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AN ATTEMPT TO MANUFACTURE CHEDDAR CHEESE CONTAINING ONLY ONE TYPE OF ORGANISM.

T H E S I S

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INTRODUCTION.

There have been many investigations on the effect of various bacteria on the ripening of hard rennet varieties of cheese. Hucker (1922) in a review of the bacteriological aspects of cheese ripening summarizes the position as follows: "As it stands to-day the investigations have clearly demonstrated that the breaking down of the insoluble casein compounds is due to enzymes, either natural or bacterial; while characteristic flavors are produced by the action of certain groups of bacteria (Bacterium casei or coccus group), which depend on the products of B. lactis acidi present in large numbers during the manufacture and early ripening stages." The effect of lactic acid bacteria upon the flavour of Cheddar cheese has been studied by Hastings, Evans and Hart (1912), Evans, Hastings and Hart (1914), Evans (1918), Leitch (1923) and Hucker and Marquardt (1926).

A study of the effect of any organism on the ripening process in cheese is attended by many technical difficulties. The contamination with bacteria which normally takes place at innumerable points during the production of the milk and the manufacture of the cheese complicates any investigation. Even when milk has a low bacterial count after production it contains several species of organisms, some of which may multiply and play a significant role in the ripening of the cheese. For instance, although lactobacilli are usually present in milk only in small numbers, they are capable of multiplying to such an extent that they become the predominant group in the ripened cheese. Again, the fact that certain organisms are present only in small

numbers and are unable to multiply in the cheese, is no proof that they have little or no influence on the product, for the bacterial enzymes liberated after death of the organisms may be capable of producing significant changes. In some investigations that have been recorded, no details of the manufacture of the cheese are given, while in others it is apparent that the method of manufacture was so modified that it was impossible to produce a normal cheese. It is well known that small differences in the details of manufacture, even with milk of high quality, may lead to abnormal characteristics in the product.

A study of the literature has failed to reveal any work on the effect of an organism on the ripening of Cheddar cheese, in which a strict bacteriological control was maintained throughout the production of the milk and the manufacturing process. It was felt that before the effects of specific organisms could be demonstrated such a control was necessary. It was therefore with the object of elaborating a technique whereby the bacteriological aspects could be followed carefully and the number of variable factors reduced to a minimum, that this investigation was undertaken. At the same time it was hoped to gain information on the following points:

(1) The effect of Streptococcus cremoris on the rate of casein breakdown of Cheddar cheese.

(2) The effect of Streptococcus cremoris on the flavour of Cheddar cheese.

(3) The source of the lactobacilli commonly found in mature Cheddar cheese.

MEDIA EMPLOYED.

The medium used most extensively was a tryptic digest of casein. This was prepared by digesting 100 grms.

of sugar-free casein with 20 c.c. of Allen & Hanbury's Liquor Trypsin Co. The casein was mixed with 1000 c.c. of water, the trypsin added and the reaction adjusted to pH 8.1 by addition of 40% NaOH. Chloroform was added to prevent the growth of bacteria. The mixture was incubated for 6 days at 37°C., the bottle being shaken at intervals. The reaction was readjusted to pH 8.1 on the second and fourth days. After six days the liquid was filtered and the filtrate was diluted until it contained 0.5% of nitrogen as determined by the Kjeldahl method. Magnesium sulphate (0.1%) and dipotassium phosphate (0.2%) were then added, the reaction was adjusted to pH 6.8 and the medium was filtered.

The resulting broth formed the basis of the casein agar and sugar media. Casein agar was prepared by adding 1% of dextrose and 2% of agar to the broth. When the agar was to be used for the preservation of stock cultures over long periods the dextrose content was reduced to 0.25%.

For the determination of fermentation reactions 1% of each sugar and 1% of Andrade's indicator were added to the broth.

All the casein digest media were sterilised by three successive steamings.

Other media used were:

Yeast Whey Agar.

Standard Agar.

Nutrient Gelatin.

MacConkey's Broth.

Peptone Water.

Peptone Water Sugars.

The sugar media used for the fermentation tests of the micrococci consisted of peptone water to which 0.5% of each sugar and 1% of Andrade's indicator were added.

BACTERIOLOGICAL METHODS.

Except where it is otherwise stated all cultures were incubated at 30°C.

Determination of Acid and Volatile Acid Production.

200 c.c. of sterilised skim milk (three successive steamings) were inoculated with the organism or mixed culture to be tested and incubated at 22° C. for ten days. The acidity was then determined on 20 grms. of the culture and after subtraction of the control value the result was expressed as percentage of lactic acid.

150 c.c. of the culture were distilled in steam after the addition of 7.5 c.c. 2N sulphuric acid, 5 grams of sodium sulphate and a piece of paraffin wax to reduce frothing. Before commencing distillation the distilled water used in the steam can was allowed to boil for several minutes. One litre of distillate was collected and titrated with N/10 NaOH using phenolphthalein as indicator. The titration given by the control milk was subtracted and the result expressed as a percentage in terms of lactic acid. The amount of volatile acid as a percentage of the total acid was then calculated. Evans (1918) found that after seven days' incubation at 30°C., cultures of lactic acid streptococci showed little or no increase in volatile acid production.

