PSYCHROPHILIC ORGANISMS IN QUEENSLAND BUTTER

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SUMMARY

Choice and first quality butters submitted for grading in Brisbane were examined for psychrophilic count at the same time as they were examined by the routine tests used in the Butter Improvement Service. Eighty-two per cent. of butters did not contain psychrophilic organisms in 1/10 g and only 7 per cent. contained more than 100 psychrophiles per g. However, one or more samples of butter from 25 of 35 factories sampled contained psychrophiles.

Although coliforms and psychrophilic organisms occurred as a result of post-pasteurization contamination, one type of contamination occurred frequently without the other. However, there was a general relationship between the two tests, as significantly larger numbers of coliform positive tests occurred in butters with higher psychrophilic counts.

The majority of the isolates were classified as *Pseudomonas*, with strains of *Vibrio*, *Achromobacter*, *Aeromonas*, *Flavobacterium*, *Arthrobacter* and *Aerobacter* also being found, as well as 2 yeasts. Over 90 per cent. of the isolates were lipolytic and over 70 per cent. proteolytic. When representative strains were inoculated into cream which was then made into butter, many produced serious flavour defects, including rabbito, rancidity, tallowiness or mouldy flavour.

Psychrophilic organisms isolated from 10 butters which had developed rabbito or rancidity were similar to many strains isolated from normal butters.

I. INTRODUCTION

Various workers have investigated defects in butter which have been caused by organisms capable of growth at low temperatures. Wagenaar (1952) reviewed the literature concerning the surface-taint or rabbito defect in butter, which can be caused by species of *Pseudomonas*. Psychrophilic organisms have also been found responsible for the development of rancidity in butter (Hammer and Collins 1934; Morrison and Hammer 1941) and for pigment production (Hiscox 1936; White 1940). An extensive review of the literature concerning psychrophilic bacteria and their importance in foodstuffs has been prepared by Witter (1961).

There have been few studies to determine the incidence of psychrophiles in butter. A survey of psychrophilic organisms in milk and milk products, but not including butter, was reported by Schultze and Olsen (1960*a*). Jezeski and Macy (1946) isolated and identified caseolytic and lipolytic organisms from butter, which were capable of growth at 8°C. Druce and Thomas (1959) determined psychrophilic colony counts on butter, using an incubation temperature of 3–5°C for 14 days, and suggested that such counts should not exceed 1000 per g. These authors did not attempt to isolate or classify the psychrophilic organisms in their samples.

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In the last two years there have been several instances of Queensland butter being degraded due to rabbito or rancid defects which were of bacterial origin. The investigations reported in this paper were undertaken to determine the incidence of psychrophilic organisms which could cause such defects. Butter submitted for grading in Brisbane was examined for psychrophilic count and the predominant types of such organisms determined.

II. EXPERIMENTAL PROCEDURE

(i) *Sampling.*—Butters sampled at the grading floors for examination under the Queensland Butter Improvement Service (Muller and Nichols 1950) were plated at the same time for psychrophilic count. Samples were taken of 404 churnings from 84 consignments, including butter from 35 factories. In addition, 10 samples of butter which showed rancid or rabbito defects were examined to determine the numbers and types of psychrophilic organisms in such butters. These butters were from four factories.

Methods of examination for the Bacteriological Quality Index were those outlined by Muller and Nichols (1950), except that total bacterial counts were made on Difco tryptone/glucose/yeast extract (TGE) agar, and casein-digester counts were not made on the majority of the samples.

(ii) *Bacteriological Examination of Butters.*—Considerable variation is to be found in the incubation times and temperatures used by various authors for determining psychrophilic organisms in dairy products (Thomas 1958). American Public Health Association (1960) recommends incubation at 5–7°C for 7–10 days for psychrophilic counts. In the present work incubation was 5°C for 14 days, using TGE agar, and isolates which were capable of growth under these conditions were regarded as psychrophiles.

