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Significance of Psychrotolerant Aerobic Sporeformers in Food Spoilage and Methodologies for their Detection and Identification*

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Introduction

In order to optimize its own logistics and to satisfy the trading companies and consumer demands, the food industry continuously endeavors to increase the shelf-life of refrigerated foods. Furthermore, the consumer's demand for high quality foods which require a minimum amount of preparation, has led to the introduction of ready-to-eat convenience foods; products that are preserved by mild methods only. Refrigeration is the main mild preservation technique that these perishable foods rely upon. By using such techniques, however, spoilage of these products by psychrotolerant sporeformers, such as *Bacillus* and *Clostridium,* is of increasing concern. Due to their ubiquitous nature, psychrotolerant spore-forming bacteria can be found in a wide range of foodstuffs. Inevitably, the route of entry for these bacteria into such products is numerous, arising from either initial contamination of the raw material or recontamination during food processing. Psychrotolerant aerobic sporeformers survive pasteurization temperatures and more severe heat treatment in the form of endospores. Ironically, this heat treatment can even trigger the germination of the spores and thus contribute to fast spoilage (1). The hurdle concept of heat treatment and subsequent cold storage, reduces and hinders the competitive flora in the final product. Therefore, refrigerated food quite often resembles an ideal environment for psychrotolerant bacilli to proliferate.

In this paper we discuss the contribution to food spoilage made by psychrotolerant aerobic sporeformers, with particular emphasis being placed upon *Bacillus cereus* and this bacterium's adulteration of milk and dairy products. In addition, we

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review recent methodologies for the detection and identification of such bacteria and speculate upon their application in contamination route analysis and the prediction of a product's shelf life.

General aspects

Taxonomy

For many years the genus *Bacillus* comprised all rod shaped aerobic sporeformers. During the last decade, however, *Bacillus* taxonomy has experienced some major changes. Currently there are eight different genera described. Besides the original genus *Bacillus* (2), there are the genera *Amphibacillus* (3), *Alicyclobacillus* (4), *Paenibacillus* (5), *Aneurinibacillus* and *Brevibacillus* (6), *Halobacillus* (7) and *Virgibacillus* (8). The psychrotolerant aerobic sporeformers isolated from food belong to several of these genera, including *Brevibacillus brevis, Bacillus circulans, Bacillus coagulans, Bacillus lentus, Bacillus licheniformis, Paenibacillus polymyxa, Bacillus pumilus,* and *Bacillus sphaericus* (9–11). In this context, the recent description of *Bacillus weihenstephanensis* sp. nov. (12) is of major importance. This new species comprises psychrotolerant strains of the well known pathogenic food spoilage organism *B. cereus.* In this review we have maintained the nomenclature of the original articles for literature published before this new species description.

Mode of spoilage

Usually, food spoilage is due to the enzyme activity of microorganisms, which cause a variety of flavour and/or texture defects. Proteases, lipases and phospholipases are the major spoilage enzymes produced by psychrotolerant aerobic sporeformers. Enzymes of psychrotrophs, both Gram-negative and Gram-positive, have been comprehensively reviewed by *McKellar* (13). Sporeforming psychrotrophs and their enzymatic activity in dairy products have also been discussed in the reviews of *Sørhaug* and *Stepaniak* (14), *Meer* et al. (15), and *Müller* (16, 17).

B. cereus/B. weihenstephanensis are recognized to be the major sporeforming spoilage organisms in milk and milk products, setting the limits for the keeping quality of many products (18). For example, in pasteurized milk without Gram-negative recontamination, the latter bacteria are responsible for two types of spoilage: sweet curdling, caused by a rennet-like protease (19, 20), and «bitty cream» defect in non-homogenized milk, caused by phospholipase C (21, 22).

Foodborne illness

Besides the above organoleptic changes that occur in the milk, the presence of these pathogens in food frequently mean that such products are not fit for human consumption. *B. cereus* is the major aerobic sporeforming food pathogen (23). The food poisoning toxins of *B. cereus,* which have been reviewed recently by *Granum* and *Lund* (24), cause two types of food poisoning; an emetic and a diarrheal type.

Both types of illness (table 1) are significantly underreported, as they are relatively mild and usually last for less than 24 h (23, 25). The causative agent of the emetic syndrome has been named cereulide. It is a heatstable compound that is preformed in the food (26). The diarrheal type food poisoning is caused by complex enterotoxins (27, 28) which are produced during growth of the organism in the small intestine (29). Furthermore, although the majority of the above studies have not discriminated between mesophilic and psychrotolerant *B. cereus (B. weihenstephanensis)* as the causative agent of disease, a number of additional studies have shown that psychrotrophic strains of *B. cereus* are indeed capable of producing enterotoxin (30, 31).

The food poisoning potential of psychrotrophic bacilli other than *B. cereus/B. weihenstephanensis* is not clear. Foodborne illness was associated with *B. lichenifor-*

Table 1
Examples of *Bacillus cereus* food poisoning outbreaks

Year	*Country*	*Product*	*Number of patients/ percentage of total food-poisoning outbreaks*	*Symptoms*	*Reference*
1987	UK	ice cream	6	not reported	48
1989	USA/ California	Cornish game hens	140	diarrhea	49
1990	Thailand	eclair	485	nausea, vomiting, diarrhea, abdominal pain Mixed culture: *B. cereus* and *Staph. aureus*	50
1990	Nether-lands	pasteurized milk	280	nausea, vomiting	30
1991	Japan	heated milk	201	vomiting, diarrhea, abdominal pain	51
1993	USA/ Virginia	fried rice	14	not reported	52
1994	USA	barbequed pork	139	diarrhea	53
1995	Norway	stew with meat & vegetables	152	diarrhea	54
1992	Germany	various foods	0,9 %		55
1994	Taiwan	various foods	14 %		56
1995	Finland	various foods	19 %		57
1996	Finland	various foods	9 %		57

mis, B. subtilis and *B. pumilus,* characterized by vomiting and/or diarrheal symptoms (23). It is not clear, however, whether these non *B. cereus/B. weihenstephanensis* isolates were psychrotolerant. Furthermore, *B. thuringiensis,* usually characterized as mesophilic, has been isolated together with *B. cereus* from a gastroenteritis outbreak. All isolates showed cytotoxic effects characteristic of enterotoxin-producing *B. cereus* (32). In natural isolates of *B. thuringiensis,* the *B. cereus*-type enterotoxin also was detected using PCR (33, 34).

Incidence

Due to their ubiquitous nature, psychrotolerant aerobic sporeformers can be found in a wide range of foodstuffs. Some products are frequently contaminated due to their raw materials, while other products are contaminated only occasionally. In general, it is the food-type and its individual handling that determines whether these organisms can proliferate to cause spoilage.

Milk and dairy products

Because it is not possible under practical conditions to produce and collect raw milk without contamination by psychrotolerant aerobic sporeformers, milk and dairy products are systematically contaminated with these bacteria. Since 1969, when *Grosskopf* and *Harper* (35) reported finding psychrotolerant aerobic sporeformers in milk, their incidence in milk and dairy products, especially in liquid milk, has been extensively studied globally (36–42). While post-pasteurization recontamination with Gram-negative bacteria has decreased, spoilage problems associated with psychrotolerant aerobic sporeformers have increased (reviewed by 15).

Published data concerning this subject varies tremendously. *Griffiths* and *Phillips* (9), for example, detected < 0.5 to 170 psychrotrophic spores/liter with an average of 17 spores/liter, whereas *Rama Raju* et al. (40) reported an average psychrotrophic spore count of 3.5×10^4 spores/liter. Surveys on the composition of this partial flora of the milk revealed international differences, too (9, 11). Therefore, data gained in one country cannot be applied to describe the situation of another country. Such diversity might be caused by different housing and feeding conditions of the cows (43, 44) or alternatively by different analytical methods. Despite these discrepancies, *B. cereus* and *B. weihenstephanensis* are generally recognized as the major spoilage organisms of pasteurized milk. Today, the spoilage rate of milk and many milk products is determined by the levels of contamination by these two species (18). However, when the storage temperature of pasteurized milk is lowered, *B. circulans* is more likely to cause deterioration of the milk (10, 45).

