Evaluation of a commercial fluorochromic system for the rapid detection and estimation of wine lactic acid bacteria by DEFT

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J.A. COUTO AND T. HOGG. 1999. A commercial fluorochromic system was evaluated for the rapid detection of lactic acid bacteria in fortified wines by the epifluorescent filter technique (DEFT). The viability test used, employing the fluorescence dyes SYTO 9 and propidium iodide, was able to detect and clearly differentiate viable from non-viable cells (killed with a 50% v/v ethanol solution). A good overall agreement ($r = 0{\cdot}92$) was obtained between the DEFT count and the plate count in the range studied (5×10^2–4×10^9 cells ml^{-1}). Wine components which might otherwise interfere with the method could be removed by including simple wash steps in the protocol. This measure proved critical to the success of the procedure. For practical purposes, the rapid method studied seems to be a good alternative to the traditional cultural methods as part of quality control programmes in wine making. It may also be useful when studying the efficacy of certain treatments in the elimination of wine bacterial contaminants.

INTRODUCTION

Apart from their association with malolactic fermentation, lactic acid bacteria (LAB) have been isolated at various stages of wine making, i.e. grapes, fermenting musts and stored wines (Costello *et al*. 1983; Wibowo *et al*. 1985), being also frequently associated with wine spoilage (Ribéreau-Gayon *et al*. 1975; Couto and Hogg 1994; de Revel *et al*. 1994). The detection of bacterial populations at different stages of vinification plays an important role in wine quality control. Detection in wines relies mainly on simple microscopic observations or cultural methods. Difficulties have been encountered in growing these organisms in culture media, especially when isolating directly from fortified wines; even when cultural isolation is possible, the process is often too long to be useful. It is recognized that in some cases, cells may be present in their natural environments in a dormant state which would not form colonies on nutrient media but which would be considered viable by a direct method such as the epifluorescent filter technique (DEFT) (Pettipher *et al*. 1980). This technique often includes acridine orange as the stain of choice. However, it has been found in this laboratory that there are some inconsistencies in the evaluation of the viability of a population when using DEFT with this dye. In this work, a bacterial viability procedure was studied for the detection of wine lactobacilli which utilizes a mixture of two fluorescent nucleic acid stains: SYTO 9 (green) which labels all bacteria in a population, and propidium iodide (red/orange) which penetrates only bacteria with damaged membranes competing with the former for nucleic acid binding sites. The applicability of this procedure to challenge–survival experiments of wine bacteria submitted to heat and ethanol challenges was also evaluated. The above two dyes have already been successfully tested in *Escherichia coli*, *Bacillus cereus* and *Staphylococcus aureus* for measuring bacterial viability and antibiotic susceptibility (Roth *et al*. 1997).

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MATERIALS AND METHODS

Organism and medium

Lactobacillus hilgardii 5, a strain isolated in this laboratory from Douro fortified wine (Couto and Hogg 1994), was used. For the viability tests, cells were grown, non-agitated, at 25 °C in a medium composed of MRS broth (Lab M) + TJ broth (Difco) (50% of each) supplemented with 5% (v/v) ethanol at pH 4·5 to late exponential growth phase.

Viability assays

Bacterial culture (2 × 20 ml) was pelleted by centrifugation at 8000 *g* for 10 min. One cell pellet was resuspended in 20 ml Port wine (19·5% v/v ethanol) and the other in 20 ml of the same wine with its alcoholic strength adjusted to 50% (v/v) ethanol. Both were allowed to stand for 30 min to obtain viable and dead cells, respectively. Viability of the suspensions was established by plating onto MRS broth (Lab M) + TJ broth (Difco) (50% of each) +2% agar (no. 1, Lab M) supplemented with ethanol (5% v/v) and pH was adjusted to 4·5 after appropriate decimal dilutions in sterile Ringer's solution. Mixtures of 11 different proportions of viable and dead cells of *Lactobacillus hilgardii* 5 were prepared from the suspensions.

Survival tests were performed in order to assess the suitability of the DEFT methodology for this type of study. The temperature used was based on previous work on the survival and enhancement of apparent resistance to ethanol by *Lact. hilgardii* (Couto *et al.* 1997). For the heat tolerance experiments, a culture of *Lact. hilgardii* 5 in the late exponential phase of growth (approximately 10^8 cells ml^{-1}) was centrifuged at 8000 *g* for 10 min, resuspended in Port wine heated to 40 °C and kept at this temperature for 5 min in a waterbath. After 2 and 5 min, samples were removed and appropriately diluted in sterile Ringer's solution for determination of viability by standard plate count and fluorescence microscopy after staining as described above. A sample at time 0 was removed from a suspension of cells in Port wine at room temperature.