(iii) *Isolation and Identification of Cultures.*—Representative colonies were picked from the psychrophilic plates, streaked on BBL nutrient agar plus 0·5 per cent. yeast extract and incubated at 22°C. Isolates were purified by repeated picking and streaking and then retested for psychrophilic growth in peptone water plus 0·5 per cent. NaCl at 3–5°C for up to 14 days. If more than one isolate was obtained from the same source with identical characteristics or differing only in a few minor details, replicates were discarded.

Determinations of morphology and Gram reaction were made both on cultures on yeast extract/nutrient agar and on peptone water cultures. Gram-staining technique was based on the Hucker modification, but 0·3 per cent. $NaHCO_3$ was incorporated into the iodine solution, water washes were standardized at 5 sec and counterstain was applied for 30 sec. Cell morphology was checked on smears stained by Newman's stain.

Motility tests were performed by the oil-drop method on cultures from peptone/sodium chloride medium which had been incubated for 24–48 hr at 22°C. Negative cultures were subcultured and retested at intervals. Flagella strains

were made on all cultures, whether found to be motile or not, on the cultures recently tested for motility. The Casares–Gil and Leifson methods (Society of American Bacteriologists 1957) were both used, with the former being adopted as the most suitable for routine purposes. The stain was modified to contain 20 ml of a saturated solution of ammonium alum, 10 ml of 1·5 per cent. aqueous solution of NaCl and 10 ml distilled water in addition to the normal ingredients of the Casares–Gil stain. This stain was found to keep well for months in the refrigerator.

Cultural characteristics were recorded from cultures incubated on yeast extract/nutrient agar and cultures in peptone water after 3 days at 22°C. Growth temperatures were determined by inoculating 1 drop of a 1 in 100 dilution of a broth culture to peptone water and incubating at 5°C, 22°C, 30°C and 37°C till growth or 28 days. The catalase test was performed on cultures on slopes.

Sensitivity to antibiotics was tested using "Sentests" (Evans Medical Ltd.) containing 2·5 I.U. penicillin and 80 μg streptomycin.

Litmus milk was inoculated with 1 drop of broth culture and reactions read after 2, 7 and 28 days at 22°C.

Methyl red and V.P. tests were performed on cultures grown in Difco M.R.–V.P. medium incubated at 22°C for 5 days, using the O'Meara modification for the V.P. test. Indole tests were performed by the Arnold and Weaver method, using the Kovacs reagent, and by the Goré modification of the Ehrlich-Böhme method (Society of American Bacteriologists 1957). Utilization of citrate was tested in Koser's citrate broth (Difco).

The oxidase test was performed by the method of Kovacs (1956). All tests for carbohydrate utilization were made using the medium of Hugh and Leifson (1953) and utilizing 5 ml of 1·5 per cent. agar to seal the "closed" tubes.

Lipolysis was demonstrated on yeast extract/nutrient agar containing 1 per cent. tributyrin or butteroil. Plates were streaked and lipolysis shown by clearing on the tributyrin plates or fatty acid precipitation on the fat plates after incubation for 15 days at 30°C. Caseinolysis was demonstrated by clearing on plates of yeast extract/nutrient agar containing 10 per cent. sterile skim-milk incubated at 30°C for 2 days. The production of pigment, particularly the yellow pigment of *Flavobacterium* strains, was also noted on this medium.

Cultures were tested for their reaction in the arginine medium used by Thornley (1960) and for pigment production and fluorescence in the asparagine medium of Georgia and Poe (1931).

(iv) *Examination for Production of Defects in Butter.*—Representative cultures were tested for their ability to produce defects in butter.

Choice grade neutralized, vacreated cream was divided into 100 ml quantities in 250 ml ground-glass-stoppered flasks and laboratory-pasteurized at 73°C for 15 sec. The cream was then inoculated with 1 ml of a 3-day culture in peptone

water + salt, which had been standardized to a reading of 10 on a colorimeter. After incubation for 1 day at 30°C the creams were cooled to 10°C and churned in the flasks, using a laboratory stirrer. The buttermilk was drained off, the butter washed with 30 ml sterile tap water, 0·8 g salt added and the butter worked in parchment paper.

