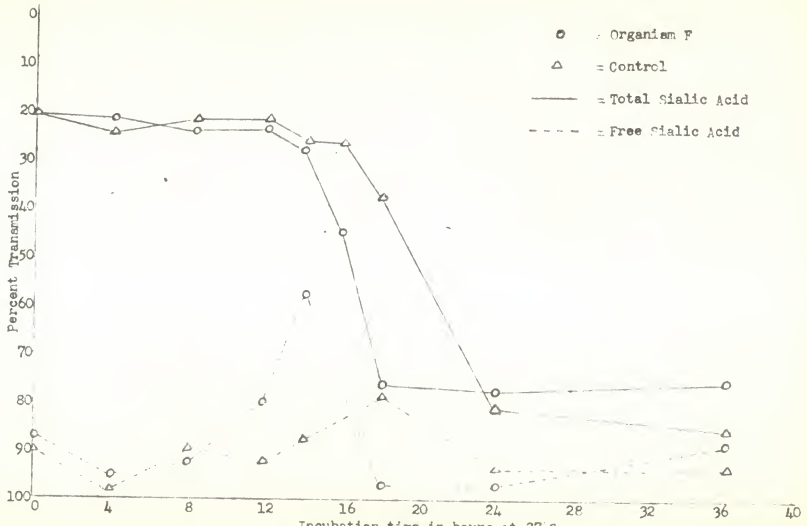

Fig. 4. Effect of isolated bacterium "F" on orally collected saliva. This group was designated CL-3 (see table 6). Uninoculated saliva served as the control.

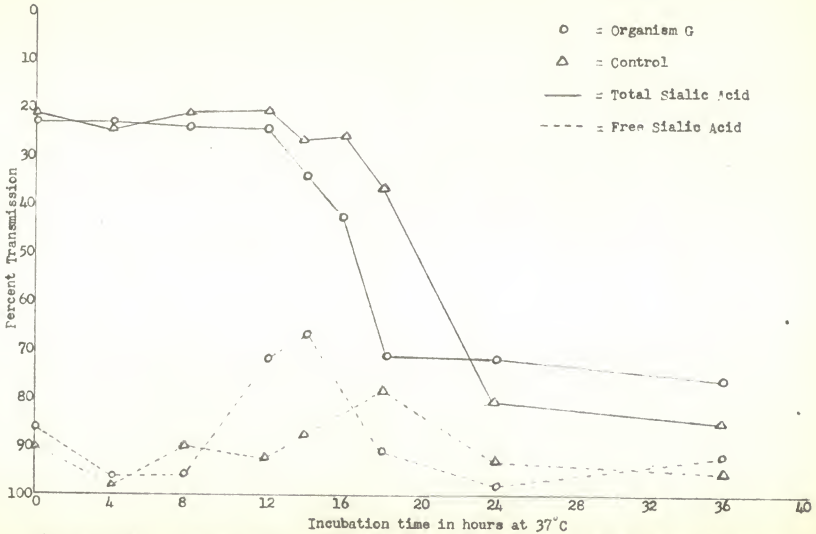

Fig. 5. Effect of bacterium "G" (Serratia marcescens) on orally collected bovine saliva. This group was designated SL-3 (see table 6). Uninoculated saliva served as the control.

