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Studies on the Alimentary Tract of Merino Sheep in South Africa. XVI.—On the Identity of *Schizosaccharomyces ovis.* Part I.—Some Yeast-like Organisms Isolated from the Rumen Contents of Sheep fed on a Lucerne Diet.

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INTRODUCTION.

In a recent publication by Quin, a description is given of a fermentative process occurring in the forestomachs of sheep fed on certain sugar-rich diets namely, green lucerne or lucerne hay and molasses. Large amounts of gas, up to 9 litres in 90 minutes, were produced. Gas production increased rapidly after feeding, reaching a maximum in 30 minutes after which a steady decline followed. Dosing of sucrose or glucose directly through the ruminal fistula, had exactly the same effect as feeding lucerne. Animals kept on a poor quality veld hay did not possess the ability to ferment the sugar at all or only very slowly without any sign of a peak.

Furthermore it was found that fluid taken from the rumen was capable of fermenting glucose at a rate comparable to that which took place in the rumen. Microscopic examination revealed a rich microflora consisting of bacteria, yeastlike cells, mould spores and infusoria, the yeastlike cells being often particularly numerous and prominent. By employing various methods of precipitation it was found that the yeastlike cells and not the accompanying bacteria, might reasonably be held responsible for the rapid fermentation of sugar. These 'yeasts' were clear, dense, oval to oblong-oval cells, $8 \times 4\mu$ in size, usually without marked internal structure, seemingly capable not only of causing rapid fermentation of glucose, but also of storing part of it simultaneously in the form of glycogen. Evidence was obtained that reproduction probably took place by binary fission instead of budding, although no cell was actually seen dividing, nor was chain formation observed. On the basis of biochemical behaviour, morphology and method of reproduction, this organism was provisionally named '*Schizosaccharomyces ovis*', and was regarded as playing an important rôle in the early stages of the digestive process, synthesizing not only glycogen which might later serve as a source of energy, but possibly also members of the Vitamin B complex.

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It became a matter of great interest to attempt to isolate the 'yeast' in pure culture and to study its fermentative and other capabilities when unmixed with bacteria. The following is an account of such attempts, all based on the assumption that the organism really resembles a known yeast.

METHODS.

The routine procedure was as follows: Rumen ingesta was aspirated from the fistula of a sheep which was known to have high concentrations of 'yeasts' in the rumen, then squeezed through a cloth to separate as much plant debris as possible from the liquid which was allowed to stand for some time during which a dense green mass usually rose to the top, leaving a turbid yellow liquid which contained large numbers of yeast cells and bacteria. The yeasts were concentrated by centrifugation at 2,000 r.p.m. for 10 minutes after which they formed a thick yellowish deposit at the bottom of the tube. The supernatant was then poured off and the yeasts were washed twice with sterile distilled water. In this way, dense suspensions of yeast cells, relatively free from bacteria were obtained and used as inocula.

Plate cultures were made either by streaking the suspension over the surface of an appropriate agar medium, or by inoculating 10 c.c. of sterile molten agar at 43° with a loopful of the suspension, and pouring a plate after mixing well. Anaerobic cultures were also made by sucking up such inoculated agar into pipettes which were later sealed at the tip. Some plate cultures were also incubated in an atmosphere of CO_2. The temperature of incubation was 37° C. After 1, 2, 3 days etc., portions of colonies which at all resembled a normal yeast colony in appearance were examined in wet mounts stained either with methylene blue or iodine. Promising isolates were purified by repeated plating in the usual way.

In general, media were chosen which were known not to encourage bacterial growth unduly while being favourable to the growth of yeasts. The composition of these media was as follows:

*GC*4 *and* 7 (glucose-Czapek agar at pH4 and pH7 respectively): glucose, 5%; $NaNO_3$, 0·2%; KH_2PO_4, 0·1% (for GC4); K_2HPO_4, 0·1% (for GC7); $MgSO_4$, $7H_2O$, 0·05%; KCl, 0·05%; $FeSO_4$, $7H_2O$, trace; agar, 2%.

MN. (mannitol-nitrate agar): Mannitol, 2%; KNO_3, 0·2%; agar 2%.

ME 1, 2, 3, 4, 5 & 6. These were all based on malt extract made as described by Fulmer & Grimes (1923). ME 1 & 3 contained no added sugar, but their pH values were adjusted to 4·4 and 7 by appropriate addition of KH_2PO_4 (0·1%) and Na_3PO_4 respectively, before addition of agar. ME 2 & 4 were similar to ME 1 & 3 save that 5% glucose was added in each case.

*ME*5 contained no added sugar and was adjusted to pH3 by means of H_3PO_4.

