QUALITY CONTROL IN THE DAIRY INDUSTRY

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14.1 INTRODUCTION

The quality of food, such as milk and dairy products, may be defined as that sum of characteristics which enables the food to satisfy definite requirements and which determines its fitness for consumption (Molnar, 1993). In this sense, quality can be judged by means of sensory evaluation, its nutritive value, and according to its chemical, physical, and microbiological characteristics.

When referring more specifically to the microbiological characteristics of a food, it implies the measurement of the hygienic quality of that food. The concept of hygienic quality, in turn, requires that undesirable microorganisms or residues do not gain access to milk or dairy products because these may prove harmful to human health, cause spoilage or deterioration of the food product, or simply be frowned upon from an aesthetic point of view.

Cases of food-borne disease and food poisoning are becoming more and more common throughout the world. Both of these public health problems and the microbiological spoilage of foods can be minimized by the careful choice of raw materials and correct manufacturing and storage procedures. Achievement of such objectives requires, in many cases, monitoring at various stages to assess microbiological load or to look for particular microbial types.

In this chapter the accent will be on monitoring procedures and microbiological analytical methods, and no attempt will be made to
discuss concepts such as good manufacturing practice (GMP), ISO 9000 certification, or hazard analysis critical control points (HACCP) (Harrigan and Park, 1991).

Monitoring procedures to be dealt with will include the air and water supplies in a factory environment, the hygiene of packaging material, and the sampling and testing of raw materials and end products.

The analytical procedures will include standard and rapid methods for assessing microbiological load and for enumerating and detecting specific microbial genera or groups. The principles of these methods will be outlined, and their advantages and disadvantages will be discussed.

14.2 CONTROL OF AIRBORNE MICROORGANISMS IN DAIRY PLANTS

The demand for extended shelf life and safety of dairy products has put increased emphasis on the microbial quality of air in dairy environments. Air quality in the processing and packaging areas is a critical control point in the processing of dairy products because airborne contamination reduces shelf life and may serve as a vehicle for transmitting spoilage organisms and, if pathogens are present, transmission of diseases (Anonymous, 1988; Kang and Frank, 1989a). Every precaution should therefore be taken to prevent airborne contamination of the product during and after processing (Kang and Frank, 1989b; Lück and Gavron, 1990; Hickey et al., 1993). Air quality in processing areas, the factory environment (e.g., walls, floors, drains), and air used in the manufacturing of dairy products should be monitored on a regular basis. Airborne microorganisms in dairy plants include bacteria, molds, yeasts, and viruses. Data on air counts and types of different microorganisms in processing areas, reported by various authors, are comprehensively reviewed by Kang and Frank (1989b). The generic composition and the levels of microorganisms can vary widely within and among plants, and on a day-to-day basis within the same plant. The variations can consequently be attributed to differences in plant design, airflow, personnel activities, and status of factory hygiene. Installation of air filters, application of UV-irradiation, and regular chemical disinfection (bactericidal, fungicidal, and viricidal agents) of air can be applied to critical areas to control airborne microorganisms (Singh et al., 1986; Homleid, 1997; Rockmann, 1998; Arnould and Guichard, 1999).
14.2.1 Sources and Routes of Airborne Microorganisms

Airborne microorganisms can be attached to solid particles like dust, are present in aerosol droplets, or occur as individual organisms due to the evaporation of water droplets or growth of certain mold species. The main sources of airborne microbes may include the activity of factory personnel, ventilation and air-conditioning systems, inflow of outdoor air, and packaging materials (Heldman et al., 1965; Hedrick and Heldman, 1969; Hedrick, 1975; Kang and Frank, 1989b; Lück and Gavron, 1990). Frontini (2000) found that factory personnel, dairy equipment, building materials, and ventilation systems are responsible for 50–60%, 25–35%, 10–20%, and 1–5%, respectively, of airborne contaminants. An increase in viable aerosols have been detected during flooding of floor drains (Heldman and Hedrick, 1971) and after rinsing the floor with a pressure water hose, thereby illustrating the ability of microorganisms to be disseminated from drains and wet surfaces as a result of physical activities (Kang and Frank, 1990; Mettler and Carpentier, 1998). Whenever possible, wet cleaning should not be used during the processing of milk products in areas in which the product is exposed and can be contaminated by aerosols. Once a high concentration of viable aerosol is generated, it can take more than 40 min to return to the normal background level. It is important to minimize the generation of aerosol droplets from bubbles bursting at a water surface—for example, during rinsing activities or during raw milk handling. It has been shown that drains, floors, and standing or condensed water can be a source of pathogens in dairy plants (El-Shenawy, 1998). The potential contamination from bacterial biofilms is of major concern because microbial cells may attach, grow, and colonize on open exposed wet surfaces—for example, floors, floor drains, walls, and conveyor belts (Wong and Cerf, 1995; Carpentier et al., 1998; Mettler and Carpentier, 1998). Floors in dairy plants are one of the main reservoirs of *Listeria monocytogenes* (Davis et al., 1996; Fenlon et al., 1996).

Water used in open circulation systems is another significant source of airborne microbial populations (Lighthart and Frisch, 1976). These authors found that as many as $10^{10}$ viable bacteria per second can be released into the air from a 15-m-high cooling tower. It should be recognized that in a cooling tower, not only does evaporation of water occur, but also the formation of small water droplets. This spray from cooling towers, if contaminated, may be a possible source of certain pathogens (Hiddink, 1995) and consequently airborne contamination. Microorganisms and small particles are commonly found in the immediate vicinity of water surfaces (Al-Dagal and Fung, 1990).
14.2.2 Outdoor Environment

The control of airborne microorganisms in the immediate surroundings of dairy premises is more difficult than in closed, indoor environments where more controlled measures can be taken. According to Al-Dagal and Fung (1990), one aspect that could be helpful in reducing the microbial load outdoors is the control of organic materials. Natural agents such as UV light, humidity, temperature, wind direction, and speed have a significant influence on the total number of airborne microorganisms in the outdoor atmosphere.

14.2.3 Processing Rooms

The most positive approach to controlling airborne contaminants indoors is to remove all contamination sources from the area where the product might be exposed to air. Good ventilation is necessary to remove moisture released during the processing of dairy products. It will also prevent condensation and subsequent mold growth on surfaces. More attention is given nowadays to air cleaning in food plants, among other things, by establishing air flow barriers against cross-contamination from the environment (Jervis, 1992; Kosikowski and Mistry, 1997a,b). In modern dairy plants, the air entering processing rooms is chilled and filtered to remove practically all bacteria, yeasts, and molds. It is essential that filtered sterilized air be supplied to areas where sterile operations are to be carried out. Rigid frame filters or closely packed glass fibers are available to achieve contamination-free air for (a) culture transfer and (b) manufacturing and packaging of sterilized milk and milk products (Shah et al., 1996, 1997). The use of high-efficiency particulate (HEPA) filters will remove 99.99% of airborne particles 0.3μm and larger (Everson, 1991), while new ultra HEPA (ULPA) filters remove 99.999% of particles as small as 0.12μm (Shah et al., 1997). Passage of air through a combined HEPA/ULPA filter is usually considered suitable for use where contamination-free work is to be carried out. Standard high-efficiency air filter systems allow more air into the room than normal, thereby establishing a positive air pressure. Upon opening a door, filtered air flows out, thus blocking the entry of untreated air and minimizing microbial contamination. For optimal ventilation, sufficient air changes have to be made to prevent the buildup of condensation on surfaces. This is usually achieved by not less than 10 changes per hour and often up to 20 changes per hour in rooms where moisture is generated. The ventilation unit must also be able to accommodate
extreme conditions—for example, during cleaning periods (Jervis, 1992).

Rooms in which direct exposure to outside air is inevitable can have air flow barriers installed, mounted over open doorways to secure a significant downward velocity of air flow, preventing contamination from outside (Kosikowski and Mistry, 1997a). Outside air should be filtered and free of condensate.

Compressed air is commonly used in various processing operations and can contribute to contamination of products by dust and microorganisms and, in the case of lubricated compression systems, by oil fumes (Guyader, 1995; Wainess, 1995a). Whenever air under pressure comes into direct contact with the product (pneumatic filling, agitation, or emptying of tanks) or is directed at milk contact surfaces, it should be of the highest quality. Sterile compressed air can be obtained by drying the air after compression in adsorption filters (e.g., chemically pure cotton, polyester, or polypropylene) and by installing a series of filters with 0.2-μm pore size downstream, immediately preceding the equipment where the air is needed (Bylund, 1995; Guyader, 1995; Anonymous, 1997).

Walls and ceilings must also be of the highest standard with no opportunity for accumulation of dust and other deposits. To achieve the required standards, suitable sealants and sterilants, as well as coatings with antimicrobial properties, are available with effectiveness against a wide range of bacteria, molds, and yeasts (Vedani, 1996; Russell, 1997a; Botta, 1998). The use of clean-room clothing, head covering, masks, and gloves largely eliminates the release of microorganisms into the processing environment. A good hygiene training program for factory personnel will contribute to reducing contamination by workers (Al-Dagal and Fung, 1990).

Standards for airborne counts in various processing areas have been proposed by various authors (Kang and Frank, 1989b). Proposed standards in this regard are presented in Table 14.1. Although these standards are relatively strict, experience has shown that they are achievable (Lück and Gavron, 1990).

### 14.2.4 Methods of Air Sampling

The main reason for sampling air in the dairy plant is to evaluate its quality and to obtain information about the hygienic condition in certain critical areas where microorganisms may contaminate the product directly, or indirectly. Samples may be taken from the following locations:
### TABLE 14.1. Suggested Standards for Air Counts in Various Processing Areas

<table>
<thead>
<tr>
<th>Processing Area</th>
<th>Plate Count (cfu m⁻³)</th>
<th>Yeasts and Molds (cfu m⁻³)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Satisfactory</td>
<td>Unsatisfactory</td>
<td>Satisfactory</td>
</tr>
<tr>
<td>Cultured milk and cream, cottage cheese</td>
<td>&lt;150</td>
<td>&gt;1500</td>
</tr>
<tr>
<td>Milk and cream</td>
<td>&lt;150</td>
<td>&gt;1500</td>
</tr>
<tr>
<td>Butter</td>
<td>&lt;100</td>
<td>&gt;1000</td>
</tr>
<tr>
<td>Powdered milk</td>
<td>&lt;200</td>
<td>&gt;2000</td>
</tr>
<tr>
<td>Ripened cheese</td>
<td>&lt;200</td>
<td>&gt;2000</td>
</tr>
</tbody>
</table>

*Source: Adapted from Lück and Gavron (1990).*

1. At openings of processing equipment that may be subjected to potential contamination by air currents.
2. At selected points in a room e.g. where products are filled and packed.
3. In areas where employees are concentrated (IDF, 1987).

Although various air samplers have been designed for sampling airborne organisms (Kang and Frank, 1989b; Al-Dagal and Fung, 1990), none of these recover viable particles without some inactivation or losses during or after sampling. The effectiveness of air quality monitoring depends on the type of sampler used, as well as on the nature of air in the specific environment to be monitored (Kang and Frank, 1989a). There are two main principles by which airborne microbes can be sampled:

(a) Collection onto solid and semiliquid media or filters.

(b) Collection into a liquid solution or medium.

The objective in each case is to determine the number of organisms on the plates, in the filter, or in the liquid media. Sampling time for all collection methods is usually standardized at 15, 30, and 60 min (IDF, 1987). The basic methods include techniques such as sedimentation (gravitation settling), impaction on solid surfaces, and impingement in liquids, as well as centrifugation and filtration (Kang and Frank, 1989b; Al-Dagal and Fung, 1990; Hickey et al., 1993; Neve et al., 1995). Comparative studies of air sampling devices have indicated that there is often no obvious choice of the correct sampler to use. Results in this regard are discussed by Kang and Frank (1989a–c, 1990).
14.3 MICROBIAL CONTROL OF WATER SUPPLIES

Water has many applications in the dairy industry and the quality requirements vary with different applications. Because water is an important commodity for dairy product manufacture, special attention should be paid to the supply and quality of water. The dairy industry consumes large quantities of water for various purposes, such as direct preparation of products, cleaning and disinfection, cooling, and steam generation. Without sufficient good-quality water, it is impossible to produce high-quality dairy products. Water systems can present a hazard if the microbiological quality is not monitored and appropriate water treatment applied (Hiddink, 1995). Water used in dairy operations must be safe and must be practically free from organisms that could contaminate the product and initiate spoilage. Spoilage of refrigerated milk and milk products by water-borne organisms—for example, psychrotrophs (Witter, 1961)—can occur either directly, through product contact with the water itself, or indirectly, by microbes metabolizing nutrient residues on improperly cleaned equipment surfaces (Hickey et al., 1993). It is necessary to check the quality of water regularly and to incorporate such practices into the quality management system, specifying the frequency and parameters to be monitored.

14.3.1 Water Used for Processing

Process water is water that can come into contact with the product, either directly or indirectly. Therefore, it must be of the highest quality, meeting the requirements for drinking water quality (Table 14.2) or, preferably, exceeding these standards. It should consequently be clear, free from odor, color, and taste, soft, and virtually sterile (Bylund, 1995). In the dairy industry process water is, for example, used for the direct preparation of products, for starting-up pasteurizers and evapo-

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**Table 14.2. Proposed Standards for Drinking Water**

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Count (cfu)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total bacterial count (22°C)</td>
<td>&lt;100 ml⁻¹</td>
</tr>
<tr>
<td>Total bacterial count (37°C)</td>
<td>&lt;10 ml⁻¹</td>
</tr>
<tr>
<td>Total coliforms</td>
<td>&lt;1 100 ml⁻¹</td>
</tr>
<tr>
<td>Fecal coliforms</td>
<td>&lt;1 100 ml⁻¹</td>
</tr>
<tr>
<td>Fecal streptococci</td>
<td>&lt;1 100 ml⁻¹</td>
</tr>
<tr>
<td>Sulfite-reducing clostridia</td>
<td>&lt;120 ml⁻¹</td>
</tr>
</tbody>
</table>

*Source: Adapted from Hiddink (1995).*
rators, for flushing-out product from process equipment at the end of production, for rinsing equipment after cleaning, for washing cheese and butter, for CIP-cleaning for post-finishing of process equipment, for regeneration of water treatment equipment, and for air-conditioning humidity control in stores (Hiddink, 1995).

Water with suitable microbiological quality (very low counts) should be used for the final rinsing of equipment after cleaning; otherwise recontamination of the cleaned surface can take place. In this case, disinfection of the water is necessary. Suitable standards for process water are usually <100 and a maximum of 1000cfuml⁻¹ for total viable count at 22°C and absence of coliform organisms in 100ml of water. Any deviation from this standard should be investigated to identify the source of contamination. Whatever the origin of water, it should be routinely examined microbiologically at point of entry and especially at the most critical place—that is, at the point of use (Hiddink, 1995; Jervis, 1992).

The sources of water supply to the dairy industry are surface water, ground water, condensate from evaporators, and public mains or tap water. Public mains or tap water can mostly be used without further treatment. However, in some countries, additional disinfection at the factory may be required. Considerable attention should be given to meet quality requirements if process water is produced at the factory itself from sources such as surface water and ground water. Recovered water (e.g., evaporator condensate) is usually not recommended for use in food contact applications, including the final rinse in cleaning (Jervis, 1992). Since most of the micro-organisms in water are destroyed by chlorination or heat, many plants treat all water to keep contamination at a minimum (Hickey et al., 1993). Stored water is usually chlorinated in order to maintain suitable microbiological quality, levels of up to 1 ppm available chlorine usually being sufficient although up to 2 ppm available chlorine is advised if water is softened (Jervis, 1992). Present-day techniques for the treatment of water include disinfection with chlorine, chlorine dioxide, ultraviolet-light, ozone, microfiltration, and other processes (Hiddink, 1995).

14.3.2 Water Used in Cooling Systems

Cooling water is used for the removal of heat from process streams and products. The quality requirements for cooling water used in plate heat exchangers to cool milk products is critical, because with this type of equipment there is a risk of failure and leakage of cooling water to the product. In such situations, cooling water should be of drinking water
quality. Mains or tap water, ground water, surface water, and condensate can be used. To obtain drinking water quality, the same treatments as for process water are applicable. To prevent problems like corrosion and fouling in cooling systems by microbial biofilms, chemical conditioning is usually applied (Mattila-Sandholm and Wirtanen, 1992; Assink and Van Deventer, 1995; Hiddink, 1995).

14.3.3 Microbiological Tests

The type of sample taken depends on the purpose of sampling. General bacteriological sampling (for *E. coli* and coliforms) involves the collection of relatively small volumes of water (500ml to 3 liters), whereas samples for detection of specific pathogens involve larger volumes (10 to 1000 liters) (IDF, 1987; Clesceri et al., 1989; Fricker, 1993). It must be stressed that only sterile sampling containers should be used and that these should be completely filled. The samples should be examined as soon as possible after collection, preferably within 6h (Fricker, 1993).

The methods for the microbiological examination of water are intended to give an indication of the degree of contamination and to ensure the safety of supply. In general, the tests are based on indicator organisms, the presence or absence of which provides a measure of the microbiological quality of the water. Detailed procedures for the sampling and testing of the microbiological quality of water is outlined in the 17th edition of *Standard Methods for the Examination of Water and Waste Water* (Clesceri et al., 1989).

14.4 ASSESSMENT OF DAIRY EQUIPMENT HYGIENE

Hygiene monitoring of dairy equipment is a routine exercise that must be carried out to verify that the cleaning and sanitation/sterilization operations have been properly conducted. Plant hygiene/sanitation is dependent on the efficiency of cleaning (removal of residual soil from surfaces) as well as on the effective destruction of most (sanitation) or all (sterilisation) of the remaining microorganisms. Verification of cleaning and sterilization of dairy equipment surfaces usually comprises sensory (sight, feel, smell) and bacteriological examination. Although modern dairy plants are highly automated and processing lines seldom assessable for visible inspection, it is often very useful in detecting inadequately cleansed equipment (IDF, 1987; Zall, 1990; Tamime and Robinson, 1999a).
14.4.1 Biofilm Formation on Dairy Equipment Surfaces

Failure by cleaning procedures to adequately remove residual soil from surfaces (especially from milk/product contact surfaces), or the ineffective destruction of the residual microorganisms, may have serious implications. Microorganisms remaining on equipment surfaces may survive for prolonged periods, depending on the amount and nature of residual soil, temperature, and relative humidity. Milk is a highly nutritious medium, hence any residue not removed can promote bacterial growth, bacterial adhesion to the surface, and consequently biofilm development (Wong and Cerf, 1995). Biofilm is a convenient term to designate microorganisms adhering to and growing on wet surfaces and acquiring, within a matter of hours, resistance to adverse environmental conditions (Carpentier et al., 1998).

