ENZYMES *of* PSYCHROTROPHS *in* RAW FOOD

Robin C. McKellar

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Enzymes of Psychrotrophs in Raw Food

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PREFACE

The food industry is committed to improving and maintaining the quality of the product offered to consumers. The practice of storing raw foods such as milk, meat, and vegetables under refrigerated conditions leads to spoilage, resulting from the growth of cold-tolerant microorganisms (psychrotrophs). This process renders the raw material unacceptable for consumption, and unsuitable for further processing. Spoilage can, at least in the case of dairy products, be attributed to the production by psychrotrophs of extracellular hydrolytic enzymes.

Psychrotrophs and their enzymes pose a considerable threat to the quality of dairy products, and have thus been studied extensively. This is reflected in the emphasis placed on dairy products in this review. While psychrotrophs play a role in spoilage of fresh meat, their hydrolytic enzymes have not been clearly implicated (Chapter 11). Growth of psychrotrophs on fruits and vegetables may also lead to spoilage; however, these products will not be dealt with here.

The role of extracellular proteinases and lipases in spoilage has been reviewed on several occasions; however, no comprehensive treatise has been presented summarizing all aspects of enzyme production and activity. This is essential to provide a perspective on a very diverse field of study, and to furnish new ideas and directions for future research. This is particularly important at times when resources are strained to the limit, and priorities must be assigned.

I am indebted to the fine scientists who made a commitment to this book, and who provided excellent reviews of the literature as well as results from their own research. The authors had a difficult task; while the focus of the book was fairly narrow, much of the necessary information was not available for psychrotrophic microorganisms and had to be obtained for other related strains. Thus, some of the authors spent a great deal more time than they had originally planned. I feel quite confident that we have produced a high quality review which will be of value to researchers and food technologists alike.

THE EDITOR

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Dr. McKellar received his B.Sc. and M.Sc. degrees from the University of Waterloo in 1971 and 1972, respectively. In 1976, he received his Ph.D. in microbiology from Ottawa University. After doing postdoctoral work at Cambridge University and the National Research Council of Canada, he joined Agriculture Canada in 1979 and is presently a member of the Dairy Research Group.

Dr. McKellar was the recipient of several NRC Scholarships between 1971 and 1975, and was awarded a NATO Postdoctoral Fellowship in 1976 and an NRC Associateship in 1977. Dr. McKellar is a member of the Canadian Institute of Food Science and Technology, and serves as a Director of the Ottawa Section. He also serves on two International Dairy Federation Expert committees.

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Milk and Dairy Products

Chapter 1

PRODUCER MICROORGANISMS

Gertraud Suhren

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I. DEFINITION

In 1887, Forster[1] first observed bacterial growth at 0°C, and since then the terminology used for microorganisms which are able to grow and multiply at refrigerating or freezing temperature has been confused. Terms such as psychrophile, facultative psychrophile, cold tolerant, psychrotolerant, and psychrotroph were used synonymously. The term psychrophile was introduced by Schmidt-Nielsen[2] in 1902. This term etymologically implies that these microorganisms are cold loving and have a preference for growing at low temperatures. Actually, the optimum temperature for growth of milk-spoiling microorganisms is in the range of 25 to 35°C, similar to that for many mesophilic microorganisms. Ingraham and Stokes[3] defined psychrophiles as bacteria which grow appreciably and often abundantly at 0°C within 2 weeks. The minimum temperature is close to − 10°C, the optimum temperature in the range of 20 to 40°C, and the maximum may be as high as 45°C. In 1960 Eddy[4] recommended the use of the term "psychrophilic" only when a low optimum temperature is implied, and "psychrotrophic" for bacteria able to grow at 5°C or less, whatever their optimum temperatures. According to Witter,[5] psychrophilic bacteria grow at a relatively rapid rate at or below 7.2°C and are capable of forming visible colonies on plates incubated for 10 d at 7 ± 0.5°C. Kandler[6] suggested the use of the terms psychrophilic, mesophilic, and thermophilic only in those cases when the optimum temperature condition for the rate of growth is to be defined. Subsequent to the suggestion made by Eddy,[4] the term "psychrotrophic" is used for those organisms which are considered mesophilic with respect to their growth optimum but which are still able to grow well at temperatures below 10°C. Nakae[7] classified psychrotrophic bacteria which grow at 5°C within 10 d into three groups on the basis of the optimum and maximum growth temperature: true psychrotrophs, mesotrophic psychrotrophs, and psychrotrophic mesophiles.

In 1976, the International Dairy Federation (IDF) adopted the following definition of psychrotrophs, which is substantially in agreement with that agreed at the IDF Seminar on psychrotrophs in 1968. A psychrotroph is a microorganism which can grow at 7°C or less, irrespective of its optimum growth temperature (growth in this context includes not only multiplication but also those metabolic processes which necessarily precede multiplication).[8]

II. PRODUCER MICROORGANISMS

A. Taxonomic Aspects

The psychrotrophs do not constitute a specific taxonomic group of microorganisms; psychrotrophic bacteria strains belonging to about 15 different genera have been isolated from milk and dairy products. These genera include Gram-negative and Gram-positive bacteria, rods, cocci or vibrios, sporeformers or nonsporeformers, and aerobic and anaerobic microorganisms. In addition, several genera of molds and yeasts contain psychrotrophic representatives which can cause defects in dairy products. In the following only psychrotrophic bacteria genera will be dealt with; pathogenic genera are excluded.

In Table 1 some taxonomic properties of Gram-negative psychrotrophic bacteria are summarized.

1. Pseudomonadaceae

The genus *Pseudomonas* are the most common psychrotrophs isolated from milk. The most important nonpsychrotrophic type is *P. aeruginosa*, which can easily be separated from other pseudomonads by its growth at 41°C and by its ability to produce pyocyanin. Among the psychrotrophic pseudomonads of dairy origin a number of different species are described: *P. fluorescens*, *P. fragi*, *P. putida*, and *P. putrefaciens*. *P. fluorescens* again is divided into five biovars and *P. putida* into two biovars. Fluorescent pseudomonads from soil and water were first described in 1886. Two biotypes, distinguished by gelatin liquefaction, which later bear the names *P. fluorescens* (liquefying) and *P. putida* (nonliquefying) were recognized.[9,17,18]

Numerous papers on taxonomic aspects of pseudomonads have been published in the last 30 years.[19-26] The principle of Stanier[21] to differentiate *Pseudomonas* sp. by testing their ability to utilize different compounds as sole carbon source seems functional. However, it calls for a rather large number of utilizing tests which may be inconvenient for studies not of strictly taxonomic nature. This is probably the reason why in food microbiology the system of Shewan et al.,[27] which is based on the ability to produce fluorescent pigments and the reaction on the medium of Hugh and Leifson,[28] is widely applied.

Taxonomic studies showed that some strains could be clearly identified despite a few variations from the ideal phenotype, whereas others were intermediate between *P. fluorescens* and *P. putida* or *P. fluorescens* and *P. fragi*.[23-25,29] The distinction between the intermediate strains was not, however, confirmed with DNA homology studies.[26] Kwan and Skura[30] observed no similarity between the fluorescent group and *P. fragi* from raw milk. The *P. fragi* isolates did not produce fluorescent pigments or phospholipase and did not liquefy gelatin. Isolates of the fluorescent group — *P. aeruginosa*, *P. fluorescens*, *P. putida* — can easily be distinguished from isolates of nonfluorescent species by their fluorescent pigments, although some of the former group sometimes fail to synthesize pyoverdin *P. fluorescens* biovar II and biovar V).[9]

The biochemical diversity of psychrotrophic pseudomonads defies taxonomic classification beyond the species level. Patel and Jackman[31] demonstrated that pseudomonads can be differentiated on the basis of a distinct pattern of susceptibility to phages (i.e., lysotypes).

