Quick Counting Method for Estimating the Number of Viable Microbes on Food and Food Processing Equipment

F. H. WINTER, G. K. YORK, AND HAMZA EL-NAKHAL

Department of Food Science and Technology, University of California, Davis, California 95616

Received for publication 29 March 1971

A rapid method for estimating the extent of microbial contamination on food and on food processing equipment is described. Microbial cells are rinsed from food or swab samples with sterile diluent and concentrated on the surface of membrane filters. The filters are incubated on a suitable bacteriological medium for 4 hr at 30°C, heated at 105°C for 5 min, and stained. The membranes are then dried at 60°C for 15 min, rendered transparent with immersion oil, and examined microscopically. Data obtained by the rapid method were compared with counts of the same samples determined by the standard plate count method. Over 60 comparisons resulted in a correlation coefficient of 0.906. Because the rapid technique can provide reliable microbiological count information in extremely short times, it can be a most useful tool in the routine evaluation of microbial contamination of food processing facilities and for some foods.

Several methods are commonly used to determine the extent of microbial contamination on foods and equipment: standard plate count, spread plates, shake tubes, most probable number, and dilution to extinction. These methods share the disadvantage of the time required for the microbial cells to grow to visible concentrations, usually 24 to 72 hr.

Attempts have been made to shorten the length of incubation of colony-counting procedures. Such modifications have included the Frost “little” plate (5), agar strips, roll, and oval tube methods. Although these methods have been accepted in certain areas of microbiology, they have not supplanted the standard plate count method with its reliance upon 24 to 72 hr of incubation.

Microscopic methods of Breed and Brew (1-3), although applicable to milk, are difficult to apply with vegetables where food particles are difficult to distinguish from the bacterial cells. In addition, there is the limitation of being unable to distinguish living from dead cells.

Frazier and Gneiser (4), reporting on use of the membrane filter, reduced the time to 8 to 18 hr of incubation; however, they concluded that only limited application of their method was feasible in routine examination of fresh and frozen vegetables. In the studies reported here, the use of membrane filters to concentrate microbial cells, plus the use of a vital stain, makes it possible to observe microcolonies which develop after 4 to 5 hr of incubation.

MATERIALS AND METHODS

Reagents. Janus B green (60 mg per 100 ml in distilled water) was filter-sterilized. The medium employed was standard plate count agar (Difco).

Preparation of sample. Fifty grams of sample was aseptically added to 450 ml of sterile saline solution and shaken vigorously for 1 min. A 10-ml amount of this diluent was then filtered through an 8.0-µm membrane to remove larger particles of vegetable material and yet permit passage of the bacteria. A washing with sterile water followed. The diluent and wash water were caught in a 1-liter sterile vacuum flask. The entire contents of the flask were filtered through a sterile 0.45-µm membrane, which was then cut into three strips which were incubated on a sterile absorbant pad pretreated with 2 ml of medium. The strips were removed at intervals of 4, 5, and 6 hr, fixed in an oven at 105°C for 5 min, and stained with Janus green, taking care not to dislodge the microcolonies. After drying in an oven at 60°C, the strips were rendered transparent by immersion oil and examined under various magnifications. An average count per field was obtained and converted to units per gram. A simple method of accomplishing this was to determine the number of microscopic fields, for the magnification used, on the total membrane. This was done by lining up the edge of one field with the edge of a grid mark on the corner of one of the squares of the membrane. The grid marks appear as a series of dots,
and by scanning across a line, with the dots of the line as a guide, the number of fields along one side was counted. It can be determined mathematically that the relationship of the area of the microscopic field to one grid square is \(14n^2/11\), where \(n\) is the number of fields along one side of the square. As there are 100 squares in the membrane covered, multiplying by 100 gives the number of fields on the membrane.

The colonies in the first strip were usually easily identifiable; however, in cases where longer incubation was necessary, the examination was facilitated by using the second or third strip.

RESULTS AND DISCUSSION

Because the membrane filter can be rendered transparent by immersion oil, it was assumed that the entrapped, living bacteria could be identified microscopically provided they could be differentially stained from dead cells. Although some success was realized by counting individual cells, it was difficult to differentiate between particles of food and bacterial cells, especially cocci, even with magnifications of 1,000 to 1,250×. Preliminary filtration through filters of larger pore sizes was attempted to remove larger food particles. Since the use of filters having a pore diameter of 1.2 μm, as suggested by Nobile (6), resulted in the entrapment of most of the bacterial cells, larger pore filters were used. It was found that an 8.0-μm membrane would filter out the larger particles of vegetable without trapping the bacteria; however, difficulty remained in differentiating between bacteria and vegetable matter.

Attempts were then made to allow the bacteria to grow sufficiently to form microcolonies, which could be more readily identified microscopically. The membranes were prepared as previously described, but, before staining, the membranes were placed on absorbant pads saturated with a nutrient medium (glucose-tryptone-yeast extract), incubated at 30 to 35 C for various periods of time, stained, dried, rendered transparent, and examined microscopically.