Biofilm formation is not a new phenomenon and has been and is being studied extensively (Mattila-Sandholm and Wirtanen, 1992; Carpentier and Cerf, 1993; Austin and Bergeron, 1995; Lindsay et al., 2000). It has been established that biofilm accumulation in the dairy environment and especially on milk/product contact surfaces has, for example, the following potential implications:

- Postpasteurization contamination, decreased shelf life, or potential spoilage of products (Koutzayiotis, 1992; Koutzayiotis et al., 1992; Austin and Bergeron, 1995) and, if pathogens are present, transmission of diseases (Dunsmore et al., 1981; Ronner and Wong, 1993; Blackman and Frank, 1996; Miettinen et al., 1999).
- Adhered cells in a biofilm are more resistant to adverse conditions than planktonic (free-living) cells and have, for example, increased resistance to antibacterial agents (antibiotics, disinfectants), chemical shock, desiccation, starvation, inconsistent nutrient supply, and extreme heat or cold (Frank and Koffi, 1990; Costerton et al., 1995; Wong and Cerf, 1995). The existence of viable but nonculturable cells within a biofilm, which survive these stressful conditions, are also possible (Leriche and Carpentier, 1995; Mettler and Carpentier, 1997). The occurrence of bacteria in such a state would not be easily detected under normal microbiological culture conditions (Wong and Cerf, 1995) (see Section 14.11.1).
- Attached cells become irreversibly adsorbed to the surface, which enables the organisms to resist mechanical and chemical cleaning procedures (Lundén et al., 2000).
- Biofilms can be found in apparently extreme environments, such as crevices between gaskets and pasteurizer plates where they
survive repeated cycles of pasteurization, cleaning, and sanitation (Austin and Bergeron, 1995; Mettler and Carpentier, 1997; Lindsay et al., 2000).

Areas in which biofilms most often develop are those that are the most difficult to rinse, clean, and sanitize, and are also more difficult to sample, regardless of the method used. Dead ends, joints, grooves, surface roughness, bypass valves, sampling cocks, overflow siphons in filters, and corrosion patches, and so on, are hard-to-reach areas (Wong and Cerf, 1995). Chemical cleaning and sanitation/sterilization are indispensable tools for dairy plant hygiene operations; however, other means of ensuring the hygiene of contact surfaces, at least at critical points, are needed. New ideas to improve surface hygiene are, for example, the modification of surfaces by incorporation of biocides, antimicrobial agents, or catalysts; improved/new processes and methods for sanitation/sterilization; and biocontrol (Carpentier et al., 1998).

14.4.2 Methods for Assessment of Dairy Equipment Hygiene

Different methods and/or techniques have been devised to monitor the hygiene of dairy equipment surfaces (IDF, 1987; BSI, 1991; Hickey et al., 1993; Wong and Cerf, 1995; Tamime and Robinson, 1999a), thus contributing to maintaining production of high-quality products and at the same time ensuring compliance with legal requirements. Whatever tests are employed, it is essential that they be applied routinely, because individual observations are in themselves meaningless; only when values for a typical, high standard of hygiene have been established for a given plant, along with acceptable tolerances, do the results of any microbiological/hygiene test become valuable (Tamime and Robinson, 1999b).

Enumeration of total counts of bacteria, coliforms, yeasts, and molds are the most common microbiological examinations carried out to assess the bacteriological contamination of surfaces. The types of microorganisms present reflect to some extent the standard of plant hygiene (Tamime and Robinson, 1999a). Selective and differential culture media may also be used to test specifically for given groups of organisms. Although a given assessment method may not remove all the organisms from the surface being tested, its consistent use in specific areas can still provide valuable information as long as it is realized that not all organisms are being removed (Jay, 1992). The most commonly methods for surface assessment are presented below:
Swab/Swab-Rinse Method. The swab method is applicable to any surface (flat or curved, horizontal, vertical, or sloped) that can be reached with hand-held sticks containing either cotton or alginate gauze swabs (or other approved alternatives). The swab technique can be used for hard-to-reach areas such as surfaces with cracks, corners, or crevices (Hickey et al., 1993). A sterile swab, moistened in an appropriate solution, is rubbed over a designated area of the contact surface. Sterile templates, with openings corresponding to the size of the area to be swabbed, are often very useful. The swab is transferred to its holder (test tube) with a known volume of a physiological neutral solution and vigorously agitated (preferably in an automated shaker to ensure reproducibility). When calcium alginate swabs are used, the organisms are released into the diluent after dissolving the alginate, for example, in 3% sodium hexametaphosphate solution. Samples of the solution, or decimal dilutions if necessary, are examined by, for example, the plate count method (Jay, 1992; Wong and Cerf, 1995).

The cellulose sponge swab method (Hickey et al., 1993) is another technique that could be used to assess dairy equipment hygiene. Little pieces of sponge (free from bacterial inhibitors) held by tweezers or by hand (using sterile gloves) are used to sample surfaces. This technique is particularly useful to examine large surface areas. Numbers of organisms recovered by alginate swabs are reportedly higher than those obtained by cotton swabs (Jay, 1992). The reproducibility of the swab/swab-rinse techniques is variable due to the unreliable efficiency of swabbing, and the proportion of bacteria removed from the surface is unknown. Furthermore, it is highly operator-, day-, and time-dependent (Wong and Cerf, 1995). The swab method is, despite its limitations, very useful and almost universally applied in the dairy industry (Tamime and Robinson, 1999b). The swab and rinse methods may also be supplemented by a bioluminescence test for total adenosine-5-triphosphate (ATP) (Pettipher, 1993; Anonymous, 1995; Werlein and Wucherpfennig, 1999) (see Section 14.8.3.1) whereby an indication of the state of hygiene of the plant surface is acquired. Obviously, the readings are not intended to correlate with the microbial count, but there is an excellent correlation between clean surfaces and low levels of ATP (Tamime and Robinson, 1999b).

Surface Rinse Method. The effectivity of cleaning and sanitation of containers and equipment can be assessed by rinsing the container or equipment with a measured volume of sterile water or Ringer's solution and analyzing the sample for total bacterial numbers or the pres-
ence of different types of organisms. The rinse (solution) method is more appropriate for assessing internal surface contamination of containers (Lück and Gavron, 1990). In cases where the volume of the rinse is large, or the microbial load is low, it is advisable to use the membrane filter technique (see Section 14.3.3) whereby a known volume of the rinse sample is filtered through an appropriate membrane (generally 0.45μm), retaining any microorganisms that may be present. The membrane is placed onto the surface of a pre-poured agar plate and inoculated, and visible colony growth is observed between 48 and 72 h. Rinse water could also be examined by the direct epifluorescent filter technique (DEFT), using fluorescent dyes and fluorescence microscopy (Holah et al., 1988; Jay, 1992; Tamime and Robinson, 1999a) (see Section 14.7.1.2). An advantage of the DEFT is that results can be obtained within 25–30 min.

**Agar Flooding Method.** The agar flooding method is used for assessing the hygiene of internal surfaces of pieces of equipment (tubing, valves, pumps, etc.), cans, and bottles. A molten nutritive agar medium is poured into the item, which is immediately closed and rolled by hand, or by an automated system to form a thin and continuous layer, until the agar sets. After incubation, the colony-forming units are counted visually through the wall if it is transparent, or with an endoscope (Wong and Cerf, 1995).

**Agar Contact Plate Methods.** Flat or slightly bent surfaces that are smooth and nonporous can be sampled by applying a solidified piece of appropriate nutritive agar medium. Microcolonies detached from the sampled surfaces and sticking to the agar can grow and form visible colonies when the agar is incubated. A number of commercial products are also available in this regard:

**RODAC Plate Count.** The replicate organism direct agar contact (RODAC) method employs special commercially available plastic plates in which the agar medium protrudes slightly above the rim. The agar surface is pressed onto the test area, removed, the lid replaced and incubated (Lück and Gavron, 1990; Jay, 1992; Hickey et al., 1993).

**Agar Slice Methods.** A sterile 100-ml syringe (modified by removing the needle end to create a hollow cylinder) is filled with agar medium. A portion of the agar is pushed out to make contact with the test surface, cut off, and placed into a petri dish and incubated (Jay, 1992). Similarly, an artificial (plastic) sausage casing can also be used in this
way (Ten Cate, 1965). Drawbacks to these methods are, for example, the covering of the agar surface by spreading colonies and its ineffectiveness for heavy surface contamination (Jay, 1992). Unless caution is taken to apply agar to the sample surface with constant pressure and time, reproducibility of sampling can be questionable (Wong and Cerf, 1995).

**Dry Rehydratable Film Method.** The dry rehydratable film (Petrifilm aerobic count) method provides a simple direct-count technique for detecting bacterial contamination on both flat and curved surfaces (Jay, 1992; Hickey et al., 1993). Petrifilm methods exist for the detection and enumeration of specific groups, such as coliforms (see Section 14.7.3.1.6). This procedure is less applicable for surfaces with cracks or crevices (Hickey et al., 1993) or when surfaces are heavily contaminated (Wong and Cerf, 1995).

**Other Methods.** Various other methods are described in the literature—for example, the adhesive (sticky) tape method (Tamminga and Kampelmacher, 1977) and rapid methods for monitoring the hygiene of dairy equipment surfaces (Russell, 1997b).

### 14.4.3 Suggested Standards

Some suggested standards for dairy equipment in contact with products prior to pasteurization/heat treatment are shown in Table 14.3. With improved cleaning and sanitation regimes, a total colony count of 200cfu 100cm⁻² would be expected nowadays, and a value of <50cfu 100cm⁻² would be expected for any equipment containing pasteurized product (Lück and Gavron, 1990).

Reliable methods for sampling and enumeration of microorganisms remaining on dairy equipment surfaces, especially techniques to

<table>
<thead>
<tr>
<th>cfu 100cm⁻²</th>
<th>Conclusion</th>
</tr>
</thead>
<tbody>
<tr>
<td>500 (coliforms &lt; 10)</td>
<td>Satisfactory</td>
</tr>
<tr>
<td>500–2500</td>
<td>Dubious</td>
</tr>
<tr>
<td>&gt;2500 (coliforms &gt; 100)</td>
<td>Unsatisfactory</td>
</tr>
</tbody>
</table>

*Source: Adapted from Harrigan and McCance (1976); Tamime and Robinson (1999b).*
detect and enumerate adhering bacteria, will contribute toward the effective monitoring of dairy equipment hygiene (Wong and Cerf, 1995). At the same time, research into the microbial ecology of surfaces in the dairy industry to minimize biofilm formation (e.g., inhibiting/preventing colonization, adhesion, and/or growth of unwanted bacteria), as well as the removal of biofilms, is also needed (Carpentier et al., 1998).

14.5 HYGIENE OF PACKAGING MATERIAL

The primary purpose of packaging is to ensure that milk and dairy products reach the ultimate consumer in a safe, sound, and convenient condition. Packaging is in fact an integral part of modern production processing and is usually considered to be the key to successful plant operation. During the last 25 years, the packaging of dairy products has made tremendous strides in improving the hygienic quality and shelf life of the product. Packaging equipment requires special attention because the product reaching this equipment will no longer be treated to reduce its microbial content. Ideally, equipment design should not allow any contamination to either the product or the package (Wainess, 1995a).

14.5.1 Manufacturing of Packaging Materials

Basically, the same general hygiene requirements that apply to dairy plants should apply to plants manufacturing packaging materials (also those in which containers are formed and filled). Requirements for the hygienic manufacture of packaging materials, containers, and closures, especially for single-service containers, have been suggested by Wainess (1995b). Uncoated paper stock, prior to lamination, should meet a microbiological standard of not more than 250 cfu per gram as determined by a disintegration test (Hickey et al., 1993). Where a rinse test can be used, the residual microbial count should not exceed 50 cfu per package except that in packages of less than 100 ml the count should not exceed 10. Where the swab test technique is used (e.g., laminated board, sheet, wrapping, etc.) the microbial count should not exceed 1 cfu cm$^{-2}$ of product contact surface. Product contact surfaces should be free from coliform organisms. It is evident that packages or packaging material must arrive at the dairy plant with an “acceptable” low microbial count and be formed, filled, and sealed employing the proper hygienic measures to preclude additional contamination.
14.5.2 Retail Packaging

Although the use of returnable containers is largely restricted to liquid milk, it may extend to other products such as cream and fermented milks. For retail volumes, the container can be made of glass, polycarbonate, or polyethylene and sealed by single-service aluminum, paper, or plastic caps. For wholesale quantities, stainless steel or aluminum cans are used. With modern mechanical bottle washing operations, the combination of jetting and the bactericidal power of the cleaning solution normally gives a very high standard of cleanliness. The residual colony count should not exceed 50 per container. In containers of less than 100 ml, the colony count should not exceed 10 cfu per container. The number of residual microorganisms on the inner surface of returnable metal cans, normally used for distribution of pasteurized milk or cream in bulk (5–45 liters), should not exceed 50 cfu per container (Wainess, 1995b).

With the advent of plastic-coated packages and closures and the development of vacuum-formed and blow-molded plastic packages, plastic bags, and extruded and fabricated sheets of plastic for packaging, hygienic problems that could not be solved by treatment after forming the package have become evident. It is obvious that physical impurities such as dust or particles released from the material should not gain access to the product. The influence of packaging on the contamination of dairy products may be direct, due to the presence of microorganisms on the material, or indirect due to the permeability of the material to bacteria. The packaging material used for heat-treated milk should first of all be free from pathogenic bacteria, but also from other microorganisms that are able to multiply in the milk or product under the prevailing conditions (Ronkilde Poulsen et al., 1995). The trend toward extended shelf-life products demands that special attention be devoted to the microbial content of air in packaging areas and filling areas. Radmore (1986) found that a correlation \((r = 0.93)\) existed between the number of airborne organisms present in a packaging environment and the number of organisms contaminating the final product. He calculated that during a 60s exposure period, 2.2% of the organisms in 1 m³ air would be able to contaminate 1 liter of a product that is being packed in a container with an opening of 100 cm². The microbial count of plastics and plastic-coated cartons is about 0.1 cfu cm⁻², provided that no recontamination has taken place after manufacture (Kelsey, 1974).

In aseptic packaging, only one spore originating from the packaging material is admissible per 10,000 containers. The surface of 10,000
containers (e.g., 1-liter Tetra Brik) equals 800 m². Assuming a level of 0.1 organism cm⁻², this surface comprises a total of 800,000 organisms before it is sterilized, of which 24,000 are spores (Cerny, 1976). Consequently, sterilization of the packaging material must reduce the spore count by at least four decimals (4D reduction). The most widespread technique to obtain sterile milk contact surfaces using thermolabile packaging material is by in-line sterilization using 15–35% hydrogen peroxide (H₂O₂). In practice, removal of H₂O₂ residues from the packaging material surface is usually achieved at temperatures exceeding 100°C, with the result that the 4D reduction could take place within a few seconds. Wetting agents improve the sporicidal effect of H₂O₂ (Kelsey, 1974). Ultraviolet and high-energy irradiation are other alternatives for the sterilization of packaging materials during aseptic filling and packaging (Flückiger, 1995; Van den Berg, 1995).

New developments in the use of high-intensity pulsed light technology to sterilize packaging materials without chemicals provide new possibilities in terms of quality, monitoring, and controlling the destruction of microorganisms (Harrysson, 1998). The innovative development of bioactive packaging material to inhibit pathogens, mycotoxin-producing molds, and spoilage organisms is also very promising, although further work is necessary to evaluate the performance of these materials in food systems (Scannell et al., 1999; Floros et al., 2000; Han, 2000). The wrapping of retail quantities of butter and cheese in coated paper, aluminum, plastic, and many combinations has changed very little in recent years. Nevertheless, the development of new materials, laminates, cups, and pots has widened the choice, improved hygiene, and provided better protection for various products.

14.5.3 Methods for the Assessment of Hygiene

Detailed information on the sampling of packaging material, containers and closures is outlined by Grace et al. (1993). Methods for the assessment of microorganisms on packaging material must reliably detect bacteria, molds, and yeasts. Various methods—that is, the disintegration test, rinse test, coating technique, membrane filter, and direct inoculation techniques—are used for this purpose (Hickey et al., 1993; Wainess, 1995b; Tacker and Hametner, 1999). The following methods that can be used are described by Hickey et al. (1993):

**Disintegration Method.** The disintegration method comprises the blending of paper, paperboard, or molded pulp samples in sterile phos-
phate dilution water using a disintegrator blender. Ten milliliters of the
disintegrated suspension (representing 0.1 g of the sample when, e.g.,
3 g of packaging material is blended in 300 ml of dilution water) is
equally divided among three Petri dishes and pour-plated with stan-
dard methods agar or appropriate differential media to determine spe-
cific microorganisms or groups of organisms. After incubation, the sum
of the colonies developed on the three plates from 0.1 g of sample is
multiplied by 10, and the result is reported as the number of colonies
per gram of packaging material. A total count of not more than 250 cfu
g⁻¹ is usually regarded as acceptable (Wainess, 1995b).

**Rinsing Methods.** A suitable method for containers is the rinse solu-
tion test in which a measured volume of a sterile buffer solution or
nutrient broth is repeatedly flushed over the interior surfaces and the
bacterial population is determined by plating or membrane filter tech-
niques (Clesceri et al., 1989). Various amounts of rinsing solution (20,
50, or 100 ml) are used, depending on the size of the container. Con-
tainers smaller than 1 liter are, for example, rinsed with 20 ml solution,
whereafter 5 ml is divided between two Petri dishes and pour-plated
with standard methods agar. After incubation at 32 °C for 48 h, the resid-
ual bacterial count (RBC) per specified container size is calculated by
multiplying the number of colonies by the volume of the rinse solution
divided by the volume of the sample plated. For example, if the volume
of the rinse solution is 20 ml, the volume of sample plated is 5 ml, and
the number of colonies is 15, the RBC is

\[
15 \times (20 \div 5) = 60
\]

For coliforms, 10 ml of rinse solution is divided among three plates. 
After incubation the coliform count per specified container capacity is
calculated by multiplying the number of coliform colonies by the
volume of rinse solution divided by the volume of the sample plated.
The direct epifluorescent filter technique (DEFT) system could also be
used to examine rinse water (Pettipher, 1993).

**Screening Method for Retail Milk Containers.** Retail milk contain-
ers can also be evaluated by rinsing the interior surfaces of, for example,
50 containers thoroughly with 20-ml portions each of nutrient broth.
The containers with nutrient broth are then incubated at 32 °C for
48 h, and the percentage of containers showing growth is calculated
(Hickey et al., 1993).
Membrane Filter Technique. Coliforms, yeasts, molds, proteolytic bacteria or other specific microorganisms can also be determined by the membrane filter technique using appropriate differential media, prescribed temperatures, and incubation temperatures (Clesceri et al., 1989; Hickey et al., 1993).