P. putrefaciens, later called *Alteromona putrefaciens*, was isolated by Kundrat[32] and Kielwein[22] from milk. The DNA-base composition excludes these organisms from *Pseudomonas*.[33]

2. Enterobacteriaceae

Some lactose-fermenting genera of the family Enterobacteriaceae contain psychrotrophic species. The frequent occurrence of these organisms in raw milk is due to the ubiquitous distribution in natural habitats such as soil, grass, hay, cereals, manure, and surface water. These genera do not dominate at temperatures below 8 to 10°C in a population which develops spontaneously in milk. Some isolates from refrigerated milk show atypical characteristics. This may point to a selection of particular biotypes and variants in populations developing at low temperatures.[17] In their taxonomic study, Kleeberger et al.[34] found certain subgroups which deserve taxonomic rank. These forms were not identifiable with routine diagnostic methods. It appeared that some enterobacteria from milk do have specific traits which should be included in a diagnostic scheme for milk enterobacteria. For example, the examination of the fermentation of lactose by *Enterobacter hafniae* strains — synonym to *Hafnia alvei* — isolated from milk and milk products revealed 84% positive strains, whereas according to Sakazaki lactose is not fermented by *Hafnia* strains.[35,36]

Table 1
TAXONOMIC PROPERTIES OF GRAM-NEGATIVE PSYCHROTROPHIC BACTERIA

Genera/family	Cell shape	Motility	Flagella	Oxidase	Catalase	O/F	Penicillin susceptibility	O_2	Nitrate reduction	Ref.
Pseudomonas	Rods 0.5—1.0 × 1.5—5.0 μm	+	Polar	+	+	O	–	Aerobic	–	9
Enterobacteriaceae	Rods 0.3—1.5 × 1.0—6.0 μm	D	Peritrichous	–	+	F		Facultative Anaerobic	+	10
Flavobacterium	Rods 0.5 × 1.0—3.0 μm	–	—	+	+	O	–	Aerobic	–	11
Cytophaga		+[a]				O			–	11,12
Aeromonas	Rods to coccid 0.3—1.0 × 1.0—3.5 μm	+	Single polar	+	+	F		Facultative anaerobic	+	13
Acinetobacter	Rods 0.9—1.6 × 1.5—2.5 μm[b]	–	—	–	+	O	–	Strict aerobic	–	14,15
Alcaligenes	Rods, coccal rods, or cocci 0.5—1.0 × 0.5—2.6 μm	+	Peritrichous	+	+	O		Obligately aerobic	D	16

Note: D = different reactions.

[a] Gliding motility, spreading growth.
[b] Approaching coccus shape in stationary phase.

Confusion exists about *Aerobacter*, which had originally been a separate genus. Since the 7th edition of *Bergey's Manual of Systematic Bacteriology*, the nonmotile *Aerobacter* strains are placed in the genus *Klebsiella* and the motile ones in *Enterobacter*. Therefore, *K. pneumoniae* includes the former *A. aerogenes*.[37] Consequently, literature references to *Aerobacter*, *Klebsiella*, or *Enterobacter* may refer to the same group of organisms.

3. Flavobacterium

Yellow-orange-pigmented Gram negative bacteria, easily identified as *Flavobacterium* spp., have often been isolated from raw milk. Two of four *Flavobacterium* strains tested had psychrotrophic tendencies; they were nevertheless unable to produce proteinase at 7°C at a sufficient rate to be of practical significance. At ambient temperatures (22°C) these strains were highly proteolytic. It is suggested therefore that the practical importance of dairy flavobacteria lies not so much in their psychrotrophic growth in refrigerated milk, as in their contamination of milk via poorly sanitized pipelines and equipment.[38]

Taxonomic studies of flavobacteria in dairy products have largely been limited to the past decade.[12,39-41] The flavobacteria, especially in the earlier studies, proved to be a heterogenous group of pigmented bacteria, and the majority of isolates could not be identified to species level.[12] The identification on the basis of the description in various editions of *Bergey's Manual of Systematic Bacteriology* was very difficult. The recent propositions of Holmes et al., however, seem to clarify that taxonomic problem.[11,41]

4. Cytophaga

Gliding motility and spreading growth are two characteristics which are important in differentiating between *Flavobacterium* and *Cytophaga*. There is considerable biochemical and chemotaxonomic evidence particularly mol% G + C contents, respiratory quinones, and cellular fatty acid composition for the similarity between *Flavobacterium* and *Cytophaga*. These similarities lead to problems when one attempts to distinguish between the two genera.[11]

5. Aeromonas

Aeromonas sp. share some properties with members of the Enterobacteriaceae and some with members of the genera *Pseudomonas* and *Vibrio*, but members of the genus *Aeromonas* are now clearly separated. The *Aeromonas* sp. which are important in dairy bacteriology belong to the motile group.[13] All *Aeromonas* strains tested by Eddy[4] were caseolytic and lipolytic. Water is one of the main natural habitats of these organisms. Therefore, it is not suprising that *Aeromonas* is consistently found in the microflora of dairy products.[18]

6. Acinetobacter, Alcaligenes, and Achromobacter

The classification of *Acinetobacter*, *Alcaligenes*, and *Achromobacter* was clarified by a taxonomic study of Thornley,[15] who recommended that the nonmotile coccoid rods should be placed in the genus *Acinetobacter*, while the genus *Alcaligenes* should be retained for motile, peritrichously flagellate-type species. *Achromobacter* should be reserved for any motile peritrichous strains which may prove suitable for inclusions.

Phenotypically, the genera *Acinetobacter* and *Moraxella* are very similar. They can be differentiated by oxidase test and penicillin sensitivity.[14]

The *Alcaligenes* sp. mentioned often in dairy bacteriology are *A. viscolactis* (or *viscosus*) and *A. tolerans*. According to Gyllenberg and Eklund[17] they are easily distinguished from each other by the pronounced heat resistance and presence of oxidase in *A. tolerans*. *A. tolerans* grows at 37°C, fails to grow at 2°C, and grows very slowly at 5°C. *A. viscolactis* is described as distinctly psychrotrophic and markedly lipolytic.[18] In 1968 Gyllenberg[42] suggested a discussion of the taxonomic relationship of this bacterium. As well *A. viscolactis* and *A. tolerans* are not mentioned in *Bergey's Manual of Systematic Bacteriology*.[43]

The name *Achromobacter* is used rather frequently in the literature of dairy bacteriology. Suprisingly enough, taxonomic research has failed to recognize taxa which would correspond to earlier descriptions of *Achromobacter*. The distinction between *Achromobacter* and *Alcaligenes* should be that *Achromobacter* contains peritrichously flagellated bacteria which oxidize glucose, whereas *Alcaligenes* contains nonmotile organisms which fail to oxidize glucose. However, peritrichous types which do not attack glucose are actually known, and also nonmotile Gram-negative short rods which are capable of oxidizing glucose and even other sugars have been described.[17] The two genera are very similar, and because no type culture of the genus *Achromobacter* exists, this genus was not described in the 8th edition of *Bergey's Manual of Systematic Bacteriology*.[16]

7. Thermoduric Psychrotrophs

Thermoduric psychrotrophs are a group of bacteria capable of withstanding high temperatures similar to those used in pasteurization (72 to 74°C) and also growing at refrigeration temperatures (4 to 7°C).[44] They belong to the genera *Bacillus, Clostridium, Arthrobacter, Microbacterium, Streptococcus, Micrococcus,* and *Corynebacterium*. Most belong to the genus *Bacillus*, which are Gram-positive spore forming, aerobic rods. Compared to the psychrotrophic Gram-negative rods, the thermoduric psychrotrophs are characterized by rather long generation times and/or lag phases. Within the mixed raw milk flora these genera are quantitatively of minor importance since milk is normally stored raw for only short periods of time. Their importance lies in their influence on the keeping quality time of pasteurized, nonrecontaminated food.

B. Growth Rate

Bacterial multiplication in stored milk is dependent on types and growth phase of bacteria present, the temperature conditions, and period of storage. When the contaminating bacteria have overcome the lag phase, the generation time — or doubling time — is a suitable parameter for the estimation of bacterial multiplication to be expected. In Table 2, information about generation times of psychrotrophs at refrigeration temperatures are summarized.

