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Qualitative and Quantitative Validation of the Micro Biological Survey Method for *Listeria* spp., *Salmonella* spp., Enterobacteriaceae and *Staphylococcus aureus* in Food Samples

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ABSTRACT

The objective of the present study was the preliminary validation of the qualitative MBS method for *Salmonella* spp. and *Listeria* spp. and the quantitative MBS method for Enterobacteriaceae and *Staphylococcus aureus*. The MBS method is a rapid alternative method for the detection and selective counting of bacteria in agro-food, in water and in environmental samples. It is based on a colorimetric survey in mono-use disposable reaction vials that can be filled with the samples without any preliminary treatment (e.g., homogenization, dilution, etc.); the greater the number of bacteria present in the sample, the faster the color changes. For the qualitative validation of the MBS method for *Salmonella* spp. and *Listeria* spp., selectivity, relative accuracy, relative specificity and relative sensitivity were determined. Selectivity, linearity and accuracy were analyzed for the quantitative validation of the MBS method for Enterobacteriaceae and *Staphylococcus aureus*. The validation here reported has shown that the MBS method for *Listeria* spp., *Salmonella* spp., Enterobacteriaceae and *S. aureus* give similar results and are in agreement with the reference method.

Key words: Food microbiological analysis, food safety, alternative microbiological method, *Listeria* spp., *Salmonella* spp., enterobacteriaceae, *S. aureus*

INTRODUCTION

Microbial food safety and food-borne infections are an important public health concern worldwide. Contaminated food consumption often results in an illness which is called food borne illness or food poisoning (Saikia and Joshi, 2010; Arzinz *et al.*, 2011).

The hygiene process criteria for many foodstuff include tests for *Salmonella* spp., *Listeria* spp., Enterobacteriaceae and *Staphylococcus aureus* (*S. aureus*). Members of the genus *Salmonella* are Gram-negative and facultative anaerobic, rod-shaped bacteria (Malkawi and Gharaibeh, 2004). *Salmonella* is a major food and water borne pathogenic bacterium which causes an intestinal infection, accompanied by fever, abdominal cramps and diarrhea which is commonly known as salmonellosis (Rathnayaka, 2011). *Salmonella* is more often associated with any raw food of animal

origin which may be subject to fecal contamination, such as raw meat, poultry, fish/seafood, eggs and dairy product (Fadel and Ismail, 2009; McGuinness *et al.*, 2009). *Listeria* spp. are ubiquitous bacteria widely distributed in the environment. Among the seven species of *Listeria*, only *Listeria monocytogenes* is commonly pathogenic for humans. *L. monocytogenes* is a Gram-positive rod that is catalase positive and shows a characteristic tumbling motility (Enan, 2006). It has been recognized as a veterinary pathogen and in humans it causes a disease known as listeriosis that could be very dangerous in older adults, persons with weakened immune systems, pregnant women and newborns. Food implicated in outbreaks of listeriosis have included various types of products such as dairy, meat, vegetable and sea food (Jalali *et al.*, 2007; Adetunji and Arigbede, 2011).

Bacteria belonging to the Enterobacteriaceae family are the most common bacterial pathogens associated with gastrointestinal infections, particularly diarrhea. This bacterial family consists of a large heterogeneous group of facultative anaerobic, Gram-negative rods and includes species in the groups *Escherichia coli, Salmonella* and *Shigella*, the most common causative agents of intestinal infections (Rustam *et al.*, 2006). Therefore, detection of Enterobacteriaceae, rather than for the traditional Coliform group, is advantageous because Enterobacteriaceae includes some potentially pathogenic species. Unlike the Coliform group, some Enterobacteriaceae species are often present in the processing environment. For this reason, enumeration of Enterobacteriaceae in foods now shows evidence of increasing interest (Rustam *et al.*, 2006; Feinberg *et al.*, 2009). *S. aureus* is a Gram-positive, catalase positive, coagulase positive non motile coccus bacterium (Ugbogu *et al.*, 2007). *S. aureus* is a common cause of a bacterial food borne disease worldwide. However, quantitative evaluation of *S. aureus* contamination of food is not a simple task. Several studies have found *S. aureus* strains unable to produce black colonies with a clear halo on the Baird Parker Agar selective medium in dairy products and many *S. aureus* display peculiar biochemical properties (Da Silva *et al.*, 2000). Therefore, the analysis of *S. aureus* eventually contaminated food stuff is made difficult by the risk of overlooking potentially pathogenic strains of *S. aureus* just because they have atypical morphological and/or biochemical characters. To avoid this danger, Harvey and Gilmour prescribe the coagulase test for all colonies with a diameter greater or less than 1 mm with or without halo or areas of clarification (Harvey and Gilmour, 1985).