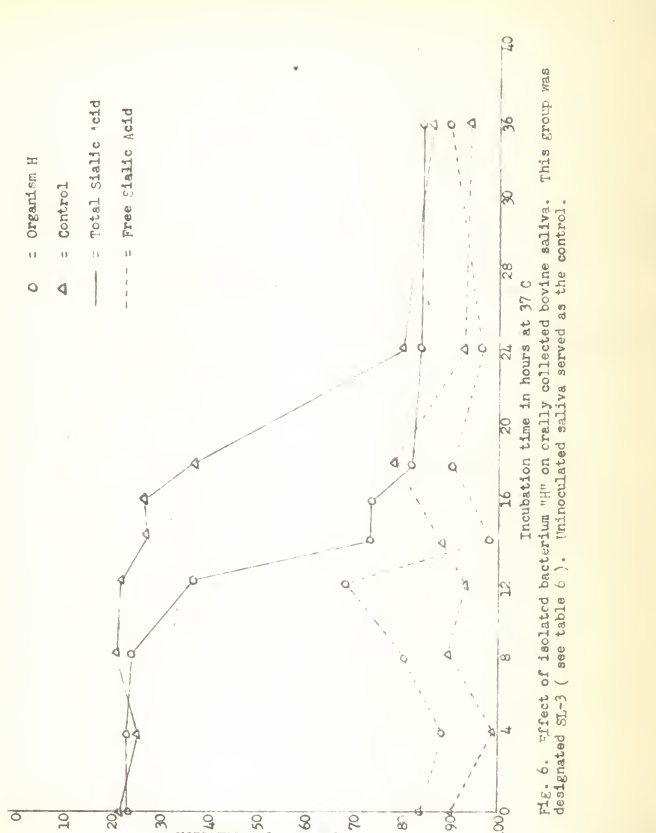

Fig. 6. Effect of isolated bacterium "H" on orally collected bovine saliva. This group was designated SL-3 (see table 6). Uninoculated saliva served as the control.

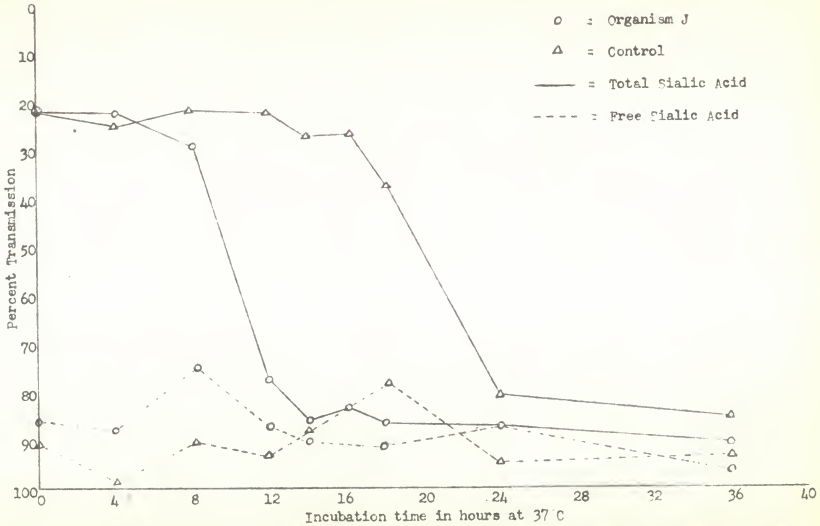

Fig. 7. Effect of isolated bacterium "J" on orally collected bovine saliva. This group was designated SL-3 (see table 6). Uninoculated saliva served as the control.

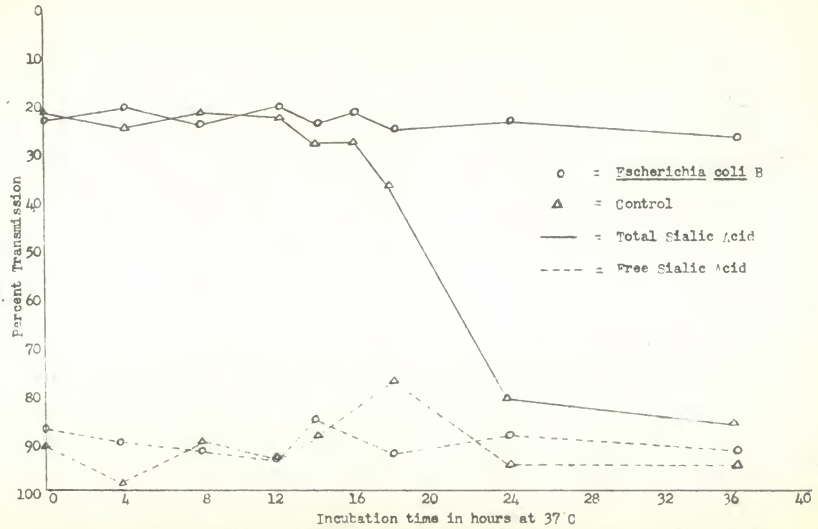

Fig. 8. Effect of Escherichia coli B on orally collected bovine saliva. This group was designated SL-3 (see table 6). Uninoculated saliva served as the control.