With 1×10^n actively multiplying contaminating bacteria per milliliter, the bacterial content increases within a 48-h storage period by about $4 \times 10^{n+3}$ ml^{-1} if the generation time is 4 h, whereas the increase is just $1.6 \times 10^{n+1}$ ml^{-1} if the generation time is 12 h (Figure 1). As can be seen from Table 2, psychrotrophic pseudomonads and Enterobacteriaceae multiply rather quickly even under cold storage conditions, whereas the Gram-positive representatives have much longer doubling times. It could be demonstrated that the specific growth rates of *Bacillus cereus, Streptococcus lactis,* and *Pseudomonas* spp. were the same whether they were present alone or in combination, provided the concentration of cells is too low to change the composition of the growth medium, which will not normally occur if the bacterial content is below 10^6 to 10^7 ml^{-1}.[49]

Aeration influences the generation time of *Pseudomonas* spp.; the higher the O_2 content, the shorter are the doubling times. This influence is more distinct at lower incubation temperature.[46,57,58] Under practical conditions milk is exposed to different temperatures during the storage period, for example, by addition of warm milk after each milking into the storage tank. An experiment with changing temperatures of 7 and 25°C revealed that the test organisms *Enterobacter hafniae* and *Pseudomonas* spp. responded immediately to a change in temperature. Assuming that the microorganisms were out of the lag phase, a fair agreement was found between experimental results and computer simulation using the growth characteristics of the bacteria as expressed by the Arrhenius equation.[49]

C. Heat Resistance

In 1953 Andrews and Kaufmann[59] reported that none of 66 psychrophilic organisms

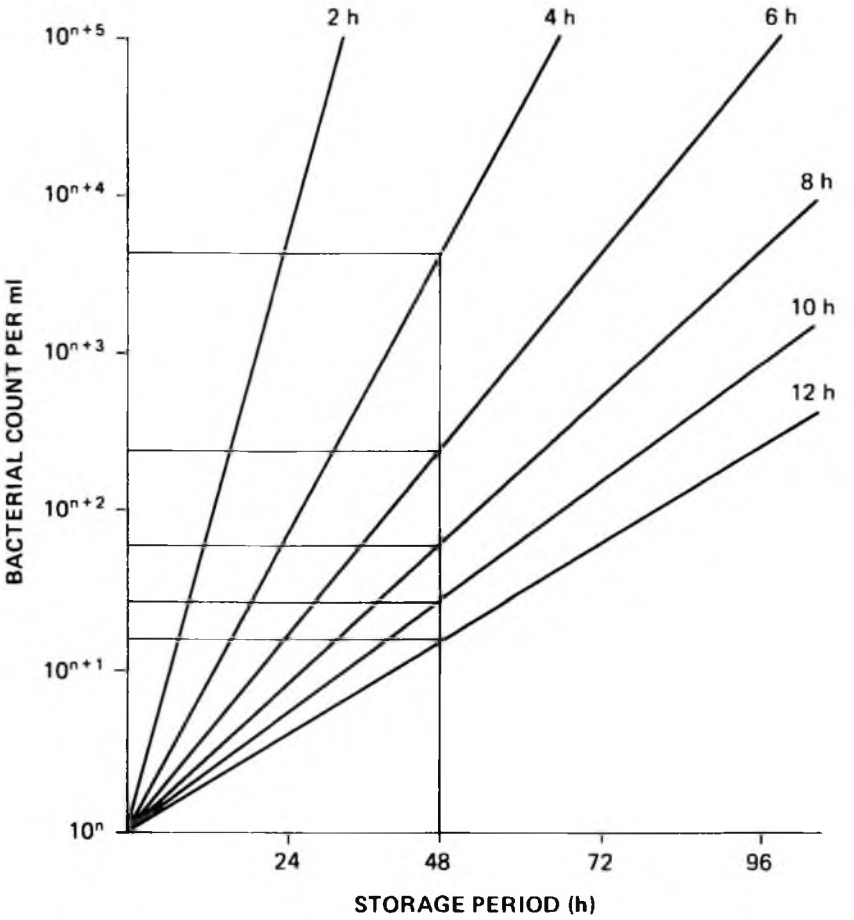

FIGURE 1. Increase in bacterial counts as a function of storage period and generation time for logarithmic growth. h = hours and n = logarithm (base 10) of bacterial count at the beginning of storage period.

isolated from milk and water supplies survived heating at 62.5°C for 25 min. In the review of Witter,[5] 15 references show evidence that psychrotrophic bacteria do not survive laboratory or commercial pasteurization and a further 9 references indicate that psychrotrophic pseudomonads are quite sensitive to heat.[5] Dabbah et al.[60] found nonlogarithmic survivor curves for *P. fluorescens*, *P. fragi*, and *Pseudomonas* sp. heated in whole milk at 55, 60 and 65.5°C, and therefore D values would not accurately describe the heat resistance of these organisms.[60] Members of the *Pseudomonas* group probably would not survive at 62.5°C for 30 min, even when recovery methods were considered and the prepasteurization bacterial number exceeded 10^7 ml^{-1}. Mottar,[61] using temperatures between 50 and 60°C, found a first-order inactivation curve for *Pseudomonads* sp. and *Flavobacterium* sp. and suggested the following equations for the relationship between decimal reduction (D) and temperature (T, °C): (1) *Pseudomonas* sp.: $\log D = 7.7142 - 0.1273\ T$ ($r = -0.996$) and (2) *Flavobacterium* sp.: $\log D = 10.6790 - 0.1706\ T$ ($r = -0.983$).

Pseudomonas spp. were able to recover and grow normally in milk after a heat treatment of 55°C for 30 min, but not after a heating to 60°C for 30 min. Some bacteria that appeared to be killed by heat could recover by a resuscitation process.[62] Heat resistance and recovery were affected by the physiological state and the number of the bacteria, the nature of heating, and the recovery media. More complex heating media, including milk whey, stabilized the

Table 2
GENERATION TIME OF PSYCHROTROPHIC BACTERIA (h)

Bacterium	Test medium	Incubation temperature (°C)				Remarks[a]	Ref.
		3	3—5	5—7	7—9		
Pseudomonas fluorescens	Aseptically drawn raw milk	12.5—16.5[b]	5.5—10.5				45
P. fluorescens	Raw milk	31.0 19.0			8.7 5.0	1—3 9—12	46
P. putida	Raw milk	31.0 16.0			9.4 5.4	1—3 9—12	46
Pseudomonas spp.	Tryptone soya broth		7—10	3.5—6.5			47
	Tryptone soya broth		13.9—19.2		5.9—6.0		48
	Pasteurized milk	8—12	6—7.5	4.5—5	3.5—4		49
Serratia liquefaciens	Raw/pasteurized milk		16.1	13.7			51
Enterobacter sp.	Pasteurized milk	17	7.5	5	3.2		49
Klebsiella aerogenes	Pasteurized milk	—	—	17	6.5		49
Achromobacter	Pasteurized milk	—	9	7	5.5		49
Alcaligenes tolerans	Pasteurized milk	—	—	—	13		49
Lactobacillus casei	Sterile whole milk	—	—		21.6		52
Micrococcus flavus	Sterile whole milk	—	26.2		20.9		52
Streptococcus faecalis	Sterile whole milk	26.4	23.3		18.5		52
S. lactis	Pasteurized milk		—	—	9		49
Bacillus circulans	Pasteurized milk				5.0—7.0		53
B. laterosporus *B. coagulans* *B. subtilis* *B. circulans* *B. coagulans*	Tryptone soya broth		7.5—14	6—9			53
B. laterosporus *B. coagulans*	Tryptone soya broth			24—36			53
Psychrotrophic sporeformers				22—26		Lag phase 8—14d	54
B. subtilis	Tryptone soya broth	26.8	20.9		6.7		52
B. cereus	Pasteurized milk	—	—	—	7		49
B. circulans	Pasteurized milk	—	22	18	12		49
B. cerus *B. pumilus* *B. licheniformis*	Buffalo milk		5.7—6.4	4.0—4.8			55
Clostridium hastiforme	Thioglycolate		73		39		56

[a] mg O_2 kg^{-1}.

[b] 1°C.

bacteria to heat, and favored recovery.[63,64] The use of 15 s at 72°C appeared more efficient in destroying *Pseudomonas* sp. than 5 s at 95°C.[64] When cultures of psychrotrophic bacteria were partially destroyed by heat, the survivors differed from the nonheated controls in being more fastidious in their requirement for nutrients, in being more sensitive to pH of the plating medium, and in having an increased lag phase at low temperatures and thus requiring much longer incubation times before visible colonies could be detected.[65] *Alcaligenes tolerans* was the only Gram-negative rod occurring in milk after HTST pasteurization.[66]

Members of the genera *Clostridium*, *Arthrobacter*, *Microbacterium*, *Streptococcus*, *Corynebacterium*, and *Bacillus* belong to the group of heat-resistant psychrotrophs. Among these genera the aerobic sporeformers are most often isolated.[67] In most cases the D values of spores produced at 20°C were greater than those for spores produced at 2°C, although z values were the same. In contrast to Shehata and Collins,[68] Laine[69] found no marked differences between the thermal resistance of mesophilic and psychrotrophic strains. Sporulation at lower temperatures gave spores of rather lower thermoresistance.[70] HTST pasteurization probably promotes spore germination and, under acceptable conditions, outgrowth of spores and vegetative cell multiplication proceed.[71]

In Table 3 some data about heat resistance of psychrotrophic microorganisms in milk are summarized.