The butters were stored at 5°C, analysed after 9 days, and graded after 10 and 13 days.

III. RESULTS

(a) Incidence

The results of psychrophilic counts on 404 samples of choice and first quality butter sampled at grading floors are shown in Table 1. In these samples, 82 per cent. did not contain psychrophilic organisms in 1/10 g and only 7 per cent. contained over 100 per g. Although this was quite a low incidence, psychrophiles were present in 1 or more samples from 25 of the 35 factories.

TABLE 1

INCIDENCE OF PSYCHROPHILIC ORGANISMS

	Normal Butters			Butters Showing Rancid or Rabbito Defects
	Apr.–Sept.	Oct.–Mar.	Total	
No. of samples	138	266	404	10
No. with psychrophilic counts/g:				
<10	112	220	332	..
10–<100	19	25	44	..
100–<1,000	5	9	14	..
>1,000	2	12	14	10
No. of factories	35	20	35	
No. of factories with butter containing psychrophiles	19	11	25	

The results were considered in two groups, the winter period April to September, and the summer period October to March. The incidence was similar for the two periods.

The 10 samples of butter which had been degraded for rabbito or rancid defects all had high counts of psychrophiles, with most of these counts being greater than 10^5 per g.

(b) Comparison of Results of Coliform Tests and Psychrophilic Counts

The relationship between the coliform test result on 1/10 g and the psychrophilic count is shown in Table 2. A X^2 test on these figures gave a value of 11·63 (3 degrees of freedom). This value is significant at the 1 per cent. level, showing that the distribution of positive coliform tests was not

homogeneous. Because of the small numbers of tests in two of the groups the results were rearranged to give the number of psychrophilic counts with logarithm less than 2 and 2 or greater. A X^2 test on these figures gave a value of X^2 (1 degree of freedom) of 9·57. This is significant at the 1 per cent. level. This shows the increase in positive coliform tests with the higher psychrophilic count.

TABLE 2

COMPARISON OF COLIFORM TESTS AND PSYCHROPHILIC COUNTS ON BUTTERS

Psychrophilic Count–log	Coliform Test			
	No. Negative		No. Positive	
<1	261		71	
1–<2	36	297	8	79
2–<3	9		5	
3 and >3	6	15	8	13

(c) Classification of Isolates

Representative colonies were picked from the psychrophilic plates, purified and classified by the use of the tests detailed above.

A summary of the reactions of the various groups is shown in Table 3. The groups had the following characteristics:

(1) *Pseudomonas.*—Gram-negative rods, slender, straight or curved. Mostly motile by polar flagella but sometimes non-motile. Oxidative utilization of glucose, sometimes sucrose and rarely lactose. Kovacs oxidase positive. Resistant to penicillin and sensitive to streptomycin. Usually an alkaline reaction in the arginine medium of Thornley (1960). Catalase positive. Usually hydrolysing fat and casein. Growth on desoxycholate agar but colonies not of the coliform type. Green or yellow-green soluble, fluorescent pigment sometimes produced. Usually methyl red negative, VP negative. Litmus milk usually digested, sometimes acid, frequently reduced and coagulated.

(2) *Aeromonas.*—Distinguished from *Pseudomonas* by producing acid and gas in glucose, sucrose and usually lactose. Reaction in arginine medium usually acid. Usually coliform growth on desoxycholate agar. No pigments produced. Usually methyl red positive. Litmus milk digested, usually reduced and coagulated.

(3) *Vibrio.*—Distinguished from *Pseudomonas* by fermentation of glucose (and frequently sucrose and lactose) with production of acid only. Reaction in arginine medium always alkaline. Methyl red usually positive. Action on litmus milk usually reduction and coagulation sometimes with digestion.

