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Rapid Enumeration of Microorganisms in Foods by the Direct Epifluorescent Filter Technique

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Filtration of "stomachered" food suspensions through nylon filters (pore size, 5 μm) removed most of the food debris without affecting the recovery of microorganisms. Two to ten milliliters of these prefiltered suspensions could be filtered in the direct epifluorescent filter technique (DEFT). The technique takes less than 30 min to complete and has a lower sensitivity of <60,000 microorganisms per g for all products examined. Vegetative bacterial cells, spores, fungal hyphae, and yeasts could be distinguished with the technique. For fresh meat and fish, the DEFT count of prefiltered suspensions agreed well with the plate count of unfiltered suspensions over the range of 10^4 to 10^{10}/g (correlation coefficient of 0.91). For frozen meat and fish and frozen vegetables, the two counting methods had correlation coefficients of 0.87 and 0.66, respectively. The poor correlation for frozen vegetables was due to the inclusion in the DEFT count of nonviable bacteria killed by the blanching process used to inactivate enzymes. Good agreement was obtained between the prefiltered DEFT count and unfiltered plate count for cooked meats, cream doughnut, and whole peppers. Possible reasons for the poor agreement between the DEFT count and plate count for certain products are discussed.

Compared with conventional culture techniques, the use of rapid methods for estimating microbial numbers in foods speeds quality control and release of products and may facilitate public health investigations. Most rapid methods are indirect, estimating a constituent or product of the microorganisms, e.g., measurement of ATP, pyruvate, or impedance changes (5). Direct methods are generally more sensitive and precise than indirect methods, but those based on viable count take 1 to 3 days to obtain a result. Microscopy has always been a prominent technique in the search for rapid direct methods for enumerating microorganisms, one of the first to be used by the food industry (2). Some microscopic techniques suffer from a number of major disadvantages: lack of sensitivity, operator fatigue after prolonged use of the microscope, and bothersome debris which may affect counting.

Recently we developed the direct epifluorescent filter technique (DEFT) for the rapid enumeration of bacteria in milk (6). The technique, which uses membrane filtration and epifluorescence microscopy (incident illumination), takes less than 25 min to complete and is suitable for milk containing 5×10^3 to 5×10^8 bacteria per ml. The method is about 100 times more sensitive than the Breed smear, and the bacteria are easily distinguishable from the small amount of debris present on the membrane filter. Bacteria on DEFT slides can be counted automatically by the use of television image analysis, thereby reducing operator fatigue (G. L. Pettipher and U. M. Rodrigues, J. Appl. Bacteriol., in press). In this paper we describe a prefiltration method which allows the DEFT to be used for the enumeration of microorganisms in suspensions prepared from meat, fish, vegetables, and other foods.

MATERIALS AND METHODS

Food samples. All foods, with the exception of whole peppers, were purchased locally. Whole peppers were obtained from the country of origin. Frozen foods were thawed before bacteriological examination. All food samples were analyzed within 2 h of arrival at the laboratory, and in addition, fresh meat and fish, frozen meat and fish, and frozen vegetables were examined during storage at 7°C.

Preparation of food suspensions. Samples of food (10 g) were "stomachered" (8) for 2 min in 90 ml of sterile Ringer solution with a Colworth Stomacher 100 (Seward Ltd., London, England). DEFT counts and plate counts were made on food suspensions both before and after prefiltration through nylon mesh (pore size, 5 μm; R. Cadisch & Sons, Finchley, London, England). For prefiltration of suspensions, 25-mm-diameter disks punched from sheets of nylon material (pore size, 5 μm) were mounted in Swinnex filter holders (Millipore Ltd., London) and autoclaved before use. A sample of the stomached food suspension was drawn

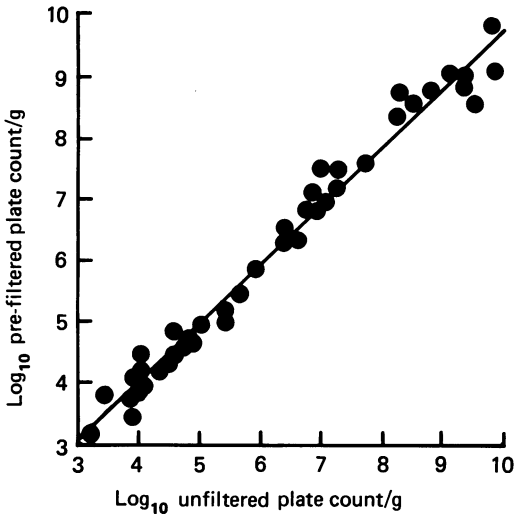

FIG. 1. Relationship between plate counts of prefiltered (y) and unfiltered (x) stomachered suspensions for all foods tested. Line represents fitted regression line ($y = 0.18 + 0.96x$; $r = 0.99$).

into a sterile disposable plastic syringe and expressed through the Swinnex filter unit into a sterile glass container. A new nylon filter was used for each sample.

