SOME FACTORS INFLUENCING THE CHOICE OF LACTIC STREPTOCOCCI FOR USE AS STARTERS IN CHEESEMAKING

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ABSTRACT

Strains of lactic streptococci characterized as bitter cheese starters multiplied rapidly during normal cheesemaking and reached high populations in the curd before salting. The multiplication of starter strains which are typically non-bitter was inhibited by the cooking temperatures used in normal Cheddar cheesemaking, even though acid production continued. The populations reached were substantially lower than with bitter starters. If manufacturing conditions were altered so that non-bitter strains reached high numbers in the curd, cheeses were bitter. Conversely, bitterness was absent, or of reduced intensity, when growth of conventionally bitter starters was restricted during cheese manufacture, either by the use of raised cooking temperatures, or by bacteriophage attack. The results of 60 cheesemaking trials with 10 starter strains confirmed this association between the size of starter population reached during cheesemaking and the presence or absence of bitterness.

A new model is proposed to account for the development of bitterness in Cheddar cheese. Unlike other schemes where the production of bitter peptides is attributed directly to rennet action on casein, the new hypothesis considers that the starter strain is responsible for the formation of bitter-flavoured peptides and does not merely act to remove them. All starters are potentially able to produce either bitter or non-bitter cheese, depending upon the conditions of manufacture (including bacteriophage development) and, hence, the population reached. The response to selected manufacturing conditions rather than any single difference between particular starter strains determines the likelihood of bitterness development.

The key cheesemaking trials were repeated using aseptic techniques under controlled bacteriological conditions. Previous studies of aseptically manufactured cheese utilized starters which gave little Cheddar flavour or exhibited pronounced flavour defects. In this investigation, cheeses

made aseptically with Streptococcus cremoris AM_2 alone, possessed full Cheddar flavour and no off-flavours provided that normal manufacturing procedures were followed. Cheeses made with AM_2 at a low cooking temperature (33°C) and ML_8 cheeses made at the normal cooking temperature (38°C) were extremely bitter and lacking in Cheddar flavour. When appropriate levels of bacteriophage were present during manufacture, bitterness was abolished entirely from both the ML_8 and low-cook AM_2 cheeses. There were accompanying increases in Cheddar flavour scores.

A search for temperate bacteriophages and lysogenic strains in the lactic streptococci used as starters in cheesemaking showed that the occurrence of lysogeny or defective lysogeny could be relatively widespread. A temperate bacteriophage was inducible from Str.cremoris R_1 by ultraviolet irradiation or mitomycin C treatment. Induced lysates produced plaques on lawns of 3 closely related Str.cremoris strains, AM_1, SK_{11} and US_3. Strain SK_{11} was readily lysogenised. Str.cremoris AM_1 was the most reliable indicator strain, although the age of the culture used for seeding plates was critical. Zones of lysis, but no plaque formation were observed on lawns of 9 additional Str.cremoris strains. Several other strains of both Str.cremoris and Str.lactis were inducible by either treatment but no indicator strains for the induced phages were found. The significance of lysogeny in cheesemaking cannot yet be assessed.

PREFACE

These studies were undertaken to investigate the role of the starter streptococci in the development of bitterness and cheese flavour in Cheddar cheese. Because of the seasonal nature of the work and long periods required to observe the outcome of cheesemaking experiments a second project, the incidence of lysogeny amongst the strains of starter streptococci, was undertaken concurrently with the cheese investigations. Most previous studies had considered only virulent bacteriophages whereas temperate bacteriophages had been virtually ignored. There was no confirmed report of lysogeny in group N streptococci when this investigation was commenced. It was possible that some cheesemaking characteristics of particular starter strains were due to their lysogenic nature.

The results of conventional cheesemaking trials carried out to investigate bitterness development are reported in Section I. Section II describes experiments based on cheesemaking using aseptic techniques under controlled bacteriological conditions. The investigations of temperate bacteriophages and lysogeny in the starter streptococci are contained in Section III.

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INTRODUCTION

Single strains of Streptococcus cremoris and Str.lactis have been characterized according to their tendency to produce bitter flavour when used as starters in the manufacture of Cheddar cheese (Emmons et al. 1962; Lawrence & Pearce, 1972). While there is general agreement that bitterness in cheese is caused by the accumulation of bitter peptides resulting from the action of proteolytic enzymes on casein, the relative contributions of rennet and starter proteinase activity to the formation and degradation of bitter-tasting peptides have not been resolved.

Czulak (1959) suggested that the formation of bitter peptides was primarily due to the action of rennet on casein, and that these peptides were degraded by proteolytic enzymes of "non-bitter" lactic streptococci but not by "bitter" strains. Hence, the starter streptococci played no significant part in the development of bitterness. Bitter starters, according to Czulak, had an entirely neutral role and non-bitter strains degraded the bitter peptides formed by rennet. Other investigators have interpreted their data to support this hypothesis (Czulak & Shimmin, 1961; Emmons et al. 1962; Jago, 1962; Stadhouders, 1962). Sullivan & Jago (1970a, 1970b) suggested that the possession of a pyrrolidone carboxylyl peptidase by non-bitter, but not by bitter starters, was the critical difference between strains in their ability to degrade bitter peptides. The detailed investigation of Exterkate & Stadhouders (1971) and subsequent studies (Sullivan et al. 1973) however, showed that pyrrolidone carboxylyl peptidase activity was not only present, but was often higher in cell extracts from bitter strains than from non-bitter strains.

An alternative to Czulak's hypothesis is that bitter strains of lactic streptococci are directly responsible for the formation of bitter peptides from casein, or from products of rennet action on casein. Gordon & Speck (1965) reported that bitter starters, in contrast to non-bitter strains,

could form bitter compounds from casein in the absence of rennet, which they attributed to greater proteolytic activity by bitter strains. Klimovsky et al. (1970) have also suggested that bitter starters possess greater proteolytic activity than non-bitter strains. The results of Harwalkar & Seitz (1971), who have conclusively shown that proteolysis of casein by bacterial enzymes alone is sufficient to produce bitter flavour components, also suggest a positive role for bitter starters in the production of bitterness in cheese.

In the present study, the growth characteristics of bitter and non-bitter strains were investigated, initially using a laboratory activity test (Pearce, 1969) and subsequently in cheesemaking trials. The results show that there is a close association between the starter population reached during cheese manufacture, and the presence or absence of bitterness, suggesting that starter streptococci contribute directly to the formation of bitter-flavoured components.

METHODS

The cheesemaking procedures, manufacturing records and methods for chemical and bacteriological analyses and flavour evaluation are reported in detail in Appendix I. The following is a summary of these methods.

Starters and bacteriophages

Streptococcus cremoris strains AM_2, HP and SK_{11} and Str. lactis strain ML_8 were from the Institute culture collection. Starter strains were subcultured (1% inoculum) twice weekly in autoclaved reconstituted skim milk (9.5% solids), held at 4°C and incubated at 22°C overnight before sub-culture. Bulk starters for cheesemaking were grown in steamed (1.75 h) whole milk (0.25-0.5% inoculum, 16 h, 24°C).

Whey stocks of bacteriophages were prepared by infecting the appropriate host strains with low levels of the homologous bacteriophage (10^2-10^3 plaque forming units/ml) during early logarithmic growth in autoclaved skim milk. Phage-infected

cultures were incubated at 30°C for 5 h, acidified (0.1 ml 10% lactic acid/ml culture), and centrifuged (12,000 x g, 10 min). The supernatants were assayed on the homologous hosts by soft agar overlay on M16 agar plates containing 0.005M calcium borogluconate (Lowrie & Pearce, 1971). Whey stocks contained 1-5 x 10^9 plaque-forming units (PFU)/ml.

Cheese manufacture

Cheddar cheeses were manufactured from pasteurized (72°C/15 sec) commercial whole milk, in the Institute's Processing Hall by a small scale (320 l) simulation of conventional commercial methods. The same calf vell rennet level (22 ml rennet/100 l milk) and setting temperature (31.7°C) were used in all experiments. The titratable acidities at which the vats were run, dried, milled and salted were, as far as possible, kept constant between experiments. The size of cubes formed when the coagulum was cut and the extent of dry-stirring had to be altered when different cooking temperatures were used, in order to keep the moisture content of the cheeses within normal limits. After overnight pressing, cheeses (18 kg) were film wrapped and matured for 14 days at 13°C and subsequently at 7°C.

Analyses of the cheeses 14 days after manufacture showed that the moisture contents (31.1-35.7%), fat contents (34.5-39.0%), salt-in-moisture values (3.84-5.44%) and pH (4.88-5.16) were all within the accepted limits for commercial New Zealand Cheddar. Compositional details are recorded in Appendix I.

Bitterness evaluation

The occurrence and intensity of bitterness in cheeses, 3 and 6 months after manufacture were assessed as described by Lawrence et al. (1972).

Laboratory activity tests

Laboratory activity tests of starter strains were based on the method of Pearce (1969). Pasteurized whole or reconstituted skim milk was chilled, inoculated with the appropriate starter (2%) and divided into 10 ml quantities in 19 x 150 mm test tubes. Cultures were incubated over the Cheddar cheesemaking temperature profile for 5-6 h. Rennet (1:5000) was added after 20 min incubation. At each sampling time two cultures were chilled in ice water, and the curd and whey macerated. The viable colony count was determined from one culture, and pH from the other. The time taken by a starter strain to reach pH 5.2 in the activity test is almost the same as the "set to salt" time in actual Cheddar cheesemaking (Pearce, 1969). In the standard activity test inoculated cultures were incubated initially at 31.7°C for 70 min with rennet addition after 20 min incubation. The temperature was raised gradually over 30 min to reach 37.8°C at 100 min, and the cultures held at this temperature until the time equivalent to running of the whey in cheesemaking (180 min), when the cultures were cooled slowly to 36.7°C over the next 30 min. After 60 min at 36.7°C cultures were again slowly cooled over 30 min to 31.7°C. If necessary, incubation was continued at 31.7°C until the cultures reached pH 5.2.

Starter colony counts

Milk or curd samples (10 g) were chilled and added to 90 ml chilled 2% trisodium citrate in an AtoMix blender (Measuring and Scientific Equipment Ltd, Crawley, England). Blending at full speed (13,000 rpm) for 2 min homogenized the samples and reduced chains of streptococci to an average colony forming unit (CFU) of 2.3-3.0 cells depending upon the strain (Martley, 1972). The efficiency of the blending was checked microscopically. Blended samples were diluted in 1/10 strength M16 broth and plated for colony counts by soft agar overlay on M16 agar plates (Lowrie & Pearce, 1971). Plates were incubated at 30°C for 18-24 h. Assays for bacterio-

phage were carried out on all samples in cheesemaking trials. The colony count data was disregarded if the bacteriophage titre was greater than one hundredth of the dilution at which the sample gave adequate starter colony counts.

In cheesemaking experiments the colony counts/g sample were multiplied by the ratio of the solids content of finished cheese to solids content of sample. All colony counts are therefore expressed as colonies/g finished cheese, thereby overcoming the problems associated with the changing moisture contents of samples from different stages of the cheesemaking process. In activity tests the sample comprised the entire contents of a 10 ml culture. Therefore, no correction for the changing moisture content was necessary in these experiments.

RESULTS

Laboratory activity tests

Laboratory activity tests, which simulate cheesemaking conditions, were used to compare the growth characteristics of the non-bitter strain Str.cremoris AM_2 with Str.lactis ML_8, a typically bitter starter. ML_8 grew rapidly throughout the standard activity test to a final population of greater than 10^9 CFU/ml and reached pH 5.2 in less than 5 h (Fig.1). The growth pattern of AM_2, on the other hand, was quite different. The colony count increased steadily in the early stages of the activity test and reached 10^8 CFU/ml after 30 min at the cooking temperature of 37.8°C. Even though the pH continued to fall, there was no further increase in the viable colony count until the temperature was reduced to 31.7°C at 270 min. In the final hour required for the cultures to reach pH 5.2 there was a small but regular increase in colony count (Fig.1).

In activity tests carried out over the normal Cheddar cheesemaking temperature profile, the population of AM_2 was never greater than one-tenth that of ML_8 at the time equivalent to salting (pH 5.2). Furthermore, microscopic examination

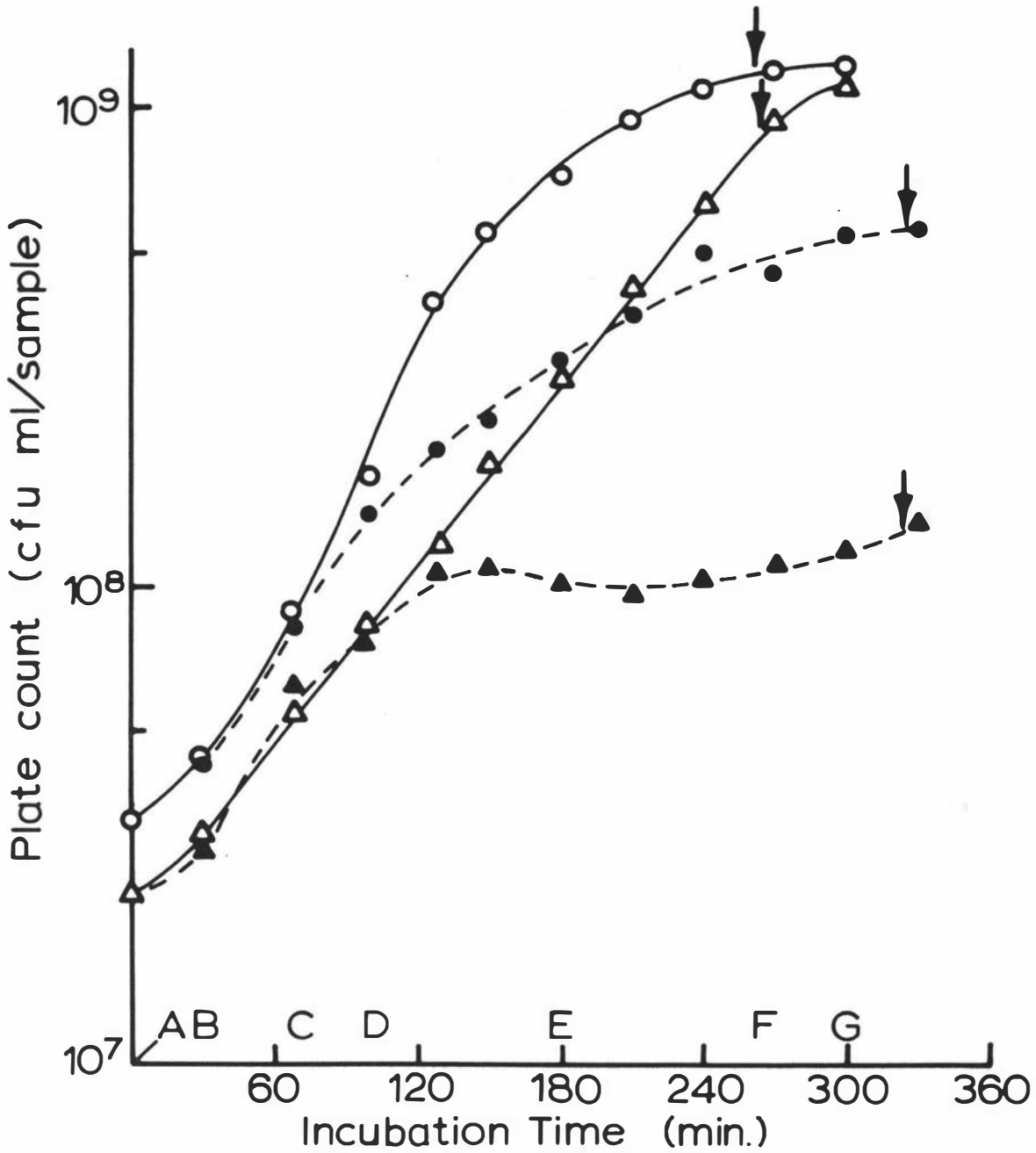

Fig. 1. Growth of *Str. cremoris* AM_2 and *Str. lactis* ML_8 during laboratory activity tests at different cooking temperatures.

In standard activity tests at the normal cooking temperature, incubation was initially at 31.7°C (A). Rennet was added at 20 min (B) and at 70 min (C) the temperature was raised gradually over 30 min to 37.8°C (D). After 80 min at the cooking temperature of 37.8°C (E) cultures were cooled slightly to 36.7°C, then further cooled (F) over 30 min until the original setting temperature of 31.7°C (G) was reached.

The times at which cultures reached pH 5.2 are indicated by the arrows.

The high temperature cooking experiments differed from the standard activity test in that cultures were at 39.4°C from D to E, instead of 37.8°C. In low cooking temperature experiments, the temperature from D to F was 33.3°C.

▲ , AM_2 at cooking temperature of 37.8°C; △ , AM_2 at cooking temperature of 33.3°C; ○ , ML_8 at cooking temperature of 37.8°C; ● , ML_8 at cooking temperature of 39.4°C.

showed that AM_2 cocci, in contrast to ML_8, were abnormal and distorted in appearance towards the end of the activity test. The cooking temperature of normal cheesemaking (37.8°C), used in these activity tests, was some 3°C above the laboratory-determined optimum for growth of AM_2. It was not unexpected, therefore, that AM_2 under these conditions did not multiply as well as ML_8 which has a higher temperature optimum.

The results obtained in activity tests when AM_2 was incubated at a low cooking temperature and ML_8 at a high cooking temperature are also shown in Fig.1. AM_2 behaved as a fast starter at 33.3°C, the lowest cooking temperature at which Cheddar cheese can be readily manufactured. Under these conditions AM_2 grew to the same high populations found with ML_8 in the standard activity test, and also reached pH 5.2 in less than 5 h. Conversely, the multiplication of ML_8 was retarded when the cooking temperature was increased to 39.4°C. This inhibition of ML_8 was less severe than observed with AM_2 at normal cooking temperatures (Fig.1). The response of ML_8 in activity tests to cooking temperatures even higher than 39.4°C was not investigated since the adverse effects of excessively high cooking temperatures on the physical properties of the curd could have resulted in somewhat abnormal cheese when the experiment was extended to actual cheesemaking trials.

Cheesemaking trials

Cheesemaking trials were undertaken to determine whether the changes induced in the growth characteristics of AM_2 and ML_8 by the use of different cooking temperatures would influence the development and intensity of bitter flavour. AM_2 was used as starter in cheeses cooked at 33.3°C and 37.8°C, and ML_8 in cheeses cooked at 33.3°C, 37.8°C and 39.4°C. The manufacturing details and the starter colony counts in samples taken at setting, drying, milling, before salting and after overnight pressing are shown in Table I.

TABLE 1. CHEESE MANUFACTURE[a] AT LOW, NORMAL AND HIGH COOKING TEMPERATURES

Starter strain	"Slow, non–bitter" strains						"Fast bitter" strains		
	AM_2		SK_{11}		ML_8			HP	
Cooking temperature (°C)	33.3	**37.8**	33.3	**37.8**	33.3	**37.8**	39.4	**37.8**	39.4
Inoculum (%)	2.2	**2.0**	2.0	**2.3**	1.8	**1.8**	2.2	**1.8**	1.8
Time (h:min)									
set to dry	2:45	**2:55**	2:35	**2:50**	2:35	**2:45**	2:55	**2:50**	3:10
dry to salt	2:00	**2:45**	2:55	**3:40**	2:25	**2:25**	3:10	**2:00**	2:55
Total make time (h:min)	4:45	**5:40**	5:30	**6:30**	5:00	**5:10**	6:05	**4:50**	6:05
Titratable acidity (%)									
dry	0.16	**0.16**	0.15	**0.16**	0.15	**0.15**	0.15	**0.17**	0.17
mill	0.56	**0.54**	0.52	**0.51**	0.52	**0.55**	0.51	**0.54**	0.55
salt	0.72	**0.72**	0.66	**0.67**	0.71	**0.71**	0.62	**0.71**	0.67
Starter colony counts[b] $\times 10^{-6}$									
initial	170	**150**	200	**220**	170	**160**	190	**87**	91
dry	800	**530**	2,400	**480**	2,400	**3,400**	2,100	**750**	540
mill	2,500	**350**	3,600	**360**	5,800	**4,700**	3,200	**1,100**	360
before salting	5,300	**280**	4,400	**270**	5,200	**4,400**	2,700	**980**	230
after overnight pressing	260	**7.2**	280	**5.9**	4,300	**3,700**	840	**370**	78

[a] Cheeses were manufactured by a small-scale simulation of normal commercial methods (Appendix I). The rennet level was 22 ml/100 l milk and the setting temperature was 31.7°C.

[b] Samples were blended and plated for colony counts which are expressed as colonies/g finished cheese.

The effects of the selected cooking temperatures on the growth of AM_2 and ML_8 in cheesemaking were similar to those observed in activity tests. At the normal cooking temperature of 37.8°C AM_2 apparently underwent only 2 population doublings, and the viable count decreased slowly from drying until salting. ML_8 doubled some 5 times giving a cell population before salting over 20 times greater than that of AM_2. It is possible that AM_2 reached its highest population before drying, but this was not determined because of difficulties in obtaining representative samples from the cheese vat between cutting and drying.

At the low cooking temperature of 33.3°C, AM_2 behaved as a typical fast starter in cheesemaking. In contrast to manufacture at normal temperatures, AM_2 continued to divide throughout the whole cheesemaking process undergoing 5 population doublings to give the high colony count in the curd before salting that is characteristic of bitter starter strains. In addition, the "set to salt" manufacturing time was reduced by almost 1 h. The growth of a second slow non-bitter starter, Str.cremoris SK_{11}, was also investigated in cheesemaking trials with normal and low cooking temperatures (Table I). Both the response to the lowered cooking temperature, and the colony counts recorded were almost identical to those obtained with AM_2. The population of ML_8 (Table I) was also increased in cheese cooked at 33.3°C. However, in this case the differences in colony count was relatively small, the manufacturing time only 10 min faster, and the growth pattern the same as at the normal temperature of 37.8°C.

Cheese manufacture with cooking and cheddaring at the higher than normal temperature of 39.4°C caused ML_8 to behave as a slow starter in terms of cheesemaking time. Although the colony counts were reduced at all but the first sampling time, the counts were still greater than those observed in normal AM_2 cheesemaking, even with a higher temperature during cheddaring (Table I). A growth pattern more like that of AM_2 in normal manufacture was observed when cheeses made with

the bitter Str.cremoris strain, HP, was cooked but not cheddared at 39.4°C. The effect on acid production was unnecessarily severe if HP, like ML_8, was cooked and cheddared at this high temperature. From Table I it can be seen that when the cooking temperature was raised from 37.8°C to 39.4°C the HP colony counts at drying, milling and salting were altered to the pattern observed with AM_2 and SK_{11} under normal cheesemaking conditions.

Flavour evaluation

Bitterness scores of cheeses, 3 and 6 months after manufacture, are shown in Table II. Cheeses made with ML_8 and HP under standard manufacturing conditions were consistently bitter, and those made with AM_2 and SK_{11} consistently non-bitter, in all cheesemaking trials where the curd was cooked at the normal temperature of 37.8°C.

Without exception, AM_2 and SK_{11} cheeses manufactured at a low cooking temperature were, in contrast to those made at normal cooking temperatures, very bitter when tasted at 3 months. The taste panel members unanimously assigned high bitterness scores to the 3 month cheeses, but at 6 months the range of scores from individual tasters was wider. The average scores (Table II) show, however, that the intensity of bitterness in AM_2 and SK_{11} cheeses manufactured at a low cooking temperature was significantly lower when cheeses had matured for 6 months. ML_8, a bitter starter at 37.8°C, gave increased bitterness in cheeses manufactured at the lower cooking temperature. The intensified bitterness was evident at both 3 and 6 months (Table II).

The typically bitter starters, ML_8 and HP, showed a reduced tendency to produce bitterness if cheeses were manufactured at a high cooking temperature. Bitterness was almost absent from HP cheeses and significantly reduced in ML_8 cheeses cooked and cheddared at 39.4°C both 3 and 6 months after manufacture.

