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Microbiological safety and quality of Mozzarella cheese assessed by the microbiological survey method

Francesca Losito,* Alyexandra Arienzo,*[1] Giorgia Bottini,†[1] Francesca Romana Priolisi,† Alberto Mari,† and Giovanni Antonini*‡[2]
*Department of Science, Roma Tre University, 00146 Rome, Italy
†MBS S.r.l., 00131 Rome, Italy
‡National Institute of Biostructures and Biosystems (INBB), 00136 Rome, Italy

ABSTRACT

Dairy products are characterized by reduced shelf life because they are an excellent growth medium for a wide range of microorganisms. For this reason, it is important to monitor the microbiological quality of dairy products and, in particular, the total viable count and concentration of *Escherichia coli*, as they are indicators of the hygienic state of these products. In addition, in dairy products such as Mozzarella cheese, it is important to monitor the concentration of lactic acid bacteria (LAB), as they are the major components of starter cultures used in cheese production, contributing to the taste and texture of fermented products and inhibiting food spoilage bacteria by producing growth-inhibiting substances. For these reasons, to ensure the quality and safety of their products, cheese makers should monitor frequently, during fresh cheese production, the concentration of LAB and spoilage bacteria. However, usually, small- to medium-size dairy factories do not have an internal microbiological laboratory and external laboratories of analysis are often too expensive and require several days for the results. Compared with traditional methods, the microbiological survey (MBS) method developed by Roma Tre University (Rome, Italy) allows faster and less-expensive microbiological analyses to be conducted wherever they are necessary, without the need for a microbiological laboratory or any instrumentation other than MBS vials and a thermostat. In this paper, we report the primary validation of the MBS method to monitor LAB concentration in Mozzarella cheese and the analysis, using the MBS method, of total viable count, *E. coli*, and LAB concentrations in the production line of Mozzarella cheese as well as during the shelf life of the product stored at 20°C. The results obtained indicate that the MBS method may be successfully used by small- to medium-size dairy factories that do not have an internal microbiological laboratory. Using the MBS method, these dairy factories can monitor autonomously the microbiological safety and quality of their products, saving both time and money.
Key words: Mozzarella cheese, lactic acid bacteria, microbiological analysis

INTRODUCTION

Microbial contamination, causing approximately one-fourth of the world's food supply loss, has become an enormous economic and ethical problem worldwide (Huis in 't Veld, 1998). Dairy products are an excellent growth medium for a wide range of microorganisms and, thus, display a reduced shelf life (Ruegg, 2003). The microbiological quality of dairy products is influenced by the initial flora of raw milk, the processing conditions, and post-heat treatments. Spoilage bacteria and various bacteria of public health concern can be found in these products and their concentrations should be kept as low as possible (Varga, 2007). In contrast, lactic acid bacteria (**LAB**), occurring in the indigenous microflora of raw milk and being the major components of starter cultures used in fermentation, contribute to the quality of fermented cheese products by improving the taste and texture and inhibiting food spoilage bacteria by producing growth-inhibiting substances and large amounts of lactic acid (Jana and Mandal, 2011). Thus, to be confident of fermented cheese quality, LAB concentration should be monitored during cheese production.

Traditional Mozzarella cheese made from water buffalo milk is one of the most highly valued unripened pasta filata cheeses in Italy, certified with the European Protected Designation of Origin. Although mainly produced in Italy, it is widely exported and it is also industrially produced in other countries. The specific taste characteristics of Mozzarella cheese mainly arise from the raw milk used, the area of production, the environmental conditions, the traditional tools, and manufacturers (Kindstedt and Fox, 1993; Mauriello et

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[1]These authors contributed equally to this work.
[2]Corresponding author: giovanni.antonini@uniroma3.it

al., 2003). Mozzarella cheese belongs to the category of stretched curd, or pasta filata cheeses. Pasta filata cheeses are distinguished by a unique plasticizing and kneading treatment of the fresh curd in hot water, which imparts to the finished product its characteristic fibrous structure and melting and stretching properties (Mijan et al., 2010). As for other typical cheeses, its manufacture and composition is regulated by law (DPR 54/97, DPR, 1997; Reg. EC 2527/98; EC, 1998). Traditionally, water buffalo Mozzarella cheese is manufactured from raw milk using natural whey cultures as starters. Starter cultures fulfill several important functions in cheese making: acid production from lactose, inhibition of spoilage and pathogenic microorganisms, improvement of cheese-keeping quality, and direct and indirect contributions to flavor and aroma (Coppola et al., 1990). In Mozzarella cheese, the main function of starter cultures is to ensure rapid acidification of the curd, which promotes the transformation of dicalcium paracasein into monocalcium paracasein during stretching in hot water. The amount and variety of microorganisms in the starter depend on the technological process and, in particular, by the use of natural whey starters (Suzzi et al., 2000).