DEFT count. The apparatus and reagents used, the treatment and filtration of samples, and the staining and counting of bacteria were as described previously by Pettipher et al. (6), except where indicated. The freeze-dried enzyme trypsin (Difco Laboratories, Detroit, Mich.), reconstituted according to the manufacturer's instructions, was used throughout for the treatment of food suspensions before filtration in the DEFT. In a preliminary study, the effect of various combinations of Bactotrypsin, α-amylase (Sigma Chemical Co., St. Louis, Mo.; A 6880), pectinase (Sigma, P 5146), and cellulase (Sigma, C 7502) on the quality of the final microscopic preparations was examined.

Plate count. Decimal dilutions of food suspensions in sterile ¼-strength Ringer solution were plated (1) with plate count agar (Oxoid Ltd., Basingstoke, England). Colonies were counted after 3 days of incubation at 30°C. Unless stated otherwise, plate counts given in the Results section are for unfiltered food suspensions.

RESULTS AND DISCUSSION

Food debris in suspensions of samples prepared for microbiological analysis can be troublesome, blocking pipettes and obscuring colonies on plates prepared from low dilutions (4). In microscopic preparations, debris may make the counting of microorganisms difficult or impossible. Thus, the removal of debris from food suspensions is preferable for microscopic techniques and may also be a useful preparative separation step for other rapid methods. After removal of food debris, microorganisms can be concentrated by a number of methods, e.g., membrane filtration, centrifugation, or use of ion-exchange resins. This may lead to an improvement in the sensitivity of the techniques and possibly, in the case of impedance, a more rapid result. To be of practical use, any method for removing debris from food suspensions should not affect the recovery of microorganisms.

For suspensions of most foods tested, enumeration of microorganisms in the DEFT proved difficult because of the presence of food debris. Prefiltration of the suspensions through nylon filters (pore size, 5 μm) removed most of the food debris and only slightly reduced the recovery of microorganisms, as determined by the plate count (Fig. 1). The recovery rates fell as the microbial content of the food increased, e.g., about 100% at 10^4/g, 87% at 10^6/g, 72% at 10^8/g, and 60% at 10^{10}/g. The loss of microorganisms during prefiltration is unlikely to substantially affect the count of microorganisms in the food. Similar results were obtained with reusable stainless steel filters (pore size, 4 μm; Don Whitley Scientific Ltd., Baildon, Shipley, West Yorkshire, England), which gave a relationship of $y = 0.04 + 0.99x$ ($r = 0.99$), where y represents $\log_{10}$ prefiltered plate count per gram, and x represents $\log_{10}$ unfiltered plate count per gram. Nylon filters were used in preference to stainless steel filters because they were sufficiently inexpensive to be disposable. This was more convenient; there was no need to wash filters before assembly of the Swinnex units.

Prefiltration of food suspensions has been used to facilitate the counting of colonies on hydrophobic grid membrane filters (5) and to clarify food suspensions without reducing the recovery of bacteria (3). Our results support these observations. After filtration through either a nylon (pore size, 5 μm) or a stainless steel (pore size, 4 μm) filter, most of the debris was removed from suspensions without a large reduction in the plate count of the liquid. This suggests that the majority of microorganisms in stomachered food suspensions are not firmly attached to large particles of food. The action of the stomacher may be to wash organisms free of the food (8). The type of material used for prefiltration is important, since microorganisms can adhere to membrane filter materials with pore size diameters much larger than the particles themselves (9).

