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Distribution of Pseudomonas fragi

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NOTE TO USERS

DISTRIBUTION OF *Pseudomonas fragi*

by

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TABLE OF CONTENTS

INTRODUCTION

While many species of bacteria produce changes in milk or other dairy products rapidly, a comparatively small number of species cause most of the practical difficulties. Pseudomonas fragi appears to be one of these. Its isolation has been reported from numerous samples of milk, cream, butter and other dairy products. Many of these samples had a distinctive ester-like odor, similar to that of the May apple, (Podophyllum peltatum) or were rancid.

Pseudomonas fragi is of especial interest to the dairy industry because, in addition to its ability to hydrolyze fat, it is psychrophilic and able to grow at temperatures of 5°C. or lower. This ability renders its presence particularly important in butter or other dairy products stored at temperatures considered low enough to prevent growth of many species of microorganisms.

The detection of Pseudomonas fragi in many samples of dairy products, some of which originated at widely separated points geographically, suggests that it is widely distributed. Its frequent occurrence in milk indicates that it probably is found close to the source of the milk supply. In order for the dairy industry to combat or control this organism more effectively, detailed information is needed regarding its habitat and distribution. This investigation was undertaken primarily to supply such data.

REVIEW OF LITERATURE

Eichholz, in 1902 (4), isolated an organism from milk held 5 days at 3.5° to 7°C. which he described as a non-spore forming, short-rod that was motile by means of a single polar flagellum. He named it Bacterium fragi. Later investigators (7), (10), (11), (12), (15) have called attention to the fact that it should have been placed in the genus Pseudomonas because of its single polar flagellum. The most striking biochemical characteristic of this organism was its ability to produce a strawberry odor and flavor on all media used except potato.

The same year, Gruber (6) published a description of an organism producing a strawberry aroma. It was isolated from beets and was also a non-spore forming, short-rod, but was motile by means of one to nine polar flagella. He named it Pseudomonas fragariae. It differed from Bacterium fragi in the type of colony and in the production of a fluorescent pigment.

In 1905, Gruber (7) isolated from pasteurized milk an organism which was so similar to Pseudomonas fragariae that he named it Pseudomonas fragariae II. It produced a strawberry aroma but was not fluorescent. The colonies on gelatin were larger and more raised than those of his earlier organism.

Hussong (11) isolated from a sample of rancid butter an organism that produced an odor which he described as resembling that of a flower of the May apple. When he attempted to purify his organism by plating methods, he found several colony variants. Resorting to single cell isolation technique, he found five distinct colony types developing from cultures grown from a single cell. He designated these as S (smooth), O (intermediate) and R_1, R_2 and R_3 (types of rough) colonies. Hussong concluded

that the three species described by Eichhols and Gruber were in reality variants of a single species. His investigation indicated that Bacterium fragi Eichhols corresponded to his R. type, Pseudomonas fragariae Gruber to his O type and Pseudomonas fragariae II Gruber to his S type. In Bergey's Manual of Determinative Bacteriology, 5th edition, (2) the organism is listed as Pseudomonas fragi (Eichhols) Hucsong, Long and Hammer.

An organism described by Conn, Esten and Stocking (3) agrees very closely with Pseudomonas fragi and probably should be included in the same group. It was isolated from milk preserved for several weeks at 1°C. and was a short monotrichic rod, which, in milk, gave rise to a pronounced odor that was not described fully. It was named Pseudomonas lactis varians.

The early investigators who isolated and described Pseudomonas fragi did not give data on the distribution or habitat of this organism. The sources from which it was isolated were raw milk held at low temperatures, (3) (4) pasteurized milk (7) and sugar beets (6). After encountering Pseudomonas fragi in rancid butter, Hucsong (11) and Hucsong, Long and Hammer (12) examined a variety of normal and abnormal dairy products to determine its presence in them. It was isolated from occasional samples of normal milk, cream, butter and ice cream. Many samples of abnormal dairy products also yielded the organism. In several cases Pseudomonas fragi was isolated from samples of raw or pasteurized milk and cream which developed either a May apple odor or rancid flavor and odor on being held at low temperatures. Many samples of rancid butter or butter with a May apple odor, especially those with a low salt content,

were found to contain the organism. Often the numbers of Pseudomonas fragi per gram were very high. Cottage cheese and bulk condensed milk with defects suggesting the action of Pseudomonas fragi were found to contain this organism in several cases. The isolation of Pseudomonas fragi was also reported from such sources as homogenizer packing, water from a lake on the Iowa State College campus and sheep fat that had been held in the laboratory for several weeks.

METHODS

Collection of Samples

a. Milk samples were taken by means of sterile milk thief pipettes directly from cans of milk being delivered to milk plants by producers. They were placed in sterile, cotton stoppered test tubes.

b. Butter samples were obtained by removing small pieces of butter from a print or lot of butter with a sterile wire loop or small knife.

c. Dairy plant samples included water and swabs from various pieces of plant equipment. Water samples were collected in sterile, screw cap glass bottles. Samples from equipment were obtained by swabbing portions of the surface of equipment with moistened sterile cotton swabs.

d. Farm samples included water, soil, bedding, feed and hay and swabs from such things as milk utensils, milk stools, barn floors, dusty ledges, stanchions and flanks of cows. The former were collected in sterile, screw cap glass jars. Moistened sterile cotton swabs were used in collecting material from the equipment and cows.

Isolation of Cultures

a. An enrichment process was regularly used because of the high bacterial content of many of the samples. As suggested by Hussong, Long and Hammer (12), milk and cream samples were kept at 5° to 10°C. for a period of 7 to 10 days before plating. Small portions from samples of water, soil, hay, bedding and like materials were added to tubes of litmus milk which, in turn, were subjected to the enrichment process.

In the case of swabs, the entire swab was placed in a tube of litmus milk for enrichment.

b. The enriched samples were plated on nile blue sulfate medium with fat emulsion added after the method of Hammer and Collins (8) and of Long and Hammer (13). Either beef infusion or beef extract agar was used. Butterfat was employed regularly for the emulsions. Plates were incubated at room temperature for 2 to 3 days.

c. Colonies of lipolytic organisms which were suggestive of Ps. fragi were picked into tubes of litmus milk. Characteristic colonies do not take up much dye from the medium and appear almost white when examined from the bottom of the plate with a hand lens. The litmus milk cultures were incubated at room temperature. Usually after 2 or 3 days, cultures of Ps. fragi developed a May apple odor. Quite commonly they developed a pink or acid ring at the surface of the milk next to the wall of the tube. On extended incubation this acid area deepened somewhat. Cultures exhibiting characteristics of Ps. fragi were preserved for more detailed identification studies.

Identification of Cultures

Cultures were finally identified on the basis of shape, size, gram stain, motility, lack of spores, lipolysis, gelatin liquefaction, production of a May apple odor in milk and production of acid from arabinose, dextrose, galactose, glycerol, lactose and levulose.

EXPERIMENTAL

General considerations of isolation procedure

While the isolation procedure used in detecting Ps. fragi is relatively simple and, judging from the results, reasonably effective, undoubtedly in some instances it fails to detect the organism. Of necessity, the amounts of material examined are small. In milk samples which contain very few Ps. fragi, the organism may be missed entirely. Sampling of butter and cheese presents difficulties because of the probable uneven distribution of organisms throughout these products. Soil and similar materials present difficulties because of the large numbers of other organisms present. Many other species of bacteria, especially those present in soil, are capable of growing at the temperatures used in the enrichment process for Ps. fragi. After holding milk inoculated with other material at 5° to 10°C. for 7 to 10 days, platings usually must be made at dilutions of 1-1,000,000 or higher in order to obtain plates suitable for picking colonies. Thus the proportion of the total original flora represented by Ps. fragi is important. On the other hand, the distinct, pleasant aroma produced by this organism is a marked aid in determining its presence, both in milk and on agar plates. The presence of nile blue sulfate in the medium is also advantageous because it tends to inhibit the growth of some other organisms while apparently it does not greatly inhibit Ps. fragi. In considering the results reported in this study, it should be recognized that failure to isolate the organism from a sample does not preclude the possibility of its presence in that sample.