(4) *Achromobacter.*—Short, stout Gram-negative rods and Gram-negative coccoid rods or even cocci. Motile by peritrichous flagella or non-motile. Oxidative utilization of glucose and sometimes of sucrose and/or lactose. Rods

TABLE 3

SUMMARY OF MAIN REACTIONS USED IN THE IDENTIFICATION OF CULTURES

Genus	Gram	Morphology	Motility	Flagella if Motile	Utilization of						Kovacs Oxidase	Penicillin	Streptomycin	Arginine medium	Fat	Casein	Pigment Produced
					Glucose		Lactose		Sucrose								
					Open	Closed	Open	Closed	Open	Closed							
Pseudomonas	–ve	Rods, slender straight or curved	+/–	Polar	A (few–)	–	A/–	–	A/–	–	+	R	S	Alk/A	+/–	+/–	Green or yellow green soluble fluorescent or none
Aeromonas ..	–ve	Rods ..	+ (few–)	Polar	AG	AG	AG (few–)	AG (few–)	AG (few–)	AG (few–)	+	R	S	A/Alk	+/–	+/–	–
Vibrio ..	–ve	Rods, slender straight or curved	+ (few–)	Polar	A	A	A/–	A/–	A/–	A/–	+	R	S	Alk	+/–	+/–	–
Achromobacter	–ve	Short, stout rods,	+/–	Peri	A/–	–	A/–	–	A/–	–	–	S	S	A/Alk	+/–	+/–	–
		coccoid rods	+/–	Peri	A/–	–	–	–	–	–	+	R	S	A/Alk	+/–	+/–	–
Aerobacter ..	–ve	Rods ..	+	Peri	AG	AG	AG	AG	AG	AG	–	S	S	A	–/+	+/–	–
Flavobacterium	–ve	Rods ..	–/+	Peri	A	–	–	–	A	–	+/–	S	S	Alk/–	+/–	–/+	Yellow
Arthrobacter	+ve	Rods and cocci	–	–	A	A	–/A	–/A	A	A	–	R	S	Alk	–	–/+	Yellow

A —acid
AG —acid and gas
Alk —Alkaline
Peri—peritrichous

+/– —generally +
R —resistant
S —susceptible

oxidase negative, coccoid and cocci forms oxidase positive. Rods sensitive to penicillin, cocci resistant. Acid or alkaline reaction in arginine medium. Fat and casein usually hydrolysed. Growth on desoxycholate agar but not of coliform type. No pigment produced. Catalase positive. Action on litmus milk usually reduction and coagulation.

(5) *Aerobacter*.—Distinguished from *Achromobacter* by fermentation of glucose, sucrose and lactose with production of acid and gas. Oxidase negative, penicillin sensitive. Acid reaction in arginine medium. Casein hydrolysed. Growth on desoxycholate agar of coliform type. Methyl red positive, VP negative. Motile by peritrichous flagella. Litmus milk surface digestion, reduction and coagulation.

(6) *Flavobacterium*.—Colonies yellow. Usually non-motile. Oxidative utilization of glucose and lactose. Oxidase positive. Penicillin sensitive. Alkaline or no reaction in arginine medium. Fat hydrolysed and usually casein. Litmus milk reduced and coagulated.

(7) *Arthrobacter*.—Gram-positive rods and cocci. Non-motile. Fermentative attack on glucose, lactose and sucrose, with production of acid only. Oxidase negative. Resistant to penicillin and sensitive to streptomycin. Alkaline reaction in arginine medium. Catalase positive. Fat and casein not hydrolysed. Growth on desoxycholate agar but colonies not of coliform type. Colonies on nutrient agar yellow. Methyl red negative and VP negative. Reduction and coagulation in litmus milk.

(8) *Yeasts*.—Typical yeast morphology, cells elliptical, and showing budding and the development of a rudimentary pseudomycelium.

(d) Distribution of Bacterial Types

The distribution of bacterial types among isolates, samples and factories is shown in Table 4.