Table 8. Bloat and stable foam produced in fistulated cows receiving cultures of mucinolytic bacteria.

Date	Cow	Inoculum		Estimation of		Time of
		Organism	Volume	Bloat	Foam	Estimation
7/28	88	F	400 ml	2	5	½ hour
	15	-	-	0	0	
	16	-	-	0	4	
	22	-	-	0	0	
	23	-	-	0	0	
7/31	88	-	-	0	2	3 hours
	15	-	-	0	0	
	16	F	400 ml	0	2	
	22	F	400 ml	0	2	
	23	-	-	0	0	
8/1	88	-	-	2	5	½ hour
	15	-	-	0	2	
	16	F	400 ml	2	5	
	22	F	400 ml	0	2	
	23	-	-	0	3	
8/4	88	-	-	0	2	2 hours
	15	F	800 ml	0	2	
	16	-	-	0	0	
	22	DEFHJ	800 ml	0	0	
	23	-	-	0	0	
8/5	88	-	-	0	0	1 hour
	15	F	800 ml	0	2	
	16	-	-	0	0	
	22	DEFHJ	800 ml	0	1	
	23	-	-	0	2	
8/6	88	-	-	0	0	1 hour
	15	F	one gal	0	3	
	16	-	-	0	0	
	22	F	one gal	0	1	
	23	-	-	0	0	
8/9	88	DEFHJ	800 ml	0	2	1 hour
	15	F	1200 ml	0	1	
	16	-	-	0	1	
	22	F	1200 ml	0	3	
	23	-	-	0	0	
8/10	88	-	-	0	0	3 hours
	15	J	800 ml	0	3	
	16	-	-	0	3	
	22	DEFHJ	800 ml	0	1	
	23	-	-	0	0	
8/11	88	J	800 ml	0	5	1 hour
	15	DEFHJ	3000 ml	1	5	
	16	-	-	0	4	
	22	F	3000 ml	0	5	
	23	-	-	2	5	

15 and 16 were Holstein twins, 22 adn 23 were Guernsey twins, 88 was a Jersey.

responded by bloating each time she was inoculated subsequently. Not shown in the tables are trials attempted on very hot days in which no bloat or foam occurred in any cow on pasture because they refused to eat.

DISCUSSION

The fact that there are mucinolytic bacteria in the rumen is clearly demonstrated. Organisms were isolated which are able to survive and grow using bovine saliva as the sole source of nutrients. In the preliminary characterization of the organisms, bacterium F, a gram negative short rod capable of using glucose and which produced a green diffusable pigment, was placed tentatively in the genus Pseudomonas (Breed et al., 1957). This is a possibility as Bryant (1959) lists Pseudomonas as one of the genera of facultative anaerobes isolated from the rumen.

By measuring the total and free sialic acid of bovine saliva as organisms grew in it, and graphing the results in per cent transmission, it was possible to show that sialic acid is first released and then utilized by the bacteria tested. Actual concentrations of the total and free sialic acid were not known. However, it was possible to calculate the range of concentrations by making use of the molar extinction coefficient at 560 m given by Whitehouse and Zilliken (1960). Thus light transmission in the range of 20-100 per cent corresponds to $0\text{-}35 \times 10^{-5}$ millimoles per ml of sialic acid.

It is evident from the graphs that organisms D, E, H and J can use sialic acid rapidly. Culture F utilizes sialic acid more slowly than the others but significantly faster than the control. Why

E. coli effects little or no decrease in sialic acid of the medium was not answered. It is possible that this strain of E. coli contributes to the sialic acid already present. Barry (1959) tested 19 strains of E. coli K_1 and found that at least one produced sialic acid abundantly in the form of a high polymer of repeating units of neuraminic acid.

