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REVIEW PAPER

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Methodologies for the characterization of microbes in industrial environments: a review

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Abstract There is growing interest in research and development to develop novel tools to study, detect, and characterize microbes and their communities in industrial environments. However, knowledge about their validity in practical industrial use is still scarce. This review describes the advantages and limitations of traditional and molecular methods used for biofilm and/or planktonic cell studies, especially those performed with *Listeria monocytogenes*, *Bacillus cereus*, and/or *Clostridium perfringens*. In addition, the review addresses the importance of isolating the microorganisms from the industrial environment and the possibilities and future prospects for exploiting the described methods in the industrial environment.

Keywords Biofilm · Culture · Molecular techniques · Fingerprinting

Introduction

Microorganisms inhabiting the food and processing industries are mostly benign, but some can be harmful to the processing and safety of the product. Therefore, the control of harmful microorganisms is essential. Industrial processes that deal with any biological material provide nutrients and conditions for microorganisms to grow, either in the shelter of sessile biofilms on surfaces or as planktonic cells in the circulating process waters. Moreover, in most natural and industrial systems where the supply of nutrients is sufficient, microorganisms grow as spatially organized, matrix-enclosed, multispecies communities in biofilms. Besides a solid surface, the microbes need only water to initiate biofilm formation [72, 73, 270]. Microbial biofilms and biofouling of surfaces and interfaces within the industrial environment are major problems. In industry, the first step is identifying the problem of biofilms and biofouling in a particular process or site. Subsequently, it is important to determine the best possible methods for detection of biofilms in situ, so that they can be characterized and possibly further studied in the laboratory. Finally, this information can be used to define strategies for controlling biofilm formation in that specific environment [208].

Microorganisms in food and in industrial environments are distributed unevenly; and there is a great variation in the cell density and composition of microbial population over space and time. Typically, the microbial cells are located in the surfaces of the food matrix and process equipment; and the cell density and species distribution may vary in different parts of a food product [77, 156, 158]. Changes in the ecosystem cause continuous qualitative and quantitative variation in the composition of the microbial community over time [152]. Both intrinsic (e.g. chemical composition, natural microbiota) and extrinsic factors (e.g. processing, storage conditions) affect microbial growth [272] and consequently the composition of the microbial community. All these factors affect the actual sampling of the industrial environment, making it a demanding task to perform.

Published data on microbial detection and characterization from industrial environments is abundant and therefore the following review is restricted to the most relevant and widely applied techniques and to just a few specific bacterial species important in the food and process industrial environments, namely *Listeria monocytogenes*, *Bacillus cereus*, and *Clostridium perfringens*. Both traditional and molecular identification and characterization methods for bacteria and their communities are discussed, in addition to typing methods for bacterial isolates obtained from the industrial environment and/or foodstuff. Future prospects for the exploitation of the

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described methods in the industrial environment and industrial samples are also addressed.

Hygiene and safety problems in the industrial environment

Problems caused by bacterial biofilms

Biofilms protects microbes from hostile environments and act as a trap for nutrient acquisition [270]. In multispecies biofilms, mixed-species microcolonies are formed by a part of the sessile population when cells of metabolically cooperative species are juxtaposed and are thus in a position to benefit from interspecies substrate-exchange and/or mutual end-product removal. This level of structural organization and metabolic specialization explains the remarkable metabolic efficiency of microbial biofilms and their universal and inherent resistance to antimicrobial agents [72]. In practice, a biofilm on improperly cleaned surfaces is a barrier between microbes and disinfectants, antibiotics, or biocides [54, 228, 298, 458]. Biofilm components can also protect microbes from the effects of steam: e.g. the bacterial slime of *Bacillus* sp. improves the heat resistance of the bacterium, extending the autoclaving time required for efficient sterilization to several hours [270]. Besides causing problems in cleaning and hygiene [184], biofilms can cause energy losses and blockages in condenser tubes, cooling fill materials, water and wastewater circuits, and heat exchangers [64]. Biofilms can occasionally cause health risks by releasing pathogens into drinking-water distribution systems [44, 453]. In food processing water-supply systems, biofilms cause problems in granular activated carbon columns, reverse osmosis membranes, ion exchange systems, degasifiers, water storage tanks, and microporous membrane filters [132, 287]. Commonly found microbes in the food industry and on food contact surfaces are enterobacteria, lactic acid bacteria, micrococci, streptococci, pseudomonads, and bacilli [458]. The formation of resistant spores that can contaminate process equipment and food products is a special concern for the food processing industry and for the consumer [17].

The level of hygiene in the paper and board industry is important, since the end-products are often in contact with foodstuffs. The microbial isolates from the paper and board industry are mainly bacilli, enterobacteria, pseudomonads, or actinomycetes, but moulds, yeasts, anaerobic sulfate-reducing bacteria, and clostridia may also be detected [168, 333, 392]. The growth of *B. cereus*, clostridia, coliforms, and staphylococci in the paper-making process is detrimental to product hygiene [323, 379]. Aerobic and anaerobic spore-forming bacteria, such as bacilli and clostridia, which are not killed during the drying stage of paper-making, are the most important microbes from the safety point of view [194, 324, 341, 407]. Slime build-up in paper-processing machines, caused by microbial biofilms, may cause significant economic losses, mainly due to machinery-running problems in addition to spots, holes, and quality problems in the end-product. The machinery slime can also contain polymers of microbial origin, fibers, and inorganic precipitates. Common bacteria detected and identified from paper-processing machinery slimes include enterobacteria, bacilli, pseudomonads, and *Clavibacter* spp. The total number of microbes in the slime can reach 10^{12} colony-forming units (cfu)/ml. Pathogens, such as *B. cereus*, can also be found in these machinery slimes. Anaerobic bacteria, such as sulfate-reducing bacteria, can be involved in the initiation and progress of corrosion [36, 168, 408]. Also, heat-stable microbial metabolites, mainly enzymes and toxins, can cause problems if migration occurs from a packaging material into a foodstuff. Volatile metabolites, such as the fatty acids produced by many *Clostridium* spp. and the hydrogen sulfide produced by sulfate-reducing bacteria, can cause organoleptic problems in end-products [107, 168, 341].

Prevention of hygiene and safety problems

The elimination of biofilms is a very difficult and demanding task, because many factors affect the detachment, such as temperature, time, mechanical forces, and chemical forces [453]. Harmful microorganisms may enter the manufacturing process and reach the end-product in several ways, e.g. through raw materials, air in the manufacturing area, chemicals employed, process surfaces, or factory personnel [193, 323].

The target of microbial control in a process line is two-fold: to reduce or limit the number of microbes and their activity and to prevent and control the formation of deposits on process equipment. The present most efficient means for limiting the growth of microbes are good production hygiene, a rational running of the process line, and a well designed use of biocides and disinfectants. Novel means to control slime formation are constantly sought, e.g. through the control of environmental factors on the process line and the use of surface-active agents, (bio)-dispersants, enzymes, and new biocidal chemicals, in addition to non- or minimally toxic chemicals [36, 108, 215]. The cleanliness of surfaces, the training of personnel and good manufacturing and design practices are important in combating hygiene problems in the food industry [179]. Disinfection after the removal of biofilms, using suitable cleaning procedures, is also required in food plants where wet surfaces provide favorable conditions for microbial growth [116, 286].

In the food industry, equipment design and choice of surface materials are important in fighting microbial biofilm formation [179, 454]. The most practical material in processing equipment is steel, which can be treated with mechanical grinding, brushing, lapping, and electrolytic or mechanical polishing. Dead ends, corners, cracks, crevices, gaskets, valves, and joints are

vulnerable points for biofilm accumulation [67, 115, 322]. Poorly designed sampling valves can destroy an entire process or give rise to incorrect information, due to biofilm formation at measuring points. Valves are vulnerable to microbial growth and thus constitute a hygiene risk [67]. Also, hoses, tubes, filters, etc. containing polyvinylchloride increase the risk of contamination, due to this material: it is more easily contaminated and it deteriorates more easily than steel [331]. Problems with the accumulation of particulates and cells occur whenever cleaning is inappropriate for any reason [278]. Inadequate cleaning and sanitation of surfaces coated with biofilms presents a source of contamination within the process [458].

Achieving a clean food plant must be the aim of the plant managers, who have to invest the necessary time and money to accomplish it. Monitoring methods and cleaning procedures, including the program, cleaning agents, disinfectants, and cleaning equipment, must be carefully planned [454]. Cleaning in the process industry should be based on systematic planning. The knowledge that microbes grow differently on surfaces, compared with suspensions, is the first step in developing advanced regimes in process hygiene [178]. Biofilm formation in industrial systems reflects a disturbance in the process [270]. Biofilms are less likely to accumulate in well designed systems, which are effectively cleaned. Results indicate that low-pressure cleaning in itself is not effective enough to remove biofilms unless the cleaning agent is effective [457]. The efficiency of cleaning agents is assessed by their ability to remove biofilms from process surfaces together with their ability to kill the bacteria present in the biofilm [457]. The cleaning effect in open systems can be enhanced using double-foaming or through scrubbing. In closed processes, the removal of biofilms from surfaces can be performed using efficient flow conditions in combination with effective cleaning agents [457]. Strong oxidizing and/or disinfective agents are used to combat microbial deposits on equipment surfaces in problem areas. Satisfactory elimination of biofilms using only disinfectant treatment cannot be achieved, even if the agent is very effective against freely suspended cells [286]. Sources, problems, and control of microbial contaminants in industrial processes are presented in Fig. 1.