III. CONTAMINATION OF RAW MILK

Psychrotrophic bacteria are ubiquitous and therefore water, soil, plants, and animals are the natural sources of these microorganisms. The contact of milk with these sources may lead to a contamination with psychrotrophs.[3,5,74-78]

A. Environment

1. Water

Many farms rely on untreated water supplies from boreholes, wells, lakes, springs, and rivers; some of these may be contaminated at the source with microorganisms of fecal origin, and a wide variety of saprophytic microorganisms derived from the soil or from vegetation including *Pseudomonas* spp. and other Gram negative rods. Numbers of these contaminants will vary widely.[79] Mains water can become contaminated by storage tanks or from hoses.[80]

In 1947 Thomas[75] found that of 126 farm water supplies 70% had psychrotrophic counts $>10^2$ colony-forming units (cfu) per milliliter while 14% gave counts $>10^4$ cfu per milliliter. The psychrotrophic microflora was dominated by *Pseudomonas*, *Achromobacter*, *Alcaligenes*, and *Flavobacterium*. The number of psychrotrophs of farm water supplies collected from three different Canadian localities ranged from 10 to 270,000 cfu per milliliter. Depending on location the median counts were 10, 300, or 560 cfu per milliliter.[81] Very large numbers (10^7 cfu per milliliter) of psychrotrophs, mainly flavobacteria, may be found in chilled water in ice banks of milk can coolers, when the water was not chlorinated or changed frequently.[75]

It is thus evident that the use of contaminated water for the final rinse of farm dairy equipment may contribute to the contamination of raw milk with psychrotrophs. Even from relatively heavily contaminated water, the contamination may be insignificant in terms of cfu per milliliter of milk, but the multiplication of some of the waterborne bacteria in any residual water in the equipment will result in more serious contamination, and may lead to the establishment and development of psychrotrophs in the milking equipment.[82]

2. Soil

Psychrotrophic colony counts of dry soil range from 3 to 200 $\times$ 10^6 g^{-1}. Coryneform types, often resembling *Arthrobacter*, constitute 60% of the psychrotrophic isolates; the

Table 3
HEAT RESISTANCE OF PSYCHROTROPHIC MICROORGANISMS IN MILK

Bacterium	Inactivated by	Resistant to	D-value		Remarks	Ref.
			°C	Time		
Pseudomonas			60	1.19 s		61
			70	0.063 s		
P. fluorescens	30 min/65.5°C					60
P. fragi,						
Pseudomonas sp.						
Pseudomonas spp.	30 min/60°C					62
			71	0.001—0.324 s		72
Flavobacterium			60	2.77 s		61
			70	0.05 s		
Gram-negative psychrotrophs	15min/58°C					73
Alcaligenes tolerans		15 s/72°C				66
Psychrotrophic		30 min/63 or 70°C				73
Microbacterium						
Streptococcus faecalis						
Psychrotrophic sporeformers			110	4.32—7.62 min	Sporulation temp 20°C	69
			110	0.38—2.42 min	Sporulation temp 2°C	
Psychrotrophic sporeformers			85	16.5—20.5 min		68
			90	4.4—6.6 min		
			82	6 min		56
Clostridium hastiforme			90	1.6 min		

remainder were *Achromobacter, Alcaligenes, Pseudomonas*, and *Flavobacterium*.[76] *Citrobacter* sp. and *Klebsiella* sp. are the dominant coliform species isolated from soil samples.[83] The soil microflora probably forms the reservoir from which much of the psychrotrophic bacterial content of contaminated surface water is derived although the psychrotrophic *Flavobacterium* of pure, deep well, and borehole waters appear to be natural water organisms.[75]

3. Vegetation

Grass, hay, and barley and oat grains contain 5×10^5 to 2×10^8 g^{-1} psychrotrophic organisms.[75] The psychrotrophic spore counts were higher in the soil (10^5 g^{-1}) than on grass (10^2 to 10^3 g^{-1}).[84]

4. Air

Air is not an important source of microorganisms in farm milk. The main sources of airborne microorganisms in dairy plants are worker activity, ventilating fans, drains, and dust.[85] Calculations based on recorded levels of aerial contamination and on the volume of air passing into the milking machine indicate that normally airborne bacteria would account for less than five cfu per milliliter of milk produced. Bacterial counts of air in cowsheds or parlors seldom exceed 200 cfu per liter. The flora consisted to a small extent of Gram-negative rods. The bacterial level in the air of cowsheds was reported to be 6 to 45 cfu per liter. The distribution of microorganisms was Gram-positive cocci 50 to 70%; Gram-positive rods 10 to 40%; Gram-negative rods 2 to 8%; aerobic sporeformers 7 to 9%, and molds 4 to 10%.[79,80,86-88]

The following mean psychrotrophic colony counts per square meter surface of sterile milk exposed for 5 min during winter months were evaluated: clean dairies 10×10^3, clean cowhouses 50×10^3, and dusty milking parlors $10{,}500 \times 10^3$. Dust collected from the outside of milking pipelines in these dusty milking parlors contained very large numbers of psychrotrophic flavobacteria, pseudomonads, and enterobacteria.[76]

5. Bedding Material

Bedding materials on which cows are housed in winter may have very high bacterial counts although the bedding may appear relatively clean and dry. On the average the showings contained 1.1×10^9 psychrotrophs (cfu) per gram. The corresponding numbers for straw and sand were 9.8×10^7 and 1.4×10^9 Hg^{-1}.[79]

Geometric means ($\bar{x}_G$) of 3.0×10^4 and 5.0×10^3 cfu per gram psychrotrophic sporeformers were found in winter bedding and on pasture, respectively.[84]

B. Udder

The milk in the upper parts of clinically healthy udders of cows is usually sterile.[89,90] The microflora of the streak canal resembles that of the surface of the udder and the composition is no doubt influenced by the udder surface flora and by the environment.[91] Most of the milk from individual udder quarters was free of Gram-negative bacteria. These results suggested that Gram-negative organisms in raw milk may be considered to be contaminants rather than part of the normal udder flora.[92]

When cows are milked by milking machines the microflora of the teats is of more consequence for the contamination of the milk than the rest of the udder surface. The flora contains psychrotrophs as well as thermodurics. The contamination of the teats is often high; the total counts ranged from 10^5 to 10^7 cfu per teat and psychrotrophs ranged from 10^3 cfu per teat for washed teats of cows at pasture to 10^6 cfu per teat for unwashed teats of cows bedded on sand.[79] The psychrotrophic microflora of teat surfaces (accounting for about 10% of the total microflora) consisted of coryneforms and Gram-negative rods, which do not appear to multiply readily in raw milk.[93-95] In-line samples from two commercial herds which

were taken from well-cleaned but unsterilized milking units during winter had on average ($\bar{x}_G$) 106 and 38 psychrotrophs per milliliter.[91] Coliform counts rarely exceeded 10^2 cfu per teat. Approximately 15% of the coliforms were psychrotrophic.[79,96,97] Particles of the bedding material adhere, sometimes unobtrusively, to teat surfaces; psychrotrophic counts of teat apex swabs of unwashed cows kept in sand were in the range of 10^6 cfu per teat apex whereas the corresponding numbers for cows on pasture were in the range of 10^3 to 10^4 cfu per teat apex. Hosewashing, using a solution of hypochlorite, followed by drying teats with paper towels reduced the psychrotrophic counts for cows bedded on sand to 10^4 to 10^5 cfu per teat apex. The washing of teats reduced the number of psychrotrophs of bulk tank milk from a herd bedded on sand from 1500 to 990 cfu per milliliter and from a herd on pasture from 280 to 270 cfu per milliliter.[79] Chatelin and Richard[98] noticed a psychrotrophic count ($\bar{x}_G$) of 3.7×10^2 cfu per milliliter in the milk of cows with carefully washed udders compared with 6.7×10^3 cfu per milliliter in the milk of cows with poorly washed udders.