Food borne pathogens are a growing concern for human illness and death. Therefore, there is an increasing demand to ensure a safe food supply and an urgent need to implement programmes such as Hazard Analysis Critical Control Points (HACCP) to monitor the quality of the products concerning produced for the presence of the pathogens (Nicolas *et al.*, 2007). New method for the rapid and reliable detection of food borne pathogens are continuously proposed and improvements in the fields of immunology, molecular biology, automation and computer technology continue to have a positive effect on the development of faster, more sensitive and more convenient method in food microbiology (Biswas *et al.*, 2011; Mandal *et al.*, 2011).

Standardized method (e.g., ISO described method) are acknowledged as the reference analytical method for official control. They rely on traditional microbiological culture standard method that are widely used in food analysis laboratories. These traditional method involve the following basic steps: pre-enrichment, selective enrichment, selective plating, biochemical screening and serological confirmation (Mandal *et al.*, 2011). These conventional method present several difficulties, such as subjectivity in the interpretation of some biochemical or morphological tests, the possible interference of matrices, especially when they present high levels of contamination, intense labor, high cost of supplies and above all, the prolonged time (from 3 to 7 days) needed to give definitive results, depending on the ability of the organisms to multiply into visible colonies (Thomas *et al.*, 2009).

In this context, MBS srl (a spin-off of Roma Tre University, Rome, Italy) has developed an alternative rapid method, called Micro Biological Survey (MBS) method. It is a fast colorimetric fast system for the detection and the selective counting of bacteria present in agro-food, in water and in environmental samples. This method consists of an analytical kit containing disposable, ready-to-use reaction vials for fast microbiological analyses. The analysis is based on the change of color of the vial content which is induced by the presence of bacteria. The analyses can be carried out by untrained personnel and anywhere they are necessary, without the need for any instrumentation other than a thermostat which is provided on request. The MBS method measures the catalytic activity of the redox enzymes in the main metabolic pathways of bacteria (Shultz and Chan, 2001; Slater, 2003; Antonini *et al.*, 2007) and allows an unequivocal correlation between the observed enzymatic activity and the number of viable cells present in the samples. The time required for a color change is inversely related to the log of bacterial concentration; like an enzymatic reaction, the greater the number of bacteria, the faster the color changes (Berlutti *et al.*, 2003). In a previous study, we carried out the primary validation of the MBS method for Total Viable Count and for *Escherichia coli* with almost perfect agreement between reference method (Bottini *et al.*, 2011). The objective of the present study was the primary validation of the qualitative MBS method for *Salmonella* spp. and *Listeria* spp. and the quantitative MBS method for Enterobacteriaceae and *S. aureus* in accord with ISO 16140 (2003). Qualitative method are method of analysis whose responses are either the presence or absence of the analyte detected either directly or indirectly in a certain amount of sample as indicated by ISO 16140 (2003). Quantitative method are method of analysis whose responses are the amount of the analyte measured either directly or indirectly in a certain amount of sample as indicated by ISO 16140 (2003). The validation here reported provide evidence that the new MBS method gave similar results and are in agreement with the reference method, confirming the reproducibility and specificity of MBS method.

MATERIALS AND METHODS

This study was conducted at the department of Biology, University Roma Tre during the period from 2009 to 2011.