TABLE II. BITTERNESS[a] OF CHEESES GRADED 3 AND 6 MONTHS AFTER MANUFACTURE

Starter strain	No. of trials	Cook Temp. (°C)	Bitterness at 3 months			Bitterness at 6 months		
			mean[b]	min.	max.	mean[b]	min.	max.
AM_2	3	33.3	3.5	3.1	3.8	2.7	2.0	3.7
	6	37.8	1.1	1.0	1.1	1.1	1.0	1.1
SK_{11}	2	33.3	3.6	3.5	3.7	2.8	2.5	3.2
	2	37.8	1.1	1.0	1.1	1.1	1.0	1.1
ML_8	2	33.3	3.0	2.5	3.5	3.8	3.8	3.8
	2	37.8	2.7	2.4	2.9	3.2	3.0	3.3
	2	39.4	1.7	1.1	2.3	2.3	2.2	2.3
HP	3	37.8	3.2	3.0	3.3	2.9	2.6	3.2
	3	39.4	1.9	1.4	2.5	1.5	1.0	2.4
HP	2	37.8	3.2	3.1	3.3	3.4	3.0	3.8
	2	37.8[c]	2.5	2.3	2.7	2.5	2.5	2.5
	2	37.8[c]	1.9	1.8	2.0	1.8	1.5	2.1
	2	37.8[c]	1.4	1.3	1.5	1.4	1.3	1.4

a Cheeses (randomized coded samples) were graded for bitterness by a panel of 8 – 10 experienced tasters on the following scale: 1, bitterness absent; 2, bitterness possibly present; 3, definite bitterness; 4, intense bitterness; 5, extreme bitterness. Individual scores were averaged to give a mean panel bitterness score for each cheese.

b Most cheesemaking trials were carried out twice. The maximum, minimum and mean panel scores of the cheeses are shown.

c Increasing amounts of bacteriophage hp added to each vat (see Table III).

In all experiments, cheeses were bitter when the cooking temperature permitted either bitter or non-bitter strains to grow to a high population in the curd. Conversely, when starter growth had been limited, bitterness was absent or of reduced intensity in the cheeses. It seemed likely, therefore, that reduction of the population of a normally bitter starter by bacteriophage action might also prevent or reduce the development of bitterness.

Bacteriophage infection during cheesemaking

A series of cheesemaking trials was carried out in which the bitter starter Str.cremoris HP was infected with small but increasing numbers of its homologous, virulent bacteriophage immediately after the starter was added to the cheese vat. Infection with low levels of bacteriophage had little effect on acid production, and presumably on the starter population, until late in manufacture. From Table III it can be seen that the required titratable acidities for running, drying and milling were reached in similar times in the phage-infected and the control vats. The time required to reach the salting acidity increased progressively with the level of initial bacteriophage infection indicating that significant lysis of the starter was occurring after milling. It was not possible to obtain meaningful starter colony counts because high bacteriophage levels near the end of manufacture interfered with the plate count assays. An attempt to overcome this difficulty, by plating for HP colonies on agar containing an antiserum to the bacteriophage, was unsuccessful because the neutralizing activities of the available antisera were too low.

The flavour evaluation results (Table II) show that even very low infection with bacteriophage was sufficient to reduce the bitterness of HP cheeses made under otherwise normal conditions. Increasing levels of bacteriophage infection brought about a progressive decrease in the intensity of bitterness. The HP cheese in which acid production had been

TABLE III.

CHEESE MANUFACTURE[a] WITH *STR. CREMORIS* HP IN THE PRESENCE OF ADDED BACTERIOPHAGE

Bacteriophage (PFU/ml milk) added before rennet	0	50	500	2000
Time (h:min)				
set to dry	2:45	2:50	2:50	2:50
dry to mill	1:30	1:30	1:35	1:45
mill to salt	0:35	0:35	0:45	1:05
Total time (h:min)				
set to salt	4:50	4:55	5:10	5:40
Titratable acidity (%)				
run	0.15	0.15	0.15	0.15
dry	0.17	0.17	0.17	0.17
mill	0.54	0.53	0.53	0.52
salt	0.72	0.72	0.72	0.72

[a] Details of cheese manufacture are given in Appendix I.
The starter inoculum was 1.5%, the rennet level 22 ml/100 l milk, and the cooking temperature, 37.8°C.

most affected by bacteriophage, although non-bitter, was slightly abnormal in body and pH.

Additional experiments

The findings shown in Tables I and II have been confirmed in studies with 10 starter strains in a total of 60 cheesemaking experiments. Regardless of the previous classification of a strain as bitter or non-bitter (Lawrence & Pearce, 1972) there was an unmistakeable trend observed in every experiment. If the starter populations were increased, bitterness was produced or intensified. If the starter populations were decreased, by bacteriophage action or as a result of manufacturing conditions, bitterness in the cheese was reduced or eliminated. The results are summarized in Table IV and detailed data is given in Appendix I.

DISCUSSION

Starter populations and bitterness development in Cheddar cheese

There is a striking difference between bitter and non-bitter strains of lactic streptococci in their growth characteristics both in laboratory activity tests and in actual cheesemaking trials. Bitter strains grew rapidly under normal cheesemaking conditions and reached high populations in the cheese curd prior to salting. The multiplication of non-bitter strains, on the other hand, was inhibited at the normal cooking temperature in cheesemaking. The highest viable colony count was recorded at drying and the count progressively decreased in the latter half of the manufacturing process. Hence, the populations of non-bitter starters were substantially lower than those of bitter strains before the curd was salted.

When the cooking temperature was reduced to 33.3°C, non-bitter strains were able to continue multiplication throughout the making process and reached the higher populations characteristic of bitter starters in normal cheesemaking.

TABLE IV. SUMMARY OF EFFECTS OF MANUFACTURING CONDITIONS DURING CHEESEMAKING ON BITTERNESS PRODUCTION IN CHEESES MADE WITH 10 DIFFERENT STARTER STRAINS.[a]

Starter			Cheese manufacturing conditions			Mean bitterness scores	
Strain	classification	special characteristics	object	technique used	no. of trials	3 months	6 months
AM_2	non-bitter	consistently produces good-flavoured cheese	control	normal manufacture	7	1.1	1.0
			increase starter no.	low cooking temp.	3	3.5	2.8
E_8	variable	attacked by rapidly multiplying phages	control	normal manufacture	2	2.3	1.8
			increase starter no.	low cooking temp.	2	3.3	2.8
HP	bitter	typically bitter strain	control	normal manufacture	5	3.2	3.0
			decrease starter no.	high cooking temp.	3	1.9	1.5
			decrease starter no.	bacteriophage	6	1.9	1.9
			increase starter no.	reduced cooking temp.	1	3.3	3.6
KH	bitter	similar to HP	control	normal manufacture (phage affected ?)	1	1.9	2.1
132	bitter	phage-resistant derivative of KH	control	normal manufacture	3	2.8	2.9
			increase starter no.	low cooking temp.	1	4.1	3.8
			decrease starter no.	high cooking temp.	2	1.5	1.5
ML_1	non-bitter variable	atypical in several properties	control	normal manufacture	2	1.9	1.9
			increase starter no.	low cooking temp.	2	4.0	3.6
ML_8	bitter fruity	strain of *Str. lactis*	control	normal manufacture	2	2.7	3.2
			increase starter no.	low cooking temp.	2	3.0	3.8
			decrease starter no.	high cooking temp.	2	1.7	2.3
SK_{11}	non-bitter	derivative of AM_1 used as AM_1 substitute	control	normal manufacture	2	1.1	1.1
			increase starter no.	low cooking temp.	2	3.6	2.8
Z_8	bitter		control	normal manufacture	1	2.9	3.6
			increase starter no.	lower cooking temps.	2	3.6	3.9
			reduce starter no.	high cooking temp.	1	2.0	2.9
			bacteriophage affected during make		4	1.4	1.1
166	bitter	new starter: no phage detectable	control	normal manufacture	1	3.1	3.3
			reduce starter no.	high cooking temp.	1	2.8	2.9

[a] Details of manufacturing procedures and analyses are given in Appendix I.

The cheeses completely normal in analysis 14 days after manufacture, were very bitter when tasted at 3 months by the flavour evaluation panel. The bitterness scores were, however, significantly lower when cheeses had matured for 6 months.

A high cooking temperature during cheese manufacture with conventionally bitter starters simulated the temperature inhibition imposed on non-bitter strains during normal Cheddar manufacture. The recoverable colony count was reduced at all sampling times. The growth pattern of the bitter Str.cremoris strain, HP, in cheeses cooked at 39.4°C was very similar to that of the non-bitter strain AM_2 in normal cheesemaking. The viable colony counts of ML_8 in cheese cooked and cheddared at 39.4°C were reduced to only two-thirds of the values found under normal manufacturing conditions. It was not unexpected that this cooking temperature inhibited ML_8 less severely than HP, since the optimal growth temperatures of Str.lactis strains are higher than those of most Str.cremoris strains. Nevertheless, cheeses made with HP or ML_8, in which the starter population had been reduced were consistently less bitter than control cheeses made on the same day.

Bitterness development in cheese was always prevented or reduced when the population of a bitter starter was limited either by a high cooking temperature or by bacteriophage action during manufacture. When the cooking temperatures were varied to alter the size of the starter population the cheesemaking times also varied. The results of Lawrence & Gilles (1969), however, have clearly demonstrated that the development of bitterness in cheese is not pre-determined by the rate of acid production. Furthermore, there were significant reductions in bitterness intensity in cheese made with HP in the presence of added bacteriophage even though the rates of acid production were nearly identical to rates in control vats which gave bitter cheeses.

These experiments indicate the need for an extensive revision of the hypothesis proposed by Czulak (1959) to account for bitterness in cheese, and a re-evaluation of the data interpreted to support it. Czulak (1959) assigned to non-bitter starters the positive role of degrading bitter peptides produced by rennet action on casein. In our experiments, cheeses were bitter if the population of a non-bitter starter was increased to levels found with bitter strains. The Czulak hypothesis would predict that such cheeses should have remained non-bitter. Conversely, if bitter starters have the negative and passive role implied by this hypothesis, cheeses should have still been bitter when the population of a bitter starter was reduced. Again, the experiments reported here show the opposite result.

If the cheesemaking conditions permitted starter strains to grow to high populations in the cheese curd the cheeses were invariably bitter, regardless of the bitter or non-bitter characteristics of the strain in normal Cheddar manufacture. All strains must therefore be potentially capable of contributing directly to the formation of bitter-flavoured components in cheese. The standard cooking temperature in Cheddar manufacture (37.8°C), is near the optimum growth temperature of conventionally bitter starters, but substantially above that of non-bitter strains. Indeed, many of the fast (and bitter) strains in commercial use in New Zealand today were selected originally for their ability to rapidly reduce methylene blue at 37°C. It is possible that the limitation of growth of non-bitter starters in normal Cheddar manufacture may be related to the loss of a single temperature-sensitive enzyme activity. Alternatively the response of these strains to other changing factors during cheesemaking, for example pH, may be altered under conditions of temperature stress, thus influencing subsequent activity during cheese maturation.

A new hypothesis to account for the development of bitterness in Cheddar cheese

A simple hypothesis which can accomodate all of the available evidence on bitterness production in Cheddar cheese is shown schematically in Fig.2. It is based upon the findings of Lawrence et al. (1972), Martley & Lawrence (1972) and the results reported in this section, all of which show that the starter streptococci are predominantly responsible for the development of bitterness in cheese. The proposed mechanism consists of 2 major steps followed by a third step which may be of lesser importance, as follows:

(i) degradation of casein by rennet produces a pool of high molecular weight (MW) peptides which are mostly non-bitter.

(ii) some of these peptides are hydrolysed by the proteinases of the starter streptococci to low MW bitter peptides.

(iii) these may in turn be further degraded to non-bitter peptides and amino acids by peptidases from the starter streptococci.

The essential feature of this hypothesis is that all starter streptococci are potentially either bitter or non-bitter. The presence or absence of bitterness in cheese depends upon the influence of manufacturing conditions (including the amount of bacteriophage) on particular starter strains. Evidence for the sequence shown in Fig.2 is given below.

Action of rennet (1). The simplest interpretation of the data of Lawrence et al. (1972) suggests that the major role of rennet in flavour development is to provide high MW, predominantly non-bitter peptides which are the precursors of the bitter peptides. The formation of bitter peptides by calf vell rennet (1a, 2a in Fig.2) is certainly possible. However, in view of the low concentration of rennet normally used for cheesemaking, and the relatively high specificity of the enzyme, its importance in bitterness development in Cheddar cheese is considered to have been over-emphasized.

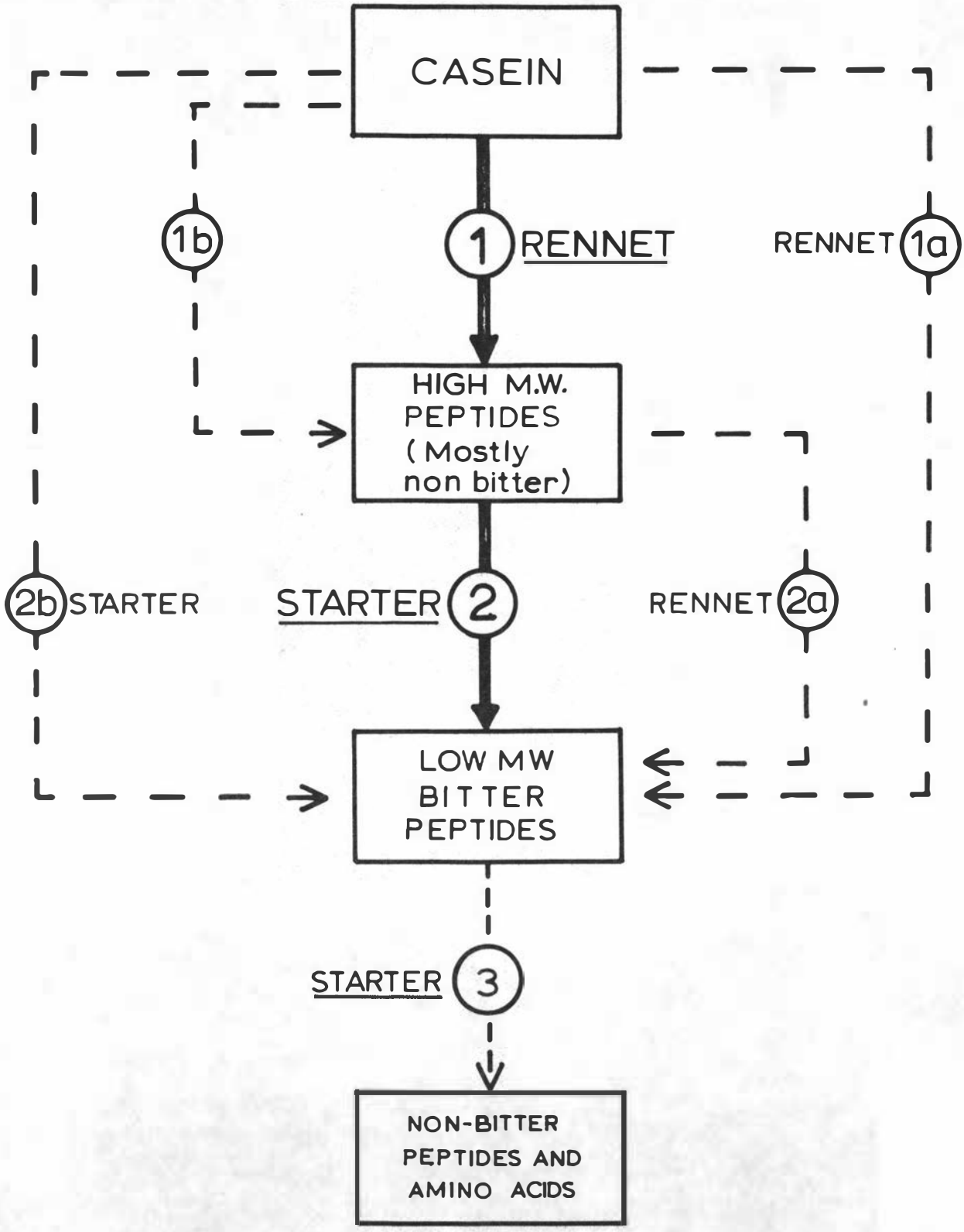

Fig. 2. A model for bitterness development in Cheddar cheese. The bold arrows show the progressive sequence that is considered to be most important. The broken arrows show steps which are possible but unlikely to be significant in contributing to bitterness intensity. The intensity of bitterness in cheese made under normal conditions is dependent primarily on step 2, but may depend to a greater or lesser extent upon the balance between steps 2 and 3 in cheese made under less normal conditions.

Formation of bitter peptides (2). The results of this investigation have shown that in cheeses made using the same rennet level, the development of bitterness was related to the starter population reached during manufacture. Regardless of the bitter or non-bitter classification of the starter strains, all of the strains tested had the potential to produce bitterness when the manufacturing conditions permitted the starter to reach sufficiently high numbers in the cheese curd. When the population of a normally non-bitter strain was increased, the cheese was bitter. Conversely, if the growth of a normally bitter strain was restricted, bitterness was absent from the cheese or of reduced intensity. Therefore, in Cheddar cheesemaking the starter strains contribute directly to the formation of bitterness. The starter strains obviously need not differ in specificity since non-bitter strains could be readily induced to give bitter cheeses.

A rapid rate of acid production in cheesemaking was associated with high starter populations in the curd before salting. Stadhouders (1962) has suggested that high rates of acid production during Gouda cheese manufacture cause a greater retention of rennet in the curd and hence increased bitterness. However, for Cheddar cheese this possibility is eliminated by the results of the fast and slow cheesemaking experiments of Lawrence & Gilles (1969), the reduction of bitterness by bacteriophage action in otherwise normal cheesemaking (Tables II and III), and the analytical data of Lawrence et al. (1972).

It seems likely therefore, that bitterness results from the conversion by the starter proteinases of some of the high MW, predominantly non-bitter peptides to bitter peptides. These appear to be of low MW (Hodges, Kent & Richardson, 1972; Matoba et al. 1969). In addition to exhibiting lower proteolytic activity than bitter starters (Martley & Lawrence, 1972), non-bitter strains do not reach the high populations in the curd before salting that are characteristic of bitter lactic streptococci. These observations suggest that

degradation of high MW peptides in cheese made with non-bitter strains under normal manufacturing conditions is limited. Low MW bitter peptides may not then be produced in sufficient amounts to cause detectable bitterness.

Gordon & Speck (1965) and Harwalkar & Seitz (1971) have isolated bitter peptide fractions from cultures of lactic streptococci grown in the absence of rennet in reconstituted skim milk. Formation of bitter flavoured components from casein by lactic streptococci is therefore possible (steps 1b, 2b in Fig.2). However, changes in either the concentration or type of rennet used in cheesemaking greatly affected bitterness development, particularly by bitter starters (Lawrence et al. 1972). Such evidence, although somewhat circumstantial, does suggest that pathways which do not involve rennet in any way are likely to be of lesser importance.

Degradation of bitter peptides (3). A striking feature of the consistently non-bitter starters is their poor survival in cheeses made under normal conditions, in contrast to the prolonged survival of bitter strains. Martley & Lawrence (1972) concluded that the poor survival resulted from the high sensitivity of non-bitter strains to salt concentrations of 4-5% at pH values between 5.0 and 5.2. Sensitivity to salt and pH may be accentuated when the cook temperature during cheesemaking is above the optimum growth temperature of the strain and reduced if the growth temperature is lowered. Because a similar response may be induced if cheese made with bitter strains is manufactured at a high cooking temperature, or when high bacteriophage levels develop, it is not necessary to postulate that any specificity be associated with individual strains. The question is only important if it is assumed that poor survival of starters in Cheddar cheese is accompanied by cell lysis when there might be a release of cell-bound peptidases which could further degrade any bitter peptides previously produced. Some evidence of lysis and release of peptidases has been reported by Law, Sharpe & Reiter (1974).

Relative importance of the formation and degradation of bitter peptides. Degradation of bitter peptides almost certainly occurs to some extent in all cheeses but may assume major importance only in the phenomenon described by Czulak (1959) as transient bitterness. This has also been noted in trials at this Institute, but only with cheeses made with non-bitter strains at low cooking temperatures or with unusually high concentrations of calf rennet.

In this investigation (Table II) it was found that cheeses in which the populations of non-bitter strains had been increased by manufacturing at a relatively low cook temperature (33.3°C) were very bitter at 3 months, but there was a significant reduction in bitterness after the cheeses had matured for 6 months. The early bitterness was considered to result from an excessive production of bitter peptides, due to the increased starter numbers. The proportion of non-survivors was also relatively low in the early stages of ripening, suggesting that bitter peptides may be formed at a rate considerably faster than their degradation. The subsequent disappearance of bitterness may be associated with the increase in the numbers of non-survivors found on prolonged ripening, since it is possible that peptidase activity is related to the absolute number of non-survivors. On the other hand at normal cook temperatures the proportion of survivors to non-survivors always remains relatively high in cheeses made with bitter strains. Such cheeses were almost always more bitter 6 months after manufacture than at 3 months.

Low levels of bitterness were also found at 3 months in cheeses made with non-bitter starters and excessively high rennet concentrations. In these circumstances the rennet may have contributed directly to the formation of bitter peptides. Alternatively the increased pool of bitter peptide precursors resulting from the high rennet levels used, may have allowed the production of bitter peptides by the starter to initially exceed their subsequent degradation.

Czulak's hypothesis. The model proposed in Fig.2 differs substantially from the hypothesis proposed by Czulak (1959) in which the starter used in Cheddar cheesemaking plays no role in the formation of bitter peptides. Bitter peptide formation was attributed solely to the action of rennet. Non-bitter starters were considered by Czulak to possess specific enzymes which allowed them to degrade bitter peptides formed by rennet. While Czulak's hypothesis has been supported by several other investigators (Stadhouders, 1962; Emmons et al. 1962; Jago, 1962), differences between starter strains either in the possession of, or in the specificity of, critical enzymes have yet to be shown unequivocally.

A numbers hypothesis. The classification of starter strains as bitter or non-bitter appears somewhat arbitrary. The incidence of bitterness in Cheddar cheese depends not on a single characteristic of the starter strain, but upon the response to the conditions of manufacture. The major factor that determines whether a starter strain produces bitter or non-bitter cheese is its ability to multiply under particular cheesemaking conditions and the population reached. The size of the starter population may be controlled by manufacturing procedures such as the choice of cooking temperature. Sullivan et al. (1973) have pointed out that the control of bitterness is essentially the control of total proteinase activity, and that as well as limiting the amount of starter proteinase by restricting starter populations, the activity may also be controlled by attention to pH and salt concentration. Such a view accepts that the starter streptococci have a positive role in bitterness formation and are not merely acting to remove it. Bacteriophage provide an additional control on starter populations. It is possible in practice that the potentially high levels of starter are seldom reached because of bacteriophage multiplication during cheesemaking.

SECTION II

THE INFLUENCE OF BACTERIOPHAGE AND COOKING TEMPERATURE ON FLAVOUR DEVELOPMENT IN CHEESE MANUFACTURED UNDER CONTROLLED BACTERIOLOGICAL CONDITIONS

The findings have been prepared for publication:
Lowrie, R.J., Lawrence, R.C. & Peberdy, M.F. (1974).

submitted to the Journal of Dairy Research.