Udder washing did not reduce the psychrotrophic spore counts on the teats as well in winter as in summer. Approximately 10^3 psychrotrophic spores were found per teat; thus, the teat surface is probably the main source for this group of psychrotrophs in milk.[84]

C. Milking Equipment

Considerable variation in the microflora of milking equipment occurs from farm to farm particularly in cases where inadequate cleaning prevails. This variation in microflora has been shown to be related to the type of detergent-sterilizer, cleaning method and solution temperature, the design of the milking equipment, the condition of the rubber components, and the magnitude of the bacterial content.[99]

It has to be kept in mind that bacteriological rinses used to assess bacterial numbers in milking machines do not recover from the machine all the bacteria available to contaminate the milk throughout milking. The percentage of bacteria assessed by rinse examinations depends on the method used. The number of bacteria removed from the surfaces by a rinsing technique may be only 10 to 40% of the total removed during milking.[100,101]

1. Milking Machine

The incidence of different types of bacteria in rinses of milking machines varies according to cleaning and disinfection methods applied and the complexity of the plant. The temperature of solutions used for cleaning and disinfection influences the microflora. In farm dairy equipment sterilized with steam, the psychrotrophic count rarely exceeded $10^5(m^2)^{-1}$.[75] When steam or boiling water immersion were used, <10% of the flora were Gram-negative rods whereas with a quaternary ammonium sanitizer (QAC) Gram-negative rods predominated.[102] The effect of a high initial circulation temperature (>82°C) was compared with a much lower temperature (<60°C); the percentages of colony counts $\leq 10^3(m^2)^{-1}$ were 50 and 20%, respectively. The corresponding percentage of coli-aerogenes positive rinse samples were 6.9 and 48.7%.[99]

Pipeline milking machines subjected to properly applied acidified boiling water (ABW) cleaning are, in effect, pasteurized and thus only thermoduric organisms should survive. In practice, pasteurizing temperatures are not always achieved but heat-resistant types predominate following hot cleaning treatments, and Gram-negative rods are relatively infrequent. If the microflora recovered by rinsing is predominantly heat labile, then it is evident that parts of the machine have not been adequately heated. Use of solutions at temperatures of only 40 to 50°C might be expected to permit development of a heterogenous microflora.[79] Psychrotrophic rinse counts of pipeline milking plants were higher for circulation cleaning than for ABW cleaning; 57% of the former compared with 37% of the latter had psychrotrophic colony counts $>10^3$ $(m^2)^{-1}$.[103]

Strains of *P. fluorescens* and *P. putrefaciens* are usually susceptible to destruction by

hypochlorite, being rapidly killed with concentration of 5 mg kg^{-1} even when present in comparatively large numbers. Some strains of *P. fluorescens* are relatively chlorine resistant, requiring 30 mg kg^{-1} available chlorine for destruction in 30 s. Pseudomonads are equally susceptible to detergent sterilizers containing QACs or iodophors.[18] Clusters efficiently cleansed and disinfected ($<1 \times 10^5$ $[m^2]^{-1}$) by means of detergent hypochlorite solutions had a microflora with 4% Gram-negative rods whereas this group predominated in high-count rinses ($>2.5 \times 10^7$ $[m^2]^{-1}$). The low-count rinses of the clusters cleansed by immersion in caustic soda had a microflora rather similar to that of steam sterilizing, whereas high-count rinses contained 36% Gram-negative rods. The flora of poorly cleansed milking plants were predominantly Gram-negative rods.[102]

Coliform counts in rinses were influenced by the cleaning method used, and increased with increasing complexity of the plant. Approximately 66% of the coliform isolates were psychrotrophic.[97]

A study of the thermoduric and psychrotrophic bacterial content of milking equipment showed that the incidence of thermoduric organisms in pipeline milking machines was slightly lower than that of psychrotrophs; none of the machines had thermoduric counts $>1 \times 10^7$ $(m^2)^{-1}$ as compared with 7.5% having psychrotrophic counts above this level.[103] The psychrotrophic spore count in rinses was low; in five of the eight rinses none were detectable in 1 ml of rinse solution.[84]

An investigation into the cleaning of large bore (50 to 100 mm diameter) milking pipeline machines in milking parlors on commercial farms showed that it is possible to achieve a satisfactory standard of cleaning. In practice, the standard is similar to that for recorder milking machines. The counts ($\bar{x}_G$) $(m^2)^{-1}$ for 20 machines were 5.1×10^5 psychrotrophs (range 2.6×10^4 to 1.1×10^7).[104]

Teat cup clusters of bucket milking machines showing a buildup of milk residues and milkstone were often found to have a high thermoduric bacterial count. These organisms tend to be less prevalent in pipeline milking machines.[79] When milk deposits are allowed to accumulate on surfaces, the associated bacteria may be protected from the detergent-disinfectant solutions used in the cleaning process and may continue to multiply.[80] One third of the deposits of milk residues scraped from milk tubes contained $>1 \times 10^9$ cfu per gram.[76]

The importance of milking machine rubberware as a source of bacterial contamination of milk has been emphasized by several workers. The milking machine rubberware added 10 to 117 times the number of contaminants contributed by the metal parts on farms in which the milk supply was found bacteriologically unsatisfactory.[80] In contrast, the rubber and metal parts contributed similar degrees of contamination when the rubberware was in satisfactory mechanical condition and the milk supply was bacteriologically sound. Bacteria can be protected from the heat of sterilizing process in the pores of the rubber.[105]

Stainless steel of an approved grade presents few problems with cleaning and disinfection; however, acids such as hydrochloric acid or low-pH chlorine solutions can cause severe corrosion, and corroded or pitted surfaces may harbor bacteria.[80,106]

Even if every single component of the machinery can be drained, residual water may be found in an installation after milking cleaning. Bacteria proliferate in this water and contribute heavily to the contamination of milk. Further, they are in an actively growing state and high counts of Gram-negative psychrotrophic rods could be detected.[82]

2. *Bulk Tank*

The total surface contamination of farm bulk tanks is lower than that of milking machines. The thermoduric bacterial content is very low, $<1 \times 10^5$ cfu per square meter; this is because most thermoduric bacteria cannot multiply in the cold environments of the tank. The proportion of Gram-negative rods and psychrotrophs in tanks is, however, much higher than in milking machines.[79,103] In recently installed bulk milk tanks the psychrotrophic

bacterial content of 8% of rinse samples was $>10^5$ $(m^2)^{-1}$ whereas a third of the rinse samples of pipeline milking plants exceeded this count. The rubber outlet plugs were often heavily contaminated, however, with 70% giving psychrotrophic counts $>10^5$ $(m^2)^{-1}$.[77]

3. Car Tanker

Insufficiently cleaned car tankers are potential sources of psychrotrophic contamination. Although car tankers offer the advantage of smaller surfaces per liter of milk their design is more complicated.[107] An examination of 69 car tankers revealed that more than 35% of the tested connections, the covers and their outlet gaskets, the environment of the entries, the intake connections, and the chamber bottoms were bacteriologically unsatisfactory after the routine cleaning and sanitizing procedure.[108]

In the trials of Dommett et al.,[109] each tested tanker was used for two trips a day with sanitizing procedure applied prior to the first load and a shorter rinsing process between loads. The examination revealed that this procedure was effective in reducing contamination from the tanker to negligible levels.

IV. QUALITATIVE AND QUANTITATIVE ASPECTS OF INCIDENCE

The incidence and development of psychrotrophic bacteria in raw milk is highly variable and depends on type, number, and physiological status of the microorganisms present; conditions of milk production and milk contacting surfaces; and temperature and duration of refrigerated storage prior to processing. In Table 4, quantitative and qualitative aspects of the contamination of raw milk with psychrotrophs are summarized.

A. Incidence

In milk produced under sanitary conditions, the typical bacteria of the udder surface, mainly Micrococcaceae, predominate and less than 10% of the total microbial flora are psychrotrophs. With increasing total count, the flora of milking equipment becomes a more important source of the milk flora. Under such unsanitary conditions milk can contain more than 75% psychrotrophs.[77,87]

The most commonly occurring psychrotrophs in fresh raw milk are Gram-negative rods. *Pseudomonas* spp. account for about 50% of the Gram-negative genera and *P. fluorescens* predominates. As pointed out by Law[125] this may be due to the ease with which this species may be tentatively identified, rather than to its true distribution. Other species which include *P. putida, P. fragi, P. putrefaciens, P. aeruginosa, Flavobacterium, Acinetobacter, Achromobacter, Alcaligenes*, and coliforms, e.g., *Serratia*, comprise most of the remaining psychrotrophic Gram-negative genera.[24] At storage temperatures of 5°C and below, pseudomonads, especially *P. fluorescens* and *P. fragi* and flavobacteria, were detected. *P. putrefaciens* was also isolated.[22,32]

Some coliform species are psychrotrophic, and constitute 10 to 30% of the microflora isolated at 5 to 7°C from raw milk. The majority of these coliforms were *Aerobacter* spp.[79] Orange-pigmented Gram-negative bacteria, generally identified as *Flavobacterium* spp., have often been isolated from raw milk, but as a minor component of the microflora.[40] *Arthrobacter* and other Gram-positive species, e.g., streptococci, have also been reported as components of the psychrotrophic microflora, but the latter probably require about 14 d at 7°C to form countable colonies on agar media.[79]

In bulk milk samples the number of psychrotrophic spore formers was generally <1 ml^{-1}. During transport and storage at the dairy during summer there was no increase in psychrotrophic spore-forming bacteria. The species found include *Bacillus coagulans, B. circulans, B. cereus*, and *B. subtilis*.[84,126]

Mol and Vincentie[50] found a significant correlation between psychrotrophic and mesophilic