General sources of samples

Samples examined in studying the distribution of Ps. fragi came from rather widely separated geographical areas. Most of the samples originated in Iowa but a considerable number were obtained in Kentucky and a few in other states. Because of this general relationship the data on distribution of Ps. fragi are presented in three sections.

Distribution of Ps. fragi in Iowa

Distribution in normal dairy products. Samples of milk from the regular supply delivered by patrons to the Iowa State College milk plant were examined for the presence of Ps. fragi. Milk was received daily from 14 farms within a radius of 7 miles. A truck route served the majority of these farms while a few producers delivered their products individually.

From 5 to 21 samples were obtained at intervals from the milk supplies of the various farms, as shown in Table 1. Samples of milk from 10 (71.4 per cent) of the 14 farms yielded Ps. fragi in one or more instances. The percentages of samples from these 10 farms that yielded Ps. fragi ranged from 7.7 per cent to 42.9 per cent and averaged 20.6 per cent. The organism was found in 7 (7.7 per cent) of 91 samples obtained in October, in 16 (22.9 per cent) of 70 samples collected in November and in 6 (40.0 per cent) of 15 samples obtained from December to May. Of the total 176 milk samples examined, 29 or 16.5 per cent yielded Ps. fragi.

One sample of raw cream, separated in the Iowa State College milk plant from milk delivered by some of the above producers yielded the

Table 1. *Ps. fragi* in milk delivered to the Iowa State College milk plant.

Date of delivery	Producer number													
	1	3	4	5	6	7	8	9	10	11	12	13	14	15
9-30-38		-*	-		-	-	-		-	-	-	-	-	-
10- 5-38		-	-	-	-	-	-			+		-	-	-
10- 6-38		-	-	-	-	-	-			-		-	-	-
10- 8-38		-	-	-	-	-	-		-	-		-	-	-
10-13-38		-	-		-	-	-			-	-	-	-	+
10-14-38		-	-		-	-	-	-	+		+		-	-
10-22-38	-	-	-		-	-	+		-	-	-		-	
10-28-38		-	-		-	-	+		-	-			-	-
10-29-38		-	-	-	+	-	-		-		-		-	-
11- 3-38	-	-		-	-	+	+		-	-	+			-
11- 5-38		-		-	-	-	-	-	+	-		+		
11-11-38		-	-		-		+		-	+	-			-
11-17-38	-	-	-		+	-	-	-	-	+	-	-	-	
11-18-38		-	+		+	-	+	-	-		-		-	
11-19-38				-				-	-		-			-
11-22-38		-	+	-	-	-	+	-	-	-		-	-	-
11-26-38							+		-		-			
11-30-38					+		-							
12- 1-38					-		-				-			
12- 3-38					-		-							-
1- 7-39					+									
1-14-39							+							
2- 6-39	-													
4-29-39						+							+	
5- 5-39					+		+							
5-12-39	-			-										
Summary														
Samples examined	5	15	13	9	20	15	21	6	14	12	12	8	13	13
Samples yielding *Ps. fragi*														
Number	0	0	2	0	6	2	9	0	2	3	2	1	1	1
Per cent	0.0	0.0	15.4	0.0	30.0	13.3	42.9	0.0	14.3	25.0	16.7	12.5	7.7	7.7

*Samples not yielding *Ps. fragi* designated -, samples yielding *Ps. fragi* designated +.

organism.

Distribution in defective dairy products. Samples of defective butter are often received at the Dairy Industry Section of the Iowa Agricultural Experiment Station for examination. Several such samples were examined for the presence of Ps. fragi. Some of them had been subjected to keeping quality tests and criticised as being rancid; others had been criticised as being putrid, tallowy or as having some other defect. Several samples of defective experimental butter was included. Defective butter serum, pasteurized cream and raw skim milk were also examined.

Results of the examinations are presented in Table 2. Of the 28 butter samples examined, 16 or 57.1 per cent yielded Ps. fragi. The organism was isolated from the sample of rancid butter serum, from both samples of rancid pasteurized cream and from the sample of raw skim milk which had developed a May apple odor on holding in a refrigerator for some time. Thus, 20 or 62.5 per cent of 32 samples of defective butter and other dairy products yielded Ps. fragi. It was isolated from 14 (70.0 per cent) of 20 samples of dairy products described as rancid or having a May apple odor. Presumably, these defects were caused by Ps. fragi. Putrid and tallowy defects have not been attributed to this organism and its presence in some samples of butter with those defects was probably of secondary importance. While the number of samples is not large, the high percentage of samples yielding the organism is of significance.

Distribution in dairy plant equipment. The possibility of dairy plant equipment harboring Ps. fragi was studied by means of swabs from various equipment, especially churns, used in creameries and milk plants.

Table 2. Ps. fragi in defective dairy products.

Dairy product	Defect	No. of Samples examined	Samples yielding Ps. fragi No.	Per cent
Butter	Rancid	15	9	60.0
"	Putrid	6	3	50.0
"	Tallowy	1	1	100.0
"	May apple	1	1	100.0
"	No specific criticism	5	2	40.0
Butter serum	Rancid	1	1	100.0
Cream (past.)	Rancid	2	2	100.0
Skim milk (raw)	May apple	1	1	100.0
Summary		32	20	62.5

Samples of this nature were obtained in 39 Iowa creameries and in 1 Iowa milk plant.

As indicated in Table 3, churns were the main type of equipment examined but a few swabs were obtained from printing and wrapping equipment, vats and miscellaneous pieces. Samples from 60 churns in 37 creameries yielded Ps. fragi in only two cases or 3.3 per cent. Three samples from printing or wrapping tables and one sample from a printer belt each yielded the organism. It was not isolated from any of the other samples. In all, Ps. fragi was found in only 6 or 5.8 per cent of 104 samples examined.

More detailed examinations of a variety of pieces of equipment were made in one creamery and one milk plant. Swabs were obtained from 24 pieces of equipment in the creamery. These included nearly all the pieces of equipment with which cream or butter comes in contact. However, the only swab yielding Ps. fragi was one obtained from the top of the table on which the butter was wrapped.

In the milk plant, 20 swabs were obtained from various pieces of equipment and from various locations, such as the weigh room floor and the loading platform. The only swab in this group yielding Ps. fragi was that obtained from the floor in front of the weigh tank.

Following the isolation of Ps. fragi from two samples of rancid pasteurized cream, a batch of cream was followed through the processing operations in the plant where the defect occurred. Ps. fragi was isolated from a sample of the raw cream in the vat before pasteurization was begun. None of the samples taken after the cream had reached the pasteurization temperature (155°F.) yielded the organism.

Table 3. Ps. fragi in dairy plant equipment.

Equipment examined	No. of dairy plants from which samples were obtained	No. of pieces of equipment	Samples yielding Ps. fragi	
			No.	Per cent
Churns	37	60	2	3.3
Printing tables	2	3	3	100.0
Printer belt	1	1	1	100.0
Friday printing equipment	1	3	0	0.0
Wrappers	1	2	0	0.0
Vats	2	12	0	0.0
Pumps	1	2	0	0.0
Miscellaneous equipment	2	21	0	0.0
Summary	40	104	6	5.8

Distribution in dairy plant water supplies. Samples of water from 37 creameries were examined. The majority of these samples represented the regular water supply of the creamery although five were samples of wash water, one was water in which parchment was soaking and two were brine used for the same purpose.