INTRODUCTION

The first investigators to utilize aseptic cheesemaking techniques were uncertain as to the role played by the starter streptococci in the production of cheese flavour (Perry & McGillivray, 1964). Other investigators, also studying aseptically manufactured cheese, were more specific. Starter was considered to be the main agent responsible for the development of basic Cheddar flavour (Reiter et al. 1966; Lawrence, 1966) and the starter organisms themselves were thought to make a direct contribution (Reiter et al. 1967). Because flavour was mild and developed slowly, supplementary, non-essential roles in enhancing flavour were, however, ascribed to non-starter bacteria, probably lactobacilli. The other flora and milk enzymes were believed to play only a minor part, possibly accelerating maturation (Reiter et al. 1966; Reiter et al. 1967). More recently, greater significance has been attributed to extraneous variables such as the composition, enzymes, and initial flora of the milk and adventitious contaminating bacteria (Reiter & Sharpe, 1971; Sharpe, 1972; Reiter, 1973) even though few details of additional experiments have been reported.

Extensive cheesemaking trials carried out at this Institute using conventional methods of manufacture showed that the strain of starter used had a greater effect on cheese flavour than any of the other factors examined (Lawrence et al. 1972). Only a limited number of related strains consistently gave cheese of good flavour (Martley & Lawrence, 1972). Most starters produced serious flavour defects especially bitterness and fruitiness (Lawrence & Pearce, 1968) but the potential for expression of these defects in the cheese depended upon the manufacturing conditions. Measures which prevented the starter from reaching high populations in the cheese curd, such as raised cooking temperature or the development of high levels of bacteriophage during manufacture, had a significant effect on cheese flavour by reducing or eliminating bitterness production (Table IV).

The findings obtained when the key cheesemaking trials were repeated under bacteriologically controlled conditions are reported in this section. The results support the view that the starter streptococci are the only bacteria required for the complete development of full Cheddar flavour.

MATERIALS AND METHODS

Starters and bacteriophages

Str.cremoris strain AM_2, Str.lactis strain ML_8, and virulent bacteriophages of AM_2 (am_2, NZDRI 799) and ML_8 (ml_8, NZDRI 646) were from the Institute culture collection. Starter cultures were maintained as described on page 13. Bacteriophage stocks were prepared as previously described (page 13) and filtered through Ford Sterimats in Beaumaris Standard Hemmings Filters.

Milk supply

Cheeses were manufactured during January and February 1973 from mid-season bulk herd commercial whole milk. The average total plate count of raw milk samples was 1.58×10^5 (range, 2.2×10^4 to 5.2×10^5) colony forming units (CFU)/ml on Standard Methods Agar (BBL). After pasteurization ($69^{\circ}C$, 5 min) average count was 4.84×10^4 (range, 6.0×10^3 to 9.7×10^4) CFU/ml.

Cheese manufacture

The apparatus and techniques for Cheddar cheese manufacture under controlled bacteriological conditions were essentially as described by Perry & McGillivray (1964). Several minor modifications, including the starter inoculation method of Chapman, Mabbitt & Sharpe (1966), were incorporated into the procedure to improve aseptic technique and facilitate some mechanical manipulations during manufacture. The amount of membrane filter-sterilized (0.45 µm pore size, Millipore Corp.) calf vell rennet (9 ml/50 l milk) and the setting temperature ($32^{\circ}C$) were the same in all experiments. As

far as possible the titratable acidities at running (0.13-0.14%), drying (0.15-0.17%), milling (0.50-0.56%) and salting (0.62-0.70%) and the time from setting to drying (2 h 40 min-2 h 50 min) were kept constant. Since cheeses were made with 2 starter strains at 2 cooking temperatures (33°C and 38°C) and in the presence and absence of bacteriophage, some variations in the starter inoculum (1.75-2.25%) and the extent of dry-stirring of the curd were necessary to keep moisture levels within normal limits and to give cheeses of comparable composition.

After overnight pressing cheeses were removed from the hoops and their surfaces painted lightly with 0.2% (v/v) pimaricin solution, before film wrapping and sealing. All possible aseptic precautions were taken during these manipulations. Cheeses were matured at 13°C. At intervals, core samples for microbiological and compositional analyses were withdrawn aseptically from one half of each cheese. The remaining half was kept intact for flavour evaluation.

Bacteriological analyses

Milk samples were taken aseptically from the vat before and after pasteurization and between the addition of starter and rennet. Curd samples were obtained immediately before salting. Cheeses were sampled at 1, 3, 7, 10 and 14 days then at 4, 6, 12 and 26 weeks from date of manufacture. In some experiments the earlier samples were taken at 1, 4, 9 and 14 days. Cheeses manufactured aseptically in the presence of bacteriophage were sampled the day after manufacture, then at monthly intervals. Samples were processed and plated for starter colony counts and checked for bacteriophage, as described in Appendix I. Samples were also plated on Standard Methods Agar for total plate count and on LBS agar (BBL). Lactobacilli or pediococci were never detected in the pasteurized milk samples. Their presence on the LBS agar plates after 5 days incubation at 30°C was used, therefore, as a test for any contamination by adventitious organisms of the cheeses during manufacture or maturing.

Compositional analyses

Compositional analyses of cheeses were carried out 14 days after manufacture. Average values were, moisture, 36.5%; fat, 35.0%; salt, 1.4%; salt-in-moisture, 3.8%; moisture in solids-not-fat, 56.2%; pH 4.95. The cheeses possessed relatively good body and texture and their moisture levels were similar to those reported by Perry & McGillivray (1964).

Flavour evaluation

Aseptically manufactured cheeses were assessed for bitterness and Cheddar flavour development at 3 and 6 months by a panel of 6 experienced judges. Randomized coded samples were submitted to the panel under controlled conditions together with a labelled standard sample of conventionally manufactured cheese previously established as having good flavour. Bitterness scores were assigned on the same scale used in the grading of conventionally manufactured cheese: 1, absent; 2, possibly present; 3, definitely present; 4, intense; 5, predominant. Cheddar flavour was scored on an increasing intensity scale from 1-5. In practice, cheeses deficient in Cheddar flavour scored 2 or less. Cheeses with well-developed Cheddar flavour were graded at 3 or 4. Individual scores were averaged to give mean bitterness and Cheddar flavour panel scores for each cheese. Panel reliability was checked periodically by random duplication of samples.

RESULTS

Effectiveness of aseptic cheesemaking techniques

All 12 aseptically manufactured cheeses considered in this study had plate counts on LBS agar of less than 10 CFU/g in samples taken throughout maturation, indicating that likely adventitious contaminants had been excluded. Unless bacteriophage had been added deliberately, tests for bacteriophage were negative. Cheeses which did not meet these requirements were rejected. The low background level of thermoduric bacteria remained relatively constant at about 10^4/g during the period over which samples were taken.

Effect of starter strain on flavour development

Two starter strains, Str.lactis ML_8 and Str.cremoris AM_2, were selected because of known differences in their flavour characteristics. Strain ML_8 has been used frequently in flavour studies by other workers (Perry, 1961; Robertson & Perry, 1961; Perry & McGillivray, 1964). It normally produces bitter cheese (Lawrence & Gilles, 1969) and may also give other off-flavours variously described as fruity, malty or burnt (Lawrence & Pearce, 1972). Cheese manufactured under normal conditions with AM_2 consistently has full Cheddar flavour and no off-flavours (Lawrence & Pearce, 1972). From Table V it can be seen that these known flavour characteristics were exhibited in the cheese manufactured aseptically at the normal cooking temperature. ML_8 cheeses were intensely bitter and appeared lacking in Cheddar flavour. Cheeses made under identical conditions with AM_2 were not bitter and possessed well-developed Cheddar flavour. Furthermore, intensity of Cheddar flavour in these aseptically-made AM_2 cheeses was approximately equivalent to that in AM_2 cheeses produced by conventional 'non-aseptic' methods (Table V).

Effect of cooking temperature on flavour development

Differences in flavour characteristics as great as those between ML_8 and AM_2 were found when the same starter, AM_2, was used in aseptic cheesemaking at different cooking temperatures. At the low cooking temperature of 33°C, AM_2 behaved as a typical fast cheesemaking starter and produced in the cheese the flavour defects associated with fast starter strains and predictable from the results in Tables II and IV. These aseptically made low-cook AM_2 cheeses were similar to normal-cook ML_8 cheese, intensely bitter and apparently deficient in Cheddar flavour (Table V) in contrast to the full-flavoured non-bitter AM_2 cheese manufactured at the standard cooking temperature of 38°C.

TABLE V.

INFLUENCE OF BACTERIOPHAGE AND COOKING TEMPERATURE ON BITTERNESS AND CHEDDAR FLAVOUR DEVELOPMENT IN CHEESE MANUFACTURED UNDER CONTROLLED BACTERIOLOGICAL CONDITIONS

Starter strain	Cooking temperature	Special conditions	Number of trials	Average bitterness score [a]		Average Cheddar flavour score	
				3 months	6 months	3 months	6 months
ML_8	normal (38°C)	–	2	3.7	4.1	1.9	1.7
ML_8	normal	ml_8 phage added [b]	2	1.0	1.0	3.0	3.3
AM_2	normal	–	2	1.5	1.1	2.9	3.1
AM_2	normal	am_2 phage added	1	1.3	1.2	3.0	3.0
AM_2	low (33°C)	–	3	3.9	4.1	2.0	1.7
AM_2	low	am_2 phage added	2	1.1	1.0	3.3	3.1
AM_2	normal	conventional manufacture [c]	2	1.3	1.1	3.1	3.4

a Bitterness and Cheddar flavour scores determined on increasing intensity scales of 1–5.

b Bacteriophage added to milk immediately before starter addition to give 2–5 x 10^2 PFU/ml milk.

c Small scale (320 l) simulation of conventional commercial manufacture (not 'aseptic').

Effect of bacteriophage on flavour development

Using aseptic techniques, cheeses were made with ML_8 at the normal cooking temperature and with AM_2 at both normal and low cooking temperatures in the presence of low initial amounts of the respective homologous bacteriophages. Phage levels added to the vats had little effect on acid production by the starter until late in manufacture. The required titratable acidities for running, drying and milling were reached in times comparable with those in the corresponding phage-free trials (Table VI). However, acid production was retarded after milling, and salting was delayed by 20-25 min in the phage-infected vats. The high phage titres in the whey at milling (Table VI) confirmed that phage levels had closely approached the limits which permit almost normal cheesemaking.

Flavour evaluation results (Table V) substantiated earlier findings that the presence of bacteriophage during cheesemaking markedly influenced the flavour potential of certain starter strains (Tables II and IV; see also Lawrence & Gilles, 1973). The intense bitterness (score 3.7-4.1) characteristic of both normal ML_8 and low-cook AM_2 cheeses was eliminated almost entirely from cheese made in the presence of bacteriophage. Equally striking were the accompanying increases in average Cheddar flavour scores, from 1.7-2.0 to average values of 3.0-3.3 which equalled those of standard AM_2 cheeses and compared favourably with conventionally manufactured "non-aseptic" AM_2 cheeses. The presence of phage during the aseptic manufacture of AM_2 cheese at the normal cooking temperature had no obvious effect upon the expected well-developed Cheddar flavour.

Starter survival

Survival characteristics of starters during cheese ripening have been implicated both directly and indirectly in the development of Cheddar flavour as well as various off-flavours (Dawson & Feagan, 1957; Perry, 1961; Perry & McGillivray,

TABLE VI.

CHEESE MANUFACTURE UNDER BACTERIOLOGICALLY CONTROLLED CONDITIONS IN THE PRESENCE OF ADDED BACTERIOPHAGE

Strain and cooking temp.	AM_2 normal (38°C) cook		AM_2 low (33°C) cook		ML_8 normal cook	
homologous phage (PFU/ml milk) added before rennet	0	500	0	250	0	200
Time (h:min)						
set to dry	2:45	2:45	2:50	2:50	2:40	2:45
dry to mill	2:10	2:15	1:50	2:00	1:50	1:40
mill to salt	0:25	0:40	0:15	0:35	0:20	0:45
Total time (h:min)						
set to salt	5:20	5:40	4:55	5:25	4:50	5:10
Titratable acidity (%)						
run	0.14	0.15	0.14	0.14	0.13	0.14
dry	0.17	0.17	0.18	0.17	0.16	0.17
mill	0.54	0.51	0.54	0.55	0.56	0.55
salt	0.66	0.64	0.68	0.63	0.64	0.66
detectable phage (PFU/ml) in whey at milling x 10^{-6}	0	120	0	8200	0	6700

1964; Vedamuthu et al. 1966; Reiter et al. 1967; Martley & Lawrence, 1972). Survival of ML_8 and AM_2 in cheeses manufactured aseptically at normal cooking temperatures (37-38°C) followed the trends found in earlier studies (Perry, 1961; Martley & Lawrence, 1972). Strain ML_8 exhibited typical Str.lactis properties of high maximum populations combined with prolonged survival in the cheeses (Fig.3). Immediately before salting (sample S, Fig.3) the ML_8 population in the curd had reached 5×10^9 organisms/g. The day following manufacture, after salting and overnight pressing ML_8 cheeses still had a count of about 5×10^9 CFU/g, of which more than 50% could be recovered after 14 days maturation, some 20% after 6 weeks and still over 1% at 3 months. In AM_2 cheeses manufactured at the same cooking temperature of 37-38°C maximum populations were low initially, and survival of the starter was extremely poor (Fig.3). Colony counts of AM_2 fell from approximately 10^8 CFU/g in the curd before salting to between 5×10^6 and 10^7 organisms/g in the salted and pressed 1 day-old cheese, a decline of some 90-95% in viable count. The viable count of AM_2 continued to fall rapidly and after 14 days was only 0.2% of the count before salting, in marked contrast to the ML_8 cheeses. At 6 weeks and 3 months from manufacture plate counts of AM_2 were about 10^4/g, just above the almost static background of thermoduric non-starter bacteria.

The survival curves of AM_2 in cheeses made at the low cooking temperature (33°C) had features in common with both ML_8 and AM_2 survival in cheeses manufactured at the normal cooking temperature. Like ML_8 at 38°C, the maximum population reached by AM_2 at 33°C was high (>10^9/g). Before salt addition the starter population in the cheeses cooked at 33°C was 10-20 times greater than in AM_2 cheeses made at the usual (38°C) cooking temperature. However, unlike ML_8, after salting there was a rapid decline in viable count of AM_2 although this was initially not as marked as in normal AM_2 cheese. Viable count fell by only 55-65% between salting and removal from the presses. The sensitivity of AM_2 to salt

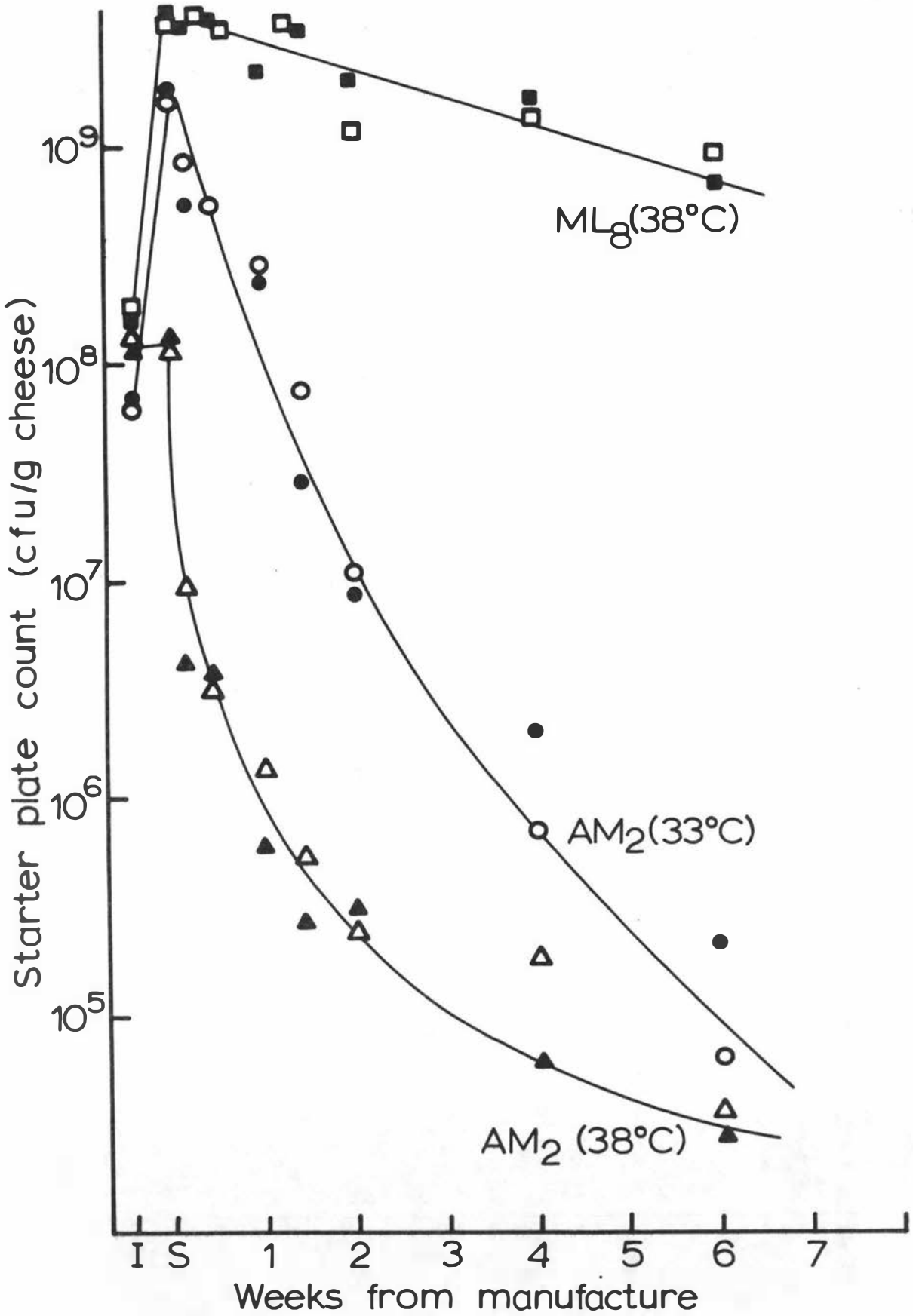

Fig. 3. Starter colony counts in maturing Cheddar cheese made using aseptic techniques.

Plate counts of samples I (milk after inoculation with starter) and S (curd immediately before addition of salt) were multiplied by the ratio of the solids content of the finished cheese to the solids content of the sample. These adjusted values are equivalent to CFU/g cheese. Open and closed symbols indicate separate experiments.

□ ,■, ML_8 at cooking temperature of 38°C; △ ,▲, AM_2 at cooking temperature of 38°C; ○ ,●, AM_2 at cooking temperature of 33°C.

and pH appeared to decrease when cooking temperature during cheesemaking was below instead of above optimum growth temperature of the strain. The combination of higher maximum population and initially improved survival gave a viable count in the low-cooked AM_2 cheeses that was 100-fold greater at one day than at the corresponding time in normal AM_2 cheeses. Thereafter, even though starter counts fell at comparable rates in both types of AM_2 cheeses, the low-cooked cheeses maintained the approximately 100 times higher starter count for 10 days. The difference in count reduced progressively until between 4 and 6 weeks from manufacture when starter counts in AM_2 cheese manufactured at either cooking temperature were at similarly low levels.

DISCUSSION

Earlier aseptic cheese studies (Perry & McGillivray, 1964; Reiter et al. 1967) were carried out with starter strains which caused marked flavour defects in the cheese and normally gave little Cheddar flavour. In this investigation cheeses made with Str.cremoris strain AM_2 consistently possessed the characteristic flavour distinctive of natural Cheddar, and no off-flavours provided that normal cooking temperatures were used. Moreover, the multiplication of bacteriophage during manufacture had a pronounced effect on the outcome of cheese which would otherwise have been of poor flavour. Bitterness could be eliminated and Cheddar flavour apparently improved. These findings establish conclusively that the restriction of starter numbers, either by the appropriate combination of starter strain and making procedures, or by the presence of bacteriophage throughout manufacture, will minimize off-flavour production and ensure full Cheddar flavour development even in aseptically produced cheese. There is no need to postulate that non-starter flora or native milk enzymes, in either the raw milk or the ripening cheese, have preliminary or auxiliary roles in the production of characteristic Cheddar flavour.

The striking, but indirect, effect of bacteriophage on flavour has some important implications. Perry & McGillivray (1964) attributed the increased flavour of cheese made in an open vat, over that of cheese made aseptically, to the presence of adventitious non-starter bacteria. They overlooked the possibility that the comparative enhancement of flavour resulted from bacteriophage infection rather than fortuitous bacterial contamination. Indeed, growth of bacteriophage during cheesemaking to levels which restrict starter numbers without markedly retarding rates of acid production may provide a general explanation for the anomalous results obtained in many of the flavour trials carried out in commercial factories.

Reiter et al. (1967) concluded that the flavour products in Cheddar cheese must have been produced by the enzymes of the dead starter organisms, a concept first put forward by Orla-Jensen (1921) and recently supported by the findings of Law et al. (1974). At first sight the poor survival of strain AM_2 and the effects of bacteriophage appear to support the hypothesis that death, autolysis or lysis of the starter cells by phage is a prerequisite for flavour development. However, the decline in starter numbers occurred at almost equally rapid rates in either the normal (38°C) cook, good-flavoured AM_2 cheeses or the low (33°C) cook, very bitter AM_2 cheeses. Since the maximum starter population reached in the low cook AM_2 cheeses was some 10-20 times higher than in the AM_2 cheeses made at 38°C the total mass of non-recoverable and presumably dead starter bacteria was always very much greater in the poor-flavoured cheese. Death or lysis of starters during cheese maturation would therefore appear unimportant in preventing bitterness or promoting cheese flavour.

Normal Cheddar flavour will develop if starter numbers are controlled so that only sufficient acid is produced to give cheese of the required moisture and pH, and if the manufacturing procedures used and percentage of salt added ensure that net survival of the starter organisms is low but nevertheless

adequate for utilization of residual lactose in the young cheese. Acidity development by the starter thus partly determines the environment in which the maturation process occurs as well as controlling the growth of non-starter bacteria and hence any off-flavours that they might produce. Should the starter itself reach too high a population or survive too long, flavour defects such as bitterness which mask or detract from cheese flavour are produced. Accumulated circumstantial evidence suggests strongly that the reduction or removal of unpleasant flavours is associated with improved perception of the Cheddar flavour. The main role of the starter organisms may therefore be limited merely to providing a suitable environment which allows the elaboration of characteristic cheese flavour. Whether the chemical compounds that contribute to this desirable flavour are also produced by the starter has yet to be determined.

SECTION III

TEMPERATE BACTERIOPHAGES AND LYSOGENY IN LACTIC STREPTOCOCCI USED AS STARTERS IN CHEESEMAKING.

Some of the results of this investigation have been published:

Lowrie, R.J. (1974).
Applied Microbiology 27, 210-217.

INTRODUCTION

Virulent bacteriophages of lactic streptococci used as starters in cheesemaking have been studied extensively since their discovery nearly 40 years ago (Whitehead & Cox, 1935). The effects of bacteriophage have always been considered undesirable because they were quickly shown to be responsible for impaired or erratic acid production by cheese starters and frequently for total starter failure. The literature on lactic streptococcal bacteriophages has been regularly reviewed (Whitehead, 1953; Collins, 1962; Reiter & Møller-Madsen, 1963; Robertson, 1966; Reiter, 1973). Most investigations have concerned virulent bacteriophages, with the emphasis upon preventing or reducing their interference in the cheesemaking process. Many difficulties have been encountered in the propagation and assay of these phages resulting in numerous attempts to develop satisfactory plating procedures and consequent variation in assay techniques (Cherry & Watson, 1949; Deane & Nelson, 1952; Potter & Nelson, 1952; Pette, 1953; Mikolajcik, 1964; Robertson, 1966; Lowrie & Pearce, 1971; Douglas, Qanber-Agha & Phillips, 1974).