*ME*6 was adjusted to pH7 plus 15% glucose added.

PRUNE AGAR. Made as described by Smith (1946).

LE, A & B. Good quality dry lucerne (100 gm.) was soaked in tap water (800 ml.) for 24 hours at 55° C. After removing leaves and debris by filtration, the filtrate was boiled for 30 min. and glucose (5%) added. The solution was divided into two parts which were brought to pH4·6 (LEA) and 6·8 (LEB) by addition of KH_2PO_4 (0·1%) and Na_3PO_4 (0·5%) respectively, and then solidified by appropriate addition of agar.

CSL (Corn steep liquor medium). The following were dissolved in commercial corn steep liquor: glucose, 5%; KH_2PO_4, 0·1%; $MgSO_4$, $7H_2O$, 0·02%; $CaCl_2$, 0·1%; agar, 2%. It had pH 7·3.

GYC (Glucose-yeast extract agar): as GC7, save that yeast extract (10%) was added.

Results.

The most common yeast-like organisms forming colonies were either small and atypical or else corresponded to the accepted description of the genus *Monilia*. Mucor-like moulds also commonly appeared (see Table on p. 122). A more detailed description of these organisms and their fermentative powers follows:—

(*a*) *The Small Yeast-like Organisms.*—Although multiplying by binary fission, they were much smaller than '*Schizosaccharomyces ovis*' as seen in rumen ingesta, they were more rectangular and tended to form chains. Furthermore they readily stained deep blue with methylene blue in wet preparations, while '*Schizosaccharomyces ovis*' rarely took the stain at all under these conditions. In general their colonies on GC4 and 7 and MN were yeast-like, being white, translucent, smooth and round with more or less irregular outline. In liquid malt extract medium none of these organisms showed marked fermentative powers or the ability to store glycogen from glucose.

(*b*) *Mucor sp. forming yeast-like spores or oidia.*—Not only do Mucor spores, as ordinarily formed, often resemble yeast cells in size and shape, but it is wellknown that *Mucor racemosus* can grow in the form of yeast-like cells (oidia) in submerged liquid culture [see Henrici (1930)]. It was therefore necessary to consider whether the rumen 'yeasts' really consisted of resting spores of a mucor. Mucor spores were present on the dried lucerne fed to the sheep, as was easily shown by the usual cultural methods for moulds, so that it may be assumed that the mucors appearing on the plates were not aerial contaminants, but really arose from spores present in the rumen ingesta. To determine whether Mucor spores derived from rumen ingesta could assimilate glycogen and cause fermentation in a sugary substrate, a solution containing glucose (5%) and KNO_3 (0·1%) was inoculated heavily with such spores obtained by dragging a platinum loop over an old culture containing many sporangia on a plate which had been streaked with rumen ingesta. The culture was incubated at 37° for 30 min., when the spores still stained yellow with iodine and not reddish brown. After a further 2 hours incubation, the appearance was the same and no fermentation had taken place.

A young growing colony of a typical Mucor derived from rumen ingesta was next transferred to the depths of a sterile glucose-nitrate liquid medium in order to determine whether the mycelium could in fact break up into yeast-like cells. After 2 days incubation the turbid liquid was found to contain numerous yeast-like cells, many of which were budding. Propagation by binary fission was not observed. Furthermore these cells did not stain red-brown with iodine, and no visible fermentation had taken place in the culture. They showed little or no resemblance therefore to '*Schizosaccharomyces ovis*'.

Micro-Organisms isolated from Rumen Ingesta.

Date on which Rumen ingesta was Drawn.	Media.	pH.	Culture Method.	Results.
2/7.....	GC7 GC4 MN	7·0 4·4 7·0	Streaked plate.	Various bacteria and small yeast-like organisms.
6/7.....	ME1 ME2 ME4 ME5	4·4 4·4 7·0 3·0	Streaked plate.	Bacteria and Mucor.
29/7.....	ME2 ME4 Prune	4·4 7·0 —	Streaked plate.	Bacteria and Mucor.
11/8.....	ME2 ME4 Prune	4·4 7·0 —	Streaked plate.	Yeastlike organism (Monilia) and Bacteria.
14/8.....	ME4 Prune	7·0 —	Pipette deep cultures	Bacteria.
22/8.....	LEA LEB	4·6 6·8	Streaked plate. Pipette cultures.	Monilia, Mucor; Bacteria. Bacteria.
1/9.....	ME6 C.S.L.	7·0 7·0	Streaked plates. Poured plate.	Monilia; Bacteria.
15/9.....	LEB ME6 Prune Glucose nutrient GYC	7·0 7·0 — — 6·8	Poured plates.	Bacteria on all plates. Monilia on LEB and ME6. Prune and GYC.
24/9.....	LEB. ME6		Streaked and Poured plates in CO_2 and CO_2 and Air 1 : 1.	Bacteria only on streaked plates.