Foodstuffs other than dairy products

Psychrotolerant aerobic sporeformers can be found in a broad range of non-milk-products e.g., wheat flour desert, lobster paté, sausages, and shellfish (23,

46). A high titer of these bacteria in such products, however, is not necessarily associated with tremendous organoleptic chances.

In contrast to dairy products, the contamination of other foods with psychrotolerant aerobic sporeformers appears to be less systematic. For example, spices have been shown to contribute significantly to the contamination of foods with sporeformers (23).

Due to consumer trends towards convenience foods, cooked chilled foods, also known as REPFEDs (Refrigerated Processed Foods of Extended Durability), are becoming more and more popular throughout Europe. In REPFEDs containing vegetables, *B. cereus, B. subtilis, B. licheniformis* and *B. pumilus* have been identified as hazardous psychrotolerant aerobic sporeformers (30, 47). The general importance of sporeformers in foods has been stressed by *Todd* (46). In a 10-year summary he found that among food pathogens, *B. cereus* was the most frequently isolated organism causing illness associated with food service locations. Its food poisoning potential is also documented in the selection of outbreaks, given in table 1.

Detection

Detection by classical methods

Detection of psychrotolerant aerobic sporeformers in food products is at present extremely slow with conventional microbiology. This is due to an inability to discriminate these cold adapted strains from their mesophilic counterparts by means other than growth at low temperature, which takes two weeks or longer (10, 15, 38). In freshly prepared/processed food, the titer of psychrotolerant aerobic sporeformers is often very low (9, 11, 38). In these cases, a pre-enrichment step at low temperatures is necessary for detection, with enumeration by the MPN-method. For these steps the incubation time cannot be generally prescribed for all investigations, but depends on the food, the growth parameters in the samples, the incubation temperature and the organism being detected. Furthermore, selective media are available for presumptive *B. cereus/B. weihenstephanensis* only, namely mannitol-egg yolk-polymyxin agar (MYP) (58), polymyxin-pyruvate-egg yolk-mannitol-bromothymol blue agar (PEMBA) (59) and polymyxin-pyruvate-egg yolk-mannitol-bromocresol purple agar (PEMPA) (60).

Detection and identification by polymerase chain reaction (PCR)

In light of recent molecular biological advances, the detection and identification of foodspoilage/pathogenic microorganisms has improved significantly in terms of rapidity, accuracy and reliability by applying nucleic acid based techniques. Among these, PCR (polymerase chain reaction) is one of the more established techniques. Considerable development has already taken place in applying PCR methods to routine medical assays and, more recently, the food industry has begun to recognize the benefits of this technique.

Major applications of PCR in microbiological food quality control will comprise routine detection and quantification of bacterial strains in the food matrix, as well as rapid identification of individual isolates by PCR-fingerprinting (61). This will enable the shelf-life of food products and the tracing of contamination routes to be more easily monitored. A number of PCR protocols for the detection and identification of food relevant bacteria, including *Bacillus* spp. have already been published. However, many of these have only been evaluated using spiked samples, rather than with naturally contaminated food. Furthermore, many of the methodologies used involve costly or protracted procedures for the concentration of the bacteria from the food matrix and the subsequent liberation of their nucleic acids; the latter two steps being necessary before the actual PCR itself can be set-up. The analysis of the PCR products can either take place during the PCR (real-time-PCR) or may involve further processing steps.

In the following section a selection of current techniques available for the detection and identification of psychrotolerant sporeformers by PCR is reviewed.

Recovery of the microorganisms and their nucleic acids from the food matrix

PCR has been used to detect most of the important foodborne bacterial pathogens using pure cultures or purified nucleic acids. However, there has been little research into methodologies for the rapid and sensitive recovery of the template nucleic acids from the food matrix using minimal sample processing steps. Basically, concentration of the target organisms can be achieved via an enrichment culture, or directly, e.g. by using immunocapture or centrifugation. In the case of spores, direct approaches require subsequent liberation of the nucleic acids from the spores, which can be attained by either germination or by mechanical rupture.

1. Recovery of spores from the food matrix

Prior to the recovery of the spores from the food matrix, the latter may have to be processed/pretreated, depending on its physical character. For textured foods (e.g. meat or cheese) protocols include cutting (62) or homogenization (63), whereas suspensions (e.g. milk) often need to be clarified (64).

a) Enrichment culture

PCR can be directly performed from a washed or diluted enrichment culture without the necessity of further nucleic acid (template) isolation steps (62, 65). This technique is very easy to perform and at the same time avoids the detection of nucleic acids from non-viable cells. However, enrichment usually takes overnight (62) and may last for up to five days (66). If only spores are of interest, a vital part of this technique is a foregoing heat treatment to kill vegetative cells and allow only spores to grow. However, spores of some strains are more heat sensitive than vegetative cells of other strains. This demands optimization of the time-heat combination for each strain to be determined (67). Furthermore, plasmid encoded PCR targets (e.g. toxin genes) may be lost during selective enrichment, leading to false negatives (65).

b) Immunocapture

The target organisms (i.e. the spores) can be directly concentrated by immunocapture. Spore antibodies, attached to magnetic beads, are incubated with the sample and are collected with a magnetic particle concentrator (68). Alternatively, the antibody can be linked to a matrix. After its contact with the sample, spores bound to the antibody can be eluted (69). The entire immunocapture procedure usually lasts between one and two hours (68, 70, 71). Protocols for the concentration of *Bacillus stearothermophilus* spores by polyclonal antibodies (68) and the enrichment of *B. cereus* spores by recombinant (70, 72) and monoclonal antibodies (69) are available. A variety of antibodies against spores of *Bacillus* or *Clostridium* have been described in the literature (e.g. 71, 73).

Although the feasibility of these immunological enrichment techniques has been demonstrated, their applicability strongly depends on the specificity of the individual antibodies. In particular, antibody crossreactivity with vegetative cells (71, 73) has to be excluded, since the subsequent PCR cannot discriminate between spores and vegetative cells. Although immunomagnetic beads have been demonstrated to recover spores in a quantitative manner from a phosphate buffer system within one hour, this ability decreased to 40 % in a whole milk, where proteins block the antigen binding site of the spores (70).

c) Centrifugation

Due to their high density, bacterial spores can be concentrated by centrifugation. For milk (64, 74, 75) and cheese (63) this approach is relatively fast and recovers a very high percentage of the spores. However, the sample processing involves caustic reagents such as NH_3, diethyl ether and petroleum ether. *Lindqvist* and colleagues (76, 77) have described the use of buoyant density centrifugation for the concentration of vegetative bacteria from comparatively small spiked food sample volumes. Whether this approach is applicable for the recovery of low spore numbers from naturally contaminated sample of larger volume remains to be investigated.

d) Other approaches

Membrane filtration can be applied for the collection of spores from solutions (e.g. drinking water) that do not contain significant amounts of suspended particles. *Isoelectric focussing* (78) as well as *aqueous two-phase separation* (79) have also been investigated to concentrate bacteria from food for subsequent PCR detection. The latter of these techniques has the added advantage of being able to separate spores from both vegetative cells and soil (80). However, with respect to sensitivity, the results of these studies are not encouraging.