Temperate bacteriophages and the possibilities of lysogenic strains of lactic streptococci have been considered but received relatively little attention. Phage-carrying cultures which were stable for many months but could be freed of phage by careful colony re-isolation had been found by Hunter (1947) and subsequently used as cheese starters (Hunter & Whitehead, 1949). These cultures, which could be confused with lysogenic cultures, probably carried a virulent phage which multiplied on sensitive segregants from a mainly resistant population. There had been no rigorous demonstration of lysogeny in group N streptococci when the present study was commenced. During the latter part of the investigation, Kozak *et al*. (1973) demonstrated inducible temperate phages in *Str.lactis* and *Str.diacetilactis*. Problems in the routine detection and enumeration of

of virulent phages, together with the complexity of their interactions with the host strains, undoubtedly hindered the characterization of temperate phages, lysogenic strains and indicator hosts.

Despite similar difficulties (Zabriskie, Read & Fischetti, 1972) lysogenic strains have been demonstrated unambiguously in streptococci of other serological groups. Lysogeny appears tc be widespread in groups A (Kjems, 1958, 1960) and C (Fox & Wittner, 1965). Some lysogenic strains have also been found in groups G (Colon, Cole & Leonard, 1971) and H (Parsons et al. 1972).

Suspected lysogenic starter streptococci were first recorded by Reiter (1949). Czulak & Naylor (1956) and Crawford & Galloway (1962) referred to cultures of starter streptococci as lysogenic but no details were reported. Preliminary results indicating possible lysogeny were described by Sandine et al. (1962) but no report confirming the observations was published. Oram & Reiter (1968) stated that they knew of no proven lysogenic host-phage system in the group N streptococci.

Induction of lysis by ultraviolet (UV) irradiation of Str. cremoris C11-56 was found by Keogh & Shimmin (1969), although they were unable to show the presence of a typical temperate phage. Lysates from strain C11-56 produced clear zones on 9 of 12 strains of Str.cremoris when spotted on appropriately seeded plates. Striking features of the lytic spectrum were that strain C11-56 was itself sensitive to the lysate and that plaque formation was not observed. Electron micrographs showed that the lysate contained phage-like particles, mostly empty heads. The antibacterial activity of lysates was attributed to the presence of an induced defective bacteriophage or a lethal component of it. McKay & Baldwin (1973) observed complete bacteriophages in electron micrographs of UV-induced lysates of Str.lactis C2, but they failed to find an indicator strain.

Kozak *et al*. (1973) examined 87 strains of lactic streptococci as potential lysogens. They found that UV treatment caused lysis and liberation of phages, which could be detected on indicator strains, from 5 of 46 *Str.lactis* and 2 of 24 *Str.diacetilactis* strains. Lysates from one of the inducible *Str.lactis* and both of the *Str.diacetilactis* strains contained phages able to multiply on several indicators belonging to all three species of lactic streptococci. The other four inducible strains had a single indicator which was a strain of *Str.lactis*. No lysogens were found among the 17 strains of *Str.cremoris* examined even though various induction procedures were attempted.

There are numerous reasons why lysogenic strains of starter streptococci might be important:

(i) such strains may be a source of phages virulent on other strains used in starter rotations.

(ii) lysogens may act as a reservoir of phages with the potential to attack newly introduced strains, to mutate to virulence, or to recombine with virulent phages affecting, for example, host range or multiplication rate.

(iii) lysogenic strains may possess additional properties of bacteriophage resistance, as well as resistance to the particular temperate phage.

(iv) some conditions encountered during cheesemaking, such as raised cooking temperatures, may induce phage development by the lysogens resulting in unpredictable acid production. Alternatively, induction might control starter populations and hence prevent the occurrence of starter-associated off-flavours in the cheese.

(v) genetic exchange and variability amongst strains may be increased through temperate phages and lysogeny.

Because of the obvious practical and theoretical significance of lysogenic starter strains, a selection of single strains

of group N streptococci used as starters for cheesemaking in New Zealand were investigated as potential lysogens. An inducible strain of Str.cremoris was found, as well as strains upon which the lysates could form plaques. Bacteriophage particles were seen in electron micrographs of the lysates and some characteristics of the temperate phage and the lysogenic host were investigated. Conditions which gave most obvious lysis and highest temperate phage titres in this strain were used to screen other starter strains for lysogenicity. Several strains were found to be inducible but only one more strain was found which gave plaque formation on an indicator strain. Lysates from another strain which was readily induced produced zones of lysis but no plaques on lawns of 7 strains. The remaining inducible strains showed neither plaques nor lytic zones when lysates were tested on lawns of 34 potential indicator strains.

MATERIALS AND METHODS

Strains of lactic streptococci

All of the cultures used in this study were cheese starter strains of Str.lactis and Str.cremoris from the collection of the New Zealand Dairy Research Institute (NZDRI). Strains were obtained as freeze-dried powders which were dissolved in autoclaved reconstituted skim milk (RSM), incubated overnight at 30°C, then streaked out on M16 agar plates. Single colonies were inoculated into autoclaved RSM to check their ability to coagulate milk and acid-producing activity. Isolates were transferred to M16 broth, sub-cultured daily for 3 days then maintained in deep-stab cultures in M16 agar at 4°C. Stab-cultures were sub-cultured every 6 weeks.

Bacteriophages

Virulent bacteriophages, the propagating host strains and the catalogue number under which the phages are deposited in the NZDRI collection are detailed in Table VII. The temperate bacteriophage inducible from Str.cremoris R_1 by UV

TABLE VII.

BACTERIOPHAGES USED IN PHAGE–SENSITIVITY TESTS

trivial name	propagating host		NZDRI Catalogue number	EOP[a]	
	strain	species		AM_1	R_1
am_1	AM_1	cremoris	601	+++[b]	r
am_2	AM_2	cremoris	602	++	r
ba_1	BA_1	lactis	604[c]	r	±
ba_2	BA_2	lactis	611[c]	r	+
br_4	BR_4	cremoris	614[c]	r	+
c_{13}	C_{13}	cremoris	615	±	+++
			616	±	+++
			617	±	+++
			733	±	+++
e_8	E_8	cremoris	618	r	++
h_1	H_1	lactis	623[c]	r	r
hp	HP	cremoris	633	r	r
kh	KH	cremoris	640	r	r
ml_3	ML_3	lactis	643[c]	r	+
ml_8	ML_8	lactis	668	r	+++
			670	r	+
			671	r	+++
			672	r	+++
			673	±	++
p_2	P_2	cremoris	651	r	+++
			694	r	+++
			695	r	+++
			696	r	+++
r_1^v	R_1	cremoris	652	±	+++
r_1	R_1	cremoris	720	±	+++
r_6	R_6	cremoris	688	±	++
sk_{11}	SK_{11}	cremoris	690[c]	+++	r
z_8	Z_8	cremoris	667	r	r

[a] $\text{EOP} = \frac{\text{titre on test strain}}{\text{titre on propagating strain}} \times 100$

[b] Symbols for EOP : +++, 100% – 1%; ++, 1% – 0.01%; +, 0.01% – 0.0001%; ±, inconsistent response; r, resistant to $> 10^8$/ml.

[c] neutralized by anti-serum to phage ml_3 (643).

or mitomycin C (MC) treatment was designated r_1t. A virulent phage (652) of Str.cremoris R_1 was referred to as r_1v.

Media and growth of cultures

Lactic streptococci were grown routinely at 22°C or 30°C without shaking in M16 broth from a 2% inoculum of an overnight (22°C, 16 h) broth culture.

M16 broth (Lowrie & Pearce, 1971) was prepared from media products of Baltimore Biological Laboratories Inc., and contained per litre:

Polypeptone	5.0 g
Phytone peptone	5.0 g
Beef extract	5.0 g
Yeast extract	2.5 g
sodium acetate trihydrate	3.0 g
ascorbic acid (sodium salt)	0.6 g

The pH was adjusted to 7.0-7.1 with 2 N NaOH and autoclaved at 15 psi for 15 min. Autoclaved lactose or dextrose solution was added to a final concentration of 0.5% before use.

For M16 agar the above ingredients (except lactose or dextrose) were dissolved in 450 ml water and added to 500 ml hot dissolved 2% Davis New Zealand agar. Sterile lactose or dextrose solution (5 g in 50 ml) was added when the autoclaved medium had cooled to about 50°C. Agar for plates was normally supplemented with calcium ions. To avoid visible precipitation of components of the medium, calcium ions were added as calcium borogluconate (Veterinary grade, May and Baker Ltd, Dagenham, England). A stock solution (0.5 M) was diluted 10-fold into the autoclaved energy source solution, which was carefully added to the warm agar. The final concentration of calcium borogluconate was 0.005 M.

M16 soft agar contained 0.45% Davis agar. Calcium borogluconate was added (1 ml/100 ml agar, from a 0.5 M stock

solution) after soft agar had been melted and cooled to 46°C.

Colony counts

Samples were first diluted with chilled 0.14 M NaCl to a final volume of 100 ml and blended at full speed (13,000 rpm) for 1 min in an AtoMix blender. This treatment reduced chains of lactic streptococci to an average colony-forming unit (CFU) of 2.2-2.6 cocci. Str.cremoris R_1 had chains of up to 12-16 cells long during logarithmic growth. Microscopic examination of samples after the blender treatment showed that 65-70% of cells were in diplococci, 25-30% in fours and 3-4% in chains of 6.

After blending, samples were diluted in 1/10 strength M16 broth and appropriate dilutions plated on M16 agar plates by soft agar overlay. Plates were incubated at 30°C for 16-24 h.

Turbidity measurements

Optical density (OD) of cultures was measured in a Bausch and Lomb Spectronic 20 colorimeter as an estimate of cell concentration. A standard curve was prepared for Str. cremoris R_1 in M16 broth by measuring the turbidity, then plating samples from cultures growing at 30°C. This procedure was used, instead of diluting a concentrated cell suspension, to avoid possible complications from changes in the optical properties of the bacteria during growth. The relationship between OD at 580 nm and colony count on M16 agar (after blending) was nearly linear over a hundred times range of OD readings (Fig.4). An OD value of 0.2 at 580 nm represented approximately 10^8 CFU/ml.

Assay of bacteriophages

Phage was assayed by a soft agar layer method (Adams, 1959) using M16 agar containing 0.005 M calcium borogluconate. In most cases, plates were seeded with 0.1-0.2 ml of the

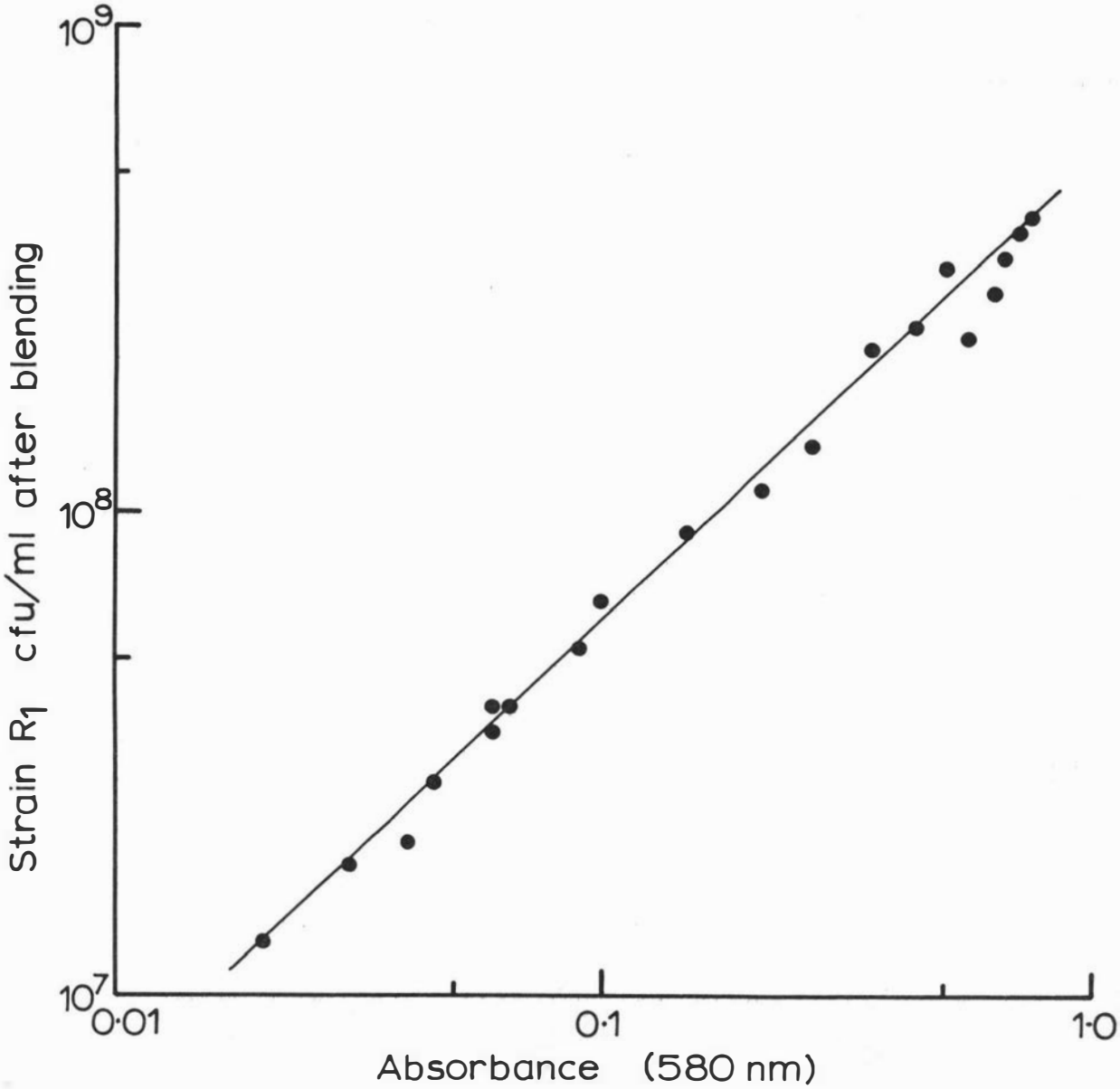

Fig. 4. Optical density readings and colony counts in M16 broth cultures of *Str. cremoris* R_1. OD_{580} was measured in a Bausch and Lomb Spectronic 20 colorimeter. Colony counts of blender-treated samples were determined by plating on M16 agar plates.

required overnight (22°C, 16 h) culture. When Str.cremoris AM_1 was used as an indicator for phage r_1t, plates were inoculated with 0.1 ml of a 30°C, 24 h culture. Plates were seeded with mid-logarithmic cultures of Str.lactis strain ML_8 and derivative strains SK_3, WM_1 and WM_2 because stationary phase cultures contained non-dispersable clumps of cells and produced uneven lawns.

The lytic spectrum of phage-containing lysates was determined by spotting 10 µl quantities of membrane-filtered (Millipore HA membrane, 0.45 µm pore size) lysates on plates previously seeded with the selected strains by soft agar overlay. Lysates were normally tested undiluted and at several decimal dilutions. Plates were incubated at 30°C for 16 h.

Mitomycin C (MC) treatment of cultures

Selected cultures were grown in M16 broth at 30°C to an OD_{580} of about 0.1 when MC (Sigma Chemical Co., St. Louis, Mo.) was added, normally to a final concentration of 1 µg/ml. Incubation was continued at 30°C and the OD_{580} was followed until completion of lysis or until the culture entered the stationary phase of growth. Stock cultures of MC (100 µg/ml) were stored at 4°C and renewed weekly.

Ultraviolet (UV) irradiation of cultures

Cultures in the mid-logarithmic phase of growth in M16 broth at 30°C were harvested by centrifugation (10,000 x g for 10 min) resuspended in 100 ml chilled buffer (0.05 M phosphate buffer in 0.14 M NaCl, pH 6.5) and blended in an AtoMix blender as described above (page 56). After blending, cells were twice centrifuged and resuspended in buffered saline. After the second cycle of centrifugation, cells were suspended in sufficient buffered saline to give an OD_{580} of 1.0 ($\sim 5 \times 10^8$ CFU/ml). Quantities of 1 ml were UV irradiated in in petri dishes of 5.5 cm diameter, 29 cm from a Hanovia bactericidal UV lamp for intervals of 5 sec to several min duration. After UV irradiation suspected lysogens were diluted 10-fold into M16 broth, incubated at 30°C and examined at intervals for lysis.

Propagation of phage

Virulent phages were propagated on their homologous hosts (Table VII) in M16 broth containing 0.005 M calcium borogluconate. Phage was added in a 1:100 ratio (PFU to CFU) to early logarithmic cultures (5×10^6 CFU/ml) which were incubated at 25°C or 30°C until lysis occurred. Lysates were centrifuged (10,000 x g for 10 min) and filtered through Millipore HA membrane filters. Phage stocks prepared in this way were stored in small quantities (without chloroform) at 4°C.

MC- or UV-induced lysates of Str.cremoris R_1 were plated on Str.cremoris AM_1 to obtain plaques of phage r_1t. Single plaques were picked into 1 ml quantities of M16 broth, from which 0.1 ml volumes were inoculated into early logarithmic phase (about 10^6 CFU/ml) 10 ml cultures of Str.cremoris AM_1. Infected cultures were incubated at 25°C for 16 h, when titres on strain AM_1 were 10^6-10^7 PFU/ml. Several variations of standard procedures for preparing phage stocks were used. Attempts to prepare high titre stocks were unsuccessful. The best stocks obtained routinely, contained about 2×10^7 PFU/ml when assayed on strain AM_1.

Concentration of phage r_1t

Phage r_1t was concentrated from MC-induced lysates of Str. cremoris R_1 because the titre of these lysates was similar to the best obtainable by propagation on strain AM_1. Logarithmically growing cultures (2000 ml) of strain R_1 in M16 broth at 30°C were induced with MC (0.5 µg/ml). After lysis was complete the induced phage was concentrated by a dextran sulphate-polyethylene glycol 2 phase separation system (Albertsson, 1967). A crude phage concentrate was obtained exactly as described for coliphage T2 (Albertsson, 1967). Polyethylene glycol 6000 (laboratory grade) was a product of BDH Chemicals Ltd, Poole, England. Dextran sulphate 500 was obtained from Sigma Chemical Co., and dried over P_2O_5 in a desiccator.

Phage concentrates from 2000 ml lysates (total volume, approximately 25 ml) were centrifuged (28,000 rpm, 2 h) using the 30 rotor in a Spinco L2-65 ultracentrifuge. Pellets were resuspended gently in 1-2 ml 0.1% bovine serum albumin solution and centrifuged (15,000 rpm, 5 min) in a Servall RC-2 centrifuge. Several phage preparations were pooled and the cycle of high and low speed centrifugations repeated. Approximately 1 ml of temperate phage r_1t of greater than 10^{10} PFU/ml on strain AM_1 was obtained from each litre of R_1 lysate.

Electron microscopy

High titre bacteriophage preparations were diluted 10-fold into 0.1% bovine serum albumin solution and negatively stained with an equal volume of 2% neutralized phosphotungstic acid, ammonium molybdate or uranyl acetate. Aerosols of stained preparations were sprayed on to carbon film grids and examined using a Phillips EM200 electron microscope.

RESULTS

Search for lysogens

Initially bacteria-free filtrates from overnight M16 broth cultures of 32 strains of lactic streptococci (22 *Str. cremoris* and 10 *Str.lactis*) were spotted (10 µl) on lawns of these 32 strains on M16 agar supplemented with calcium ions. No obvious instances of lysis, phage-reactions or growth inhibition were observed in the 1024 strain combinations examined.

Treatment with inducing agents, however, gave more positive results. When MC was added to logarithmically growing (CD_{580} of 0.1) M16 broth cultures at 30°C, at final concentrations of 0.5- and 1.0 µg/ml, overt lysis resulting in complete clearing of the cultures was observed in 4 (C_{13}, BK_5, R_1 and TR) of 22 *Str.cremoris* strains and 2 (BA_1 and H_1) of 7 *Str.lactis* strains which were tested. Samples of crude lysates from MC-treated cultures of these 6 strains

were stained with ammonium molybdate, potassium phosphotungstate and uranyl acetate and examined in an electron microscope. Structures resembling bacteriophage or phage components were seen only in lysates from Str.cremoris R_1. In addition, in preliminary lytic spectrum determinations the lysate from strain R_1 showed plaque formation on some strains. Str.cremoris R_1 was, therefore, selected for further study.

MC induction of lysis in Str.cremoris R_1

The effects of MC additions to cultures of Str.cremoris R_1 are shown in Fig.5. Neither time of addition nor final concentration of MC was a particularly critical factor in inducing lysis of cultures. Cultures incubated at 30°C were inducible by a range of MC concentrations from 0.1-4.0 µg/ml, provided that cultures were growing logarithmically and contained less than about 10^8 CFU/ml. MC concentrations below 0.1 µg/ml caused no observable departure from the control OD_{580} curve. Addition of MC at levels of higher than 2.0 µg/ml reduced the initial increase in OD_{580} and there was progressively less lysis. At 5.0 µg/ml there was almost no growth and no lysis.

In the standard procedure adopted for MC induction of lysis, MC was added at a final concentration of 0.5-1.0 µg/ml to logarithmically growing cultures which had reached an OD_{580} of 0.1 ($\sim 5 \times 10^7$ CFU/ml after blending). Under these conditions there was little effect on growth for some 30 min; then the rate of increase in OD_{580} slowed gradually over the next 60-90 min, followed by marked and rapid lysis of the culture (Fig.5). Higher or lower MC concentrations gave less complete lysis of cultures.

UV induction of lysis in Str.cremoris R_1

Survival of Str.cremoris R_1 and of an apparently non-inducible strain of Str.lactis, ML_3 after UV irradiation of blender-treated cultures under the conditions described in Methods (page 58) are shown in Fig.6. Str.lactis ML_3 (NCDO 763;

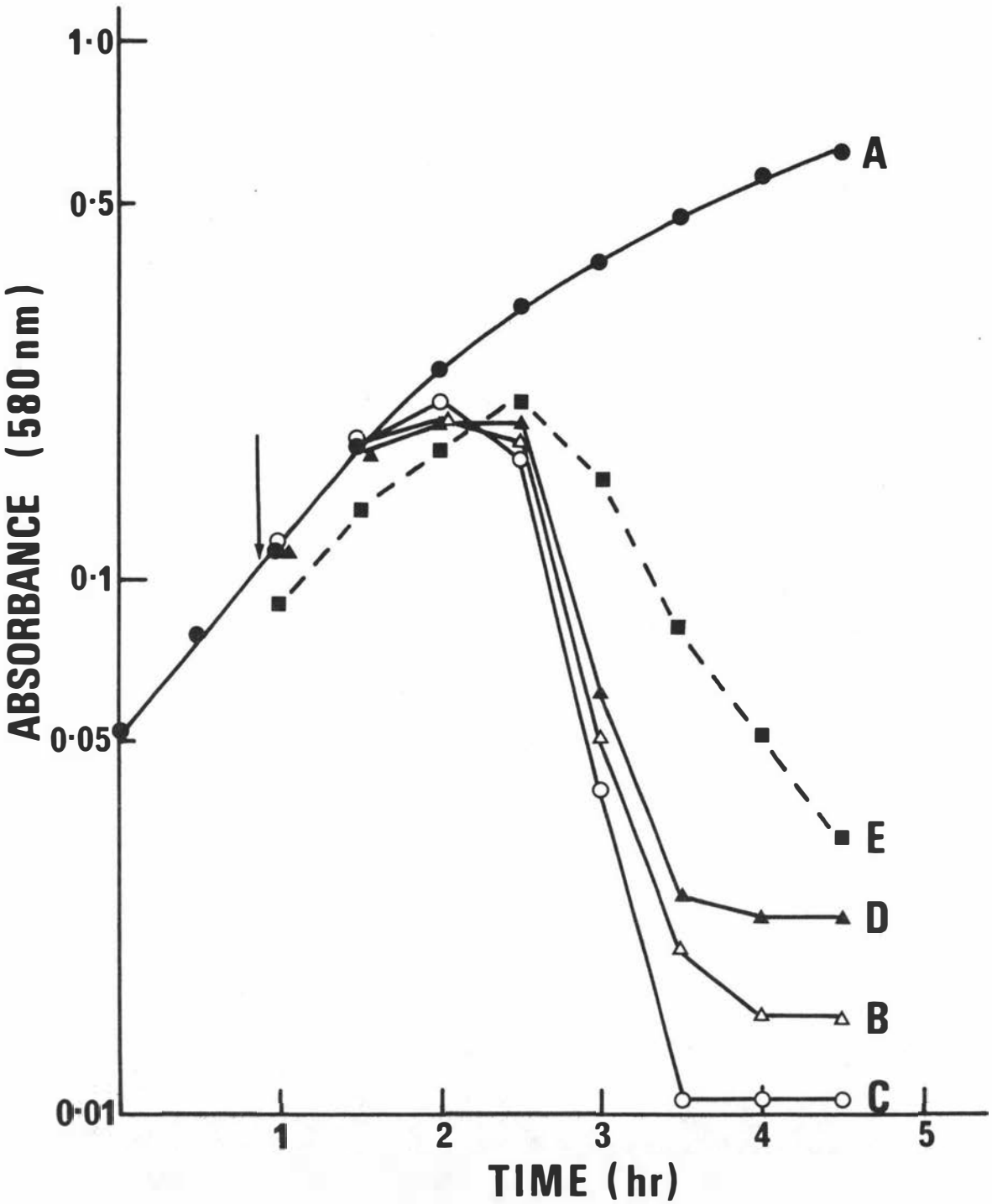

Fig. 5. Induction of lysis in *Str. cremoris* R_1 with mitomycin C.