Sugar Fermentations.

The inoculated tubes were incubated at 30°C. for 14 days (28 days in the case of lactobacilli) and were examined at 3 days, 7 days, and 14 days. The degree of fermentation was estimated by the intensity of colour produced through the action of the acid on the indicator. In the Tables the results are indicated by the following symbols:

\- no fermentation.

\+ very slight fermentation.

L slight fermentation.

\+ fermentation.

\+ strong fermentation.

ISOLATION OF THE STARTER ORGANISM.

The difficulty of preparing and maintaining an active "starter" consisting of a pure culture of a lactic streptococcus was realised very early in the work, so an attempt was made to isolate from two commercial "starters" one of the strains most active in producing acid. These two "starters" were being successfully used at the time for the manufacture of Cheddar cheese in the College factory.

The starters were plated out in duplicate on casein agar and incubated at 30°C. for 4 days. By the use of the 1/3" objective, representatives of the several types of colonies were picked off and inoculated into tubes containing 10 c.c. of sterile milk. The rate of acid production of the cultures was determined by titration with N/10 NaOH after incubation for 24 hrs. at 30°C. By this means, five of the most active acid producers (two from starter X and three from starter K) were isolated for further study. For comparative purposes a stock strain of Streptococcus lactis was added to the series.

The six cultures were purified twice by re-isolation from casein agar plates and their characteristics were then determined by repeated observations. Table I gives typical results obtained by sowing one loopful of culture into 10 c.c. of sterile milk, incubating at 30°C. for 24 hours and titrating with N/10 NaOH to phenolphthalein. In each case the control value was subtracted and the result expressed as a percentage in terms of lactic acid.

TABLE I.

Acid Production of Streptococci isolated from Starters

24 hrs. at 30°C.

Culture	Acid Produced expressed as % lactic acid
BX 1	0.43
CX 1	0.59
BK 1	0.46
CK 11	0.31
DK	0.25
Sc. lactis	0.38
Starter X	0.69
Starter K	0.68

It will be observed that none of the isolated strains was as rapid an acid producer as either of the original starters. Of the pure cultures, CX 1 consistently produced the largest amount of acid in 24 hours, although the other cultures gave higher values after several days' incubation, as will be observed in Table II which gives the Total Acid and Volatile Acid results.

TABLE II.

Acid and Volatile Acid Production of Streptococci isolated from Starters.

Culture.	Total Acid Produced as % lactic acid.	Volatile Acid Produced	
		as % lactic acid.	as % Total acid
BX1	0.67	0.011	1.6
CX1	0.55	0.015	2.7
BK1	0.66	0.016	2.4
CK11	0.59	0.015	2.6
DK	0.56	0.010	1.7
Sc. lactis	0.58	0.018	3.0
Starter X	0.78	0.095	12.1
Starter K	0.78	0.079	10.1

The results in Table II show that, whereas all the pure cultures had a low volatile acid production the starters contained organisms capable of producing large amounts of volatile acid.

Table III gives the results of the determination of sugar fermentations.

TABLE III.

Sugar Fermentation Results of Streptococci isolated from Starters X and K.

Culture	Time of Incubation	Glycerin	Xylose	Arabinose	Rhamnose	Sorbite	Mannite	Dulcite	Laevulose	Dextrose	Mannose	Galactose	Saccharose	Maltose	Lactose	Raffinose	Inulin	Dextrin	Starch	Salicin
BX1	3 days	-	-	-	-	-	-	-	+	+	+	+	-	-	+	-	-	-	-	-
CX1		-	-	-	-	-	-	-	+	+	+	+	-	-	+	-	-	-	-	-
BK1		-	-	-	-	-	-	-	+	+	+	+	-	-	+	-	-	-	-	-
CK11		-	-	-	-	-	-	-	+	+	+	+	-	-	+	-	-	-	-	-
DK		-	-	-	-	-	-	-	+	+	+	+	-	-	+	-	-	-	-	-
Sc.lactis		-	-	-	-	-	-	-	+	+	+	+	-	+	+	-	-	L	-	L
BX1	7 days	-	-	-	-	-	-	-	+	+	+	+	-	-	+	-	-	-	-	-
CX1		-	-	-	-	-	-	-	+	+	+	+	-	L	+	-	-	-	-	-
BK1		-	-	-	-	-	-	-	+	+	+	+	-	-	+	-	-	-	-	-
CK11		-	-	-	-	-	-	-	+	+	+	+	-	-	+	-	-	-	-	-
DK		-	-	-	-	-	-	-	+	+	+	+	-	-	+	-	-	-	-	-
Sc.lactis		-	-	-	-	-	-	-	+	+	+	+	-	+	+	-	-	+	L	+
BX1	14 days	-	-	-	-	-	-	-	+	+	+	+	-	-	+	-	-	-	-	-
CX1		-	-	-	-	-	-	-	+	+	+	+	-		+	-	-	-	-	-
BK1		-	-	-	-	-	-	-	+	+	+	+	-	-	+	-	-	-	-	-
CK11		-	-	-	-	-	-	-	+	+	+	+	-	-	+	-	-	-	-	-
DK		-	-	-	-	-	-	-	+	+	+	+	-	-	+	-	-	-	-	-
Sc.lactis		-	-	-	-	-	-	-	+	+	+	+	-	+	+	-	-	+	L	+

