

OCCURRENCE OF FERMENTS IN THE STERILE MILK  
COLLECTED BY MILKING TUBE FROM COWS AND  
GOATS.

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THE literature upon the presence of various ferments in milk is extremely large and dates back through many years. Up to the beginning of 1911, however, when the experiments described in this paper were undertaken, no observer had dealt with milk uncontaminated by bacteria.

Many observers had collected milk with very great care, but it did not appear that anyone had obtained sterile milk for the purpose of investigation of the ferments, although the method of obtaining sterile milk had been described by Lister (1878) and frequently employed for various purposes. It was decided to adopt the method described by Swithinbank and Newman (1903), for the collection of sterile milk, by means of a milking-tube or catheter.

The apparatus used by these observers consists essentially of two portions; the first is a milking-tube connected by about 3 or 4 ft. of rubber-tubing to a piece of glass tubing. The milking-tube and glass tube are both enclosed in test-tubes the open ends of which are blocked by plugs of cotton wool, so that the whole can be sterilised and preserved sterile.

The second part of the apparatus is a filter flask, through the cork of which passes a piece of glass tubing, closed by a short piece of rubber-tubing, and a clamp. Both the end of the rubber-tubing and the side tube of the flask are plugged with cotton wool, so that this also can be kept sterile.

The teat of the cow's udder which is to be used is carefully cleaned with 2% lysol, as are also the hands of the experimenter. A little

milk must be milked away so as to remove that portion of the milk which had been stagnating in the mouth of the teat. The milking-tube is now removed from the test-tube and inserted into the teat, when the milk flows into the test-tube at the other end of the rubber-tubing. As a rule the milk runs freely but occasionally the udder has to be "milked" a little, as the cow "holds the milk back."

The milk which flows into the test-tube should be discarded or used for boiled controls. The rubber-tube is now disconnected from the glass tube, and (the cap of india-rubber having been removed from the tube of the filter flask) connected to the filter flask, so that the milk runs into the flask, and the requisite quantity can be collected.

This apparatus was used throughout the experiments, with satisfactory results. While the work was in progress, a paper by Rullmann (1911) appeared, covering the ground upon which we were working, the results obtained by him agreeing in almost every particular with those which we were obtaining.

We decided however to continue the experiments, especially as the method which we were employing for the estimation of catalase was different from that used by him.

Both goats' and cows' milk were used, the goats being kept on the premises, and thus easily accessible. The cows' milk was obtained from a neighbouring dairy where cows were kept under good conditions. We are much indebted to Mr E. J. Walker the owner of the dairy for allowing us to use his cows for the purpose of these experiments and desire to take this opportunity of thanking him for his kindness.

#### EXPERIMENTS UPON GOATS' MILK.

The ordinary milking-tube used for cows was found to be rather too large for the goat's teat and a smaller size of silver catheter was obtained. Immediately after collection plates were made of the milk. The amount taken was either 1 or 2 c.c. and the medium used was nutrient agar, and also whey-litmus-agar. Some of the plates were incubated at 37° C. and others at 22° C. for four or five days.

In no case did we succeed in getting all the plates free from any growth, although not infrequently some of the plates remained sterile. The count varied from 0·3–9·7 per c.c., the organisms being for the most part moulds which had evidently obtained entrance from the outside air, and possibly during the process of plating. In a few cases the goats were difficult to catheterise, and kicked out the catheter, the slight

contamination observed being probably introduced in this way. The ferments investigated were peroxidase, catalase and both direct and indirect reductase (Schardinger, 1902), and the results obtained for each ferment are dealt with separately.

*Peroxidase.*

Para-phenylene-diamine and hydrogen peroxide were used for the detection of this ferment, 1 c.c. of a 1% solution of the para-phenylene-diamine, and 0.4 c.c. of a 1% solution of the peroxide being employed. The reaction was in all cases instantly positive, the controls with boiled milk being always negative. It seems clear therefore that goats' milk while in the udder contains peroxidase.