At the time indicated by the arrow, an M16 broth culture (1000 ml) of strain R_1 at 30°C was divided into 4 cultures of 250 ml, which received, no additions (curve A), 0.5 μg/ml MC (curve B), 1.0 μg/ml MC (curve C) and 2.0 μg/ml (curve D). Incubation was continued at 30°C, and 10 ml samples were withdrawn at 30 min intervals for OD readings.

The induction of lysis after UV irradiation of *Str. cremoris* R_1 for 10 sec (from Fig. 7A) is shown for comparison (curve E).

NZRCC 20030) which has a mean chain length of 2.64 cocci under a variety of cultural conditions (Thomas & Batt, 1968) was considered to represent a typical non-inducible strain of lactic streptococcus, and UV doses could be operationally defined from the lethal effects on this strain. The survival curve appeared to be linear for at least 30 sec of exposure to UV irradiation. There were no visible signs of lysis when UV-irradiated samples of ML_3 were diluted into M16 broth and incubated at 30°C for several hours.

Str.cremoris R_1 showed an unusual UV survival curve (Fig.6) with a marked discontinuity in the plot of plate count against UV exposure between 10 and 20 sec. The change in slope of the curve occurred at the doses of UV which gave most pronounced lysis when samples were subsequently diluted into M16 broth and incubated at 30°C (Figs 7a and 7b). Under the conditions used for UV irradiation, 5 sec was the minimum exposure required to cause visible lysis of strain R_1. Consistent and extensive lysis of cultures resulted from 10-15 sec of UV irradiation. Longer exposures to UV brought about progressively less lysis. After 30 sec of UV irradiation strain R_1 showed little growth and no lysis during 4 h of subsequent incubation in M16 broth at 30°C. The OD_{580} readings showing growth and lysis of R_1 transferred to M16 broth after 5, 10, 15, 20 and 30 sec of UV treatment are shown in Figs 7A and 7B. The recoverable colony count at 0 time after inoculation into M16 broth provided the data for the R_1 survival curve in Fig.6. The results of 10 sec of UV irradiation are also included in Fig.5. The pattern of induction after this exposure to UV irradiation was very similar to that brought about by MC addition.

Lytic spectrum determinations

MC- and UV-induced lysates of strain R_1 were spotted undiluted, and at decimal dilutions on lawns of 34 strains of Str.lactis and Str.cremoris. Table VIII lists the 12 strains, all Str.cremoris which gave a positive response. Serial dilutions of the MC-induced lysate were plated by the

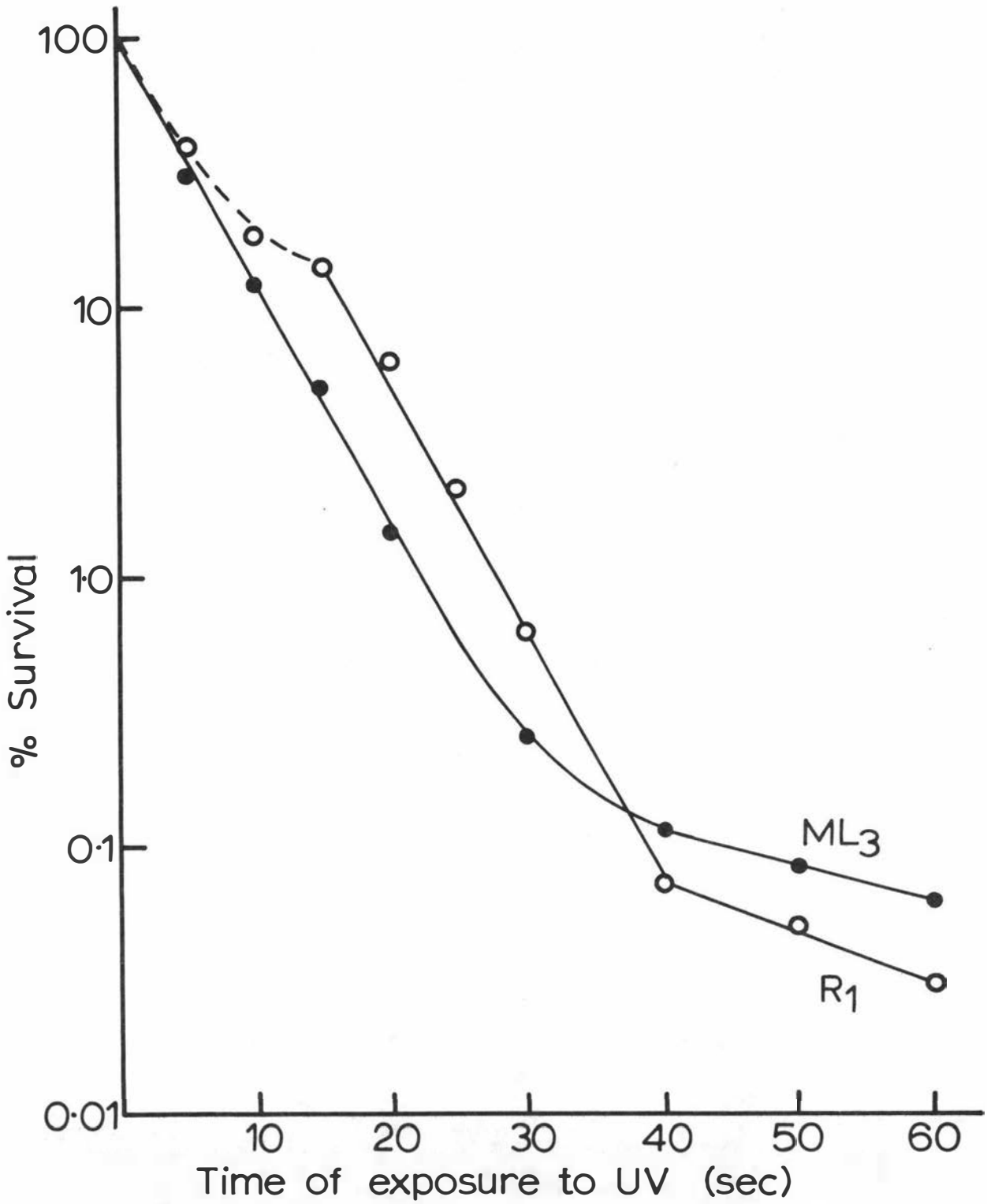

Fig. 6. Survival of *Str. cremoris* R_1 and *Str. lactis* ML_3 after exposure to UV irradiation. Cultures were washed by cycles of centrifugation and resuspension in phosphate-buffered saline solution, and subjected to blender treatment (to reduce chains of streptococci to mainly diplococci) before UV irradiation. Irradiated samples were diluted in 1/10 strength M16 broth and plated on M16 agar plates as soon as possible after irradiation.

● , *Str. lactis* ML_3; ○ , *Str. cremoris* R_1.

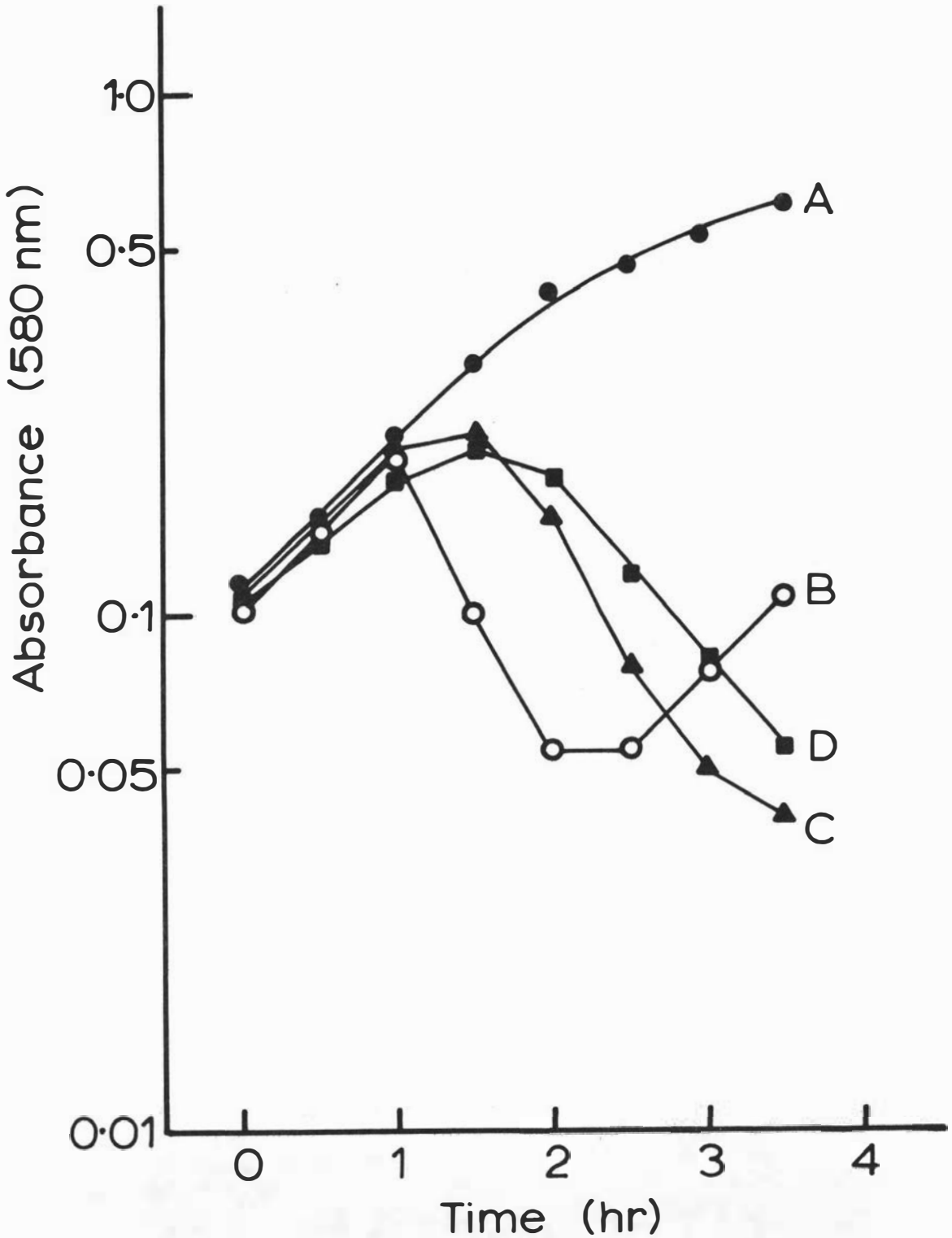

Fig. 7A. Induction of lysis of *Str. cremoris* R_1 by exposure to UV irradiation.

Logarithmically growing cultures of R_1 in M16 broth at 30°C were washed, blended and irradiated with UV light as described in the text. A non-irradiated sample (curve A) and samples irradiated for 5 sec (curve B), 10 sec (curve C) and 15 sec (curve D) were diluted into M16 broth and incubated at 30°C. Readings of OD_{580} were made at 30 min intervals.

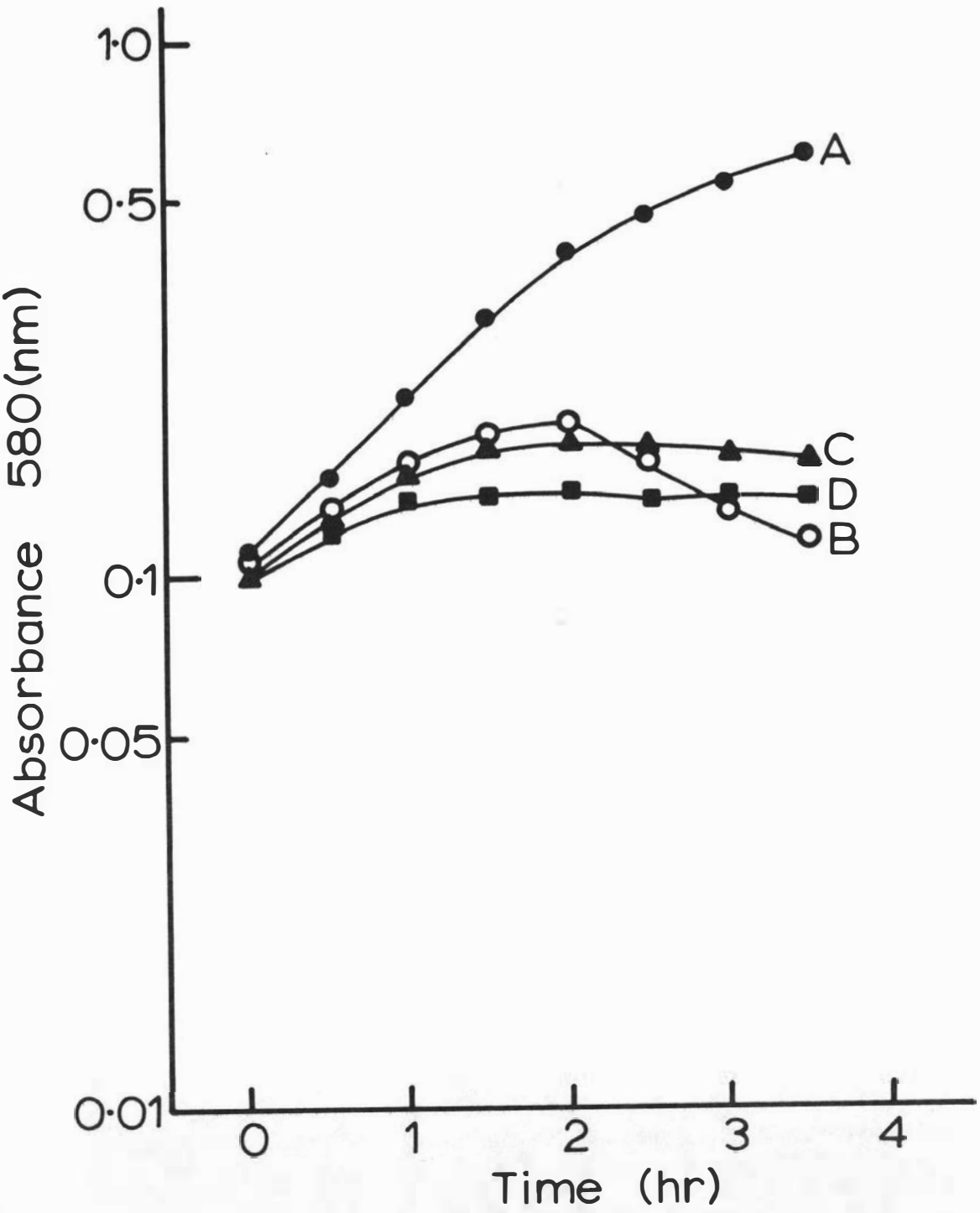

Fig. 7B. Induction of lysis of *Str. cremoris* R_1 by exposure to UV irradiation.

The same cell suspension used in Fig. 7A was irradiated for 20 sec (curve B), 25 sec (curve C) and 30 sec (curve D). The same non-irradiated control sample is shown in curve A.

soft agar layer technique on all 12 strains which had shown either lytic zones, turbid zones or plaques in the spot tests. Plaque formation occurred on only 3 strains, AM_1, SK_{11} (an AM_1 derivative) and a closely related strain, US_3. The zones of lysis observed on the remaining 9 strains were not caused by sensitivity of some strains to MC still present in the MC-induced lysates, because lysates obtained by UV treatment of R_1 showed the same combination of plaquing and non-plaquing reactions in the lytic spectrum (Table VIII). Furthermore, 6 of these 9 strains showed no signs of induction by MC under conditions which caused lysis of strain R_1 (Table XI).

The lytic spectrum of a concentrated MC-induced R_1 lysate was also tested. The concentration procedures (page 59) raised PFU on AM_1 from $\sim 10^7$/ml to $\sim 10^{10}$/ml, but no changes were found in the range of strains showing zones of lysis, and there was no increase in the number of strains exhibiting plaque formation. The concentrated R_1 lysate was diluted to a titre (PFU/ml) on AM_1 similar to that in crude R_1 induced lysates. The lytic spectrum was still virtually unchanged (Table VIII) thus eliminating the possibility that the lytic zones resulted from some relatively non-specific reaction such as a phage-induced lysozyme.

The phage present in induced R_1 lysates was isolated from plaques produced on AM_1 and propagated on this strain and on US_3. Phage preparations of greater than 10^7 PFU/ml on AM_1 were difficult to obtain. From Table VIII it can be seen that only 2 (C_{13}, AM_2) of the 9 strains which showed positive but non-plaquing reactions to R_1 lysates were retained in the lytic spectrum after the phage had been propagated on AM_1. Plaque formation and relative efficiency of plating (EOP) on strains AM_1, SK_{11} and US_3 were unchanged. Phage preparations grown on US_3 rarely exceeded 10^6 PFU/ml on AM_1 or US_3. Titres were too low for lytic spectrum tests.

TABLE VIII.

LYTIC SPECTRUM[a] OF TEMPERATE AND VIRULENT BACTERIOPHAGES OF *STR. CREMORIS* STRAIN R_1.

Strain of *Str. cremoris* [b]	lytic spectrum						
	R_1 culture filtrate [c]	induced R_1 lysates				$r_1t.AM_1$ [f]	$r_1v.R_1$ [g]
		UV	MC	MC,conc. [d]	MC, dil. [e]		
R_1	–	–	–	–	–	–	1.4×10^9 [h]
AM_1	–	3.7×10^7	3.0×10^7	1.2×10^{10}	1.8×10^7	2.8×10^7	4.0×10^2
AM_2	–	±	±	±	–	+	+
BR_4	–	+	+	+	+	–	–
C_{13}	–	++	++	++	++	++	–
HP	–	++	++	++	++	–	–
KH	–	++	++	++	+	–	–
ML_1	–	±	+	+	±	–	–
P_1	–	++	++	++	+	–	–
P_2	–	++	++	++	++	–	–
R_6	–	+	+	+	+	–	5.3×10^2
SK_{11}	–	2.0×10^6	2.3×10^6	6.7×10^9	8.3×10^5	2.2×10^6	8.0×10^1
US_3	–	1.8×10^7	1.4×10^7	6.9×10^9	9.2×10^6	1.1×10^7	3.8×10^2

[a] Symbols: +, lysis; ++, pronounced lysis; ±, weak or inconsistent lysis; –, no lysis.

[b] No lysis was observed on 10 *Str. lactis* (BA_1, BA_2, C_{10}, H_1, ML_3, SK_1, ML_8, SK_3, WM_1, WM_2) and a further 11 strains of *Str. cremoris* (AM_3, BK_5, E_8, H_2, K, SK_4, TR, Z_8, 166, 158, 924) which were tested.

[c] Phage preparation tested.

[d] Concentrated MC– induced R_1 lysate ($\sim 10^{10}$ PFU/ml).

[e] X 1000 dilution of concentrated MC–induced lysate.

[f] Temperate phage from *Str. cremoris* R_1 propagated on strain AM_1.

[g] Virulent phage of *Str. cremoris* R_1 propagated on strain R_1.

[h] PFU/ml in preparations which showed plaquing reaction in spot tests.

Characteristics of strain R_1 and its verification as a lysogen.

History. The strain was obtained in 1937 from United Dairies Ltd., England, by H.R. Whitehead who named the culture UD_1. An isolate from this culture, $UD_{1/2}$ (22.3.38) was renamed R_1. The strain was maintained at this Institute by daily sub-culture in skim milk with periodic selection and subsequent sub-culture of active acid-producing colonies (Whitehead et al. 1956). Reserve cultures were kept deep-frozen and in the freeze-dried state. A new sub-culturing regimen was adopted about 1968. Sub-culturing in autoclaved RSM was reduced to 1-2 times weekly. Freshly inoculated cultures were held at 4°C and incubated at 22°C for 16-24 h before sub-culture. Purity and acid-producing activity of these cultures were checked semi-annually by plating on a citrated-milk agar medium (Pearce, Skipper & Jarvis, 1974) and testing isolates in an activity test (Pearce, 1969). The strain may give rise to slow variants (Pearce, 1970) although some of these may be lactose-negative derivatives.

Virulent bacteriophages. There are early reports of phages virulent on Str.cremoris R_1 (Hunter, 1939). One virulent phage of R_1 (NZDRI 652, originally isolated from cheese whey produced when R_1 was the starter in use) is referred to in this study as phage r_1v. The sensitivity of strain R_1 to r_1v and 27 other virulent phages of different host range, isolated from cheese whey samples, is shown in Table VII. Strain R_1 was attacked by 13 isolates at high efficiencies which approached those at which they attacked their propagating hosts. Medium or low sensitivity (EOP, 0.1-0.0001%) to 8 other phages was found. Strain R_1 appeared resistant to 7 of the phages tested.

Lysogenic state of strain R_1. The interactions of some virulent phages with their hosts may superficially simulate lysogeny (Hayes, 1968). The possibilities of pseudolysogeny and the carrier state were considered because phage-carrying cheese and lactic casein starters were well known (Hunter,

1947; Hunter & Whitehead, 1949; Pette, 1953; Crawford & Galloway, 1962; Thomas & Lowrie, 1974a, 1974b). The following were adopted as tests to show conclusively that Str.cremoris R_1 was a lysogenic strain:

(i) stability on repeated purification and sub-culture and inducibility by different induction procedures.
(ii) plaque formation on an indicator strain.
(iii) lysogenisation of another strain with the phage induced from R_1.
(iv) curing of the suspected lysogen.
(v) presence of bacteriophage in electron micrographs of lysates.

Stability of the lysogenic character of strain R_1 with repeated re-isolation and sub-culture

The R_1 culture which was currently available for distribution to cheese factories, as well as freeze-dried stock cultures of R_1 prepared in 1968, 1967, 1962 and 1955 were obtained, inoculated into autoclaved RSM and incubated at 30°C until coagulation occurred. Clotted cultures were purified by 3 cycles of plating at high dilution (10-20 colonies/plate) on M16 agar, followed by inoculation of single colonies into tubes of autoclaved RSM. These milk cultures were examined microscopically and tested for acid production in an activity test (Pearce, 1969; and page 15). Isolates from the 5 different sources were indistinguishable and showed no obvious differences from the R_1 isolate upon which the initial observations were made.

Broth cultures were tested for susceptibility to 5 different virulent phages (r_1v, c_{13}, p_2, sk_{11}, z_8) and subjected to the previously described MC and UV induction procedures. Patterns of phage susceptibility and responses to the induction treatments were identical. Lysates were of similar titre on strain AM_1 (approximately 10^7 CFU/ml) and there were no differences observed in the lytic spectrum results when lysates were spotted on lawns of the 12 Str.cremoris strains listed in Table VIII.