Table 4
PSYCHROTROPHIC FLORA IN RAW MILK

Substrate	Reference count	Psychrotrophic flora	Flora analysis		Ref.
Raw milk	Total flora	95—98.8%	*Flavobacterium*	22%	110
			Escherichia coli	16%	
			Achromobacter	12%	
			Citrobacter	9%	
			Alcaligenes	5%	
			Aeromonas	3%	
			Pseudomonas	3%	
Freshly drawn milk	Total flora	Low count: 3.5% Gram-negative rods High count: 21.2% Gram-negative rods			87
Raw milk	Total flora	98% Gram negatives	*P. fluorescens* *Aeromonas* *Achromobacter* Coliforms	84%	111
Raw milk stored 4 d at 2—4°C	Total flora	Increase from 35 to 85% Gram negative, nonfermentative			112
Bulk raw milk	Total flora	13.1%			113
Raw milk	Total flora		*Flavobacterium*	3.6%	39
Raw milk	Total flora	65.5% Gram negatives			114
Raw milk	Total flora	18%	*Aeromonas*	10.2%	115
			Pseudomonas	1.8%	
			Acinetobacter, Alcaligenes, Flavobacterium	6.0%	
Raw milk	Total flora	90.4% Gram negatives	*Pseudomonas*	70.2%	116
			Enterobacter	7.7%	
			Acinetobacter	5.6%	
			Chromobacter	2.0%	
			Flavobacterium	3.6%	
			Alcaligenes	1.4%	

Table 4 (continued)
PSYCHROTROPHIC FLORA IN RAW MILK

Substrate	Reference count	Psychrotrophic flora	Flora analysis		Ref.
Bulk tank milk	Total flora	85.4% Gram negatives	*Pseudomonas*	50.5%	117
			Flavobacterium	14.4%	
			Corynebacter	9.0%	
			Acinetobacter/Moraxella	8.1%	
Raw milk	Total flora		*Pseudomonas*	27.8%	118
			Coliforms	22.2%	
			Proteus	14.4%	
			Alcaligenes	13.3%	
			Bacillus	11.1%	
			Flavobacterium	5.6%	
			Micrococcus	3.3%	
			Streptococcus	2.2%	
Raw milk	Psychrotrophs	30% Coli-aerogenes	*K. cloacae*	95%	119
			K. aerogenes	2.5%	
			E. coli-type II	2.5%	
Bulk raw milk	Psychrotrophs		*Pseudomonas*[a]		32
			Alcaligenes		
			Proteus		
			Acinetobacter		
			Flavobacterium		
			Serratia		
Milk and dairy products	Psychrotrophs		*Pseudomonas*	52.0%	7
			Alcaligenes	4.6%	
			Achromobacter	22.6%	
			Flavobacterium	2.2%	
			Enterobacteriaceae	4.0%	
			Gram positive	9.6%	
Bulk raw milk	Psychrotrophs		*Pseudomonas*	70.0%	46
Raw milk	Proteolytic psychrotrophs	96.7% Gram negatives	*Cytophaga*	53.3%	120
			P. fluorescens	32.5%	
			Coliforms	6.2%	
			Aeromonas	3.5%	

Raw milk	Proteolytic psychrotrophs		*Pseudomonas*	54.9%	121
Raw milk	Caseolytic psychrotrophs	96.7% Gram negatives	*Flavobacterium*	53.3%	40
			P. fluorescens	32.5%	
			Serratia marcescens	6.2%	
			Micrococcus	6.2%	
			Acinetobacter	1.1%	
			Enterobacteriaceae	0.1%	
Cooled raw milk	Gram-negatives		*Pseudomonas*	31.2%	122
			Enterobacteriaceae	20.5%	
			Aeromonas	17.7%	
			Flavobacterium	14.9%	
			Acinetobacter	12.5%	
			Moraxella	3.3%	
Raw milk	Enterobacteriaceae	50%	*E. cloacae*		51
			K. ozaenae		
			S. liquefaciens		
Raw milk stored 4 d at 4°C	*Pseudomonas*	Increase from			123
	Achromobacter	11.2—35.1%			
	Aeromonas	0—0.6%			
	Enterobacteriaceae	1.1—0%			
		5.3—7.3%			
Raw milk	Psychrotrophic aerobic sporeformers	In 25—35% of samples			124
Raw milk	Psychrotrophic aerobic sporeformers	In 8% of samples			56
Raw milk	Psychrotrophic sporeformers	In 83.3% of samples	*B. firmus*		54
			B. megaterium		
			B. brevis		
			B. coagulans		
			B. polymixa		
			B. marcerans		
			B. circulans		
			B. cereus		
Raw milk	Psychrotrophic sporeformers	In 23.3% of samples	*B. cereus*		71
			B. megaterium		
			B. coagulans		
			B. licheniformis		
			B. firmus		

[a] Descending quantity.

counts in Dutch raw milk samples. A correlation of 0.66 between these two colony counts of bulk tank milk was reported when mean results from individual farms were compared. In that survey, the geometric mean psychrotrophic count, 1.3×10^3 ml^{-1}, was only 7% of the geometric mean total count of 2×10^4 ml^{-1}. However, 25% of the 5000 samples examined had psychrotroph counts of $>5 \times 10^3$ ml^{-1}, and 25% of farms had at least one psychrotroph count of $>5 \times 10^4$ ml^{-1} during the course of the year.[79]

B. Seasonal Effect

Thomas et al.[96] found a slightly higher incidence of psychrotrophs in daily collected bulk tank milk in summer; psychrotrophic counts were $>10^3$ ml^{-1} in 12% of the summer milks compared with 7% in winter. Søgaard and Lund[127] detected in individual raw milk supplies on average ($\bar{x}_G$) ten fold higher psychrotrophic counts in summer (553 ml^{-1}) than in winter (52 ml^{-1}). The total counts in the farm bulk milk was almost the same in the summer and winter examination but psychrotrophic counts were considerably higher, despite the fact that temperatures in the bulk tanks were lower in winter. There was no obvious seasonal pattern in the *Pseudomonas* group, although the proportion of fluorescent types fell markedly during September. Organisms of the Enterobacteriaceae group were found more frequently in the summer months while Gram-positive organisms reached a peak in the autumn.[116] Part of the seasonal effect may be explained by delayed cooling of farm bulk tank milk, especially in summer when milking is carried out in pastures situated a long way from the farm.

C. Multiplication

Milk produced under sanitary conditions usually does not have a rapid increase in psychrotrophs when held at 4°C or less. Milk that is produced under unsanitary conditions, however, has a rapid increase in psychrotrophic microorganisms. The increase is not the result of the initial number of psychrotrophs but rather of the presence of actively multiplying psychrotrophs.[76]

Stadhouders[128] found that farm milk supplies cooled to 4°C immediately after production did not usually show much increase in total colony counts at 4°C for 72 h, whereas there was a significant increase in bacterial numbers when the commencement of cooling was delayed for 2 to 3 h. He further demonstrated that the multiplication of five Gram-negative psychrotrophic strains in sterile drawn milk held at 4°C was stimulated by preincubation of the inoculated milk at 30°C for 3 h. The phenomenon is probably due to a reduction of the lag phase by this preincubation. Experiments of Stadhouders revealed a lag time of approximately 72 h for *Pseudomonas* if incubated at 4°C. Only exceptionally rapid psychrotrophic bacteria which started growing immediately after inoculation were found. These belonged to the family of Enterobacteriaceae. In accordance with these findings was the observation that the number of psychrotrophs in bulk-collected milk consisting of six milkings, increased only slowly during the first 24 h after delivery.[1,29]

Von Blockelmann and Swartling investigated the influence of repeated warming and cooling of milk in the tank on bacterial growth in connection with alternate day delivery of milk. A slight reduction in bacteria took place during storage of the first milk below 5°C. At the second milking a slight increase in the bacterial count was observed and at the third milking a slight reduction in bacteria was found. All together a very slight rise in the total bacterial count from the first to the fourth milking was found.[130,131] Von Bockelmann came to the conclusion that the critical storage period was between 60 and 72 h when the tank temperature was 2 and 4°C, respectively. On the 1st day 1 to 10% of bacteria were psychrotrophs. After 2 and 3 d of refrigerated storage these bacteria were completely dominant.[112]

As the successive warm milkings during alternative day collection are added, the cold milk in the farm tank undergoes periodical increases in temperature. Successive rises in temperature from 4 up to 15.5°C did not appear to influence the psychrotrophic content of

bulk milk, provided that the resulting blend temperature is lowered again to 4°C within 2 h. A blend temperature of 21°C caused a slight, but significant, increase in the bacterial content of the milk.[96]