Of the 33 samples from regular water supplies, 5 or 15.2 per cent yielded Ps. fragi (Table 4). However, three of these samples were from one creamery; therefore, Ps. fragi was isolated from the regular water supply of 3 or 10.0 per cent of the creameries whose supplies were examined. One of five samples of wash water yielded the organism, as did one sample taken from brine used for soaking parchment. In all, Ps. fragi was isolated from 7 or 17.1 per cent of the 41 water samples examined.

Distribution on farms. The presence of Ps. fragi in milk supplies from a considerable percentage of farms delivering milk to the Iowa State College milk plant suggested the source of this organism to be on the farms. Accordingly, the farms of 6 of the 14 producers were visited and samples obtained for examination.

In the absence of definite information regarding the prevalence or distribution of Ps. fragi on farms, the first two visits were made to farms whose milk supplies had yielded the organism. The results of these visits as well as of an additional visit to each farm are shown in Table 5. At the first visit to the farm of Producer 6, 21 samples were obtained, 8 of which yielded Ps. fragi. It was found in 13 of 20 samples obtained on the first visit to the farm of Producer 8. The samples which yielded the organism on both farms were dirt from the barn floor, bedding, milk (not aseptically drawn) and swabs from milk pails, milk

Table 4. *Ps. fragi* in water supplies of dairy plants.

Samples	No. of dairy plants from which samples were obtained	No. Samples examined	Samples yielding *Ps. fragi* No.	Per cent
Regular water supply	30	33	5*	15.2
Wash water	5	5	1	20.0
Water for soaking parchment	1	1	0	0.0
Brine for soaking parchment	1	2	1	50.0
Summary	37	41	7	17.1

*3 of these samples came from one creamery.

Table 5. Ps. fragi in samples from farms whose milk supplies had frequently yielded the organism.

Type of sample	Samples obtained at farm of Producer number 6				8				All samples	
	1-6-39		5-4-39		1-13-39		5-4-39			
	No. examined	No. yielding Ps. fragi	No. examined	No. yielding Ps. fragi	No. examined	No. yielding Ps. fragi	No. examined	No. yielding Ps. fragi	No. examined	No. yielding Ps. fragi
Water from										
tap	1	0			1	0			2	0
stock tank	1	0	1	1	1	1	1	0	4	2
soil surface	1	1							1	1
Dirt from										
barnyard			2	1	1	1	2	0	5	2
barn floor	1	1			1	1			2	2
Feed										
hay			2	1	1	1	1	0	4	2
grain	1	1	1	0	1	0	1	1	4	2
Bedding	1	1	1	1	1	1	1	1	4	4
Animals										
coat	2	0	1	1	3	2			6	3
milk	4	1			2	1			6	2
Milking utensils										
milking machine	3	0							3	0
pails	1	1			1	1	1	0	3	2
strainers	1	0			1	0			2	0
cans					1	0			1	0
Misc. barn equipment										
stanchions	1	0	1	1	1	1	1	1	4	3
milk stools	2	1	1	1	2	1	1	0	6	3
scrapers, shovels, etc.			1	0	1	1	1	1	3	2
Floors			1	1			1	1	2	2
Ledges	1	1	1	1	1	1	1	1	4	4
Summary - Number	21	8	13	9	20	13	12	6	66	36
Per cent		38.1		69.2		65.0		50.0		54.5

stools and ledges. In addition, on the former farm, it was found in the grain mixture and soil surface water from the barnyard, and on the latter farm, it was isolated from water from a stock watering tank, dirt from the barnyard, hay and swabs from coats of cows, a stanchion and a floor scraper handle.

The later visit to each of the farms resulted in the isolation of Ps. fragi from 9 of 13 samples obtained at the farm of Producer 6 and from 6 of 12 samples obtained at the farm of Producer 8. The samples which yielded the organism on both farms at the second visit were bedding and swabs from the barn floor, a stanchion and dusty ledges. On the farm of Producer 6, samples of water from a stock watering tank, barnyard dirt, hay, the coat of a cow and a milk stool also yielded Ps. fragi, while on the farm of Producer 8, other samples yielding it were grain and a scraper handle.

Ps. fragi was isolated from 36 (54.5 per cent) of 66 samples obtained on the two farms. All the six swabs from floors and ledges yielded it, as did all the four samples of bedding, and it was also isolated from 61.5 per cent of the samples from miscellaneous barn equipment, 57.1 per cent of the samples of dirt, 50 per cent of the samples of feed, 42.9 per cent of the samples of water, 41.7 per cent of the samples from the animals themselves and 22.2 per cent of the samples from milking utensils.

Five visits were made to farms from whose milk supplies Ps. fragi had not been isolated. Results of the examination of samples obtained on these visits is given in Table 6. Two visits were made to the farms of Producer 1. On the first visit 24 samples were obtained, 12 of which yielded the organism. On a second visit 5 of 12 samples were found to

Table 6. Ps. fragi in samples from farms whose milk supplies had not yielded the organism.

	Samples obtained at farm of Producer number										All samples	
	1		3		5		14					
	2-5-39		5-11-39		4-28-39		5-11-39		4-28-39			
	No. examined	No. yielding Ps. fragi	No. examined	No. yielding Ps. fragi	No. examined	No. yielding Ps. fragi	No. examined	No. yielding Ps. fragi	No. examined	No. yielding Ps. fragi	No. examined	No. yielding Ps. fragi
Water from												
tap	1	0									1	0
stock tank	2	2	2	2	1	1	1	1	1	0	7	6
barn floor	1	1									1	1
soil surface	1	0					1	1			2	1
cooling tank									1	0	1	0
Dirt from barnyard	1	1	1	0	2	1	1	1	2	1	7	4
Feed												
hay	1	1	1	1			4	0	1	1	4	3
grain	1	0	1	1	1	0	1	0			4	1
silage	1	0									1	0
Bedding	1	1	1	0	1	1	1	1	1	1	5	4
Animals - coat	2	0					1	0			3	0
Milking utensils												
Milking machine	1	0									1	0
cooler	1	0									1	0
cans					1	0			1	0	2	0
pails	1	0									1	0
Misc. barn equipment												
stanchions	5	3	2	0	1	1	2	1	1	0	11	5
milk stools					1	1	1	0	1	1	3	2
scrapers, shovels, etc.					1	1			1	0	2	1
Floors	2	2	3	1	1	0	1	1	1	0	8	4
Ledges	1	0	1	0	1	0	1	1	1	1	5	2
Manure	1	1									1	1
Summary - Number	24	12	12	5	11	6	12	7	12	5	71	35
Per cent		50.0		41.7		54.5		58.3		41.7		49.3

harbor it. Samples from this farm that yielded Ps. fragi included water from stock watering tanks and from the barn floor, barnyard dirt, hay, bedding and swabs from stanchions, floors and manure.

On each of the other three farms Ps. fragi was isolated from samples of barnyard dirt and bedding. On two of the three farms water from stock watering tanks and swabs from stanchions, milk stools and ledges also yielded it. Samples from which it was isolated on only one of the three farms included water from soil surface, hay, and swabs from a scraper handle and a dusty ledge. The number of samples examined from the farms of Producer 3, 5 and 14 was 11, 12 and 12 respectively and the per cent of samples from these farms yielding Ps. fragi was 54.5, 58.3 and 41.7 per cent.

In all, 71 samples obtained on four farms, from the milk supplies of which Ps. fragi had not been isolated, yielded it in 35 (49.3 per cent) of the instances.

A summary of the results of examination of samples obtained on all nine visits to the six farms is shown in Table 7. Of the materials and equipment sampled, bedding, floors and ledges, water, dirt, miscellaneous barn equipment, feed, the cows themselves and milk handling utensils harbored Ps. fragi in the order named. Bedding, water from stock watering tanks, hay, general barn equipment and dust and dirt on ledges and floors in the barn as well as outside were especially important as sources of this organism. Milking utensils were relatively free of it.