The R_1 culture in which the temperate phage was originally found was, therefore, a true lysogen and unlikely to be an artifact, a chance contaminant, an atypical isolate or a phage-carrying pseudolysogenic culture.

The indicator strain

Of the 3 strains on which R_1 lysates produced plaques, Str. cremoris AM_1 consistently gave the highest plate counts for the phage present in induced R_1 lysates. Strain AM_1 was used routinely as the indicator for this phage (designated r_1t). Initially, the plaque counts of phage r_1t on AM_1 showed considerable variation. It soon became apparent that the age of the culture used for seeding plates had a marked effect on plating efficiency. Highest EOP on AM_1 was found when plates were seeded from cultures grown well into the stationary phase of growth by incubation at 30°C for 24 h. Plaque counts were always lower, frequently reduced to 10%, 1% or even 0.1% if cultures grown at 22°C or still in logarithmic growth were used as host cells for plating r_1t from induced R_1 lysates.

Table IX shows the results of experiments carried out to determine if the special conditions necessary for highest EOP on AM_1 were a requirement for stationary phase host cells, some supernatant factor, or simply a low pH in the culture used for seeding plates. The titre of r_1t was clearly characteristic of the stage of growth of the culture. Changes in pH, suspending medium or cell concentration appeared to have no significant effect.

The age of seed culture also affected EOP of r_1t phage on strains SK_{11} and US_3. The effects were not as pronounced as with AM_1, possibly because with these hosts the assay was already at such low efficiency that this factor was not significant.

Variations in titre of induced R_1 lysates depending upon plating conditions suggested that the indicator for r_1t phage was itself becoming lysogenised. Specific attempts to

TABLE IX.

EFFECT OF AGE, pH AND SUSPENDING MEDIUM OF STRAIN AM_1 SEED CULTURES ON TITRES OF INDUCED r_1t PHAGE

culture	treatment	OD_{580}	pH	PFU/ml phage r_1t detected
stationary phase AM_1 (30°C, 16 h)	untreated	0.70	4.6	2.04×10^8
	centrifuged, resusp. in original volume of own supernatant	0.70	4.7	2.41×10^8
	centrifuged, resusp. in original vol. of supernt. from log. culture	0.65	5.9	2.21×10^8
	centrifuged, resusp. in original volume of new broth	0.62	6.6	2.34×10^8
logarithmic phase AM_1 (30°C, 4 h)	untreated	0.29	5.7	3.30×10^6
	centrifuged, resusp. in 1/3 vol. of own supernatant	0.78	5.5	2.30×10^6
	centrifuged, resusp. in 1/3 vol. supernt. from stat. culture	0.68	4.7	1.24×10^6
	centrifuged, resusp. in 1/3 vol. of new broth	0.81	6.3	2.02×10^6

demonstrate lysogenisation of AM_1 by phage r_1t were inconclusive. Attempts to characterize isolates of AM_1 which were suspected r_1t lysogens were complicated by difficulties of consistently demonstrating resistance to r_1t and also by the possibility that AM_1 was already lysogenised and inducible under certain conditions.

Attempted lysogenisation of other strains

The 9 strains showing turbid zones, or zones of lysis in their reaction to spot tests with induced R_1 lysates (Table VIII) but never exhibiting plaque formation, may have become lysogenised by phage in the lysates. Colonies were isolated from the lytic zones and turbid areas on all 9 strains and tested as suspected lysogens. Isolates and control cultures were screened for phage susceptibility and resistance, and inducibility with MC treatment. Results were negative, inconclusive or insufficiently different from the response of control cultures to clearly demonstrate lysogenisation by r_1t.

Lysogenisation of strain SK_{11}

Strain SK_{11}, which exhibits better growth characteristics on synthetic media than its parent strain AM_1, was examined in detail for evidence of lysogenisation by phage r_1t because EOP on this strain remained lowest, regardless of age of seed culture. Isolations were made from the turbid zones formed when a MC-induced lysate of R_1 was spotted ($\sim 2 \times 10^5$ PFU on AM_1/10 µl drop) on a lawn of SK_{11} on M16 agar. Material from the turbid zones was streaked on to M16 agar plates which were incubated for 48 h. Twenty clearly separated colonies were transferred into M16 broth. These cultures were tested for inducibility with MC, the presence of phage in induced lysates and their phage susceptibility and resistance patterns were examined.

In MC-induction tests there was a slowing of rate of increase in turbidity in all cultures, including the controls. After incubation for 5 h all culture supernatants or lysates

(including the control SK_{11} cultures) were tested for r_1t phage on lawns of AM_1. Of the 20 isolates treated as suspected lysogens, 4 produced lysates which gave plaque formation on AM_1. None of the other isolates or the SK_{11} controls (some of which had lysed after MC treatment) gave any signs of phage reactions or lysis on AM_1.

The results of phage sensitivity tests are shown in Table X. Two isolates, code-named I601 and I604, proved to be resistant to r_1t phage in contrast to the susceptibility shown by control SK_{11} cultures and other isolates from the same turbid spot. These isolates appeared to be derivatives of SK_{11} which had become lysogenised by phage r_1t because:

(i) cultures were inducible.
(ii) an increase in detectable r_1t phage coincided with induction.
(iii) cultures showed the same pattern of sensitivity as SK_{11} to virulent phages.
(iv) cultures were resistant to phage r_1t.

One of these r_1t phage-resistant SK_{11} isolates was purified by sub-cloning through 3 cycles of plating, selecting colonies and culturing. A control SK_{11} culture was treated in an identical manner. Induction with MC and the detection of phage demonstrable on AM_1 are shown in Fig.8. The behaviour of the control SK_{11} culture is also shown. Induction of lysis was less obvious than in strain R_1 and the level of spontaneously induced phage much higher. Nevertheless, there was a definite increase in the level of r_1t phage after MC treatment. No phage was detectable on AM_1 in samples from either untreated or MC-treated SK_{11} control cultures.

Attempts to derive a cured strain of R_1

Cultures of R_1 were exposed to UV irradiation for periods of up to several minutes under the conditions previously described (page 58) to allow only 0.01%, 0.001%, 0.0001% and 0.00001% survival. Irradiated cultures were plated on M16

TABLE X.

PHAGE SENSITIVITY[a] OF STRAIN SK_{11} AND SUSPECTED LYSOGENIC ISOLATES

isolate	reason for testing	phage preparation tested [b]				
		am_1 1×10^8 [c]	sk_{11} 1×10^9	r_1t MC–induced 2×10^7	r_1t UV–induced 2×10^7	r_1v 1×10^9
SK_{11}	control	s	s	s	s	±
I-601	) suspected	s	s	r	r	r
I-614	) r_1t lysogens	s	s	r	r	r
I-605	) other	s	s	s	s	r
I-608	) isolates from same	s	s	s	s	r
I-617	) turbid spot	s	s	s	s	±
AM_1	r_1t indicator	s	s	s	s	s
R_1	source of r_1t	r	r	r	r	s

a Symbols: s, sensitive; r, resistant; ±, inconsistent reaction.

b spot tests (10 μl) on lawns of test strain on M16 agar plates.

c PFU/ml in phage preparation.

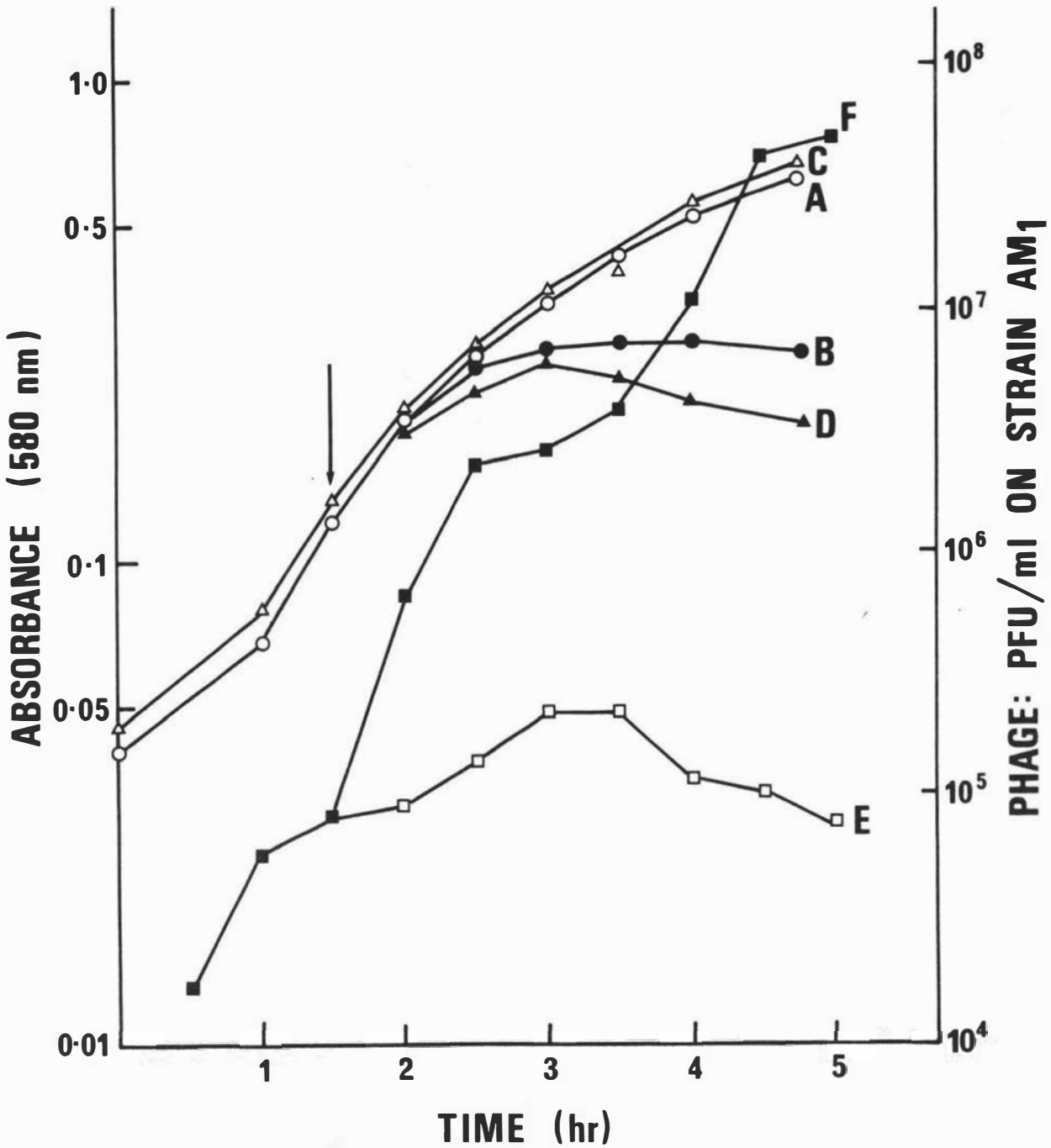

Fig. 8. Induction of suspected SK_{11} lysogens with mitomycin C.

MC (1.0 μg/ml) was added to logarithmically growing cultures of SK_{11} (curve B) and the suspected r_1t phage-lysogenised derivative (curve D) at the time indicated by the arrow. OD_{580} readings of untreated control cultures of SK_{11} (curve A) and of the suspected lysogen (curve C) are also shown.

Samples were plated for PFU of phage r_1t on strain AM_1 from control (curve E) and MC-treated (curve F) cultures of the suspected lysogen. No evidence of phage could be detected in corresponding samples from the SK_{11} cultures.

agar plates (without added calcium ions) and single colonies inoculated into M16 broth. Cultures isolated in this way were tested as follows:

(i) sensitivity to phage r_1v at high dilutions to confirm that isolates were R_1 cultures and not contaminants.
(ii) sensitivity to phage r_1t.
(iii) inducibility with MC.
(iv) presence of r_1t in induced lysates.
(v) phage sensitivity and resistance pattern.

Over 100 isolates were tested. No obviously cured strain which gave the expected results from the first 4 tests was found. Lysis of cultures of all the isolates was readily inducible with MC treatment. There was some variation in the level of r_1t phage detectable on AM_1 in these induced lysates, but this could have been due to assaying procedures (page 71).

It was possible that strain R_1 was a multiple lysogen. If only one of several prophages were lost, gross inducibility might remain unchanged. Some evidence of loss of a prophage might be detected in the response to other phages besides r_1t which could be re-lysogenising any cured isolates. To test this hypothesis the parent culture, R_1, and isolates from heavily UV-irradiated cultures were tested for their sensitivity or resistance to 6 serologically related phages (Table VII) of markedly different host range, ml_3, ba_1, ba_2, br_4, h_1 and sK_{11}. The control R_1 culture was known to be sensitive to only 3 of the phages. Isolates were examined for changes in overall susceptibility or in the level of sensitivity to these phages. No indications of an altered phage immunity pattern were detected in any R_1 isolate.

Characteristics of the induced r_1t phage

Once Str.cremoris AM_1 had been established as the indicator strain for phage r_1t it was possible to re-investigate the

induction of strain R_1 and assay for the presence of this temperate phage in induced and non-induced cultures. It can be seen from Fig.9 that there was a low but consistent rate of spontaneous induction during logarithmic growth of strain R_1 in M16 broth. Detectable r_1t phage was at a PFU to CFU ratio of 10^{-6} until the mid-logarithmic phase of growth. This ratio increased slightly as the culture entered late logarithmic growth then fell suddenly with the onset of stationary phase. Since the levels of phage r_1t detectable in non-induced R_1 cultures never exceeded 5×10^2 PFU/ml on strain AM_1 at any time, it is not surprising that the lytic spectrum tests with culture filtrates gave negative results (Table VIII).

The increase in detectable r_1t phage in a MC-induced R_1 culture is shown in Fig.10. This increase, often greater than 10^4 PFU/ml over that found in untreated control cultures, coincided with the slowing of increase in OD_{580} and commencement of lysis. The induced culture reached a maximum OD_{580} equivalent to 1.7×10^8 CFU/ml (from 6×10^7 CFU/ml at MC addition) before lysis of 90% of the cells. The phage titre in this lysate of 5×10^6 PFU/ml on the indicator strain, AM_1, represented a burst of less than 1 detectable phage/10 induced cells.

Very recently, careful assay procedures have raised EOP on AM_1 by some 5-10 times (cf. Tables VIII and IX). Similar results have been achieved also by using a different assay medium (personal communication from L.E. Pearce). Even so, the level of detectable phage in induced lysates is still relatively low.

Relationship between virulent and temperate phages of Str.cremoris R_1

The host range of a virulent phage of R_1, r_1v propagated on R_1 is shown in Table VIII. A low incidence of plaque formation on strain R_6 together with the expected virulence on R_1 were the only differences in the range of hosts on which the virulent and temperate phages could form plaques.

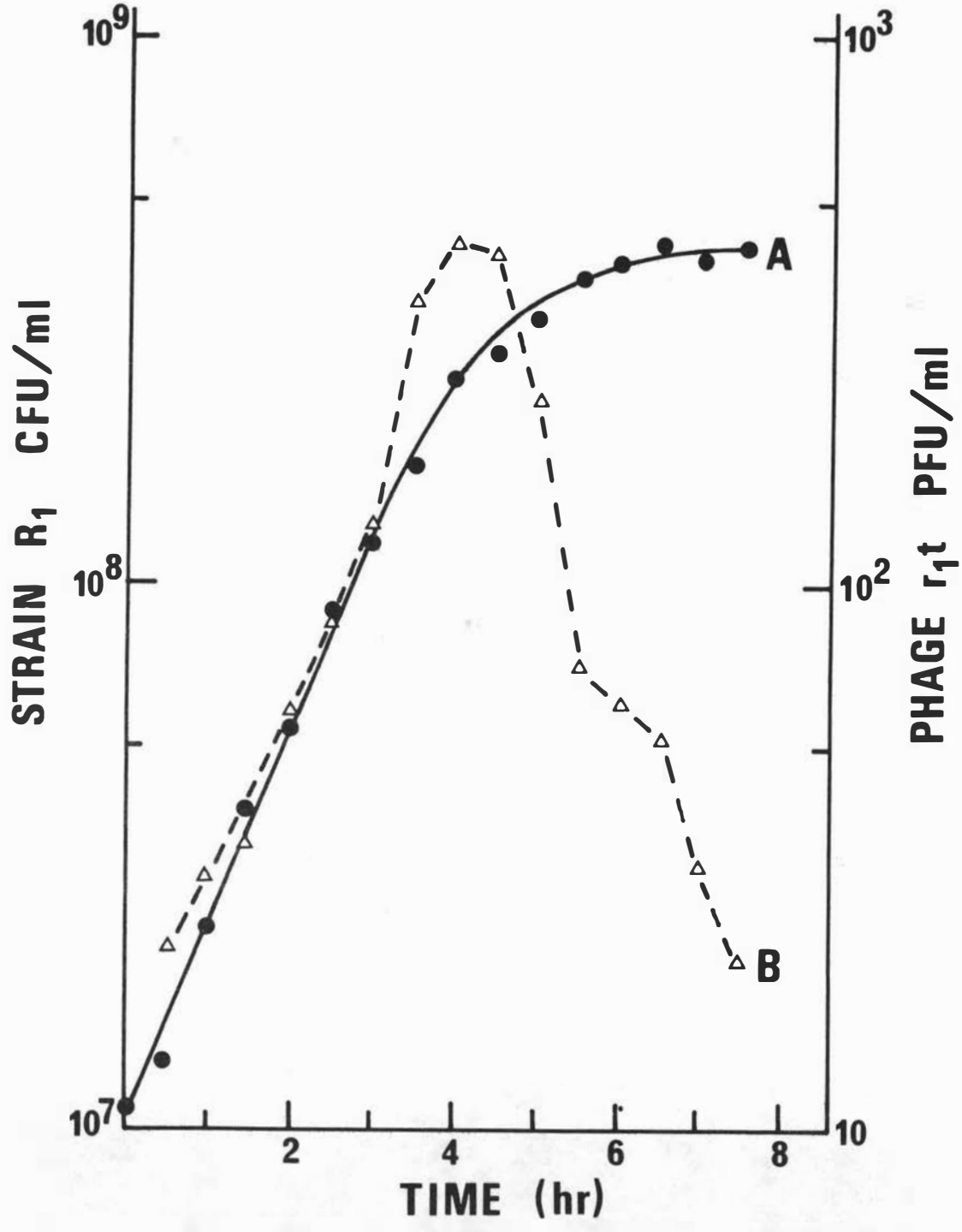

Fig. 9. The presence of phage r_1t in a non-induced culture of *Str. cremoris* R_1.

M16 broth (500 ml) was inoculated (2%) from an overnight culture of strain R_1 and incubated at 30°C. Samples were withdrawn at 30 min intervals and CFU/ml or R_1 on M16 agar plates determined following blender treatment (curve A). The balance of the sample was chilled and centrifuged (10,000 x g for 10 min). Supernatants were assayed for phage r_1t using *Str. cremoris* AM_1 as the indicator strain (curve B).

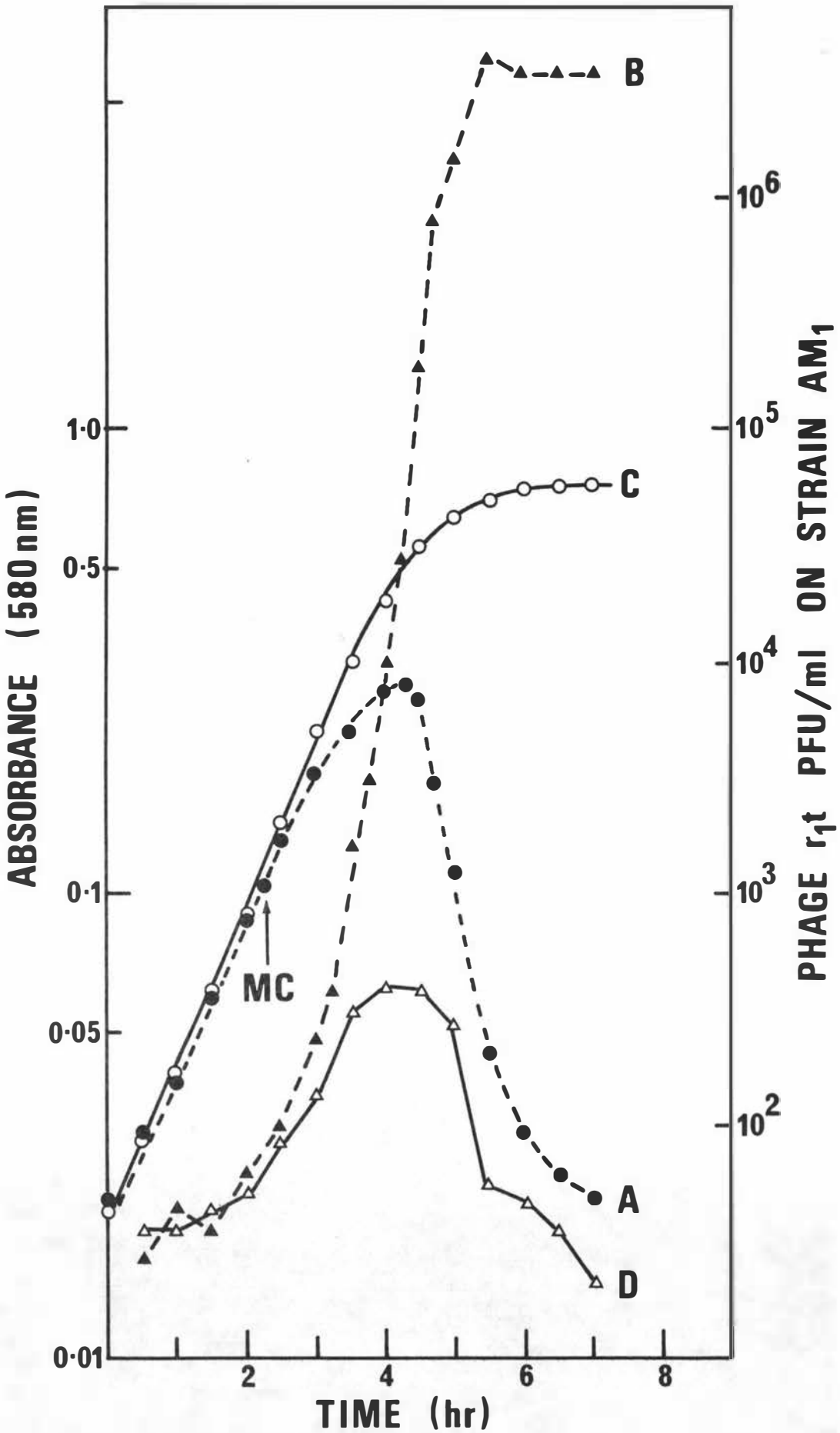

Fig. 10. Phage r_1t in a MC-induced culture of *Str. cremoris* R_1.

A M16 broth culture prepared as in Fig. 9 was incubated at 30°C. MC (1.0 μg/ml) was added when the culture reached an OD_{580} of 0.1 (arrow). Samples were withdrawn at intervals for OD readings (curve A). The balance of each sample was centrifuged and supernatants were assayed for phage r_1t as in Fig. 9 (curve B). The OD profile (curve C) and r_1t phage levels (curve D) of an untreated control culture are also shown.

