Microbiological analysis of total viable count and E. coli in food samples

by Giorgia Bottini, Francesca Losito, Alessio De Ascentis, Giovanni Antonini, Department of Biology, University Roma Tre, 00146 Rome, Italy, Francesca Romana Priolisi, Alberto Mari, MBS srl, 00131 Rome, Italy and Annie Tu, Philip EC Huang, Royal Biotech GmbH, 14469 Potsdam, Brandenburg, Germany.

The hygiene process criteria for many foodstuffs now include a test for total viable count (TVC) and Escherichia coli (E. coli). TVC gives a quantitative idea of the presence of mesophilic aerobic micro-organisms of animal origin.

It serves as important criteria for evaluating the microbial quality of various foods and also degree of freshness of food. E. coli is part of the normal microflora of the gastrointestinal tract of mammals and birds. As early as the 19th century, E. coli was recognised as a good indicator of faecal contamination. Standardised methods (for example ISO methods) are acknowledged as the reference analytical methods for official control.

These standardised methods are based on traditional microbiological culture standard methods that are widely used in food analysis laboratories.

These techniques present several difficulties and above all, by the long time needed to obtain definitive results (from 3-7 days). In this context, we have developed an alternative method, called the Micro Biological Survey (MBS) method. It is a colorimetric fast system for the detection and the selective counting of bacteria present in agro-food, in water and in environmental samples. The MBS method consists of an analytical kit utilising disposable, ready-to-use reaction vials for fast microbiological analyses.

The analysis is based on the colour change of the vial content which is induced by the presence of bacteria. The analyses can be carried out by untrained personnel and anywhere where they are necessary, without the need for any other instrumentation than a thermostat provided on request.

The MBS method measures the catalytic activity of the redox enzymes in the main metabolic pathways of bacteria, which allows an unequivocal correlation between the observed enzymatic activity and the number of viable cells present in the samples. The time required for a colour change is inversely related to the log of bacterial concentration; like an enzymatic reaction, the greater the number of bacteria, the faster the colour change.

Statistical analysis

Sterile water was initially used to avoid any chemical interference due to organic matrices. In MBS colorimetric method the change of the starting colour of the vials from blue for TVC and reddish for E. coli to yellow colour indicates a positive result, presence of micro-organisms.

The time occurred for colour change is inversely related to bacteria content of analysed samples. The water samples were artificially contaminated. The statistical analysis for the MBS method on TVC and on E. coli vials was carried out according to ISO/TR 13843 (2000) using as reference method the plate counting method ISO 9998 (1991) on 10 different dilutions of 10 different samples.

MBS reliable operating limits were comparable to the reference methods for plate counts at concentrations between $1x10^7$ and <10CFU/ ml. The results of the statistical analysis were expressed in terms of:

- Estimate of precision.
- Coefficient of variance.
- Uncertainty.

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Fig. 1. Linearity: correlation line between analytes (TVC and E. coli) with the time occurred for colour change in the vials MBS, on all five food matrix of the two methods. A linear inverse relationship between the MBS method and the bacteria concentration. The correlation factor is (R2) 0.95 for TVC and 0.98.

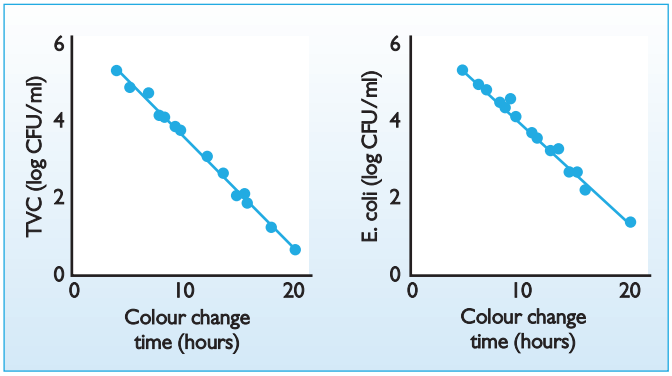
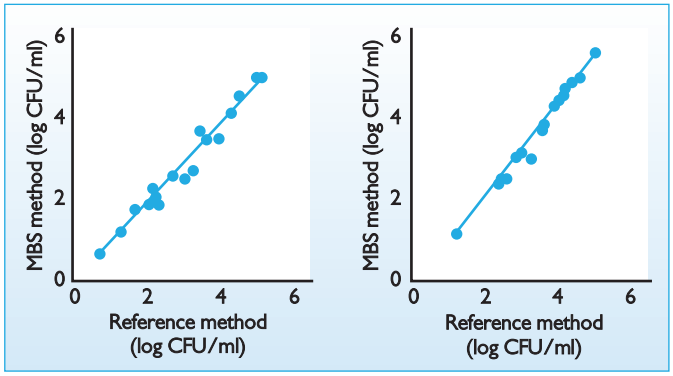


Fig. 2. Accuracy: correlation line between alternative MBS method and reference method (TVC and E. coli). A good correlation between the bacteria number (expressed as log CFU/ml) obtained with the traditional counting method and the alternative MBS method could be observed. In fact the slope is close to the theoretical value 1.00 (1.00 for TVC and 0.99 for E. coli). The correlation factor is (R2) 0.94 for TVC and 0.99 for E. coli.