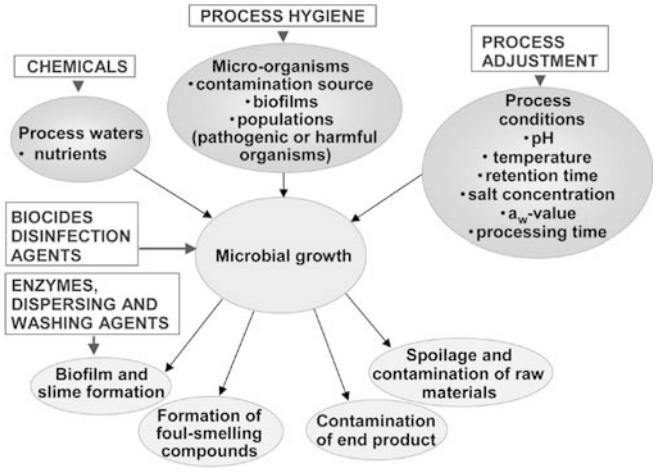

Fig. 1 Sources, problems, and control of microbial contaminants in industrial processes

Monitoring hygiene and safety problems

Monitoring practices based on sampling of the liquid phase do not reflect the location or extent of microbes growing in biofilms on surfaces [69]. The methods used for monitoring process hygiene are often based on conventional cultivation, using various types of agar plates or adenosine triphosphate (ATP) measurement. Conventional cultivation requires several days before the result can be obtained and it enumerates cells able to form colonies on the given agar [457]. The measurement of ATP is an often used method for detecting biological growth [4, 147], e.g. for the measurement of total hygiene [457]. The detection limit of ATP measurement for bacteria is 10^3–10^4 cfu/ml [39].

The detection of deposit build-up on equipment surfaces at an early stage enables effective countermeasures and thus results in an improvement in the process hygiene. Successful on-line monitoring of microbiological deterioration in the process industry has great beneficial impact, of both economic and environmental value. On-line monitoring saves both expense and the environment when gentle cleaning methods can be used and unnecessary procedures avoided.

A reliable identification of industrial microbial isolates is often difficult to obtain. Over the past decade, many improvements have been seen in both conventional and modern methods for the detection and identification of microorganisms from the industrial environment. Phenotypic analyses (e.g. the fatty acid methyl ester test, or sodium dodecyl sulfate–polyacrylamide gel electrophoresis) have traditionally played an important role in microbial identification and classification. Genotypic analyses (e.g. partial 16S rDNA sequencing, ribotyping) have proved very useful and accurate in the identification and classification of industrial microbial isolates, since the physiological properties of the industrial microbes may be different from those of the reference strains. Industrial strains are usually well adapted to their specific environments and do not often possess the typical characteristics of any species hitherto described. Thus, the effective use of molecular methods requires the development of extensive identification libraries. Furthermore, in many cases, the results of phenotypic and genotypic tests are not in good agreement, which further hampers identification [333, 413].

L. monocytogenes, *B. cereus*, and *C. perfringens* in the industrial environment

L. monocytogenes, *B. cereus*, and *C. perfringens* are important pathogens in industrial environments,

especially due to their ability to endure adverse/harmful process conditions.

L. monocytogenes is a significant food-borne pathogen and may cause epidemic and sporadic outbreaks. The infective dose of *L. monocytogenes* is related both to the level of contamination of the food product and to the host susceptibility. *L. monocytogenes* is able to grow across a wide range of temperatures (including very low temperatures) and pHs; and it is extremely salt-tolerant. Due to this good tolerance to environmental stress-factors, *L. monocytogenes* is difficult to remove from the factory environment once it has become a part of the house microbiota. The typical food vehicles for *L. monocytogenes* are dairy, meat, and fish products [for reviews, see 119, 251].

B. cereus is widely distributed in nature (soil contains 10^5–10^6 spores/g) and is extremely tolerant to different environmental stresses. *B. cereus* is a non-competitive bacterium. However, food processes can select for it, since pasteurization is insufficient to kill the spores and many of the strains are psychrotrophic [18]. Spores of *B. cereus* are very hydrophobic [196] and adhere tightly to surfaces. Vegetative cells, especially cells in the late stationary growth phase, are also hydrophobic [318]. *B. cereus* strains may produce emetics and/or enterotoxins, which leads to food poisoning when a toxin-producing strain is present at levels $>10^5$ cells/g [200, 417]. But strains causing food poisoning at lower cell levels (10^3–10^4 cells/g) have also been found [18].

C. perfringens is mainly restricted to meat products, since this bacterium is unable to synthesize several amino acids. *C. perfringens* spores survive insufficient heating of the food product, vegetative cells reproduce at temperatures between 10 °C and 47 °C, and the generation time in optimal growth conditions is short [18]. Wild strains of *C. perfringens* are mainly enterotoxin-negative. However, enterotoxin-positive *C. perfringens* strains may cause food poisoning, especially through cooked food [283]. Aerotolerant vegetative cells survive for some time under aerobic conditions, but do not multiply.

Characteristics of *L. monocytogenes*, *B. cereus*, and *C. perfringens* are presented in Table 1.

Isolation of microorganisms from the industrial environment

Sample collection and processing

Due to the spatial and temporal heterogeneity and technical problems related to sampling in the industrial environment, obtaining a representative sample from certain foods and food-related industry is a demanding task. Microbes are often tightly attached to surfaces and the process equipment may contain parts that are hard to access, such as dead-ends or bends in pipework. Different methods, such as swabbing, rinsing, agar-flooding, and contact agar methods, have been employed for sampling in the industrial environment [141, 333, 352, 456, 457]. The conditions during sample transportation have a great impact on sample quality; and the time between sampling and processing should be limited to the minimum.

Table 1 Typical characteristics of *Listeria monocytogenes*, *Bacillus cereus*, and *Clostridium perfringens*. *cfu* Colony-forming units, *ND* not detected

Characteristic	*L. monocytogenes*	*B. cereus*	*C. perfringens*
Minimum water activity (a_w; for growth)	0.92	0.94	0.93
Growth temperature	−1.5 °C to 50 °C	4–55 °C	12–50 °C
pH (for growth)	pH 4.1–9.4	pH 4.3–9.3	pH 5.5–9.0
Heat resistance of spores (at 100 °C)	–	3–8 min	0.3–13.0 min
Number of bacteria per gram in food reported in infective cases	<10–10^4 cfu/g (high risk groups) 10^5–10^9 cfu/g (normal population)	10^5–10^7 cfu/g (diarrheal type) 10^5–10^8 cfu/g (emetic type)	10^6–10^7 cfu/g
Incubation period	18–20 h (diarrheal type), in other types even 2 months	8–16 h (diarrheal type)0.5–5.0 h (emetic type)	8–24 h
Symptoms	Diarrhea, fever (healthy adults)Sepsis, meningitis, fecal infection (adult high risk groups)Meningitis, sepsis, pneumonia, fecal infections (new-borns) Fever, preterm delivery, stillbirth (during pregnancy)	Abdominal pain, nausea, vomiting (emetic type) Abdominal pain, diarrhea, nausea (diarrheal type)	Abdominal pain, nausea, acute diarrhea
Food vehicles	Ready-to-eat foodstuffs with long shelf-life (fish, meat products, soft cheeses) and vacuum-packed products	Meat products, soups, vegetables, pudding, milk, and milk products (diarrheal type) Rice, pasta and noodles (emetic type)	Incompletely cooked or slowly cooled food products, meat, poultry, shellfish, fish, and dairy products
Occurrence in food biofilms	Yes (dairy, fish, meat)	ND (dairy)	ND
Occurrence in environmental site biofilms	Yes (plentiful)	Yes (non-food)	ND

Recovery of microbial cells from the sample matrix is a critical step and may lead to biases in the qualitative and quantitative estimation of the microbial community [for a review, see 131]. In most cases, samples need to be macerated and homogenized to liberate the microbial cells from the sample matrix. The cells in natural samples, e.g. naturally contaminated food, can be tightly attached to the matrix [62] and may need vigorous processing. Maceration of a food matrix may change the chemical environment of the sample and release substances that are toxic or inhibitory to some microbes. Dilution is also a potential bias-causing step and needs standardization [131]. Several methods have been developed to concentrate the sample, either to decrease the detection limit of the target microbes or to overcome the inhibitory effect of the matrix on the detection method, e.g. polymerase chain reaction (PCR). Selective or non-selective enrichment cultures are widely used for amplifying populations of food pathogens before detection by cultivation or molecular techniques [62]. However, the enrichment step is not always optimal for the recovery of the target species and may lead to false-negative results, especially when a too-selective enrichment medium is used for a sample containing injured cells [131]. Moreover, enrichment precludes attempts to quantitate results and, due to differences in growth rate between different populations, enrichment may lead to a bias in the recovery of different species [105]. Immunomagnetic separation (IMS) is a technique in which magnetic particles coated with specific antibodies are used to capture the target cells [312]. IMS has been used to concentrate food pathogens from sample homogenates or enrichment cultures, followed by detection with cultivation, immunological, or molecular methods [62, 102, 188, 312]. In addition, centrifugation and filtration techniques are commonly used in concentrating cells from certain types of sample.

Microbial cells are exposed to several environmental stresses during food processing and storage, which may change the physiological status of the cells. In addition to culturable and metabolically active cells and autolysing dead cells, microbial cells in many other physiological states can be found in samples [222]. In several food matrices, the dominant cells are those in a stationary growth phase, which are still metabolically active [131]. Adverse conditions, such as nutrient depletion and low temperature, can lead to viable but non-culturable cells (VBNC), which do not produce colonies on media that normally support their growth. However, VBNC cells remain metabolically active and infective [222, 273]. Two different types of cells contribute to the silent but active majority: (1) known species for which the applied cultivation conditions are just not suitable or which have entered a non-culturable state and (2) unknown species that have never been cultured before, due to a lack of suitable methods [16]. Sub-lethally injured cells, which do not grow on selective media but grow on non-selective media, may be present in processed samples and processed foods [152, 202]. In addition, cells in certain microbial groups have the ability to form spores, which are extremely resistant dormant states [222]. VBNC and injured cells may resuscitate or recover under appropriate conditions [58, 391]. The recovery of injured cells is highly dependent on the chemical composition of the enrichment medium and on the degree of the injury and the presence of accompanying microbes [244, 374, 391]. Besides culture, VBNC and injured cells can be more easily detected by fluorescent staining or molecular techniques [for reviews, see 152, 223, 274], which do not rely on the viability of the target cells.

