A Study of the Distribution of Strains of *Streptococcus lactis* Which Are Sensitive to a Filterable Inhibitory Principle from Slow Starters[1]

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BUTTER cultures which undergo a sudden loss of vitality and require excessive periods of time to ripen to the desired degree of acidity are encountered rather frequently in dairy plants, especially where considerable quantities of culture are ripened in large containers. The longer ripening period which is necessary under these conditions is associated commonly with a decrease in desirable flavor and aroma and with characteristic off-flavors. Butter cultures of this type are usually designated as "slow" cultures and may be the cause of considerable difficulty in the manufacture of butter, cheese and cultured milks. Some butter cultures are more subject than others to this type of retarded acid development. This study was undertaken in an effort to determine the distribution of types of *Streptococcus lactis* sensitive to the inhibitory action of bacteria-free filtrates from slow butter cultures.

HISTORICAL

In 1933 the Iowa Agricultural Experiment Station (1) reported that a bacteria-free filtrate from a slow butter culture would delay the coagulation of a normal one, resulting in a characteristic slow culture. The restraining action was found to be destroyed by boiling the filtrate.

Harriman (3) made an exhaustive study of the suddenly-appearing type of slow butter culture and found that the growth of some freshly inoculated butter cultures could be markedly restrained by the addition, at the time of inoculation, of certain other cultures, especially those which

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had previously shown slow acid development or had been grown in large lots. By preliminary filtration of the coagulated cultures through filter paper, followed by filtration through grade N Berkfeld ultrafilters, bacteria-free filtrates were obtained from some of the cultures which showed the inhibitory effect when added to other cultures. Using seven butter cultures and one culture of *S. lactis* as test organisms, 11 of the 19 filtrates from mother cultures and 22 of the 23 filtrates from large lots of butter culture were found to have a marked inhibitory effect on the acid production of one or more of the test cultures after a 16-hour period of incubation at 21° C. These filtrates were shown to cause a marked inhibition of growth, the number of organisms in the cultures at the 16-hour interval being markedly less than the number in the check cultures to which no filtrate had been added. The ratio of citric acid-fermenting streptococci to *S. lactis* frequently was unusually high in the retarded cultures. The surviving *S. lactis* strains were apparently normal in their ability to grow and coagulate milk, the result being that at 24 hours the acidities and counts of the retarded starters approached those of the control cultures. Using pure *S. lactis* cultures and inhibitory filtrates, counts under 500,000 per ml. were obtained frequently at the 16-hour interval, when the check cultures without added filtrate gave counts ranging from 219,000,000 to 1,750,000,000 per ml. Since the filtrates varied in their action toward the butter cultures and the *S. lactis* culture used as test organisms, Harriman (3) suggested that possibly different filtrates had varying degrees of restraining ability and that the ability to resist the action of filtrates varied among cultures, the inference being that resistance was a quantitative rather than a qualitative characteristic. Studies of the filtrates themselves showed that some of them had a marked restraining effect in dilutions as great as 1 part of filtrate in 20,000 parts of milk. The inhibitory principle was found to be completely inactivated by heating to 60° C. for five minutes in all but one case, in which 30 minutes were required. Holding at 50° C. for 30 minutes resulted in a decrease in the "virulence" of the filtrate but not in complete inactivation. Attempts to increase the titers of the filtrates by adding them to butter cultures and recovering them following coagulation were unsuccessful. The active principle apparently was closely related to a bacteriophage, but this investigator did not designate it as a bacteriophage.

Whitehead and Cox (6) succeeded in isolating from a slow starter a bacteriophage active against *Streptococcus cremoris* and considered this bacteriophage to be the causative agent of slowness in starters of the type which they were then investigating. After several propagations at the expense of a sensitive culture of *S. cremoris* the bacteriophage was obtained in such strength that one part in several hundred million parts of milk "sufficed to show the characteristic lysis with a susceptible streptococcus." Characteristic plaques were obtained on solid media. These investigators suggested that the isolation of nonsensitive strains of lactic acid strepto-

cocci for use in butter cultures might be the means of solving the problem of slow butter cultures.

The literature contains a few other references to bacteriophages active against *S. lactis* and related organisms. In 1926 Hadley and Dabney (2) reported the isolation of a bacteriophage active against *S. lactis*. They obtained their bacteriophage from sewage. Another bacteriophage from the same source was active against *Streptococcus fecalis* and was inhibitory toward *S. lactis* and toward *Bacterium coli* also. McKinley (5) mentioned *S. lactis* and *S. fecalis* among the organisms susceptible to bacteriophage activity. Klingmüller (4) was unable to isolate bacteriophages inhibitory toward *S. lactis* or *S. mastiditis* from old cultures of the organisms or from dung filtrates.