From Table III it is evident that none of the isolated pure cultures corresponded with the stock strain of Streptococcus lactis and also that CX1 differed from the other isolated strains by reason of a slow fermentation of maltose. It was concluded that all the five strains isolated from the two starters were types of Streptococcus cremoris according to the classification of Orla Jensen (1919). Strain CX1 was selected as most suitable for use as a starter in the present investigation, because of its more rapid acid production.

CHARACTERS OF THE STARTER ORGANISM.

Morphology.

After 24 hours' growth in milk at 22°C. or at 30°C. the organism appeared as slightly oval Gram-positive cells in pairs and in short chains. Occasionally long chains were formed. Milk cultures incubated at 37°C. showed chiefly diplococci with a small number of single cells, short chains and involution forms. When grown on casein agar at 22°C. or at 30°C. the microscopical appearance of the organism was similar to that seen in the milk cultures at these temperatures. A number of swollen and rod-shaped cells, however, were also present. On occasions, long and very long chains were formed on casein agar plates. At 37°C. the organism tended to form long chains.

The colonies formed on casein agar and yeast whey agar plates were small, spherical, translucent to opaque, with entire margins.

Biochemical Characters.

Growth in milk was rapid and a clot was formed within 24 hours. The rate of acid production was consistently more rapid than was the case with the stock strain of Streptococcus lactis. This may have been due to the fact that CX1 was of recent isolation from a mixed culture, while

the Streptococcus lactis had been growing for several years on artificial media in pure culture. Immediately after isolation the first two transfers of CX1 in milk showed slight gas formation. This property rapidly disappeared, however, and has not been observed since.

It has already been mentioned that CX1 produces only a small proportion of volatile acid.

The sugar fermentation reactions have been given in Table III. They were later determined by the method of Orla-Jensen (1919) in which the amounts of acid formed are measured by titration. The results are given in Table IV and it will be observed that they are in close agreement with those given previously.

TABLE IV.*

Nitrogen Source.	Glycerin	Xylose	Arabinose	Rhamnose	Sorbite	Mannite	Laevulose	Dextrose	Mannose	Galactose
Casein-digest.	1.3	1.1	1.4	1.4	1.4	2.0	6.3	6.1	5.9	4.3

Nitrogen Source.	Saccharose	Maltose	Lactose	Raffinose	Inulin	Dextrin	Starch	Salicin	Milk	Milk + Yeast Extract
Casein-digest	1.6	2.0	5.2	1.3	1.3	1.8	1.4	1.3	5.3	5.1

(results given as grms. of lactic acid per mille)

The proteolytic action of the organism‡ was determined by growth in a trisodium phosphate solution of rennet casein at 22°C. for 14 days. Formol titrations and determinations of the percentage of protein soluble at pH 4 showed that the organism had negligible proteolytic power.

* Figures kindly supplied by Mr.H.R.Whitehead, Dairy Research Institute (N.Z.)

‡ Determined by Mr.H.R.Whitehead, Dairy Research Institute (N.Z.)

A consideration of all the characteristics led to the conclusion that CX1 was most probably a strain of Streptococcus cremoris (Orla-Jensen 1919). This conclusion derives support from a recent paper by Knudsen (1931) who, in discussing Orla-Jensen's classification of the lactic acid bacteria, states: "The deciding factor between the two species (Sc. lactis and Sc. cremoris) is, however, that Streptococcus lactis can ferment maltose and dextrin while Streptococcus cremoris cannot."

For the whole period of the investigation the organism was resown daily in 200 c.c. of sterile milk and incubated at 22°C. During the first month after isolation the rate of acid production decreased until it became necessary to use an inoculum of 1 c.c. daily in order to induce clotting in 24 hours. During the next month the rate of acid production increased until only 4 drops of inoculum were required. The activity of the organism thereafter remained constant and its cultures received the same amount of inoculum as did five cheese "starters" which were being maintained in the laboratory under similar conditions.

Great care was taken to prevent contamination of the starter throughout the whole period. Between the manufacture of Cheese II and Cheese III the starter was examined thoroughly. No contamination could be detected either by plating out on casein and standard agar or by direct microscopical examination. The sugar fermentation reactions proved to be identical with those originally obtained. Determinations of the total and volatile acid production gave values of 0.60% and 0.013% respectively.

It was evident that after several months' cultivation the starter organism had not altered its characteristics in any detail which it was possible to detect.