Staining of bacteria

Wine bacterial suspensions were centrifuged (8000 *g* for 10 min) in microfuge tubes, the cells were washed twice with filter-sterilized distilled water and finally resuspended in 1 ml sterile distilled water. SYTO 9 and propidium iodide stains were obtained commercially from Molecular Probes (Eugene, OR, USA). Equal volumes of the two stains were combined in a microfuge tube and 3 μl of this solution was added to 1 ml bacterial suspension (the concentration of dyes for cell staining was 5 and 30 μmol l^{-1} for SYTO 9 and propidium iodide, respectively). These were mixed thoroughly and incubated at room temperature in the dark for 15 min as recommended by the manufacturer. Stained bacteria were then immobilized in a 16 mm diameter polycarbonate filter membrane with 0·2 μm pores (Costar) under vacuum (Pettipher *et al.* 1980).

Epifluorescence microscopy

Each membrane with immobilized stained bacteria was air dried and mounted on a microscope slide on top of a water droplet. Immersion oil was added on top of the filter over which a coverslip was placed. Samples were observed by epifluorescence microscopy with an oil immersion objective at 1000× magnification. The microscope used was a JENAMED-2-fluorescence (Carl Zeiss, Jena, Germany) equipped with a mercury HBO-50 Watt lamp.

Estimation of number of cells in each sample was calculated as follows:

$$N = CA/av$$

where N = cells ml^{-1}; C = mean number of cells per observation field; A = filtration area (mm^2); a = observation field area (mm^2); v = volume of filtered sample (ml).

The sample was diluted and the counts repeated when more than 100 cells per field occurred. The number of fields counted in each estimation was 10.

RESULTS AND DISCUSSION

Non-viable lactobacilli were obtained by treatment of cells in Port wine with added ethanol to a final concentration of 50% (v/v). This method resulted in a >99·99% reduction in viable cell number as determined by standard plate count. The inactivated cells (treated with ethanol) stained fluorescent orange whereas nearly all the cells of the non-treated population stained green. The two cell types could be clearly distinguished when mixtures were observed (not shown). It should be emphasized that, as noted in this work, wine components may interfere with the staining reaction by binding to SYTO 9 and propidium iodide, resulting in unacceptable variations in staining. Thus, care must be taken to remove medium components. The washing steps with filter-sterilized distilled water were sufficient to remove any such interfering components of the wine from the bacterial suspensions.

Live and dead cell suspensions were used to achieve various proportions of live : dead cells. The percentage of green cells in each mixture was determined by fluorescence microscopy. A good correlation ($r = 0{\cdot}99$) was found between bacterial viability determined by this method and expected viability (Fig. 1). The concentration and relative proportion of the stains used appeared to be appropriate for the discrimination of live and dead cells when present in different ratios.

DEFT counts were compared with plate counts for a variety of contaminated wines. The numbers of green-fluorescing cells determined by DEFT correlated well with the plate counts in the range of 5×10^2–4×10^9 cells ml^{-1} (Fig. 2). The relationship between the DEFT and the plate counts appeared to be dependent on cell density. The reason for this is not easy to explain. It should be noted that the assumption as to what constitutes the signal (counted as one unit) in DEFT is far more subjective than that in plates (bacterial colony). The DEFT underestimation at high cell

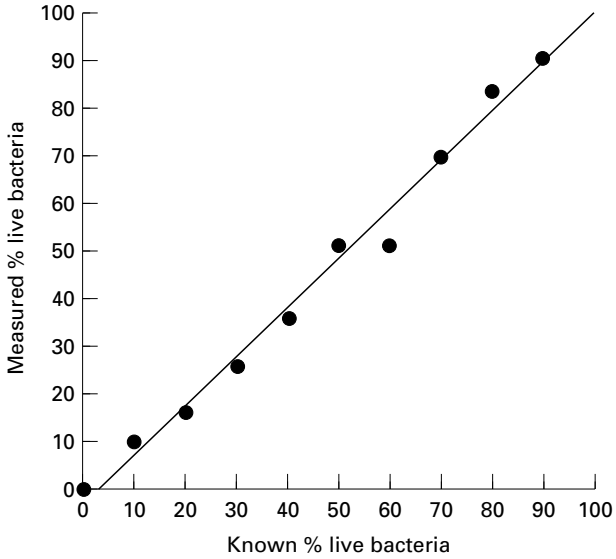

Fig. 1 Relationship of viability determined by fluorescence microscopy (percentage of cells staining fluorescent green) with percentage of viable cells present in suspensions obtained from mixing different proportions of live and dead cells (killed with ethanol (50%)). Regression line equation is $y = 1{\cdot}0229x - 2{\cdot}5929$; $r^2 = 0{\cdot}98796$