Bacterial strains: All the strains used in these validations were available at ATCC (American Type Culture Collection): *E. coli* (ATCC 25992), *E. coli* O157:H7 (ATCC 35150), *C. freundii* (ATCC 43864), *K. pneumoniae* (ATCC 13883), *E. cloacae* (ATCC 13047), *E. sakazakii* (ATCC 51329), *S. enteritidis* (ATCC 13076), and *S. enterica* ser. Typhimurium (ATCC 14028), *Y. enterocolitica* (ATCC 19543), *B. cereus* (ATCC 11778), *B. stearothermophilus* (ATCC 24567), *B. subtilis* (ATCC 6633), *L. innocua* (ATCC 33090), *L. ivanovii* (ATCC 19119), *L. monocytogenes* (ATCC 7644), *S. aureus* (ATCC 12600), *S. epidermidis* (ATCC 12228), *S. lentus* (ATCC 29070), *P. aeruginosa* (ATCC 27853), *R. equi* (ATCC 31543), *E. faecalis* (ATCC 29212), *L. delbrueckii* subsp. *lactis* (ATCC 12315), *C. perfringens* (ATCC 13124), *A. niger* (ATCC 9642) and *S. cerevisiae* (ATCC 9763).

Preparation of naturally contaminated food samples with different levels of contamination: Naturally contaminated food samples were randomly selected among those found positive by reference method. Four different food matrices were selected for validation of qualitative method: Raw meat products, vegetables, pastry and dairy products. Three different food matrices

were selected for validation of quantitative method: Raw meat products, pastry and dairy products. Sterilized "Baby foods" of the same foodstuff typology were used as a negative control. Different levels of contamination of naturally contaminated samples were obtained as follows: 10±0.5 g of naturally contaminated samples were homogenized in 90 mL of peptone water by a stomacher according to ISO 16140 (2003). Then homogenates were incubated for different times at different temperatures obtaining different levels of contamination. To verify the equivalence between the MBS method and the reference method, these samples were simultaneously tested.

Artificially contaminated food samples: To achieve the number of samples required for statistical evaluation of the chosen parameters, artificially contaminated samples were also used. Food samples, found negative by reference method, were contaminated with a mixture of the above indicated microorganisms from overnight cultures with serial dilutions in sterile saline solution up to 10^{-8}. Ten different dilutions of ten different samples were analyzed for *Listeria* spp., *Salmonella* spp., Enterobacteriaceae and *S. aureus*. Each dilution was tested in duplicate with both the MBS method and the plate counting reference method.

Colorimetric MBS method procedure: The analytical procedure for qualitative and quantitative MBS method is based on colorimetric survey, using a redox indicator of the change of the oxidoreductive state in the reaction medium. For the analysis by the MBS method, ready-to-use MBS vials, sterilized and containing the reagent for the analysis were used. Four different kinds of vials were used: The vials for *Listeria* spp., *Salmonella* spp., Enterobacteriaceae and *S. aureus* analysis. To carry out the analysis, 10 mL of sterile distilled water and 1 mL of the samples were added to a vial of one or another type, depending on the type of analysis to be carried out. The vial was shaken until the entire reagent was dissolved. Later on, the vial was incubated at 37°C for all bacteria. The starting color is blue for vials for *Listeria* spp. and red for vials for *Salmonella* spp., Enterobacteriaceae and *S. aureus*. In the presence of the microorganisms of interest, the colors of vials changed to yellow indicating a positive result. The time taken to turn yellow is inversely related to the bacterial content of the analyzed sample. The persistence of the starting color after 36 h for *Listeria* spp. and *S. aureus*, 32 h for *Salmonella* spp. and 24 h for Enterobacteriaceae indicates a negative result, that is an absence of microorganisms.