2. Nucleic acid liberation

In order to exclude false positives during PCR detection of spores, it is necessary to lyse the remaining vegetative cells and destroy their nucleic acids prior to releasing the spores nucleic acids. Liberation of nucleic acids from spores has been accomplished using a number of different techniques including microwave treatment,

mechanical rupture and germination, with the latter two techniques being equally efficient and sensitive enough to identify single spores (81).

a) Microwave treatment

Vaid et al. (82) have investigated DNA liberation from bacterial spores using a high pressure microwave apparatus which heats samples to temperatures as high as 200 °C. However, no particular efforts were made to evaluate the sensitivity of the technique. Similarly, *Herman* et al. (63, 64, 83) have reported the liberation of DNA from spores by a microwave treatment. However, they also found that mechanical disruption of spores by a minibead beater led to higher amounts of liberated DNA than a microwave treatment did (64).

b) Germination

The efficiency of spore germination depends on a variety of factors including the strain, heat activation, temperature, the availability of triggering nutrients (germinants) and finally the age of the spore (75, 84–88). This means that a germination protocol has to be optimized for the individual group of strains to be detected. If, for example, quantitative PCR is to be performed in order to estimate the ratio between mesophilic and psychrotolerant *B. cereus* spores, it is vital to achieve equivalent germination efficiencies. According to *Griffiths* and *Phillips* (9), psychrotolerant *B. cereus* spores have germination optima at 5 and 15 °C, whereas mesophilic spores have their optima at 15 and 30 °C. Hence, a temperature of 15 °C could be chosen to ensure optimal germination for both species. Apart from these individual requirements, the major drawback of using germination protocols is the long incubation time involved. *Reif* et al. (81), for example, report that it is necessary to incubate the spores for a period of 4.5 hours, followed by a lysis step of 30 minutes.

c) Mechanical rupture

Mechanical disruption of spores in a mini-bead beater for 10 minutes with 0.1 mm glass or zirconia beads, yielded similar results to germination but in a much shorter time. This was indicated by equivalent sensitivity being obtained in PCR assays for germinated and mechanically disrupted *B. anthracis* spores (81, 85). Similarly, *Herman* et al. (64) were able to detect a single *C. tyrobutyricum* spore in raw milk after chemical extraction, centrifugation, mechanical rupture and a subsequent two-step PCR.

Specific primed PCR

Specific primed PCR uses one, or multiple pairs (multiplex PCR) of primers to identify a defined organism by targeting a nucleic acid sequence that is unique to that organism. This technique enables an organism to be detected even in the presence of 10^6-fold higher background flora (89). Many primers specific for the detection of sporeformers that are frequently associated with the spoilage of refrigerated food or with food poisoning have been published (table 2).

PCR performed using primers that target enterotoxin genes, cannot necessarily be used to establish whether a strain produces toxins, since these genes may not be

Table 2
PCR primers for genes of pathogenic or food spoilage sporeformers

Strains	*Target gene*	*Reference*
all *B. cereus*-group strains	cerolysin AB	90
all *B. cereus*-group strains	cold shock protein F (*cspF*)	91
psychrotolerant *B. cereus*-group strains	cold shock protein A (*cspA*)	91, 92
psychrotolerant *B. cereus*-group strains	16S rDNA	93
mesophilic *B. cereus*-group strains	16S rDNA	93
enterotoxic *B. cereus*	haemolysin *(hblA)*	94
enterotoxic *B. cereus*	haemolysin *(hlb)*	62
enterotoxic *B. cereus*	haemolysin BL (B component)	95
enterotoxic *B. cereus*	enterotoxin *(bceT)*	96
B. cereus, B. thuringiensis	phosphatidylinositol-specific phospholipase C *(pl-plC)*	97

expressed under all conditions. Milk, for example is an unsuitable medium for enterotoxin production by *B. cereus,* as it lacks glucose and free amino acids (98). Hence, screening refrigerated dairy products for toxin producing strains using the aforementioned PCR will give false positives, and is therefore not practical.

In order to asses the microbiological quality of dairy products with respect to their shelf-life, we have designed two PCR assay for the detection of *B. weihenstephanensis* (psychrotolerant *B. cereus* group strains) (91, 93). These assays could prove to be valuable tools for the dairy industry, taking into consideration that psychrotolerant *B. cereus* are the dominant spoilage organisms in pasteurized refrigerated dairy products. The assays were developed around two independent gene sequences that encode molecular determinants believed to be involved in psychrotolerance, namely 16S rRNA (93) and the major cold-shock protein CspA (91).

The latter assay, called *cspF/cspA* assay (91), is designed as a duplex PCR that is based on the major cold-shock protein genes *cspF* and *cspA,* and incorporates discriminatory primers specific to these two genes. The *cspF* primers are capable of detecting strains of the entire the *B. cereus* group, whereas the *cspA* primers are specific for the psychrotolerant strains of this group *(B. wheihenstepanensis).* Figure 1 illustrates the principle of this assay.

Our second assay, the 16S-assay (93), is a duplex PCR based on two 16S rDNA signatures which allow to discriminate psychrotolerant and mesophilic strains of the *B. cereus* group. Figure 2 explains how this assay works.

Currently we are extending the range of available primer systems to other cold-tolerant sporeformers known to cause problems for the food industry.

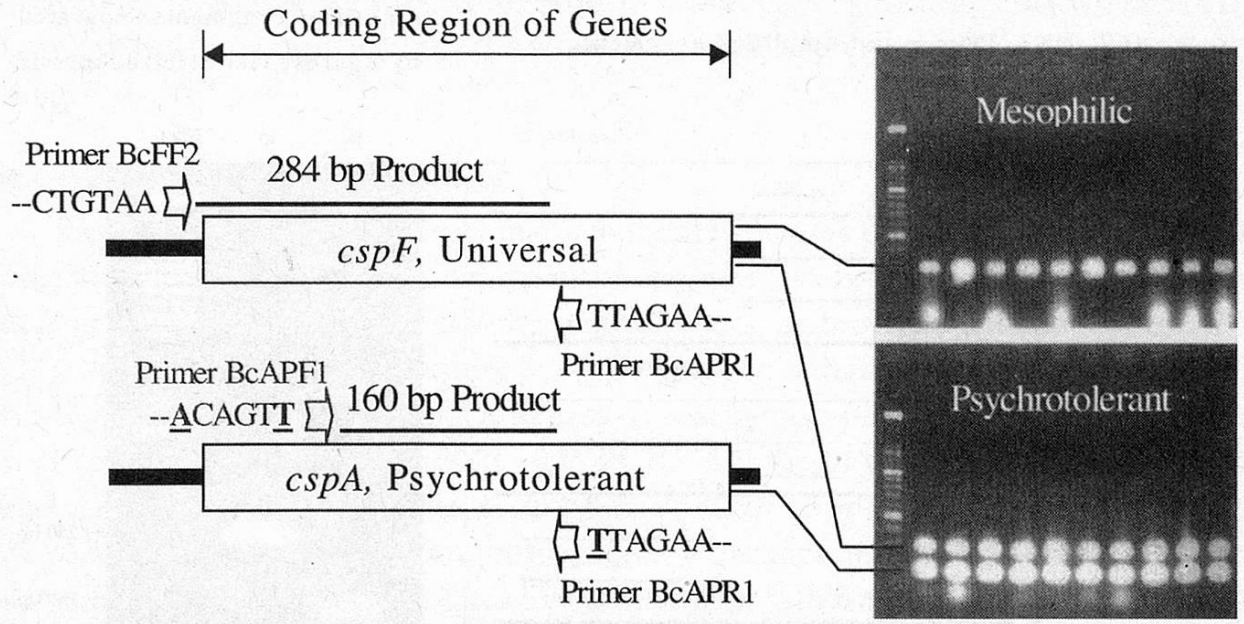

Figure 1 **The *cspA/cspF* assay to detect the psychrotolerant species *B. weihenstephanensis***

Left of the figure shows a schematic diagram of *cspA* and *cspF*, the two genes used as PCR target sequences for the differentiation of psychrotolerant and mesophilic strains of the *B. cereus* group. The approximate positions at which each of the three primers hybridizes to each of the genes and the size of the resulting PCR products are indicated accordingly. Signature bases, allowing PCR amplification of psychrotolerant *cspA* only, are underlined and in grey. Two pictures of an ethidium bromide stained agarose gel with examples of DNA amplification from psychrotolerant and mesophilic strains of the *B. cereus* group gained using this PCR assay are shown (taken from ref. 91).