Direct Plating Method. According to Lück and Gavron (1990) the surface count of nonabsorbent packing materials based on paper, cardboard, plastics, aluminum foil, etc. can also easily be determined by the direct surface agar plating method. A specified area of the packing material is aseptically placed on the solid agar medium of a Petri dish and then overlaid with the same medium. After incubation, the colonies on both sides of the material can be counted. The use of selective media also allows the counting of coliforms, yeasts, molds, or other organisms.

14.6 SAMPLING OF PRODUCTS FOR MICROBIOLOGICAL EVALUATION

Correct sampling procedures for microbiological analysis require careful attention during sampling, storage, and transport of samples before analysis. Special precautions to prevent direct contamination by microorganisms and subsequent growth of such contaminants have to be taken. Sampling should therefore only be undertaken by experienced persons trained in the appropriate techniques. Emphasis cannot be too strongly placed on the necessity of obtaining a representative sample, using appropriate aseptic techniques. It is imperative that the sample drawn gives a true reflection of the compositional and microbiological quality of the product from which it has been selected. Detailed information on the general requirements and technical instructions for sampling, sampling equipment, and sampling techniques, as well as guidance on the storage and transport of milk and milk product samples, is described in various national and international standards [e.g., IDF (1995a)] and in Standard Methods for the Examination of Dairy Products (Grace et al., 1993). Reference methods for sampling of milk and milk products are summarized in Table 14.4. Sampling equipment for milk collection tankers (IDF, 1990a) and the sampling of milk for quality payment schemes (IDF, 2000) are described by the International Dairy Federation.
<table>
<thead>
<tr>
<th>Product</th>
<th>Description</th>
<th>IDF</th>
<th>ISO</th>
<th>AOAC</th>
</tr>
</thead>
<tbody>
<tr>
<td>Butter</td>
<td>General instructions</td>
<td>50C: 1995</td>
<td>707: 1997</td>
<td>970.29/930.31</td>
</tr>
<tr>
<td>Cheese</td>
<td>Collection of sample</td>
<td></td>
<td></td>
<td>920.122</td>
</tr>
<tr>
<td>Condensed milk (sweetened)</td>
<td>General instructions</td>
<td>50C: 1995</td>
<td>707: 1997</td>
<td>970.30</td>
</tr>
<tr>
<td>Cream</td>
<td>Collection of sample</td>
<td></td>
<td></td>
<td>970.27</td>
</tr>
<tr>
<td>Edible ices, ice cream, frozen desserts</td>
<td>General instructions</td>
<td>50C: 1995</td>
<td>707: 1997</td>
<td>935.41A/970.28</td>
</tr>
<tr>
<td>Evaporated milk</td>
<td>General instructions</td>
<td>50C: 1995</td>
<td>707: 1997</td>
<td>985.30</td>
</tr>
<tr>
<td>Infant formula (milk-based)</td>
<td>Collection of sample</td>
<td></td>
<td></td>
<td>985.30</td>
</tr>
<tr>
<td>Milk and milk products</td>
<td>Automated method (bulk tanks)</td>
<td></td>
<td></td>
<td>970.26</td>
</tr>
<tr>
<td>Milk and milk products</td>
<td>Attributes sampling schemes</td>
<td>113A: 1990</td>
<td>5538: 1987</td>
<td></td>
</tr>
</tbody>
</table>

Source: Adapted from Webber et al. (2000).
14.6.1 Sampling Equipment and Containers

All sampling equipment and instruments must be clean, sterile, and dry prior to use. Sterilization by hot air (170–175°C for at least 2h) or steaming (121 ± 1°C for at least 20min in an autoclave) is normally recommended, although the following alternative methods can also be used if these methods are unpractical (IDF, 1995a):

- Direct exposure of sampler surfaces to a suitable flame
- Immersion in at least 70% (v/v) ethanol solution
- Ignition after immersion in 96% (v/v) ethanol
- Exposure to sufficient gamma-radiation

After thermal sterilization, the equipment should be allowed to cool down before using for sampling. It is essential that the sample containers and closures should be clean, sterile, and dry and that they should be securely closed to prevent contamination from external sources. Any deviations from the prescribed sampling instructions, abnormal sampling conditions, or additional information concerning the samples to be tested should be noted in the sampling report to ensure scientifically sound interpretation of the test results.

14.6.2 Sampling Techniques

Samples for microbiological examination are always taken first and, whenever possible, from the same product containers as those for chemical, physical, and sensory evaluation. Specific sampling techniques for milk and milk products are described in various standard methods (Grace et al., 1993; IDF, 1995a). The mixing of milk and liquid milk products, for example, can be achieved by pouring the milk from one container to another, by using a stirrer (plunger) of a suitable design, by mechanical agitation, or, sometimes, by clean, filtered compressed air. When air is used, care should be taken to avoid foaming of milk because it may cause oxidative lipolysis. The milk is usually agitated for 5min when the tank capacity is 500–4000 liters, for at least 10–15min when the volume is more than 4000 liters, and for 30–60min in large factory storage tanks (Lück and Gavron, 1990). If tanks are equipped with time-programmed agitation systems, samples may be taken after agitation for shorter periods.

The collection of a representative sample from large vessels, storage tanks, and tankers may present problems. In a large vessel with a
bottom discharge outlet, samples should preferably be taken through the manhole. If taken from the discharge outlet valve or the sampling cock, sufficient milk must be discharged to ensure that the sample is representative of the whole. Proportionate sampling is done by taking representative quantities from each container and mixing the portions in amounts that are proportional to the quantity in the container from which they were taken. With raw milk, bulk portions must be split with care, and the homogeneity of the samples must regularly be validated. A useful method is to determine the butterfat levels because the distribution of microorganisms in raw milk closely follows that of the fat (Reuter and Quente, 1977).

Special procedures must be followed for sampling other dairy products (Grace et al., 1993; IDF, 1995a). The recommended sample sizes for various products are shown in Table 14.5.

**In-Line Sampling.** Flow lines of modern dairy plants are complex, and improper designs may cause recontamination of heat-treated products (Dickerson, 1987). A quality control program may include sampling of milk at different sites after pasteurization to assess the microbiological quality or possible postpasteurization contamination. Samples can be withdrawn from different critical points in the processing line using modern sampling devices. Various commercially available devices (e.g., membrane/rubber septums for syringe sampling, valves, and cock-types) of different designs may be obtained for in-line sampling. In general, septa are used, for microbiological sampling and valves for chemical sampling. Sampling devices should be hygienically designed without dead spaces or difficult-to-clean areas. The seal design should not harbor bacteria and should be drainable. Devices that can be cleaned or sterilized independently of flow-line (or tank) cleaning are ideal for sampling (Anonymous, 1998). Multiple samples from the same batch, or taken at specific time intervals, can consequently be obtained without the risk of cross-contamination of samples. Great care should be taken when sampling unmixed milk, because significant carry-over from one sample to the next can invalidate sensitive tests. Stepaniak and Abrahamsen (1995) found no effect of the type of seven different sampling valves on the total plate count of freshly pasteurized milk or on the count of cold-stored samples. However, the sample volume and sample storage container influenced the plate count of cold-stored samples.

**Automatic Sampling Systems.** With automatic sampling it is also essential that the sample taken is sterile and representative of the milk
<table>
<thead>
<tr>
<th>Product</th>
<th>Storage temperature (°C)</th>
<th>Maximum Time Before Examination</th>
<th>Minimum Sample Size</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nonsterilized milk and liquid milk products</td>
<td>0–4</td>
<td>24 h</td>
<td>100 ml or g</td>
</tr>
<tr>
<td>Sterilized milk, UHT milk and sterilized liquid milk products in unopened containers</td>
<td>Ambient, max. 30</td>
<td>7 days</td>
<td>100 ml or g</td>
</tr>
<tr>
<td>Sterilized milk, UHT milk and sterilized liquid milk products after sampling from the production line</td>
<td>0–4</td>
<td>24 h</td>
<td>100 ml or g</td>
</tr>
<tr>
<td>Evaporated milk, sweetened condensed milk, and milk concentrates in unopened containers</td>
<td>Ambient, max. 30</td>
<td>7 days</td>
<td>100 g</td>
</tr>
<tr>
<td>Semisolid and solid milk products (except butter and cheese)</td>
<td>0–4</td>
<td>24 h</td>
<td>100 g</td>
</tr>
<tr>
<td>Edible ices and semifinished ice products</td>
<td>–18 (or lower)</td>
<td>7 days</td>
<td>100 g</td>
</tr>
<tr>
<td>Dried milk and dried milk products</td>
<td>Ambient, max. 30</td>
<td>7 days</td>
<td>100 g</td>
</tr>
<tr>
<td>Butter, butter products, butter fat (butteroil), and similar products</td>
<td>0–4 (in the dark)</td>
<td>48 h</td>
<td>50 g</td>
</tr>
<tr>
<td>Fresh cheese</td>
<td>0–8</td>
<td>48 h</td>
<td>100 g</td>
</tr>
<tr>
<td>Processed cheese</td>
<td>Ambient, max. 30</td>
<td>7 days</td>
<td>100 g</td>
</tr>
<tr>
<td>Other cheeses</td>
<td>4–8</td>
<td>48 h</td>
<td>100 g</td>
</tr>
</tbody>
</table>

Source: Adapted from Prentice and Langridge (1992); IDF (1995a).
or milk product from which the sample is taken. Automatic sampling systems should be constructed in such a way that it is guaranteed that there are no milk residues left from the previous sample, which might lead to carry-over from one sample to the next, leading to erroneous results (IDF, 1990a; Lück and Gavron, 1990). With modern automatic sterile sampling systems it is, however, possible to automatically clean and sterilize the system between samplings. Several samples could also be taken simultaneously at various sampling points from pipelines, production tanks, and storage tanks. One of the advantages of these systems is that sampling valves can be fitted, for example, at normally inaccessible sampling points.

14.6.3 Numerical Selection of Samples

It is normal practice that the producer and buyer should come to an agreement as to what the quality of the product should be. The critical major and minor defects (IDF, 1992) should be clearly defined before selection of a sampling plan, so that they are unambiguously understood by all users of the contract or specification, when referring to the sampling plan. A single test on any individual sample may suggest that the product is better or worse than it actually is. Therefore, an element of risk is introduced, because only 100% sampling will give 100% certainty. In practice, this is not possible, and for normal quality control procedures the producer and the buyer accept a certain range of error—for example, 5% and 10%, respectively (Lück and Gavron, 1990). When the quality of dairy products is tested, the number of units to be sampled from a bulk consignment or batch depends on the size of the unit (large containers or small retail units) and the purpose of the test (determination of qualitative or quantitative characteristics). The batch is accepted or rejected according to the sampling plan that is based on the batch or lot size and acceptable quality levels (e.g., 1–10%). An acceptable quality level is considered to be the average of quality which, if maintained by a provider, could result in the acceptance of most of his production (IDF, 1990b). Tables are available which show how sample-taking should be carried out in order to obtain a statistically reasonable basis for the assessment of quality—that is, the number of consumer units that should be taken (randomly) from a consignment (batch) of a certain size. Sampling plans for milk and milk products are usually based on inspection by attributes (IDF, 1990b) or inspection by variables (IDF, 1992).
**Attribute Sampling.** Attribute sampling is used to qualitatively classify whether a unit is "good" or "defective." A "good" unit is one that meets the requirements of a specification, while a "defective" unit is one that does not. There are therefore only two answers when checking for an attribute (characteristic). For example, a UHT product can either be sterile or not.

**Inspection by Variables.** A variable may be described as a characteristic that can be measured quantitatively and that may have any value within certain limits—for example, total bacterial count, coliform count, percentage butter fat, and so on. Inspection by variables should not be used for critical defects—for example, one that would make the product unacceptable. The large variation in microbiological properties necessitates that more units have to be sampled to determine the microbiological quality than to determine the chemical quality of a batch (Lück and Gavron, 1990). The latter authors suggested the following number of samples (per batch) to be taken for routine microbiological control purposes.

- Pasteurized milk and pasteurized milk products: at least 10.
- Condensed milk, evaporated milk: at least 20.
- Dried milk, dried milk products: at least 10.
- UHT products: at least 75 packages per product per machine.

It is also recommended that the number of samples be increased should any drop in quality be noted. Samples could also be drawn over a period of time (for example, at the start, midway through and at the end of production) to identify time-related problems during production.

**14.6.4 Storage and Transport of Samples**

The most effective way to stabilize the microbial content of milk and perishable milk product samples prior to analysis is by storage and transport of the samples in crushed ice (0–4°C). At these temperatures, especially between 0°C and 2°C, the microbial numbers will remain virtually unchanged for up to 24–36h (Harding, 1995). This ideal is sometimes difficult to achieve, especially in less temperate countries. To prevent microbiological, physical, or chemical deterioration of the milk, consideration can be given to the use of chemical
preservatives for samples to be tested for compositional quality (Heeschen et al., 1969; Lück et al., 1982; Grace et al., 1993; De Wet, 1998). In many countries, however, the use of preservatives are not permitted.

Samples must reach the laboratory as quickly as possible, preferably within 24h. If cooling is necessary, the minimum requirements to be met are the temperature ranges that are either legally required or prescribed by the manufacturer. General guidelines that can be used in this regard are also presented in Table 14.5. Thermally insulated containers are used for the storage of cooled, frozen and quick-frozen samples to the laboratory. Crushed ice, pre-frozen icepacks, or dry ice (solid CO$_2$) may be used as refrigerant agents (IDF, 1995a). For most analyses, however, freezing should be avoided because it can cause disruption of bacterial cells.

14.6.5 Preparation of Samples for Microbiological Testing

The preparation of samples, prior to microbiological examination, is just as important as taking representative samples. The correct standard procedures—for example, those prescribed by IDF (1996a)—should consequently be followed carefully. The precise procedure for the preparation of the test portion varies with the nature of the product. All samples should be thoroughly mixed by shaking and inverting, using a rotary blender, a peristaltic blender (stomacher), or glass beads, depending on the type of product. Only specified diluents for general or special purposes are used for primary and further decimal dilutions. Damaging of microorganisms by sudden changes in temperature should be avoided, for example, when transferring a portion of the test sample to a diluent. The normal aseptic precautions during weighing and mixing of test portions, or transferring suspensions, should always be taken.

A myriad of microbiological tests are described in the literature that can be used in the dairy industry to assess the quality of milk and milk products. The selection of a method for a specific test should be carefully considered, and aspects such as the purpose of analysis and the required sensitivity of the method will, for example, determine which method is to be used. It is, however, recommended that officially prescribed or generally recognized procedures such as those of the IDF/ISO/AOAC or other standard procedures—for example, those recommended by the APHA (Marshall, 1993)—be used. Reference methods for determining the microbiological quality of various dairy
products are given in Table 14.6. These and other methods will be dealt with in more detail in the ensuing sections.

14.7 PROCEDURES FOR THE DIRECT ASSESSMENT OF THE MICROBIAL CONTENT OF MILK AND MILK PRODUCTS

The procedures described in this section are referred to as "direct" methods on the basis that they are able to give an estimate of the microbial numbers in the food product by counting the cells directly or the colonies developing from viable cells on a nutrient medium.

14.7.1 Cell Counting Procedures

14.7.1.1 The Breed Microscopic Count. The Breed smear or direct microscopic count (DMC) was developed as a rapid method for counting bacterial cells in milk, and the procedure is outlined in *Standard Methods for the Examination of Dairy Products* (Packard et al., 1993). This method suffers from major disadvantages:

- The staining method does not distinguish between dead and viable cells.
- The small sample volume renders the technique insensitive and subject to considerable error.
- For most microscopes a single organism per field represents $3–6 \times 10^5$ organisms ml$^{-1}$ of milk.

Because of this detection limit the DMC is unsuitable as a quality test for dairying nations that have milk quality standards of less than 100,000 per milliliter (Hill, 1991a). The DMC is therefore mainly of historical interest. Nevertheless, the technique may be of some use as a diagnostic tool where rapid screening of milk supplies with high bacterial counts is required. It has a further advantage that a skilled operator can recognize the morphological characteristics of the bacterial cells and infer whether the contamination has arisen from improperly cleansed utensils, dirty cows, aged or stale milk, or an udder infection.

14.7.1.2 The Direct Epifluorescent Filter Technique (DEFT). The direct epifluorescent filter technique is a modern approach to the direct microscope count (Hill, 1991b). With this technique the milk is first pretreated with a proteolytic enzyme and a surfactant which lyses the
<table>
<thead>
<tr>
<th>Product</th>
<th>Test</th>
<th>Principle</th>
<th>IDF</th>
<th>ISO</th>
<th>AOAC</th>
</tr>
</thead>
<tbody>
<tr>
<td>Butter</td>
<td>Lipolytic microorganisms</td>
<td>Colony count (30°C)</td>
<td>41: 1966</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Contaminating microorganisms</td>
<td>Colony count (30°C)</td>
<td>153: 1991</td>
<td>CD 13559</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Molds</td>
<td>Colony count (25°C, 5d)</td>
<td>94B: 1990</td>
<td>6611: 1992</td>
<td></td>
</tr>
<tr>
<td>Cheese</td>
<td><em>Escherichia coli</em></td>
<td>Colony count (44°C, using membranes)</td>
<td>170A: 1999</td>
<td>11866–3: 1997</td>
<td></td>
</tr>
<tr>
<td>Cheese (fresh)</td>
<td><em>Staphylococcus aureus</em></td>
<td>Colony count (37°C)</td>
<td>145A: 1997</td>
<td>CD 11 867</td>
<td></td>
</tr>
<tr>
<td>Cheese (powders)</td>
<td><em>Salmonella</em></td>
<td>Membrane filter</td>
<td>153: 1991</td>
<td>CD 13 559</td>
<td></td>
</tr>
<tr>
<td>Dried milk</td>
<td><em>Salmonella</em></td>
<td>MSRV medium</td>
<td></td>
<td></td>
<td>987.42A</td>
</tr>
<tr>
<td>Dried milk products</td>
<td><em>Staphylococcus aureus</em></td>
<td>Colony count (37°C)</td>
<td>138: 1986</td>
<td>CD 8869</td>
<td>995.07</td>
</tr>
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<td>Milk</td>
<td><em>Bacillus cereus</em></td>
<td>MPN</td>
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<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Coliforms</td>
<td>Colony count (30°C)</td>
<td>73B: 1998</td>
<td>5541–1: 1986</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Colony count (30°C)</td>
<td>73B: 1998</td>
<td>5541–2: 1986</td>
<td></td>
</tr>
<tr>
<td>Milk products</td>
<td>Microorganisms (enumeration)</td>
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<td>6610: 1992</td>
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</tr>
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<td>Dried milk (nonfat)</td>
<td><em>Salmonella</em></td>
<td>Membrane filter</td>
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<td></td>
<td>985.48</td>
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<td>Dried milk (whole)</td>
<td><em>Salmonella</em></td>
<td>Selective broth</td>
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<td></td>
<td>967.25</td>
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<td>Fermented milks</td>
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<td>Reference</td>
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<tr>
<td><strong>Bacterial and coliform counts</strong></td>
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<td>5541-1: 1986</td>
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<td>143A: 1995</td>
<td>10560: 1993</td>
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<td>6785: 1985</td>
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<td>6611: 1992</td>
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<td><em>Staphylococcus aureus</em></td>
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<td>5541-2: 1986</td>
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<td>Coliforms</td>
<td>Colony count (37°C)</td>
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<td>DIS 7889</td>
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<td><em>Lactobacillus delbruekii</em> spp. <em>bulgaricus</em></td>
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<td><em>Streptococcus salivarius</em> spp. <em>thermophilus</em></td>
<td>Colony count (37°C)</td>
<td>117B: 1997</td>
<td>DIS 7889</td>
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</table>

Source: Adapted from Webber et al. (2000).
somatic cells and modifies fat globules sufficiently to filter the sample through a 0.6-μm pore size polycarbonate filter. The filtration concentrates bacteria on the surface, after which they are stained with acridine orange. The mounted filter is examined through an epifluorescence microscope. Metabolically active bacteria fluoresce orange-red, while inactive bacteria fluoresce green.

