The Action of B.coli in Milk.

by

Herbert B. Foote, M. A.

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F. H. Billings
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The Action of B.coli in Milk.

The Purpose of the Work.

Altho B.coli of various strains will ferment lactose and dextrose with the formation of gas of varying amounts, those same strains will not ferment milk with any appreciable amounts of gas being produced, though there is in solution in this medium as high as 4% and 5% of lactose in its pure form. B.coli finds in milk a favorable medium and the curd is thrown in down in a short time after inoculation, but there will be but little gas produced. The purpose of this work was to find out if possible why no gas was produced. There were several possibilities; the lactose might not be in a form accessable to the organism, the milk being a substance which readily absorbs foreign substances might absorb any gas produced, or the proteins present were not the proper ones or were not accessable to the organism. The purpose of the experiments was to find out if possible if any or all of the above reasons were correct.

The Media Used.

Milk being the material in question it was used in one form or another throughout the work. The milk was obtained as fresh as possible and of high grades.

Sterilization was accomplished in the "Arnold" by the successive method, the milk being heated for from twenty to thirty minutes once a day for three days. This slower method was employed in order to avoid the charing and consequent browning of the milk which often is dome in the autoclav. In the first part of the work the whey was obtained by precipitation of the curd with dilute acetic acid, filtration and repeated washings of the curd with water to remove all the sugar. The whey thus obtained was neutralized with dilute NaOH and sterilized in the arnold.

The curd which was obtained was washed two or three times to free it from all the sugar and other soluble substances, and was redissolved in dilute NaOH. Approximately the original amount of albumen was dissolved in water and added to the redissolved curd. In preparing this medium the amount of water normally found in milk was used. The albumen used was taken from fresh eggs.

The redissolved casein was neutralized with dilute HCl, care being taken not to acidify the solution.

Where broth was used as a control or with milk the standard nutrient beef broth was employed with I% peptone and I% of whatever sugar was desired, except when otherwise stated.

The peptone broth was made as follows;

Witte's Peptone --------------- I gm.

Water ----------------------- IOO cc.

At first only the small inverted tubes were used inside the larger test tubes. While they were sufficient for mere qualitative determinations they were found to be entirely inadequate for any exact quantitative work. These then were replaced with the Smith type of fermentation tubes. In these it was possible to determine the amount of gas and also the composition. The larger tubes did away with the choking up of the apparatus with the curd such as took place with the smaller tubes.

Organisms Employed.

Different strains of B.coli were employed. These were obtained from various sources. Strain No.I2 was a culture isolated from a sample of human faeces. Culture No. I4 was isolated from milk. They were tested as to gas producing power in dextrose and lactose, especially the latter, and kept activated by transfers on to agar plates and into broth. Strain No.I4 was B.coli communior.

Tabulation of Data.

In the following tables the results obtained are set down in the order in which the experiments were done.

To ascertain whether the milk would absorb gas produced a set of tubes was inoculated in which there had been placed varying amounts of I% lactose nutrient broth. The following data was obtained.

Table No. I.

Media	.I cc.	.5 cc.	I cc.	5 cc.
Plain Milk	* +	+	+	+
Sugar Free Milk	+	+	+	+
I% Lactose in redissolved curd	+	+	+	+

* + designates production of gas.

One of the possible inhibitors of gas production in milk was here eradicated, namely that milk would absorb the gas produced, for here was obtained as much gas as with straight lactose broth.

In order to determine whether the organism used the sugar of the broth introduced in the above case, or the lactose normally found in the milk, another set of tubes was treated with 0.5 cc. of plain nutrient broth and inoculated. The following results were obtained.

Table No. 2.

Media	.I cc.	.5 cc.	I cc.	Check*
Whey	—	—	—	—
Plain Milk	+	+	+	—
Sugar free Milk	—	—	—	—
I% Lactose in redissolved curd	—	—	slight +	—

*plain milk inoculated as the others.

As here no new sugar was introduced it is plain that only that of the milk was used in the formation of gas.

Another inhibitor was thus eradicated, namely that the sugar normally found in the milk was not available for the metabolism of the organism in the formation of gas.

It was now left to determine if possible the role of the various proteins already present in the normal milk and of those introduced.

It being suspected that the peptone in the broth might somehow be active in the gas formation, a set of tubes was inoculated in which there was milk which had been treated with varying amounts of peptone broth of varying strengths.

Table No.3 shows the results obtained.

Table No.3.

