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DETECTION OF VIABLE BUT NONCULTURABLE CELLS OF *LISTERIA MONOCYTOGENES* WITH THE USE OF DIRECT EPIFLUORESCENT FILTER TECHNIQUE

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ABSTRACT

Direct epifluorescent filter technique was studied as a method to enumerate *Listeria monocytogenes* in artificially contaminated cheese. Occurrences of viable but nonculturable (VBNC) cells of *L. monocytogenes* prompted us to investigate the viability of this pathogen in hard cheese during refrigerated storage under different packaging conditions. This study compared the results of two enumeration methods: epifluorescent microscopic counting and plating. The microscopic enumeration was based on fluorescent staining with carboxyfluorescein diacetate (CFDA) for tracing cells that display metabolic activity. In this study, the *L. monocytogenes* persisted well throughout a prolonged storage of cheese, regardless of the packaging conditions. Based on a discrepancy between CFDA and plate count results, a dormant fraction of VBNC cells was distinguished, thus greater quantities of VBNC cells were estimated along with storage time. We conclude that direct epifluorescent filter technique may be an attractive approach for assessing bacterial viability, especially for pathogens with the ability to enter a dormant state.

PRACTICAL APPLICATIONS

Listeria monocytogenes is a foodborne pathogen with a remarkable long-term ability to persist in a variety of ready-to-eat foods. The plating method for enumeration of *L. monocytogenes* in food is well established, but it is time consuming and relies only on cell reproduction, so that injured and nonculturable cells have no chance to be counted. As a solution, we describe a rapid method for the enumeration of *L. monocytogenes* in cheese using the direct epifluorescent filter technique. This method is based on fluorescent staining of cells with a metabolic activity indicator and cell counting with the help of epifluorescence microscopy. The method presented in this study allows the rapid enumeration of both active and nonculturable cells of *L. monocytogenes* and could be used by predictive microbiologists for precise estimation of the safety of dairy products with respect to pathogenic bacteria.

INTRODUCTION

Precise determination of bacterial viability in food products remains a challenging task for virtually all techniques because of, e.g., the complexity of food matrices and composition, the low number of distributed cells in foods, the stress encountered by cells during food processing and storage (López-Campos *et al.* 2012). These especially concern pathogenic species, including *Listeria monocytogenes*. *L. monocytogenes* appears as facultative anaerobe and psychrotroph, and its adaptability suits growth and survival in processed, refrigerated foods (Oliveira *et al.* 2004; Raghu 2013). *L. monocytogenes* spreads by a wide range of ready-to-eat (RTE) foods, including dairy products, and

causes foodborne disease listeriosis (Ikeda *et al*. 2009; Raghu 2013). The occurrence of *L. monocytogenes* is usually due to post-pasteurization contamination from plant environments (Gougouli *et al*. 2008; Rosshaug *et al*. 2012). Contaminated RTE products may therefore establish a health hazard for consumers. Hence, it would be of interest to assess the viability of *L. monocytogenes* in diverse RTE products under different conditions.

As traditional culture methods for assessing bacteria in foods are based on the use of synthetic media, they are laborious and time consuming. Traditional methods are also not fully sufficient in viability assessment because they refer only to bacterial multiplication, which can be hampered because of environmental stresses (Papadimitriou *et al*. 2006). An alternative approach to traditional methods is to use quantitative molecular tools, e.g., fluorescence *in situ* hybridization (FISH) and quantitative real-time polymerase chain reaction (qPCR). FISH can be applicable, while it detects targeted microbial cells based on their specific rRNA sequences. Therefore, it can be used to identify various bacteria and analyze complex bacterial communities (Ikeda *et al*. 2009). The downside aspect of this method is connected with limited capabilities in bacterial viability assessment, depending on rRNA decay after cell death. Although rRNA decays more rapidly after death in comparison with DNA, the levels of rRNA can remain high enough for dead cells to be false-positively detected as live (Lahtinen *et al*. 2006). qPCR also has the potential to detect nonviable cells because of the already mentioned DNA stability after cell death. In view of these disadvantages, a more promising molecular method is the quantitative real-time reverse transcriptase polymerase chain reaction based on mRNA detection, but as it is strongly dependent upon sufficient production of targeted mRNA, a lack of its expression in living cells makes it difficult to distinguish from food matrices (Zhang *et al*. 2011). In spite of the importance of molecular tools, there is still a need to develop simple, cost-effective procedures to monitor bacterial viability and understand their behavior in different food matrices. The application of direct epifluorescent filter technique (DEFT) in conjunction with fluorescent viability staining may be an answer for increased demands for assessment of bacterial viability and effectiveness. Fluorescent dyes reflect structural and functional features of bacteria distinct from bacterial culturability, e.g., membrane integrity, intracellular enzymatic activity, membrane potential and may also be helpful in estimating dormancy state (called viable but nonculturable [VBNC] or active but nonculturable) (Oliver 2005a). The clear resolution of cells in different physiological states (live, dead and dormant) is highly required for food safety assurance, especially when it comes to human pathogens, which may remain VBNC and retain virulence, e.g., upon resuscitation (Oliver 2009).