The clumps of orange-red fluorescing bacteria are counted in the field of view, and a DEFT count ml⁻¹ of the milk sample is calculated over several fields. The technique has the advantage over the DMC that viable cells can be distinguished from inactive ones. The detection limit is also enhanced by filter concentration of the bacterial cells to a level that is useful for assessing the quality of milk produced by modern dairy industries. In line with the DMC a "same-day" result makes it possible to give producers rapid feedback of information.

Disadvantages of the method include the number of samples that can be examined because the DEFT is a microscopic count. A semi-automated system for slide examination overcomes this problem, but the sample preparation is also a limitation (Hill, 1991b). Another drawback is that the DEFT cannot be applied to heat-treated products due to some nonviable bacteria in such products fluorescing orange (Kroll, 1989).

14.7.1.3 Automatic Fluorescent Microscopic Count of Bacteria (Bactoscan 8000). Both the DEFT and the Bactoscan 8000 method belong to the group of direct microscopic counting methods (Suhren, 1989). In both methods, samples are pretreated with lysing and proteolytic reagents. Where separation and concentration with the DEFT technique is achieved by membrane filtration, gradient centrifugation is used in the Bactoscan method. In the latter method, cells are also stained with the fluorochrome acridine orange but are counted electronically as light impulses of single bacteria.

The Bactoscan technique has been found (Suhren et al., 1991) to be an acceptable alternative to the standard plate count in that it can, depending on the mode of estimation, reliably analyze milk samples with a colony-forming unit content of 40,000 to 80,000 cfu ml⁻¹.

The main advantages of the Bactoscan 8000 method is its speed (80 samples/hour) and the rapid availability of the results (approximately 15 min). Samples can also be preserved (Suhren et al., 1991) for not more than 7 days by addition of chemicals such as boric acid or sodium azide. A possible disadvantage is the fact that the statistical relationship between the standard plate count and Bactoscan values at lower
plate counts (e.g., <10,000 cfu ml\(^{-1}\)) are less consistent than at higher cfu levels.

### 14.7.1.4 Flow Cytometry (Bactoscan FC)
Flow cytometry is the science of measuring components (cells) and the properties of individual cells in liquid suspension. In essence, suspended cells are brought to a detector by means of a flow channel (Jay, 1992). Flow cytometric analysis of bacteria usually requires staining of the cells with fluorescent dyes binding to specific cell constituents in order to distinguish cells from other particulate matter (Suhren and Walte, 1998). In the Bactoscan FC technique the DNA/RNA of the bacteria is stained with the fluorescent dye ethidium bromide, and “disturbing” milk constituents are reduced/dispersed by buffers, detergents, and enzymes during sample preparation. Fluorescence is excited by laser [usually an argon ion laser (Suhren and Walte, 1998)]. The light emitted is detected when the stained particles pass in a hydrodynamically focused stream by a fluorescence detector and are indicated as Bactoscan counts (BC-FC). With respect to sample preparation, staining, and measuring, the Bactoscan-FC method differs principally from the Bactoscan 8000 (BC-8000) procedure. The accuracy of the estimation by which the SPC ml\(^{-1}\) can be estimated from the results of the routine method was slightly superior for BC-FC over BC-8000 and is improved when not single results but the average of two samples of dairy farm milk taken at different times is considered (Suhren and Walte, 1998).

Advantages of this method are similar to those of the BC-8000. Disadvantages are the cost of the equipment and the fact that somatic cell counts exceeding 1 million ml\(^{-1}\) might influence the counts.

### 14.7.2 Electronic Counting of Microcolonies
In this method (Suhren and Heeschen, 1991a) the milk to be tested is mixed with a liquid nutrient gelatin solution to give a milk dilution of 1:500. The mixture is pipetted into tubes (±3-cm depth) and overlayed with 1 ml of the nutrient gelatin. The mixture is allowed to solidify and incubated at 20 ± 1 h at 21°C. The developed colonies in the medium are fixed by overlaying the medium with a 2 ml formaldehyde—hydrochloric acid mixture or, alternatively, glutaraldehyde/hydrochloric acid or potassium dichromate. This step is followed by liquefaction of the medium in a waterbath at 35°C for 30 min. A volume of 7 ml of electrolyte (sodium chloride and formaldehyde) is added and the mixture is stirred gently. The microcolonies are then counted using a Coulter counter. The counter is adjusted to count all particles >600 μm\(^3\).
Calibration is done using 20.5-μm³-diameter latex particles or by com-
paring the electronic and direct microscopic count of the microcolonies
(Suhren and Heeschen, 1991a).

Milk samples can be preserved before testing by adding a mixture
of orthoboric and sorbic acid (final concentrations 0.6% m/v and
0.009% m/v, respectively) for 24h at 5–20°C and for a further 24h at
refrigeration temperature.

The correlation coefficient between the microcolony count and the
standard plate count (SPC) was $r = 0.85$, and 100,000 microcolonies
ml⁻¹ corresponded to a SPC of 170,000 cfu ml⁻¹.

Advantages of this method are that the counts are available within
24h and the electronic counter also speeds up the counting procedure.
A disadvantage is the fact that the growth of aerobic bacteria such as
*Pseudomonas* is suppressed in the tubed medium compared to facult-
tive anaerobes (Suhren and Heeschen, 1991a). This might lead to
the microcolony method underestimating counts of samples in which
pseudomonads predominate.

### 14.7.3 Macrocolony Count Procedures

#### 14.7.3.1 “Total” Counts

14.7.3.1.1 *Conventional Standard Plate Count.* Most standards and
regulations refer to macrocolony counts determined by a reference or
official method (Suhren, 1989). The problematic nature of this para-
meter will continue to be debated. Sharpe as quoted by Suhren (1989)
has stated that: “the plate count is a totally unique datum; nothing in
the physical, chemical, biochemical or immunological world corre-
sponds to it and no test based on these properties can ever correlate
with it reliably.” Nevertheless, the macrocolony count remains the inter-
nationally accepted standard.

In the standard plate count method, those microorganisms that are
able to produce colony-forming units in a specific growth medium are
enumerated after decimal dilutions of the sample have been plated and
incubated aerobically at 30°C ± 1°C for 72 ± 2h (Webber et al., 2000)
or at 30–32°C for 48h (Houghtby et al., 1993). Bacterial colonies that
grow on the surface and in the various depths of the solid growth
medium are counted, and calculations are done according to the speci-
fied procedures to determine the number of colony-forming units
(cfu’s) per milliliter of the original samples (Brazis, 1991).

The method described in IDF (1991a) is recommended for a wide
range of dairy products such as milk, liquid and dried milk products,
lactose, caseins, caseinates, processed cheese, butter, frozen milk products, custard, desserts, and cream. Plate count standards have been developed with a view to ensuring satisfactory production hygiene and to ensure that the product is safe (Brazis, 1991). This method has also been used as a valuable adjunct to guide sanitarians in correcting sanitation failures and in improving the bacteriological quality of milk. A few disadvantages of the standard plate count include the following:

1. The long incubation time, often yielding counts after the product has been processed.
2. Inherent limitations brought on by a specific culture medium, the aerobic incubation conditions, and temperature of incubation.
3. The fact that both viable single cells and clumps of cells are counted as single colony-forming units.

14.7.3.1.2 Surface Count Technique. Attempts to speed up the standard plate count or decrease the amount of agar medium or number of plates and dilutions have resulted in a range of modifications. One of these modifications is the surface count technique (Lück and Gavron, 1990). Surface counts result in more rapid development of colonies, and these can be counted after 24h. In producing such colonies, the spread or drop method can be applied. In the spread method, 0.1 ml of the 10-fold dilutions are transferred to and spread over the dry surface of a solid agar medium. After incubation, countable plates, selected according to the usual procedures, are counted. Advantages of this method are as follows:

1. Plates of media can be prepared and dried beforehand.
2. All colonies are on the surface and easily visible.
3. Aerobic colonies develop more rapidly.

Disadvantages are as follows:

1. Only 0.1 ml of dilution can be plated per conventional Petri dish.
2. Undetected contaminant colonies occurring on the prepared plate are spread over the entire plate during the spreading procedure, resulting in a film of growth that masks the development of colonies to be counted.
3. The spreading technique itself is time-consuming.
14.7.3.1.3 Plate Loop Technique. The plate loop technique (Hill, 1991c) substantially decreases the time it takes to process a milk sample (from the sample bottle to the Petri dish). Because of reduced media requirements and the elimination of the necessity for serial dilutions, a single operator can process a greatly increased number of samples compared with the reference method. Disadvantages of this method are as follows:

1. The test result is still governed by the incubation time (e.g., 72h at 30°C).
2. Several features of the technique itself influence the precision and accuracy of the final count obtained.

These factors relate to the loop itself, the manner in which the loop is used by the operator, and the characteristics of the milk under analysis (Hill, 1991c).

14.7.3.1.4 Roll Tube Method. The roll tube method (Slaghuis, 1991) has been developed to save labor and money and is in fact a mechanization of the plate count method. The principle of the method entails transferring a fixed volume of the milk dilution into a thin-walled glass "roll tube" in which the pre-sterilized, melted medium is contained. The tube is sealed by means of a special rubber stopper, and the tube is placed in a horizontal position on an apparatus that spins the tube on its horizontal axis while cold water is sprayed onto the external surface of the tube. The medium solidifies and adheres to the inside surface of the tube. After incubation for 3 days at 30°C, the colonies are counted and the cfuml⁻¹ count is determined as usual.

A modification of this method in which the loop method and the roll-tube method is combined has been applied in the Netherlands in the quality payment scheme of that country (Slaghuis, 1991). The results of the roll tube method have been found to be virtually identical to those of the standard plate count (Slaghuis, 1991). Limitations of the roll tube method are:

1. Similar to those related to colony count methods in general.
2. Those attributable to the roll tube method itself, namely a higher agar concentration necessary to attach the thin layer of medium to the wall; the fact that water does not evaporate in the sealed tube as compared to a Petri dish, which results in occasional surface spreaders; and no replenishment of oxygen as compared
to the Petri dish method. This may result in strict aerobes not developing as well in the roll tube (Slaghuis, 1991).

14.7.3.1.5 **Spiral Plate Count.** The spiral plate count method (Harding, 1995) is another version of the SPC in which a spiral plating instrument inoculates the surface of a prepared agar plate in such a way that between 500 and 500,000 cfu ml\(^{-1}\) of sample can be counted. The instrument deposits a decreasing amount of milk on the surface of the agar plate by means of an Archimedean spiral, such that the volume of the sample deposited on any portion of the plate is known. Colonies on a portion of the plate are counted using a special grid that associates a calibrated volume with each area. An advantage of this method is that it removes the need for multiple dilutions necessary in the SPC techniques. In collaborative studies (Jay, 1992) on milk sample testing, the spiral count compared favorably with the SPC. Spiral plating is an official Association of Official Analytical Chemists method (AOAC, 1990). Other advantages of the method are that less agar is used as well as fewer plates, dilution blanks, and pipettes. Three to four times more samples per hour can be examined, compared to the conventional method (Jay, 1992).

A disadvantage of the method is the expense of the device, and it is not likely to be available in laboratories that do not analyze large numbers of plates; it is also more suited to liquid food such as milk, because more particulate foods can lead to blockage of the dispensing stylus (Jay, 1992).

14.7.3.1.6 **Dry Rehydratable Film Technique.** The dry rehydratable film consists of two plastic films attached on one side and coated with culture medium ingredients and a cold water-soluble jelling agent. The film was developed by the 3M Company and designated *Petrifilm* [McAllister et al. (1984) as quoted by Jay (1992)].

In applying the film, 1 ml of diluent is placed in the shallow 6-cm-diameter well and is sandwiched in the nutrient area by pressing the two plastic sheets together. Following incubation the microcolonies appear red on the nonselective film due to the presence of a tetrazolium dye in the nutrient phase (Jay, 1992). *Petrifilm* test methods are available for the aerobic ("total") plate count, the coliform count, and the *Escherichia coli* count.

Use of this method to date indicates that it is an acceptable alternative to the conventional plate count methods (Ginn et al., 1984) and has been approved by the AOAC, the ISO, and the International Dairy Federation (IDF, 1996b).
14.7.3.1.7 Hydrophobic Grid Membrane Technique. The hydrophobic grid membrane filter (HGMF) combines desirable features of the conventional plate count methods, the principles of membrane filtration, and most probable number (MPN) counts and offers additional benefits (Sharpe, 1989).

"Conventional" membrane filtration became the main tool for analytical water microbiology after World War II. Its success was due not only to its permeability to nutrients, its chemical stability, and, to a moderate extent at least, its ability to keep developing colonies separate, but to the improved limits of detection it allowed by concentrating the bacteria in a liquid (Sharpe, 1989).

The HGMF is a square membrane filter (60 × 60mm, pore size 0.45μm) printed on one side with a black hydrophobic ("waxy") grid outlining 1600 (40 × 40) small squares. Its unique properties result from the confining of colony growth to the grid cells in which the individual cell/cell clump was captured originally. The typical appearance after incubation is of a grid bearing "square" colonies distributed among the 1600 available locations (Sharpe, 1989). The additional advantages offered by HGMF include the following:

1. An automated counter and a variety of filtration equipment and items supplied by two Canadian companies.
2. With agar plates and ordinary membrane filters, colony overlaps at high cfu densities limit the numerical range necessitating sequential dilutions. The HGMF counts, however, follow a most probable number mathematical principle (Sharpe, 1989) where each grid cell can be linked to one tube in an MPN count done at a single dilution, thus

\[ \text{MPNGU} = 1600 \log \frac{(1600 - X)}{X} \]

where \( X \) is the number of positive grid cells and MPNGU is the most probable number of growth units filtered onto the HGMF. There is therefore no need to prepare serial dilutions of the sample.
3. Plating of duplicate HGMFs is unnecessary because the precision is better than the plate count,
4. The HGMF regiments colonies into arrays that electronics can deal with more easily.
5. HGMF-based analyses are available for all the common foodborne organisms and many of the HGMF techniques (e.g., aerobic
plate count, coliforms, fecal coliforms, *E. coli*, *E. coli* 0157, and *Salmonella* enjoy AOAC official action (Sharpe, 1989).

HGMFs do have some disadvantages (Sharpe, 1989):

1. Each grid (ISO-GRID HGMF) is relatively expensive.
2. An automated colony counter is recommended if large numbers of HGMFs are to be counted, which also brings in a cost factor.
3. For some food suspensions, filterability needs to be improved by means of enzyme digestion.

### 14.7.3.2 Contaminating Organisms.

The so-called “contaminating organisms” count is a version of the standard plate count. It differs from the conventional SPC technique in that the culture medium is carbohydrate-free. Lactic acid starter organisms requiring this carbon source are consequently not able to develop in the medium or at best are only capable of developing pinpoint colonies. The rationale is that non-lactic acid organisms (e.g., Gram-negative spoilage organisms) can be selectively detected in products such as butter, fermented milks, and fresh cheese in which beneficial (flavor- or acid-producing) organisms such as viable lactic acid starter bacteria may be present.

This method was developed by a joint IDF/ISO/AOAC group of experts and has been published as an international standard (IDF, 1991b). The medium consists of peptone from casein, peptone from gelatin, sodium chloride, agar, and water, and all medium components are carbohydrate-free. The incubation temperature/time is 30°C for 72 h. Pinpoint colonies do not represent typical “contaminants” and should not be counted. All other colonies should be counted. The results are reported as “contaminating microorganisms per gram of product.”

Advantages of the method include the detection of nonstarter contaminants in fermented milk products. Disadvantages are that pinpoint colonies may erroneously be counted as contaminants.

### 14.7.3.3 Psychrotrophic Bacteria.

Psychrotrophic bacteria are those bacteria able to grow at 7°C or less regardless of their optimal growth temperature (Frank et al., 1993). Psychrotrophic bacteria commonly isolated from dairy products belong to a variety of Gram-negative and Gram-positive genera. The most detrimental of these are the oxidative Gram-negative rods belonging to the genus *Pseudomonas.*
This is also the psychrotroph genus most commonly isolated from milk (Frank et al., 1993). In raw milk, high counts of this group of bacteria are related to unsanitary conditions during production and to temperature abuse during storage before pasteurisation. These organisms are inactivated by pasteurization, and their presence in pasteurized milk indicates either improper pasteurization or postpasteurization contamination. In pasteurized milk these organisms severely limit the shelf life of the milk. They also produce proteases and lipases that, when produced in the raw milk, can survive heat treatment and cause sensory and textural defects in the processed dairy product (Frank et al., 1993).

Reference methods for counting psychrotrophic bacteria in milk include the 7°C 10-day incubation period of the American Public Health Association standard method (Frank et al., 1993) and the IDF international standard (IDF, 1991) (6.5°C for 10 days). The culture media and plating procedures are as for the standard plate count.

The reference methods are of limited use in practice because of the 10-day incubation period they require. Consequently, more rapid methods have been developed. One set of methods is based on using higher temperatures of incubation such as 21°C for 25h (Griffiths et al., 1980) or 18°C for 45h (Oehlrich and McKellar, 1983). Lück et al. (1984) found that no significant differences existed between the standard psychrotrophic counts and the 21°C/25-h counts when poor-quality raw milk that contained a high percentage of psychrotrophs was tested. Significant differences did, however, exist in good-quality raw milk that contained relatively low numbers of psychrotrophs. They are of the opinion that the 21°C/25-h regime can be recommended as a method by which an estimate of the psychrotrophic population can be obtained. This method has been approved as an International Dairy Federation Standard (IDF, 1985).