It was observed that microcolonies could be identified after 2 to 4 hr of incubation under magnifications of 800 to 1,000× (Fig. 1) and after 4 to 6 hours when a magnification as low as 80× was used (Fig. 2). During the staining procedure, many colonies were found to be washed from the filter surface. The problem was solved by application of mild heat (105 C) for 1 to 2 min to fix the colonies without adversely affecting the membrane.

If the rapid method is to be of practical value in estimating microbial populations, the results must be comparable to results from established methods of bacteriological quantification. Comparisons were made between the rapid method and the standard plate count method by running parallel determinations on several series of samples of fresh and frozen vegetables and on

**Fig. 1.** Microcolony after 4 hr of incubation. ×800. (a) Cocci and (b) rods.

**Fig. 2.** Group of microcolonies fixed on membrane after 6 hr of incubation. ×80.
### Table 1. Comparison of rapid (QC) and standard plate count (SPC) methods

| Product         | Sample | SPC     | QC      | Product         | Sample | SPC     | QC      |
|-----------------|--------|---------|---------|-----------------|--------|---------|---------|
| Broccoli        | 1      | $3.4 \times 10^4$ | $2.3 \times 10^4$ | Green beans     | 4      | $1.5 \times 10^5$ | $1.3 \times 10^5$ |
|                 | 2      | $4.2 \times 10^4$ | $2.4 \times 10^4$ |                 | 5      | $4.7 \times 10^5$ | $6.7 \times 10^5$ |
|                 | 3      | $3.5 \times 10^4$ | $2.2 \times 10^4$ |                 | 6      | $4.8 \times 10^5$ | $7.3 \times 10^5$ |
|                 | 4      | $3.2 \times 10^4$ | $2.2 \times 10^4$ |                 | 7      | $3.9 \times 10^5$ | $2.3 \times 10^5$ |
|                 | 5      | $1.5 \times 10^5$ | TNC³       |                 | 8      | $4.3 \times 10^5$ | $2.9 \times 10^5$ |
|                 | 6      | $2.1 \times 10^5$ | TNC³       |                 |        |         |         |
|                 | 7      | $2.6 \times 10^5$ | $1.1 \times 10^5$ | Lima beans      | 1      | $2.8 \times 10^4$ | $3.0 \times 10^4$ |
|                 | 8      | $7.3 \times 10^5$ | $1.7 \times 10^5$ |                 | 2      | $2.5 \times 10^4$ | $2.7 \times 10^4$ |
|                 | 9      | $4.4 \times 10^5$ | $1.2 \times 10^5$ |                 | 3      | $6.8 \times 10^5$ | $6.0 \times 10^5$ |
|                 | 10     | $3.3 \times 10^5$ | $1.2 \times 10^5$ |                 | 4      | $5.9 \times 10^5$ | $7.3 \times 10^5$ |
|                 | 11     | $2.8 \times 10^5$ | $4.1 \times 10^5$ |                 | 5      | $3.1 \times 10^5$ | $3.0 \times 10^5$ |
|                 | 12     | $2.3 \times 10^5$ | $4.1 \times 10^5$ |                 | 6      | $3.4 \times 10^5$ | $3.1 \times 10^5$ |
|                 | 13     | $1.7 \times 10^5$ | $4.0 \times 10^5$ |                 | 7      | $6.9 \times 10^5$ | $5.1 \times 10^5$ |
|                 | 14     | $1.6 \times 10^5$ | $4.0 \times 10^5$ |                 | 8      | $6.8 \times 10^5$ | $7.4 \times 10^5$ |
| Brussel sprouts | 1      | $8.2 \times 10^5$ | TLC³       | Mixed vegetables| 1      | $7.0 \times 10^4$ | $4.3 \times 10^4$ |
|                 | 2      | $3.1 \times 10^5$ | TLC³       |                 | 2      | $6.7 \times 10^4$ | $4.5 \times 10^4$ |
|                 | 3      | $6.7 \times 10^5$ | $6.6 \times 10^4$ |                 | 3      | $7.1 \times 10^4$ | $5.1 \times 10^4$ |
|                 | 4      | $2.4 \times 10^5$ | $2.3 \times 10^5$ |                 | 4      | $7.2 \times 10^4$ | $5.2 \times 10^4$ |
|                 | 5      | $1.1 \times 10^5$ | $2.0 \times 10^4$ | Peas            | 1      | $2.3 \times 10^4$ | $1.5 \times 10^4$ |
| Carrots         | 1      | $2.3 \times 10^4$ | $4.1 \times 10^4$ |                 | 2      | $3.4 \times 10^4$ | $2.2 \times 10^4$ |
|                 | 2      | $6.1 \times 10^4$ | $6.9 \times 10^4$ |                 | 3      | $6.8 \times 10^4$ | $7.1 \times 10^4$ |
|                 | 3      | $6.7 \times 10^4$ | $6.8 \times 10^4$ |                 | 4      | $7.1 \times 10^4$ | $5.0 \times 10^4$ |
|                 | 4      | $6.3 \times 10^4$ | $6.5 \times 10^4$ |                 | 5      | $8.0 \times 10^4$ | $7.1 \times 10^4$ |
|                 | 5      | $2.9 \times 10^4$ | $2.2 \times 10^4$ |                 | 6      | $1.0 \times 10^5$ | TLC³       |
|                 | 6      | $5.1 \times 10^4$ | $5.2 \times 10^4$ |                 | 7      | $3.5 \times 10^5$ | $3.9 \times 10^5$ |
|                 | 7      | $7.3 \times 10^4$ | $6.7 \times 10^5$ |                 | 8      | $5.1 \times 10^4$ | $5.5 \times 10^5$ |
|                 | 8      | $8.0 \times 10^4$ | $6.9 \times 10^7$ | Peas and carrots| 1      | $3.0 \times 10^4$ | $1.9 \times 10^4$ |
|                 |        |         |         |                 | 2      | $4.0 \times 10^4$ | $2.9 \times 10^4$ |
|                 |        |         |         |                 | 3      | $3.1 \times 10^4$ | $3.5 \times 10^4$ |
|                 |        |         |         |                 | 4      | $5.2 \times 10^4$ | $4.9 \times 10^4$ |
| Cauliflower     | 1      | $2.1 \times 10^4$ | $2.2 \times 10^4$ | Spinach         | 1      | $2.3 \times 10^4$ | $1.8 \times 10^4$ |
|                 | 2      | $3.0 \times 10^4$ | $2.5 \times 10^4$ |                 | 2      | $2.2 \times 10^4$ | $2.0 \times 10^4$ |
|                 | 3      | $2.5 \times 10^4$ | $2.6 \times 10^4$ |                 | 3      | $1.6 \times 10^4$ | $9.1 \times 10^4$ |
|                 | 4      | $2.8 \times 10^4$ | $3.0 \times 10^6$ |                 | 4      | $2.0 \times 10^4$ | $7.7 \times 10^4$ |
| Green beans     | 1      | $4.2 \times 10^4$ | $4.1 \times 10^4$ |                 |        |         |         |
|                 | 2      | $6.1 \times 10^4$ | $5.8 \times 10^4$ |                 |        |         |         |
|                 | 3      | $2.0 \times 10^4$ | $1.8 \times 10^4$ |                 |        |         |         |