The sample-processing method is dependent on the properties of the target microbial groups and on the method used in the subsequent detection step. If detection is performed by culture-based methods, the target strains must retain viability and culturability during sample processing, whereas the inhibitory components of the sample matrix play an important role in PCR-based detection. The sample matrix studied plays an important role when deciding which method to use for microbial detection and identification.

Cultivation

An effective cultivation procedure for the detection of food pathogens should suppress competitive microorganisms to the extent that the diagnostic system allows easy and reliable detection of the target genera/species. Several selective culture-based techniques are available for the detection and enumeration of *L. monocytogenes*, *B. cereus*, and *C. perfringens* in environmental and food samples; and the international standard methods for the detection and enumeration of these food pathogens are based on cultivation [77, 417]. The cultivation and subsequent identification of isolates using conventional techniques are time-consuming. It may take more than one week to obtain the complete results [77, 338]. During the past decade, much effort has been put into the development of more rapid culture techniques, many of which are based on the use of fluorogenic and/or chromogenic culture media.

Microbial community analysis by cultivation

An ideal method for studying microbial communities would detect and enumerate all microbial species present in the samples with equal efficiency. It was speculated that many microbial communities are too diverse to be counted exhaustively, which led to the application of statistical approaches for the estimation of diversity [192]. In food microbiology, especially in food hygiene surveys, cultivation has usually been aimed at the detection of selected groups/species of microorganisms, rather than the assessment of the complexity and dynamics of the microbial community. Culture-independent, DNA-based methods have also had limited applications in the investigation of microbial

communities in foods and food-related industries; and the diversity of industrial microbial populations is therefore poorly known [152].

Microbial community analysis by cultivation is extremely laborious, especially when complex samples with high diversity are studied. When cultivation is used for microbial community analysis, several non-selective and selective culture media should be included, in addition to different growth conditions (different temperatures, atmospheres), followed by accurate identification of a large number of isolates from each medium, to get an overview of the diversity of the microbial population in the sample. The dominant cultivable population is recovered from non-selective media, whereas selective media allow the detection of groups or species that are present at lower numbers. Traditional methods for the identification of isolates are based on the assessment of several phenotypic features, which is often inaccurate and may lead to underestimation of the species diversity. It is generally known that conventional cultivation methods recover less than 1% of the total species of microbes present in environmental samples [16, 150, 440], partly due to the poor ability of the routinely used culture media and growth conditions to recover a large fraction of the microbial population [315]. In industrial environments and in food matrices, the processing parameters are likely to select for certain types of microbes; and the composition of the dominant microbial groups or species may be predicted more easily than in complex natural ecosystems. The development of culture media and conditions on the basis of the chemical and physical parameters of the environment investigated could enhance community analysis by cultivation. Combining an optimal cultivation technique with accurate identification of isolates with molecular microbiological methods would yield useful information on the diversity and the culturability of the microbes present in food and industrial environments. Knowledge of the function of the microorganisms in the ecosystem is also of utmost importance [315], which necessitates assessment of the properties of isolates.

Detection of *L. monocytogenes* by cultivation

In most countries, there is a requirement for the absence of *L. monocytogenes* in most food products. However, since several countries have established quantitative guidelines for *L. monocytogenes* in certain types of foods, such as raw meat and some ready-to-eat products, convenient enumeration methods are also needed. Several standard methods are available for the detection of listeria in foods [77, 171, 172, 438]. The detection of *L. monocytogenes* in food and environmental samples by cultivation typically includes enrichment step(s) for resuscitation of injured cells and concentration of the cells, followed by plating on selective media and confirmation of the tentative identifications of suspected colonies by biochemical tests [for a review, see 77]. Current conventional culture techniques take approximately one week to complete [77, 338]. The recent methodology development has focused on the optimization of enrichment steps and the development of new differential culture media to obtain faster and more reliable detection of *L. monocytogenes* in food and environmental samples [338].

An ideal enrichment medium facilitates the recovery of injured cells and the enrichment of *L. monocytogenes* over the competing microbiota [177]. The selective agents in commonly used *Listeria* spp.-selective agars are lithium chloride (LiCl), polymyxin B or colistin, acriflavine, and cephalosporins [for a review, see 77]. Most conventional selective enrichment broths rely on nalidixic acid and acriflavine as selective agents; and cycloheximide and LiCl have also been used in the enrichment step [77, 139, 416, 439]. In a more selective enrichment broth, L-PALCAMY, nalidixic acid is substituted by ceftazidime and polymyxin [416]. The selective enrichment step used in conventional procedures can be inadequate in facilitating the recovery of injured cells. The delayed recovery of injured cells in selective media [202, 435] and the inhibitory effect of selective agents, e.g. LiCl on some *L. monocytogenes* strains [74, 202], have been reported. Optimization of the composition of enrichment media and the use of a two-stage enrichment procedure, where selective agents are added after non-selective pre-enrichment, facilitate the recovery of injured cells [202, 338, 391]. Enrichment broths may include an indicator system, e.g. aesculin–ferric iron, which can be used for presumptive indication of the presence of *Listeria* spp. in the sample. Most agars have an aesculin–ferric iron indicator system and, additionally, a second indicator system based on mannitol fermentation can be added to the media [77]. Besides *L. monocytogenes*, all other *Listeria* spp. and some interfering microbes produce aesculinase, which complicates the use of aesculin hydrolysis as a differential characteristic [177].

Highly selective enrichment media are useful for the detection of *Listeria* spp. from samples that are heavily contaminated with interfering organisms [77]. The detection of *L. monocytogenes* after enrichment is complicated by the fact that other faster-growing species of *Listeria*, such as *L. innocua*, may overgrow during enrichment, which may lead to an underestimation of the presence of *L. monocytogenes* [40, 257, 319]. In food samples, non-pathogenic *Listeria* spp. typically outnumber *L. monocytogenes*; and it is probable that *L. monocytogenes* may not be detected on media that do not allow differentiation by colony appearance [218]. A selective agar medium containing sheep blood was developed to differentiate *L. monocytogenes* from non-pathogenic *Listeria* spp. on the basis of hemolysis [40, 75, 214, 319]. Pathogenic and non-pathogenic *Listeria* spp. can be distinguished on the basis of phosphatidylinositol-specific phospholipase C (PIPLC) activity [305]. Chromogenic culture media based on PIPLC activity were recently developed and applied for the detection of

L. monocytogenes in food and environmental samples [177, 218, 338].

The success of enumerating *L. monocytogenes* by direct plating is dependent on having a sufficient number of *L. monocytogenes* cells in the samples, compared to the number of interfering organisms [158]. For the direct enumeration of *Listeria* spp. from food and environmental samples, spread-plates on selective agar media [90, 143, 156, 172, 249] or most probable number (MPN) counts on standard enrichment media [118, 171] can be used. In addition, a hydrophobic grid membrane filter, a technique that allows fast presumptive enumeration of *L. monocytogenes* from environmental and food samples, has been developed [113].

Restaino et al. [338] described a *L. monocytogenes*-selective detection system (LMDS) containing optimized steps of pre-enrichment, enrichment, and selective differential plating. LMDS allows the specific detection of *L. monocytogenes* in food and environmental samples [177, 338]. Presumptive results for the presence of pathogenic *Listeria* spp. in the sample can be obtained by the detection of fluorescence in the enrichment broth (containing a fluorogenic substrate based on PIPLC activity) within 1 day; and the complete detection and identification takes just 4–5 days [177, 338]. Restaino et al. [338] reported higher specificity and sensitivity for isolation of *L. monocytogenes* from naturally contaminated sites by LMDS than by a USDA standard method. The ability of different conventional standard procedures to detect *L. monocytogenes* has proved comparable [439]. However, the recovery of *L. monocytogenes* can be enhanced by using several parallel procedures in the analysis of samples [77, 171].

Detection of *B. cereus* by cultivation

Several selective and non-selective culture media have been developed for the detection of *B. cereus* from foods [for a review, see 417]. The enumeration of *B. cereus* in food and industrial samples is commonly based on a plate-counting culture technique, except for samples with low cell numbers (< 10 cfu/g) or dehydrated starchy foods, for which the MPN method is preferred. Direct-plating on a non-selective medium such as blood agar is suitable for the detection of *B. cereus* in samples with a high number of target cells, e.g. in foods implicated in outbreaks, whereas selective media are needed for the enumeration of *B. cereus* from food and industrial samples, which typically contain higher numbers of interfering organisms. The selective agents employed in *B. cereus* media are polymyxin B or colistin, LiCl, and actidione. Color indicators, such as phenol red, bromocresol purple, or bromothymol blue, are added to help in the assessment of colony appearance [417]. Lecithinase production, negative mannitol fermentation, and nitrate reduction are the key properties used in the identification of *B. cereus* in standard procedures [325]. However, lecithinase-negative isolates or isolates showing weak lecithinase activity or other aberrant biotypes, such as negative nitrate reductase, have been detected from food and industrial samples and may lead to false-negative results [182, 325, 417]. Since the selective agents in various *B. cereus* selective isolation media are similar, the performance of different media in the enumeration of *B. cereus* from food samples is comparable [182, 200, 417]. A selective and differential chromogenic medium based on PIPLC activity has been developed for the enumeration of *B. cereus* and *B. thuringiensis* [260].