Reference method: For *Listeria* spp. the reference method was plate count on Agar *Listeria* Ottaviani and Agosti (ALOA; Sigma, St. Louis, MO, USA) and *Listeria* Palcam Agar (PALCAM; Liofilchem, Roseto degli Abbruzzi, Italy) after 24 h of incubation at 37°C according to ISO 11290-1:1996/Adm 1 (2004). For *Salmonella* spp. it was plate count on Xylose Lysine Desoxycholate Agar (XLD, Sigma, St. Louis, MO, USA) and Brilliant Green Agar (BGA; Sigma, St. Louis, MO, USA) after 24-48 h of incubation at 37°C according to ISO 6579:2002/COR 1 (2004). For Enterobacteriaceae the reference method was plate count on Violet Red Bile Glucose Agar (VRBGA; Liofilchem, Roseto degli Abbruzzi, Italy) after 24 h of incubation at 37°C. For *S. aureus* it was plate Baird-Parker Agar (BPA; Sigma, St. Louis, MO, USA) after 46-48 h according to ISO 6888-1:1999/Adm 1 (2003). However, it should be kept in mind that, when a food sample is analysed with a reference method, a pretreatment is always required, according to the above reported ISO rules. Such pretreatment may vary from sample homogenization and dilution up to an additional enrichment, according to the different analysis to be carried out. The whole procedure for analysing a food sample may therefore last from a minimum of 36 h up to a maximum of 72 h.

Data analysis for qualitative validation: The primary validation of the qualitative MBS method for *Listeria* spp. and *Salmonella* spp. was made according to ISO 16140 (2003). The relative performance parameters indicated by the ISO 16140 (2003): accuracy, specificity and sensitivity were determined. They were calculated as follows: accuracy AC = ((PA+NA)/N) x 100%; specificity SP = (NA/N$_-$) x 100%; sensitivity: SE = (PA/N$_+$) x 100%. Where, PA is the agreement for positive results; NA is the agreement for negative results; N is the total number of samples; N$_-$ is the total number of negative results with the reference method (N$_-$=NA+PD); N$_+$ is the total number of positive results with the reference method (N+ = PA+ND); PD is positive deviation (i.e., false positive result); ND is negative deviation (i.e., false negative result) (ISO 16140, 2003).

Data analysis for quantitative validation: Data analysis was carried out according to ISO 16140 (2003). Two parameters were analyzed: linearity and accuracy. The linearity of the method was assessed by analyzing the correlation using a plot of bacteria concentrations (expressed as CFU mL^{-1}) against the time taken to change color. The accuracy was assessed by analyzing the correlation using a plot of bacteria concentrations (expressed as log CFU mL^{-1}) obtained with the reference method and with the alternative MBS method (ISO 16140, 2003).

***S. aureus* confirmative coagulase test:** Preparation of samples for coagulase test was carried out as follows: selected colonies grown on BPA agar were transferred each with a sterile inoculation loop to different culture tubes containing Brain Heart Broth (Sigma, St. Louis, MO, USA) and were incubated at 37°C for 20-24 h; 10 mL of the supernatants of MBS method for *S. aureus,* that have changed color from red to yellow, were inoculated each within a vial containing 0.5 g of Amberlite MB-150 Mixed Bed Exchanger (Sigma, St. Louis, MO, USA), moderately shacked and left rest for 5-10 min. The confirmative coagulase test was carried out as follows: a vial with lyophilized rabbit plasma with EDTA (Sigma, St. Louis, MO, USA) was rehydrated with 3 mL of distilled water and 0.3 mL of the rehydrated rabbit plasma were pipetted into a sterile culture tube using a sterile pipette; 0.1 mL of the sample (either coming from culture tubes containing Brain Heart Broth or from MBS supernatants) was carefully mixed with the plasma in the sterile culture tube and then incubated at 37°C; the tubes were checked every hour for coagulation by gently tipping to the side; the coagulase test was positive if more than 75% of the tube contents had formed a coherent clot. If the test was negative after 4-6 h, the tube was left in the incubator and a final assessment was made after 24 h.

RESULTS
Selectivity of MBS method: Preliminary experiments were carried out to determine whether the food matrices may interfere with the MBS method. For this purpose tests on food samples artificially contaminated with target ATCC strains (*L. monocytogenes* ATCC 7644, *S. enterica* ser. Typhimurium ATCC 14028, *E. coli* ATCC 25922 and *S. aureus* ATCC 12600) were carried out (data not shown). A perfect agreement between reference method and MBS method was observed for all the strains, indicating that no interference came from any of the food matrices utilized (raw meat products, vegetables, pastry and dairy products).