Testing the organisms in vivo presented many problems. Factors beyond the control of the experiment were always in evidence. Such things as sudden weather changes from hot, humid days to rainy, and the sudden appearance of several hot days made "natural" bloat provoking conditions extremely variable. However, because 10 of 16 trials in which control animals were used resulted in more foam-bloat in the test animal and the results obtained with 88, it is suggested that mucinolytic bacteria may be one of the factors in frothy bloat. It is interesting to note that cow 88 previous to the addition of the mucinolytic bacteria was bloat resistant. This is not to say that bacteria are the only cause of bloat, but merely that they are able to contribute to bloat and are yet another factor in the bloat problem.

SUMMARY

Mucinolytic bacteria were isolated from frothy rumen fluid in media containing saliva as the sole source of nutrients. These short Gram negative rods were tested for their ability to degrade and utilize sialic acid complexes in vitro. Their mucinolytic ability was studied in an effort to elucidate the role of mucin in bloat.

The effect in vitro of the the isolated organisms upon oral bovine saliva was measured by following the release and utilization of neuraminic acid. Stimulation of salivation was accomplished by the

use of carbachol. The free sialic acid was determined by Aminoff's pyrrole test, and the total sialic acid found by using the "direct" Ehrlich method. Comparison of the rate of decrease of total sialic acid as a result of inoculation with an isolated organism, and the rate of control decrease, showed that the isolated bacteria were able to degrade the mucin in saliva. Of the five isolated, four were able to reduce the amount of sialic acid markedly within 12 hours when incubated at 37° C. Also included and tested for comparison were Serratia marcescens and Escherichia coli B. These and the fifth organism isolated utilized neuraminic acid at a slower rate.

The possible bloat provoking action of the isolated organisms was tested in vivo. Organisms were grown 24 hours on nutrient broth at 37° C and poured into rumen of fistulated twin cows just prior to pasturing on mature alfalfa. Estimations of bloat and foam were made one-half to 3 hours later. Two pairs of twins and one lone twin were used. In 10 of 16 trials in which control and test animals were used, the test animals inoculated with individual isolated organisms or a mixture of all showed more bloat symptoms than did the control twins. In three of the trials no difference was observed, and in the other three the control had more symptoms. The lone twin 88 could not be directly compared but significantly, prior to the addition of the mucinolytic bacteria, it had never bloated. Also, it should be further noted that once she did bloat on July 28, after one inoculation, she continued to bloat daily even when uninoculated until August 4 or for at least one week. From this work one can conclude that the possibility exists that mucinolytic organisms can contribute to frothy bloat.

ACKNOWLEDGMENT

The author wishes to express his sincere appreciation to Dr. L. R Fina for his aid and advice and under whose guidance the work in this manuscript was accomplished.

Gratitude is also expressed to Dr. E. E. Bartley, Department of Dairy Husbandry, for his advice and assistance and for providing the fistulated animals for these studies; and to Dr. T. J. Claydon, Department of Dairy Husbandry.

Appreciation is expressed to Doctors W. E. Ruliffson and R. E. Clegg, Department of Chemistry, for their advice and criticisms.

The author wishes also to acknowledge the support of the United States Department of Agriculture, North Central Regional Research Project 25, which provided the graduate assistantship and research funds which made this study possible.

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APPENDIX

Appendix Table 1. Media and supplements used in isolation.

Ashby's Salts

K_2HPO_4	7.2 gm
KH_2PO_4	2.8
$MgSO_4$	0.8
NaCl	0.8
CaCl	0.8
Micro element solution - 0.5 per cent aqueous of following: $CuSO_4.5H_2O$, $MnCl_2$, MoO_3, H_3BO_4, and $ZnCl_2$	4.0 ml
Iron solution (sat'd FeCl)	0.1
Distilled water	2000.0