The importance of the types and/or physiological status of bacteria rather than the numbers present can be emphasized by the following results: at storage conditions of 4 d at 5°C the psychrotrophic count increased from 370 ml^{-1} to $<1 \times 10^4$ ml^{-1} in one sample and from 900 ml^{-1} to 1×10^6 ml^{-1} in another sample.[132]

About 70 samples of raw milk (mixture of 2 milkings refrigerated at 4 to 5°C) from 33 farms, at which 5 milking machine cleaning methods were practiced, were examined after sampling and again after a further storage of 4 d at 4 to 5°C. Despite a low initial psychrotrophic count ($<10^4$ cfu per milliliter), the milk contained more than 10^6 psychrotrophs per milliliter after the storage period, whatever cleaning method was used. The correlation between the counts in milk before and after storage was poor. The characterization of the dominant psychrotrophic flora of the milk before and after storage revealed a marked change; after storage more than 90% of the isolates were identified as varieties of *P. fluorescens* while before storage these bacteria were much fewer than the other psychrotrophs. In practice it seems very difficult to minimize the milk contamination by *Pseudomonas* to the very low level needed (<10 ml^{-1}) for an extension of the storage time, e.g., 5 d at 4 to 5°C. It seems easier and more reliable to cool the milk at a lower temperature (e.g., 1°C).[45]

At 3°C, a reduction in oxygen level from 9 to 12 to 1 to 3 mg/kg in milk resulted in a 63% increase in generation time for *P. fluorescens* and *P. putida* (see Table 2). However, the reduction in growth temperature from 9 to 3°C at 9 to 12 mg O_2 per kilogram produced a 280% generation time increase for *P. fluorescens*. Similarly, psychrotrophs had a longer lag phase and lower growth rate at 4°C in N_2-flushed milk than in aerobically stored milk.[133] O_2 saturation of 90 and 50% had no effect on the growth rate of a fluorescein-producing *Pseudomonas* sp. at 4°C.[121] Under practical conditions psychrotrophic growth patterns were similar whether storage involved large air-agitated silos or small paddle-agitated vats.[134] In view of the influence of aeration on generation times, increased attention should be focused on sources of aeration in milk-handling systems such as dropline inlets of bulk tanks, leaky gaskets, and improper operation of pumps.[46]

D. Transportation

The percentage of psychrotrophic counts compared to total counts increased from 4.1% on the farm to 6.2% on the tank lorry to 13.9% in the dairy bulk tank in winter time. The corresponding values for the summer period were 16.7, 21.9, and 78.1%, respectively. During transportation the increase in psychrotrophs was moderate. The final counts of psychrotrophic organisms in the dairy bulk milk were 5.8×10^3 and 9.6×10^4 psychrotrophs per milliliter for winter and summer, respectively, due to a fivefold higher initial psychrotrophic contamination in farm bulk milk in summer. These results confirm that a high initial contamination results in rapid multiplication because a larger proportion of bacteria are actively growing. The higher level of contamination with psychrotrophs during summer was not due to lack of cooling capacity in the bulk tanks since temperatures in the milk were almost identical during the two periods of examination.[127]

About half of the Enterobacteriaceae isolated from raw milk collected from refrigerated bulk tank trucks at the entry of a milk processing plant were psychrotrophs, and these consisted mainly of *Enterobacter cloacae*, *Klebsiella ozaenae*, and *S. liquefaciens*.[54] Samples of 3-day-old refrigerated raw tanker milk with a total proteolytic count of $<10^5$ cfu per milliliter contained a wide variety of Gram-negative organisms as pseudomonads, Enterobacteriaceae, *Aeromonas*, *Flavobacterium*, *Chromobacterium*, and *Alcaligenes*. The proteolytic colonies from samples with counts $>10^5$ cfu per milliliter were mainly *P. fluorescens*.[135] The ratio of psychrotrophs to total counts in the milk of car tankers at arrival at the dairy

plants was higher in milk from alternate day delivery (76%) than in those car tankers which collected milk every day (30%).[136]

V. CONTROL OF PSYCHROTROPHS

The most direct way to prevent spoilage of milk by psychrotrophic bacteria is to avoid contamination; however, this is difficult under practical conditions of milk production. As shown in Section III the contamination of milk by psychrotrophs is mainly from two sources: (1) teat and under surface and (2) inadequately cleaned and disinfected milking equipment. Therefore, good hygienic practice in relation to the equipment and method of milking will reduce the level of contamination by psychrotrophic bacteria.[79]

The potential spoilage activities of psychrotrophic bacteria in refrigerated milk and the increasing tendency to store milk under conditions conducive to the growth of these organisms led to the development of methods for their control. These include destroying psychrotrophic organisms or inhibiting their growth by refrigeration, thermization, or the addition of chemicals or lactic cultures.

A. Refrigeration

Refrigeration on the farm and in the dairy plant is important in delaying the multiplication of psychrotrophic bacteria. The influence of temperature on generation time of pure cultures and on the development of psychrotrophic flora in milk can be derived from Table 2. The prolongation of the lag phase of the psychrotrophic bacteria by cooling is probably the main factor influencing the keeping quality of raw milk. Raw milk of good initial quality cooled to 4 to 5°C immediately after production can be held for up to 3 d without significant increase in bacterial number or decrease in final product quality. When the cooling of milk was postponed for some time, there was a significant increase in the number of bacteria under the same conditions of storage. Thus, raw milk received at a dairy and not cooled immediately after production showed a higher increase in bacterial number during storage at 4°C than raw milk cooled on the farm immediately after production. The phenomenon is probably due to a reduction of the lag phase by preincubation at the higher temperature. In practice these circumstances arise particularly in summer when the cows were milked on pastures that are situated a long way from the farm.[126,128,129,137]

B. Thermization

Even when raw milk is cooled to 4°C after delivery, and the systems of milk storage at the farm and of milk collection are satisfactory, its keeping quality at 4 to 5°C is limited to 1 to 3 d, depending on the contamination level.[126,129,137] This may be insufficient in a modern dairy operating on a 5- or 6-d week. In such cases, thermization — a heat treatment less severe than pasteurization — is a usual treatment in the Netherlands. Milk heated for 10 to 15 s at 60 to 70°C could be kept for at least 3 d without any appreciable increase in the bacterial counts. Time of heating had little effect on survival, whereas the effectiveness increased with increased temperature.[129,138,139] Humbert et al.[140] recommended a treatment of 65°C for 20 s since this was the minimum which provided a shelf-life extension of 4 d. The treatment not only reduces bacterial counts, but also results in subsequent delayed growth, presumably due to thermal shocking of the cells.[141] Another effect of thermization is that it activates the germination of *B. cereus* spores during subsequent cold storage of the milk more effectively than does cold storage alone and thus the germinated spores are killed at the final pasteurization.[129]

C. Additives

Many food preservatives have been investigated for their effectiveness in inhibiting psy-

chrotrophs in milk. The legal aspects of applying such additives in milk, however, have to be regarded. Low temperature ripening by the addition of 0.1 to 0.5% selected starter organisms (lactic acid-producing streptococci, *Leuconostoc cremoris)* to milk for the manufacture of cultured dairy products has been suggested as a method of inhibiting the growth of psychrotrophs.[142,143] By the use of this low level of starter and at the low storage temperature used, no significant amount of lactic acid is produced. However, the starter organisms prevent the growth of psychrotrophs by lowering the redox potential or by the production of H_2O_2 which participates in the lactoperoxidase system.[142,144-149]

The antimicrobial lactoperoxidase system, consisting of three components — lactoperoxidase, thiocyanate (SCN^-), and hydrogen peroxide (H_2O_2) — may be utilized to inhibit spoilage of raw milk as it has been shown to be effective against psychrotrophic bacteria. To activate the system, H_2O_2 is required together with thiocyanate to give final molar ratio H_2O_2 to SCN^- of 1:1 in the raw milk. The final component, lactoperoxidase, is normally present in excess. The lactoperoxidase system is most effective at refrigeration temperatures and at a pH value of approximately 6.6. It is also inactivated by heating at 60°C for 15 min and thus will only be effective before pasteurization.[148,150,151]

Activation of the lactoperoxidase system combined with cooling at 5°C suppressed the growth of the psychrotrophic microflora for 5 d in contrast to the normal lag period of only 48 h.[150] With increasing storage temperatures the bactericidal effect becomes shorter.[152,153]

Carbon dioxide has been proven successful in inhibiting the growth of *Pseudomonas* spp. and other psychrotrophs and is a more powerful inhibitor of psychrotrophs than is N_2. CO_2 is particularly effective in milk in the range 4 to 7°C. The bacteriostatic action of 20-m*M* CO_2 on pure cultures of selected psychrotrophs inoculated into sterile milk held at 7°C could be demonstrated by counts 10^3 to 10^5 cfu per milliliter smaller than in untreated control milk samples.[154-160] CO_2 has the advantage of being cheap, safe, and easily removable by warming under vacuum. The storage time can be extended by about 3 d at 4°C or 2 d at 7°C for poor quality milk and considerably longer for good quality milk.[155,161]