Detection of *C. perfringens* by cultivation

Detection and enumeration of *C. perfringens* from food and environmental samples is usually based on cultivation on agar plates, but a MPN technique can also be applied. Several selective culture media for the detection and enumeration of *C. perfringens* are based on sulfite reduction as a differential characteristic and cycloserine as a selective agent [21, 161, 276]. Selectivity of cultivation can be increased by using an elevated incubation temperature, since *C. perfringens* is able to grow rapidly at 45 °C [2, 355]. The shortcoming of the selective culture and growth conditions is the poor recovery of injured vegetative cells of *C. perfringens* [161, 183, 355]. Fluorogenic and chromogenic substrates have been applied to culture media to enable more reliable presumptive identification of *C. perfringens* [21, 260, 355]. Routine methods described for confirming the identification of *C. perfringens* isolates are based on testing the gas production from lactose and sulfite reduction on lactose–sulfite medium, or alternatively testing motility and nitrate reduction on motility–nitrate medium in combination with lactose fermentation and gelatin liquefaction on lactose–gelatin medium [110]. In a study by Eisgruber et al. [110], the lactose–sulfite approach enabled the identification of less than 50% of pure cultures of *C. perfringens*; and motility–nitrate combined with lactose–gelatin procedures also failed to identify some *C. perfringens* strains. The reverse adenosine 3′,5′-cyclic phosphoric acid test and acid phosphatase reaction proved to be easy to perform and confirmation reaction tests were reliable [110]. Also, Adcock and Saint [5] reported acid phosphatase in combination with beta-galactosidase activity as a reliable and extremely fast test for the confirmation of *C. perfringens*.

Fluorescence-based, non-specific detection of microorganisms

Fluorescence microscopy is widely used in microbial ecology. There are several advantages in its use. It is fast and rather easy to use, it allows the visualization of spatial distribution of cells in the sample and, with a suitable combination of fluorescent stains, differentiation between viable and dead cells is possible. However, direct identification of microbes is not possible with

conventional fluorescent stains. Distinguishing cells on the basis of morphology is therefore important, because fluorochromes are not specific for bacterial species or genera [223].

Microscopy

There are five major attributes of fluorescence as a tool in microscopy: specificity, sensitivity, spectroscopy, temporal resolution, and spatial resolution [398]. Important phenomena in fluorescence microscopy are fading, photo-bleaching of the fluorochrome, and fluorescence-quenching, the loss of fluorescence due to the interaction of the fluorochromes with other molecules in the environment. The use of antifading agents can at least partly solve fading problems [398, 449]. Epifluorescence microscopy [327] and confocal scanning laser microscopy (CSLM) [50] are used for studying specimens that fluoresce. The excitation spectrum in epifluorescence microscopy is the product of the emission spectrum of the light source, the bandpass of the filter, and the reflectance spectrum of the dichroic mirror [449]. Excitation filters are band-pass filters chosen to pass light at the absorption spectrum of fluorochrome, while blocking the longer wavelength light of the fluorescence spectrum. In contrast, emission filters are chosen to pass the light in the emission spectrum, while blocking the light of the excitation spectrum [398]. In epifluorescence microscopy, multilayered samples, e.g. biofilms, can only be analyzed two-dimensionally [459]. In CSLM, a specimen is scanned with a focused laser beam and fluorescent signals are detected by a photomultiplier. A confocal pinhole allows only the signals arising from a focused plane to be detected. CSLM allows detailed, non-destructive examination of thick microbial samples, e.g. biofilms. Thus, the impact of various biocides (for example) can be studied at different optical sections in more detail than with epifluorescence microscope [24, 60, 72, 88, 234; for a review, see 235]. In addition, determination of the three-dimensional relationship of cells and three-dimensional computer reconstruction from optical thin sections becomes possible [60, 234].

The analysis of fluorescence from samples under the microscope can be evaluated either manually or with computer-aided image analysis programs. Manual evaluation is usually chosen when evaluating whether microbes are present in the sample or not. When more information is needed, image analysis is normally used. Image analysis includes image acquisition, processing, and segmentation, object recognition and measurement, and data output [60]. The image analysis systems allow rapid quantification of many parameters, which could hitherto only be described qualitatively, e.g. fluorescence intensity, quantification of different sizes of microorganisms, and percentage of area covered by biofilm [45, 221, 284, 450]. However, the analysis of particles with different brightness is still problematic and thresholds have to be established for deciding what is a bacterium and what is background [45].

Flow cytometry

Flow cytometry (FCM) combines the advantages of microscopy and biochemical analysis for the measurement of the physical and chemical characteristics of individual cells as they move in a fluid stream past optical or electronic sensors [76, 98, 99, 100]. Emitted light is detected by photodetectors and data are analyzed by computer-aided means. FCM permits simultaneous measurement of multiple cellular parameters, both structural and functional, such as cell size and DNA content [211, 402, 433], and allows rapid characterization of individual cells (more than 10^3 cells/s) in homogeneous and heterogeneous populations [52, 99, 211, 402, 433]. FCM has a higher throughput and can more readily be automated than microscopic quantification of microbial populations [433]. Furthermore, FCM requires only small sample sizes [29].

Fluorescent stains

Fluorochromes are stains or probes that are added to cells to obtain a fluorescent signal [398]. Detection of labeled molecules depends on both the intensity of fluorescence and the ability to resolve specific fluorescence from background fluorescence [109]. The use of fluorescent stains, with the ability to distinguish between living and dead bacteria, is becoming increasingly important [for a review, see 274]. The use of fluorogenic indicators of metabolic activities in microscopy provides information on the physiological status of individual cells [271, 464]. Moreover, the fluorescent nature of the compounds greatly facilitates their use in studying bacteria associated with optically nontransparent surfaces [271, 342, 362, 368, 457, 464].

Enumeration of bacteria

Numerous fluorescent stains are used for the detection of both biofilm and suspension samples, to study the viability and/or the total number of microorganisms. The most commonly used stains for the detection of total number of bacteria are acridine orange (AO) and 4′,6-diamidino-2-phenylindole (DAPI) [223].

AO binds to DNA and RNA [390]. The distribution of dead, metabolically inactive but living, and living cells cannot be determined by the standard technique of AO staining, because DNA retains its staining properties in nonviable cells [223, 455]. The emission spectra of AO upon binding to nucleic acids are highly dependent on substrate structure, i.e. AO complexed with single- and double-stranded nucleic acid emits red and green fluorescence, respectively [284]. Since AO is known to stain

all organic material—e.g. food residues—it has been used for studying biofilms and environmental samples [87, 180, 181, 455] and to enumerate the total number of bacteria, while using a culture technique to determine the number of viable and culturable bacteria [42, 68, 106]. AO has also been used for enumeration of *L. monocytogenes* cells, both in biofilms [185, 254, 457] and in suspension [37].

DAPI is a nonintercalating, DNA-specific stain [223, 328, 402], which fluoresces blue or bluish-white when bound to DNA. When unbound or bound to non-DNA material, e.g. polyphosphates, it may fluoresce in various shades of yellow. As with AO, DAPI cannot be used for viability-staining [223]. DAPI is rapidly replacing AO as the most commonly employed bacterial stain for a wide range of sample-types. With both DAPI and AO, bacteria are identified on the basis not only of color but also of size and shape. DAPI has been used for the enumeration of *L. monocytogenes* [37, 63] and *B. cereus* [31, 431] cells. DAPI has also been used as a counterstain with Evans blue [26], erythrosine B (ERB) [219], 5-cyano-2,3-ditolyl tetrazolium chloride (CTC) [49, 51, 190, 271, 342, 362, 388, 457, 463], and iodonitrophenyltetrazolium (INT) [103].

Viability stains

There are several stains that target either viable bacteria [e.g. CTC, INT, rhodamine 123, fluorescein diacetate (FDA), carboxy-FDA, ChemCrome B] or nonviable bacteria [e.g. rhodamine B, calcofluor white, Evans blue, bis-1,3-dibutylbarbituric acid trimethine oxonol (oxonol dye), propidium iodide (PI), ERB]. The two most commonly used viability-staining systems for industrial samples are CTC-DAPI and the LIVE/DEAD *Bac*Light viability kit (Molecular Probes, Eugene, Ore.).

CTC is a monotetrazolium redox stain that produces a red-fluorescent formazan when it is chemically or biologically reduced [342, 463]. CTC can be chemically reduced in a low-redox environment and hence its use is restricted to aerobic or microaerophilic systems. Usually, CTC is used in connection with DAPI [342]. With computerized image analysis, it is possible to scan a colonized surface and rapidly quantify the respiratory activity of CTC-stained cells [362]. The utilization of CTC allows the clear resolution of individual cells by epifluorescence microscopy [464]. CTC can also be used for non-destructive analysis of the architecture and distribution of physiological activity within a biofilm [362]. The CTC method provides a convenient and rapid approach for e.g. quantification of the effect of biocides [51, 190, 362, 388, 457, 460, 464]. CTC-DAPI has been used for viability analysis of *L. monocytogenes* cells in biofilms [271, 457] and in suspension [271].

Molecular Probes′ LIVE/DEAD *Bac*Light viability kit provides a two-color fluorescence assay of bacterial viability. It has been proven useful for a diverse array of bacterial genera, including both Gram-negative and Gram-positive species [20]. The stains in the LIVE/DEAD kit are a membrane-permeating green fluorescent nucleic acid dye, SYTO 9, which stains viable cells, and a red fluorescent nucleic acid dye, PI, which does not permeate membranes, but stains dead cells. The background remains virtually nonfluorescent. Due to the interference of biofilm matrix polysaccharides and slime with the stain, LIVE/DEAD staining is not usually suitable for biofilms attached to a surface [271]. LIVE/DEAD samples should be analyzed immediately, whereas samples stained with CTC-DAPI and thereafter filtered on a microscopic membrane can be stored for several weeks. Therefore, CTC-DAPI offers a more convenient tool for viability investigation [271]. The LIVE/DEAD kit has been used for viability analysis of *L. monocytogenes* cells in suspension [203, 271, 457].

Molecular techniques in bacterial detection and identification

A molecular technique used for the detection of pathogens must be capable of detecting low numbers of target bacteria in samples which may contain a considerable background of interfering microorganisms and several matrix-derived compounds that may hamper the detection. In microbial community analysis, the method should allow the detection of different groups or species present in the ecosystem with similar efficacy, to avoid biases in the evaluation of species distribution and the complexity of the microbiota. Biases may be introduced by initial sample-handling [294] and during the extraction of nucleic acids from microbes in the sample.