Ashby's $KHPO_4$ Solution

K_2HPO_4 (M/10)	900.0 ml
KH_2PO_4 (M/10)	380.0

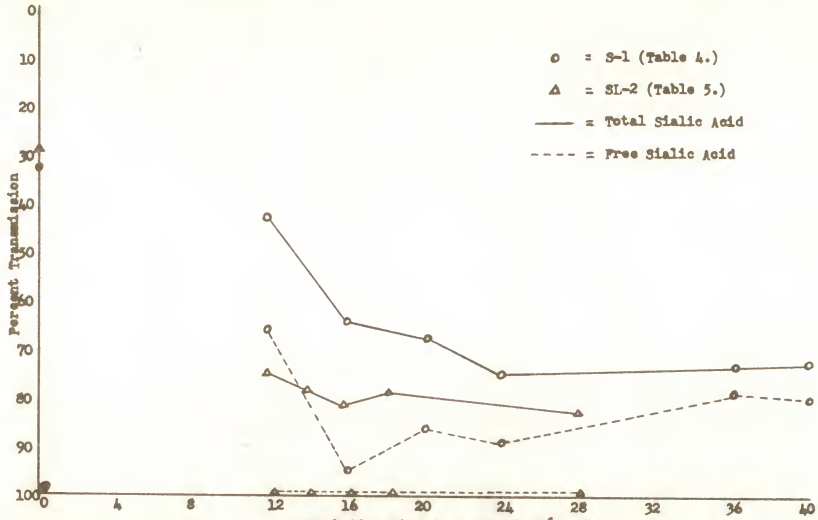
Adjust to pH 7.0

Ashby's Mineral Salts Solution (AMS)

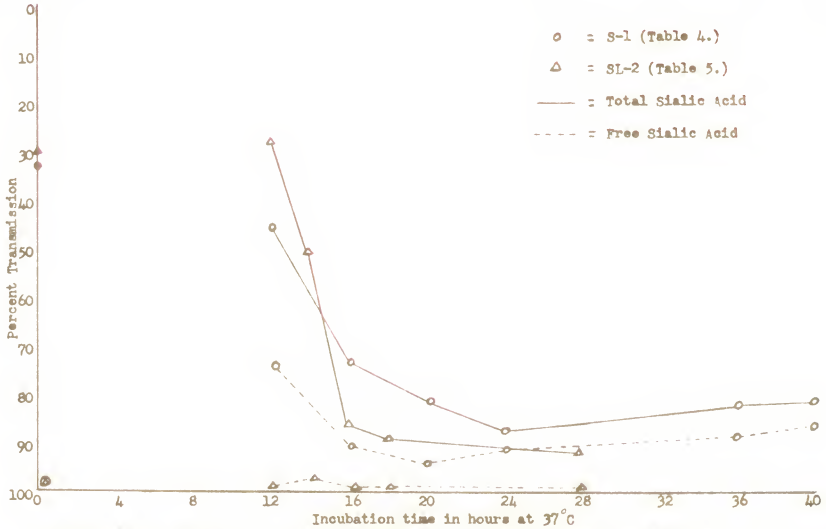
$KHPO_4$ solution (M/10, pH 7.0)	1000.0 ml
Ashby's Salts (supernatant; no precipitate)	1000.0
Distilled water	1000.0
$(NH_4)_2SO_4$	6.0 gm

Isolation Agar Medium

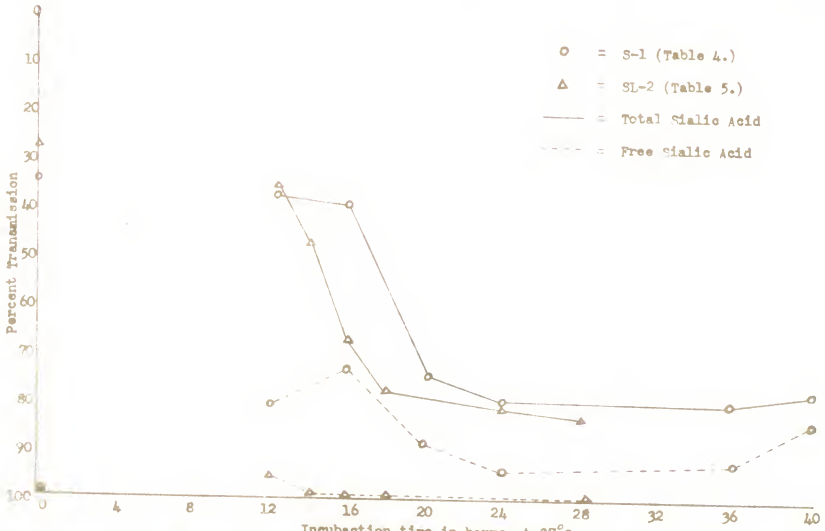
Lord's Carbon-free Agar	100.0 ml
Extracted bovine mucoprotein	0.5 gm
or	
Bovine saliva unaltered	50.0 ml