Further preliminary tests were carried out to determine the selectivity of the different MBS method. The selectivity is defined as the ability of an alternative method to detect the target analyte from a wide range of strains and the lack of interference from a relevant range of non-target strains of the alternative method (ISO 16140, 2003). Table 1 reports the selectivity tests

Table 1: Selectivity tests

Bacteria strains		Lowest detection limits for the different reagents (CFU mL^{-1})			
		Listeria spp.	*Salmonella* spp.	*Enterobacter* spp.	*S. aureus*
Enterobacter cloacae	ATCC 13047	$>10^6$	$>10^5$	1	$>10^6$
Enterobacter sakazakii	ATCC 31329	$>10^6$	$>10^5$	1	$>10^6$
Pseudomonas aeruginosa	ATCC 27853	$>10^6$	$>10^6$	$> 10^6$	$>10^5$
Salmonella enteritidis	ATCC 13076	$>10^6$	1	1	$>10^6$
Salmonella enterica ser. Typhimurium	ATCC 14028	$>10^6$	1	1	$>10^6$
Yersinia enterocolitica	ATCC 19543	$>10^6$	$>10^6$	1	$>10^6$
Citrobacter freundii	ATCC 43864	$>10^6$	$>10^6$	1	$>10^6$
Klebsiella pneumoniae	ATCC13883	$>10^6$	$>10^6$	1	$>10^6$
Escherichia coli	ATCC 25922	$>10^6$	$>10^6$	1	$>10^6$
Escherichia coli O157:H7	ATCC 35150	$>10^6$	$>10^6$	1	$>10^6$
Enterococcus. faecalis	ATCC 29212	$>10^6$	$>10^6$	$>10^6$	$>10^5$
Bacillus cereus	ATCC 11778	$>10^6$	$>10^6$	$>10^6$	$>10^5$
Bacillus stearothermophilus	ATCC 24567	$>10^6$	$>10^6$	$>10^6$	$>10^5$
Bacillus subtilis	ATCC6633	$>10^6$	$>10^6$	$>10^6$	$>10^5$
Listeria innocua	ATCC 33090	1	$>10^6$	$>10^6$	$>10^5$
Listeria ivanovii	ATCC 19119	1	$>10^6$	$>10^6$	$>10^5$
Listeria monocytogenes	ATCC7644	1	$>10^6$	$>10^6$	$>10^5$
Rhodococcus equi	ATCC 31543	$>10^6$	$>10^6$	$>10^6$	$>10^6$
Staphylococcus aureus	ATCC 12600	$>10^6$	$>10^6$	$>10^6$	1
Staphylococcus epidermidis	ATCC 12228	$>10^6$	$>10^6$	$>10^6$	$>10^4$
Staphylococcus lentus	ATCC 29070	$>10^6$	$>10^6$	$>10^6$	$>10^4$
Lactobacillus delbrueckii subsp. *lactis*	ATCC 12315	$>10^6$	$>10^6$	$>10^6$	$>10^6$

indicating the minimum detection limit (expressed as CFU mL^{-1}) of the MBS method for *Listeria* spp., *Salmonella* spp., *Enterobacteriaceae* and for *S. aureus* towards different ATCC bacterial strains suspended in protonated water.

PRIMARY VALIDATION OF MBS METHOD FOR *Listeria* spp. AND *Salmonella* spp. IN QUALITATIVE ASSAYS

The primary validation of the qualitative MBS method for *Salmonella* spp. and *Listeria* spp. was performed according to ISO 16140 (2003).

Table 2 shows the results of analysis on both naturally and artificially contaminated food matrices (raw meat products, vegetable, pastry and dairy products) obtained with MBS method and reference method for *Listeria* spp. and *Salmonella* spp. These results indicate the concordance between results obtained with MBS method and reference method to detect *Listeria* spp. and *Salmonella* spp. Out of 71 positive samples obtained with the reference method for *Listeria* spp. 67 were found to be positive (N_+) and 4 were found to be negatives (ND) by the MBS method. Out of 17 negative samples with the reference method, 17 were found to be negative (N_-) by the MBS method and no positives were found (PD). For *Salmonella* spp. out of 81 positive samples obtained with the reference method, 79 were found to be positive (N_+) and 2 were found to be negatives (ND) by the MBS method. Out of 15 negative samples obtained with the reference method, 12 were found to be negative (N_-) and 2 were found to be positives (ND) by the MBS method.