Table 7. *Ps. fragi* in samples from six Iowa farms. Summary of Tables 5 and 6.

Type of sample	No. Samples examined	Samples yielding *Ps. fragi* No.	Per cent	No. Samples examined	Samples yielding *Ps. fragi* No.	Per cent
Water from						
tap	3	0	0.0			
stock tank	11	8	72.7			
barn floor	1	1	100.0	19	11	57.9
cooling tank	1	0	0.0			
soil surface	3	2	66.7			
Dirt from						
barnyard	12	6	50.0			
barn floor	2	2	100.0	14	8	57.1
Feed						
hay	8	5	62.5			
grain	8	3	37.5	17	8	47.1
silage	1	0	0.0			
Bedding	9	8	88.9	9	8	88.9
Animals						
coat	9	3	33.3			
milk	6	2	33.3	15	5	33.3
Milking utensils						
milking machine	4	0	0.0			
pails	4	2	50.0			
strainers	2	0	0.0	14	2	14.3
cooler	1	0	0.0			
cans	3	0	0.0			
Misc. barn equipment						
stanchions	15	8	53.3			
milk stools	9	5	55.6	29	16	55.2
scrapers, shovels, etc.	5	3	60.0			
Floors	10	6	60.0			
Ledges	9	6	66.7	19	12	63.2
Manure	1	1	100.0	1	1	100.0
Summary				137	71	51.8

Distribution of Ps. fragi in Kentucky

Distribution in normal dairy products. Three sets of samples from milk delivered by patrons to a Lexington, Kentucky, milk plant were examined for the presence of Ps. fragi. One set was obtained in June and the other two sets in December. The samples represented the milk supplies of 26 farms in three Kentucky counties. Six farms supplied one sample each, nine farms two samples each and eleven farms three samples each. In all, 57 samples were examined.

None of the seventeen samples obtained in June yielded the organism, as shown in Table 8. It was found, however, in 16 of the 40 samples obtained in December. Including summer and winter samples, Ps. fragi was isolated from 16 or 28.1 per cent of the 57 samples examined.

Of the 17 farms represented by the June samples, only 12 were represented by the samples obtained in December. Samples from nine of these 12 dairies yielded Ps. fragi, while samples from only three did not. The organism was isolated from samples representing milk supplies from 13 of the 26 farms.

Distribution in defective dairy products. Only a few samples of defective dairy products were available for examination at Lexington. Each of two samples of skim milk, which developed a May apple odor in the University of Kentucky Dairy Department refrigerator, yielded the organism. It was not isolated from seven samples of butter following keeping quality tests at a Lexington creamery; none of these samples were criticised as being rancid or having a May apple odor.

Distribution in dairy plant equipment. A swab from a churn in a Lexington dairy plant did not yield Ps. fragi. Two swabs obtained in

Table 8. Ps. fragi in milk delivered to a Lexington, Kentucky, milk plant.

Producer No.	Date of delivery 6-27-39	12-6-39	12-22-39	Samples examined	No. Samples yielding Ps. fragi
238	-*	-	+	3	1
239**		+		1	1
241	-	-	+	3	1
242	-			1	0
243		-	-	2	0
244	-	-	-	3	0
245		-	-	2	0
246		-	-	2	0
247**	-		+	2	1
248	-			1	0
251	-	+	-	3	1
255	-	+	-	3	1
256	-	-	-	3	0
259		+	+	2	2
264		-	-	2	0
265	-	+	-	3	1
266	-			1	0
268	-	+	+	3	2
269	-	-	+	3	1
270		-	+	2	1
271	-	+	+	3	2
274	-			1	0
275	-			1	0
277		-	-	2	0
333		+	-	2	1
334	-	-	-	3	0
Summary					
Samples, No.	17	20	20	57	
Samp. yielding Ps. fragi					
No.	0	8	8		16
Per cent	0.0	40.0	40.0		28.1

*Samples not yielding Ps. fragi designated -, samples yielding Ps. fragi designated +.
**Samples were also obtained at farm of these producers.

June and December from the receiving room floor of the same plant yielded the organism. It was also isolated from a swab obtained in December from the floor of a covered truck delivering milk to this plant, while a similar sample obtained in June failed to yield it.

Distribution in water supplies. Samples of water from dairy plants in Kentucky were not examined. A sample of pond water received at the Kentucky Agricultural Experiment Station from a farmer who questioned its fitness for cattle to drink was found to contain Ps. fragi. Another water sample yielding the organism was taken from a portable ice box in which several bottles of milk and cream were shipped from Louisville to Lexington. Water containing some milk solids, that remained in this shipping box after removal of milk and cream samples developed a May apple odor; Ps. fragi was readily isolated from it.

Distribution on farms. Forty nine samples representing water, dirt, feed, miscellaneous barn equipment, floors and ledges were obtained on ten Kentucky farms during the summer of 1939 (Table 9). Only two of these samples (4.1 per cent) yielded Ps. fragi. Both samples represented feed; one grain and one hay. Most of the samples were obtained in July and August when the weather was hot and dry.

Ninety nine samples were obtained from nine Kentucky farms in December. Of these 37 (37.4 per cent) yielded Ps. fragi (Table 10). It was isolated from 9 of 12 water samples obtained in December, as compared with none of 13 obtained in the summer. Eleven of 14 dirt samples obtained in December yielded the organism, as contrasted with none of 12 collected in the summer. There was little difference between the percentages of feed samples yielding Ps. fragi in December and in

Table 9. Ps. fragi in samples from ten Kentucky farms during the summer season.

Type of sample	No. Samples examined	No. Samples yielding Ps. fragi	No. Samples examined	Samples yielding Ps. fragi No.	Samples yielding Ps. fragi Per cent
Water from					
stock watering tank	6	0			
drinking cup	2	0			
tap	1	0			
cooling tank	1	0	13	0	0.0
wash tank	1	0			
barn floor	1	0			
rinse from utensils	1	0			
Dirt from					
barnyard	10	0	12	0	0.0
barn floor	2	0			
Feed					
grain	12	1	19	2	10.2
hay	7	1			
Misc. equipment					
stanchions	2	0	3	0	0.0
drinking cup	1	0			
Floor	1	0	2	0	0.0
Ledge	1	0			
Summary			49	2	4.1

Table 10. *Ps. fragi* in samples from nine Kentucky farms during the winter season.

Type of sample	No. Samples examined	No. Samples yielding *Ps. fragi*	No. Samples examined	Samples yielding *Ps. fragi* No.	Percent
Water from					
stock watering tank	5	5			
drinking cup	1	1			
creek	1	1			
cistern	1	1			
cooling tank	2	0	12	9	75.0
barn floor	1	0			
rinse from utensils	1	1			
Dirt from					
barnyard	9	9			
barn floor	2	1	14	11	78.6
mangers	2	1			
gutter	1	0			
Feed					
grain	6	1			
hay	9	2	18	3	16.7
silage	1	0			
Bedding	6	3	6	3	50.0
Utensils					
milk pails	3	0			
strainer	1	0			
cooler	2	0	9	0	0.0
can	1	0			
bottler	1	0			
bottle	1	0			
Misc. barn equipment					
stanchions	5	1			
milk stools	6	2			
scrapers	4	1	21	4	19.0
mangers	2	0			
wash tank (dry)	1	0			
Ceiling	1	0			
Floors	6	4			
Ledges	10	3	19	7	36.8
Cobwebs	2	0			
Summary			99	37	37.4

summer. The presence of this organism was demonstrated in three of the six samples of bedding obtained during December; samples of bedding were not obtained during the summer season. Likewise, samples were not obtained in summer from utensils for handling milk, but of nine samples obtained from utensils in December none yielded the organism. It was isolated from four of 21 samples obtained from miscellaneous dairy barn equipment, such as stanchions, milk stools, scraper and broom handles, etc., in December, but not from any of three obtained from such equipment in the summer. Two samples obtained from floors and ledges in summer did not yield Ps. fragi while it was found in seven of nineteen such samples obtained in December.