Detection limits of PCR

The detection limit of a conventional one-step PCR with subsequent agarose gel electrophoresis and ethidium bromide staining, is about 100 copies of the target gene (81). After an overnight culture enrichment, PCR protocols of relatively low sensitivity can be used (62). However, to obtain higher sensitivities other approaches must be taken. Firstly, PCR fidelity can be greatly increased by applying a two-step amplification procedure incorporating the use of nested primers. This technique enables the detection of a single copy of the target gene, which is a 100-fold increase in sensitivity compared to the one-step PCR protocol (62–64). Secondly, the detection of the amplified DNA can be enhanced about 100-fold (in comparison to ethidium bromide staining) by using labeled probe hybridization techniques (81, 89, 90, 99).

Quantitative PCR

After the pre-enrichment of an organism, quantitative PCR is rarely feasible. In this case, the use of MPN-pre-enrichment techniques with subsequent PCR of the

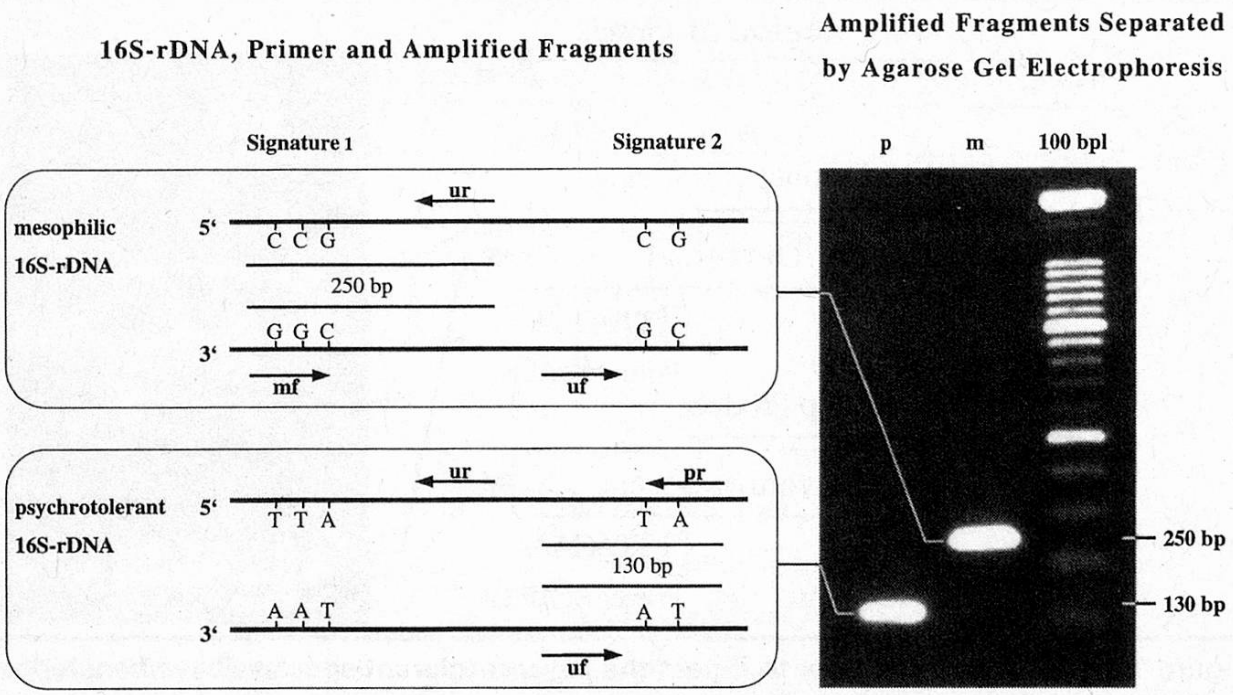

Figure 2 **The 16S-rDNA assay for detection of the psychrotolerant species *B. weihenstephanensis***

The mesophilic-specific forward primer (mf) only anneals when a mesophilic signature 1 is present, the psychrotolerant-specific reverse primer (pr) only anneals if a psychrotolerant signature 2 is present. The universal reverse and forward primers (ur, uf) anneal in either case. The distance of their binding site to the mesophilic or psychrotolerant signature, respectively, determines the length of the amplified DNA. A 250-bp fragment detected on the agarose gel indicates a mesophilic target (lane m), a 130-bp fragment indicates a psychrotolerant target (lane p). The molecular weight marker is a 100-bp ladder (100 bpl) (taken from ref. 93).

aliquots has been introduced, especially with regard to the small numbers of spores that are usually present in naturally contaminated samples (66).

Direct, real-time-quantitative PCR provides the possibility of estimating contamination level rapidly and with a high throughput (100). This is achieved by continuously monitoring DNA amplification using one of three different fluorescence techniques, in which fluorescence is monitored from (i) the double-strand-DNA specific dye SYBR Green I, (ii) a decrease in fluorescein quenching by rhodamine after 5'-exonuclease hydrolysis of a dual-labeled hydrolysis probe and (iii) Fluorescence Resonance Energy Transfer between adjacent probes, labelled with a donor and an acceptor dye respectively (101, 102). Complete amplification and analysis requires only 10–20 minutes if a microvolume fluorimeter with rapid temperature control is available (103). The precision of SYBR Green I detection is limited to a minimum of 100 copies of a target by nonspecific product formation, although the presence or absence of a template can be distinguished (104). Real-time-quantitative PCR can be performed either using Boehringer Mannheim's LightCycler™ which is extremely fast but has a capacity for only 32 samples, or PE Applied Biosystem's

Sequence Detection Systems, Gene Amp®5700 and ABI PRISM®7700, which are relatively slow but compatible to the 96-well-format, allowing high throughput screening.

Contamination route analysis

Fast and accurate identification of bacterial isolates is becoming increasingly important to accelerate contamination route analysis. Traditional identification protocols involve slow and cumbersome methods such as biochemical characterization (44), or phage typing, and require the use of reagents that are only available in reference laboratories (105). Rapid and easy-to-use genotypic fingerprinting methods, such as Arbitrarily Primed PCR (AP-PCR) (106) may overcome these problems and are applicable in industrial settings. In contrast to specific primed PCR, which detects the organism being sought from a high background flora, these methods are designed to classify single isolates by comparison with fingerprints of other strains. This way, differentiation at the subspecies level is possible and contamination routes of individual food contaminating strains can be assessed.

Three techniques were originally described to discriminate organisms by creating amplification based fingerprints: Arbitrarily Primed PCR (AP-PCR) (106), Random Amplified Polymorphic DNA (RAPD) (107) and DNA Amplification Fingerprinting (DAF) (108). These techniques have meanwhile been widely applied. Like *Bassam* et al. (109), we found the DAF approach of *Caetano-Anollés* (108) to give a higher resolution than the more common RAPD approach (105, 106), due to altered amplification conditions, such as primer concentration and the modified thermal cycling profile.

Given below are some examples of RAPDs which have been performed to type *Bacillus* isolates from food commodities. *Lechner* et al. (12) were able to classify *B. cereus*-group isolates from pasteurized refrigerated milk into a mesophilic and a psychrotolerant cluster. *Ronimus* et al. (110) applied RAPD to trace over 2000 mesophilic and thermophilic *Bacillus* species from feedstock to the final product of a food processing line. *Nilsson* and *Christiansson* (111) traced contamination routes of *B. cereus* in a dairy plant, finding that most of the strains originated from the raw milk. In order to make large-scale typing of *B. cereus* easier, *Nilsson* et al. (112) introduced a rapid DNA preparation method for RAPD.

Alternative PCR-based fingerprinting methods not as common as RAPD, are PCR-Restriction Fragment Length Polymorphisms (PCR- RFLP) (e.g. applied by 113), Amplification Fragment Length Polymorphism (AFLP) analyses (114), Repetitive Element sequence based PCR (REP-PCR) (e.g. applied by 115), and PCR-Single-Strand Conformation Polymorphism Analysis (PCR-SSCP) (116).

Considering the variety of different fingerprinting techniques it becomes clear that data exchange between different user groups remains impossible. In order to allow the exchange of fingerprinting data, the implementation of a standardized fingerprinting system would be inevitable.