Media	.I cc.	.5 cc.	I. cc.	Check
Plain Milk with .5% peptone	+	+	+	−
Plain Milk with I% peptone.	+	+	+	−

With this experiment it was found that the mere trace of peptone was sufficient for the gas activity to be set up. With as low as 0.I cc. of a 0.5% solution of peptone in 7 cc. of milk there was a positive reaction.

In order to determine if the peptone alone was productive of gas a set of tubes was treated with varying amounts of "peptone free" nutrient broth. The following data show the results.

Table No. 4.

Media	.I cc.	.5 cc.	I cc.	Check*
Plain Milk with "peptone free" broth	+	+	+	−
	+	+	+	−

*Plain milk was run as a check.

It was thus found that the other proteins were also capable of stimulating this gas activity on the part of the colon organism.

It was thought best to discard the inefficient inverted tubes so far used and employ only the Smith type of fermentation tubes.

Essentially the same experiments were performed using the following various forms of media: whey treated with peptone broth; straight milk treated with peptone breth; whey and straight milk treated with nutrient broth. With these was run a set containing lactose and dextrose broth of varying composition. Following are the results.

Table No. 5.

Media	Tubes	Gas.	
		24 hrs.	48 hrs
Peptone I%. Lactose 2%. Water	I - 2.	22%	35%
Lactose I%	I		I2%
Broth (peptone free)	2.		I2%
	I		I2%
Plain Milk.	2		none
Milk	I		25%
.I cc. peptone Broth.	2		20%
Milk with .Icc.	I		28%
peptone free broth	2		I5%
*Whey with Icc.	I		I5%
peptone broth.	2		I2%
Dextrose I%	I		50%
Peptone I% Broth.	2		45%
	I		22%
Lactose peptone broth	2		22%
Water 99%			none
Lactose I%			none

*The whey was obtained by precipitation with acetic acid/.

The effect of peptone upon lactose was tried and compared with the same without peptone . As will be readily seen the peptone and lactose gave a considerable amount of gas, while lactose and water gave no gas. The peptone seems to be sufficient for the metabolism of the organism.

The data above also shows that milk with peptone broth gave more gas than the same with only the "peptone free" nutrient broth.

The whey while giving gas with the addition of peptone broth, was slow in action and even at 48hrs. when the other media were producing more gas was still low in production.

The other media, i.e. the lactose and dextrose broths were used as checks upon the milk and also the organism.

In the following tables are shown the results obtained with various media such as whey, nutrient broth, and peptone broth, milk with the same ingrediants, milk made alkaline before inoculation, and lactose broth of varying strenghths and of I% alkalinity before inoculation. The final reaction of the media was determined at the time of final reading.

The casein used was precipitated with rennet and redissolved with dilute NaOH..The milk being fresh, produced a neutral whey which was used as obtained as the filtrate from the curd.

Table NO.6.

Media	Tubes	Gas (%) 48 hrs.	ave	Acidity (final)
Milk with .5 cc. of nutrient broth	I - 2 - 3 4 - 5 - 6	80-50	65	
Milk with .5 cc. of peptone broth	I - 2 - 3 4 - 5 - 6	55-35-85 70-60-75	63	
Milk with.5cc. of nutrient broth (I% alk)	I - - 3 4 - 5 - 6	60-40-40 30-35-80	5I	5.6%
Milk (I% alk) with.5cc of peptone broth	I - 2 - 3 4 - 5 - 6	5035-80 50-70-40	54	2.2%
Milk(.5%alk)with .5cc. of nutrient broth	I - 2 - 3 4 - 5 - 6	I0-40-40 55-35-80	43	3.6%
Milk(.5%alk)with.5cc. of peptone broth.	I - 2 - 3 4 - 5 - 6	35-50-75 60-60	56	II.2%
Whey with .5cc. of nutrient broth.	I - 2 - 3 4 - 5 - 6	30-35 30-25-35	3I	
Whey with .5cc. of peptone broth	I - 2 - 3 4 - 5 - 6	30-30-35 50-25	32	
Lactose broth (I%)	I - 2x- 3 4 - 5 - 6	I0-I0)I5- I5-20	I4	
Lactose broth (2%)	I - 2 - 3 4 - 5 - 6	I5-20-25 25-50	23	
Lactose broth (3%)	I - 2 - 3 4 - 5 - 6	30-40-25 25-I0	26	
Lactose broth (4%)	I - 2 - 3 4 - 5 - 6	30-20-20 28-38-25	27	
Lactose broth (5%)	I - 2 - 3 4 - 5 - 6	25-30-35 30-30-30	30	
Lactose broth (5%) I% alkaline	I - 2 - 3 4 - 5 - 6	25)35-30 25-40-25	30	I.2% I.2
Lactose broth (I%) I% alkaline	I - 2 - 3 4 - 5 - 6	25-20-30 70-40-40	38	2.2%

It is apparent that the proteins normally present in milk are not such asare needed for the gas producing metabolism of the organism. If however peptone is added it is apparent also that the proper element or elements have been suppl plied. Peptone is a very complex molecule, its composition is still a matter of study and therefore the element or elements furnished for the metabolism could not be determined. A simplier substance was sought for the purpose.