Oehlrich and McKellar (1983), who proposed an 18°C/45-h temperature–time combination, found that this method led to a more uniform and visible colony size than was the case at 21°C for 25h. With the latter method the majority of colonies were found to be pinpoint colonies. The 18°C/45-h method was also found by Fischer et al. (1986) to be well-correlated with the reference method ($r = 0.911$), and the percentage distribution of bacterial types in the 18°C/45-h method corresponded very well with those in the reference method.

Media that are selective for Gram-negative bacteria have also been used for estimating the psychrotrophic population. An SPC medium containing crystal violet and tetrazolium is recommended by the American Public Health Association (Frank et al., 1993) with an incubation time and temperature of 48h and 21°C, respectively. Red
colonies are counted on this medium. Fischer et al. (1986) found that a count made on SPC medium containing alkylidimethyl benzyl ammonium chloride ("Merquat") and incubated at 18°C for 45h was well-correlated with the psychrotrophic count reference method ($r = 0.920$). Lück and Gavron (1990) are of the opinion that differences in counts between the reference method and the methods in which inhibitory substances are added to the media to selectively suppress the growth of Gram-positive organisms are often rather great.

The counting of oxidase positive colonies at elevated temperatures has also been proposed as a rapid test to determine the presence of potential psychrotrophs. This test is based on the ability of certain bacteria, which contain a strong cytochrome C oxidase system, to oxidize chemicals and to form dyes (Lück and Gavron, 1990). For estimating the psychrotrophic count, the oxidase-positive count at 27°C or 32°C ($r = 0.59$ and 0.57, respectively) showed no advantage over the SPC method ($r = 0.61$ and 0.61, respectively; Lück et al., 1971).

### 14.7.3.4 Proteolytic and Lipolytic Bacteria

Many bacteria responsible for spoilage of refrigerated dairy products are highly proteolytic and/or lipolytic and can cause flavor defects. Proteolytic enzymes produced by psychrotrophic bacteria during growth in milk often remain active after HTST and even UHT heat treatment and reduce the quality of stored, heat-treated products. Two methods for the detection or enumeration of proteolytic bacteria are recommended by the American Public Health Association (Frank et al., 1993).

The first method entails using standard plate count medium with 10% added skim milk. Plates are incubated at 32°C for 48–72h. After incubation, plates are flooded with 1% hydrochloric acid and are left for 1 min before decanting the excess acid and counting the colonies surrounded by clear zones. A disadvantage of this method is that acid-producing bacteria can produce false-positive reactions on this agar. In the author’s opinion a better counting procedure is the standard methods caseinate agar method. This method is based on the addition to standard methods agar of sodium caseinate dissolved in a citrate solution (Frank et al., 1993). After sterilization and cooling of the medium to 45°C, a sterile calcium chloride solution is added to the molten agar and mixed immediately. Dilutions of the milk sample are surface-plated and spread on the solidified and pre-dried plates of caseinate agar and incubated at 32°C for 48–72h. Colonies surrounded by a white or off-white zone of casein precipitate are proteolytic. Highly proteolytic colonies will also produce a clear inner zone with an outer opaque precipitate zone.
Disadvantages of this method are that only uncrowded plates (fewer than 80 colonies per plate: Frank et al., 1993) can be read accurately. Calcium chloride added at too high a concentration or temperature results in turbid plates, rendering the plates useless. The caseinate plates must be completely clear after solidification.

Growth of lipase-producing microorganisms can contribute to flavor defects in milk and high-fat dairy products. Some of the free fatty acids released by the action of lipolytic enzymes have a low flavor threshold and can impart a rancid flavor at low concentrations. The less volatile fatty acids are more susceptible to oxidation following hydrolysis, which leads to oxidative flavor defects (Frank et al., 1993). Heat-stable bacterial lipases are of particular concern because they affect products stored for long periods such as cheese, butter, and UHT products.

Several different methods are available for the enumeration of lipolytic microorganisms. The growth medium recommended by the American Public Health Association is preferred over the Victoria blue butter-fat agar (Lück and Gavron, 1990) because of difficulties in preparing the latter medium and problems in interpreting the reaction (Shelley et al., 1987).

The method described by Frank et al. (1993) employs spirit blue agar. This medium is commercially available; and after cooling the melted sterile agar, 3% v/v of lipase reagent (containing tributyrin as a substrate) is added to the medium and thoroughly mixed to emulsify the reagent uniformly in the agar.

The prepared and dried plates are surface-inoculated with 0.1 ml of the milk sample dilutions and incubated at 32°C for 48 h. For psychrotrophic lipolytic organisms, plates are incubated at 21°C for 72 h. The complete medium is light blue and translucent when prepared as above. Colonies of lipolytic microorganisms develop a clear zone and/or a deep blue color around or underneath each colony.

14.7.3.5 Thermoduric Bacteria and Sporeformers. In the dairy industry the term thermoduric bacteria refers to microorganisms that survive pasteurization but do not grow at pasteurization temperatures (Frank et al., 1993). Thermoduric bacteria isolated from milk usually include spore-formers such as Bacillus and Clostridium and also non-spore-forming cocci (e.g., Micrococcus spp. and Streptococcus spp.) and rods such as Microbacterium and other members of the coryneform group (Thomas et al., 1967).
Primary sources of contamination of milk by thermoduric microorganisms are poorly cleaned and sanitized udders, utensils, and equipment. Because high thermoduric counts are consistently associated with unhygienic production practices, the thermoduric or laboratory pasteurization count is used to indicate the thoroughness of equipment sanitation and to detect milk supplies that can be responsible for high-count pasteurized milk products (Frank et al., 1993). The laboratory pasteurization count (LPC) is performed by heating a 5-ml sample of milk to 62.8°C for 30 min. Plating and incubation of plates are the same as for the conventional standard plate count method (Frank et al., 1993). The LPC simulates low-temperature long-time (LTLT) pasteurization. For enumeration of these bacteria according to the HTST (high-temperature short-time) method of pasteurization, a standardized loopful of milk sample is mixed with 10 ml of melted plate count agar at 74°C in a water bath. After exactly 15 s, the medium is poured into a Petri dish. The solidified plates are incubated at 30–32°C for 48–72 h (Lück and Gavron, 1990). The above thermoduric count techniques (LPC and the HTST methods) yield counts that are significantly different (Lück and Gavron, 1990).

The spore-forming component of the thermoduric population can be more selectively counted by applying higher temperature treatments of the milk sample. Various temperature–time intervals have been recommended—for example, 80°C for 10 min, 85°C for 10–15 min, and 100°C for 12 min. According to Lück and Gavron (1990), a combination of 80°C for 10 min is not sufficient to kill all vegetative bacteria in milk, and hence a treatment of 85°C for 10 min is recommended. After this treatment the milk sample is immediately cooled to 10°C and the SPC dilution and plating technique is applied with incubation of the plates at 30°C for 72 h.

When spores of anaerobic bacteria are to be counted, a broth of reinforced clostridial medium, plus a “seal” of 2% sterile water agar containing 0.5 g liter⁻¹ sodium thioglycolate, is recommended (Lück and Gavron, 1990). Because of the low numbers of anaerobic spores usually found in dairy products, the most probable number method should be applied. The inoculated and sealed (25-mm-thick seal) tubes are incubated at 30°C for 14 days. The tubes are examined on a daily basis, and those showing evidence of gas production are positive. For enumerating hydrogen sulfide-producing clostridia, differential reinforced clostridial medium is used (Lück and Gavron, 1990). Tubes that show blackening of their contents are positive and can be used to isolate and further characterise the clostridia.
14.7.3.6 **Thermophilic Organisms.** In the dairy industry, the term *thermophilic bacteria* refers to organisms that grow in milk or milk products held at elevated temperatures (55°C or higher), which include LTLT pasteurization conditions. Thermophilic bacteria may also accumulate in certain areas of HTST pasteurizers that have been in continuous operation for extended periods (Frank et al., 1993).

Thermophilic bacteria are usually species of *Bacillus*, which enter into milk from various sources on the farm or from poorly cleansed equipment in the processing plant. These bacteria rapidly increase in numbers when present in milk or dairy products that are held at high temperatures for long periods.

To enumerate these organisms in milk and milk products, prepare dilutions the same as for the standard plate count, but use 15–18 ml of agar medium per plate. Incubate the inverted plates for 48 h at 55°C and maintain humidity during incubation to prevent drying of agar medium. Report the colony count as "thermophilic bacterial count per milliliter" or "per gram" (TBC ml⁻¹ or g⁻¹).

14.7.3.7 **Enterococci.** The *Enterococcus* count is more reliable than the coliform count as an index of the sanitary quality of cultured butter [Blankenagel et al. (1967) and Saraswat et al. (1965) as quoted by Frank et al. (1993)]. This is because enterococci are better able than coliforms to survive in the unfavorable microenvironment of the butter. In addition, the *Enterococcus* count may be a more reliable indicator of the sanitary quality of yogurt than the coliform count because coliforms are inactivated in the low-pH environment whereas enterococci are not [Jordano Salinas (1984) as quoted by Frank et al. (1993)].

Citrate azide agar has been recommended by the American Public Health Association (Frank et al., 1993) for enumerating enterococci in dairy products. Petri dishes are inoculated and poured using citrate azide agar in the usual way. After the medium has solidified, a thin (3–4 ml) overlay of the same medium is poured onto the surface and tilted to allow the overlay to cover the surface of the solidified agar completely. The plates are incubated at 37°C for 48–72 h and only the blue colonies are counted. A white sheet of paper placed under the Petri dish on the illuminated colony counter enhances the contrast between the colonies and the background (Frank et al., 1993). An *Enterococcus* count of fewer than 10 colonies per gram of butter is not considered too stringent for a well-managed butter manufacturing plant [Saraswat et al. (1965) as quoted by Frank et al. (1993)].
14.8 PROCEDURES FOR THE INDIRECT ASSESSMENT OF THE MICROBIAL CONTENT OF MILK AND MILK PRODUCTS

14.8.1 Most Probable Number Method

The MPN method makes use of a statistical technique to detect low counts of bacteria in dairy products (Lück and Gavron, 1990). Liquid media are usually employed, with the nature of the medium depending on the nature of the microorganisms to be counted. Three sets of three or five tubes, each containing the sterile medium, are prepared and inoculated from each of three consecutive 10-fold dilutions. Tubes showing bacterial growth after incubation are positive. From the number of positive tubes in each set of three or five tubes, the most probable number of bacteria per unit of sample is read off from McCrady’s tables (McCrady, 1918). When more than three dilutions (more than three sets of tubes) are made, only the results from any three consecutive dilutions are significant. The highest dilution that gives positive results in all tubes, along with the next two succeeding (higher) dilutions, should be chosen. When the mass or volume of sample in the first dilution is 10 or 100 times less than the mass or volume listed in McCrady’s tables, then the count tabled must be multiplied by 10 and 100, respectively (Lück and Gavron, 1990).

This method of analysis has gained popularity. According to Jay (1992) the advantages that it offers are the following:

1. It is relatively simple.
2. Results from one laboratory are more likely than SPC results to agree with those from another laboratory.
3. Specific groups of organisms can be determined by use of appropriate selective and differential media.
4. It is the method of choice for determining fecal coliform densities.

Among the drawbacks to its use is the large volume of glassware required (especially for the five-tube method), the lack of opportunity to observe the colonial morphology of the organisms, and the method’s lack of precision (Jay, 1992).

14.8.2 Methods Based on the Metabolic Activity of the Microorganisms

14.8.2.1 Dye Reduction. The principle of these tests is to add oxidation–reduction-sensitive dyes, such as methylene blue, resazurin,
or triphenyltetrazolium chloride, to milk or liquid dairy products and to measure the color change after incubation. The color change is based on the dehydrogenase activity of the bacteria present. Dehydrogenases (i.e., mainly flavine enzymes) transfer hydrogen from a substrate to biological acceptors or to the dyes added. The period of time needed to change or to decolorise the dye is an index of the bacterial load of the product (Lück and Gavron, 1990).

Dye reduction tests are, however, of little value as an index of the bacterial count of refrigerated milk, because this relationship is poorly correlated $[r = 0.36 \text{ to } -0.62 \ (\text{Luck et al., 1970a;Luck, 1972})]$. The reason is that most of the bacteria in refrigerated milk are in a dormant state. Furthermore, a relatively large proportion of the bacteria present are psychrotrophs. These microorganisms have, compared to lactic acid bacteria, a low dehydrogenase activity, a characteristic that contributes to the low correlation between bacterial count and methylene blue reduction time or resazurin disc reading. In order to achieve the same reliability of, say the methylene blue test for nonrefrigerated milk, approximately twice as many samples of refrigerated milk have to be tested (Lück and Andrew, 1975). Preincubation (13–18°C for 16–24 h) has been shown to be unsuccessful in improving the relationship between bacterial count before incubation and the results of a metabolic activity test after preincubation (Lück and Gavron, 1990).

Advantages of the dye reduction tests are that the results are available within a few hours and that the test can be carried out in a small laboratory without expensive equipment (Lück, 1991a). Reduction tests are, therefore, suitable to improve the bacteriological quality of milk in developing countries.

Disadvantages of dye reduction tests are that they are of very little value for the estimation of the plate count of cold-stored milk, especially when the “total” bacterial count is below 100,000 ml⁻¹, no matter whether the samples are or are not preincubated before testing (Lück, 1991a). The tests are also imprecise, because some bacteria have a high rate of reduction whereas others have a low rate. Furthermore, somatic cells are also capable of reducing the dyes (Harding, 1995).

### 14.8.2.2 Nitrate Reduction

The nitrate reduction test (NRT) makes use of the ability of several bacterial species to reduce nitrate to nitrite, which is a multienzyme reaction catalyzed by different flavine enzymes (Lück, 1991b).

The test entails adding 1 ml KNO₃ solution (0.3% w/v) to 10 ml milk. The milk is then incubated at 30°C for a specified period, and a spot test is done for nitrite formation—that is, 1 drop of milk plus 1 drop of
nitrite reagent \((5 \text{ g sulfanilamide} + 1 \text{ g } \alpha\text{-naphthylamine} + 100 \text{ ml glacial acetic acid} + 100 \text{ ml } \text{H}_2\text{O}; \text{ the solution is made up weekly and refrigerated in an amber glass bottle})\). A pink to red color indicates a positive reaction.

The NRT is suitable as a method for detecting raw milk samples with psychrotrophic or other contaminating bacteria in numbers exceeding \(200,000 \text{ ml}^{-1}\). For this purpose a 5-h NRT at 30°C without preincubation or a 3-h NRT at 30°C after preincubation at 15°C for 16 h can be used (Lück et al., 1972).

The advantages of the NRT is that it gives a better indication of the psychrotroph and coliform content in cold-stored raw milk than the dye reduction tests and that it can be used in smaller laboratories anywhere.

A disadvantage of the NRT is that it is not suitable for testing milk with a bacterial count of less than \(100,000 \text{ ml}^{-1}\).

### 14.8.2.3 Pyruvate Determination

Pyruvate is an intermediate metabolite in a wide variety of metabolic pathways and is therefore a constituent of all microbial cells. Tolle et al. (1972) suggested the measurement of pyruvate as an indication of the hygienic quality of milk. Immediately after milking, the pyruvate level in normal milk is \(0.5–1.5 \mu\text{g ml}^{-1}\), with much of this pyruvate being derived from sources other than bacteria e.g. leucocytes, (Easter and Prentice, 1989). There is therefore a background level of pyruvate in milk, which limits usefulness for determining low levels of microorganisms. The level of microbial pyruvate also varies according to the storage conditions of the milk (Easter and Prentice, 1989). For these reasons, pyruvate measurement is not a method of choice for determining the hygienic quality of milk with low microbial load.

If it is only necessary to detect milk with high levels of bacteria (e.g., \(>10^6 \text{ ml}^{-1}\)), the pyruvate test has the advantage of being rapid, easily automated, and able to be carried out on preserved milk. For the latter reason, the pyruvate test has been used in a quality control test program in the Federal Republic of Germany (Easter and Prentice, 1989).

### 14.8.2.4 Catalase Production

The psychrotrophic bacterial population in milk consists largely of Gram-negative, catalase-producing bacterial genera. The enzyme catalase catalyzes the following reaction:

\[
2\text{H}_2\text{O}_2 \underset{\text{catalase}}{\rightleftharpoons} 2\text{H}_2\text{O} + \text{O}_2
\]
It may be argued that the amount of oxygen released by catalase activity in milk is related to the microbial load of the milk (Easter and Prentice, 1989). Lück (1991c), however, came to the conclusion that the catalase test cannot be recommended as an accurate index of the bacterial load of raw milk. The activity of the catalase present was found not to be correlated well enough with the number of microorganisms in the milk.

### 14.8.2.5 Oxygen (O<sub>2</sub>) Tension Measurement.

Another metabolic activity test described by Lück and Gavron (1990) is the oxygen (O<sub>2</sub>) test carried out by means of an O<sub>2</sub> electrode. The amount of dissolved oxygen in the milk decreases as the initial bacterial count increases.

Different procedures of handling milk and liquid dairy products affect the dissolved oxygen content. Such procedures include time lapses until testing, temperature of the milk, and/or the time and speed of stirring or agitation, and so on. For this reason the direct measurement of the O<sub>2</sub> content of the milk does not give a clear indication of the bacterial count.

The milk samples have to be saturated with air first (e.g., by shaking), followed by a specified period of incubation to obtain a close relationship between O<sub>2</sub> content and bacterial count. The multiple regression relationship between O<sub>2</sub> content and bacterial counts under these conditions was consistent enough ($r = -0.70$) to recommend the 6-h O<sub>2</sub> test as an index of the total mesophilic and psychrotrophic count (Lück et al., 1970b; Lück, 1991d). The necessity to saturate the samples with air followed by a 6-h incubation period at 25°C, together with the necessity for having an oxygen meter, has so far discouraged the routine implementation of the O<sub>2</sub> depletion test (Lück, 1991d).

### 14.8.2.6 Impedance.

It is known that growth of microorganisms in a medium alters the electrical properties of the growth medium (Easter and Prentice, 1989). This occurs in two ways:

1. The catabolic activity of microorganisms breaks down large molecules into small molecules that are frequently charged (e.g., undissociated sugars are broken down into lactic and acetic acids, and proteins are broken down into fatty acids). These metabolic products frequently have a greater charge than the larger molecule from which they originate.

2. The smaller molecules are more mobile than the larger molecules and are able to conduct electricity more readily in a solvent.
Microbial growth in a growth medium tends to increase the ability of a medium to conduct electricity; that is, conductance is an increasing function of microbial growth. Because conductance is inversely dependent on impedance, there is an inverse relationship between microbial growth and impedance (Easter and Prentice, 1989).