Molecular techniques can be utilized in the detection and identification of microbes in two ways: (a) identification is performed directly from sample material, or (b) identification is based on combined culture and molecular detection. The sample matrix studied plays an important role when the decision between the two choices is made. If the matrix is known to contain factors that can inhibit a PCR reaction (for example) and are difficult to remove, it is often best to use the combination of a culture technique and a suitable molecular technique. There are two major techniques applied in the molecular detection and identification of bacteria: PCR and hybridization. When molecular tools were first introduced for the detection and identification of microbes, hybridization methods were widely applied. The rapid evolution of PCR techniques led to the present situation, where hybridization is mainly used in combination with PCR. However, a technique called in situ hybridization, in which bacteria are detected in their natural microhabitat, proves useful in applications where enumeration of the target organisms is warranted. Through its automation of the procedure, the recent development of DNA microarrays allows the simultaneous identification of a huge number of specific sequences by hybridization [165].

Release of nucleic acids from sample material

The reliable and reproducible lysis of microbial cells and the extraction of intact nucleic acids from environmental and industrial habitats is a demanding task [423, 446]. In addition, the removal of substances which may interfere with hybridization or PCR amplification, such as food components or process additives, may be difficult [446]. The procedures for cell lysis can be enzymatic (e.g. lysozyme, lyticase, proteinase), chemical (e.g. detergents, guanidium isothiocyanate), or mechanical (e.g. freeze–thaw/freeze–boil cycles, bead-beating, microwave heating) [for reviews, see 62, 347]. In many cases, e.g. in the identification or fingerprinting of isolates obtained by culture, the crude cell lysate can be used directly in a subsequent molecular analysis. However, since food and environmental samples may contain inhibitory compounds [233, 350, 364], further processing of the cell lysate is often necessary when direct molecular detection methods are applied. Processing steps include the removal of proteins, which is commonly done by phenol–chloroform extraction [432] or salt saturation [169], followed by precipitation of nucleic acids by ethanol, isopropanol, or polyethylenglycol precipitation, and purification of the nucleic acids [for a review, see 347]. When the metabolically active fraction of the community is of interest, the analysis should be performed with RNA rather than DNA. However, while extracting RNA from industrial and environmental samples, special attention should be paid to avoiding the degradation of RNAs with RNAses during the extraction procedure [for a review, see 423]. There are several articles describing different RNA extraction procedures [e.g. 123, 195, 285]. Several commercial kits are also available for DNA and RNA extractions.

Each step included in the sample preparation reduces the nucleic acid yield and decreases the sensitivity of detection. In food microbiology, a large effort is put into optimizing sample manipulation prior to cell lysis, to concentrate the target cells and to remove inhibitory substances from the sample matrix. A short enrichment culture and harvesting the bacterial cells from the sample by centrifugation, filtration, and immunomagnetic beads are applied to sample-processing in the detection of food-borne pathogens [177, 232, 307, 312, 437; for a review, see 62]. The sensitivity of the molecular method can be improved by an enrichment culture [6, 437], but this also precludes attempts to quantitate the number of target organisms in the sample. It should also be noted that some enrichment media might contain substances inhibitory to PCR [437].

Target sequences for molecular detection

Environmental microbiological studies are often based on ribosomal RNA (rRNA) or rDNA sequences. rDNA and rRNA are ideal targets for nucleic acid probes and primers for several reasons: (1) they are functionally conserved and present in all organisms, (2) 16S and 23S rDNA are composed of sequence regions with higher and lower evolutionary conservation, (3) 16S rDNA sequences have already been determined for a large fraction of the validly described bacterial species, and (4) the natural amplification of rRNA with high-copy numbers per cell (usually more than 10,000) greatly increases the sensitivity of rRNA-targeted techniques [384]. 16S rDNA sequences can be used to infer phylogenetic relationships and to identify unknown microbes by database comparisons [310]. Due to the patchy evolutionary conservation of rDNA sequences, the specificity of rDNA- or rRNA-targeted detection or identification can be tailored to the needs of the investigator, reaching from the subspecies to the kingdom level [10, 386]. It has also been proposed that rRNA content is appropriate for assessing changes in metabolically active bacterial populations, since rRNA content depends on bacterial activity [430]. In contrast to 16S rDNA, the intergenic spacer region (ISR) between 16S and 23S rDNA is highly variable in length and often shows species-specific sequence traits useful for designing molecular markers. Hence, in many cases, the ISR sequences are more applicable targets for diagnostic PCR-amplification than 16S rDNA [35, 210; for a review, see 163]. In addition, ISR amplicons can be separated into fingerprints by conventional electrophoresis [122].

Besides rDNA, other target genes can be used for the molecular detection of selected microbial groups/species from food and industrial samples. Genes associated with virulence factors, such as the toxin-producing listeriolysin O (*hlyA*) gene in *L. monocytogenes* [38], are commonly used for the detection of food-borne pathogens. In addition, genes coding for physiological properties, e.g. the cold-shock protein genes present in psychrotrophic *B. cereus*-group strains [137], can be used as target molecules for detection. In addition to known genes, species-specific sequences selected on the basis of random amplified polymorphic DNA (RAPD) analysis can be used [114]. However, this approach is hampered by the fact that relatively little sequence data is available from genomes, which results in difficulties in both creating the specific primer pairs and evaluating their specificity.

Techniques

PCR amplification

In PCR, a thermostable DNA polymerase enzyme is used to exponentially amplify a target DNA sequence defined by two oligonucleotide primers [288, 289, 290, 351]. The amplified DNA fragment can be visualized either by agarose gel electrophoresis, which allows size-determination of the PCR product, or by hybridizing the PCR product with a labeled probe. Combining PCR with a hybridization step improves the sensitivity and

specificity of the assay. PCR is very sensitive and small amounts of contaminating DNA carried from one run to the next (for example) can give false-positive results.

Many types of sample matrix (e.g. foods) contain factors which can either totally inhibit the PCR reaction or cause partial inhibition, leading to non-exponential amplification of the target DNA [233, 350, 364]. Inhibition may be avoided or reduced by pre-PCR sample manipulations, such as dilution of the sample material, short enrichment culture, extraction of the DNA from the sample, or harvesting the bacterial cells from the sample by centrifugation, filtration, or immunomagnetic beads coated with monoclonal antibodies specific to the target organism. However, even partial inhibition of the PCR reaction inevitably leads to reduced sensitivity and excludes the possibility of performing quantitative PCR. To minimize the risk of obtaining false-negative amplification results, suitable external standards should be used, which are coamplified together with the target DNA in the PCR reaction [337]. The sensitivity of the PCR assay can be improved by enrichment culture prior to PCR [6, 437], but this also precludes attempts to quantitate the number of target organisms in the sample. Thus, amplification of target DNA sequences from sample materials containing inhibitory factors for PCR can provide information on the presence, but not on the numbers and usually not on the viability of target organisms (except in the case where an enrichment step is included). It should also be remembered that PCR detects nonviable cells, as long as intact target nucleic acid sequences are available as templates [216].

When PCR is applied to environmental or industrial samples, several problems arise, including inhibition of PCR amplification by co-extracted contaminants, differential PCR amplification, formation of PCR artifacts, e.g. chimeric molecules (leading to the description of non-existing species), and DNA contamination. It should also be noted that 16S rDNA sequence variations due to *rrn* operon heterogeneity can interfere with the analysis [for a review, see 423]. When PCR is used in direct bacterial detection from sample materials containing other microbes, validation of the protocol applied is of utmost importance. The chosen method has to be tested on a large panel of strains representing the target species, closely related species, and other microbes commonly present in the sample material. This, together with the fact that different methods have to be applied to overcome the inhibitory effects of different sample matrixes, necessitates the use of tailor-made approaches for each microbe–sample matrix pair. A positive control for each analysis is important for confirming that inhibitory substances do not interfere with the detection and cause false-negative results [177].

Quantification of the initial amount of target is not possible in traditional end-point PCR, because the amount of PCR product is determined when the reaction has already reached the plateau phase. In real-time PCR, the amount of PCR product is measured at each cycle and also during the exponential phase, which enables the quantification of the initial template amount. The real-time measurement is based on fluorescent dyes that either bind to double-strand DNA or hybridize to a specific sequence. Since real-time PCR is especially vulnerable to inhibitory compounds, internal standards should always be used when complex sample matrixes are studied [337].

There are numerous articles reporting the identification of *L. monocytogenes* by PCR amplification. Most of the reported studies used PCR primers specific for fragments of the listeriolysin O (*hlyA*) gene [6, 34, 38, 41, 43, 47, 71, 96, 130, 133, 140, 175, 176, 191, 207, 241, 256, 299, 300, 302, 303, 316, 349, 401, 429, 437] and/or PCR primers specific for the invasion-associated protein (*iap*) gene [6, 55, 56, 164, 264, 265, 266, 267, 299, 300, 426]. A short enrichment period before PCR amplification greatly improves the sensitivity of the assay [6, 130]. Other PCR protocols using *inlA* [9, 207] and genes encoding flagellin (*flaA*) [389], fibrinectin-binding protein (*fbp*) [149], aminopeptidase [452], transcription activation protein (*prfA*) [71, 101, 353, 376, 443], and 16S rRNA [233, 434] as targets for specific detection of *L. monocytogenes* have also been introduced. Another approach is the use of 16S–23S rDNA spacer regions for *Listeria* genus-specific and *L. monocytogenes* species-specific PCR assays [155]. Multiplex-PCR targeting different sequences of *iap* [56], *hlyA* and 23S rDNA [191], or *hlyA* and 16S rRNA [445] have been developed for the rapid identification of *L. monocytogenes*. PCR protocols have been used to identify *Listeria* spp. from water, skimmed and raw milk, ice-cream, cheese, soft cheese, mozzarella cheese, cooked sausage products, fermented sausage, ham, pork, ground beef, minced beef, chicken skin, turkey, raw and cooked poultry products, seafood, raw fish, cold smoked fish, coleslaw, cabbage, lettuce leaves, and vegetables [6, 41, 47, 71, 101, 130, 133, 140, 164, 175, 176, 186, 191, 233, 236, 265, 267, 299, 300, 302, 303, 316, 353, 376, 401, 434, 437, 445].