The main performance parameters which the alternative method must demonstrate are the relative accuracy, specificity, sensitivity and selectivity.

Table 2: Results of analysis of food samples (raw meat products, vegetable, pastry and dairy products) either naturally or artificially contaminated obtained with MBS method and reference method for (a) *Listeria* spp. and (b) *Salmonella* spp.

	Reference method		
MBS method	Present	Absent	Total
***Listeria* spp.**			
Positive	67 (PA)	0 (PD)	67
Negative	4 (ND)	17 (NA)	21
Total	71 (N+)	17 (N-)	88 (N)
***Salmonella* spp.**			
Positive	79 (PA)	3 (PD)	82
Negative	2 (ND)	12 (NA)	14
Total	81 (N+)	15 (N-)	96 (N)

PA: Positive agreement, NA: Negative agreement, ND: Negative deviation (false negatives), PD: Positive deviation (false positives), N: Total number of samples (NA+PA+PD+ND), N_+: Total number of positive results obtained with reference method, N_-: Total number of negative results obtained with reference method

Table 3: Paired values of relative accuracy (AC), relative sensitivity (SE) and relative specificity (SP) for MBS method and reference method

	MBS method		Reference method	
	Listeria spp.	*Salmonella* spp.	*Listeria* spp. (ISO 11290-1:1996, 2004)	*Salmonella* spp. (ISO 6579:2002, 2004)
(%)			(%)	
AC = (PA+NA)/N	95.4	94.8	88.7	89.1
SE = PA/N+	100.0	80.0	85.2	94.4
SP = NA/N-	94.4	97.5	97.4	88.8

Relative accuracy is the degree of correspondence between the results obtained by the reference method and the results obtained by the alternative method on identical samples (ISO 16140, 2003). 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".

Relative sensitivity is the ability of the alternative method to detect the analyte when it is detected by the reference method.

Relative specificity is the ability of the alternative method to not detect the analyte when it is not detected by the reference method.

Table 3 shows the values of the performance parameters for MBS method calculated using the positive and negative results shown in Table 2 and the same performance parameters for the reference method as reported in the literature (ISO 11290-1:1996/Adm 1, 2004 for *Listeria* and ISO 6579:2002/COR 1, 2004 for *Salmonella* spp.).

PRIMARY VALIDATION OF QUANTITATIVE MBS METHOD FOR ENTEROBACTERIACEAE AND *S. aureus*

The primary validations of the quantitative MBS method for *S. aureus* and Enterobacteriaceae were performed according to ISO 16140 (2003). The main performance parameters which the alternative method must demonstrate are: linearity and accuracy.
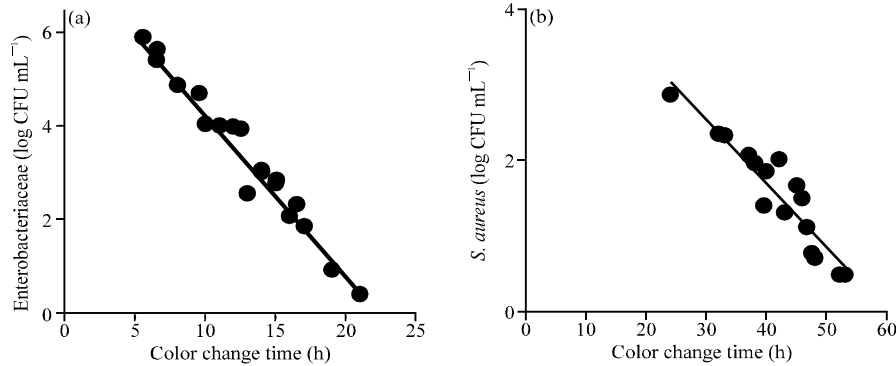
Fig. 1 (a-b): Linearity: Correlation line between analyte (a) Enterobacteriaceae and (b) *S. aureus* concentrations with the time taken to change color with in the MBS method