The electrical impedance in a culture medium remains fairly consistent until a threshold of $10^6$-$10^7$ cells ml$^{-1}$ is reached, when major changes in impedance start to occur. The time taken to reach the threshold value is indicative of the initial bacterial load in raw milk (Cady et al., 1978).

An advantage of this method is that a result can be obtained in 8.5 h compared to 72 h using a conventional SPC (Easter and Prentice, 1989). In addition, this method can be used to detect specific microorganisms in the food. By designing media that will inhibit the growth of all but the test organism, while still allowing satisfactory growth curves of the latter organisms, it is possible to detect a wide range of indicator organisms and pathogens—for example, enterococci, coliforms, and Salmonella (Easter and Prentice, 1989).

The impedance detection time (IDT) can, however, also be used to screen raw milk samples, and in a specific study a 7-h cutoff time (equivalent to $10^5$cfu ml$^{-1}$) was successfully used to screen the samples (Gnan and Luedecke, 1982). Impedimetry was also used to determine the potential shelf life of pasteurized whole milk (Bishop et al., 1984).

Attention must be paid to culture media for impedimetry because not all media sustain satisfactory growth of a given organism. In brain heart infusion (BHI) broth, Pseudomonas spp. exhibited a triphasic type growth curve, making it difficult to determine the true IDT (Firstenberg-Eden and Tricarico, 1983). The incubation temperature is also important (Easter and Prentice, 1989). A method for estimating the bacterial content of a milk sample by impedimetry is described in Standard Methods for the Examination of Dairy Products (Marshall, 1993).

### 14.8.3 Methods Based on Specific Cellular Components

#### 14.8.3.1 Adenosine Triphosphate (ATP) Bioluminescence.

All living cells contain ATP, which acts as a substrate in the bioluminescence firefly enzymes system luciferin–luciferase, giving rise to light emission (Harding, 1995). This very sensitive light emission reaction can be used as a measure of low levels of bacteria via their ATP content according to the following general reaction:
\[
\text{ATP} + \text{luciferin} + \text{oxygen} \xrightarrow{\text{luciferase}} \text{reaction products} + \text{light}
\]

The light emitted as a result of the above reaction is measured using a liquid scintillation spectrometer or luminometer. The amount of light produced by firefly luciferase is directly proportional to the amount of ATP added (Jay, 1992).

Most foods, however, contain both free ATP and ATP associated with plant and animal cells from which that food was derived. ATP from these sources is often present in amounts greatly in excess of ATP from any contaminating bacteria that may be present (Van Crombrugge and Waes, 1991). The usefulness of the ATP determination as a means of detecting contamination in food depends on the efficiency with which the bacterial ATP can be separated from the nonbacterial ATP. In milk the principal source of ATP is somatic cells and the calcium phosphate–citrate–caseinate micelles (Van Crombrugge and Waes, 1991).

In a review article on the application of ATP bioluminescence in the food industry, Griffiths (1996) refers to various authors who agree that ATP determination can be used successfully as a rapid assay method for the microbial loads in raw milk. Reybroeck and Schram (1995), for example, outlined a test that took less than 6 min. In this test the milk is incubated in the presence of a somatic cell-lysing agent and then filtered through a bacterial cell-retaining membrane. The concentrated cells on the membrane are then lysed using a second extraction agent (specific for the extraction of bacterial ATP) and assayed by measurement of the resultant bioluminescence. Griffiths (1996) stressed that using this method, microbial populations down to \(10^4\text{cfu mL}^{-1}\) can be detected with greater precision than when using the standard plate count. Other workers have also reported that the use of the ATP-bioluminescence method is a practical and reliable screening test for assessing the hygienic quality of bovine raw milk (Bell et al., 1996; Brovko et al., 1999).

Using the bioluminescence principle, the hygienic status of tankers, plants, and equipment can be assessed in as little as 2 min (Anonymous, 1995). Two methods are available, one to check surfaces using swabs and the other to check rinse waters from CIP systems. Both tests determine not only the microbial contamination but also product residues left behind on surfaces and in closed systems. Apart from the undesirable aesthetic nature of the remaining residues, they also serve as nutrients for the growth of microorganisms. Because of the measurement of
both soiling and microbial contamination by this method, direct correlation with more traditional methods is not always possible.

In the swabbing procedure (Anonymous, 1995) an area of 100 cm² is swabbed, the swab is vortexed in a cuvette containing the ATP extraction reagents, and the luciferin–luciferase reagent is subsequently added to produce the bioluminescence. The measurement by a luminometer is recorded in relative light units (RLU). Less than 500 RLU is regarded as a “clean” value, whereas more than 500 RLU is regarded as a “contaminated” value (Anonymous, 1995).

The rinse water test (Anonymous, 1995) is a rapid miniaturized filtration system, and end results are obtained in approximately 5 min. A volume of 20 ml of the rinse water is filtered through an 8-mm-diameter filter by applying vacuum. The filter is removed aseptically and transferred to a flat-bottom cuvette. The reagents to release the microbial ATP and to catalyze the bioluminescence reaction are added, and the light output is measured in the luminometer. Two cutoff limits based on correlation with traditional plate counts have been established (Anonymous, 1995). A value of above 200 RLU indicates residual contamination, while a result above 1000 RLU should necessitate re-cleansing of the equipment.

The advantages of the bioluminescence methods are in keeping with other rapid methods in that reference or conventional methods usually give a result after the milk has been processed or the equipment to be monitored has been used. The bioluminescence method, on the other hand, gives a rapid and also reliable result that will assist in ensuring good manufacturing practice proactively.

Limitations of the bioluminescence system that should be kept in mind are that the bioluminescence assay of ATP is affected by certain interfering factors causing a reduction in measurable photons (Van Crombrugge and Waes, 1991). First, it is important to adhere to the pH optimum of 7.75. Lower or higher pH affects the reaction rate and decreases the light emission. Temperatures higher than 25°C may inactivate the luciferase, and at lower temperatures the reaction is progressively slower. Certain ionic and other compounds can interfere with the reaction, causing a decreased light output. In practice, however, the interfering factors can be minimized by dilution and be corrected for by internal standardization (Van Crombrugge and Waes, 1991).

14.8.3.2 Gram-Negative Endotoxins (Limulus Test). Gram-negative bacteria are characterized by their production of endotoxins, which consist of lipopolysaccharides (LPS) of the outer membrane of the cell envelope (Jay, 1992). The LPS is pyrogenic and responsible for
some of the symptoms that accompany infections caused by Gram-negative bacteria.

The *Limulus* amebocyte lysate (LAL) test employs a lysate protein obtained from the hemolymph or blood cells (amebocytes) of the horseshoe crab (*Limulus polyphemus*). The lysate protein is the most sensitive substance known for the detection of endotoxins (Jay, 1992). A freeze-dried extract of the *Limulus* amebocytes forms a clot or a gel when dissolved and brought into contact with the lipopolysaccharides of Gram-negative bacteria (Easter and Prentice, 1989).

The assay technique involves the use of tubes or microtiter plates (Suhren and Heeschen, 1991b) that are prepared by loading the tubes or wells with freeze-dried LAL. For quantitative determination, the milk sample is diluted with pyrogen-free water and 30μl of the milk or dilution thereof pipetted into the wells of the microtiter plates. Raw milk samples are prepared by heating to 80°C for 10 min and cooling before dilution. The inoculated microtiter plates are then incubated for 1 h at 37°C. Reading the plates involves detecting those wells that contain a gelled or clotted reaction mixture. The detection can be performed by adding a dye solution (toluidine blue) and applying moderate suction using a capillary tube. In the case of positive wells the colored gel is firm and resists suction. Negative reactions are represented by empty wells (Suhren and Heeschen, 1991b). The concentration of the LPS can be calculated by obtaining the reciprocal of the highest positive dilution.

Because this test provides an estimate of the Gram-negative bacterial content of the milk, one nanogram (ng) of LPS can be said to approximate a Gram-negative colony count of 20,000 cfu ml⁻¹ with a standard deviation ranging from 5600 to 80,000 cfu ml⁻¹ (Suhren and Heeschen, 1991b). This method has been found suitable for the rapid evaluation of the hygienic quality of milk before or after pasteurization (Terplan et al., 1975; Zaadhof and Terplan, 1981; Jaksch et al., 1982; Jay, 1992).

Commercial substrates are available that contain amino acid sequences similar to *Limulus* coagulogen (Jay, 1992). These substrates are rendered chromogenic by linkage to *p*-nitroaniline. When the endotoxin-activated enzyme attacks the chromogenic substrate, free *p*-nitroaniline is released and can be read spectrophotometrically at 405 nm. The amount of chromogenic compound liberated is proportional to the quantity of endotoxin in the sample. An automated method for endotoxin assay was devised by Tsuji et al. (1984), and the method was shown to be sensitive to as little as 30 pg of endotoxin ml⁻¹.

Advantages of the *Limulus* test are as follows:
1. The speed at which results can be obtained.

2. Bacterial lipopolysaccharide is heat-resistant and will even withstand UHT time and temperature treatment (Easter and Prentice, 1989). The application of the *Limulus* test to heated milk therefore gives an indication of the bacteriological quality of the milk prior to processing.

A “disadvantage” of the test is that the relationship between the lipopolysaccharide concentrations and “total” bacterial numbers is dependent on the composition of the microbial population. However, because this test per se is aimed at the Gram-negative component of the population, which would include important spoilage organisms such as the psychrotrophs or indicator organisms such as coliforms, this attribute can also be regarded as a positive one.

### 14.9 METHODS FOR DETERMINING THE SHELF LIFE OF MILK

An important factor today is the open dating of perishable dairy products to indicate when the products were packed or when they should be removed from the supermarket shelf (Lück and Gavron, 1990). Many dairy factories consequently need accelerated tests to determine the shelf life or keeping quality of milk. The shelf life of milk is defined as the period between manufacture or processing and that point when the milk becomes unsuitable for use by the consumer. The milk is deemed to be unsuitable due to the presence of flavor defects or change of physical appearance (Manners, 1993).

The key to predicting shelf life is not the “total” bacterial count immediately after processing, because this count does not differentiate between contaminants and these bacteria surviving pasteurization (Lück and Gavron, 1990). The shelf life is, however, dependent on the number of postpasteurization contaminants and especially those that multiply rapidly at refrigeration temperatures namely the Gram-negative psychrotrophs (and especially the pseudomonads). Numbers of such microorganisms as low as one or two bacteria per liter can frustrate any attempt to manufacture a product with an extended shelf life.

The shelf life is also related to the time–temperature history of the product. In doing shelf-life tests it is necessary to simulate the marketing conditions to which the product will actually be subjected. For this reason a storage temperature of 5–7°C is recommended (Lück and
Regardless of the test itself, the key to predicting the shelf life of milk and milk products (White, 1998) is that the method must be rapid. Reliable and meaningful results must be obtained within 72 h and ideally within 24 h. For this reason the classic Moseley count [e.g., the plate count after 5 days of storage at 7°C minus the plate count directly after processing. (Moseley, 1958)] has not proved a popular shelf-life predictive test for pasteurized milk in practice. This despite the fact that it has been shown to be a good index of postpasteurization contamination (Lück and Gavron, 1990).

**Determination of the Actual Shelf Life.** It is important to note that tests to predict shelf-life must be evaluated against the actual product shelf life. The actual shelf life can be determined by incubating the milk sample at the relevant temperature (e.g., 7°C) and testing at regular intervals until a definite off-flavor is detected by sensory evaluation (White, 1998). More objective tests for determining the shelf-life end point include the alcohol test and the clot-on-boiling tests (Jooste and Groeneveld, 1971). The alcohol test entails the rapid mixing of 2 ml of 68% ethanol (alizarin can be added to serve as indicator) with a 2-ml milk sample previously pipetted into a test tube. Any definite evidence of precipitation is regarded as positive (Jooste and Groeneveld, 1971). With the clot-on-boiling test, 5 ml of the milk sample in a test tube is placed into boiling water for 5 min. Positive results vary from a loose curd to a solid coagulum. The alcohol test was found to be highly correlated with both the clot-on-boiling \((r = 0.9488)\) and the sensory evaluation \((r = 0.9072)\) test results (Jooste and Groeneveld, 1971). The latter study also showed that at an incubation temperature of 18°C, the milk often became alcohol-positive simultaneously with the appearance of a taint in the milk. Clot-on-boiling usually occurred 1 or 2 h later. At that time most of the samples had become completely unpalatable.

**Preincubation of the Milk Sample Followed by Plating.** Because of the low initial numbers of bacteria in freshly pasteurized milk, most predictive shelf-life tests consist of preincubating the product (in its original container) at 21°C for 18 h followed by some rapid bacterial detection test (White, 1998). Such detection methods include the standard plate count, the accelerated psychrotrophic count (21°C for 25 h or 18°C for 48 h) described previously, or plating on agar plates containing crystal violet and triphenyl tetrazolium chloride (White, 1998). The American Public Health Association (White et al., 1993) recom-
mends the 21°C/25h count method using plate count agar or 21°C for 48h using the Petrifilm aerobic count method following preincubation (PI) of the sample at 21°C for 18h. The relationship between these PI counts and sensory tests (White et al., 1993) gave rise to the following shelf-life estimates:

<table>
<thead>
<tr>
<th>PI cfu count ml⁻¹</th>
<th>Estimated Shelf Life (days)</th>
</tr>
</thead>
<tbody>
<tr>
<td>&lt;1,000</td>
<td>&gt;14</td>
</tr>
<tr>
<td>1,000–200,000</td>
<td>9–14</td>
</tr>
<tr>
<td>&gt;200,000</td>
<td>&lt;10</td>
</tr>
</tbody>
</table>

Preincubation of Milk Sample Followed by DEFT. The DEFT microscopic count referred to earlier can also be used as a rapid test to predict shelf life after PI at 21°C for 18h. Alternatively milk with added benzalkonium chloride and crystal violet (for inhibiting Gram-positive bacteria) can be pre-incubated at 30°C for 18h (White et al., 1993) before subjecting the milk to the DEFT microscopic count.

Impedance Method. The use of impedance detection time (DT) with milk samples has been shown to be superior to a traditional keeping quality method such as the Moseley test (Marshall, 1993). Results correlated better with actual keeping quality ($r = 0.94$ vs. 0.75 for the Moseley test) and are available within 48h. In this test, 5ml of milk sample is added to 5ml of sterile broth medium, and the mixture is subsequently incubated for 18h at 18°C. Modified plate count agar (White et al., 1993) is added and impedance testing is done at 21°C for up to 48h.

According to White (1998), there is no single ideal test for predicting the shelf life of fluid milk products. They suggest that processors should carefully select one or two tests that best fit their overall quality assurance program. The key points regarding shelf-life prediction (White, 1998) include the following:

1. Know the actual shelf life of the product as measured at 7°C.
2. Select the test to predict shelf-life that best fits the total program.
3. Routinely do the tests and develop a history categorizing the results.
4. Define a course of action in case product failure is projected by these tests.
14.10 STERILITY TESTS

The aim of the quality control of ultra-high-temperature (UHT)-treated milk products that have been aseptically processed and packed is to limit the number of containers spoiled by microbial growth to a level acceptable to the market (Lück and Gavron, 1990). The term “sterility test” in this context implies stability rather than sterility in the microbiological sense. Even though a small number of bacteria may have survived the heat treatment, the product is regarded as commercially sterile as long as they do not grow during a commercially acceptable period. Commercial sterility therefore means the absence of all pathogenic or toxigenic microorganisms and the absence of any microorganisms that are capable of multiplication under the conditions of storage and distribution (Lück and Gavron, 1990).

Spore-forming bacteria are usually the cause of microbiological spoilage in UHT-treated milk products. The spore-forming anaerobes, principally of the genus *Clostridium*, are less thermoresistant and are therefore of less concern than the spores of mesophilic and thermophilic aerobes of the genus *Bacillus* (Lück and Gavron, 1990).

UHT treatment reduces the number of spores by $8–10\log_{10}$ cycles. This means that after treatment, milk with an original spore content of 100 spores ml$^{-1}$ ($10^3$ spores liter$^{-1}$) will contain 0.001 to 0.00001 spores per liter pack, and thus the number of nonsterile packs will be approximately 1 in 1000 to 1 in 100,000. This level is acceptable (Lück and Gavron, 1990).

To detect unsterile packages, the plate count method per se will not be practically feasible with survivors at a level of less than one per milliliter or as low as one per liter directly after the heating and aseptic packaging. For this reason, incubation of the packaged product at different incubation times and temperatures have been suggested. A 2-week incubation period as suggested by the International Dairy Federation (IDF, 1969) is commercially not practicable (Lück and Gavron, 1990). Tests carried out on UHT milk revealed that 18% of unsterile packs were detected after 3 days at 30°C, 41% after 5 days, and 68% after 7 days (Lück et al., 1978). The results indicated that incubation times of 3 and 5 days were not sufficiently long, and an incubation period of 7 days was consequently deemed advisable (Lück and Gavron, 1990).

With regard to incubation temperature, a thermophilic incubation temperature of 55°C is not necessary because most of the thermophilic spores causing spoilage at 55°C remain dormant at ambient storage temperatures. Only one incubation temperature is consequently
METHODS FOR DETECTING PATHOGENIC MICROORGANISMS AND THEIR TOXINS

needed (Lück and Gavron, 1990), namely in the region of 35–37°C. Facultative thermophiles may be able to grow at 35°C, but one should bear in mind that damaged (stressed) bacteria recover and grow more readily at 27°C than at 35°C.

On termination of the incubation period the UHT product is adjudged defective or not. Defectiveness or spoilage is recorded when the sensory properties are different from those normally obtained by prolonged incubation, when the titratable acidity differs from what it was before incubation by more than 0.02% (expressed as grams of lactic acid per 100 g of milk), or when the colony count exceeds 10 per 0.1 ml of milk. Normal inspection should consist of 50–100 samples per machine per production run per day (IDF 1969; IDF 1981; Lück and Gavron, 1990).

Other tests that have been recommended for testing the sterility of UHT treated milks include the impedance method on milk preincubated at 25–35°C (time not specified; White et al., 1993) and the bioluminescence method (Anonymous, 1995). In the latter method a dairy products sterility kit is used. A representative sample (0.1–0.3% of total production run) is taken. The unopened packs are incubated at 28–30°C for 48 h. The milk is then tested for bioluminescence as recommended in the sterility kit instructions (Anonymous, 1995). Sterile samples have an RLU reading of <2 × RLU background, while unsterile samples have a RLU reading of >2 × RLU background.

The possibilities of ultrasound imaging as a nondestructive quality control method was tested among others on UHT milk packages (Mattila et al., 1989). The results showed that spoilage could be detected in all the milk products tested within 2–3 days. The ultrasound could penetrate all the common plastic packaging materials. Cardboard packaging material, however, suppressed penetration. Lower frequencies were used, but this decreased the sensitivity.