For the PCR detection of *B. cereus*, various sequences are used as targets, including genes encoding 16S rRNA, hemolysin BL, cereolysin AB, non-hemolytic enterotoxin, enterotoxin T, gyrase B, IS*231*, and 16S-23S rDNA spacer region [166, 167, 173, 187, 226, 261, 404, 422, 461]. Recently, Bach et al. [31] developed a neutral metallopeptidase gene-based real-time quantitative PCR assay for quantification of *B. cereus*. It was noted that PCR analysis of the 16S–23S rDNA spacer region reveals identical patterns for *B. cereus* and *B. thuringiensis* [166] and that discrimination between *B. cereus* and *B. thuringiensis* is difficult when *gyrB* gene-based primers are used [66]. PCR is used to discriminate psychrotolerant and mesophilic strains of the *B. cereus* group [422], to investigate the growth, sporulation, and germination of *B. cereus* strains isolated from dairy and meat products [17], and to detect *B. cereus* from milk [226, 367]. Tsen et al. [404] developed a multiplex-PCR assay targeting simultaneously both the enterotoxin and 16S rRNA genes of *B. cereus*.

There are PCR assays targeting 16S rDNA and genes encoding alpha-, beta-, epsilon-, tau-, and enterotoxins for the rapid identification of *C. perfringens* strains from food, animal, and clinical specimens [22, 23, 57, 217, 220, 230, 253, 268, 326, 395, 406, 436]. There is a duplex PCR assay targeting alpha-toxin or the phospholicase C and enterotoxin (*cpe*) genes for the rapid detection and identification of enterotoxigenic *C. perfringens* strains in food and fecal samples [25, 117, 395, 396]; and there is a multiplex PCR assay targeting simultaneously five toxin genes for the analysis of clinical *C. perfringens* isolates [381].

Also, broad-range PCR primers, targeting many bacterial species of interest, have been developed for the detection of pathogenic bacteria, including *L. monocytogenes* and *Bacillus* spp. The essential part of this assay is the confirmation of the target species/genera with specific probes [159]. There are also PCR assays for determining the total bacterial load, using real-time PCR with a universal probe and primer set [297]. Another new approach to quantitate environmental DNA sequences involves a multiplexed, bead-based method with flow cytometry [382].

Hybridization

Hybridization techniques can be used in bacterial identification either alone or combined with a preceding PCR step. In hybridization, a labeled probe (a denatured DNA fragment varying in size between tens of basepairs to kilobasepairs) anneals to a denatured target DNA (genomic DNA or PCR amplification product) with sequence homology [10, 380]. Target DNA can be directly blotted onto a membrane, or if size information of the hybridization target is warranted, the target DNA is first run through agarose gel and then transferred to a membrane. Detection of hybrids is based on a radioactive signal, fluorescence, or color reaction, depending on the type of the label. By determining the intensity of the hybridization signal, the number of target organisms can be estimated [123, 122, 336, 364, 366, 386; for a review, see 311]. With dot-blot hybridization, nucleic acids can be fairly rapidly analyzed for the presence of specific sequences [for reviews, see 357, 365]. This technique is commonly used to confirm the identity of PCR products [38, 43, 79, 245, 367, 437]. A miniaturized and automated form of dot-blot hybridization is called a microarray (see DNA microarray, below).

Hybridization probes targeting 16S rDNA, the listeriolysin or enterotoxin genes, *iap*, *inlA*, *prfA*, the 16S–23S rRNA spacer region, or genomic sequences related to the expression of surface antigens have been developed for the detection and identification of *L. monocytogenes* [47, 83, 84, 154, 164, 242, 320, 339, 426, 434, 443]. Detection of *B. cereus* with hybridization is mainly performed to confirm the results obtained with specific PCR [79, 367, 437]. 16S rDNA, phospholipase and cereolysin AB genes, and 16S–23S rDNA spacer region sequences are used as probes in *B. cereus* detection [79, 334, 367, 399]. Hybridization is used e.g. to detect *B. cereus* from traditional Indian foods [334] and for enterotoxic *B. cereus* detection [261]. DNA hybridization has also been used to identify enterotoxic *C. perfringens* strains [85, 411] and to detect enterotoxic *C. perfringens* from Mexican spices and herbs [343] and from the feces of Mexican subjects [418].

Fluorescent in situ hybridization

The detection of whole-bacterial cells via labeling of specific nucleic acids with fluorescence-labeled oligonucleotide probes is called fluorescent in situ hybridization (FISH). FISH requires no cultivation and cells can be fixed before analysis, enabling the storing of samples prior to analysis [11, 12, 13, 14, 15, 94, 262, 383]. The whole-cell or in situ hybridization technique is now a much-used molecular tool in environmental microbiology, since organisms or groups of organisms can be identified with minimal disturbance of their environment and spatial distribution. Due to the fact that environmental conditions influence the cellular rRNA content, the amount of rRNA is considered to correlate with the growth rate [329]; and in situ hybridization using rRNA-targeted oligonucleotides can therefore be a powerful tool for the assessment of bacterial activities.

FISH in combination with epifluorescence microscopy is a widely applied method to analyze microbial communities [16]. The sensitivity and objectivity can be greatly enhanced by digital image analysis [335]. The application of FISH combined with conventional fluorescence microscopy for the analysis of complex microbial biofilms can be impaired by biofilm thickness, background fluorescence caused by humic substances or detritus, and the inherent autofluorescence of phototrophs. These problems can be circumvented by using FISH with CSLM [425; for a review, see 427]. The advantage of CSLM for the study of complex environments is that undisturbed samples can be analyzed without removal or homogenization of biofilm or other material [234]. Sample thickness is not limiting, since light from out-of-focus planes is excluded [263].

There are only a few published studies in which FISH has been used to identify *Clostridium* spp., *Listeria* spp., or *Bacillus* spp. This is most likely caused by the fact that FISH is much more difficult to perform with Gram-positive bacteria than with Gram-negative bacteria, due to the permeability problems associated with Gram-positive bacteria [138, 258]. Furthermore, FISH cannot be used to quantify bacterial spores [377]. FISH has been used to study the growth of *B. cereus* inoculated on tomato seeds [377] and to detect an uncultured *Bacillus* sp. from Dutch grassland soil [126]. FISH has also been used to identify the *Clostridium histolycum*-group, including *C. perfringens* [138] and *Clostridium* spp. [205] from human fecal samples. In addition, FISH has been used to detect *Clostridium* spp. from rice straw in anoxic paddy soil [441].

Recently, a microscopic method combining FISH and microautoradiography was developed [240, 314]. With this combination, it is possible to simultaneously determine the identities, activities, and specific substrate-uptake profiles of individual microbial cells within complex microbial communities under different environmental conditions [240].

DNA microarray

DNA microarrays facilitate the study of large numbers of genes simultaneously by hybridization of DNA or mRNA to a high-density array of immobilized probes [134, 248, 363]. The DNA microarray is basically a miniaturized form of dot-blot hybridization in a high-throughput format. There are two major types of DNA microarrays: an oligonucleotide-based array and a PCR product-based array. Microarrays allow the production of a gene expression profile or signature for particular organisms under certain environmental conditions. These can be used to study variability between the same or related species and between ancestor and descendants. As a result, microarrays provide information on the molecular basis of microbial diversity, evolution, and epidemiology [for reviews, see 32, 95, 145, 252, 462]. To our knowledge, there is a single published study indicating the successful use of an oligonucleotide microarray for the differentiation of closely related *Bacillus* spp. [247] and there is one study in which microarray was used for the identification of *C. perfringens* [451]. However, the genome projects of *L. monocytogenes* [153] and *C. perfringens* [375] are now complete, which will help the build-up of specific microarrays for these species.

Genetic fingerprinting techniques

Genetic fingerprinting techniques can be used to characterize bacterial communities or single bacterial isolates. The genetic fingerprinting of microbial communities provides a pattern or profile of the community diversity, based upon the physical separation of unique nucleic acid sequences [385]. Community analysis techniques are relatively easy and rapid to perform and they allow simultaneous analysis of multiple samples, enabling the comparison of the genetic diversity of microbial communities from different habitats, or the study of the behavior of individual communities over time. Community analysis can be performed with techniques such as denaturing-gradient gel electrophoresis (DGGE), temperature-gradient gel electrophoresis (TGGE), and single-stranded conformational polymorphism (SSCP). There is also a new approach based on heteroduplex mobility analysis of 16S rDNA fragments for targeted detection of sub-populations of bacteria within diverse microbial communities [405].

Fingerprinting of bacterial isolates can be performed by a variety of techniques, including e.g. ribotyping, amplified ribosomal DNA restriction analysis (ARDRA), pulsed-field gel electrophoresis (PFGE), RAPD, repetitive element sequence-based PCR (rep-PCR), and amplified fragment length polymorphism (AFLP). All these techniques aim at differentiating bacterial isolates at the subspecies level, preferably even at the strain-level.

An overview of the genetic fingerprinting techniques described in this review, with their advantages and limitations, is presented in Table 2.