To determine the viability of *L. monocytogenes*, we used two methods of enumeration. These included plate counting and epifluorescent microscopic counting with fluorochrome labeling, carboxyfluorescein diacetate (CFDA). CFDA was used to determine the intracellular activity of nonspecific esterases of cells. CFDA is a nonfluorescent precursor which, when hydrolyzed with esterases inside the cell, is transformed into fluorescence product carboxyfluorescein (Papadimitriou *et al*. 2006). Cheese samples contaminated with *L. monocytogenes* and kept under refrigeration in different packaging conditions were used as models of food storage. This paper presents the viability of *L. monocytogenes* in cheese under different packaging conditions and cell parameters: intracellular enzymatic activity (CFDA counts) and culturability (plate counts) were chosen for a description and VBNC-state investigation.

MATERIALS AND METHODS

Experimental Design

Cheese samples of long-ripened hard cheese from retail market were prepared by artificial contamination with *L. monocytogenes*. To do so, cheese was shredded and transferred aseptically to sterile packaging bags with an overall weight of 10 g of cheese and then inoculated with *L. monocytogenes* ATCC 19112 culture to a final concentration of 6–7 $\log_{10}$ cfu/g. Contaminated cheese samples were packaged in four different ways, absorbent packaging (AP), modified atmosphere packaging (MAP), vacuum packaging (VP) – all variants based on removal of air from the packages – and seal packaging (SP), the only variant without removing air from the package. All packaging variants were prepared in triplicate and stored for 3 months under refrigeration at 6C. Sampling was performed at selected time intervals: 0, 30, 60 and 90 days after homogenization in stomacher bags with 2% (w/v) sodium citrate solution (Poch S.A., Gliwice, Poland) providing a 1:10 cheese–solution ratio.

Microbiological Analysis

Fluorescent Staining. Prior to staining with CFDA (5-[and-6-]-carboxyfluorescein diacetate) (Biochemika Fluka, Buchs, Switzerland), cell suspensions from homogenized cheese in sodium citrate solutions were prepared in phosphate-buffered saline (0.1 M, pH 7.8). Cell suspensions were incubated with 50 µM CFDA delivered in anhydrous dimethyl sulfoxide (Sigma, Poznań, Poland) for 35 min in the dark at 37C. The stained cell suspensions were filtered on black polycarbonate filters: Ø 13 mm, 0.22 µm pore size (Millipore, Billerica, MA) using a vacuum filtering device (Millipore). The air-dried filters were coated with

nonfluorescent immersion oil (Molecular Probes, Invitrogen, Eugene, OR) on microscopic slides underneath cover slips and stored at –20C until microscopic analysis. Microscopic analysis was performed with the OLYMPUS BX51 epifluorescent microscope equipped with Digital Color Camera XC10 (Olympus, Hamburg, Germany) and a FITC (U-MNB2, 470–490 nm) filter (Olympus, Hamburg, Germany). Images were analyzed using the CellSens Dimension System (Olympus). The mean values of green cell counts from 10 to 20 fields per assay were further calculated as $\log_{10}$ cells/g. Beforehand, an optimizing procedure was established in order to avoid overestimation of *L. monocytogenes* cells in a heterogeneous environment of cheese by native cheese microflora. Cell suspensions from serial dilutions of cheese both with and without *L. monocytogenes* were fluorescently stained and analyzed by epifluorescent microscopy. Micrographs were taken from all cheese dilutions and the dilution where no green cells per field from noncontaminated with *L. monocytogenes* cheese samples and single green cells per field from contaminated with *L. monocytogenes* cheese samples was proven to be suitable for estimation of active *L. monocytogenes* cells in cheese storage models.

Plate Counting

Appropriate artificially contaminated with *L. monocytogenes* ATCC 19112 cheese dilutions prepared from homogenized cheese in sodium citrate solutions were plated on the Ottaviani and Agosti agar (Merck, Darmstadt, Germany) in duplicate. The culturable cell counts were determined after 48 h of incubation at 37C under aerobic conditions and calculated as $\log_{10}$ cfu/g.