METHODS

The *S. lactis* cultures used in this study were isolated from butter cultures from various sources, from raw milk allowed to sour spontaneously and from raw-milk whey. The samples were plated on tomato juice agar and representative colonies picked and inoculated into sterile litmus milk. Unless a culture appeared as a typical *S. lactis* organism in litmus milk and coagulated the milk within 48 hours at room temperature, it was discarded.

Inhibition of the separate strains of *S. lactis* was determined by the addition of 0.1 ml. of filtrate and one drop of a 24-hour litmus milk culture to approximately 8.0 ml. of litmus milk in a test tube. Included in each series were controls from which the filtrate had been omitted. The cultures were incubated at room temperature (about 22° C.) during these experiments. When the reduction or coagulation of the litmus milk cultures to which filtrate had been added required an appreciably longer period than was necessary for the reduction and coagulation of the control cultures to which no filtrate had been added, the filtrate used was considered to be inhibitory to the test organism. Occasionally delayed reduction of the litmus was the only means of detecting inhibition, for the secondary growth was sufficiently rapid to cause coagulation to occur as rapidly in the inhibited culture as in the control. This was especially true of cultures which required more than 30 hours to coagulate under normal conditions.

When a preliminary run on a group of organisms from a single source showed no differences among the members of the group, only one or two representative organisms were used for further studies.

The filtrates used in testing any one group of organisms varied from time to time because an endeavor was made to use the greatest possible variety of filtrates for each group and the filtrate collection was constantly increasing in numbers because of new additions. The origins of the various filtrates used are indicated in table 1. These filtrates were, with three exceptions, originally isolated from butter cultures which suddenly

TABLE 1. *The sources of the bacteria-free filtrates*

Filtrate	Date obtained	Source	Remarks
1	10-19-34	B. C. 122 + 232	Slow in 10-gal. can in butter laboratory
2	10-19-34	B. C. 232	Slow in 10-gal. can in butter laboratory
10	10-25-34	B. C. 232	Slow in 140 ml. aerated sterilized milk
13	10-27-34	B. C. 15/3+F191	
14	10-27-34	B. C. 232+F1	
21	11-13-34	B. C. 103	Slow in laboratory
29	11-23-34	F10	Propagated one generation on *S. lactis* 42
30	11-23-34	F14	Propagated one generation on *S. lactis* 42
32	11-23-34	F191	Propagated one generation on *S. lactis* 42
35	12-16-34	F10	Propagated two generations on *S. lactis* 42
41	1-16-35	B. C. 15/3	Slow in 10-gal. can in butter laboratory
51	1-25-35	B. C. 15/1	Normal but gave slow transfer in laboratory
52	1-25-35	B. C. 15/1	Slow in 140 ml. past. milk in laboratory
53	1-25-35	B. C. 26	Slightly slow in 140 ml. past. milk in laboratory
54	1-25-35	B. C. 232	Slow in 140 ml. past. milk in laboratory
55	1-25-35	B. C. 15/3	Slow in 140 ml. past. milk in laboratory
61	2-13-35	B. C. 15/3	Slow in 1-gal. can of past. milk in laboratory
80	3- 9-35	F191	Propagated once on *S. lactis* 163 and once on *S. lactis* 42
82	3- 9-35	F21	Propagated once on *S. lactis* 97 and once on *S. lactis* 42
124	4-11-35	F191	Propagated once on *S. lactis* 99
134	5- 2-35	F54	Propagated once on *S. lactis* 111
135	5- 2-35	Commercial starter	Propagated once on *S. lactis* 97
157	5-31-35	F191	Propagated four times on *S. lactis* 99
158	5-31-35	F191	Propagated three times on *S. lactis* 147
159	9-26-35	B. C. 232	Slow in 400-gal. vat
191	.....	B. C. 15	Slow (filtrate from Harriman)
206	11- 5-35	F61	Propagated five times on *S. lactis* 97
207	11- 5-35	B. C. 232	Plaque isolation propagated five times on *S. lactis* 97
208	11- 5-35	F191	Propagated five times on *S. lactis* 147
209	11- 5-35	Slow starter	Bacteriophage R from Whitehead
210	11- 5-35	Slow starter	Bacteriophage RW from Whitehead
256	12-12-35	F191	Propagated once on *S. lactis* 153A
257	12-12-35	F54	Propagated once on *S. lactis* 111 and once on *S. lactis* 153-0.
315	2- 6-36	B. C. 122F	Slow in 10-gal. can in butter laboratory

B. C. = butter culture.
F = filtrate.

had become slow, either in the laboratory or in the college dairy plant where they were used for buttermaking purposes. The coagulated cultures first underwent a preliminary filtration through filter paper to remove the coagulated casein and were then filtered through grade N Berkfeld filters to remove all bacteria. The utensils used in handling the filtrates both before and after filtration were sterilized before use at 15 pounds pressure for 25 minutes in an autoclave. The filtrates were stored at approximately 6° C.