Samples were obtained from only one of the farms in both summer and winter. Among fourteen samples obtained from this farm in June, only one (grain) yielded Ps. fragi while it was found in seven of eleven samples obtained in December. The winter samples from which it was isolated were water (2 samples), dirt (2 samples), bedding and swabs from the floor and from a dusty ledge. Approximately the same materials and equipment were sampled on each visit.

Samples were obtained in December at the farms of two producers listed in Table 8. Six of ten samples from the farm of Producer 239 yielded Ps. fragi. These included samples of barnyard dirt, hay, water from stock tank, and swabs from a dusty ledge, barn floor and milk stools. The samples in which it was not found included feed, silage and swabs from a stanchion and a scraper handle. The single sample of milk obtained on delivery to the milk plant yielded the organism.

Three of fifteen samples obtained at the farm of Producer 247 yielded Ps. fragi. These three samples included dirt from the barnyard and from the barn floor and water from a cistern. Samples in which it was not found included grain, dirt from a manger, hay (ground), water from a cooling tank and swabs from stanchion, cobwebs, dusty window ledge, milk stool, milk pail, strainer, milk can and dry wash tank. A milk sample from this farm obtained on delivery at the milk plant in June did not yield the organism, while it was isolated from a second sample obtained at the milk plant in December.

A summary of the results of examination of all samples obtained on Kentucky farms is given in Table 11. Ps. fragi was found in 39 or 26.4 per cent of the 148 samples examined. Bedding, dirt, water, floors and ledges, miscellaneous barn equipment and feed were found to harbor the organism in the order given. One half to one third of the samples in the first four groups yielded Ps. fragi, but it was not found in any sample from milk handling utensils.

These results are very similar to those already reported for Iowa although the percentages of Kentucky samples yielding Ps. fragi are somewhat lower. A partial explanation for this may be because approximately one third of the Kentucky samples were obtained in the summer season when Ps. fragi was found only very infrequently while all the Iowa farm samples were obtained during the winter and spring.

Table 11. *Ps. fragi* in samples from 15 Kentucky farms during both summer and winter. Summary of Tables 9 and 10.

Type of sample	No. Samples examined	No. Samples yielding *Ps. fragi*	No. Samples examined	Samples yielding *Ps. fragi* No.	Samples yielding *Ps. fragi* Per cent
Water from					
stock watering tanks	11	5			
drinking cups	3	1			
creek	1	1			
cistern	1	1			
tap	1	0	25	9	36.0
cooling tank	3	0			
wash tank	1	0			
barn floor	2	0			
rinse from utensils	2	1			
Dirt from					
barnyard	19	9			
barn floor	4	1	26	11	42.3
manger	2	1			
gutter	1	0			
Feed					
grain	20	2			
hay	16	3	37	5	13.5
silage	1	0			
Bedding	6	3	6	3	50.0
Milking utensils					
milk pails	3	0			
strainer	1	0			
cooler	2	0	9	0	0.0
can	1	0			
bottler	1	0			
bottle	1	0			
Misc. barn equipment					
stanchion	10	1			
milk stool	6	2			
scraper, broom and shovel handles	4	1	24	4	16.7
manger	2	0			
drinking cup	1	0			
wash tank (dry)	1	0			
Ceiling	1	0			
Floor	7	4	21	7	33.3
Ledge	11	3			
Cobwebs	2	0			
Summary			148	39	26.4

Distribution of Ps. fragi in the United States

In order that the distribution of Ps. fragi throughout the United States could be studied, a request for a sample of barnyard soil was sent to the Dairy Department at each State Agricultural College in the United States. Barnyard soil was requested because of the frequency with which Ps. fragi had been isolated from it and the convenience of collection and shipment. The requests were made in the late winter or early spring months as that seemed the most probable time for the easy detection of the organism. Samples were received from 33 states, representing all sections of the country.

Examination of the samples revealed the presence of Ps. fragi in those from 23 states or 69.7 per cent (Table 12). Samples from 10 states failed to yield the organism; these samples were re-examined, but with the same results.

By grouping the states into an eastern and a western division, an interesting comparison can be made (Table 13). In the eastern section are included the states from Minnesota to Louisiana and those eastward; in the western section, those from North Dakota to Texas and westward. The eastern section included 31 states, samples from 20 of which were examined. The presence of Ps. fragi was demonstrated in samples from 18 (90.0 per cent) of these 20 states. This indicates a wide distribution of the organism throughout the eastern half of the United States. In the western section are included 17 states, 13 of which furnished samples for examination. Samples from only 5 (38.5 per cent) of these 13 western states yielded Ps. fragi. While a single sample from one particular spot in a state is by no means representative of the entire state, nevertheless,

Table 12. Ps. fragi in barnyard soil from various states in the United States.

Origin of samples yielding Ps. fragi	Origin of samples not yielding Ps. fragi
Alabama	California
Arkansas	Colorado
Delaware	Florida
Georgia	Idaho
Indiana	Kansas
Iowa	Massachusetts
Kentucky	Nebraska
Louisiana	New Mexico
Maryland	South Dakota
Minnesota	Wyoming
Missouri	
Montana	
New Jersey	
North Carolina	
North Dakota	
Ohio	
Oklahoma	
Pennsylvania	
South Carolina	
Texas	
Virginia	
Washington	
West Virginia	

Table 13. Ps. fragi in barnyard soil from different sections of the United States.

	Eastern division	Western division	All states
No. of states	31	17	48
No. of states represented by samples	20	13	33
No. of samples yielding Ps. fragi	18	5	23
Per cent of samples yielding Ps. fragi	90.0	38.5	69.7

the majority of samples (8 of 13) from this section of the country which did not yield Ps. fragi probably indicates that the organism is not as prevalent in the western states as in the eastern.

A summary of results of examinations of all samples obtained on farms is given in Table 14. Of 316 samples examined, 131 (41.5 per cent) yielded Ps. fragi. It was isolated from more than half the samples from states other than Kentucky. However about one third of the Kentucky samples were obtained during the season when the relatively high temperatures are unfavorable to the wide distribution of the organism on farms; if these 49 samples are excluded, 48.3 per cent of the samples obtained on farms yielded the organism. This summary emphasizes the importance of the farms as a source of Ps. fragi in milk.

Identification of cultures of Ps. fragi

In order to establish the presence of Ps. fragi on a plate, cultures were picked and studied in some detail. The first criterion used in picking colonies into litmus milk from plates containing nile blue sulfate and butterfat emulsion was the ability of the organism to hydrolyze fat. This was indicated by the presence of fat droplets stained blue instead of pink in the vicinity of the colony. As many other organisms are capable of hydrolyzing fat, the appearance of the colony in question is important. In order to differentiate colonies, they were examined from the bottom of the plate with the aid of a hand lens. Typical surface colonies of Ps. fragi usually were round and opaque and from 2 to 5 mm. in diameter. After 2 to 3 days incubation

Table 14. Ps. fragi in all samples obtained on farms. Summary of Tables 7, 11 and 13.