*Catalase.*

In the first three experiments an Einhorn Saccharimeter was used, which is practically the method known as Koning's (Koning, 1906), and was that used by Rullmann in his experiments with cows' milk.

The results obtained are given below. The amounts used were 15 c.c. milk, 1 c.c. water and 5 c.c. of 1% H<sub>2</sub>O<sub>2</sub> incubated at 37° C. The water was used to make sufficient fluid in the saccharimeter.

	Method of procuring milk	Bacterial count per c.c.	Amount of oxygen given off in c.c.	Control
1.	Taken by catheter	5.0	In 45 mins. = 0. In 1½ hrs. 0.25. In 17 hrs. = 0.5	Negative.
2.	Milked into sterile flask	350.0	In 3 hrs. = 0. In 21 hrs. = 0.25	„
3 a.	Milked into sterile flask	Plates spoiled by sporing	In 3 hrs. = 1 c.c. In 4 hrs. = 1.2 c.c.	„
3 b.	Taken by catheter	3.0	In 1 hr. = 1 c.c. In 4 hrs. + 1 c.c.	„

These experiments showed that the Einhorn saccharimeter was not reliable for the estimation of the gas evolved, owing to the impossibility of shaking the apparatus. The importance of shaking in the estimation of catalase has been pointed out by many observers, and was brought prominently into discussion in the papers published in 1911 by Kooper (1911) and Grimmer (1911). The Lobeck apparatus was also tried but it was finally decided to use the apparatus devised by Harden, Thompson and Young (1910) in estimating the gas evolved in

alcoholic fermentation. This consists of a flask with long neck, capable of containing about 100 c.c. of fluid, which is clamped into a water-bath of desired temperature, and connected with rubber and glass tubing to a eudiometer, so that it can be taken out of the water-bath and shaken, without any disturbance of the gas content of the apparatus. The apparatus is so arranged that the pressure on the gas in the flask is always equal to that of the atmosphere.

In these experiments 50 c.c. of milk and 5 c.c.  $\text{H}_2\text{O}_2$  (1%) were used throughout both for goats' and cows' milk, control experiments with  $\text{H}_2\text{O}_2$  having shown that this quantity of  $\text{H}_2\text{O}_2$  gave the optimum evolution of gas with 50 c.c. milk at 25° C., the temperature at which the water-bath was kept.

5 c.c. of a 1% solution of hydrogen peroxide are capable of evolving about 18 c.c. of oxygen at atmospheric temperature and pressure under the influence of catalase and hence, as soon as this number was approached, a fresh quantity of  $\text{H}_2\text{O}_2$  was added.

All the apparatus with which the milk came in contact was sterilised. After the 50 c.c. of milk had been pipetted into the flasks they were allowed to remain for at least 10 minutes in the water-bath before the hydrogen peroxide was added. The eudiometer was then adjusted, the flasks shaken and a reading taken every 5 minutes.

The following were characteristic experiments and results:

#### I. Bacterial count 1 per c.c.

3.40 p.m. 5 c.c.  $\text{H}_2\text{O}_2$  added, by 5.17 p.m. 12.6 c.c.  $\text{O}_2$  given off.

5.20 p.m. 5 c.c. more added, by 6.20 p.m. 14.1 c.c.  $\text{O}_2$  given off

6.20 p.m. 5 c.c. more added, by 7.0 p.m. 8.6 c.c.  $\text{O}_2$  given off.

In 2 hours 40 minutes 35.3 c.c.  $\text{O}_2$  in all.

#### II. Bacterial count 1.6 per c.c.

2.18 p.m. 5 c.c.  $\text{H}_2\text{O}_2$  added, by 2.39 p.m. 12.8 c.c.  $\text{O}_2$  given off.

2.46 p.m. 5 c.c. more added, by 3.6 p.m. 14.1 c.c.  $\text{O}_2$  given off.

3.12 p.m. 5 c.c. more added, by 4.40 p.m. 18.0 c.c.  $\text{O}_2$  given off.