Conclusion

The food industries problems, associated with psychrotolerant aerobic sporeformers have lead to diverse research activities concerning these bacteria, ranging from ecological studies to the design of rapid PCR-based detection and identification systems. Classical detection methods involve incubation steps, which may take up to a fortnight. Thus, they are not capable to detect psychrotolerant bacteria in foods before the latter leave the processing plant. Now, PCR-technology makes rapid detection and identification of these psychrotolerant food spoilage and pathogenic microorganisms possible, paving the way for their control.

Summary

Aerobic sporeformers are present in a wide range of foodstuffs. Due to their heat resistant spores they are associated with major spoilage problems in pasteurized products. Psychrotolerant aerobic sporeformers are able to spoil food at temperatures lower than 7 °C. This spoilage flora is mainly dominated by *Bacillus cereus/weihenstephanensis* strains. Some of these strains are toxin producers and may cause food poisoning. Until now, their detection involved extended enrichment steps at low temperature. However, recent advances in molecular biology have led to the development of several PCR-assays for the rapid detection and identification of psychrotolerant sporeformers.

Zusammenfassung

Aerobe Sporenbildner sind ubiquitär verbreitet und kommen in Lebensmitteln häufig als Kontaminanten vor. Für pasteurisierte Lebensmittel stellen die hitzeresistenten Sporen aufgrund der geringen Konkurrenzflora ein besonderes Problem dar. Psychrotolerante aerobe Sporenbildner können auch bei Temperaturen unter 7 °C zum Verderb von Lebensmitteln führen. *Bacillus cereus/weihenstephanensis*-Stämme dominieren dabei häufig die Verderbsflora. Einige dieser Stämme sind Toxinbildner und können Lebensmittelvergiftungen hervorrufen. Bisher war ihr Nachweis und ihre Abgrenzung von mesophilen Varietäten nur auf dem langwierigen Weg der Kälteanreicherung möglich. Mit Hilfe neuer PCR-Verfahren ist seit kurzem die schnelle und spezifische Detektion und Typisierung von psychrotoleranten Sporenbildnern möglich.

Résumé

Les sporulés aérobies sont présents dans de nombreux produits alimentaires. En raison de leurs spores thermorésistentes ils sont associés aux principaux problèmes de détérioration des produits pasteurisés. Les sporulés aérobies psychrotolérants peuvent détériorer les produits alimentaires aux températures inférieures de 7 °C. Cette flore d'altération est principalement dominée par les souches de Bacillus cereus/weihenstephanensis. Certaines de ces souches sont productrices de toxines et peuvent causer des intoxications alimentaires. Jusqu'ici, leur détection impliquait de

longues étapes d'enrichissement à basse température. Cependant, les progrès récents en biologie moléculaire ont permis le développement de plusieurs techniques de PCR pour la détection et l'identification rapide des sporulés psychrotolérants.

Key words

Bacillus cereus, Bacillus weihenstephanensis, psychrotolerant *Bacillus,* Detection, PCR

References

1 *Kessler, H. G.* and *Horak, F. P.:* Effect of heat treatment and storage conditions on the keeping quality of pasteurized milk. Milchwissenschaft **39,** 451–454 (1984).

2 *Claus, D.* and *Berkeley, R.C.W.:* Genus *Bacillus Cohn* 1872. In: Sneath, P.H.A. et al. (ed), Bergey's manual of systematic bacteriology, Vol. 2, 1105–1139. Williams & Wilkins, Baltimore 1986.

3 *Niimura, Y., Koh, E., Yanagida, F., Suzuki, K.-I., Komagata, K.* and *Kozaki, M.: Amphibacillus xylanus* gen. nov., sp. nov, a facultativly anaerobic sporeforming xylan-digesting bacterium which lacks cytochrome, quinone and catalase. Int. J. Syst. Bacteriol. **40,** 297–301 (1990).

4 *Wisotzkey, J. D., Jurtschuk jr., P., Fox, G. E., Deinhard, G.* and *Poralla, K.:* Comparative sequence analyses on the 16S rRNA (rDNA) of *Bacillus acidocaldarius, Bacillus acidoterrestris* and *Bacillus cycloheptanicus* and proposal for creation of a new genus, *Alicyclobacillus* gen. nov. Int. J. Syst. Bacteriol. **42,** 263–269 (1992).

5 *Ash, C., Priest, F.G.* and *Collins, M.D.:* Molecular identification of rRNA group 3 bacilli (Ash, Farrow, Wallbanks and Collins) using a PCR probe test. Antonie van Leeuwenhoek. **64,** 253–260 (1993).

6 *Shida, O., Takagi, H., Kadowaki, K.* and *Komagata, K.:* Proposal for two new genera, *Brevibacillus* gen. nov. and *Aneurinibacillus* gen. nov. Int. J. Syst. Bacteriol. **46,** 939–946 (1996).

7 *Spring, S., Ludwig, W., Marquez, M.C., Ventosa, A.* and *Schleifer, K.-H.: Halobacillus* gen. nov., with descriptions of *Halobacillus litoralis* sp. nov. and *Halobacillus trueperi* sp. nov. and transfer of *Sporosarcina halophila* to *Halobacillus halophilus* comb. nov. Int. J. Syst. Bacteriol. **46,** 492–496 (1996).

8 *Heyndrickx, M., Lebbe, L., Kersters, K., De Vos, P., Forsyth, G.* and *Logan, N.A.: Virgibacillus:* a new genus to accomodate *Bacillus pantothenticus* (Proom and Knight 1950). Emended description of *Virgibacillus pantothenticus.* Int. J. Syst. Bacteriol. **48,** 99–106 (1998).

9 *Griffiths, M.W.* and *Phillips, J.D.:* Incidence, source and some properties of psycrotrophic *Bacillus* spp. found in raw and pasteurized milk. J. Soc. Dairy Technol. **43,** 62–66 (1990).

10 *Mayr, R.:* Kältetolerante *Bacillus*-Arten in pasteurisierter Trinkmilch: Flora, Verderbsanalysen und Phagentypisierung von *Bacillus cereus.* Ph.D. Thesis, Technical University of Munich 1997.

11 *Meer, R.R., Wodburn, M.J.* and *Bodyfelt, F.W.:* Identification and characterisation of heat-resistant psychrotrophic bacteria in Oregon grade A raw milk. Dairy Food Environ. San. **13,** 631–637 (1993).

12 *Lechner, S., Mayr, R., Francis, K.P., Prüss, B.M., Kaplan, T., Wiesner-Gunkel, E., Stewart, G.S.A.B.* and *Scherer, S.: Bacillus weihenstephanensis* sp. nov. is a new psychrotolerant species of the *Bacillus cereus* group. Int. J. Syst. Bacteriol. **48,** 1373–1382 (1998).

13 *McKellar, R.C.* (ed.): Enzymes of psychrotrophs in raw food. CRC Press, Boca Raton 1989.

14 *Sørhaug, T.* and *Stepaniak, L.:* Psychrotrophs and their enzymes in milk and dairy products: Quality aspects. Trends Food Sci. Technol. **8,** 35–41 (1997).

15 *Meer, R.R., Baker, J., Bodyfelt, F.W.* and *Griffiths, M.W.:* Psychrotrophic *Bacillus* spp. in fluid milk products: A review. J. Food Protec. **54,** 969–979 (1991).