	Iowa Samples		Kentucky Samples		Other states Samples		All Samples		
	No. examined	No. yielding Ps. fragi	No. examined	No. yielding Ps. fragi	No. examined	No. yielding Ps. fragi	No. examined	yielding Ps. fragi No.	Per cent
Dirt	14	8	26	11	31	21	71	40	56.3
Water	19	11	25	9			44	20	45.5
Feed	17	8	37	5			54	13	24.1
Bedding	9	8	6	3			15	11	73.3
Milking utensils	14	2	9	0			23	2	8.7
Misc. barn equipment	29	16	24	4			53	20	37.7
Floors, ledges, etc.	19	12	21	7			40	19	47.5
Animals	15	5					15	5	33.3
Manure	1	1					1	1	100.0
Summary									
Number	137	71	148	39	31	21	316	131	
Per cent		51.8		26.4		67.7			41.5

they appeared practically chalk white, while colonies of many other organisms had taken up enough dye to make them appear bluish. With some experience, the colonies of Ps. fragi could be distinguished quite accurately.

When Ps. fragi was picked into litmus milk and incubated 2 to 3 days at room temperature, a May apple odor became noticeable. If a drop of ethyl alcohol was added to the tubed milk before inoculating, the aroma was produced after only 1 to 2 days. Usually, after 3 to 4 days, a slightly pink (acid) ring appeared at the surface of the milk next to the wall of the tube. In most cultures, this was followed in a few days by coagulation of the milk with some reduction of the litmus. However, in some cases, the milk did not coagulate, even after incubating 2 to 3 weeks.

After replating for purification on beef infusion or beef extract agar containing nile blue sulfate and fat emulsion, typical colonies were transferred to agar slopes for use in inoculating various media.

Motility was determined by removing to a microscope slide a loopful of a 1 day old culture from a tube of neutral sugar medium and examining the organisms under the high dry lens of the microscope. All of the cultures identified as Ps. fragi were motile. Smears for Gram staining were also made from 1 day old cultures in a liquid medium. All of the cultures of this organism were Gram negative. They were all rod shaped, although usually short and medium length rods were found in each culture. Most of the organisms were single or in pairs, although in some cultures chains of several cells occurred. No spores were observed in any of the cultures.

The ability of different cultures of Ps. fragi to liquefy gelatin varied. Most of them did so with considerable rapidity, some slowly and a few seemed unable to do so. When liquefaction occurred, it commonly was of stratiform or crateriform type.

Action of cultures designated as Ps. fragi on the indicator media used was uniform except in the case of arabinose. They all produced acid rapidly in dextrose, but somewhat slower in galactose, 2 or more days usually elapsing before the galactose became acid. The majority of the cultures produced acid in arabinose although with many cultures nearly 7 days elapsed before the arabinose became definitely acid. None of the cultures produced acid in lactose, levulose or glycerol.

Some factors affecting aroma production

Several investigators have considered aroma production by bacteria and have attempted to identify the compounds responsible for various aromas.

In studying a fruit ester producing organism, Bacillus praepollens, which he had isolated, Maassen (14) analyzed the decomposition products formed by it. Among these he found a valeric acid ester which smelled like the amyl ester of valeric acid. He attributed the aroma to the presence of this compound. In 1902, Grimm (5) reported the isolation of Bacillus aromaticus lactis which produced a strong fruity aroma in milk. He thought the aroma was due to ethyl lactate formed in the milk. Beck (1) studied the aromatic substance formed by Micrococcus esterificans and found it to be insoluble in alcohol but soluble in ether, chloroform and carbon disulfide. Omelianski (15) reported that the addition of leucine

to meat peptone agar stimulated aroma production by Bacterium estero-aromaticum, especially in the presence of a little ethyl alcohol. He noted no stimulation from the addition of propyl or amyl alcohols. In his opinion leucine was split out of protein and further decomposed to form iso-valeric acid and iso-amyl alcohol, which united to form iso-valeric-iso-amyl ester. He thought the presence of ethyl alcohol merely intensified the aroma, as it had been found to do in artificial "apple esters". With most of the aroma producing organisms described in the literature, the pleasant aroma disappeared and was replaced by an ammoniacal or cheesy odor. Omelianski attributed the former to deaminisation in the leucine breakdown and the latter to the presence of iso-valeric acid which had been reported to be one of the causes of the odor of old cheese.

In the studies on Ps. fragi several media were investigated from the standpoint of their effect on production of the May apple odor. Nutrient broth (0.3 per cent beef extract - 0.5 per cent peptone), Uschinsky's medium, 0.5 per cent beef extract solution and 1 per cent solutions of peptone, gelatin, dextrose and lactose were inoculated and examined for growth and aroma production after 5 days. Good growth occurred in the nutrient broth, beef extract and peptone solutions and there was slight growth in gelatin. A strong May apple aroma was produced in peptone solution, a fair aroma in nutrient broth and gelatin and a slight aroma in beef extract. No growth or aroma production occurred in Uschinsky's medium, dextrose or lactose solutions. When 1 ml. of the peptone solution was added to 50 ml. of the lactose solution considerable aroma was produced.

For aroma production, Omelianski (15) recommended a medium containing: tap water 1000 ml., peptone 5 to 10 gm., potassium phosphate 1 gm., agar 20 gm. and a few drops of ethyl alcohol. Where plates were poured with this medium and 0.1 ml. of a suspension of Ps. fragi distributed over the surface with the aid of a sterile bent glass rod, abundant growth and a very strong aroma production occurred; the aroma remained typical for several days.

A medium employed for cultivation of propionic acid bacteria was also used. It consisted of: distilled water 1000 ml., sodium lactate 10 gm., yeast extract 7.5 gm., peptone 20 gm. and washed agar 12 gm. On this medium growth was abundant in 24 hours but only a slight May apple aroma was produced, together with a slight ammoniacal odor. Growth was also abundant and a very strong May apple aroma was produced in 24 hours on a plate to which 0.1 ml. of 10 per cent ethyl alcohol had been added before pouring the agar. In both these cases, however, after 48 hours the pleasant aroma had been entirely replaced by a strong ammoniacal odor.

Three series of six plates each were used in studying the effect on aroma production of peptone alone and in combination with glycerol or butterfat. Each of six plates in the first series was poured with 10 ml. of 2 per cent agar after adding the following amounts of 5 per cent peptone solution: none, 0.2, 0.4, 0.6, 0.8 and 1.0 ml. In a second series glycerol (1 ml. of 5 per cent solution) was added to each of the plates containing peptone and agar as above, and in a third series 0.5 ml. of a 5 per cent emulsion of butterfat in 0.5 per cent agar was added. After pouring, all of the plates were inoculated by spreading a suspension of Ps. fragi over the surface of the agar. The plates containing the larger amounts of peptone showed more abundant growth than did those with the smaller amounts in all three

series. The most abundant growth and aroma production occurred in the series with added glycerol and the least in the series with the peptone alone.

Another series of plates was prepared to study the effect on aroma production of glycerol esters of several fatty acids. The esters used were tri-propionin, tri-butyrin, tri-n-valerin, tri-iso-valerin, tri-caproin, tri-heptylin, tri-caprylin and tri-caprin. One tenth per cent aqueous solutions or suspensions were used. One per cent ethyl alcohol was added to each solution or suspension in the hope that the esters containing the longer chain acids would dissolve better; however, all except the tri-propionin and tri-butyrin mixtures remained cloudy to milky. One ml. of a solution or suspension was added to different plates before adding beef infusion agar. A drop of a suspension of Ps. fragi was then spread on the surface of the solidified agar. After 2 days a fair amount of aroma had been produced on each of the plates containing the esters, the greatest amount being produced on tri-heptylin. The check plate without any ester had a faint aroma. The most typical aroma was produced on plates containing tri-propionin, tri-n-valerin, tri-heptylin and tri-caprin. The plate containing tri-butyrin gave a slight butyric acid odor, that with tri-caprylin an odor resembling that of pine and those with tri-iso-valerin and tri-caproin were not typical.

Aroma production was also tested on Omelianski's agar without alcohol and with methyl, ethyl, propyl, butyl and amyl alcohols. After 2 days growth on plates, the most conspicuous and most typical aroma had been produced on agar containing ethyl alcohol. A faint aroma had been produced on the control with no alcohol but none on agar with methyl alcohol.