The filtrates which are designated as having been propagated on strains of *S. lactis* were obtained by the addition of a loopful of the designated sensitive *S. lactis* culture and 1.0 ml. of the designated filtrate to 100 ml. of sterile litmus milk, the interval between inoculation and addition of filtrate being as much as 12 hours in the cases of some of the more "virulent" filtrates. The cultures were held at room temperature for approximately 24 hours following the addition of the filtrate before they were filtered. Addition of lactic acid to cause coagulation before the preliminary filtration was necessary in most cases, the growth of the organisms having been sufficiently inhibited by the presence of the added filtrate to prevent the formation of appreciable amounts of lactic acid.

EXPERIMENTAL RESULTS

The results of the tests of the susceptibility of various strains of *S. lactis* to inhibition by a series of bacteria-free filtrates from slow butter cultures are summarized in table 2.

Strains of *S. lactis* isolated from milk or whey which had been allowed to sour without the addition of butter culture were never found to be susceptible to any of the filtrates with which they were tested. The filtrates tabulated are only representative of a considerably larger number which were used in the study in an effort to find one or more filtrates to which members of this group of organisms might be sensitive. Apparently naturally-occurring organisms are not susceptible to inhibition by filtrates obtained from slow butter cultures, although it is possible that filtrates which would inhibit organisms of this group could be obtained if a sufficiently large number of filtrates from a greater range of sources were examined.

Examination of the cultures of *S. lactis* obtained from butter cultures revealed that some butter cultures yielded only strains which were subject to inhibition by some of the filtrates used in this study, some yielded only nonsusceptible strains, while both sensitive and nonsensitive strains were obtained from others. The last group included the majority of the butter cultures examined in any detail during the studies herein reported.

The results obtained from a study of the organisms obtained from butter culture 146 will be discussed in some detail, as they are more or less characteristic of those obtained with the groups isolated from other butter cultures in the series. At the time of the first isolations four different sensitivity types were recognizable among the six cultures studied. One type represented by two cultures was not sensitive to any filtrate then available; a second type represented by a single culture was sensitive to filtrate 191 only; a third type represented by two cultures was sensitive to two filtrates, 13 and 191; a fourth type represented by a single culture was sensitive to filtrates 13, 52, 53 and 191 and not sensitive to filtrates, 21, 205, 206, 207, 208, 209 and 210, which formed the remainder of the test group. Seven days after the first isolations were made, 18 other cultures were obtained from the same butter culture (No. 146) which

TABLE 2. *Summary of tests to determine the susceptibility of S. lactis strains to inhibition by bacteria-free filtrates*

Source	Date of isolation	No. of cultures isolated	Filtrates which inhibit	Filtrates which do not inhibit
Raw milk whey	11- 5-34	8		10, 14, 30, 32, 35, 53, 61, 124, 134, 135, 157, 159, 191
Soured raw milk	11- 5-34	10		10, 14, 30, 32, 35, 53, 61, 124, 134, 135, 157, 159, 191
Soured raw milk P1	10- 6-35	5		13, 21, 52, 53, 61, 134, 135, 157, 158, 159, 191
Soured raw milk P5	10- 6-35	5		13, 21, 52, 53, 61, 134, 135, 157, 158, 159, 191
Soured raw milk P6	10- 6-35	6		13, 21, 52, 53, 61, 134, 135, 157, 158, 159, 191
Soured raw milk P7	10- 6-35	6		13, 21, 52, 53, 61, 134, 135, 157, 158, 159, 191
Soured raw milk P8	10- 6-35	6		13, 21, 52, 53, 61, 134, 135 157, 158, 159, 191
B. C. 146	11- 6-35	6 2		13, 21, 52, 53, 191, 205, 206, 207, 208, 209, 210
		1	191	13, 21, 52, 53, 205, 206, 207, 208, 209, 210
		2	13, 191	21, 52, 53, 205, 206, 207, 208, 209, 210
		1	13, 52, 53, 191	21, 205, 206, 207, 208, 209, 210
	11-13-35	18		35, 134, 157, 191, 192, 205, 206, 207, 208, 209, 210
	3- 4-36	13 7		35, 55, 134, 157, 191, 206, 208, 315
		4	55, 134, 315	35, 157, 191, 206, 208
		1	55, 315	35, 134, 157, 191, 206, 208
		1	55, 134, 206	35, 157, 198, 208, 315
B. C. 122F	11-13-35	15	134, 191	35, 157, 192, 205, 206, 207, 208, 209, 210
	3- 4-36	9 1	315	35, 134, 157, 191, 206, 208, 315
		3	55, 191, 208	35, 134, 157, 206, 315
		3	55, 191, 315	35, 134, 157, 206, 208
		2	55, 134, 191, 315	35, 157, 206, 208
B. C. 232	11- 8-34	9	10, 14, 30, 32, 35, 41, 53, 61, 134, 157, 191	2, 21, 54, 132, 135, 159
	11-15-34	5 4	10, 14, 30, 35, 54, 61, 134, 135, 157, 191	32, 159
		1	10, 14, 30, 54, 61, 134, 135, 159	32, 35, 157, 191
	1-14-35	3 2	29, 35, 53, 61, 134, 135, 157, 191	30, 159, 92
		1	41, 53, 61, 92, 134	29, 30, 35, 54, 135, 157, 159, 191