*Dolt in his work with B. coli used various combinations of amino acids and other proteins with water to determine a good medium for the growth of the organism. He found that a medium composed of

Asparagin ----------I gm.

$(NH_4)_2HPO_4$ ------ 0.2 gms.

Water -----------IOO cc.

made an excellent culture medium for the bacteria.

†Keyes made a medium composed of

Asparagine ------- IOgms.

Na_2HPO_4 ---------- 2 gms.

Water -----------IOOO cc.

which was an excellent medium for B.coli They both found

*Dolt, Journal of Inf. Diseases, vol.V. I908. p. 6I6.
†Keyes, Journal of Med. Research. Vol. XXI. I909. p.69.

that the phosphorous present was a distinct advantage to the organism although they do not say in which way. In milk there is already present a small quantity of phosphorous in the form of calcium phosphate and phosphoric acid.* Asparagin with water alone gave no growth at all. Neither did urea with either give any growth. It was evident that the organism needed both the phosphate and the protein.

For the simpler substance urea was used. This has a formula $CO(NH_2)_2$ and is easily broken up. I% of this substance was dissolved in the milk, and also in whey. For purposes of comparison I% of peptone was dissolved in samples of milk and whey and inoculated. There being always present in the protein molecule Nitrogen, some of the milk was treated with a stream of nitrogen from a generator in which were placed

NH_4Cl	--------------	I2 parts
$NaNO_2$	-------------	8 parts.
water	-------------	20 parts.

The gas resulting from the gentle heating of the mixture was led through a wash bottle filled with water to remove any acid which might have passed over. The milk thus treated was placed into Smith tubes and sterilized in the arnold for half an hour.

The following table shows the results obtained.

*Leach - Methods of Food Analysis.

Table No.7.

Media	with	Gas (%) 48 hrs.	72 hrs.	96 hrs.	H_2/CO_2	Final Acidity
Whey	urea (I%)	5 - 5 0 - 5	I5 - I5 20 - 25	I5 - I7 30 - 30	5/I 3/I	2.7%
Whey (neutral)	peptone (I%)	25 - 40 40 - 35 60	35 - 80 50 - 50 65 - 40		3/I 5/I	5% 9%-8%
Milk	urea (I%)	I0 - 25 I0 - 20 20 - I5	40 - 40 30 - 45 35 - 40	45 - 40 40 - 40 35 - 40	5/3 3/I	I.4%
Milk	peptone (I%)	80 - 85 55 - 80 75 - 40			5/2 3/I	I.4%
Milk	nitrogen	40 - 30 30 - I5 -- --	50 - 55 50 - 30 30 - 35	40 - 40 50 - 40 60 - 60	3/I 3/I 2/I	6% 7.5% 6%
Milk	peptone	45 - 75 3θ - 30 --------			2/I 4/3 2/I	

In all eases the whey was slow in the production of gas with urea, while with the peptone the gas production was on the average about as fast as with the milk and peptone. However peptone gave the better results and seemed to make the more favorable medium.

The work with the nitrogen was not repeated to verify the results. What the action and cause operative in this ca case may be must be worked out with more experiments than have been performed in connection with this work. The end products in all cases seemed to be normal at least as far a the CO_2 and H_2 were concerned.

In making a thorough study of this subject a fuller knowledge of the composition of the milk constituents is neessary than is at present at hand and also a better understanding of the composition and constituents of the protein molecule.

Much has been done along the line of synthetic media and this work has opened up the possibilities of the constituents of milk in the making of such media.

Summary and Conclusions.

I. The lactose of milk as normally found in solution there is fermentable by Bacillus coli with the production of acid and gas.

II. The milk does not absorb the gas at least to any great extent, as at first thought possible. On the contrary there seems to be more gas obtained with the organism in milk than in the standard nutrient lactose broth.

III. The proteins of the milk are somehow not available far the gas producing power of the organism used and a foreign protein must be added before this production occurs.

IV. Peptone and Urea are two substances which somehow allow of gas production, the former in greater amounts or in more available form than the latter.

V. Free Nitrogen when bubbled through milk seems too be sufficient for this action.