The aroma produced on agar with propyl alcohol was not typical and rather faint, while the odors of the butyl and amyl alcohols were so strong they covered up any aroma that may have been produced by Ps. fragi. These results confirm those of Omelianski who found that alcohols other than ethyl alcohol had little effect in increasing aroma production. The same results were obtained when 1, 2, 3 or 4 drops of the above alcohols were added to cultures of Ps. fragi in tubes of litmus milk. More aroma was produced and it was produced more quickly when 1 to 4 drops of ethyl alcohol was added per tube than in the check tube with no alcohol added or when other alcohols were used.

When 0.05 per cent of methyl, ethyl, propyl, butyl and amyl alcohols were added to 0.1 per cent peptone solutions inoculated with Ps. fragi, a typical aroma was produced only in the solution containing ethyl alcohol. When a 0.1 per cent solution of lactose was used with a similar series of solutions, the results were the same.

Various combinations of peptone, ethyl alcohol, glycerol and lactose gave the greatest aroma production in solutions containing the alcohol, and there seemed to be no advantage in including glycerol or lactose or both over peptone and ethyl alcohol alone.

The addition of 0.2 per cent of either leucine or ethyl alcohol to 0.5 per cent peptone solution increased the aroma produced by Ps. fragi, and when both were added it was increased still more. A slight atypical aroma was produced by Ps. fragi when grown in 0.2 per cent leucine alone or in 0.2 per cent leucine plus 0.2 per cent ethyl alcohol. The addition of 0.2 per cent leucine to skim milk resulted in the production of slightly more aroma than did the addition of 0.2 per cent ethyl alcohol. When both

leucine and alcohol were added, the aroma produced seemed about the same as when leucine alone was added.

The addition of 0.1 per cent tryptophane to a 0.1 per cent peptone solution did not increase aroma production. However, when 0.2 per cent ethyl alcohol was added to the above mixture, considerably more aroma was produced. Some aroma was produced by the organism in a solution containing 0.2 per cent tryptophane and 0.2 per cent alcohol but not as much as in one containing 0.2 per cent peptone and 0.2 per cent alcohol.

Glycocol, alanine or calcium acetate, together with peptone solution, when added to plates before pouring with 2 per cent agar, did not stimulate aroma production above that produced when peptone alone was used. Use of ethyl alcohol in this manner with peptone resulted in a marked increase in aroma. When glycocol or alanine was used with alcohol but without peptone, more aroma was produced than when combined with peptone but less than with the peptone-alcohol combination.

Apparently Ps. fragi thrives best near the surface of milk as changes produced by it in litmus milk begin at the surface. At first the milk usually becomes slightly alkaline after which an acid ring is formed at the surface next to the wall of the tube. In order to test the effect of volume-surface relationships, the contents of four tubes of litmus milk were emptied into four sterile, 150 ml. cotton stoppered erlenmeyer flasks. The depth of the milk in the flasks was about 0.25 cm. as compared with about 4 cm. in the tubes. The surface area of milk in each flask was approximately 28 sq. cm. as compared with about 1.5 sq. cm. in each tube. One drop of 10 per cent ethyl alcohol was added to one flask and one tube of milk and 2 drops to another flask and tube. One tube and one flask were held as checks to compare

the changes taking place in the other three flasks and tubes, which were each inoculated with 1 drop of a suspension of Ps. fragi.

After 1 day the reaction of the milk in the inoculated tubes remained unchanged while milk in all of the inoculated flasks had become alkaline (Table 15). No aroma could be detected in any of the tubes butthere was a distinct aroma in the flasks containing alcohol, the aroma being most conspicuous in the flask containing 2 drops. After 2 days the inoculated flask containing no alcohol was still alkaline, the flask with 1 drop was slightly alkaline and that with 2 drops of alcohol was about the same as the uninoculated control. In all the inoculated tubes an acid ring had begun to form at the surface, but the milk was unchanged below it. The aroma had lessened appreciably in the flasks but had become apparent in the tubes, the most aroma being present in the tube containing 2 drops of alcohol. On the third day the inoculated flask containing no alcohol was about the same color as the control, but the flask with 1 drop of alcohol was slightly acid and that with 2 drops was distinctly acid. No further change was noted in the appearance of any of the tubes. In the flasks the aroma was faintly perceptible only in that containing 2 drops of alcohol, while the aroma in the tubes appeared to remain the same as the previous day. On the fourth day, milk in each of the inoculated flasks had become more acid than the uninoculated control and eventually all became pink (acid) and coagulated with no reduction of the litmus. The milk in the tubes never turned as pink as did that in the flasks. The aroma had disappeared completely from all flasks by the fourth day but persisted in the tubes for a much longer time. Apparently, the alcohol and abundant oxygen supply combined to speed up the metabolism of Ps. fragi so that it carried its reactions

Table 15. Effect of relative amount of surface on reactions produced in litmus milk by Ps. fragi.

	After 1 day		After 2 days		After 3 days		After 4 days	
	aroma	appearance	aroma	appearance	aroma	appearance	aroma	appearance
Tubes								
No inoc., no alcohol	-*	unchanged	-	unchanged	-	unchanged	-	unchanged
Ps. fragi, " "	-	"	+	unch. below acid ring	+	unch. below acid ring	+	unch. below acid ring
" 1 drop alcohol**	-	"	++	"	++	"	++	"
" 2 drops alcohol		"	+++	"	+++	"	+++	"
Flasks								
No inoc., no alcohol	-	"	-	unchanged	-	unchanged	-	unchanged
Ps. fragi, " "	±	alkaline	-	alkaline	-	neutral	-	sl. acid
" 1 drop alcohol	+	"	+	sl. alk.	-	sl. acid	-	acid
2 drops alcohol	++	"	+	neutral	±	acid	-	"

*Aroma is designated as follows: none -, very faint ±, faint +, distinct ++ and strong +++.

**10 per cent ethyl alcohol.

through more quickly.

As a check on acid production by Ps. fragi, three series of solutions were prepared, as shown in Table 16. Before inoculating, 25 ml. portions of the solutions in series 1 and 2 were titrated with 0.05 N sodium hydroxide, using phenolphthalein as an indicator. After inoculating with Ps. fragi and incubating 11 days at room temperature, 25 ml. portions were removed and titrated. The variation in the amount of sodium hydroxide necessary in Series 1 (0.1 per cent peptone) after 11 days was rather surprising, especially because all three of the solutions in Series 2 (0.5 per cent peptone) required approximately equal amounts and these latter amounts were nearly the same as those necessary to neutralize the solutions containing alcohol in Series 1. However, the results obtained with Series 1 were corroborated by a third series made up like Series 1 but with a few drops of brom cresol purple indicator added. After 11 days the reaction of the 0.1 per cent peptone without alcohol appeared to be about neutral, while both solutions containing alcohol were acid.

Electrometric pH determinations by the quinhydrone electrode on the solutions in Series 3 after 28 days were 7.32, 5.60 and 5.69, respectively, for the peptone alone, peptone plus 0.1 per cent alcohol and peptone plus 1 per cent alcohol. These three solutions were left at room temperature until 12 weeks had elapsed. During this time they became less acid; the solution containing 0.1 per cent alcohol appeared to be about neutral and the others alkaline, the solution without alcohol appearing the most alkaline.