P = patron.

B. C. = butter culture.

TABLE 2. (Continued)

Source	Date of isolation	No. of cultures isolated		Filtrates which inhibit	Filtrates which do not inhibit
B. C. 232	12- 4-35	22	21		13, 14, 35, 41, 51, 134, 157, 191, 206, 209, 210
		1	1	41, 134, 191, 257	13, 14, 35, 51, 157, 206, 209. 210
B. C. 15/3	11-15-34	5	2	14, 32, 191	2, 10, 30
			1	10, 14, 30, 35, 41, 53, 61, 157, 191	2, 32, 159
			2	10, 32, 35, 41, 53, 61	2, 14, 30, 157, 159
	3-18-35	1		13, 53, 61, 134, 157, 191	21, 52, 135, 158, 159
	12- 3-35	25	21		13, 14, 35, 41, 51, 134, 157, 191, 206, 209, 210
			2	191, 256	13, 14, 35, 41, 51, 134, 157, 206, 209, 210, 257
			2	41, 134, 257	13, 14, 35, 51, 157, 191, 206, 209, 210, 256
	3- 4-36	5	3		35, 55, 134, 157, 191, 206, 208, 315
			2	315	35, 55, 134, 157, 191, 206, 208
B. C. A	4- 8-35	4			13, 21, 52, 53, 61, 134, 135, 157, 158, 159, 191
B. C. LOL	3- 4-36	14			35, 55, 134, 157, 191, 206, 208, 315
B. C. M_1	11-19-35	25			13, 14, 35, 41, 51, 134, 157, 191, 206, 209, 210
B. C. 103	11-13-35	25			35, 134, 157, 191, 192, 205, 206, 207, 208, 209, 210
B. C. 19/1	11-15-34	5			10, 14, 35, 41, 53, 61, 132, 134, 135, 157, 158, 159, 191
B. C. H	11-15-34	7	4		2, 10, 14, 30, 32, 52, 53, 61, 134, 135, 157, 158, 159, 191
			1	134	2, 10, 14, 30, 32, 52, 53, 61, 135, 157, 158, 159, 191
			2	10, 14, 30, 32, 53, 61, 134, 157, 191	2, 132, 135, 158, 159
B. C. S	1-14-35	4	1	13, 61, 135, 158, 191	21, 35, 51, 52, 53, 80, 82, 134, 157, 159
			1	80, 82, 191	21, 35, 52, 53, 61, 134, 157, 158, 159
			2	13, 41, 61, 80, 135, 158, 191	21, 35, 52, 53, 134, 157, 159
B. C. 233	12- 7-35	12	11		13, 14, 35, 41, 51, 134, 157, 191, 206, 209, 210
			1	13, 41, 191, 256, 257	14, 35, 51, 157, 206, 209, 210
B. C. 15/1	12-10-35	23	4		13, 14, 35, 41, 51, 134, 157, 191, 206, 209, 210
			13	13, 14, 41, 134, 157, 191, 256	35, 51, 206, 209, 210, 257

TABLE 2. (Continued)