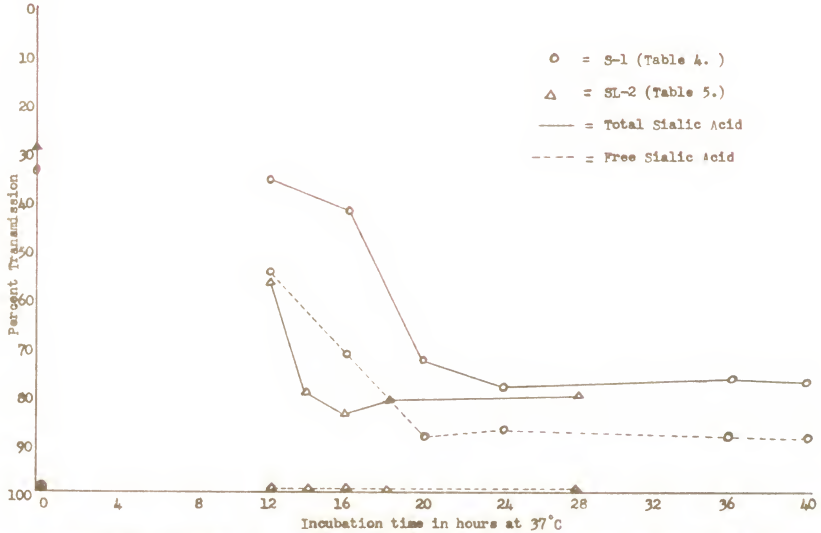
Appendix Fig. 1. Effect of isolated bacterium "D" on oral bovine saliva using two different inoculums.



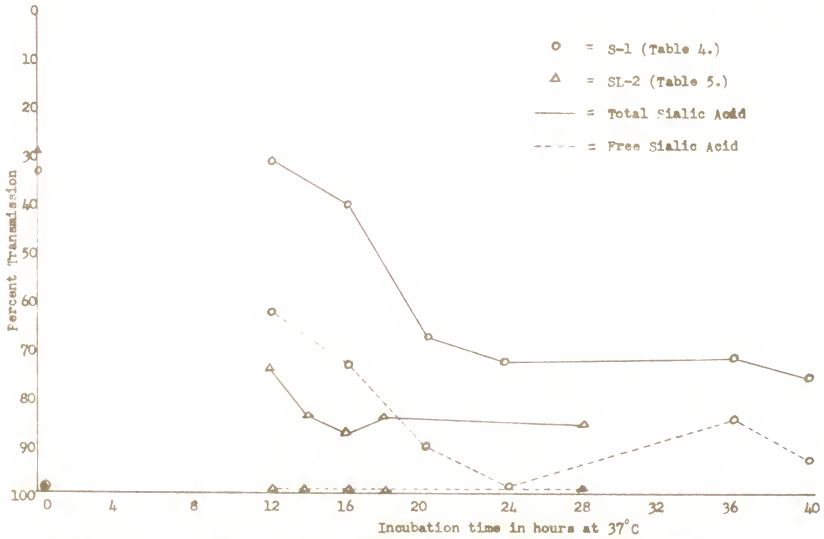
Appendix Fig. 2. Effect of isolated bacterium "E" on oral bovine saliva using two different inoculums.