It might, however, be argued that the behaviour of the *Mucor* would be different in the much more complex medium represented by the liquid part of rumen ingesta. To prepare such a medium, rumen ingesta was centrifuged at high speed for 20 min. until the supernatant appeared to be free from yeasts when examined under the microscope. It was then divided into 3 portions of 10 ml. each, to each of which was added 5 ml. of the glucose-nitrate solution previously used. One portion was then inoculated with a heavy suspension of *Mucor* spores, the second with the yeast-like cells obtained by submerged growth (see above) and the third served as control. The sediment from the

rumen ingesta, which was rich in yeast-like cells, was re-suspended in sterile distilled water (10 ml.), and 5 ml. of the glucose-nitrate solution added. Fresh rumen ingesta was similarly mixed with glucose-nitrate solution to serve as control. All were incubated at 37° and examined at half-hourly intervals with the following results:

	30 Minute Result.		3 Hour Result.	
	Fermenta	Glycogen Storage.	Fermenta-tion.	Glycogen Storage.
1. Ingesta Control	+	+	0	+
2. Sediment+Glucose	+	+	0	+
3. Supernatant+Spores	0	0	0	0
4. Supernatant+Mycelium	0	0	0	0
5. Supernatant only	0	0	0	0

It may be safely concluded, therefore, that the rumen Mucor in no circumstances behaves as does '*Schizosaccharomyces ovis*' with respect to fermentation and glycogen storage, and that it is an entirely different organism.

(*c*) *Monilia sp.*—This fungus was observed more than once, sometimes in large numbers on plates of Malt extract and Prune agar which had been inoculated with sediment of centrifuged rumen ingesta by streaking. At first, this organism was mistaken for a true yeast. The colonies were pasty, creamy white, slightly convoluted, irregular in outline, white underneath with a thin white margin. A strong, pleasant, fruity odour was given off. After a few days, fine radiating hyphae appeared round the margin.

Microscopically the cells were typically yeastlike, oval to oblong-oval or elongate-oblong with a tendency to filament-formation depending on the medium. Submerged colonies were initially spindle or lensshaped but after 2-3 days incubation, filaments were formed. Coridia were borne freely on short lateral hyphae and proliferated further by budding. It was highly pleomorphic and complied closely with the description of Monilia spp. without aerial mycelium given by Henrici (1931). It is possible but not certain that more than one species was isolated.

To determine whether this organism played any part in the fermentation in the rumen, heavy suspensions of cells in the supernatant liquid of centrifuged rumen ingesta were made in glucose-nitrate medium just as described above in the experiment with mucor spores. The results were similar in that Monilia did not seem to be able to ferment glucose quickly and assimilate glycogen intracellularly in the characteristic fashion of the rumen yeasts. In another experiment it was found that Monilia, given several days, was capable of fermenting glucose to CO_2 and a little alcohol in the presence of Malt extract.

The Monilia isolated from the rumen ingesta is therefore a different organism from the rumen yeast; because of the slow fermentation of which it is capable, it cannot be regarded as participating in the fulminating type of fermentation observed in the rumen. If, for example, *Monilia* cells are added to freshly drawn rumen ingesta, already rich in 'yeasts', and the whole incubated after addition of a little glucose, the yeast-like Monilia cells which have been added can readily be distinguished after a little while from the indigenous 'yeasts', which they resemble in size and shape, by the fact that they do not store glycogen, and are stained only pale yellow by iodine while the rumen 'yeasts' stain reddish brown.

On a later occasion, a considerable number of the typical rumen 'yeasts' were seen to be actively motile in a wet mount made from fresh rumen ingesta. It may be concluded, therefore, that 'Schizosaccharomyces ovis' is not only not a yeast, but does not belong to the *Eumycetes* at all.

Summary and Conclusions.

All attempts by the usual mycological techniques to isolate 'Schizosaccharomyces ovis', have failed although other yeast-like organisms have been isolated, often in quantity, from rumen ingesta. None of these organisms have the characteristic properties of '*Schizosaccharomyces ovis*' as present in sheep's rumen ingesta, viz. the power of quick fermentation of added glucose with simultaneous storage of massive amounts of glycogen in the cells. The rumen 'yeasts' have also been shown not to be Mucor spores or oidia or yeast like forms of Monilia which they resemble in size and shape. From this and other evidence, it is concluded that '*Schizosaccharomyces ovis*' is misnamed and is probably not a member of the Eumycetes at all.

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