Community analysis

Denaturing/thermal gradient gel electrophoresis

In DGGE [129] and TGGE [348], PCR-amplified DNA fragments of the same length but with different DNA sequences can be differentiated [70, 129, 296, 348]. Separation in DNA fragments is based on the electrophoretic mobility of a partially melted double-stranded DNA molecule in polyacrylamide gels containing either a linear gradient of DNA denaturants (a mixture of urea and formamide in DGGE) or a linear temperature gradient (TGGE). Partially melted DNA fragments are held

Table 2 Overview of genetic fingerprinting techniques described in this review. *AFLP* Amplified fragment length polymorphism, *ARDRA* amplified ribosomal DNA restriction analysis, *DGGE* denaturing-gradient gel electrophoresis, *PFGE* pulsed-field gel electrophoresis, *RAPD* randomly amplified polymorphic DNA, *rep* repetitive element sequence, *SSCP* single-strand conformational polymorphism, *TGGE* temperature-gradient gel electrophoresis

Technique	Advantages	Limitations
Community level		
DGGE/TGGE and SSCP	Community structure and dynamics can be studied, Identification of community members possible	Only those populations making up over 1% of the total community can be detected
Strain level		
Ribotyping	Can be automated, Good discriminatory power, Can be used for bacterial identification,	Expensive, laborious, and manually slow to perform
ARDRA	Fairly simple and fast	Limited discriminatory power
PFGE	Very high discriminatory power	Expensive, Slow to perform
RAPD	Fast, simple, and cost-effective	Reproducibility problems possible
rep-PCR	Fast, simple, and cost-effective	Reproducibility problems possible
AFLP	Good discriminative power	Expensive, Laborious

together with a G + C-rich oligonucleotide, a GC-clamp. Therefore, each denaturing fragment generates only a single band in the gel [for a review, see 294]. DGGE/TGGE performed after PCR gives an insight into the predominant microbial populations; and DGGE/TGGE performed after reverse transcriptase (RT)-PCR helps identify the predominant active microbial populations [104, 123, 124, 400, 466]. DGGE/TGGE can also be used in combination with quantitative RT-PCR to quantify rRNA sequences in complex bacterial communities [125].

DGGE/TGGE analysis combines a direct visualization of bacterial diversity and the opportunity to subsequently identify community members by DNA fragment sequence analysis or hybridization with specific probes [292, 293]. Sequence analysis or hybridization performed after DGGE/TGGE has detected *Bacillus*-like sequences and *Clostridium* spp. in various environmental and clinical samples [104, 121, 127, 128, 198, 201, 231, 243, 317, 345, 358, 378, 397, 441, 465].

DGGE/TGGE has some specific limitations. DGGE/TGGE can be used to separate only relatively small fragments [295] and it displays only the rDNA amplicons obtained from the predominant (over 1% of the population) species present in the community [291, 292, 294]. The presence of heterogeneous 16S rRNA genes (16S rRNA genes that exhibit small sequence variations in the genome of a given strain) can result in several bands in a DGGE/TGGE profile [125, 306, 356, 466]. Furthermore, a single band may represent more than one strain [124, 127, 356, 371, 409]. The construction of 16S rDNA clone libraries and the screening for different clones by DGGE may overcome these deficiencies [127, 128, 148, 294, 358].

Single-stranded conformation polymorphism

SSCP analysis detects sequence variations between different DNA fragments, which are usually PCR-amplified from variable regions of the 16S rRNA gene. The technique is based on the fact that a single base modification can change the conformation of a single-strand DNA molecule, altering the migration speed of the molecules in a non-denaturing gel [313, 344]. DNA fragments of the same size but with different base composition can thus be separated [170].

The limitations of the SSCP method are similar to those of DGGE/TGGE. The discriminatory power and reproducibility of SSCP analysis is usually most effective for fragments up to 400 bp in size, depending on the length of the fragment studied, the position of the sequence variations in the gene studied, and the test conditions [413]. In addition, PCR-SSCP detects bacterial populations that make up 1% or more of a bacterial community [239]. A major limitation of SSCP for community analysis is the high rate of DNA strand-annealing after the initial denaturation during electrophoresis [372].

Besides community studies, PCR-SSCP analysis can be adapted for the rapid identification of Gram-negative and Gram-positive bacteria at the genus and species levels [48, 79, 91, 239, 241, 260, 369, 415, 429, 447], to discriminate between *B. cereus* and *B. subtilis* [448], and for detecting *Listeria* spp. [241, 260, 415, 429, 447, 448], *Clostridium*-related bacteria [91], and *Clostridium* spp. [92, 447].

Typing of microorganisms

Prior to molecular techniques, phenotypic methods such as biotyping and serotyping were used for bacterial strain differentiation. These techniques are still used today, but more reliable and often less laborious fingerprinting can be achieved with molecular techniques. Regardless of whether phenotypic or genotypic techniques are applied, fingerprinting is preceded by culture and single-colony subculture steps. Thus, even though PCR and hybridization can be used both in bacterial detection and fingerprinting, the techniques applied differ in a profound way. While detection methods are able to find the target organisms in a sample containing hundreds of other bacteria, fingerprinting methods are not genus- or species-specific and can therefore only be applied to pure bacterial cultures. When molecular techniques were first applied for bacterial fingerprinting, both conventional restriction endonuclease analysis (REA) of genomic DNA and plasmid profiling were used. Both techniques have their limitations. With conventional REA, complicated patterns with hundreds of restriction fragments are obtained, which makes the profile comparison difficult. With plasmid profiling, far simpler profiles are obtained, but this technique is suitable only for bacteria carrying (several) plasmids [for a review, see 413].

Ribotyping

When conventional REA is combined with a hybridization step, a far simpler and thus more easily comparable fingerprint is obtained. This technique, where genomic restriction fragments are separated by gel electrophoresis, transferred to a Nylon membrane, and hybridized to a probe, is called restriction fragment length polymorphism (RFLP). By far the most widely applied RFLP technique is (classic) ribotyping, in which rRNA genes (usually both 16S and 23S rRNA genes, or a whole rRNA operon containing 16S, 23S, and 5S rRNA genes and their spacer regions) are used as a probe. Since a rRNA operon contains both conserved and hypervariable regions, the same probe (e.g. originating from *Escherichia coli*) can be used in ribotyping different bacterial species [160]. The strain differentiation in ribotyping is thus based on the unique hybridization pattern (fingerprint) obtained and not on the specificity of the probe. Differences in the hybridization patterns originate from restriction endonuclease recognition site-variation within variable regions of rRNA genes and their spacer regions. The discriminatory ability of

ribotyping is greatly influenced by both the probe (whole rRNA operon vs a single gene) and the restriction endonuclease applied. Obviously, the best discrimination is obtained when the whole operon is used as a probe and an optimal restriction endonuclease for each bacterial genus is selected from a panel of restriction endonucleases tested. However, when ribotyping is used as a taxonomic tool, riboprints of isolates representing different genera and species are compared; and thus the same restriction endonuclease has to be applied for all bacteria. The invention of an automated ribotyping system (Riboprinter; Dupont Qualicon, Wilmington, Del.) greatly facilitated bacterial fingerprinting, thus allowing larger numbers of bacterial isolates to be characterized and compared than when ribotyping is performed manually.

Classic ribotyping has been used to characterize *C. perfringens* isolates associated with food-borne cases and outbreaks, e.g. ground meat [225, 359, 360]. Ribotyping has also been used for *B. cereus* typing [19, 325, 332, 361]. There are several publications on *L. monocytogenes* ribotyping, including the characterization of isolates from the smoked fish, meat, poultry, and seafood industries, from different foods, and from human and animal listeriosis cases [8, 33, 89, 142, 157, 204, 207, 209, 277, 304, 308, 393, 394].

Amplified rDNA restriction analysis

In addition to classic ribotyping, rDNA-based fingerprints can be obtained by a technique called ARDRA. In ARDRA, bacterial rRNA gene(s) are first amplified by PCR, using conserved sequences of rDNA as primers. The PCR amplification product is then digested with restriction endonuclease and restriction fragments are resolved electrophoretically to obtain a fingerprint [414]. Although ARDRA fingerprinting is faster to perform than classic ribotyping, its discriminatory power is often inferior to that of ribotyping. This is due to the fact that smaller areas of the rRNA operon (and none of the sequences surrounding the rRNA genes) are targeted in ARDRA than in ribotyping. The few references on applying the ARDRA technique for *Bacillus*, *Clostridium*, or *Listeria* fingerprinting describe RFLP analysis of PCR-amplified 16S rDNA (16S rDNA-RFLP) for the characterization of psychrophilic and psychrotrophic clostridial strains associated with spoilage of vacuum-packed meats, typical and atypical *Listeria* isolates, and enterotoxic *B. cereus* [53, 261, 415].

Pulsed-field gel electrophoresis

Due to the problems encountered with conventional REA of bacterial genomes, a technique was developed for bacterial fingerprinting, using profiles consisting of fewer numbers of larger-sized genomic restriction fragments [111]. In this technique, bacterial genomic DNA is restricted in situ (in a gel block) with a rare cutting restriction endonuclease, such as *Sma*I, *Sfi*I, *Not*I, or *Bss*HII, and the restriction fragments are separated by PFGE, which is a special technique capable of the resolution of large DNA fragments. With PFGE, highly discriminative fingerprinting of bacterial isolates can be performed. Of the different molecular fingerprinting methods, PFGE has in most cases proved to be the most discriminatory. However, PFGE is a laborious technique and it is not usually applied in studies where large numbers of isolates are characterized.

PFGE has been used in the fingerprinting of *C. perfringens* clinical isolates, but has only infrequently been applied in the fingerprinting of *B. cereus*. However, there are many more data on the applicability of PFGE for *L. monocytogenes* typing. PFGE has been used to characterize *L. monocytogenes* isolates from food items, the environment, and human listeriosis cases, to trace a contamination in an ice cream plant, in fish-, seafood-, and meat-processing plants, and in pig slaughterhouses [27, 28, 65, 86, 97, 120, 151, 204, 255, 280, 281, 282, 308, 309, 373]. PFGE analysis of clostridia proves challenging, due to their endogenous DNAse activity [229]. However, PFGE was successfully applied in the characterization of *C. perfringens* strains associated with food-borne-disease or antibiotic-diarrhea, especially when cell pre-treatment steps that interfere with the DNAse activity were included [253, 269, 326, 381]. In *B. cereus* typing, PFGE was applied to investigate a bacillus pseudo-outbreak in a pediatric unit [246].