Statistical Analysis

To describe changes in both CFDA and plate counts of *L. monocytogenes* in cheese during refrigerated storage under all packaging conditions, an analysis of variance with repeated measurements was performed. A one-way analysis of variance and Tukey's HSD (Honestly Significant Difference) test were used to compare the cells cfu/g in view of different packaging conditions. Mann–Whitney *U*-test was used for comparison of CFDA and plate counts of *L. monocytogenes* in each sampling time and packaging variant. The significance of differences was analyzed with respect to $P < 0.05$ using Statistica program ver. 9 (StatSoft Inc., Tulsa, OK). The following equation was formulated for estimating the number of VBNC cells: $c = (a_x - a_y) - b$, in which c is number of VBNC cells per gram after x days of storage; a_x is $\log_{10}$ cells per gram after x days of storage based on CFDA counts; a_y is $\log_{10}$ cfu per gram after x days of storage based on plate counts; b is 1.0 $\log_{10}$ unit, a value reflecting approximate difference between colony on plates and microscopically detected cell counts.

RESULTS

Results of *L. monocytogenes* enumeration revealed significant differences between CFDA and plate counts at each sampling point during cheese storage, regardless of the packaging conditions ($p_{0days} = 0.003$; $p_{30,60,90days} < 0.001$). CFDA counts of the four packaging systems did not change significantly during storage ($p_{SP} = 0.072$; $p_{AP} = 0.086$; $p_{MAP} = 0.122$; $p_{VP} = 0.134$); on the other hand, plate counts decreased slowly and significantly during storage ($p_{SP} = 0.002$; $p_{AP} = 0.003$; $p_{MAP} = 0.006$; $p_{VP} = 0.002$) by 0.5–1.0 $\log_{10}$ unit, depending on the packaging system applied (Table 1). The longer storage time, the greater the difference between CFDA and plate counts. The magnitude of culturability loss was greater for vacuum-packaged cheese samples than for the rest of the packaging conditions. Statistical calculations confirmed these observations by indication of significant differences between vacuum-packaged and seal-packaged ($P = 0.006$), absorbent-packaged ($P < 0.001$) and modified atmosphere-packaged ($P < 0.001$) cheese samples. As a result of underestimation of the plating method compared with the DEFT method, cells in a VBNC state were estimated (Fig. 1). In our study, this refers to cells, which maintain their intracellular esterase activity but are not able to grow on synthetic media. For

TABLE 1. CHANGES IN *LISTERIA MONOCYTOGENES* ATCC 19112 COUNTS IN CHEESE DURING LONG-TERM STORAGE UNDER REFRIGERATION IN FOUR PACKAGING SYSTEMS AND ENUMERATED WITH TWO METHODS

	Sealed		Absorbent		Modified atmosphere		Vacuum	
Storage time (days)	CFDA count	Plate count	CFDA count	Plate count	CFDA count	Plate count	CFDA count	Plate count
0	7.0 ± 0.2	5.9 ± 0.2	7.0 ± 0.2	5.9 ± 0.2	7.0 ± 0.2	5.9 ± 0.2	7.0 ± 0.2	5.9 ± 0.2
30	7.3 ± 0.0	5.6 ± 0.1	7.3 ± 0.2	5.6 ± 0.1	7.0 ± 0.1	5.6 ± 0.1	7.2 ± 0.2	5.5 ± 0.1
60	6.8 ± 0.5	5.0 ± 0.1	7.2 ± 0.2	5.2 ± 0.1	7.0 ± 0.3	5.5 ± 0.1	7.0 ± 0.2	5.1 ± 0.1
90	7.2 ± 0.1	5.3 ± 0.2a	7.5 ± 0.0	5.4 ± 0.1a	7.3 ± 0.1	5.3 ± 0.0a	7.3 ± 0.1	4.9 ± 0.1a

The results are means (±SD) of three replicate storage systems, each expressed as $\log_{10}$ cells (cfu)/g.

a Statistically significant downward tendency.

CFDA, carboxyfluorescein diacetate.