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General estimate of precision was made according to ISO/TR 13843 (2000) using Analysis of Variance (ANOVA) tests.

Results obtained by both one-way analysis of variance and two-way analysis of variance have shown that there were no statistical differences on bacterial count between the results obtained with MBS method and the results obtained with the reference method.

The reliability of the bacterial count using MBS method was also assessed by statistical analysis using Coefficient of Variation (CV) analysis according to ISO/TR 13843 (2000). It appeared that the MBS method was more reliable than the reference method.

Likewise, the uncertainty of the bacterial count using MBS method was less than that of the reference method as determined by χ^2 statistical test according to ISO/TR 13843 (2000).

Primary validation

The primary validation of the MBS method for TVC and for E. coli was made according to ISO 16140 (2003). The main performance parameters which the alternative method must demonstrate are: linearity, accuracy and selectivity.

● **Linearity** is the ability of the method when used with a given matrix to give results that are in proportion to the amount of analyte present in the sample. An increase in analyte should correspond to a linear or proportional increase in results (ISO 16140:2003).

This was achieved graphically as illustrated in Fig. 1 by plotting bacteria numbers (expressed as the log of CFU/ml) obtained with the reference methods for TVC and E. coli with the time occurred for colour change of the identical samples analysed with MBS methods for TVC and E. coli.

A linear inverse relationship between the MBS methods and the bacteria concentration, with a correlation factor (R2) close to 1.00, confirming the linearity of the data, can be observed.

● **Accuracy** is the degree of correspondence between the response obtained by the reference method and the response obtained by the alternative method on identical samples. The term 'relative accuracy' used here is complementary to the 'accuracy' and 'trueness' as defined in ISO 5725-1:1994/COR 1 (1998).

This states that accuracy is 'the closeness of agreement between a test result and the accepted reference value', and that trueness is 'the closeness of agreement between the average value obtained from a large series of test results and an accepted reference value'. For the purpose of this standard, the accepted reference values are chosen as the values obtained by the reference method. Thus, the term 'relative' implies that the reference method does not automatically provide the accepted reference value as indicated by ISO 16140 (2003).

In Fig. 2 the bacteria numbers (expressed as log CFU/ml) obtained with the reference counting methods for TVC and E. coli are plotted against the bacteria numbers (expressed as log CFU/ml) obtained with the alternative MBS method for TVC and E. coli. The straight lines in both graphs show a perfect correlation between the reference methods and the MBS methods. In fact the slopes are close to theorical value of 1.00.

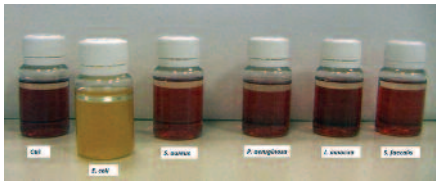

Fig. 3. Selectivity of the MBS method for E. coli. The selectivity has been observed by testing samples artificially contaminated with different bacterial strains. The vials for E. coli are inoculated with five different bacteria after 24 hours after inoculation. Only the vial inoculated with E. coli shows a change of colour from red to yellow.

● **Selectivity** is the ability of an alternative method to detect the target analyte from a wide range of strains and is the lack of interference from a relevant range of non-target strains of the alternative method as indicated by ISO 16140 (2003).

Fig. 3 illustrates selectivity of the MBS method for E. coli. The figure shows the vials for E. coli inoculated with five different bacteria strains 24 hours after inoculation. Only the vial inoculated with E. coli shows a change of the colour from red to yellow. Table 1 shows the lowest detection limit (expressed as CFU/ml) of the MBS vials for TVC and for E. coli towards different bacterial strains in artificially contaminated samples.

The lowest detection limit represents the minimal bacterial quantity required for inducing the colour change in either MBS vials for TVC or for E. coli.

The lowest detection limit is utilised to assess of the MBS vial selectivity. It could be noted that, using the E. coli vials, very high concentrations of all the bacteria other than E. coli were required for inducing the colour change (i.e. a positive result of the test), while just one E. coli cell (on average) was sufficient to induce the colour change of the same vials.

These results indicate that the E. coli vials are selective for E. coli, although the E. coli vials showed a lower selectivity towards other coliforms strains, medium level selectivity towards Enterobacteriaceae and a higher selectivity towards Gram-positive bacteria. On the contrary, using TVC vials, just one cell of all the aerobic bacteria strains (on average) was sufficient to induce the colour change, indicating the very low selectivity of the TVC vials.