Random amplified polymorphic DNA

In RAPD fingerprinting, one or two primers (usually 10–12 bp long) are arbitrarily selected and allowed to anneal to the bacterial genomic DNA template at a low stringency. In RAPD, several amplification products of varying sizes are obtained. These products are resolved electrophoretically to yield a RAPD-fingerprint [330, 442]. RAPD typing is fast to perform, especially in cases where fingerprinting can be performed directly on single-colonies growing on an agar plate. Due to the low stringency of the PCR amplification, RAPD-fingerprints can show some variation (especially in band strengths) and therefore the fingerprint comparisons have to be done visually by an experienced person. However, when strictly identical conditions (same thermocycler, reagents, etc.) are used, the method usually works well [279]. RAPD banding-pattern reproducibility can be improved by using a procedure where the same strains are exposed to three different annealing temperatures (with increasing stringency) and by identifying the stable amplicons [78]. This triplicate procedure naturally makes the RAPD fingerprinting technique more laborious.

RAPD is best suited for studies where a specific bacterial strain (e.g. a certain food-borne pathogen) is sought among large number of isolates. Bacterial isolates with fingerprints clearly different from the specific bacterial strain can quickly be identified with RAPD and

rejected. Thereafter, the remaining (fewer) strains may be further characterized with another, more laborious technique (e.g. ribotyping, PFGE, AFLP). RAPD is not well suited for interlaboratory or taxonomic studies, or studies where the aim is to develop a fingerprint database.

RAPD can be used for the fingerprinting of *B. cereus* isolates in a versatile manner [19, 80, 81, 301, 387]. For example, it proved useful in the differentiation of psychrotolerant *B. cereus* strains [238] and *B. cereus* isolates from spontaneously fermented food [354] and in tracing the source of *B. cereus* contamination in pasteurized milk, ethyl alcohol, and food-poisoning outbreaks [112, 144, 189]. There are several reports on RAPD fingerprinting of *L. monocytogenes*, including studies where RAPD was used in typing isolates from different foods (cheese, poultry products, (cold-)smoked salmon, meat products, imitation crab meat), animals, human listeriosis cases, and to trace contamination in pork slaughtering and cutting plants, in fish, seafood, and meat processing plants, in a poultry processing environment, and in a dairy environment [7, 30, 46, 59, 93, 120, 135, 136, 151, 199, 224, 237, 250, 259, 277, 428, 444]. To our knowledge, RAPD-typing has not been reported for *C. perfringens*, although another food-borne *Clostridium*, *C. botulinum*, has been fingerprinted with RAPD [197].

Repetitive element sequence-based PCR

Repetitive chromosomal elements, which are found randomly distributed in bacterial genomes, are the targets of rep-PCR amplification. In rep-PCR, primers anneal to repetitive parts of the chromosome and amplification occurs when the distance between primer binding sites is short enough to enable this [419]. The repetitive DNAs can be classified either as short sequence repeats (SSRs) or variable number of tandem repeats (VNTRs). Variations of rep-PCR include enterobacterial repetitive intergenic consensus PCR (ERIC-PCR), BOX-PCR, repetitive extragenic palindromic unit sequence PCR, and VNTR-PCR [410].

rep-PCR techniques are fairly infrequently applied for the characterization of *Clostridium*, *Bacillus*, or *Listeria* strains. To our knowledge, there is only one report of *Clostridium* rep-PCR, where *C. botulinum* strains were characterized [197]. For *B. cereus* typing, ERIC-PCR, BOX-PCR, and VNTR-PCR have been applied [3, 162, 227, 321, 361] and rep-PCR has been used for the typing of other *Bacillus* spp. [82, 174]. For *Listeria* spp., fingerprinting rep-PCR and ERIC-PCR have been used [212, 213, 370].

Amplified fragment length polymorphism

AFLP involves restriction of total bacterial DNA with two restriction enzymes of differing cutting frequencies (e.g. *Hin*dIII, *Taq*I), followed by ligation of the fragments to oligonucleotide adapters complementary to the sequences of the restriction sites (restriction-half-site-specific adapters). Selective PCR amplification of subset of fragments is achieved using primers corresponding to the contiguous sequences in the adapter and restriction site, plus a few nucleotides in the original target DNA. When only one of the primers is labeled, only a subset of amplified fragments is detected during visualization [206, 424]. A variation of this technique has been developed, where only a single restriction enzyme is used [146].

AFLP is a fairly new technique and therefore only scarce data are available on its application in *B. cereus*, *C. perfringens*, and *L. monocytogenes* fingerprinting. In the few papers published, AFLP proves a sensitive and reproducible technique for the typing of *C. perfringens* and *L. monocytogenes* [1, 275, 340]. AFLP was used to trace an outbreak of *B. cereus* infections in a neonatal intensive care unit to the balloons used in manual ventilation and to study *B. cereus* soil isolates [403, 412].

Discriminative power of different techniques

A number of studies have been performed where the discriminative power of the above mentioned techniques have been compared. For *B. cereus* fingerprinting, RAPD proved a somewhat more discriminative method than ribotyping, whereas the discriminative abilities of ribotyping and ERIC-PCR were equal [19, 361]. For *C. perfringens* typing, ribotyping and PFGE proved equally discriminative [360]. In *L. monocytogenes* fingerprinting, RAPD, PFGE, and AFLP were equally discriminative [97, 151, 224, 420, 421]. Ribotyping has proved either equally discriminative or a bit less discriminative than PFGE and RAPD in *L. monocytogenes* typing [224, 250, 308]. These results indicate that RAPD, PFGE, ribotyping, rep-PCR, and AFLP are all suitable methods for subspecies-level fingerprinting of *B. cereus*, *L. monocytogenes*, and *C. perfringens*. The discriminative capabilities of the techniques are about equal and the results obtained with different techniques are generally in very good agreement with each other. The choice of restriction endonuclease in PFGE and ribotyping and the choice of primers in RAPD have a great impact on the discriminative ability of these techniques. Therefore, the applied technique has to be tailor-made for each bacterial species, to obtain the best possible discriminative ability.

Future prospects for the exploitation of the described methods in the industrial environment

Biofilms cannot be eliminated from industrial systems by any of the current methods available. Thus, the primary challenge is to control rather than eradicate biofilms from the industrial environment [61]. Knowledge about the microbiota present in the industrial environment will help to control the formation and build-up of biofilms, since specific characteristics of each microbiota can be

considered when preventative and/or control measures are applied. Applications of the microbiological methods described in this review are presented in Table 3. It should be remembered that sampling is a crucial step in the characterization/identification procedure. If it is performed inadequately, the characterization of microbiota will inevitably be biased.

The emergence of new detection and real-time methods is linked to the need for a better assessment of the microbiological quality of products. This objective can be reached through an increase in detection specificity and a reduction in analysis time [346]. In particular, in situ techniques should enable progress in understanding the ecology of complex microbial communities in minimally disturbed samples. The most important weakness of culture-independent methods is that the taxonomic interpretation of data appears problematic [152]. Although various new detection methods are applied to detect microorganisms from the industrial environment, the use of culture techniques will persist, since the international standard methods for the detection and enumeration of pathogens are based on cultivation. In addition, in many industrial quality control laboratories, resources for the use of new molecular methods are inadequate.

In routine food control, PCR assays may shorten the time needed to identify e.g. *L. monocytogenes*, although enrichment may be necessary prior to the detection. By using virulence-associated genes as primers or probes, the presence of pathogenic species can be rapidly determined. However, dead bacterial cells may constitute a problem in basic PCR detection in hygiene control. For example, heat-treated samples may contain dead or damaged cells with no relevance to product safety, although the dead bacteria may still create positive signals due to the stability of their DNA molecules [311]. In some circumstances (when the RNAse activity of the bacterial population is not destroyed in the sample prior to analysis), RT-PCR can be applicable in assessing the viable and active populations in samples. DNA-based detection methods, especially PCR, may gradually replace traditional methods for assaying microorganisms in food. When applicable (e.g. when no enrichment step is required), real-time PCR (which enables the quantification of target sequences) can prove highly useful for the rapid analysis of food pathogens. However, PCR detection of pathogens in food samples is still time-consuming, particularly in the case of large-scale testing [364]. High-throughput methods, such as dot-blot hybridization using microarrays, have promising future potential for routine diagnostic and quality control procedures in industrial settings [245].

One of the challenges for microbial ecology is to gain more information below the bacterial community, genera, and even species level. Subspecies-level identification is especially important when a source of contamination is traced in an industrial environment. DNA fingerprinting techniques provide effective molecular tools to identify and type microorganisms to subspecies level [152]. When typing of the microbial isolates is performed, e.g. to trace a contamination source, the importance of including sufficient numbers of isolates from each sample site should be remembered. Once efficiently integrated, the typing techniques provide precise information on the heterogeneity of the target bacterial population at a given time/space combination. However, fingerprinting methods are laborious and time-consuming, since isolation and cultivation of a large number of bacterial isolates cannot be avoided. Another limitation is that the unculturable strains

Table 3 Applications of microbiological methods described in this review. *FISH* Fluorescence in situ hybridization

Task	Available methods
Detection of selected species/groups	Culture Specific PCR Specific hybridization (including microarray) FISH
Strain-level identification	Ribotyping ARDRA PFGE RAPD rep-PCR AFLP
Quantification of specific microorganisms	Culture Quantitative PCR FISH Culture hybridization
Activity of the microorganisms	Metabolic stains in combination with microscopy RT-PCR alone or combined with other techniques RNA hybridization
Community analysis	Epifluorescence/confocal laser scanning microscopy Culture DGGE/TGGE SSCP FISH Specific hybridization (including microarray)

present in natural ecosystems cannot be reached with typing methods.

In conclusion, bacterial detection, identification, and typing from industrial samples remains a laborious task, mainly due to the fact that frequently large numbers of samples need to be analyzed. The development of automated techniques that allow high-throughput analysis of large numbers of samples will greatly facilitate studies on industrial microbial ecology in the future.

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