a Statistical analysis: $x = 9.666 \times 10^4; y = 9.019 \times 10^4; \Sigma x = 5.993 \times 10^4; \Sigma y = 5.592 \times 10^4; n = 62$ (TLC and TNC disregarded); $r = \Sigma xy nxy/[(\Sigma x^2 - nx^2) (\Sigma y^2 - ny^2)]^{1/2} = 0.906$.

b Too numerous to count.

c Too low to count.

e To count.

equipment surfaces. Various amounts of contaminations of frozen vegetables were effected by varying the care in preparing the vegetables before freezing. Approximately 60 comparisons were made and analyzed statistically (Table 1). The correlation coefficient of 0.906 indicated that the rapid or quick count method is statistically valid when compared to the standard plate count method.

The value of the quick count method lies in the fact that, within a relatively short time, 4 to 5 hr, persons with minimal training can determine, by line-checks and equipment and product monitoring, whether a given product will meet specific microbial specifications when packed and frozen. The quick count method is not intended to replace the standard plate count method in all aspects of food microbiology, nor can it be expected to give the same degree of accuracy within a 4-hr period. Because it yields results within the
ranges of “low,” “moderate,” or “excessive” contamination, i.e., less than 50,000 cells per g, between 50,000 and 100,000 cells per g, and over 100,000 cells per g, it will serve as the rapid monitoring system for which it is intended.

The quick count method has been used successfully for monitoring purposes by several food processing plants that cooperated in the study.

ACKNOWLEDGMENTS

This investigation was supported by the American Frozen Food Institute.

The technical assistance of Robert Rose and Roger Norquist, Millipore Corp., and Howard Aronson, Cox Instrument, formerly with Millipore, is gratefully acknowledged. We also thank Lee Chugg, Birdseye Division of General Foods Corp., and Jerry Klink, John Inglis Frozen Foods.

LITERATURE CITED

1. Breed, R. S. 1911. The determination of the number of bacteria in milk by direct microscopic examination. Centrabbl. Bakteriol. Parasitenk. Infektionskr. Abt. 2 Orig. 30:337-340.
2. Brew, J. D. 1929. The comparative accuracy of the direct microscopic and agar plate methods in determining numbers of bacteria in milk. J. Dairy Sci. 12:304-319.
3. Brew, J. D. 1914. A comparison of the microscopical method and the plate method of counting bacteria in milk. Bull. (Geneva, New York) N.Y. Agr. Exp. Sta. 373:1-38.
4. Frazier, W. C., and D. F. Gneiser. 1968. Short-time membrane filter method for estimation of numbers of bacteria. J. Milk Food Technol. 31:177-179.
5. Frost, W. D. 1916. A rapid method of counting living bacteria in milk. J. Amer. Med. Ass. 66:889-890.
6. Nobile, J. 1967. Use of membrane filter technique in the microbiological control for the brewing industry. Appl. Microbiol. 15:736-737.