The addition of potassium sorbate to milk retarded bacterial growth and increased the shelf life. The inhibitory effect on psychrotrophic bacteria depended upon initial psychrotrophic bacterial load. Optimum concentration was between 0.075 and 0.1%. Sorbate greater than 0.1% imparted a slightly sweet flavor to the milk.[162]

VI. DETERMINATION METHODS

Numerous conditions of time and temperatures for the determination of psychrotrophic cfu are cited in the literature. The examination of differences in results caused by incubation at 3 to 5°C to 7°C for 7 to 10 d revealed highest count at 7°C for 10 d.[163] However, an incubation period up to 20 d still increases bacterial number compared to 10 d.[115] The present method recommended for the enumeration of psychrotrophs is the same as the standard plate count except that plates are incubated at 6.5 to 7 ± 1°C for 10 d.[164,165]

The reference method for the enumeration of psychrotrophic bacteria requires an incubation period of 10 d which means that the results are of historical value only. It was concluded at the IDF Seminar on Psychrotrophs in 1968 that simple and rapid routine methods for assessing psychrotrophs in milk should be developed.

An increasing amount of research has been done in the last 20 years to find more practical, less time-consuming methods. The main directions which have been pursued in these attempts are

1. Incubation at elevated temperatures and/or preincubation of the plates and/or attempts to accelerate colony formation by surface inoculation
2. The addition of inhibitory substances to inhibit Gram-positive bacteria with the use of higher incubation temperature

3. Measurement of metabolic activity or cell components

The basis of direction 2 and partly 3 is the reasonable assumption that the psychrotrophic flora is predominantly Gram negative (see Table 4) and that the measurement of the Gram-negative flora would give an indication of the number of psychrotrophs.

It is quite evident that organisms in refrigerated foods may well exist in a debilitated state as a result of rapid cooling or exposure to low temperature or a heating process. Appropriate methodology should therefore be employed which will take into consideration these injured cells. Enumeration of stressed indicator and pathogenic organisms has been the subject of intensive investigation, but little attention has been directed to enumerating injured psychrotrophic spoilage organisms. This indicates that some form of controlled preincubation/repair period should be included in the enumeration procedure if it is necessary to detect sublethally damaged cells.[166]

A. Colony Count Methods

1. Total Psychrotrophs

When evaluating colony count results it must be kept in mind that organisms present in milk are enumerated as cfu and not as individual cells. Factors which influence plate count results include operator variability, diluents, recovery media, and blending. Gram-negative rods are particularly influenced by the diluents and recovery media.[167,168] A comparison of different media revealed significant differences ($p \leq 0.1$) in psychrotrophic counts between standard plate count agar and heart infusion agar or blood agar.[115,169] As these organisms usually grow as aggregates in milk, blending can produce large increases in counts for milk samples which contain a high proportion of Gram-negative rods.[167,170]

Based on the fact that practically all psychrotrophic bacteria grow faster aerobically, Punch and Olson[171] compared surface inoculation and poured plate method. They found that the incubation period could be shortened by 2 to 3 d at an incubation temperature of 6 ± 0.5°C with surface inoculation. Using the same incubation conditions (10 d at 6.5°C) significantly ($p \leq 0.01$), higher psychrotrophic counts were obtained with surface inoculation than with poured plate method.[115] On the other hand it is doubtful whether the aerobic inoculation of petri dishes by means of a spatula is very suitable as a routine method, but spiral surface plate methods provide an alternative automated method to replace the pour plate method.[165,172,173]

Another possibility to shorten the incubation period is to count microcolonies instead of macrocolonies. Electronic microcolony counting was suggested by Heeschen et al.[174] using nutrient gelatin as medium and a 5-d incubation period at 6°C. The correlation coefficient of the relationship between electronic microscopy count and standard psychrotrophic count was $r = 0.97$.

Juffs and Babel developed a modification of the Frost little plate method: 0.1 ml of an equal mixture of milk sample and melted agar is spread over a microscope slide and incubated at 21°C for 13.5 or 16.5 h. After staining, the slide is counted under a low-power objective. A good correlation with the standard method was obtained.[175]

The method of Oliveria and Parmelee[176] used an elevated incubation temperature to make the determination of psychrotrophs more rapid. The incubation conditions are 25 h at 21°C. Most mesophiles normally present in raw milk appeared to be unable to form visible colonies under these conditions. The correlation coefficient between counts following this method and the standard method were 0.992 or 0.91 for raw milk samples or 0.97 compared to 14 d at 6°C counts in pasteurized cream.[173,176,177] Using 3 d at 15°C as incubation conditions, a coefficient of correlation of 0.967 was obtained relative to the standard psychrotroph count.[178]

Another modification using an elevated incubation temperature of 18°C for 45 h with

surface inoculation was suggested.[178] Highly significant correlations were found when the 45 h at 18°C and 10 d at 7°C results were compared in raw milk (r = 0.87). The 45 h at 18°C test is not as rapid as the 25 h at 21°C test but has the advantage of producing larger colonies of more uniform size.

Tests using the principle of preincubation of plates at elevated temperatures were developed by Leesment and Dufeu,[180] Juffs,[181] and Lück and Hopkins.[178] The experiences with the Leesment test — 16 h at 17°C + 3 d at 7°C — were variable: Waes[172] found no good agreement between the Leesment and the standard psychrotrophic counts, whereas Lund and Sørgard[182] had similar results with both methods. The coefficient of correlation between Leesment results and standard-method counts for raw milk was 0.94, but the counts differed significantly.[177]

For counting psychrotrophs by the agar plate method, Juffs[181] concluded that a preliminary incubation of plates for 24 h at 15°C, followed by incubation for 3 d at either 5 or 7°C, is a satisfactory alternative to the standard method. The coefficient of correlation between the results of Juffs' methods and of standard psychrotrophic count were 0.96 and 0.97 for 5 and 7°C, respectively.[177]

Lück and Hopkins[178] recommended the following methods: 2 d at 15°C + 1 d at 7°C and 1 d at 17°C + 3 d at 7°C. The coefficients of correlation between these methods and the standard procedure were 0.958 and 0.971, respectively.

2. Gram Negatives

Since psychrotrophs are mainly Gram-negative bacteria, the inhibitors of choice should have a greater action on Gram-positive bacteria. There are three classes of inhibitors that generally meet these requirements: surfactants, basic dyes, and antibiotics. The possible use of inhibitors for the selective determination of Gram-negative bacteria was investigated by several authors.[92,172,178,183-197] The procedures with inhibitors normally use incubation temperatures between 20 and 30°C for 2 - 3 d. One disadvantage is that Gram-negative mesophiles cannot be differentiated from Gram-negative psychrotrophs.[77,171,188]

Waes[172] compared violet red bile agar, nutrient-agar + crystal violet, plate count agar + crystal violet, and plate count agar + 1 IU penicillin per milliliter and found that the latter was by far the best. Fung and Miller[191] found that bromthymol blue, *o*-cresolphtalein, janus green, methylene blue, and safranin O allowed Gram-negative but not Gram-positive bacteria to grow. Of 17 tested inhibitory substances (surfactants and dyes), crystal violet at 2 mg/l and neotetrazolium chloride at 2 mg/l totally inhibited the nonpsychrotrophs while having no statistically significant effect on the psychrotrophs.[193] Contradictory results were obtained by Phillips and Griffiths[197] who found that neotetrazolium was ineffective in suppressing the growth of Gram-positive bacteria. Crystal violet-containing media do not inhibit all Gram-positive bacteria,[194,198,199] and such media are inhibitory to some Gram-negative bacteria.[191,196,199,200]

Phillips and Griffiths[197] tested seven systems inhibitory to Gram-positive bacteria and found that none of these systems worked perfectly. A mixture of crystal violet-penicillin-nisin or monensin had least inhibitory effect on Gram-negative isolates, whereas a mixture of Benzalkon A and crystal violet and sodium desoxycholate were the most effective inhibitors of Gram-positive bacteria. There was also evidence which suggested that the growth media could effect the results achieved especially with mixtures of benzalkonium chloride and crystal violet and also with monensin.

B. Measurement of Metabolic Activity or Cell Components and Rapid Tests

Dye reduction tests such as methylene blue and resasurin which measure metabolic activity of microorganisms have been widely used to measure the quality of milk. These tests are not applicable to evaluate psychrotrophic load, as psychrotrophs give poor reduction results.