It is well known that many substances which have a very pleasant and agreeable odor when present in minute quantities have an entirely different and often disagreeable odor when in concentrated form. This is true of musk

Table 16. Production of acid by Ps. fragi in peptone solution without and with alcohol.

	ml. 0.05 N NaOH required to neutralise 25 ml. of solution		Difference
	at start	after 11 days	
Series 1			
0.1% peptone	.15	.30	.15
0.1% " plus 0.1% alcohol	.20	1.05	.85
0.1% " " 1.0% "	.20	.90	.70
Series 2			
0.5% peptone	.90	1.70	.80
0.5% " plus 0.1% alcohol	.90	1.60	.70
0.5% " " 1.0% "	.90	1.60	.70

	General reaction		
Series 3	at start	after 11 days	pH after 28 days
0.1% peptone plus Brom cresol purple	neutral	neutral	7.32
0.1% " " 0.1% alcohol plus B.C.P.	"	acid	5.60
0.1% " " 1.0% " " "	"	"	5.69

and indol which are used a great deal in perfumes. In order to obtain an idea of the odor of the aroma material of Ps. fragi in more concentrated form, the following experiment was performed. Two 6 liter batches of 0.2 per cent peptone solution were prepared and sterilized in 12 liter flasks. Following sterilization, 12 ml. (0.2 per cent) of ethyl alcohol was added to each flask as well as 1 ml. of a suspension of Ps. fragi. The inoculated flasks were incubated 6 days at room temperature during which time a strong May apple aroma was produced. About 1600 gm. of sodium chloride were added to one of the flasks and its contents were then distilled until about 500 ml. of distillate had been collected. The aromatic material came over in the early distillate and none could be detected in the distilled culture. The contents of the other flask were distilled without adding sodium chloride and about the same amount of distillate collected. About 100 ml. of ether was added to each distillate to take up the aromatic material. The ether was left in the flasks with the distillates for 2 days, being thoroughly distributed at intervals during this time. It was recovered with a separatory funnel, dried with anhydrous sodium sulfate and then placed in small flasks which were not tightly stoppered and, accordingly, allowed the ether to evaporate slowly. When the ether had entirely evaporated, there remained in each flask only a very small amount of material. In a relatively high concentration the odor of this suggested plum blossoms, but in relatively low concentration the odor resembled the May apple odor which is so characteristic of Ps. fragi.

The results indicate that the aroma material of Ps. fragi is soluble in ether. This is also indicated by the failure to detect the May apple odor in the distillates after extracting with ether and allowing the ether odor to disappear.

DISCUSSION OF RESULTS

The existence of Ps. fragi in the United States was overlooked until it was isolated and identified at the Iowa Agricultural Experiment Station some years ago. The frequency with which it was noted in dairy products thereafter suggested that it is widely distributed in Iowa. Its isolation from a few samples of dairy products from other sections of the country was evidence that it is not confined to that state.

The examination of milk supplies delivered to plants in Iowa and Kentucky showed that Ps. fragi was present in the milk in a considerable number of instances. In the milk supplies in which it was found, the method of examination used did not detect it in all the samples studied. It was not detected at all in milk supplies from some farms, even when as many as 15 samples, representing a period of about 2 months, were examined. Perhaps, if larger samples, possibly a pint or more, could have been studied, an appreciably larger percentage of the samples would have yielded it. As it is easily killed by heat, it should not be present on well cared for milking utensils. Therefore, its presence in milk may be some indication of the care used in production. However, because of its psychrophilic nature, Ps. fragi is more prevalent in the winter when the cows must be kept in the barn for longer periods, and it may be more difficult for the dairymen to prevent contamination of milk by it during this season.

The majority of the species of the genus Pseudomonas appear to be soil and water forms. Evidently Ps. fragi is no exception. The isolation of this organism from a majority of barnyard dirt samples obtained on Iowa

and Kentucky farms in seasons favorable for its growth indicates that it is commonly present in soil in those regions. Failure to isolate it from any of 10 such samples obtained on Kentucky farms in summer emphasizes its psychrophilic nature. Since the dirt samples were taken from the surface and winter samples from some of the same locations yielded the organism, it is probable that in summer it survives deeper in the soil where temperatures remain lower than at the surface. None of the samples of tap water or water direct from wells in Iowa and Kentucky yielded Ps. fragi, but water from sources exposed to contamination by dirt, such as stock watering tanks, surface of ground, etc., harbored it in a large majority of instances. Since all of the general barn equipment, feed, bedding and even the animals themselves have ample contact with dirt, the presence of the organism on them is easily explainable. Of the dairy equipment sampled, only milking utensils are given such care or treatment as to control this organism. This is reflected in the data, since only 2 of 23 samples from such equipment yielded Ps. fragi. These two samples came from farms whose milk supplies had frequently yielded the organism, and presumably these dairymen were not caring for their milking equipment as well as some of the others.

The more frequent occurence of Ps. fragi in barnyard soil in the eastern part of the United States than in the western part is of interest. It is in the eastern part that most of the nation's milk is produced and most of the dairy products manufactured. The fact that Ps. fragi is more widely distributed in the heavy milk producing area increases the possibility of contamination of a large portion of milk and other dairy products. While the reasons for this geographical distribution are not definitely known, the weather and especially the amount of annual rainfall may offer an explanation. States in the western prairie section and the Rocky Mountains have only a compara-

tively small annual rainfall, much less than those in the eastern section. In addition, the high temperatures occurring in summer throughout much of this territory probably are detrimental to this psychrophilic organism. These conditions no doubt influence the existence or survival of an organism, such as Ps. fragi, whose habitat appears to be soil and water. Conceivably, such a comparatively dry area might form somewhat of a barrier to the westward spread of Ps. fragi.

The effect of alcohol on aroma production is of interest but probably of little practical importance. It may be of use when time is an important factor in determining the presence of Ps. fragi or in the preliminary identification of it. By its use results might be obtained a day sooner. The use of alcohol in the enrichment procedure does not seem to be particularly helpful because the aim of this procedure is to produce sufficient increase in the organism to dominate the flora so that its colonies can be readily detected after plating. It is doubtful that alcohol would speed up the reproduction of Ps. fragi appreciably at temperatures used in the enrichment procedure.

The aromatic material produced by Ps. fragi appears to be formed from protein or protein fractions. In peptone or litmus milk made from skim milk, where little or no fat is present, no rancidity detectable by smell is produced. When cultures are kept in cotton stoppered tubes, the May apple aroma eventually disappears.

SUMMARY

Data are presented on the distribution of Ps. fragi in normal and defective dairy products, dairy plant equipment and water supplies and on farms. Samples examined for the presence of this organism were obtained principally in Iowa and Kentucky, although data on samples of soil from barnyards in 31 other states are included.

Ps. fragi was found frequently in samples of normal milk and cream delivered by producers to an Iowa milk plant. It was not detected in milk delivered in June to a Kentucky milk plant, but was found in 40.0 per cent of samples delivered to the same plant in December.

Several samples of defective dairy products, especially those criticised as rancid or as having a May apple odor, yielded the organism.

In general, the dairy plant equipment examined was relatively free of it.

An appreciable percentage (10.0 per cent) of the Iowa dairy plant water supplies examined yielded the organism.

A large proportion (51.8 per cent) of dirt samples obtained on Iowa farms and samples of other materials or equipment likely to come in contact with or be contaminated by dirt was found to harbor Ps. fragi. Relatively few (4.1 per cent) similar samples obtained on Kentucky farms during the summer season yielded it, but it was found in a larger proportion (37.4 per cent) of those obtained in December.

Ps. fragi was found in 23 of 33 samples (69.7 per cent) of barnyard soil obtained from state agricultural experiment stations throughout the United States. It was found in samples from a larger proportion of states (90.0 per

cent) in the eastern half of the country than from those in the western half (38.5 per cent)

The wide distribution of Ps. fragi on farms emphasizes the importance of farms as a source of the organism.

Aroma production by Ps. fragi in milk, peptone broth and on agar media containing peptone was stimulated by the presence of ethyl alcohol but not by other alcohols tried.

The aromatic material produced by Ps. fragi was ether soluble. When concentrated, it had an odor resembling that of plum blossoms.

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