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, that is, an increase in analyte corresponds to a linear or proportional increase in results as indicated by ISO 16140 (2003). This was achieved graphically as illustrated in Fig.1 by plotting bacteria concentrations (expressed as the log of CFU mL^{-1}) obtained with the reference method with the time occurred for taken to change color with the identical samples analyzed with MBS method. A linear inverse relationship between the MBS method and the bacteria concentration, with a correlation factor (R^2) close to 1.00, confirming the linearity of the data can be observed. Using naturally and artificially contaminated food samples, bacteria concentrations (expressed as the log of CFU mL^{-1}) obtained with the reference method are plotted against the time taken to change color with the identical samples analyzed with MBS method. A linear inverse relationship between the time, taken to change color with the MBS method and the bacteria concentration could be observed with Enterobacteriaceae vials and *S. aureus* vials on three different food matrices: raw meat products, pastry and dairy products. The correlation factors (R^2) are 0.98 and 0.95 for Enterobacteriaceae and for *S. aureus*, respectively.

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 (ISO 16140, 2003). Figure 2 show a perfect correlation between the bacteria number (expressed as log CFU mL^{-1}) obtained with the traditional counting method and the alternative MBS method. The straight lines obtained were close to the theoretical y = x (slope = 1,00), with values of correlation factor (R^2), which confirm the high equivalence between the reference method and the alternative. Using naturally and artificially contaminated food samples, bacteria numbers (expressed as the log CFU mL^{-1}) obtained with the reference method are plotted against the time taken to change color with the identical samples analyzed with MBS method. A good correlation between the bacteria numbers (expressed as log CFU mL^{-1}) obtained with the traditional counting method and the alternative MBS method could be observed. In fact the slopes are close to the theoretical value of 1.00 (i.e., 0,98 for Enterobacteriaceae and 0.93 for *S. aureus*). The correlation factors (R^2) are 0.97 and 0.94 for Enterobacteriaceae and for *S. aureus*, respectively.

Confirmation tests for *S. aureus* were performed on all the positive and negative results. Results obtained using coagulase test were in full agreement with results obtained by MBS method since all the MBS positive results were positive in the coagulase tests.
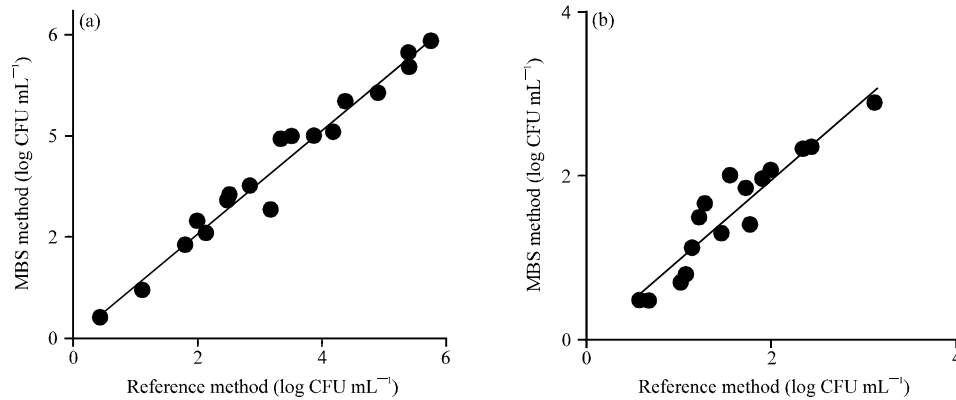
Fig. 2 (a-b): Accuracy: Correlation line between alternative MBS method and reference method (a) Enterobacteriaceae and (b) *S. aureus*

DISCUSSION

Rapid and reliable detection of microorganisms in food samples is essential for prevention of disease, but it is also important to save on the cost of storage and transportation of infected products, (Rathnayaka, 2011), therefore, the development of new method for detection and identification of microorganisms in food, water and environmental samples which give accurate results and are economically competitive, are always needed. With traditional count plate method bacteria replication can be observed with the naked eye, but greater expertise between the operators and operational complexity are required. On the other hand, alternative method often turn out to be very expensive and also require highly equipped laboratories (Settanni and Corsetti, 2007; Thacker *et al.*, 1996).