Treatment with enzyme and surfactant was necessary to achieve efficient filtration of some, but not all, prefiltered food suspensions in the DEFT. For unfiltered suspensions, the combination of amylase and cellulase produced good microscopic preparations from peas and corn,

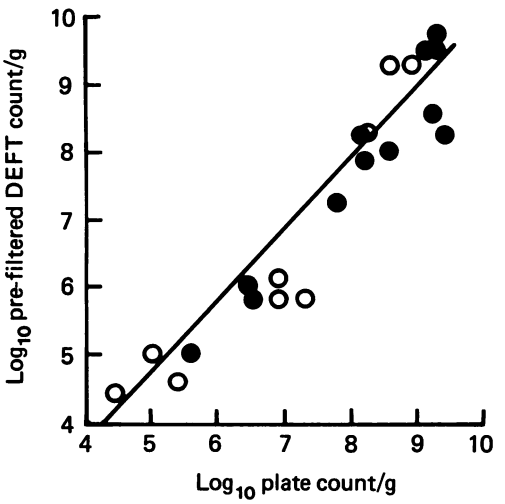

FIG. 2. Relationship between prefiltered DEFT count and plate count for fresh meat (●) and fish (○). Line represents fitted regression line ($y = 1.0x - 0.41$; $r = 0.91$).

but for prefiltered suspensions this treatment was no better than that obtained with trypsin. Since treatment with trypsin and Triton X-100 improved the quality of the microscopic preparations for all prefiltered food suspensions tested, the treatment was used throughout this study. Since this work was completed, it has been found that the use of isopropanol in place of ethanol as the final rinse in the DEFT considerably improves the intensity of fluorescence of the microorganisms.

Depending on the food, 4 to 15 ml of stomachered food suspensions could be filtered through a single nylon prefilter, and 3 to 10 ml of these prefiltered suspensions could be filtered in the DEFT. A sample size of 2 ml was routinely used in the DEFT, together with a microscope factor of 57,500 (i.e., 1 microorganism per field = 57,500 microorganisms per g if 2 ml of food suspension is filtered). Microorganisms were morphologically distinguishable as vegetative bacterial cells, spores, fungal hyphae, or yeasts. Unless stated otherwise, these organisms were included in the DEFT count if they fluoresced orange. The coefficient of variation between triplicate log DEFT counts on food suspensions was <1.0%, lower than that previously obtained for milk (6).

For fresh meat and fish, the prefiltered DEFT count agreed well with the plate count over the range of 10^4 to 10^{10}/g, with a correlation coefficient (r) of 0.91 (Fig. 2). For initial counts on frozen meat and frozen fish, the prefiltered DEFT count also agreed well with the plate count over the range 5×10^4 to 5×10^7/g (r = 0.87) (Fig. 3). The relationship between the two counting techniques for frozen fish thawed and stored at 7°C for 1 to 4 days was $y = 0.47 + 0.88x$ ($r = 0.94$), where y represents $\log_{10}$ prefiltered DEFT counts per gram, and x represents $\log_{10}$ plate counts per gram. Counts were in the range of 10^4 to 10^{10}/g.

For initial counts on frozen vegetables (range, 5×10^2 to 5×10^5/g), there was poorer agreement between the prefiltered DEFT count and the plate count. The relationship had a correlation coefficient of 0.66 (Fig. 4). This was mainly due to counts obtained for Brussels sprouts; other vegetables, e.g., peas, corn, and broad beans, had individual correlation coefficients of 0.8 or greater. This poor agreement is unlikely to be due solely to the effect of freezing, since there was reasonable agreement between the two counting techniques for frozen meat and frozen fish. Unlike meat and fish, vegetables are blanched at a temperature of about 98 to 99°C before freezing to inactivate enzymes which would otherwise lead to deterioration of the product during storage. The time of blanching is based on the heat penetrability of the product; small vegetables such as peas and corn are blanched for about 1 min, whereas larger vegetables such as Brussels sprouts are blanched for 2 to 3 min. Heat treatment is known to affect the agreement between the DEFT count and plate count, especially for products containing a large number of streptococci or micrococci (7). The DEFT count of peas, which comprised mainly streptococci, exceeded the plate count by about

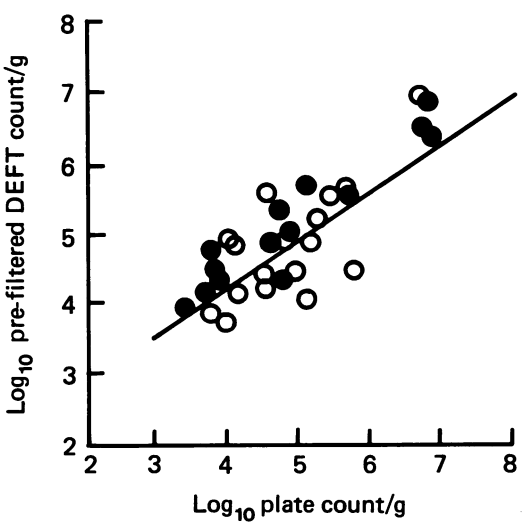

FIG. 3. Relationship between prefiltered DEFT count and plate count for frozen meat (●) and frozen fish (○). Line represents fitted regression line ($y = 1.31 + 0.74x$; $r = 0.87$).