Other results were obtained with MBS method and reference method to detect E. coli in naturally contaminated samples of five different food matrices.

Both methods have identified 100 target strains on 100 as positives, with total

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Table 1. Results of selectivity tests.

Bacteria strains	ATCC	Lowest detection limits for E. coli vials (CFU mL^{-1})	Lowest detection limits for TVC vials (CFU mL^{-1})
Enterobacter cloacae	13047	$>10^5$	1
Enterobacter sakazakii	31329	$>10^5$	1
Pseudomonas aeruginosa	27853	$>10^6$	1
Salmonella enteritidis	13076	$>10^6$	1
Salmonella, enterica ser. typhimurium	14028	$>10^4$	1
Yersinia enterocolitica	19543	$>10^6$	1
Citrobacter freundii	43864	$>10^3$	1
Klebsiella pneumoniae	13883	$>10^3$	1
Escherichia coli	25922	1	1
Escherichia coli O157:H7	35150	1	1
Enterococcus faecalis	29212	$>10^6$	1
Bacillus cereus	11778	$>10^6$	1
Bacillus stearothermophylus	24567	$>10^6$	1
Bacillus subtilis	6633	$>10^6$	1
Listeria innocua	33090	$>10^6$	1
Listeria ivanovii	19119	$>10^6$	1
Listeria monocytogenes	7644	$>10^6$	1
Rhodococcus equi	31543	$>10^6$	1
Staphylococcus aureus	12600	$>10^6$	1
Staphylococcus epidermidis	12228	$>10^6$	1
Staphylococcus lentus	29070	$>10^6$	1
Lactobacillus delbrueckii subsp. lactis	12315	$>10^6$	$>10^3$
Clostridium perfringens	13124	$>10^6$	$>10^6$

The lowest detection limit represents the minimal bacterial quantity of each bacterial strain required for inducing the colour change in the corresponding MBS vials for TVC or E. coli

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absence of false negatives; moreover both have identified 25 non target strains on 25 with a total absence of false positives.

Discussion

In recent years, the need for the food industry to rapidly assess the microbiological quality of raw materials and finished products, has led to the development and refinement of alternative microbiological methods of analysis.

Such alternative methods are quicker and easier to perform than the corresponding reference method. In this context, the goal of the present study was the primary validation of the Micro Biological Survey (MBS) method for both TVC and E. coli, defined, according to European Directive 91/492/CEE, as thermophilic coliforms that produce indole from tryptophan after incubation at 44°C±2°C for 24 hours.

Validation studies aim to compare the results obtained with an alternative method, in this case the MBS method, with the results obtained with the reference method verifying the equivalence between the two methods by looking at linearity, accuracy and selectivity. The results of the validation studies were statistically analysed and compared according to the norm ISO/IEC 17025 (2005) and ISO 16140 (2003).

All the performance parameters indicated a total equivalence between the reference method and the MBS method for detection and counting of TVC and E. coli in artificially contaminated water samples and in naturally contaminated food samples.

When a method is validated for environmental sample analysis, it is important to include naturally contaminated samples. In our studies, we have selected five different food matrices: cheese, vegetable, white meat, red meat and fruit.

The validation of the MBS method strongly support its use as an alternative method for food analysis. The linearity over a range of bacterial concentrations was excellent. The selectivity was more than satisfactory with the absence of false negatives and false positives. The accuracy, evaluated on 125 naturally contaminated samples, showed a high correlation between the MBS method and the reference methods.

Comparison of methods

With traditional count plate methods bacteria replication can be observed with the naked eye, but greater expertise in the operators and operational complexity are required.

On the other hand, alternative methods often turn out to be very expensive and also require highly equipped laboratories. The use of immunological or genetic probes (with the assistance of PCR to increase sensitiveness) had a great impact on microbial analysis. Indeed they are very quick and sensitivity can be improved by using automated or semi-automated systems. The disadvantages are not only related to the need for specialised personnel and equipment, but also for an high limit of sensitivity (immunological methods) and/or complexity and high costs of analysis (genetic methods).

In addition, the exact quantification of the number of bacteria over a large range of concentrations is not always possible.

Colorimetric methods currently available are mainly based upon micro-organisms secondary metabolism measuring. One of these methods detects the presence of E. coli on the basis of the activity of the enzyme β-glucuronidase.

However, it should be mentioned that using this method it is not possible to detect the pathogenic, although relatively uncommon forms of E. coli O157: H7 verocytotoxin producers, which do not exhibit β-glucuronidase activity.

Instead, E. coli O157: H7 is detected by the MBS method on the basis of its indole production from tryptophan. For the above reported reasons, the MBS method can represent a worthy aid in food screening without replacing the analysis carried out with traditional methods which are very precise though often long and complex. ■