In 2 hours 22 minutes 44.9 c.c. in all.

The controls with boiled milk in each case were negative.

These results show that a much higher figure is obtained when the shaking is carried out at short intervals during the experiment, than when the Einhorn tube is used, and they leave no doubt that catalase is present in the milk of goats as it leaves the gland.

*Reductase.*

The reduction of methylene blue was tested for, both with methylene blue alone and also with Schardinger's reagent, the tubes being incubated at 37° C. Ten c.c. of milk were taken, and 0·1 c.c. of the reagent. In the milk collected by catheter there was no reduction of methylene blue alone in any of the tubes, the times of observation varying from 1½ to 18 hours. The only case in which reduction occurred was in milk which was milked by the animal attendant, and had an initial bacterial count of 350 per c.c. This milk reduced the methylene blue in two hours completely.

Schardinger's reagent was not reduced on any occasion except that upon which there was reduction of the methylene blue. This however was probably merely a reduction of the methylene blue and not a specific action on the Schardinger's reagent. It seems therefore that there is no reductase either direct or indirect present in sterile milk from goats.

Koning (1906, 1907), working with carefully collected goats' milk used as soon as possible after milking, obtained similar results, although he considered that there must be traces of reductase present, since, when goats' milk was added to cows' milk, the reduction of Schardinger's reagent took place more rapidly than when the cows' milk was simply diluted with water. Sames (1910) however has found that Schardinger's aldehyde-reductase will not bear dilution, so that if this be the case Koning's results might really show a delay in the action of the ferment in the cows' milk as a result of dilution by water and not an acceleration of the action by the addition of the goats' milk.

## EXPERIMENTS WITH COWS' MILK.

The same method as was used for the detection of the ferments in goats' milk was used for the cows' milk. The milk was collected by catheter, and brought at once to the Lister Institute, the several reactions being started as soon as possible after arrival.

*Peroxidase.*

The bacterial counts of the specimens of milk used for the peroxidase reactions varied from 0-6·0 per c.c. The reaction was strongly positive on all occasions. On one occasion when the cow had calved 2-3 weeks before the milk was examined, the reaction with paraphenylene-diamine was positive before the addition of the peroxide,

indicating the presence of an oxidase. Inasmuch as the oxidases have been shown to be a mixture of a peroxide and of a peroxidase this observation has no special significance for the present work. Rullmann also found peroxidase constantly present in sterile milk from cows.

### *Catalase.*

The reaction in this case was always positive, as it was also in Rullmann's work, but the amount of  $O_2$  given off was frequently very small, and much less than that given off by the goats' milk.

The following are some of the experiments; in all cases 50 c.c. of milk were taken. Controls of boiled milk in all instances gave negative results.

#### I. Bacterial count 0.

11.55 a.m. 5 c.c.  $H_2O_2$  added, and flask shaken every 5 minutes.

12.55 p.m. 2.5 c.c.  $O_2$  given off, 5 c.c. more  $H_2O_2$  added.

3.45 p.m. 1.2 c.c.  $O_2$  given off.

3.7 c.c. in all.

In 3 hours 50 minutes 3.7 c.c.  $O_2$  given off from 50 c.c. milk.

II. This experiment was carried out in the severest heat of summer (1911), and unavoidably towards midday. The cow in this case kicked out the catheter, and the bacterial count was 47 per c.c. 6.0 c.c. were given off in 3 hours 25 minutes. It is unlikely that the rather higher amount of catalase was due to the action of the bacteria, since, even at the end of the three hours, the number present cannot have been high, and in subsequent experiments with milk practically free from organisms equally high values were obtained.

#### III. Bacterial count 2.0 per c.c.

In  $\frac{1}{2}$  hour 6.3 c.c.  $O_2$  were evolved from 50 c.c. whole milk.

In 4 hours 12.6 " " " " "

In  $\frac{1}{2}$  hour 1.2 " " " 45 c.c. skimmed milk.