In this context, rapid colorimetric MBS method may play an important role. This is a fast colorimetric system for the detection and selective counting of bacteria in agro-food, in water and in environmental samples. Colorimetric method currently available are mainly based upon microorganisms secondary metabolism measuring. On the contrary, the MBS method measures the catalytic activity of redox enzymes of the main metabolic pathways of bacteria (Antonini *et al.*, 2007; Bottini *et al.*, 2011), allowing an unequivocal correlation between the observed enzymatic activity and the number of viable cells present in the samples. The time required for color change is inversely related to the log of bacterial concentration; like an enzymatic reaction, the greater the number of bacteria, the faster the color change (Bottini *et al.*, 2011). Alternative rapid analytical method, like the MBS method, are allowed by regulatory authorities once they have been validated against the reference method according to ISO 16140 (2003) and McGuinness *et al.* (2009). A previous study provided the evidence that the MBS method for TVC and *E. coli* gave similar results and is in agreement with reference method (Bottini *et al.*, 2011). The purpose of the present study was the primary validation of qualitative MBS method for *Listeria* spp. and *Salmonella* spp. and of quantitative MBS method for Enterobacteriaceae and *S. aureus*. Qualitative method are method of analysis whose responses are either the presence or absence of the analyte detected either directly or indirectly in a certain amount of sample as indicated by ISO 16140 (2003). Quantitative method are method of analysis whose responses are the amount of the analyte measured either directly or indirectly in a certain amount of sample as indicated by ISO 16140 (2003). Both the MBS qualitative and quantitative method were demonstrated to be very selective and all showed a high reliability and correlation with traditional count plate method. No interference due to food matrices was observed.

In particular, quantitative MBS method for Enterobacteriaceae and *S. aureus* demonstrated high linearity (results are in proportion to the amount of analyte present in the sample) and accuracy (correspondence between the results obtained by the reference method and the results obtained by the alternative method on identical samples). It should also be noted that results obtained using coagulase test were in full agreement with results obtained by the MBS method on *S. aureus*, demonstrating that the well known variability of the morphological and biochemical properties of naturally occurring *S. aureus* strains did not influence the exact quantification by MBS method of *S. aureus* cells present in the food samples.

For qualitative analysis, a perfect correspondence between the MBS method and the reference method for *Listeria* spp. and *Salmonella* spp. was observed when ATCC reference strains were inoculated into different food matrices. On the contrary, a limited number of discrepancies between MBS method and reference method were observed when the same food matrices were contaminated by naturally occurring strains of *Listeria* and *Salmonella*. This phenomenon may lead to either false positive or false negative results; however these discrepancies may be attributed either to the MBS method or to the reference method. It should be kept in mind that the apparent false negatives or false positives become real false negatives or false positives only when an independent reference method has been proven to be true. The paired values for relative accuracy, sensitivity and specificity for MBS method here reported are lower than the same parameters reported for the reference method (ISO 11290-1:1996/Adm 1, 2004; ISO 6579:2002/COR 1, 2004). As a matter of fact, a small percentage of false negatives and false positives were observed using the reference method in a inter-laboratory experiments (ISO 11290-1:1996/Adm 1, 2004; ISO 6579:2002/COR 1, 2004). For these reasons, the very same ISO documents stated that when the reference method indicates a positive result, supplementary analysis to prove whether there is a real presence or not of pathogenic bacteria should be carried out (ISO 11290-1:1996/Adm 1, 2004; ISO 6579:2002/COR 1, 2004).

Although, we have here demonstrated that relative accuracy, sensitivity and specificity for MBS method for *Listeria* spp. and *Salmonella* spp. are more reliable than the respective reference method, an independent analysis should be carried out when a positive result is found.

CONCLUSIONS

The validations of the MBS method for *Listeria* spp., *Salmonella* spp., Enterobacteriaceae and *S. aureus* give similar results and are in agreement with the reference method according to ISO rules. MBS method could therefore become a valid support for the control procedures for all the food farming companies willing to do a microbiological screening of their products to ensure complete hygienic production.

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