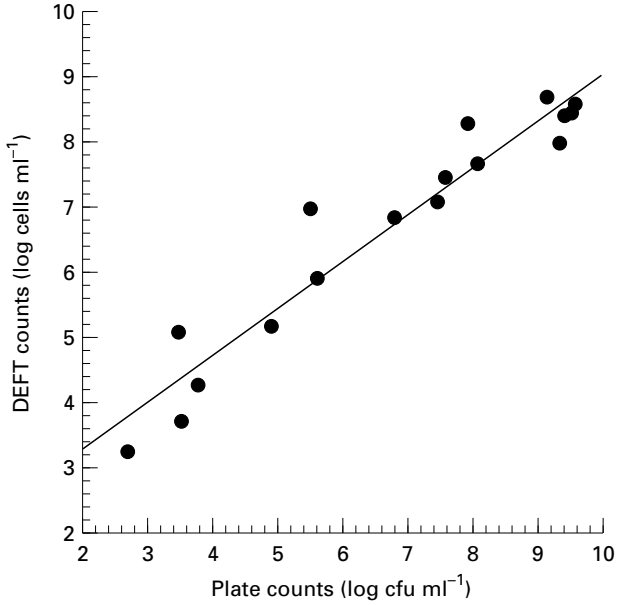

Fig. 2 Relationship between DEFT counts and plate counts of viable bacteria in wine samples. Regression line equation is $y = 1{\cdot}912 + 0{\cdot}70966x$; $r^2 = 0{\cdot}92402$

densities could be related to the disruption of cell agglomerations (filaments and clumps) during the dilution needed for plating (for the same sample of wine, higher dilutions are required for plating than for epifluorescence microscopy). The precision of the DEFT counts at low cell densities could have been enhanced either by using higher sample volumes (more cells per microscope field) or by increasing the number of microscope fields counted.

The suitability of the DEFT method described in this work for the enumeration of viable organisms in survival experiments of wine bacteria was compared with that of the cultural method. The ability of *Lact. hilgardii* 5 to survive heat challenges in wine is demonstrated in Fig. 3, where cells were submitted to 40 °C for 5 min. Percentage survival was based on counts determined either by cultural methods or by DEFT (as the ratio of colony forming units or fluorescent green cells after treatment to the number of viable cells at time 0) (Table 1). Inactivation kinetics were very similar for both methods. As discussed above, at the highest density, DEFT exceeded the plate counts whereas an overestimation was noted at the lowest density (Table 1). Thus, the survival percentages were themselves affected by this. The survival experiments showed that the rapid detection method used in this work might be extremely useful when studying the effect of specific factors on the viability of wine LAB contaminants (e.g. effect of pasteurization regimes).

A similar approach was used with the common dye, acridine orange. However, the expected differential staining

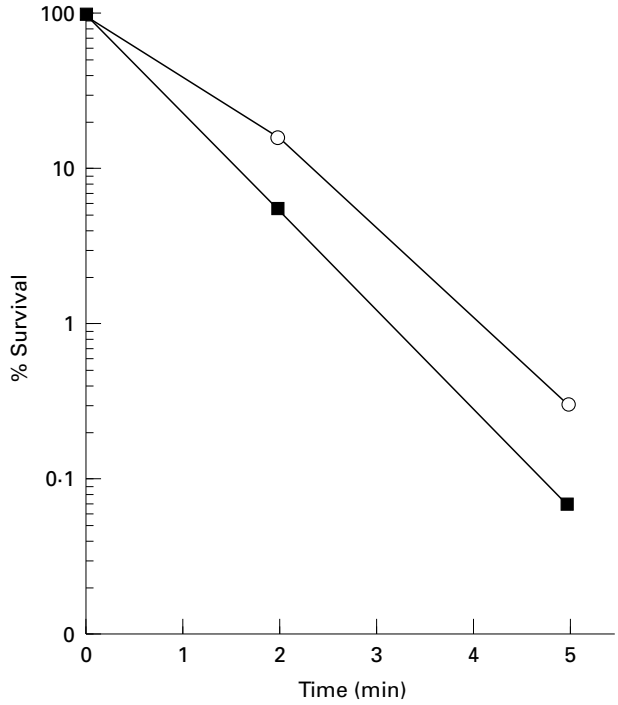

Fig. 3 Effect of heat stress at 40 °C on the survival of *Lactobacillus hilgardii* 5 determined by the DEFT (○) and plate (■) counts indicated in Table 1

Time (min)	DEFT counts (cells ml^{-1})	Survival (%)	Plate counts (cfu ml^{-1})	Survival (%)
0	$4{\cdot}5 \times 10^7$	100	$1{\cdot}2 \times 10^8$	100
2	$7{\cdot}2 \times 10^6$	16·0	$6{\cdot}5 \times 10^6$	5·4
5	$1{\cdot}5 \times 10^5$	0·3	$8{\cdot}0 \times 10^4$	0·07

Table 1 Bacterial counts of *Lactobacillus hilgardii* 5 exposed to 40 °C for 5 min

The data shown represent triplicate experiments; S.D. of the number of cells or cfu was within 34% of the mean value at each time point.

pattern, active and non-active cells fluorescing orange and green, respectively (Pettipher *et al*. 1980), could not be achieved when staining bacteria from the wine environment with this dye, as all the cells in the samples analysed stained orange despite being treated with high concentrations of ethanol or heat (results not shown). The staining procedure described in this work was found to give better results than with acridine orange in the differentiation of viable from dead wine LAB by DEFT. Such a rapid and reliable method might be useful as part of routine wine-making quality control programmes. The centrifugation and membrane filtration steps enable the sensitivity threshold of the method to be increased, as variable volumes of sample can be used for bacterial detection.

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