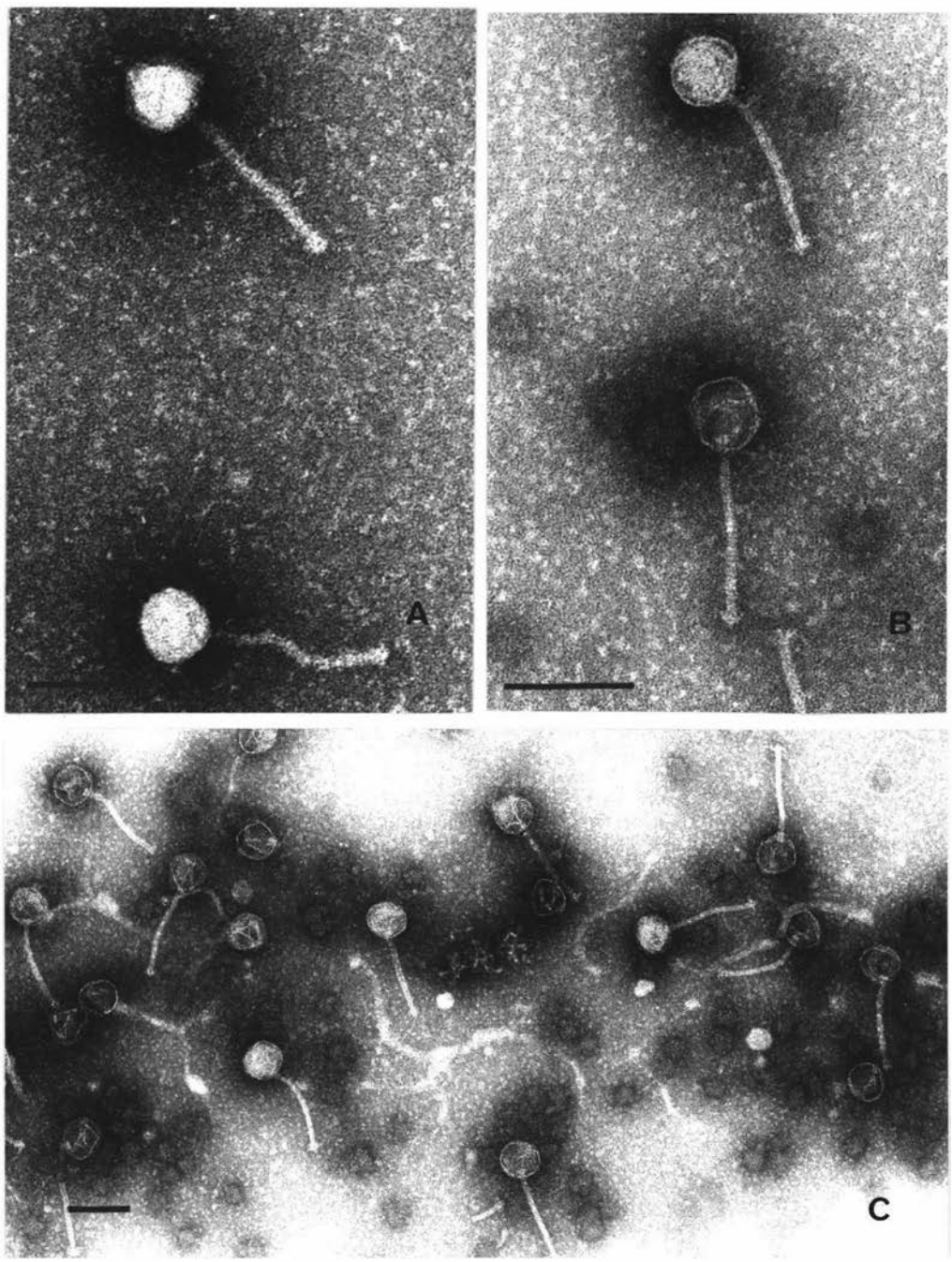

PLATE I. Bacteriophages of *Streptococcus cremoris* strain R_1 negatively stained with neutral 2% potassium phosphotungstate.
Bar markers represent 100 nm.

A. Virulent phage r_1v. Magnification, X180,000.
B. Temperate phage r_1t. Magnification, X180,000.
C. Phage, phage fragments and ghosts in a MC-induced lysate of *Str. cremoris* R_1. Magnification, X85,000.

In routine propagation of phage r_1v on R_1, lysis normally occurs when cultures are in late logarithmic growth, the time when spontaneously induced temperate phage is highest (Fig.9). Plaque formation on AM_1, SK_{11} and US_3 may merely represent the extent of temperate phage contamination of the virulent phage preparation.

The similarity in host range between the virulent phage (r_1v, 652, originally isolated from cheese whey) and the induced phage suggested that the former might be a virulent mutant of the temperate phage. An examination of electron micrographs of the 2 phages showed that they were indeed very similar in appearance and dimensions (Plate 1A and 1B). The most striking contrast between the virulent and temperate r_1 phages was the high amounts of incomplete phage particles, fragments and ghosts that were seen in temperate phage preparations (Plate 1C). Extensive searching of grids was necessary in order to find fragments or ghosts in r_1v preparations.

The high incidence of incomplete phage particles may have been an artifact of the concentration procedures or resulted from MC induction conditions, which may have been excessively rigorous. However, ghosts and incomplete particles have been observed in normal preparations of other virulent phages (Lowrie & Pearce, 1970).

Inducibility of other strains of Str.lactis and Str.cremoris

In the preliminary experiments conducted at the outset of this investigation, several strains, from which Str.cremoris R_1 was selected for detailed study, showed induction of lysis when treated with MC or exposed to UV irradiation. Conditions which gave most consistent induction of lysis with strain R_1 were used to re-test the remaining 21 strains of Str.cremoris and 10 Str.lactis strains.

The responses of Str.cremoris strains to the induction procedures used are shown in Table XI. In addition to R_1, 3 strains, BK_5, C_{13} and TR showed overt lysis after MC treatment or UV irradiation. Variable results were obtained with

6 strains AM_1, AM_2, AM_3, ML_1, SK_{11} and US_3 which were tested with MC. UV treatment failed to give more consistent results. No signs of induction were observed in 12 strains.

The 3 strains (AM_1, SK_{11}, US_3) upon which R_1 lysates produced plaques may themselves be inducible and lysogenic. Only one (C_{13}) of the 9 strains upon which R_1 lysates gave lytic zones (but no plaques) was clearly inducible. Inconsistent responses to induction treatments were found with 2 (ML_1, AM_2) of the strains. The balance of 6 strains showed no evidence of being lysogenic.

Table XII shows the results obtained when 10 strains of Str. lactis were treated with MC. Of these strains, 5 were also tested by UV irradiation. As in the preliminary experiments, 2 strains, BA_1 and H_1 gave pronounced lysis after MC or UV treatment. An additional strain, C_{10} was found to be inducible with MC. Responses to MC treatment suggesting possible induction were found in 4 strains. The similarity of response of 3 of the strains was not unexpected since 2 (SK_3, WM_1) were derivatives of the third, ML_8 (Table XII).

None of the lysates from Str.lactis MC- or UV-induced lysates showed any positive results in lytic spectrum tests. Evidence of some lytic activity on other strains was found in lysates induced from 2 of the Str.cremoris strains. Induced lysates from C_{13} produced plaques on AM_1 and US_3 and a zone of lysis on Str.lactis H_1. While the plaquing reactions were similar to those found with R_1 lysates, lytic zones on other strains were not found. Lysates from Str.cremoris TR showed lytic zones on a range of strains very similar to those affected by R_1 lysates, namely AM_1, AM_3, HP, KH, ML_1, P_1 and P_2. However, plaque formation was not observed on any strain.

DISCUSSION

Lysogeny amongst strains of group N lactic streptococci has been suspected for some years (Reiter, 1949; Czulak & Naylor, 1956; Crawford & Galloway, 1962; Sandine et al. 1962), and

TABLE XI.

INDUCTION OF LYSIS[a] IN STRAINS OF *STR. CREMORIS*

strain	inducing agent	
	mitomycin C [b]	UV irradiation [c]
AM_1	++ ; ±	++ ; ±
SK_{11} [d]	±	NT[e]
AM_2	++ ; –	++ ; ++
AM_3	– ; ±	+
BK_5	++ ; +++	+++
BR_4	–	–
C_{13}	+++	+++
E_8	–	–
H_2	–	–
HP	–	–
P_1	–	–
P_2	–	–
KH	–	–
ML_1	±	±
R_1	+++ [f]	+++ [g]
R_6	–	NT
SK_4	–	NT
TR	+++	+++
US_3	++ ; ±	±
Z_8	–	–
158	–	NT
166	–	NT

See Table XII for footnotes.

TABLE XII.

INDUCTION OF LYSIS[a] IN STRAINS OF *STR. LACTIS*

strain	inducing agent	
	mitomycin C [b]	UV irradiation [c]
BA_1	+++	+++
BA_2	+	±
C_{10}	++	NT [e]
H_1	+++	+++
ML_3	–	–
SK_1 [d]	–	–
ML_8	+	NT
SK_3	+	NT
WM_1	±	NT
WM_2	–	NT

[a] Symbols:

+++, following treatment with inducing agent, OD_{580} doubled, then fell to below the value when treatment with the inducing agent commenced.

++, at least one doubling of OD_{580} after initiation of induction treatment followed by fall in OD_{580} value to near that at which treatment was commenced.

+, initial OD_{580} doubled, followed by definite reduction but not to values as low as those when treatment commenced.

±, less than one doubling of OD_{580} followed by a slight fall in OD.

–, no indication of fall in OD_{580} after treatment with inducing agent.

Strains were tested at least twice: when different results were obtained, both are shown.

[b] MC treatment (0.5–, 1.0–, 2.0 μg/ml) carried out as in Fig. 5.

[c] UV irradiation (5, 10, 15, 20 sec) as for Figs. 6 and 7.

[d] Strains indented are derivatives of preceding strain.

[e] Not tested.

[f] Pattern of induction of lysis shown in Fig. 5.

[g] Pattern of induction of lysis shown in Fig. 7.

it is surprising in view of the increasing commercial significance of Str.cremoris strains that this presumed lysogeny has not been confirmed much earlier. Certainly, the simple procedures of looking for phages in culture filtrates and cross-streaking of suspected lysogens have, in the main, been unsuccessful. Where reactions between strains have been observed the possibilities of inhibition of growth by culture products, such as the nisin antibiotics were not specifically eliminated.

Strains of group N streptococci inducible by 2 very frequently used inducing agents, UV irradiation and MC treatment, appear to be relatively widespread. Use of either of these agents caused unambiguous induction of lysis in 4 of 22 Str.cremoris and 3 of 10 Str.lactis strains that were investigated in this study. A further 8 strains gave less clear-cut results. Kozak et al. (1973) found that 5 of 46 Str.lactis and 2 of 24 Str.diacetilactis strains were UV inducible. These workers failed to find inducible Str.cremoris strains but induction by UV treatment of strains of this species had already been shown by Koegh & Shimmin (1969) and by Reiter (1973) although no temperate phages had been characterized. It may well be that lysogeny, or defective lysogeny, is such a common feature of the group that the absence of indicator strains rather than of lysogens has impeded the study of temperate bacteriophages. For example, McKay & Baldwin (1973) failed to find an indicator for the phage revealed in electron micrographs of lysates UV-induced from Str.lactis C2 even though it was subsequently shown that this phage may be used to transduce a lactose metabolism marker (McKay, Cords & Baldwin, 1973). An alternative explanation for the absence of indicators and the seemingly high occurrence of defective phage, is that the conditions of induction with UV and particularly with MC (Levine, 1961; and review by Szybalski & Iyer, 1967) may have been unduly rigorous or too harsh and have had severely damaging effects on the induced phages.

Indicator strains on which plaque formation occurred, were found initially for only one (Str.cremoris R_1) of the 6

strains that showed overt lysis after UV irradiation or MC treatment. In addition, zones of lysis, but no plaque formation were produced on lawns of several strains. Unlike the report of Keogh & Shimmin (1969), no lysis was observed on lawns of the strain which gave rise to the lysate. Of the 3 closely related strains on which R_1 lysates produced plaques, Str.cremoris AM_1 was selected as the routine indicator for the phage induced from R_1, although strain AM_1 is an unusual indicator in several respects. In addition to being itself a potential lysogen, the history of the culture used for seeding plates was found to have a striking effect on the EOP of phage r_1t. Highest plating efficiencies were obtained with late stationary-phase cells that had grown at 30°C. The reasons why these cultures have the highest plate counts are not understood and the finding was unexpected because the use of early to mid-logarithmic cultures for seeding plates was essential for highest EOP of some group H temperate phages on their indicator, Str.sanguis strain Wicky (Parsons et al. 1972). Frequently, no plaque formation whatsoever was observed if logarithmically growing AM_1 cultures were used in r_1t assays.

It is not surprising that AM_1 was a poor propagating strain for r_1t phage. It is possible that AM_1 may become lysogenised by phage r_1t but direct evidence could not be obtained. However, strain SK_{11} which consistently showed lowest EOP for r_1t, did become lysogenised since r_1t phage-resistant colonies from which the phage could be induced by MC treatment were readily isolated from the turbid areas formed on SK_{11} lawns by lysates of strain R_1. Attempts to accomplish this using other strains were unsuccessful and the SK_{11} results were complicated by the possible lysogenicity of the parent SK_{11} cultures. The sensitivity of the r_1t-lysogenised SK_{11} isolates to 2 serologically different virulent phages (am_1 and sk_{11}) was unchanged from that of the parent strain. The resistance of strain R_1 to these 2 phages is not then specifically associated with the presence of this temperate phage.

An inadequacy of the indicator strain alone seems insufficient reason to account for the generally low level of phage r_1t detectable in either induced or non-induced R_1 cultures. Rapid adsorption of the induced phage to cell debris may cause the apparently low titres of phage, since losses as high as 99.9% of PFU have been attributed to such adsorption in a group H temperate phage system (Parsons et al. 1972; Parsons & Cole, 1973). Very recently, improved plating techniques have raised EOP of r_1t on AM_1. Nevertheless, induced lysates are still of low titre.

Lysates from strain R_1 caused lytic reactions but no plaque formation on several strains in addition to those that showed plaques. The possibilities of inhibitory substances or of some modification and restriction phenomenon have not been eliminated entirely. The similarity in lytic spectrum produced by crude and concentrated R_1 lysates and dilutions of the concentrated preparations would argue against the presence of some simple inhibitory compounds. It would seem more likely that strain R_1 is lysogenised, perhaps defectively by more than one phage, or that only a small proportion of the induced phages is infective. The high incidence of incomplete phage particles observed in electron micrographs of induced R_1 lysates is compatible with either possibility, but this could also result from the concentration procedures or from the conditions of induction.

All but one of the non-plaquing reactions were lost from the lytic spectrum when phage r_1t was propagated on strain AM_1. A host range modification of r_1t by AM_1 cannot be eliminated, but it is more likely that R_1 carries in addition to r_1t one or more inducible, perhaps defective, phages which do not attack AM_1 or cannot be carried or propagated by it. Passage through AM_1 may free r_1t lysates from these other phages induced simultaneously from R_1, causing loss of the lytic zones from the $r_1t \cdot AM_1$ phage preparations. It may be more realistic at this stage to assume that the R_1 lysates are mixed phage preparations.

Virulent phages have been a major industrial problem in the manufacture of fermented dairy foods for many years (Whitehead, 1953). Virulent phages of newly isolated strains of lactic streptococci appear rapidly when these strains are used commercially. The possibility that these previously unknown phages arise as virulent mutants of temperate phage may account for their sudden appearance. Neither the electron micrographs nor the host range data would be incompatible with this hypothesis in the case of the virulent and temperate phages of Str.cremoris R_1.

Preliminary studies of additional strains in this investigation as well as reports from other workers, indicate that lysogeny, defective lysogeny or even multiple lysogeny is widespread in the lactic streptococci, and as in other groups of bacteria, must be regarded as the normal rather than the exceptional case. At this stage, there appears to be no consistent correlation between the particular characteristics of strains as starters for cheesemaking and the lysogenic state. The relatively frequent occurrence of suspected lysogenic strains would, however, indicate the possibility of a substantial reservoir of phage from which new virulent mutants or recombinants might arise.

CONCLUSIONS

The starter streptococci contribute directly to the formation of bitter flavour in Cheddar cheese. The potential to produce bitter cheese may be reduced by controlling starter populations in the cheese by choice of starter strain, manufacturing conditions, or by bacteriophage.

The starter organisms may not have any direct role in the production of Cheddar flavour itself. Other than producing the correct environment which allows Cheddar flavour to develop, any additional effect of the starter may be detrimental, particularly if high starter populations are reached. Excessive starter numbers cause off-flavours, especially bitterness which appears to mask Cheddar flavour.

A strain of Streptococcus cremoris has been shown to be lysogenic. Lysogeny may be relatively widespread amongst strains of lactic streptococci used as starters in cheese-making. There is, as yet, no consistent correlation between the behaviour of any strain in cheese manufacture and the lysogenic state.

APPENDIX I

EXPERIMENTAL CHEESES:
DETAILS OF MANUFACTURE AND ANALYSES

MATERIALS AND METHODS

Milk supply

Cheeses were manufactured during the 1971-72 dairying season using bulk herd commercial whole milk (casein : fat ratio, 0.58 ± 0.02) which came mainly from Jersey cows. The total plate counts were approximately 10^5 organisms/ml on Standard Methods Agar (BBL). After pasteurization (72°C, 15 sec) average counts were about 2×10^4 organisms/ml. These values were within the range shown by Lawrence *et al*. (1972) to have no obvious influence on the development of cheese flavour.

Cheese manufacture

Experimental cheeses were manufactured in the Institute Processing Hall by a small scale (320 l) simulation of conventional commercial cheesemaking procedures. The number of experiments which could be carried out at once, on one day, was limited to 4. Bulk starters were grown in steamed (1.5 h) whole milk (0.25-0.5% inoculum, 16 h, 25°C). The same calf vell rennet level (22 ml/100 l milk) and setting temperature (31.7°C) were used in all experiments. As far as possible, the titratable acidities at which the vats were run, dried, milled and salted were kept constant between experiments. The size of the cubes formed when the coagulum was cut and the extent of dry-stirring of the curd before cheddaring, were altered when higher or lower than normal cooking temperatures were used. These measures were necessary for adequate control of the moisture content of the curd, and to keep the composition of the cheeses within normal limits. After overnight pressing, cheeses (18 kg) were film wrapped and matured for 14 days at 13°C and subsequently at 7°C.

Compositional analyses

Samples of cheeses were analysed 14 days after manufacture for moisture, fat and NaCl contents by standard procedures

(British Standard 770, 1963). The pH values of cheeses were determined from 10 g samples which had been ground in a mortar with the minimum amount of water (less than 2 ml) to give a smooth paste.

Bacteriological analyses

Starter colony counts were determined after inoculation of the milk with starter and the addition of rennet (setting), after most of the whey had been run off (drying), at milling, immediately before salting and after the cheeses had been removed from the hoops following overnight pressing. Milk or curd samples (10 g) were chilled and added to 90 ml chilled 2% trisodium citrate solution in an AtoMix blender (Measuring and Scientific Equipment Ltd, Crawley, England). Blending at full speed (13,000 rpm) homogenized the samples and reduced chains of streptococci to an average CFU of 2.3-2.6 cells depending upon the strain (Martley, 1972). The effectiveness of the blending treatment was checked microsccpically. Blended samples were diluted in 1/10 strength M16 broth and plated for colony counts by soft agar overlay on M16 agar plates (Lowrie & Pearce, 1971). Plates were incubated at 30°C for 18-24 h aerobically, or in a 5% (v/v) CO_2-air mixture.

As soon as the important influence of bacteriophage on flavour development was fully realized, samples taken for colony count determinations in cheesemaking trials as well as whey samples collected at running, milling and before salting were routinely assayed for bacteriophage. Appropriate dilutions were mixed with the test starter strain and plated using the soft agar layer technique on M16 agar containing 0.005M calcium borogluconate (Lowrie & Pearce, 1971).

Expression of starter colony count data

Because of the large differences in moisture contents of samples the observed starter colony counts /g of milk, curd or finished cheese were multiplied by the ratio of the solids content of the finished cheese to the solids content

of the sample. All colony counts are, therefore, expressed as colonies /g finished cheese. Presentation of the data in this way allows direct comparisons of the values recorded for samples from different stages of the cheesemaking process. Colony counts were interpreted cautiously if the bacteriophage titre of the sample, or of a whey sample taken at a similar stage in cheesemaking, exceeded 1% of the starter count.

Flavour evaluation

Cheeses were assessed for bitterness development at 3 and 6 months from manufacture by a panel of 8-10 experienced judges. Samples, which were coded and randomized, were submitted to the panel under controlled conditions together with a labelled standard sample of cheese previously established as not bitter and possessing good flavour. Bitterness scores were assigned on a 1-5 scale as follows:

1. absent
2. possibly present
3. definitely present
4. intense
5. predominant

The bitterness value for each cheese was the average of the individual scores. Panel reliability was checked periodically by random duplication of samples. Consistency of grading was maintained by re-submitting selected samples to consecutive grading sessions.

Cheese samples were also assigned scores for overall acceptability of flavour on a scale of 1-9. Poor cheeses with serious flavour defects had acceptability values of 1-3, average cheeses scored 5 and cheeses of outstandingly good flavour, 8-9.

List of experimental cheeses

Starter	Date	Special conditions	Page
AM_2 (non-bitter)[a]	17.9.71	standard procedure	98
AM_2		low cooking temperature	98
AM_2	24.9.71	standard procedure	99
AM_2		low cooking temperature	99
AM_2	28.9.71	standard procedure	99
AM_2		low cooking temperature	99
AM_2	5.10.71	)	100
AM_2	12.10.71	) control non-bitter	100
AM_2	28.1.72	) cheeses made by	100
AM_2	3.2.72	) standard procedure	100
E_8 (variable)	27.10.71	standard procedure	105
E_8		low cooking temperature	105
E_8	23.11.71	standard procedure	105
E_8		low cooking temperature	105
HP (bitter)	9.9.71	standard procedure	111
HP		bacteriophage added	111
HP		bacteriophage added	111
HP		bacteriophage added	111
HP	14.9.71	standard procedure	112
HP		bacteriophage added	112
HP		bacteriophage added	112
HP		bacteriophage added	112
HP	17.9.71	standard procedure	98
HP		high cooking temperature	98
HP	24.9.71	standard procedure	104
HP		high cooking temperature	104
HP	5.1.72	reduced cooking temperature	104
HP		standard procedure	104
HP		high cooking temperature	104
KH (bitter)	3.2.72	standard procedure	109
132[b] (bitter)	3.2.72	standard procedure	109
132	9.2.72	low cooking temperature	110
132		standard procedure	110
132		high cooking temperature	110

List of experimental cheeses (continued)

Starter	Data	Special conditions	Page
132	11.2.72	standard procedure	110
132		high cooking temperature	110
ML_1 (non-bitter ?)	4.11.71	low cooking temperature	106
ML_1		standard procedure	106
ML_1	26.11.71	low cooking temperature	106
ML_1		standard procedure	106
ML_8 (bitter)	5.10.71	low cooking temperature	102
ML_8		standard procedure	102
ML_8		high cooking temperature	102
ML_8	12.10.71	low cooking temperature	103
ML_8		standard procedure	103
ML_8		high cooking temperature	103
SK_{11} (non-bitter)	8.10.71	low cooking temperature	101
SK_{11}		standard procedure	101
SK_{11}	15.10.71	low cooking temperature	101
SK_{11}		standard procedure	101
Z_8 (bitter)	14.1.72	low cooking temperature	107
Z_8		low cooking temperature	107
Z_8		standard procedure	107
Z_8		high cooking temperature	107
Z_8	19.1.72	low cooking temperature [c]	108
Z_8		low cooking temperature [c]	108
Z_8		standard procedure [c]	108
Z_8		high cooking temperature [c]	108
166 (bitter)	15.2.72	standard procedure	109
166		high cooking temperature	109

Footnotes

[a] Tendancy to bitterness under normal conditions.

[b] *Str.cremoris* 132 was isolated from *Str.cremoris* KH. This isolate was resistant to a bacteriophage which attacked the parent strain, KH, and *Str.cremoris* strains HP and Z_8.

[c] Heavy contamination with bacteriophage.