TABLE 4

DISTRIBUTION OF BACTERIAL TYPES AMONG CULTURES ISOLATED, AND FROM SAMPLES AND FACTORIES

——	Isolates	Samples	Factories
Total	126	52	25
Number of, or containing—			
Pseudomonas	66	33	17
Vibrio	21	15	10
Achromobacter	19	15	9
Aeromonas	9	5	3
Flavobacterium	4	4	3
Arthrobacter	3	3	3
Aerobacter	2	2	2
Yeasts	2	2	2

Pseudomonas types were the most widely distributed, with approximately equal numbers of *Vibrio* and *Achromobacter* species being the next most frequent genera encountered. Of all the isolates, 90 per cent. were Gram-negative rods. The isolates were very active biochemically. Ninety-two per cent. hydrolysed tributyrin and butteroil, 72 per cent. hydrolysed casein, 84 per cent. reduced and coagulated litmus milk, and 68 per cent. peptonized it.

(e) Production of Defects in Butters

Types of psychrophiles isolated from normal butters were very similar to those isolated from the butters showing defects. Representative types were inoculated into cream which was then churned and made into butter. The results of these tests are shown in Table 5. Fifteen cultures from normal butter or from cream in addition to 3 isolates from degraded butter produced

TABLE 5

DEFECTS PRODUCED IN BUTTER BY PSYCHROPHILIC ORGANISMS

Type	Source	No. of Cultures	Average Total Count/gm–log	Grading Comments on Inoculated Butter
Pseudomonas	Normal butter ..	6	6·1	Rabbito
	Normal butter ..	3	5·8	Clean acid
	Normal butter ..	1	5·7	Tallowy
	Normal butter ..	1	5·2	No effect
	Rancid or rabbito butter	3	5·9	Rabbito
	Pasteurized cream ..	1	6·1	Tallowy, slight rabbito
	Pasteurized cream ..	1	5·8	Rancid
Achromobacter ..	Normal butter ..	1	5·5	Slight rabbito, oxidised
	Normal butter ..	1	5·7	Clean acid
	Defective butter ..	1	5·7	Clean acid
	Pasteurized cream ..	1	5·8	Mouldy
Vibrio	Normal butter ..	2	5·7	Rabbito
	Pasteurized cream ..	1	5·4	Mouldy
	Pasteurized cream ..	1	5·8	Rancid, slight rabbito
Arthrobacter	Normal butter ..	1	5·3	Slight rabbito
	Pasteurized cream ..	1	5·6	Rancid, mouldy
Aerobacter	Normal butter ..	1	5·8	Mouldy
	Pasteurized cream ..	1	5·7	Slight fermented
Aeromonas	Normal butter ..	1	5·9	Clean acid
	Pasteurized cream ..	1	5·7	No effect
Flavobacterium ..	Normal butter ..	1	5·9	Slight rancid
Yeast	Normal butter ..	1		No effect
	Pasteurized cream ..	1		Mouldy

rancid or rabbito defects. These cultures included cultures classified as *Pseudomonas, Achromobacter, Vibrio* and *Arthrobacter.* The *Flavobacterium* culture tested produced a slightly rancid butter, the *Aerobacter* cultures gave a mouldy flavour and a slightly fermented flavour and one of the yeasts produced a mouldy flavour. Nine of the cultures tested produced only clean acid flavour or no effect. These results show that a large proportion of psychrophiles can cause flavour defects in butter.

IV. DISCUSSION

The numbers of psychrophilic bacteria found in choice and first grade butter at the time of grading were generally low, and only 7 per cent. of samples contained more than 100 psychrophiles per g. However, 71 per cent. of factories produced butters containing low numbers of psychrophilic organisms, showing that the incidence, although low, was very wide. The 15 butters examined by Jezeski and Macy (1946) all showed psychrophilic counts of 1000 per g. or greater, but these butters were selected by the development of a defect of bacteriological origin in a routine keeping quality test. Several of these butters had very high counts comparable with those of butters examined in the present study which had been degraded because of rabbito or rancid defects.