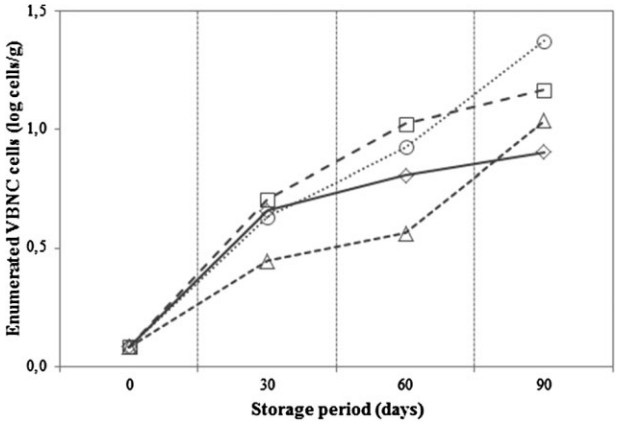

FIG. 1. ESTIMATION OF VIABLE BUT NONCULTURABLE (VBNC) CELL COUNTS OF *LISTERIA MONOCYTOGENES* ATCC 19112 IN CHEESE DURING REFRIGERATED STORAGE IN FOUR PACKAGING SYSTEMS: ◇ SEAL PACKAGING; □ ABSORBENT PACKAGING; △ MODIFIED ATMOSPHERE PACKAGING; ○ VACUUM PACKAGING

all packaging conditions, *L. monocytogenes* cells apparently entered a dormancy state as the estimated VBNC cell numbers were increasing during cheese storage. Estimation of VBNC state found the highest number of VBNC cells in vacuum-packaged cheese on the 90th day of sampling and the lowest in the modified atmosphere-packaged cheese nearly throughout the storage.

DISCUSSION

This study compares two methods of enumeration, epifluorescent microscopic counting and plating, and verifies the suitability of epifluorescent microscopy for the enumeration of *L. monocytogenes* in cheese. The enumeration method based on fluorescent staining was previously applied to various food products for several bacteria (e.g., Auty *et al.* 2001; Gatti *et al.* 2006; Olszewska *et al.* 2012; Papadimitriou *et al.* 2006; Sunny-Roberts and Knorr 2008). Its application was concluded to be a reliable tool, especially when stress conditions were taken into consideration. A similar methodology concerning *L. monocytogenes* in cheese has not been found in the reviewed literature. To date, greater emphasis has been placed on rapid detection of *L. monocytogenes* with the use of PCR and FISH (Oliveira *et al.* 2004; Ikeda *et al.* 2009). They are based on the hybridization of the genomic sequences, which makes them difficult for reliable determination of pathogen viability in food environments. Plate counting is a common method for estimation of bacterial numbers in food products. Still, for culturable cells, plating remains the most suitable tool. However, when it comes to analyzing bacterial physiology under unfavorable conditions, the plate count may be insufficient. *L. monocytogenes* focuses the interest of predictive microbiologists, describing the behavior of *L. monocytogenes* with the use of mathematical models (Gougouli *et al.* 2008; Liu and Puri 2008; Rosshaug *et al.* 2012; Lobacz *et al.* 2013). Thus, relying mostly on culture-dependent methods in this kind of research may be questionable. This paper highlights the point that culturability may not be a synonym of pathogen viability in hard cheese under different packaging conditions. Its importance is connected with VBNC cell existence and, consequently, their ability to retain virulence. *L. monocytogenes* is a bacterium with the ability to enter the VBNC state (Oliver 2005a). What is more, it appears that *L. monocytogenes* VBNC cells are more tolerant to stresses than growing cells (Kastbjerg *et al.* 2009). In several recent reviews, Oliver discussed the VBNC state of bacteria, importantly, the potential health hazards of VBNC cells present in foods (Oliver 2005a,b, 2009). The most alarming issue of VBNC cell existence is their ability to resuscitate to an actively metabolizing state and cause infections. *L. monocytogenes* has a remarkable ability to survive and persist in dairy products (Cetinkaya and Soyutemiz 2004). In this study, *L. monocytogenes* also persisted in high numbers in cheese throughout prolonged storage under rigorous conditions. Bacterial pathogens in a VBNC state are an interesting challenge to the safety of all types of foods and this study shows that the DEFT method is a suitable tool for enumerating viable cells, including VBNC cells. As well as offering many attractive features, the DEFT method is a practical method for enumerating bacteria at high concentrations, while its detection limit with food samples is around 3–4 $\log_{10}$ units (López-Campos *et al.* 2012). The foundation of any method is its accurateness. In this study, the intent of applying the DEFT method was to reliably determine the viability of *L. monocytogenes*, in particular to detect the extent of VBNC cells in cheese, and this goal was achieved. Therefore, the VBNC state of *L. monocytogenes* is a highly important consideration in developing analytical assays for foods. Furthermore, the clinical effect of *L. monocytogenes* VBNC cells has not been clarified and should be precisely studied in order to assure food safety.

CONCLUSION

The results of this study provide useful information for understanding the behavior of *L. monocytogenes* in cheese stored under different packaging conditions. Such survival data result from a comparison between methods of enumeration for *L. monocytogenes*. It was found that for certain conditions in which *L. monocytogenes* occurs, culturability may not be a reliable indicator of viability, as part of the population may become nonculturable.

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