Source	Date of isolation	No. of cultures isolated	Filtrates which inhibit	Filtrates which do not inhibit
		2	13, 41, 134, 191, 256, 257 (13 neg. for one cult.)	14, 35, 51, 157, 206, 209, 210
		4	13, 14, 134, 157, 191 (14 neg. for one cult.)	35, 41, 51, 206, 209, 210
	2- 4-36	14		35, 55, 134, 157, 191, 206, 208, 315
B. C. FL	11-25-35	26 22		13, 14, 35, 41, 134, 157, 191, 206, 209, 210
		2	41, 134, 206	13, 14, 35, 51, 157, 191, 209, 210
		2	14, 41, 134, 206, 257	13, 35, 51, 157, 191, 209, 210, 256
B. C. 22	12- 6-35	23 7		13, 14, 35, 41, 51, 134, 157, 191, 206, 209, 210
		2	13, 41, 191, 206, 256	14, 35, 51, 134, 157, 209, 210, 257
		14	41, 134, 257	13, 14, 35, 51, 157, 191, 206, 256

had been transferred daily in specially pasteurized whole milk. This second group of cultures was uninhibited by any of 11 representative filtrates, apparently being identical with the nonsensitive group obtained from the previous isolations. About four months later 13 additional cultures were obtained from the same butter culture. Seven of these cultures were uninhibited by any filtrate used; a second group of four organisms was inhibited by filtrates 55, 134 and 315 and uninhibited by filtrates 35, 157, 191, 206 and 208; a third type represented by a single culture was inhibited by filtrates 55 and 315; and a fourth type also represented by only one culture was sensitive to filtrates 55, 134 and 206. The *S. lactis* organisms in this butter culture (No. 146) were shown to change from dominantly sensitive types with only a small percentage of nonsensitive cultures to dominantly nonsensitive types with the percentage of sensitive types too small to be detected by a random picking of 18 cultures and then back to sensitive and nonsensitive types in almost equal numbers. Possibly further changes were undergone between the periods of observation. The organisms obtained at any one time showed a group similarity in that all sensitive cultures were inhibited by some one filtrate, 191 in the first group and 55 in the third group, even though their sensitivity to other filtrates varied. The organisms obtained in the first group showed no similarity in sensitivity characteristics to those in the third group, indicating that the period of nonsensitivity during which the second group of organisms was obtained was apparently a definite entity following which a new group

of sensitive organisms inhibited by other types of filtrates was developed.

The *S. lactis* strains obtained from butter cultures 15/3 and 232 were also studied quite intensively and showed a marked tendency toward variation of the sensitivity types, not only among the organisms isolated at one time but also among the organisms obtained over rather extended periods of time. Butter culture 122F is of special interest because it at one time apparently contained only *S. lactis* cultures of a single sensitivity type. This condition proved to be only transitory, however, since four different types were obtained from the next group of organisms which was isolated almost four months later. Butter cultures A, M_1, 103, LOL and 19/1 were unusual because they seemed to contain only nonsensitive organisms. Had these cultures been studied more intensively and at intervals instead of only once, sensitive strains quite possibly could have been obtained from them.

Among the butter cultures used in this study, there was no absolute relationship between the record of a culture with regard to slowness and the type of sensitive or nonsensitive organisms obtained from it. Cultures M_1, A and 103 had been typically slow at least once under laboratory conditions during the 18 months of observation and still contained no demonstrable sensitive organisms, although examination at other times or the use of other filtrates of different inhibition characteristics might have shown these cultures to contain sensitive organisms. Cultures 233, H, S and 22 had shown no tendency toward retarded acid production during the 18 months during which they were observed, but sensitive strains of *S. lactis* were obtained from them. Possibly they could become noticeably slow under some circumstances, since they apparently possessed at least part of the latent potentialities, needing only the proper supplementary conditions, which are at present unknown, to bring about considerable loss in vitality.

CONCLUSIONS

1. All of the 46 cultures of *S. lactis* isolated from spontaneously-soured raw milk and raw-milk whey were resistant to the inhibitory action of bacteria-free filtrates from slow butter cultures.

2. Of the 317 cultures of *S. lactis* obtained from 14 different butter cultures at various times, 170 were inhibited by one or more bacteria-free filtrates from slow butter cultures.

3. The cultures of *S. lactis* isolated at one time from a butter culture may be quite varied in their sensitivities to inhibitory filtrates.

4. The cultures of *S. lactis* isolated from a butter culture at different times may be entirely different in their susceptibility to inhibition by bacteria-free filtrates from slow starters

5. The range in sensitivity of the strains of *S. lactis* studied varied in an almost continuous manner from those uninhibited by any of the fil-

trates used to those sensitive to the inhibitory action of a large percentage of the filtrates against which they were tested.

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