14.11 METHODS FOR DETECTING PATHOGENIC MICROORGANISMS AND THEIR TOXINS

Classic methods of detecting and enumerating microorganisms depend on the recognition of microbial colonies, often after growth on selective agar (Waites, 1997). Such methods are time-consuming and labor-intensive and require well-trained staff able not only to carry out aseptic work but also to recognize different morphological types of microorganisms. Because most food-borne pathogenic microorganisms generally occur in low numbers, selective enrichment is also usually
required in order to allow detection and prevent overgrowth by other organisms. In addition, some cells, particularly from food processing environments, may be damaged and a pre-enrichment step becomes necessary to assist cells to recover before being exposed to selective agents (Waites, 1997).

Although standard methods based on culturing the organisms will be referred to in this section, the primary aim will be to review a range of more rapid techniques for detecting pathogenic microorganisms and their toxins. The latter methods will include a range of more recent molecular techniques (Table 14.7), immunological methods (Table 14.8), methods for dealing with stressed or damaged cells, and then currently

**TABLE 14.7. Molecular Methods for Detecting Microbial Pathogens**

<table>
<thead>
<tr>
<th>Method</th>
<th>Principle and Application</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>DNA/RNA probes</td>
<td>A probe is a nucleic acid sequence typical of the organism of interest, used to detect homologous DNA or RNA sequences in the target organism. RNA as target sequence has an advantage in having $10^4$ copies per cell versus DNA which has only one or two copies per cell. Nucleic acid fragments for testing are prepared using restriction endonucleases.</td>
<td>Wallbanks (1989)</td>
</tr>
<tr>
<td></td>
<td>--------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------</td>
<td>Jay (1992)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Waites (1997)</td>
</tr>
<tr>
<td>Polymerase chain reaction (PCR)</td>
<td>An amplification technique to increase low numbers of DNA molecules to detectable levels ($10^6$ copies of the target sequence). PCR can increase one molecule of DNA to produce $10^7$ identical copies. Advantages: speed (4 h), sensitivity (1 cell) and high specificity.</td>
<td>Waites (1997)</td>
</tr>
<tr>
<td>Amplified fragment length polymorphism (AFLP)</td>
<td>A method for the genomic typing of microorganisms based on DNA sequence polymorphism. The method is reproducible and highly discriminatory and is used for identification and classification of bacteria, fungi and yeasts. It is an advancement on related techniques such as PFGE, BRENDA, and RAPD.</td>
<td>Aarts (1999)</td>
</tr>
</tbody>
</table>
**TABLE 14.8. Immunological Methods for the Detection of Microbial Pathogens and Toxins**

<table>
<thead>
<tr>
<th>Method</th>
<th>Principle and Application</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fluorescent antibody (FA)</td>
<td>Fluorescent antibody reagent reacts with test antigen. Resultant antigen–antibody complex emits fluorescence and is detected using a fluorescence microscope. AOAC-approved method for detecting <em>Salmonella</em> yields results within 52h.</td>
<td>Jay (1992)</td>
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<td></td>
<td></td>
<td>Flowers et al. (1993)</td>
</tr>
<tr>
<td>Latex agglutination test</td>
<td>Simplest of immunoassays. Reagent consists of latex particles coated with antibodies. Specific antigens in test sample combine with antibodies. Latex particles remain in suspension compared to negative test in which latex particles form a sediment.</td>
<td>Notermans (1989)</td>
</tr>
<tr>
<td>Enzyme immunoassays (EIA)</td>
<td>Example of a so-called non-competitive EIA is the well-known ELISA (enzyme-linked immunosorbent assay). Immobilized antibodies react with specific antigens in test sample. Adsorbed antigen is measured using enzyme-labeled antibodies added to reaction mix (antigen must have two binding sites). The ELISA technique is used to detect and quantitate microorganisms or their metabolic products.</td>
<td>Notermans (1989)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Jay (1992)</td>
</tr>
<tr>
<td>Immunodiffusion</td>
<td>Gel diffusion methods are widely used for detecting and quantifying bacterial toxins and enterotoxins, for example, <em>Staphylococcus aureus</em> enterotoxin and <em>Clostridium botulinum</em> toxins. Most widely used test is the Crowle modification of the Ouchterlony slide.</td>
<td>Casman and Bennett (1965) Duncan and Somers (1972) Bennett and McClure (1976) Jay (1992)</td>
</tr>
</tbody>
</table>
TABLE 14.8. Continued

<table>
<thead>
<tr>
<th>Method</th>
<th>Principle and Application</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Radioimmunoassay (RIA)</td>
<td>A radioactive label is attached to the antigen. The labeled antigen is allowed to react with specific antibody in the test sample. The amount of antigen–antibody complex is quantified using a scintillation counter. The RIA is used to examine foods for biohazards such as mycotoxins, endo- and enterotoxins.</td>
<td>Miller et al. (1978)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Bergdoll and Reiser (1980)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Jay (1992)</td>
</tr>
<tr>
<td>Immunomagnetic separation (IMS)</td>
<td>The Dynal system uses Dynabeads®, which are superparamagnetic polystyrene beads coated with specific antibodies. The antibodies combine with the target organism in the test sample, and the bead–bacterial complexes are separated using a magnetic particle concentrator.</td>
<td>Wachsmuth et al. (1990)</td>
</tr>
<tr>
<td></td>
<td>Incorporation of the IMS step greatly reduces the isolation time for the target organism. IMS can be used for isolation of Salmonella, Listeria, and E. coli 0157.</td>
<td>Safarik et al. (1995)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Waites (1997)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Kaclíková et al. (1999)</td>
</tr>
</tbody>
</table>

recognized techniques for detecting specific pathogenic and toxigenic organisms (or their toxins) in milk and milk products.

14.11.1 Dealing with Stressed Food-Borne Pathogens

Legislation requires food manufacturers to demonstrate that certain foods they produce are free from contamination by pathogens. For many pathogens, this is interpreted to mean absence of a single cell in a 25-g sample of food. It is also interpreted to imply a single cell that is possibly in an injured or stressed state. The importance of these specifications is reinforced by the epidemiological data obtained from many well-documented outbreaks of food poisoning linked to very low levels of contamination, in some cases less than 10 organisms per portion consumed (Stephens, 1999).
When microorganisms are subjected to environmental stresses such as sublethal heat and freezing, many of the individual cells undergo metabolic injury resulting in their inability to form colonies on selective media that uninjured cells can tolerate. Whether organisms have suffered metabolic injury can be determined by plating aliquots of the sample separately on a nonselective and a selective medium and enumerating the colonies that develop after suitable incubation. The colonies that develop on the nonselective medium represent both injured and uninjured cells, while only the uninjured cells develop on the selective medium (Jay, 1992). The difference between the number of colonies on the two media is a measure of the number of the injured cells in the original sample.

Injury of food-borne microorganisms has been shown by a large number of investigations to be induced not only by sublethal heat and freezing but also by freeze-drying, drying, irradiation, aerosolization, antibiotics, and sanitizing compounds (Jay, 1992). The protection of cells from heat and freeze injury is favored by complex media or specific components thereof. Milk, for example, provides more protection than saline or mixtures of amino acids (Moats et al., 1971; Jay, 1992) and milk components that are most influential appear to be phosphate, lactose, and casein.

Metabolically injured microorganisms have increased nutritional requirements. Foods inoculated with salmonellae were frozen, and the fate of the organisms during freezer storage was studied (Jay, 1992). More organisms could be recovered on highly nutritive nonselective media than on selective media such as MacConkey, deoxycholate, or violet red bile agars. In another example, dry-injured Staphylococcus aureus cells failed to recover on the nonselective recovery medium (tryptone soya agar) but did recover when pyruvate was added to the medium (Jay, 1992). Pyruvate is well established as an injury repair agent, not only for injured S. aureus cells but for other organisms such as E. coli. Higher counts were obtained on media containing this compound when the organisms were injured by a variety of agents (Jay, 1992). Catalase is another compound that increases recovery of injured aerobic organisms. Various investigations (Jay, 1992) have shown that sublethally injured S. aureus, Pseudomonas fluorescens, Salmonella typhimurium, and E. coli effectively recovered in the presence of this enzyme. More information on cell injury and methods of recovery (Jay, 1992) can be obtained in the book of Andrew and Russell (1984).
14.11.2 Specific Pathogenic, Indicator or Toxigenic Microorganisms

14.11.2.1 Enterobacteriaceae, Coliforms, and Escherichia coli

*Enterobacteriaceae*. The presence of any member of the *Enterobacteriaceae* family is undesirable in pasteurized dairy products. Methods for detecting *Enterobacteriaceae* are much less specific than those developed for detecting coliforms. The methods, however, do provide increased sensitivity for detecting postpasteurization contamination, and results are available in 12h using the impedance method or in 24h using the modified MacConkey glucose agar plating method (Christen et al., 1993).

*Coliform Bacteria*. The coliform group of bacteria comprises all aerobic and facultatively anaerobic, Gram-negative, non-spore-forming rods able to ferment lactose with the production of acid and gas at 32°C or 35°C within 48h (Christen et al., 1993). One source of these organisms is the intestinal tract of warm-blooded animals, although certain bacteria of nonfecal origin are also members of this group. Typically, this group is represented by the genera *Escherichia*, *Enterobacter*, and *Klebsiella*, but a few lactose-fermenting species of other genera are also included in the coliform group. In proportion to the numbers present, the existence of any of these species in dairy products is suggestive of unsanitary conditions or practices during production, processing, or storage (Christen et al., 1993).

Reference methods for enumerating these organisms in milk and milk products are referred to in Table 14.6 and include a colony count at 30°C, a dry rehydratable film method, a MPN method, and a pectin gel method. Similar standard methods are described by Christen et al. (1993).

*Escherichia coli*. *Escherichia coli* is currently the best-known fecal indicator, and its recovery from dairy products suggests that other organisms of fecal origin, including pathogens, may be present. Reference methods for detecting and enumerating this organism are summarized in Table 14.6. Other standard methods, including hydrophobic grid membrane filtration, an impedance method, and a fluorogenic assay (MUG test), are described by Christen et al. (1993).

*Pathogenic E. coli*. Most *E. coli* strains are harmless commensals common to the intestinal tract of humans and animals. Some strains have, however, been found to be pathogenic. Final determination of the
enteropathogenicity of any strain of *E. coli* is based on that strain's ability to "elicit a specific, consistently positive response in two or more standardized model pathogenicity systems." These model systems demonstrate invasiveness, production of a heat-labile toxin, and production of a heat-stable toxin. These methods are fully described in the American FDA's *Bacteriological Analytical Manual* and in the AOAC's OMA (Flowers et al., 1993).

Based on distinct virulence properties, different interactions with the intestinal mucosa, distinct clinical symptoms, differences in epidemiology, and variations in O (somatic) and H (flagellar) antigens, more than 60 distinct strains causing different forms of diarrhea in humans have been identified (Ryser, 1998). These strains are grouped into the following five categories: classic enteropathogenic *E. coli* (EPEC), enterotoxigenic *E. coli* (ETEC), enteroinvasive *E. coli* (EIEC), enterohaemorrhagic *E. coli* (EHEC), and, most recently, enteroadherent *E. coli* (EAEC) (Ryser, 1998).

Selective recovery of *E. coli* 0157:H7 (EHEC) from food samples is based on the inability of typical isolates to ferment sorbitol and hydrolyze 4-methyl-umbelliferone glucuronide (MUG) to a fluorogenic product. Several selective enrichment broths containing novobiocin or other antibiotics can also be used to enhance recovery. Presumptive *E. coli* 0157:H7 must be serologically confirmed with antisera or with a serotype specific DNA probe. Verotoxin production can be confirmed using traditional cell culture techniques or the newly developed DNA probe and PCR assays for VT-1 (Ryser, 1998).

### 14.11.2.2 Salmonella.

The gastroenteric form of nontyphoid salmonellosis was not clearly linked to raw milk consumption until the mid-1940s. Interest in milk-borne salmonellosis has peaked twice since the 1940s, first in 1966 when several large outbreaks were traced to nonfat milkpowder and again in 1985 when one of the largest recorded outbreaks of food-borne salmonellosis involving more than 180,000 cases was traced to consumption of a particular brand of pasteurized milk in the Chicago area (Ryser, 1998). Today *Salmonella* and *Campylobacter* are generally recognized as the two leading causes of dairy-related illness in the United States and Western Europe, with rates of infection being particularly high in regions where raw milk is neither pasteurized or boiled.

Examination of milk and milk products according to the International Dairy Federation standard method (IDF, 1995a) begins with pre-enrichment in a buffered peptone water broth to resuscitate injured
cells. Following 16–20 h incubation at 37°C, two different media—Rappaport–Vassiliadis and selenite–cystine broths—are inoculated from the pre-enrichment broth and incubated at 42°C and 37°C, respectively, for a total of 48 h. Immunomagnetic concentration of salmonellae present in the enrichment broth can remove the need for enrichment (Waites, 1997) and decrease detection time by 2 days. Streaking out and recognition can be done on two selective media—for example, Brilliant Green/Phenol Red Agar (IDF, 1995b) and any other suitable solid selective medium (e.g., Hektoen Enteric; Xylose Lysine Desoxycholate or Bismuth Sulfite Agar; Ryser, 1998). The plates are then incubated at 35°C for 24–48 h. Biochemical or serological confirmation of isolated colonies can be done according to procedures described by Flowers et al. (1993) or IDF (1995b).

Alternatively, several rapid methods using fluorescent antibodies, immunodiffusion, enzyme immunoassays, DNA hybridization, hydrophobic grid membrane filtration, or impedance determination may be applied (Flowers et al., 1993). All positive findings must be confirmed by culturing. Commercial test kits available are described by Ryser (1998).

Standard methods for detecting Salmonella in cheese powder and dried milk are referred to in Table 14.6.

14.11.2.3 Campylobacter. Campylobacter jejuni has been recognized since 1909 as an important cause of abortion in cattle and sheep. Improved isolation strategies have also implicated this organism as a causative agent of human diarrhea. Altogether, 45 food-borne campylobacteriosis outbreaks (1308 cases) were reported in the United States between 1978 and 1986, over half of which involved ingestion of raw milk (Ryser, 1998). Similar reports linking raw or inadequately pasteurized milk to 13 outbreaks in Great Britain from 1978 to 1980 (Ryser, 1998) helped to further substantiate C. jejuni and also C. coli (Duim et al., 1999) as important milk-borne pathogens that have come to rival or even surpass Salmonella as an etiological agent of human gastroenteritis worldwide (Ryser, 1998).

Campylobacter is likely to be greatly outnumbered by the normal bacterial flora of milk. Consequently, selective enrichment under microaerobic conditions is a crucial initial step in procedures for recovering Campylobacter from raw milk (Ryser, 1998). The latter author refers to three methods that are currently recommended for detecting Campylobacter in raw milk, namely, Flowers et al. (1993), Hunt and Abeyta (1995), and Stern et al. (1992). These methods are complicated and require initial centrifugation of the raw milk sample and selective
enrichment of the pellet at 42°C under microaerobic conditions (5% O₂, 10% CO₂, 85% N₂). Subsequent plating of the enrichment medium is then done on two selective media followed by similar incubation. Additional steps are required in the FDA procedure (Hunt and Abeyta, 1995).

Phenotypic methods (Duim et al., 1999) have the disadvantage that a large proportion of C. jejuni and C. coli strains are nontypable by such methods. The latter authors reported that amplified fragment length polymorphism (AFLP) fingerprinting resulted in satisfactory and highly discriminatory identification of the campylobacters. They concluded that AFLP is suitable for epidemiological studies on Campylobacter. Mouwen and Prieto (1999) also reported that Fourier-transformed infrared (FT-IR) spectroscopy was found to be a simple, rapid, and accurate procedure for identifying, typing, and grouping Campylobacter species and subspecies. A disadvantage of the latter method is the cost of the equipment and the fact that reproducibility of the method needs to be improved.

14.11.2.4 Listeria. Listeria monocytogenes has only recently emerged as a serious food-borne pathogen that can cause abortion in pregnant women and meningitis, encephalitis, and septicaemia in newborn infants and immunocompromised adults. The outcome of listeric infection can be particularly devastating, with a mortality rate of 20–30% (Ryser, 1998). Three major dairy-related outbreaks resulting in more than 100 deaths prompted the United States to institute a policy of “zero tolerance” for L. monocytogenes in all cooked and ready-to-eat foods including dairy products (Ryser, 1998). Recalls issued for contaminated dairy products, principally ice cream and cheese, in the United States in 1994 and 1995 suggests that Listeria contamination within dairy processing plants has not yet been fully controlled (Ryser, 1998).

Reference methods for determining L. monocytogenes in dairy products (IDF, 1995c; Hitchins, 1995) both commence with a selective liquid medium containing acriflavin, nalidixic acid, and cycloheximide as selective agents. The inoculated selective medium is incubated at 30°C for up to 48h. If the immunomagnetic separation (IMS) technique is used, the selective medium is replaced by half-Fraser broth and incubated for only 24h at 30°C. This saves 24h on the selective medium step. Oxford medium as main medium and PALCAM as additional medium is recommended in the above reference procedures as isolation media.
Species identification of presumptive *Listeria* isolates is based on morphological, physiological, and biochemical reactions that can take up to 7 days to complete (IDF, 1995c; Hitchins, 1995).

The time required for phenotypic confirmation can be shortened using commercially available test kits (e.g., API 20 S, API-ZYM, API Listeria, Micro ID; Ryser, 1998). Alternatively, several DNA hybridization (Accuprobe, Gene Trak) and ELISA assays such as VIDAS can be used to screen IMS concentrates or enrichment broths for *Listeria* spp. (Ryser, 1998; see also Table 14.6). Molecular methods studied by Harvey et al. (1999) included multilocus enzyme electrophoresis (MEE), pulsed field gel electrophoresis (PFGE), repetitive element sequence-based PCR (REPPCR), and plasmid profiling. They found PFGE to be the most discriminatory typing method, followed by MEE and lastly REPPCR. MEE and PFGE were largely in agreement and tended to group the same strains together. The sensitivity of MEE could be improved (Harvey et al., 1999) by increasing the number of enzymes examined. While plasmid profiling was not useful for typing *L. monocytogenes* from a wide range of sources, the presence and stability of plasmid DNA in certain strains could serve as a useful marker in ecological studies. REPPCR proved to be a rapid and reliable method, but was less discriminatory than MEE or PFGE (Harvey et al., 1999).