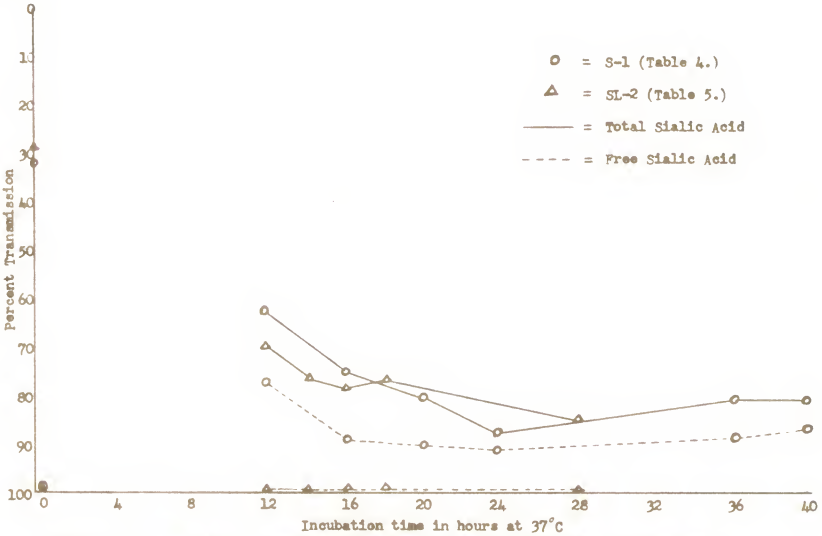
Appendix Fig. 3. Effect of isolated bacterium "FM" on oral bovine saliva using two different inoculums.



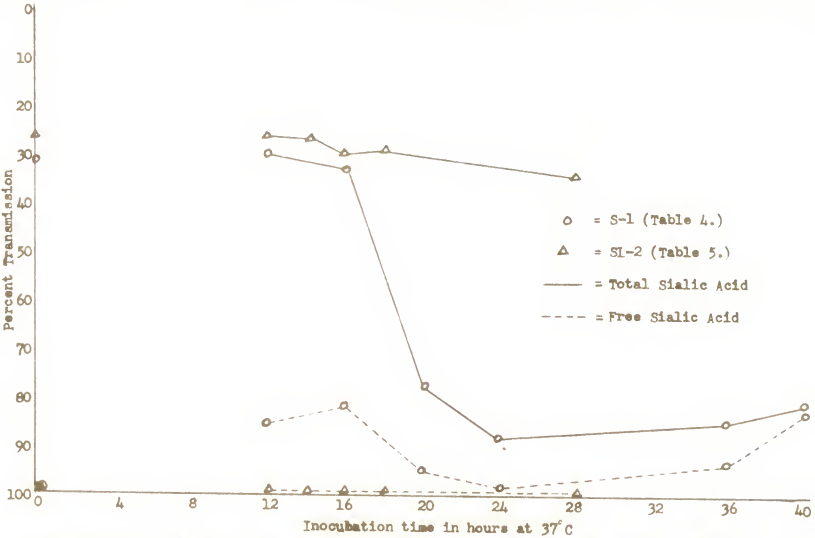
Appendix Fig. 4. Effect of bacterium "G" (Serratia marcescens) on oral bovine saliva using two different inoculums.



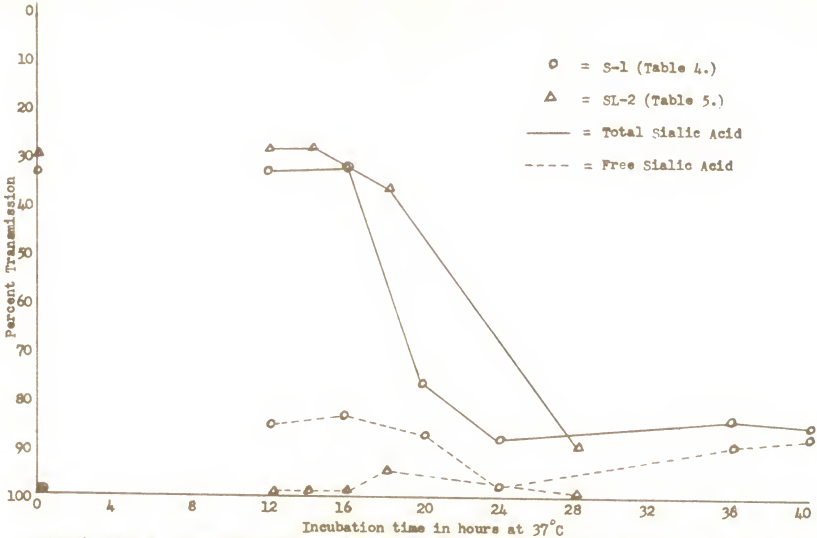
Appendix Fig. 5. Effect of isolated bacterium "H" on oral bovine saliva using two different inoculums.