16 *Müller, U.:* Mikrobiologische Produktverderbnis durch *Bacillus* spp. I: *Bacillus* spp. als bedeutender Vertreter der Mikroflora von Milch und Milchprodukten. DMZ – Lebensmittelind. Milchwirtsch. **116,** 540–550 (1995).
17 *Müller, U.:* Mikrobiologische Produktverderbnis durch *Bacillus* spp. II: Aktivität von *Bacillus*-Proteasen – eine Verderbnishauptursache. DMZ – Lebensmittelind. Milchwirtsch. **116,** 669–675 (1995).
18 *Stadhouders, J.:* Introduction. IDF Bulletin No. **275,** 3 (1992).
19 *Jayne-Williams, D.J.* and *Franklin, J.G.: Bacillus* spores in milk – Part 1. Dairy Sci. Abstr. **22,** 215 (1960).
20 *Choudhery, A.K.* and *Mikolajcik, E.M.:* Bacilli in milk: 2. Sweet curd formation. J. Dairy Sci. **54,** 321–325 (1971).
21 *Stone, M. J.:* The action of the lecithinase of *Bacillus cereus* on the globule membrane of milk fat. J. Dairy Res. **19,** 311–315 (1952).
22 *Stone, M. J.* and *Rowlands, A.:* «Broken» or «bitty» cream in raw and pasteurized milk. J. Dairy Res. **19,** 51–62 (1952).
23 *Kramer, J.M.* and *Gilbert, R.J.: Bacillus cereus* and other *Bacillus* species. In: Doyle, M.P. (ed.), Foodborne bacterial pathogens, p. 21–70. Marcel Dekker, New York 1989.
24 *Granum, P.E.* and *Lund, T.: Bacillus cereus* and its food poisoning toxins. FEMS Microbiol. Lett. **157,** 223–228 (1997).
25 *Granum, P.E.: Bacillus cereus.* In: Doyle, M.P., Beuchat, L. and Montville, T. (eds.), Fundamentals in food microbiology, p. 327–336. ASM Press, Washington D.C. 1997.
26 *Agata, N., Mori, M., Ohta, M., Suwan, S., Ohtani, I.* and *Isobe, M.:* A novel dodecadepsipeptide, cereulide, isolated from *Bacillus cereus* causes vacuole formation in Hep-2 cells. FEMS Microbiol. Lett. **121,** 31–34 (1994).
27 *Beecher, D.J.* and *Wong, A.C.L.:* Tripartite hemolysin BL from *Bacillus cereus.* Hemolytic analysis of component interaction and model for its characteristic paradoxical zone phenomenon. J. Biol. Chem. **272,** 233–239 (1997).
28 *Lund, T.* and *Granum, P.E.:* Comparison of biological effect of the two different enterotoxin complexes isolated from three different strains of *Bacillus cereus.* Microbiology **143,** 3329–3339 (1997).
29 *Granum, P.E.: Bacillus cereus* and its toxins. J. Appl. Bacteriol. Symp. Suppl. **76,** 61S–66S (1994).
30 *van Netten, P., van de Moosdijk, A., van Hoensel, P., Mossel, D.A.A.* and *Perales, I.:* Psychrotrophic strains of *Bacillus cereus* producing enterotoxin. J. Appl. Bacteriol. **69,** 73–79 (1990).
31 *Rowan, N.J.* and *Anderson, J.G.:* Diarrhoeal enterotoxin production by psychrotrophic *Bacillus cereus* present in reconstituted milk-based infant formulae (MIF). Lett. Appl. Microbiol. **26,** 161–165 (1998).
32 *Jackson, S.G., Goodbrand, R.B., Ahmed, R.* and *Kasatiya, S.: Bacillus cereus* and *Bacillus thuringiensis* isolated in a gastroenteritis outbreak investigation. Lett. Appl. Microbiol. **21,** 103–105 (1995).
33 *Asano, S.-I., Nukumizu, Y., Bando, H., Iizuka, T.* and *Yamamoto, T.:* Cloning of novel enterotoxin genes from *Bacillus cereus* and *Bacillus thuringiensis.* Appl. Environ. Microbiol. **63,** 1054–1057 (1997).
34 *Perani, M., Bishop, A.H.* and *Vaid, A.:* Prevalence of β-exotoxin, Diarrheal toxin and specific δ-endotoxin in natural isolates of *Bacillus thuringiensis.* FEMS Microbiol. Lett. **160,** 55–60 (1998).
35 *Grosskopf, J.C.* and *Harper, W.J.:* Role of psychrophilic sporeformers in long life milk. J. Dairy Sci. **52,** 897 (1969).
36 *Barkley, M.B.* and *Delaney, P.:* An occurrence of bitty cream in Australian pasteurized milk. Neth. Milk Dairy J. **34,** 191–198 (1980).

37 *Larsen, H.D.* and *Jørgensen, K.:* The occurrence of *Bacillus cereus* in Danish pasteurized milk. Int. J. Food Microbiol. **34,** 179–186 (1997).
38 *Mayr, R., Eppert, I.* and *Scherer, S.:* Incidence and identification of psychrotrophic (7 °C) *Bacillus* spp. in German HTST pasteurized milk. Milchwissenschaft – Milk Sci. Int. **54,** 26–30 (1999).
39 *Phillips, J.D.* and *Griffiths, M.W.:* Factors contributing to the seasonal variation of *Bacillus* spp. in pasteurized dairy products. J. Appl. Bacteriol. **61,** 275–285 (1986).
40 *Rama Raju, V.V., Satynarayana Chetty, M.* and *Kiran Kumar, M.:* Aerobic psychrotrophic spore forming bacteria in heat treated milk. Cheiron **18,** 15–18 (1989).
41 *te Giffel, M.C., Beumer, R.R., Bonestroo, M.H.* and *Rombouts, F.M.:* Incidence and characterization of *Bacillus cereus* in two dairy processing plants. Neth. Milk Dairy J. **50,** 479–492 (1996).
42 *Wong, H.-C., Chang, M.-H.* and *Fan, J.-Y.:* Incidence and characterization of *Bacillus cereus* isolates contaminating dairy products. Appl. Environ. Microbiol. **54,** 699–702 (1988).
43 *McKinnon, C.H.* and *Pettipher, G.L.:* A survey of sources of heat-resistant bacteria in milk with particular reference to psychrotrophic spore-forming bacteria. J. Dairy Res. **50,** 163–170 (1983).
44 *te Giffel, M.C., Beumer, R.R., Slaghuis, B.A.* and *Rombouts, F.M.:* Occurrence and characterization of (psychrotrophic) *Bacillus cereus* on farms in the Netherlands. Neth. Milk Dairy J. **49,** 125–138 (1995).
45 *Langeveld, L.P.M., Cuperus, F.* and *Stadhouders, J.:* Bacteriological aspects of the keeping quality at 5 °C of reinfected and non-reinfected pasteurized milk. Neth. Milk Dairy J. **27,** 54–65 (1973).
46 *Todd, E.C.D.:* Foodborne disease in Canada – a10-year summary from 1975 to 1984. J. Food Prot. **55,** 123–132 (1992).
47 *Carlin, F.* and *van Leusden, F.:* Quantitative risk analysis of spore-forming bacteria in cooked chilled foods containing vegetables. In: Gaukel, V. and Spiess, W.E.L. (eds), Berichte der Bundesforschungsanstalt für Ernährung BFE-R—98-02, 3rd Karlsruhe Nutrition Symposium – European research towards safer and better food – review and transfer congress, Proceedings part 1: Lectures, 51–58, Karlsruhe 1998.
48 *Burt, R.* and *Wellsteed, S.:* Food safety and legislation in the dairy industry. J. Soc. Dairy Technol. **44,** 80–86 (1991).
49 *Slaten, D.D., Oropeza, R.I.* and *Werner, S.B.:* An outbreak of *Bacillus cereus* food poisoning – are caterers supervised sufficiently. Public Health Rep. **107,** 477–480 (1992).
50 *Thaikruea, L., Pataraarechachai, J., Savanpunyalert, P.* and *Naluponjiragul, U.:* An unusual outbreak of food poisoning. Southeast Asian J. Trop. Med. Public Health **26,** 78–85 (1995).
51 *Shinagawa, K.:* Serology and characterisation of toxigenic *Bacillus cereus.* IDF Bulletin No. **287,** 42–49 (1993).
52 *Khodr, M., Hill, S., Perkins, L., Stiefel, S., Comer-Morrison, C., Lee, S., Patel, D.R., Peery, D., Armstrong, C.W.* and *Miller, G.B.J.: Bacillus cereus* food poisoning associated with fried rice at two child day care centers Virginia, 1993. Morbidity and Mortality Weekly Rep. **43,** 177–178 (1994).
53 *Luby, S., Jones, J., Dowda, H., Kramer, J.* and *Horan, J.:* A large outbreak of gastroenteritis caused by diarrheal toxin-producing *Bacillus cereus.* J. Infect. Dis. **167,** 1452–1455 (1993).
54 *Granum, P.E., Naestvold, A.* and *Gundersby, K.N.:* An outbreak of *Bacillus cereus* food poisoning during the Norwegian ski championship for juniors. Norsk Veterinaerstidsk **107,** 945–948 (1995).
55 *Zastrow, K.D.* und *Schöneberg, I.:* Lebensmittelbedingte Infektionen und Intoxikationen in der Bundesrepublik Deutschland – Ausbrüche 1992. Bundesgesundheitsblatt **37,** 247–251 (1994).
56 *Pan, T.M., Chiou, C.S., Hsu, S.Y., Huang, H.C., Wang, T.K., Chiu, S.I., Yea, H.L., Lee, C.L.:* Food-borne disease outbreaks in Taiwan, 1994. J. Formos. Med. Assoc. **95,** 417–420 (1996).