### 14.11.2.5 *Staphylococcus aureus.*

Staphylococcal food poisoning is a classic food-borne intoxication resulting from the ingestion of a pre-formed, heat-stable toxin (termed enterotoxin) and produced by the bacterium *Staphylococcus aureus*. Dairy products are well-known vehicles of staphylococcal poisoning, with cheese and raw milk linked to outbreaks before the turn of the century (Ryser, 1998). By the late 1930s, staphylococcal poisoning emerged as a major milk-borne illness accounting for 26%, 50%, and 30% of all milk-borne diseases reported in the United States during the 1940s, 1950s, and 1960s, respectively (Ryser, 1998). These cases of staphylococcal poisoning involved various dairy products including raw milk, pasteurized milk, cheese, ice cream, butter, and nonfat dry milk. Improvements in milk pasteurization and dairy sanitation standards in the United States, England, and most other industrialized countries have made dairy-related outbreaks rare (Ryser, 1998).

The significance of finding *S. aureus* in foods suspected of causing staphylococcal poisoning should be interpreted with caution. Although foods must contain at least $10^6$ enterotoxigenic *S. aureus* cfug$^{-1}$ to induce illness, small numbers of *S. aureus* present in thermally
processed foods may represent the survivors of very large populations. Consequently, actual or potential staphylococcal poisoning can only be verified by isolating enterotoxigenic staphylococci from the food or demonstrating the presence of enterotoxin in the food (Ryser, 1998).

The enumeration of coagulase-positive staphylococci in milk and milk-based products using the colony count technique is described in an IDF standard (IDF, 1997a). The principle of this technique is the inoculation of serial dilutions of product using a solidified culture medium, namely, Baird–Parker agar medium or rabbit plasma fibrinogen (RPF) agar medium. The use of the latter medium is recommended when the Baird–Parker medium is not selective enough. After incubation for 24–48 h, coagulase-positive staphylococci on RPF agar form gray or black colonies surrounded by an opaque or cloudy zone indicating coagulase activity. Confirmation of colonies is done by means of a coagulase test (IDF, 1997a). In products where staphylococci are expected to be stressed and in low numbers—as, for example, in dried milk products—the IDF recommends a MPN technique (IDF, 1997b). A 9-tube battery of Giolitti and Cantoni broth is inoculated with three consecutive dilutions of the dried milk sample. After incubation at 37°C, tubes are subcultured to Baird–Parker agar. Presumptively positive colonies are subjected to the coagulase test.

An international standard (IDF, 1998a) specifies a method for the detection of heat-stable DNase (thermonuclease) produced by coagulase-positive staphylococci in samples of milk or milk-based products. The enzyme may be used as an indicator of staphylococcal growth to hazardous levels and the potential presence of staphylococcal enterotoxins.

The enzyme is extracted by means of a procedure involving acidification, centrifugation, treatment of the supernatant with trichloroacetic acid, redissolving the resultant precipitate, heating the solution, and testing for thermonuclease activity in toluidine blue O-DNA agar. A positive thermonuclease test indicates that coagulase-positive staphylococci have grown to levels of 10⁶ or more cfu g⁻¹. This may imply toxic levels of enterotoxin, and testing for the presence of enterotoxin should be conducted.

Staphylococcal enterotoxin detection techniques include a microslide gel double diffusion immunoassay and a radioimmunoassay technique described by Flowers et al. (1993). The latter technique is rapid and highly sensitive, whereas the former involves a long and complicated extraction and concentration procedure.

An ELISA method, namely the TECRA staphylococcal enterotoxin visual immunoassay (SETVIA), has been designed for rapid detection
of staphylococcal enterotoxins in foods. The SETVIA was awarded AOAC first action approval in 1993. More recently the food extraction protocols have been simplified and sample preparation protocols have been improved. Benson et al. (1999) undertook a study to validate these changes and found the SETVIA to be a highly sensitive and specific test for staphylococcal enterotoxins. It gave results within approximately 4h including 30min for the extraction. The microtiter plate assay may be read visually with the aid of the color comparison card provided. DNA hybridization assays and latex agglutination tests are also available for identifying enterotoxins in culture fluids (Ryser, 1998).

14.11.2.6 Bacillus cereus. Bacillus cereus is an aerobic spore-forming bacterium that is involved in food intoxication as a result of the production of two enterotoxins. The first is a diarrheal enterotoxin, and the second is an emetic enterotoxin (Ryser, 1998). Bacillus cereus is a common contaminant in the dairy environment and the raw milk supply and has also been involved in spoilage phenomena such as “sweet curdling” and “bitty cream” in milk. The ability of B. cereus to persist in powdered milk and to grow in the reconstituted product, as evidenced by a large outbreak in Chile involving newborn infants (Ryser, 1998), has led to the establishment of rigid international standards for B. cereus in infant formulae (Becker et al., 1994). Most outbreaks of B. cereus poisoning have been traced to foods containing at least 10⁶cfu g⁻¹. Small numbers of surviving spores can, however, germinate and grow when milk or baby formulae are reconstituted and attain dangerous levels during storage (Ryser, 1998).

A standard MPN technique is described for the enumeration of B. cereus in dried-milk-based infant food and dried milk products intended for use as ingredients of milk-based baby or infant foods and dietary foods (IDF, 1998b). The three- by three-tube MPN method employs tryptone soya polymyxin broth. After inoculation and incubation the tubes are subcultured onto polymyxin pyruvate egg yolk mannitol bromothymol blue agar (PEMBA) or mannitol egg yolk polymyxin agar (MEPA). Identification of colonies is confirmed by biochemical and morphological tests, and a MPN count g⁻¹ is read off from the statistical tables.

To confirm that the suspect isolate is toxigenic, the strain should be tested for the diarrheal and emetic enterotoxin. A serologically based microslide gel double diffusion assay has been developed for the diarrheal enterotoxin, with several fluorescence-based immunoblot and reverse passive latex agglutination assays also available commercially
Production of the emetic (mitochondrio) toxin can be detected by means of a recently developed sensitive bioassay known as the boar spermatozoa toxicity test (Andersson et al., 1998).

**14.11.2.7 Aflatoxin.** Although mycotoxin production is not limited to aflatoxigenic molds (Ryser, 1998), only aflatoxin will be dealt with in this section. The aflatoxins are highly potent carcinogens produced by certain strains of *Aspergillus flavus, A parasiticus,* and *A. niger* and is the primary mycotoxin of public health concern (Ryser, 1998). Four major forms of aflatoxin are currently recognized, namely, AFB₁, AFB₂, AFG₁, and AFG₂. AFB₁ is the most potent and is most often found in moldy peanuts and animal feeds containing corn (maize) and other grains. When cattle ingest contaminated feed, AFB₁ is metabolized to AFM₁ (M₁), some of which is shed in the milk (Ryser, 1998). Most countries today have legislation in place specifying the maximum levels of AFM₁ that are acceptable in fluid milk and dairy products.

A provisional standard drafted by the IDF (IDF, 1995d) specifies a method for the determination of at least 0.008 μg liter⁻¹ in milk and 0.08 μg kg⁻¹ in whole milk powder. The method is also applicable to fluid low-fat and skim milk and low-fat and skim milk powder.

The principle of the method involves extraction of AFM₁ by passing the sample through an immunoaffinity column. The column contains specific antibodies immobilized on a solid support material. These antibodies bind any AFM₁ passing through the column. After washing the column to remove other sample components, AFM₁ is eluted from the column and the eluate collected. The amount of AFM₁ present is determined by high-performance liquid chromatography.

**14.12 MICROBIOLOGICAL STANDARDS FOR DIFFERENT DAIRY PRODUCTS**

Limitations of poor-quality products and health protection form the basis for food standards. Legal and voluntary bacteriological standards vary widely from country to country. Lück and Gavron (1990) proposed certain limits that may be a useful tool to improve the quality of dairy products (Table 14.9). A product complies with the bacteriological specifications when at least four out of five portions or samples contain less than the maximum bacterial count specified. Legal action should only be taken when more than one out of the five samples exceeds the upper limit.
### TABLE 14.9. Suggested Microbiological Standards for Different Dairy Products

<table>
<thead>
<tr>
<th>Product</th>
<th>Test</th>
<th>Count or Results&lt;sup&gt;a&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>Raw milk intended for further processing</td>
<td>Total bacterial count</td>
<td>&lt;100,000 (30,000) cfu ml&lt;sup&gt;-1&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>Total coliform count</td>
<td>&lt;500 (100) cfu ml&lt;sup&gt;-1&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td><em>Escherichia coli</em> (fecal type)</td>
<td>&lt;1 cfu ml&lt;sup&gt;-1&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>Thermotolerant count</td>
<td>&lt;1000 cfu ml&lt;sup&gt;-1&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>Spore-formers</td>
<td>&lt;10 cfu ml&lt;sup&gt;-1&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td><em>Bacillus cereus</em> (spores)</td>
<td>&lt;1 cfu ml&lt;sup&gt;-1&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td><em>Staphylococcus aureus</em> (coagulase-positive)</td>
<td>100 (10) cfu ml&lt;sup&gt;-1&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>Methylene blue reduction time (at 36°C)</td>
<td>Not less than 6 h</td>
</tr>
<tr>
<td></td>
<td>3-h Resazurin test (at 36°C Lovibond disc No. 4/9)</td>
<td>Not less than 4 h</td>
</tr>
<tr>
<td></td>
<td>Somatic cell count</td>
<td>&lt;750,000 (500,000) ml&lt;sup&gt;-1&lt;/sup&gt;</td>
</tr>
<tr>
<td>Raw milk or raw cream for direct consumption</td>
<td>Total bacterial count</td>
<td>&lt;30,000 (10,000) cfu ml&lt;sup&gt;-1&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>Total coliform count</td>
<td>&lt;30 (10) cfu ml&lt;sup&gt;-1&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td><em>E. coli</em> (fecal type)</td>
<td>1 in 10 cfu ml&lt;sup&gt;-1&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>Methylene blue reduction time (at 36°C)</td>
<td>Not less than 7 h</td>
</tr>
<tr>
<td></td>
<td>3-h Resazurin test (at 36°C Lovibond disc No. 4/9)</td>
<td>Not less than 4 h</td>
</tr>
<tr>
<td></td>
<td><em>S. aureus</em> (coagulase-positive)</td>
<td>&lt;10 (1) cfu ml&lt;sup&gt;-1&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>Somatic cell count</td>
<td>&lt;500,000 (250,000) ml&lt;sup&gt;-1&lt;/sup&gt;</td>
</tr>
<tr>
<td>Pasteurized market milk or pasteurized cream (after processing)</td>
<td>Total bacterial count</td>
<td>&lt;30,000 (5000) cfu ml&lt;sup&gt;-1&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>Total coliforms</td>
<td>&lt;1 (0.1) cfu ml&lt;sup&gt;-1&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td><em>E. coli</em> (fecal type)</td>
<td>Absent in 1 ml</td>
</tr>
<tr>
<td>Dried milk products (including casein and whey powder, etc.)</td>
<td>Total bacterial count</td>
<td>&lt;10,000 (1000) cfu g&lt;sup&gt;-1&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>Total coliforms</td>
<td>&lt;10 (1) cfu g&lt;sup&gt;-1&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td><em>E. coli</em> (fecal type)</td>
<td>Absent in 25 g</td>
</tr>
<tr>
<td></td>
<td><em>S. aureus</em> (coagulase-positive)</td>
<td>Absent in 25 g</td>
</tr>
<tr>
<td></td>
<td>Yeasts and molds</td>
<td>&lt;10 cfug&lt;sup&gt;-1&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>Salmonellae (and other pathogens if required)</td>
<td>Absent in 200 (500) cfug&lt;sup&gt;-1&lt;/sup&gt;</td>
</tr>
<tr>
<td>Ice cream</td>
<td>Total bacterial count</td>
<td>&lt;50,000 (5000) cfug&lt;sup&gt;-1&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>Total coliforms</td>
<td>&lt;10 cfug&lt;sup&gt;-1&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td><em>E. coli</em> (fecal type)</td>
<td>Absent in 1 g</td>
</tr>
<tr>
<td></td>
<td><em>S. aureus</em> (coagulase-positive)</td>
<td>Absent in 1 g</td>
</tr>
<tr>
<td></td>
<td>Yeasts and molds</td>
<td>&lt;10 cfug&lt;sup&gt;-1&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>Salmonellae (and other pathogens if required)</td>
<td>Absent in 25 (100) g</td>
</tr>
<tr>
<td>Cultured milk products (including yogurt, cottage cheese, cultured milk, sour cream, etc.)</td>
<td>Contaminating organisms (non-lactic acid bacteria)</td>
<td>&lt;1000 cfug&lt;sup&gt;-1&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>Total coliforms</td>
<td>&lt;10 (1) cfug&lt;sup&gt;-1&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td><em>E. coli</em> (fecal type)</td>
<td>Absent in 1 g</td>
</tr>
<tr>
<td></td>
<td><em>S. aureus</em> (coagulase-positive)</td>
<td>Absent in 1 g</td>
</tr>
<tr>
<td></td>
<td>Yeasts and molds</td>
<td>&lt;10 (1) cfug&lt;sup&gt;-1&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>Salmonellae (and other pathogens if required)</td>
<td>Absent in 15 (100) g</td>
</tr>
</tbody>
</table>


**TABLE 14.9. Continued**

<table>
<thead>
<tr>
<th>Product</th>
<th>Test</th>
<th>Count or Results*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sweetened condensed milk</td>
<td>Total bacterial count</td>
<td>&lt;100 cfu g⁻¹</td>
</tr>
<tr>
<td></td>
<td>Spore-formers</td>
<td>Absent in 1 g</td>
</tr>
<tr>
<td></td>
<td>Total coliforms</td>
<td>&lt;1 cfu g⁻¹</td>
</tr>
<tr>
<td></td>
<td>Yeasts and molds</td>
<td>&lt;1 cfu g⁻¹</td>
</tr>
<tr>
<td>Butter</td>
<td>Contaminating organisms (non-lactic acid bacteria)</td>
<td>&lt;10,000 (5000) cfu g⁻¹</td>
</tr>
<tr>
<td></td>
<td>Total bacterial count (noncultured butter only)</td>
<td>&lt;50,000 cfu g⁻¹</td>
</tr>
<tr>
<td></td>
<td>Total coliforms</td>
<td>&lt;10 (1) cfu g⁻¹</td>
</tr>
<tr>
<td></td>
<td><em>E. coli</em> (fecal type)</td>
<td>Absent in 1 g</td>
</tr>
<tr>
<td></td>
<td><em>S. aureus</em> (coagulase-positive)</td>
<td>Absent in 1 g</td>
</tr>
<tr>
<td></td>
<td>Yeasts and molds</td>
<td>&lt;10 cfu g⁻¹</td>
</tr>
<tr>
<td></td>
<td>Proteolytic organisms</td>
<td>&lt;100 cfu g⁻¹</td>
</tr>
<tr>
<td></td>
<td>Lipolytic organisms</td>
<td>&lt;50 cfu g⁻¹</td>
</tr>
<tr>
<td>Cheese (ripened— standards at 1 month)</td>
<td>Total coliforms</td>
<td>&lt;10 cfu g⁻¹</td>
</tr>
<tr>
<td></td>
<td><em>E. coli</em> (fecal type)</td>
<td>Absent in 1 g</td>
</tr>
<tr>
<td></td>
<td>Spore-formers</td>
<td>&lt;10 (1) cfu g⁻¹</td>
</tr>
<tr>
<td></td>
<td>Fecal streptococci</td>
<td>&lt;100 cfu g⁻¹</td>
</tr>
<tr>
<td></td>
<td><em>S. aureus</em></td>
<td>Absent in 1 g</td>
</tr>
<tr>
<td></td>
<td>Yeasts and molds</td>
<td>&lt;10 (1) cfu g⁻¹</td>
</tr>
<tr>
<td></td>
<td>Other pathogens (if required)</td>
<td>Absent in 25 (100) g</td>
</tr>
</tbody>
</table>

*Figures in parentheses mean standards that should be strived for.

Source: Adapted from Lück and Gavron (1990).

During recent years, the variation of counts in different samples has been taken into consideration, and the hygiene requirements are, for instance, often expressed as follows: Examine five (n) samples of dried milk, and allow two (c) samples to exceed 50,000 bacteria g⁻¹ (m), but none to exceed 200,000 g⁻¹ (M) or abbreviated: n = 5, c = 2, m = 50,000, M = 200,000.

### 14.13 RELEVANCE OF TECHNIQUES AND INTERPRETATION OF RESULTS

No single laboratory test performed on a food product can produce the full information that is required. No bacteriological test yet evolved is above criticism. There is no “best” test, and yet often much money is wasted on elaborate tests for which fictitious accuracy are claimed. It is now recognized that regular testing is of far greater importance and that any test that is better than mere haphazard classification will con-
tribute to an improvement in quality. The real value of a test is whether it can detect products of unsatisfactory quality, and in this way make a contribution to improving production hygiene. It is, therefore, not necessary that a test should give an absolute measure of the quality, nor need it be in complete agreement with the results of other tests (Lück and Gavron, 1990).

Because none of the different methods employed to determine a specified bacterial content yield exactly the same result, one test cannot be automatically substituted for another. Two different tests can be compared statistically in order to arrive at comparable standards, but not necessarily to determine their value. The only conditions of a suitable test are as follows:

1. There must be a significant correlation between the results of the test and the quality required.
2. The operator must be fully informed on how to obtain this quality.

Due to the error inherent in a single bacterial count—for example, limitations of the particular bacterial count/test, seasonal and local variation of the microflora, day-to-day variation of the bacterial count, and counting only colony-forming units or clumps instead of individual bacteria—only approximately fivefold differences in plate counts can be regarded as significant when grading dairy products. Hence the quality categories have to be established in such a way that the differences between bacterial counts are large enough to be significant. The realization of this fact led to the development of rapid screening procedures to meet the requirements of the dairy industry.

Quality control is planned and introduced by management, but diagrams indicating real or anticipated quality levels should, however, concern not only the management but also the operators. One operator should be responsible for a specific machine or a specific process, and the relevant diagrams can take the form of control charts showing, on the horizontal axis, the sequence of sample or the date of manufacture and, on the vertical axis, the quality characteristics (shelf life, log bacterial count, etc.). A line can represent, for instance, the expected average log bacterial count of a product; above this line the upper limit line is drawn, such that only 1 in 20 of the plotted points should lie outside this line (Lück and Gavron, 1990).

For shelf-life tests, a lower limit line is drawn. The number of points outside the limit lines indicates whether the process has been altered
in some way; and they are, therefore, essentially indicators of a need for corrective action.

It is often necessary to summarize results or to calculate mean counts. When there is a small variation in the bacterial counts of different samples, or when the counts are low, the arithmetic mean of the bacterial counts may be calculated. When there are, however, large variations between counts, and the counts are high, the geometric mean (logarithmic average) should be used (geometric mean = arithmetic mean of the log count, which is subsequently transformed into a bacterial count again). When the plate count method supplies positive results, more emphasis should be attached to this method than to the most probable number test, because the repeatability of the plate count method is better than that of the MPN values (Lück and Gavron, 1990).

REFERENCES


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