The types of psychrophilic organisms isolated from butter were similar to those found in other dairy products (Jezeski and Macy 1946; Erdman and Thornton 1951; Marth and Frazier 1957; Schultze and Olsen 1960*a*), with a marked predominance of *Pseudomonas* species. Earlier investigators (Thomas and Sekkar 1946) reported a predominance of *Achromobacter* types among 206 representative strains of psychrophilic bacteria, but this difference was probably one of classification, and recent changes in the classification of the Gram-negative rods would probably result in a greater percentage of these organisms being classified as *Pseudomonas.*

Psychrophilic coliform organisms have been frequently reported in dairy products (Hammer and Yale 1932; Erdman and Thornton 1951; Panes and Thomas 1959; Schultze and Olsen 1960*b*). Psychrophilic cultures of *Vibrio* and *Aeromonas* have not previously been reported from dairy products, but have been isolated from other sources (e.g. Brown and Weidmann 1958; Georgala 1958).

The characteristics used to differentiate the Gram-negative rods were largely those proposed by Shewan, Hobbs, and Hodgkins (1960) and Thornley (1960). The majority of *Pseudomonas* isolates fitted into one or other of Thornley's *Pseudomonas* groups, but a number of isolates which were non-motile, inert to carbohydrates and non-fluorescent, but resistant to penicillin and oxidase positive, were classed as *Pseudomonas.* These organisms were definite rods, and not similar in morphology to the oxidase positive, penicillin sensitive coccoid rods which were included in the *Achromobacter* by Thornley. Other Gram-negative coccoid rods or cocci with reactions similar to those reported by Thornley have been included in the *Achromobacter.*

The two *Aerobacter* isolates fitted Thornley's description except that they were sensitive to penicillin. Two non-motile isolates which were oxidase positive, penicillin resistant and fermented glucose with the production of acid and gas were considered to be *Aeromonas*. These strains appeared to be very similar to the non-motile organisms studied by Eddy (1960).

The group *Vibrio* has been taken to comprise polar flagellate fermentative rods, oxidase positive and resistant to penicillin, which produced acid but not gas from glucose. This grouping is in accordance with the scheme proposed by Shewan, Hobbs, and Hodgkiss (1960). Organisms which would be similar to *Vibrio cuneatus* or *V. percolans* with an oxidative attack on glucose would have been included among the *Pseudomonas*. Liston (1960) has shown the similarity of these organisms to *Pseudomonas*. *Pseudomonas* and *Vibrio* could not be differentiated on the basis of morphology.

The chief differentiating characteristic of the genus *Flavobacterium* appeared to be the production of a yellow colony. In other respects isolates resembled the rod forms of the *Achromobacter*.

The types of psychrophilic organisms isolated from the butters which showed defects were similar to those obtained from the choice and first grade butters. Representatives of all the Gram-negative species were obtained from high dilutions of these samples of degraded butter. The majority of isolates from the good-quality butters were lipolytic or caseolytic, and when representatives of these were inoculated into butter many produced serious flavour defects. It would appear that organisms capable of causing such flavour defects were present in many butters in low numbers. In the event of underworking, taints could occur in these butters due to growth of these organisms during cold storage.

It has been shown that psychrophilic organisms do not usually survive pasteurization and that the occurrence of psychrophiles after pasteurization of milk and cream is due to post-pasteurization contamination (Thomas 1958; Witter 1961). For this reason and the fact that these organisms are such undesirable types it has been proposed that a count of psychrophilic organisms should be included in routine butter investigations (Druce and Thomas 1959). The inclusion of such a count poses practical problems because of the long incubation period required. Low counts of psychrophiles as found in some of the butters examined in this study would not be of any importance with regard to keeping quality unless the butter was very underworked. Although the presence of psychrophiles can be taken to indicate post-pasteurization contamination, the test for coliform organisms is now used to give this information. A comparison of the results of the two tests showed that there was an increase in numbers of positive coliform tests in butters showing higher psychrophilic counts but about 50 per cent. of butters with psychrophilic counts greater than 100 per g. did not contain coliforms in 1/10 g. It is considered that the inclusion of a psychrophilic count in routine butter quality investigations is not warranted, but the psychrophilic count can prove very useful in investigating causes of defects and pin-pointing sources of contamination which may not be found by other test methods.

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