Manufacturing records and analyses

Manufacturing records and analyses of all cheeses considered in this study are recorded in a standard format in the following pages. For all cheeses the setting temperature was 31.7°C and calf vell rennet (22 ml /100 l milk) was used. Compositional, microbiological and flavour analyses were carried out as described above.

Starter strain	AM_2	AM_2	HP	HP
Manufacture date	17.9.71	17.9.71	17.9.71	17.9.71
Special conditions	cook temp.		cook temp.	
Inoculum (%)	2.1	2.1	1.6	1.6
Cut of coagulum (mm)	9.5	6.4	9.5	12.7
Cooking temp. (^{o}C)	37.8	33.3	37.8	39.4
Time (h:min)				
set to dry	2:45	2:50	2:45	2:45
dry to salt	2:40	2:30	2:45	3:20
total make time	5:25	5:20	5:30	6:05
Titratable acidity (%)				
dry	0.16	0.17	0.16	0.16
mill	0.54	0.88	0.52	0.65
salt	0.73	0.90	0.65	0.70
Salt added to curd (%)	2.50	2.50	2.25	2.75
Starter (CFU/g)$x10^{-6}$				
set		Not determined		
dry		"	"	
mill		"	"	
before salting		"	"	
after pressing		"	"	
Analysis at 14 d				
moisture (%)	34.4	34.6	33.8	33.5
fat (%)	36.0	36.0	36.0	36.0
NaCl (%)	1.36	1.33	1.52	1.49
pH	4.89	4.88	4.96	5.14
salt-in-moisture (%)	3.96	3.84	4.50	4.44
Flavour evaluation				
bitterness 3 m	1.3	3.1	2.5	1.4
(1-5) 6 m	1.0	3.7	1.9	1.0
overall score 3 m	5.4	2.7	3.8	4.5
(1-9) 6 m	6.2	1.9	4.6	4.4

Starter strain	AM_2	AM_2	AM_2	AM_2
Manufacture date	24.9.71	24.9.71	28.9.71	28.9.71
Special conditions		cook temp.		cook temp.
Inoculum (%)	2.0	2.25	2.0	2.3
Cut of coagulum (mm)	9.5	6.4	9.5	6.4
Cooking temp. (^{o}C)	37.8	33.3	37.8	33.3
Time (h:min)				
set to dry	2:55	2:45	2:45	2:40
dry to salt	2:45	2:00	3:00	1:50
total make time	5:40	4:45	5:45	4:30
Titratable acidity (%)				
dry	0.16	0.16	0.16	0.17
mill	0.54	0.56	0.53	0.52
salt	0.72	0.72	0.74	0.74
Salt added to curd (%)	2.5	2.75	2.25	2.75
Starter (CFU/g)x 10^{-6}				
set	150	170	110	140
dry	530	800	160	714
mill	350	2500	84	2550
before salting	280	5300	124	2580
after pressing	7.2	260	6.2	298
Analysis at 14 d				
moisture (%)	33.4	33.2	34.1	34.0
fat (%)	38.5	38.5	37.5	37.5
NaCl (%)	1.54	1.65	1.35	1.51
pH	4.96	4.92	5.02	4.96
salt-in-moisture (%)	4.62	4.97	3.96	4.44
Flavour evaluation				
bitterness 3 m	1.0	3.8	1.2	3.6
(1-5) 6 m	1.1	2.3	1.0	2.8
overall score 3 m	5.9	2.5	6.0	2.7
(1-9) 6 m	6.3	4.3	6.3	4.2

Starter strain		AM_2	AM_2	AM_2	AM_2
Manufacture date		5.10.71	12.10.71	28.1.72	3.2.72
Special conditions		normal manufacturing procedures			
Inoculum (%)		2.2	2.2	2.0	2.0
Cut of coagulum (mm)		9.5	9.5	9.5	9.5
Cooking temp. (^{o}C)		37.8	37.8	37.8	37.8
Time (h:min)					
set to dry		2:30	2:40	2:50	3:05
dry to salt		3:05	2:45	3:05	2:50
total make time		5:35	5:25	5:55	5:55
Titratable acidity (%)					
dry		0.15	0.15	0.14	0.16
mill		0.54	0.52	0.52	0.55
salt		0.72	0.71	0.68	0.80
Salt added to curd (%)		2.25	2.75	2.25	2.25
Starter (CFU/g)x 10^{-6}					
set		122	160	not determined	
dry		220	200	"	"
mill		300	150	"	"
before salting		57	84	"	"
after pressing		4.6	10	"	"
Analysis at 14 d					
moisture (%)		33.4	33.1	34.1	34.6
fat (%)		37.5	38.0	38.0	36.0
NaCl (%)		1.58	1.56	1.43	1.40
pH		5.01	4.97	4.98	4.91
salt-in-moisture (%)		4.73	4.71	4.19	4.04
Flavour evaluation					
bitterness	3 m	1.0	1.1	1.0	1.3
(1-5)	6 m	1.0	1.0	1.0	1.0
overall score	3 m	7.0	6.3	6.3	4.2
(1-9)	6 m	7.1	6.8	6.4	6.6

Starter strain	SK_{11}	SK_{11}	SK_{11}	SK_{11}
Manufacturing date	8.10.71	8.10.71	15.10.71	15.10.71
Special conditions		cook temp.		cook temp.
Inoculum (%)	2.3	2.0	2.3	2.6
Cut of coagulum (mm)	9.5	6.4	9.5	6.4
Cooking temp. (^{o}C)	37.8	33.3	37.8	33.3
Time (h:min)				
set to dry	2:50	2:35	2:55	2:55
dry to salt	3:40	2:55	3:30	2:25
total make time	6:30	5:30	6:25	5:20
Titratable acidity (%)				
dry	0.16	0.15	0.15	0.16
mill	0.51	0.52	0.53	0.51
salt	0.67	0.66	0.67	0.67
Salt added to curd (%)	2.75	2.75	2.75	2.50
Starter (CFU/g)x 10^{-6}				
set	220	200	160	200
dry	480	2400	150	1700
mill	360	3600	82	2300
before salting	270	4400	12	1100
after pressing	5.9	280	1.0	150
Analysis at 14 d				
moisture (%)	35.4	34.9	35.7	34.7
fat (%)	36.0	36.0	34.5	34.5
NaCl (%)	1.71	1.68	1.52	1.56
pH	4.98	4.97	4.95	4.94
salt-in-moisture (%)	4.83	4.81	4.26	4.49
Flavour evaluation				
bitterness 3 m	1.0	3.7	1.1	3.5
(1-5) 6 m	1.1	3.2	1.0	2.5
overall score 3 m	6.5	3.1	6.6	3.1
(1-9) 6 m	6.9	3.2	6.9	4.5

Starter strain		ML_8	ML_8	ML_8
Manufacture date		5.10.71	5.10.71	5.10.71
Special conditions		Cooking temperature		
Inoculum (%)		2.2	1.7	1.8
Cut of coagulum (mm)		6.4	9.5	12.7
Cooking temp. (^{o}C)		33.3	37.8	39.4
Time (h:min)				
set to dry		2:35	2:40	2:50
dry to salt		2:05	2:35	3:10
total make time		4:40	5:15	6:00
Titratable acidity (%)				
dry		0.17	0.16	0.16
mill		0.54	0.53	0.52
salt		0.74	0.73	0.60
Salt added to curd (%)		2.75	2.38	2.75
Starter (CFU/g)x 10^{-6}				
set		140	96	190
dry		4300	3500	2200
mill		4300	5800	2700
before salting		5200	4700	2400
after pressing		4300	4050	1400
Analysis at 14 d				
moisture (%)		33.4	32.7	31.3
fat (%)		37.5	37.5	37.5
NaCl (%)		1.58	1.64	1.54
pH		5.01	5.02	5.16
salt-in-moisture (%)		4.73	5.02	4.92
Flavour evaluation				
bitterness	3 m	2.5	2.4	1.1
(1-5)	6 m	3.8	3.0	2.2
overall score	3 m	2.8	3.3	4.4
(1-9)	6 m	2.1	3.1	3.7

Starter strain		ML_8	ML_8	ML_8
Manufacture date		12.10.71	12.10.71	12.10.71
Special conditions			Cooking temperature	
Inoculum (%)		1.8	1.8	2.2
Cut of coagulum (mm)		6.4	9.5	12.7
Cooking temp. (^{o}C)		33.3	37.8	39.4
Time (h:min)				
set to dry		2:35	2:45	2:55
dry to salt		2:25	2:25	3:10
total make time		5:00	5:10	6:05
Titratable acidity (%)				
dry		0.15	0.15	0.15
mill		0.52	0.55	0.51
salt		0.71	0.71	0.62
Salt added to curd (%)		2.75	2.75	2.50
Starter (CFU/g)x 10^{-6}				
set		170	160	190
dry		2400	3400	2100
mill		5800	4700	3200
before salting		5200	4400	2700
after pressing		4300	3700	840
Analysis at 14 d				
moisture (%)		33.1	33.1	31.6
fat (%)		38.0	38.0	38.0
NaCl (%)		1.61	1.59	1.42
pH		5.01	5.04	5.06
salt-in-moisture (%)		4.86	4.80	4.50
Flavour evaluation				
bitterness	3 m	3.5	2.9	2.3
(1-5)	6 m	3.8	3.3	2.3
overall score	3 m	3.6	3.0	3.7
(1-9)	6 m	2.6	2.9	3.9

Starter strain		HP	HP	HP	HP	HP
Manufacture date		24.9.71	24.9.71	5.1.72	5.1.72	5.1.72
Special conditions		Cook temp.		Cook temp.		
Inoculum (%)		1.7	1.5	1.8	1.8	1.8
Cut of coagulum (mm)		9.5	12.7	9.5	9.5	12.7
Cooking temp. (^{o}C)		37.8	39.4	36.7	37.8	39.4
Time (h:min)						
set to dry		2:45	3:05	2:50	2:50	3:10
dry to salt		2:15	3:05	1:55	2:00	2:55
total make time		5:00	6:10	4:45	4:50	6:05
Titratable acidity (%)						
dry		0.17	0.16	0.17	0.17	0.17
mill		0.52	0.50	0.54	0.54	0.55
salt		0.71	0.65	0.71	0.71	0.67
Salt added to curd (%)		2.25	2.75	2.50	2.50	2.00
Starter (CFU/g)x 10^{-6}						
set		63	59	78	87	91
dry		492	452	715	750	540
mill		not recorded		1050	1100	360
before salting		735	132	930	980	230
after pressing		269	156	340	370	78
Analysis at 14 d						
moisture (%)		33.6	31.5	32.5	32.3	32.6
fat (%)		38.5	39.0	38.0	38.0	38.0
NaCl (%)		1.55	1.49	1.56	1.62	1.43
pH		4.95	5.16	4.89	4.93	4.94
salt-in-moisture (%)		4.62	4.73	4.80	5.02	4.38
Flavour evaluation						
bitterness	3 m	3.0	2.0	3.3	3.3	2.5
(1-5)	6 m	2.9	1.1	3.6	3.2	2.4
overall score	3 m	3.8	4.0	2.5	3.0	3.5
(1-9)	6 m	3.9	5.1	2.5	2.7	3.6

Starter strain		E_8	E_8	E_8	E_8
Manufacture date		27.10.71	27.10.71	23.11.71	23.11.71
Special conditions		Cook temp.		Cook temp.	
Inoculum (%)		2.5	2.5	3.0	3.0
Cut of coagulum (mm)		9.5	6.4	9.5	9.5
Cooking temp. (^{o}C)		37.8	33.3	37.8	33.3
Time (h:min)					
set to dry		2:45	3:00	2:45	3:00
dry to salt		3:25	3:00	3:50	3:20
total make time		6:10	6:00	6:35	6:20
Titratable acidity (%)					
dry		0.16	0.16	0.16	0.16
mill		0.51	0.54	0.50	0.51
salt		0.58	0.68	0.71	0.73
Salt added to curd (%)		2.63	2.63	2.75	3.0
Starter (CFU/g)x 10^{-6}					
set		130	130	195	190
dry		510	1910	1100	1930
mill		1100	2700	1270	2600
before salting		1400	1700	900	2120
after pressing		470	1100	550	1170
Analysis at 14 d					
moisture (%)		34.2	34.1	33.7	33.7
fat (%)		36.0	36.0	36.5	36.5
NaCl (%)		1.70	1.68	1.61	1.73
pH		4.96	4.93	5.04	5.03
salt-in-moisture (%)		4.97	4.93	4.77	5.14
Flavour evaluation					
bitterness	3 m	2.5	3.5	2.1	3.0
(1-5)	6 m	2.3	3.0	1.3	2.6
overall score	3 m	3.9	3.1	4.4	3.1
(1-9)	6 m	4.5	3.2	6.1	3.7

Starter strain	ML_1	ML_1	ML_1	ML_1
Manufacture date	4.11.71	4.11.71	26.11.71	26.11.71
Special conditions		Cook temp.		Cook temp.
Inoculum (%)	3.0	3.0	3.0	3.0
Cut of coagulum (mm)	9.5	6.4	9.5	6.4
Cooking temp (^{o}C)	37.8	33.9	37.8	33.9
Time (h:min)				
set to dry	2:55	2:55	2:50	2:55
dry to salt	3:20	2:25	3:40	2:35
total make time	6:15	5:20	6:30	5:30
Titratable acidity (%)				
dry	0.17	0.18	0.17	0.17
mill	0.50	0.52	0.52	0.52
salt	0.50	0.65	0.67	0.67
Salt added to curd (%)	2.75	2.75	2.50	2.50
Starter (CFU/g)x 10^{-6}				
set	160	160	206	174
dry	1200	2500	1150	2140
mill	840	1600	525	1900
before salting	560	1500	462	2560
after pressing	450	1500	335	2020
Analysis at 14 d				
moisture (%)	33.1	33.2	33.8	32.8
fat (%)	36.5	37.0	36.5	36.5
NaCl (%)	1.80	1.74	1.64	1.70
pH	4.94	4.95	5.05	5.03
salt-in-moisture (%)	5.44	5.24	4.84	5.18
Flavour evaluation				
bitterness 3 m	1.5	4.5	2.3	3.5
(1-5) 6 m	2.5	4.2	1.3	3.0
overall score 3 m	5.5	2.0	4.3	3.4
(1-9) 6 m	4.0	1.2	5.0	3.0

Starter strain	Z_8	Z_8	Z_8	Z_8
Manufacture date	14.1.72	14.1.72	14.1.72	14.1.72
Special conditions	Cooking temperature			
Inoculum (%)	1.25	1.5	2.0	2.25
Cut of coagulum (mm)	6.4	9.5	9.5	12.7
Cooking temp. (^{o}C)	33.3	35.6	37.8	39.4
Time (h:min)				
set to dry	3:10	3:00	2:50	3:00
dry to salt	2:15	2:25	2:35	2:45
total make time	5:25	5:25	5:25	5:45
Titratable acidity (%)				
dry	0.16	0.15	0.16	0.16
mill	0.51	0.50	0.50	0.53
salt	0.67	0.66	0.66	0.56
Salt added to curd (%)	2.38	2.38	2.38	2.38
Starter (CFU/g)x 10^{-6}				
set	46	57	59	78
dry	2100	2100	1050	780
mill	2400	1850	1030	630
before salting	2800	1900	970	610
after pressing	980	685	610	205
Analysis at 14 d				
moisture (%)	33.1	32.0	31.5	32.0
fat (%)	38.0	38.0	38.0	38.0
NaCl (%)	1.39	1.42	1.41	1.48
pH	4.95	4.96	4.97	4.97
salt-in-moisture (%)	4.18	4.44	4.47	4.62
Flavour evaluation				
bitterness 3 m	3.8	3.4	2.9	2.0
(1-5) 6 m	4.0	3.8	3.6	2.9
overall score 3 m	2.5	2.8	3.4	4.4
(1-9) 6 m	2.1	2.6	2.6	3.7

Starter strain		Z_8	Z_8	Z_8	Z_8
Manufacture date		19.1.72	19.1.72	19.1.72	19.1.72
Special conditions			Cooking temperature		
Inoculum (%)		1.75	1.75	2.0	2.0
Cut of coagulum (mm)		6.4	9.5	9.5	12.7
Cooking temp. (^{o}C)		33.3	35.6	37.8	39.4
Time (h:min)					
set to dry		3:00	3:00	3:00	3:00
dry to salt		2:35	2:55	3:10	3:30
total make time		5:35	5:55	6:10	6:45
Titratable acidity (%)					
dry		0.16	0.16	0.16	0.15
mill		0.53	0.51	0.51	0.49
salt		0.70	0.70	0.72	0.65
Salt added to curd (%)		2.38	2.25	2.25	2.13
Starter (CFU/g)x 10^{-6}					
set		64	74	69	78
dry*					
mill*		phage titre in whey before salting x 10^{-9}			
before salting*		100	9.5	30	30
after pressing*					
Analysis at 14 d					
moisture (%)		32.2	31.1	31.1	32.7
fat (%)		38.5	38.5	38.5	38.5
NaCl (%)		1.64	1.58	1.58	1.36
pH		4.98	5.00	5.00	5.05
salt-in-moisture (%)		5.08	5.08	5.08	4.16
Flavour evaluation					
bitterness	3 m	1.7	1.2	1.8	1.0
(1-5)	6 m	1.2	1.2	1.1	1.0
overall score	3 m	5.6	5.7	5.0	5.4
(1-9)	6 m	6.1	5.9	5.9	6.1

*Severely contaminated by bacteriophage

Starter strain	KH	132	166	166
Manufacture date	3.2.72	3.2.72	15.2.72	15.2.72
Special conditions		no phage	no phage,	cook temp.
Inoculum (%)	2.25	2.25	2.1	2.1
Cut of coagulum (mm)	9.5	9.5	9.5	9.5
Cooking temp. (^{o}C)	37.8	37.8	37.8	39.4
Time (h:min)				
set to dry	3:25	2:45	2:30	2:35
dry to salt	3:10	2:10	2:10	2:35
total make time	6:35	4:55	4:40	5:10
Titratable acidity (%)				
dry	0.15	0.16	0.15	0.15
mill	0.48	0.52	0.50	0.52
salt	0.59	0.67	0.66	0.66
Salt added to curd (%)	1.88	2.38	2.50	2.25
Starter (CFU/g)x 10^{-6}				
set	170	145	72	72
dry	1050	1330	920	670
mill	940	1400	1500	730
before salting	1200	1350	1300	570
after pressing	700	550	1500	640
Analysis at 14 d				
moisture (%)	35.4	33.9	33.7	33.7
fat (%)	36.0	36.0	38.0	38.0
NaCl (%)	1.36	1.67	1.43	1.65
pH	4.95	4.93	4.98	4.92
salt-in-moisture (%)	3.84	4.93	4.24	4.89
Flavour evaluation				
bitterness 3 m	1.9	2.5	3.1	2.8
(1-5) 6 m	2.1	2.9	3.3	2.9
overall score 3 m	3.6	3.0	3.4	3.3
(1-9) 6 m	3.6	2.9	2.9	2.9

Starter strain		132	132	132	132	132
Manufacture date		9.2.72	9.2.72	9.2.72	11.2.72	11.2.72
Special conditions			Cook temp.			Cook temp.
Inoculum (%)		2.25	2.25	2.25	2.25	2.25
Cut of coagulum (mm)		9.5	12.7	12.7	12.7	12.7
Cooking temp. (^{o}C)		33.3	37.8	39.4	37.8	39.4
Time (h:min)						
set to dry		2:45	2:45	3:00	2:45	2:55
dry to salt		1:40	3:00	3:30	2:50	3:40
total make time		4:25	5:45	6:30	5:35	6:35
Titratable acidity (%)						
dry		0.16	0.16	0.17	0.15	0.15
mill		0.54	0.50	0.43	0.52	0.47
salt		0.69	0.70	0.46	0.66	0.56
Salt added to curd (%)		2.25	2.00	1.75	2.50	2.50
Starter (CFU/g)x 10^{-6}						
set		110	93	90	100	88
dry		2200	1100	680	660	610
mill		3100	1000	140	900	230
before salting		3000	1000	47	840	115
after pressing		3200	500	41	530	27
Analysis at 14 d						
moisture (%)		34.5	33.4	35.0	34.0	33.6
fat (%)		37.0	37.0	38.0	38.0	38.0
NaCl (%)		1.51	1.49	1.39	1.48	1.48
pH		4.88	4.95	5.12	4.95	5.01
salt-in-moisture (%)		4.38	4.46	3.97	4.35	4.41
Flavour evaluation						
bitterness	3 m	4.1	2.7	1.8	3.3	1.1
(1-5)	6 m	3.8	2.8	1.9	3.0	1.0
overall score	3 m	1.8	3.8	4.8	2.8	6.7
(1-9)	6 m	2.6	3.9	4.8	3.0	7.0

Starter Strain		HP	HP	HP	HP
Manufacture date		9.9.71	9.9.71	9.9.71	9.9.71
Special conditions		phage added to initial level of			
		no addition	10/ml	100/ml	5000/ml
Inoculum (%)		1.5	1.5	1.5	1.5
Cut of coagulum (mm)		9.5	9.5	9.5	9.5
Cooking temp. (^{o}C)		37.8	37.8	37.8	37.8
Time (h:min)					
set to dry		2:45	2:45	2:45	2:45
dry to salt		2:00	2:00	2:10	3:20
total make time		4:45	4:45	4:55	6:05
Titratable acidity (%)					
dry		0.17	0.17	0.17	0.17
mill		0.55	0.56	0.54	0.55
salt		0.70	0.72	0.71	0.65
Salt added to curd (%)		2.50	2.50	2.50	2.25
Starter (CFU/g)x 10^{-6}					
set		74	bacteriophage present		
dry		400	"	"	"
mill		1030	"	"	"
before salting		772	"	"	"
after pressing		302	"	"	"
Analysis at 14 d					
moisture (%)		33.9	33.5	33.5	34.2
fat (%)		38.0	38.0	38.0	37.5
NaCl (%)		1.45	1.48	1.45	1.45
pH		4.93	4.92	4.90	5.16
salt-in-moisture (%)		4.28	4.42	4.31	4.32
Flavour evaluation					
bitterness	3 m	3.3	2.7	2.0	1.5
(1-5)	6 m	3.0	2.5	2.1	1.3
overall score	3 m	2.1	2.5	3.4	4.0
(1-9)	6 m	2.8	3.8	5.2	4.6

Starter strain		HP	HP	HP	HP
Manufacture date		14.9.71	14.9.71	14.9.71	14.9.71
Special conditions		no addition	phage added to initial level of 50/ml	500/ml	2000/ml
Inoculum (%)		1.5	1.5	1.5	1.5
Cut of coagulum (mm)		9.5	9.5	9.5	9.5
Cooking temp. (°C)		37.8	37.8	37.8	37.8
Time (h:min)					
set to dry		2:45	2:50	2:50	2:50
dry to salt		2:05	2:05	2:20	2:50
total make time		4:50	4:55	5:10	5:40
Titratable acidity (%)					
dry		0.17	0.17	0.17	0.17
mill		0.5	0.53	0.53	0.52
salt		0.72	0.72	0.72	0.72
Salt added to curd (%)		2.50	2.50	2.50	2.50
Starter (CFU/g)x 10^{-6}					
set			Bacteriophage present		
dry			"	"	"
mill			"	"	"
before salting			"	"	"
after pressing			"	"	"
Analysis at 14 d					
moisture (%)		33.5	32.1	33.0	33.0
fat (%)		38.0	38.0	38.0	38.0
NaCl (%)		1.43	1.54	1.49	1.48
pH		5.04	5.02	5.01	5.02
salt-in-moisture (%)		4.28	4.80	4.52	4.49
Flavour evaluation					
bitterness	3 m	3.1	2.3	1.8	1.3
(1-5)	6 m	3.8	2.5	1.5	1.4
overall score	3 m	2.8	3.8	5.2	4.6
(1-9)	6 m	2.9	3.1	6.0	5.9

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