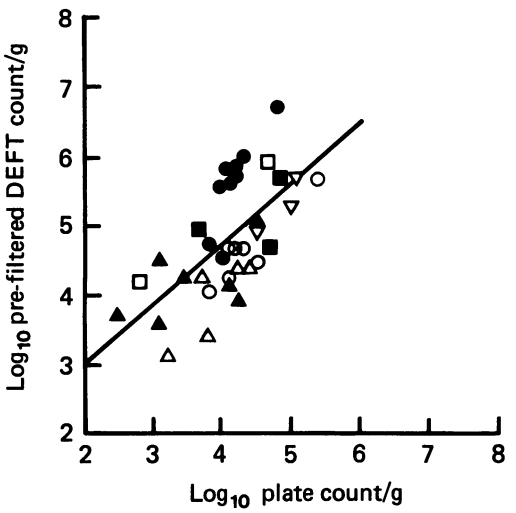

FIG. 4. Relationship between prefiltered DEFT count and plate count for frozen vegetables. Symbols: ●, peas; ○, corn; ▲, Brussels sprouts; △, broad beans; ▽, carrots; □, runner beans; ■, mixed vegetables. Line represents fitted regression line ($y = 1.22 + 0.88x$; $r = 0.66$).

1.5 log units. This is probably due to nonviable organisms fluorescing orange and therefore being included in the DEFT count. Following microbial growth after thawing and refrigerated storage, the DEFT count and plate count of frozen vegetables were in closer agreement. The relationship between the two counting techniques for frozen vegetables thawed and stored at 7°C for 1 to 4 days was $y = 0.89 + 0.86x$ ($r = 0.97$), where y represents $\log_{10}$ prefiltered DEFT count per gram, and x represents $\log_{10}$ plate count per g. Counts were in the range of 10^4 to 10^{10}/g.

Of the other foods tested, there was good agreement between the prefiltered DEFT count and the plate count for cooked meats, cream doughnut, and whole pepper (Table 1). The high plate count of the cooked chicken roll was comprised mainly of psychrotrophic bacteria. For cream doughnut, the numerous orange-red-fluorescing yeasts seen in the DEFT were not included in the count, since it was assumed they had originated from the dough and would be nonviable after baking. Colonies on plates prepared from this product were of bacterial origin. The DEFT counts of whole white and black peppers were comprised mainly of pieces of fungal hyphae and bacterial spores, respectively. This was reflected in the type of colonies seen on the corresponding plates. For most of the dry products tested, the DEFT count exceeded the plate count by 3- to 20-fold (Table 1). It is possible that certain procedures in the manufacture of these products, e.g., heating or drying, may have reduced the viable count but that these nonviable organisms are detected and included in the DEFT. In these cases the DEFT count probably reflects the microbiological quality of the food before heating.

TABLE 1. DEFT counts and plate counts of cooked meats, cream cakes, spices, and dry products

Food	$\log_{10}$ count per g	
	DEFT	Plate
Cooked chicken roll	7.63	7.80
Cooked ham	4.26	4.59
Cream doughnut	6.10	6.41
Whole black pepper	7.14	6.32
Whole white pepper	5.96	5.11
Curry powder	5.87	5.08
Flour	4.06	3.52
Desiccated coconut	5.50	4.02
Custard powder	3.24	1.90

With prefiltration, the DEFT can be used to obtain a count of microorganisms in a variety of foods in less than 30 min. As an added advantage of the technique, the organisms can be tentatively identified as bacteria, spores, yeasts, or fungi. For products for which agreement between the DEFT count and plate count is good, e.g., fresh and frozen meat and fish, the DEFT could be used to speed quality control during production and public health investigations. For products for which agreement between the two counting methods is reasonable, such as frozen vegetables, the DEFT may be useful for monitoring growth during storage and transport resulting from inadequate cooling or mishandling. For products for which agreement between the DEFT count and plate count is poor, the DEFT, because of its rapidity, may still prove useful in detecting contamination by monitoring the condition of the product at various stages during its manufacture.

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