57 *Rahkio, M., Niemi, V.-M.* and *Pakkala, P.:* Foodborne outbreaks in Finland in 1995 and 1996. In: 4th World Congress Foodborne Infections and Intoxications, Abstracts, 172–173, Berlin, 1998.
58 *Mossel, D.A.A., Koopman, M.J.* and *Jongerius, E.:* Enumeration of *Bacillus cereus* in foods. Appl. Microbiol. **15,** 650–653 (1967).
59 *Holbrook, R.* and *Anderson, J.M.:* An improved selective and diagnostic medium for the isolation and enumeration of *Bacillus cereus* in foods. Can. J. Microbiol. **26,** 753–759 (1980).
60 *Szabo, R.A., Todd, E.C.D.* and *Rayman, M.K.:* Twenty-four hour isolation and confirmation of *Bacillus cereus* in foods. J. Food Protec. **47,** 856–860 (1984).
61 *Micheli, M.R.* and *Bova, R.* (eds.): Fingerprinting methods based on arbitrarily primed PCR. Springer, Berlin 1997.
62 *Wang, R.-F., Cao, W.-W.* and *Cerniglia, C.E.:* A universal protocol for PCR detection of 13 species of foodborne pathogens in foods. J. Appl. Microbiol. **83,** 727–736 (1997).
63 *Herman, L., de Block, J.* and *van Rentetghem, R.:* Isolation and detection of *Clostridium tyrobutyricum* cells in semi-soft and hard cheese using the polymerase chain reaction. J. Dairy Res. **64,** 311–314 (1997a).
64 *Herman, L.F., De Block, J.H.G.E.* and *Waes, G.M.A.V.J.:* A direct PCR detection method for *Clostridium tyrobutyricum* in up to 100 milliliters of raw milk. Appl. Environ. Microbiol. **61,** 4141–4146 (1995a).
65 *Pillai, S.D.* and *Ricke, S.C.:* Strategies to accelerate the applicability of gene amplification protocols for pathogen detection in meat and meat products. Crit. Rev. Microbiol. **21,** 239–261 (1995).
66 *Hielm, S., Hyytiä, E., Ridell, J.* and *Korkeala, H.:* Detection of *Clostridium botulinum* in fish and environmental samples using polymerase chain reaction. Int. J. Food Microbiol. **31,** 357–365 (1996).
67 *Broda, D.M., de Lacy, K.M.* and *Bell, R.G.:* Efficacy of heat and ethanol spore treatments for the isolation of psychrotrophic *Clostridium* spp. associated with the spoilage of chilled vacuum-packed meats. Int. J. Food Microbiol. **39,** 61–68 (1998).
68 *Blake, M.R.* and *Weimer, B.C.:* Immunomagnetic detection of *Bacillus stearothermophilus* spores in food and environmental samples. Appl. Environ. Microbiol. **63,** 1643–1646 (1997).
69 *Darquea, D., Swaisgood, H.E.* and *Foegeding, P.M.:* Development and characterization of a bioselective adsorption matrix for removal of *Bacillus cereus* spores from buffer and milk. Lebensm.-Wiss. Technol. **30,** 786–792 (1997).
70 *Koo, K., Foegeding, P.M.* and *Swaisgood, H.E.:* Construction and expression of a bifunctional single-chain antibody against *Bacillus cereus* spores. Appl. Environ. Microbiol. **64,** 2490–2496 (1998a).
71 *Kim, H.U.* and *Goepfert, J.M.:* Efficacy of a fluorescent-antibody procedure for identifying *Bacillus cereus* in Foods. Appl. Microbiol. **24,** 708–713 (1972).
72 *Koo, K., Foegeding, P.M.* and *Swaisgood, H.E.:* Development of a streptavidin conjugated single-chain antibody that binds *Bacillus cereus* spores. Appl. Environ. Microbiol. **64,** 2497–2502 (1998b).
73 *Quinlan, J.J.* and *Foegeding, P.M.:* Monoclonal antibodies for use in detection of *Bacillus* and *Clostridium* spores. Appl. Environ. Microbiol. **63,** 482–487 (1997).
74 *Herman, L.F., De Block, J.H.G.E.* and *Moermans, R.J.B.:* Direct detection of *Listeria monocytogenes* in 25 milliliters of raw milk by a two-step PCR with nested primers. Appl. Environ. Microbiol. **61,** 817–819 (1995b).
75 *Herman, L.F., Vaerewijck, M.J.M., Moermans, R.J.B.* and *Waes, G.M.A.V.J.:* Identification and detection of *Bacillus sporothermodurans* spores in 1, 10 and 100 milliliters of raw milk by PCR. Appl. Environ. Microbiol. **63,** 3139–3143 (1997b).