In 4 hours 2.8 " " " " "

In  $\frac{1}{2}$  hour 2.5 " " " cream of 45 c.c. of milk.

In 4 hours 4.5 " " " " " "

The cream was made up to 45 c.c. with sterilised saline.

IV. Bacterial count about 6 per c.c. Plates spoiled by spring organisms.

In 50 mins. 5.9 c.c.  $O_2$  were evolved from 50 c.c. whole milk.

" 2.4 " " " 45 c.c. skimmed milk without sediment.

" 0.9 " " " cream from 45 c.c. of milk.

Experiments upon centrifugalised milk as well as upon whole milk were also carried out, with the following results.

V. Bacterial count 1 per c.c.

In $\frac{1}{2}$ hour	3·7	c.c. O <sub>2</sub>	were given off by	whole milk.
"	2·9	"	"	whole milk without sediment.
"	0·4	"	"	the sediment alone.

The sediment was examined for leucocytes of which only a few were present. This experiment shows that the seat of the catalase is not wholly or even chiefly in the leucocytes of the sediment, as was also found by Meyer. Jensen, Torday and others found the catalase to be associated with the fat content, and Jensen also found that the strippings contained more catalase and more leucocytes than the rest of the milk, but that the amount of catalase was very slight compared with that produced by the bacteria.

Catalase therefore is present in cows' milk collected by catheter, having a negligible bacterial content; the amount present being variable.

This agrees with the work of Rullmann except that our figures are higher than his, but this is probably accounted for by the different apparatus used, for Rullmann used the Koning apparatus which as was seen in the case of the goats' milk gave lower figures than that employed by us.

Rullmann's figures for 10 c.c. milk were as follows:

In 1 hour	from 0·0—traces of O <sub>2</sub> .
In 12–18 hours	0·2–1·8 c.c. O <sub>2</sub> .
In 24 hours	0·2–3·6 c.c. O <sub>2</sub> .

*Reductase.*

The reduction of methylene blue was negative in every case, even when the observations extended over more than 18 hours at 37°. With Schardinger's reagent the reaction was very variable.

- I. Bacterial count 0 per c.c. Positive in 3 hours at 37° C.
- II. Bacterial count 47 per c.c. Positive in 5 hours at 37° C.
- III. Bacterial count 47 per c.c. Negative after 2 hours 45 mins. at 37° C.
- IV. Bacterial count about 13 per c.c. Positive in 3½ hours at 37° C.
- V. Bacterial count 2 per c.c. Positive in 2½ hours at 37° C.
- VI. Bacterial count 1 per c.c. Negative after 28 hours at 37° C.

The irregularity of the reaction is probably to be explained by the fact that sometimes the cows had been fairly recently milked and at other times not. Jensen (1906) and Romer and Sames (1910) have shown that the strippings have the highest reducing value for

Schardinger's reagent, and the last two authors have further shown that the first milk rarely contains any aldehyde-reductase (the ferment reducing Schardinger's reagent).

Reinhardt and Seibold (1911) have also shown that the reaction with Schardinger's reagent is often negative during the first two months after calving, and that the time of its appearance is also very irregular.

No precise data could be obtained in regard to the time after calving of the cows from which the milk was obtained, and these facts are sufficient to account for the variable results obtained. The length of time taken for the reaction to develop is longer than that observed by most investigators but the optimum temperature is from 45°–50° C. and the foregoing observations were made at 37° C. which accounts for this point of difference. Rullmann also found direct reductase absent and the reaction with Schardinger's reagent positive, in catheter milk.

#### CONCLUSIONS.

1. The presence of peroxidase and catalase can be demonstrated in the milk obtained by catheter from both goats and cows.
2. The catalase content of goats' milk is apparently higher than that of cows' milk.
3. The reduction of methylene blue does not occur with catheter milk of either goats or cows, at any rate within many hours.
4. Schardinger's reagent is not reduced by goats' milk, but catheter milk from a cow frequently reduces it.

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