Appendix Fig. 6. Effect of isolated bacterium "J" on oral bovine saliva using two different inoculums.



Appendix Fig. 7. Effect of Escherichia coli B on bovine saliva using two different inoculums



Appendix Fig. 8. Free and total sialic acid in non-sterile uninoculated control saliva. See appendix figures 2 through 8 for relation of uninoculated saliva to inoculated.

THE EFFECT OF MUCINOLYTIC BACTERIA OF THE BOVINE RUMEN UPON SALIVA AND THEIR POSSIBLE ROLE IN BLOAT

by

CHARLES ALFRED HAY

B.S., Kansas State University, 1959

AN ABSTRACT OF A THESIS

submitted in partial fulfillment of the

requirements for the degree

MASTER OF SCIENCE

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KANSAS STATE UNIVERSITY
Manhattan, Kansas

1961

Most investigators now agree that the formation of stable foam is the primary cause of bloat in cattle grazing legume pastures. Some workers indicate that saliva components, for example, mucin, may be instrumental in preventing foam while others believe that it may contribute to foam in frothy bloat. The work herein reported was initiated in an effort to determine if the rumen harbored mucinolytic bacteria and, if so, whether or not the rate of degradation of salivary mucoprotein could be a factor in frothy bloat.

Five organisms were isolated using bovine saliva as the sole source of nutrients. All were found to be Gram-negative, short rods. Studies were made of these organisms to determine mucinolytic action *in vitro* and bloat provoking ability *in vivo*. In addition *Serratia marcescens* and *Escherichia coli* B were also used *in vitro* since the former had been reported as being mucinolytic.

The test organisms were inoculated by various methods into 100 ml amounts of freshly collected bovine saliva and incubated aerobically at 37° C. At zero time and subsequent intervals samples were tested for the sialic acid moiety of the mucoprotein complex. Free sialic acid was determined using Aminoff's pyrrole test and total sialic acid (free and bound) was determined by the "direct" Ehrlich method. It was found that with four of the five isolated organisms sialic acid was released from the mucoprotein and subsequently rapidly diminished in the test cultures. The fifth isolated bacterium as well as *S. marcescens* also utilized sialic acid but at a slower rate. The strain of *E. coli* used apparently was able to synthesize sialic acid.

The effect of isolated organisms upon cattle grazing a bloat provoking diet was studied in vivo. Cultures of isolated organisms were grown 24 hours in nutrient broth. They were then poured into the rumen of one of a pair of fistulated twin cows. The other twin was left uninoculated to serve as a control. The cattle were then pastured on mature alfalfa. Of 16 trials having adequate controls, 10 resulted in more bloat symptoms occurring in the inoculated cow than in the control. In the remaining 6 trials, no difference was observed in three, while three resulted in the control cow having more bloat symptoms than the test animal. One animal that previously had never bloated developed a severe case that lasted one week after only one inoculation.

These results tend to support the theory that saliva helps prevent bloat because: (1) it was found that mucinolytic bacteria do inhabit the bovine rumen and that they are able to attack the muco-protein of saliva in vitro, and (2) the isolated organisms inoculated into cattle caused an increased incidence of bloat. It must be pointed out, however, that they did not unquestionably cause bloat and, therefore, can only be considered as one more contributing factor in the production of bloat.