76 *Lindqvist, R.:* Preparation of PCR samples from food by a rapid and simple centrifugation technique evaluated by detection of *Escherichia coli O157:H7.* Int. J. Food Microbiol. **37,** 73–82 (1997).
77 *Lindqvist, R., Norling, B.* and *Lamberth, S.T.:* A rapid sample preparation method for PCR detection of food pathogens based on buoyant density centrifugation. Lett. Appl. Microbiol. **24,** 306–310 (1997).
78 *Xiang, Y.* and *Griffiths, M. W.:* Removal and concentration of bacteria from milk using isoelectric focusing. In: International Dairy Federation – Symposium on bacteriological quality of raw milk, Proceedings, 162–164, Wolfpassing 1996.
79 *Lantz, P.-G., Tjerneld, F., Hahn-Hägerdal, B.* and *Radström, P.:* Use of aqueous two-phase systems in sample preparation for polymerase chain reaction-based detection of microorganisms. J. Chromatogr. B, **680,** 165–170 (1996).
80 *Sacks, L.E.* and *Alderton, G.:* Behavior of bacterial spores in aqueous polymer two-phase systems. J. Bacteriol. **82,** 331–341 (1961).
81 *Reif, T.C., Malcolm, J., Pillai, S.D.* and *Mitchell, C.:* Identification of capsule-forming *Bacillus anthracis* spores with the PCR and a novel dual-probe hybridization format. Appl. Environ. Microbiol. **60,** 1622–1625 (1994).
82 *Vaid, A.* and *Bishop, A.H.:* The destruction by microwave radiation of bacterial endospores and amplification of the released DNA. J. Appl. Microbiol. **85,** 115–122 (1998).
83 *Herman, L., Rijpens, N., Tcherneva, E.* and *Jersek, B.:* Detection, identification and typing of foodborne bacteria with genetic technology. Cerevisia **21,** 75–77 (1996).
84 *Hashimoto, T., Frieben, F.R.* and *Conti, S.F.:* Microgermination of *Bacillus cereus* spores. J. Bacteriol. **100,** 1385–1392 (1969).
85 *Johns, M., Harrington, L., Titball, R.W.* and *Leslie, D.L.:* Improved methods for the detection of *Bacillus anthracis* by the polymerase chain reaction. Lett. Appl. Microbiol. **18,** 236–238 (1994).
86 *Senesi, S., Cercignani, G., Freer, G., Batoni, G., Barnini, S.* and *Ota, F.:* Structural and stereospecific requirements for the nucleoside-triggered germination of *Bacillus cereus* spores. J. General Microbiol. **137,** 399–404 (1991).
87 *Wilkinson, G.* and *Davies, F.L.:* Germination of spores of *Bacillus cereus* in milk and milk dialysates: effect of heat treatment. J. Appl. Bacteriol. **36,** 485–496 (1973).
88 *Rajarathinam, R., Shankar, P.A.* and *Laxminarayana, H.:* Effect of heat shock on germination of spores of *Bacillus* spp. in milk. Indian J. Dairy Sci. **40,** 309–314.
89 *Baez, L.A., Juneja, V.K.* and *Sackitey, S.K.:* Chemiluminescent enzyme immunoassay for detection of PCR-amplified enterotoxin A from *Clostridium perfringens.* Int. J. Food Microbiol. **32,** 145–158 (1996).
90 *Schraft, H.* and *Griffiths, M.W.:* Special oligonucleotide primers for the detection of lecithinase positive *Bacillus* spp. by PCR. Appl. Environ. Microbiol. **61,** 98–102 (1995).
91 *Francis, K.P., Mayr, R., von Stetten, F., Stewart, G.S.A.B.* and *Scherer, S.:* Discrimination of psychrotrophic and mesophilic strains of the *Bacillus cereus* group by PCR targeting of major cold shock protein genes. Appl. Environ. Microbiol. **64,** 3525–3529 (1998).
92 *Francis, K.P.* and *Stewart, G.S.A.B.:* Detection and speciation of bacteria through PCR using universal major cold-shock protein primer oligomers. J. Ind. Microbiol. Biotechnol. **19,** 286–293 (1997).
93 *von Stetten, F., Francis, K.P., Lechner, S., Neuhaus, K.* and *Scherer, S.:* Rapid discrimination of psychrotolerant and mesophilic strains of the *Bacillus cereus* group by PCR targeting of 16S rDNA. J. Microbiol. Meth. **34,** 99–106 (1998).
94 *Mäntynen, V.* and *Lindstrøm, K.:* A rapid PCR-Based DNA Test for enterotoxic *Bacillus cereus.* Appl. Environ. Microbiol. **64,** 1634–1639 (1998).

95 *Granum, P.E., Andersson, A., Gayther, C., Te Giffel, M., Larsen, H., Lund, T.* and *O´Sullivan, K.:* Evidence for a further enterotoxin complex produced by *Bacillus cereus.* FEMS Microbiol. Lett. **141,** 145–149 (1996).
96 *Agata, N., Ohta, M., Arakawa, Y.* and *Mori, M.:* The bceT gene of *Bacillus cereus* encodes an enterotoxic protein. Microbiology **141,** 983–988 (1995).
97 *Damgaard, P.H., Jacobsen, C.S.* and *Soerensen, J.:* Development and application of a primer set for specific detection of *Bacillus thuringiensis* and *Bacillus cereus* in soil using magnetic capture hybridization and PCR amplification. System. Appl. Microbiol. **19,** 436–441 (1996).
98 *Rowan, N.J.* and *Anderson, J.G.:* Maltodextrin stimulates growth of *Bacillus cereus* and synthesis of diarrheal enterotoxin in infant milk formulae. Appl. Environ. Microbiol. **63,** 1182–1184 (1997).
99 *Aranda, E., Rodríguez, M.M., Asenio, M.A.* and *Córdoba, J.J.:* Detection of *Clostridium botulinum* types A, B, E and F in foods by PCR and DNA probe. Lett. Appl. Microbiol. **25,** 186–190 (1997).
100 *Batt, C.H.:* Molecular diagnostics for dairy-borne pathogens. J. Dairy Sci. **80,** 220–229 (1997).
101 *Wittwer, C.T., Herrmann, M.G., Moss, A.A.* and *Rasmussen, R.P.:* Continuous fluorescence monitoring of rapid cycle DNA amplification. Biotechniques **22,** 130–131, 134–138 (1997).
102 *Heid, C.A., Stevens, J., Livak, K.J.* and *Williams, P.M.:* Real time quantitative PCR. Genome Res. **6,** 986–994 (1996).
103 *Wittwer, C.T., Ririe, K.M., Andrew, R.V., David, D.A., Gundry, R.A.* and *Balis, U.J.:* The LightCycler: a microvolume multisample fluorimeter with rapid temperature control. Biotechniques **22,** 176–181 (1997).
104 *Morrison, T.B., Weis, J.J.* and *Wittwer, C.T.:* Quantification of low-copy transcripts by continuous SYBR Green I monitoring during amplification. Biotechniques **24,** 954–958, 960, 962 (1998).
105 *Chachaty, E., Saulnier, P., Martin, A., Mario, N.* and *Andremont, A.:* Comparison of ribotyping, pulsed-field gel electrophoresis and random amplified polymorphic DNA for typing *Clostridium difficile* strains. FEMS Microbiol. Lett. **122,** 61–68 (1994).
106 *Welsh, J.* and *McClelland, M.:* Fingerprinting genomes using PCR with arbitrary primers. Nucleic Acids Research. **18,** 7213–7218 (1990).
107 *Williams, J.G.K., Kubelik, A.R., Livak, K.J., Rafalski, J.A.* and *Tingey, S.V.:* DNA polymorphisms amplified by arbitrary primers are useful as genetic markers. Nucleic Acids Research **18,** 6531–6535 (1990).
108 *Caetano-Anollés, G., Bassam, B.J.* and *Tresshoff, P.M.:* DNA amplification fingerprinting: a strategy for genome analysis. Plant. Mol. Biol. Rep. **9,** 292–305 (1991).
109 *Bassam, B.J., Caetano-Anolles, G.* and *Gresshoff, P.M.:* DNA amplification fingerprinting of bacteria. Appl. Microbiol. Biotechnol. **38,** 70–76 (1992).
110 *Ronimus, R.S., Parker, L.E.* and *Morgan, H.W.:* The utilization of RAPD-PCR for identifying thermophilic and mesophilic *Bacillus* species. FEMS Microbiol. Lett. **147,** 75–79 (1997).
111 *Nilsson, J.* and *Christiansson, A.:* A RAPD-PCR fingerprinting method for differentiation of *Bacillus cereus* strains with application to dairy milk processing lines. In: Food Associated Pathogens, **207,** Uppsala, 1996.
112 *Nilsson, J., Svensson, B., Ekelund, K.* and *Christiansson, A.:* A RAPD-PCR method for large-scale typing of *Bacillus cereus*. Lett. Appl. Microbiol. **27,** 168–172 (1998).
113 *Schraft, H., Steele, M., McNab, B., Odumeru, J.* and *Griffiths, M.W.:* Epidemiological typing of *Bacillus* spp. isolated from food. Appl. Environ. Microbiol. **62,** 4229–4232 (1996).
114 *Janssen, P., Coopman, R., Huys, G., Jean, S., Bleeker, M., Vos, P., Zabeau, M.* and *Kerstets, K.:* Evaluation of the DNA fingerprinting method AFLP as a new tool in bacterial taxonomy. Microbiol. **142,** 1881–1893 (1996).

115 *Herman, L., Heyndrickx, M.* and *Waes, G.:* Typing of *Bacillus sporothermodurans* and other *Bacillus* species isolated from milk by repetitive element sequence based PCR. Lett. Appl. Microbiol. **26,** 183–188 (1998).
116 *Widjojoatmodjo, M.N., Fluit, A.D.C.* and *Verhoef, J.:* Molecular identification of bacteria by fluorescence based PCR-single strand conformation polymorphism analysis of the 16S rRNA gene. J. Clin. Microbiol. **33,** 2601–2606 (1995).

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