Lactic acid bacteria are the major component of the starters used in fermentation, and some of them are also natural components of the gastrointestinal microflora (Coeuret et al., 2003). The term LAB is conventionally reserved for genera in the order *Lactobacillales*, which includes the genera *Lactobacillus*, *Leuconostoc*, *Pediococcus*, *Lactococcus*, and *Streptococcus*, in addition to *Carnobacterium*, *Enterococcus*, *Oenococcus*, *Tetragenococcus*, *Vagococcus*, and *Weissella* (Mack et al., 1999). Lactic acid bacteria are gram-positive, nonspore-forming cocci, coccobacilli, or rods. They generally are anaerobes, lack catalase, and ferment glucose primarily to lactic acid, or to CO_2 and ethanol (Coeuret et al., 2003). Lactic acid bacteria play an important role in the production and conservation of fermented foods, especially in the dairy industry (Loones, 1994; Moreira et al., 2000; Luquet and Corrieu, 2005). As starter cultures, LAB contribute to the development of the physical properties of cheese, particularly body and texture. The use of starter cultures that display different proteolytic characteristics can modify the stretch, melt, and color of Mozzarella cheese (Oberg et al., 1991). In addition, LAB produce several metabolic products, such as organic acids, FA, hydrogen peroxide, and bacteriocins, which have antimicrobial activity, resulting in the growth inhibition of spoilage and pathogenic bacteria and in the improvement of quality and shelf life of the product (Osman Mohamed Abdalla and Nourein Mohammed Ibrahim, 2010).

The evaluation of the microbiological quality and safety of Mozzarella can be carried out through the evaluation of 3 parameters: total viable count (**TVC**), the number of *Escherichia coli*, and the number of LAB. The TVC gives a quantitative idea of the presence of mesophilic aerobic microorganisms of animal origin. It serves as an important criterion to evaluate the microbial quality of various foods and also the degree of freshness of food (Nanu et al., 2007). *Escherichia coli* are commensal organisms that reside within the host gut, but some pathogenic strains are recognized as a cause of gastroenteritis (Callaway et al., 2003). Contamination from human and animal waste is traditionally indicated by the presence of commensal *E. coli*. Although these organisms are essentially nonpathogenic, their presence warns of the possible concurrent existence of pathogenic microbes (Sherfi et al., 2006). Regulation (EC) No. 2073/2005 on microbiological criteria for foodstuffs requires *E. coli* quantification as an indicator of the level of hygiene. In cheeses made from milk or whey that have undergone heat treatment, *E. coli* content is unsatisfactory at >1,000 cfu/g. In addition, Regulation (EC) No. 2527/98 of 25 November 1998, concerning the characteristics of Mozzarella, as per Regulation (EC) No. 2515/94, requires for Mozzarella cheese, the presence of "typical microflora resistant to curd stretching, in an amount of not less than 10^7 cfu/g in samples analyzed within three days after the date of production" (EC, 1998).

The microbiological quality of Mozzarella cheese is assessed by quarterly controls performed by external laboratories on samples chosen randomly in the production lines in Mozzarella cheese manufacture. The analyses concern the presence of pathogenic and contaminating bacteria, but are still insufficient to guarantee the constant microbiological control needed for a particular product such as Mozzarella. In this case in fact, it would be important to assess the microbiological quality before the product is sold and consumed to allow rapid intervention. Analysis should, therefore, be rapid, reliable, economical, and executable by the personnel without relying on external laboratories.

In this context, MBS S.r.l. (a spin-off of Roma Tre University, Rome, Italy) has developed an alternative method, called the microbiological survey (**MBS**) method. It is a fast colorimetric system for the detection and the selective count of bacteria present in agrofood, water, and environmental samples. This method consists of an analytical kit, using disposable, ready-to-use reaction vials for fast microbiological analyses. The analysis is based on the color change of the vial content, which is induced by the presence of bacteria. The analyses can be carried out by untrained personnel and

wherever analyses 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 (Schultz and Chan, 2001; Slater, 2003; Antonini et al., 2007), 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 color change is inversely related to the logarithm of bacterial concentration; similar to an enzymatic reaction, the greater the number of bacteria, the faster the color change (Bottini et al., 2011; Losito et al., 2012).

The ultimate goal of the research was to allow dairy factories to carry out independently microbiological analysis to assess Mozzarella cheese microbiological safety and quality. To reach this goal, a primary validation of MBS vials for the detection of LAB was carried out and afterward the MBS method was used directly in a small artisanal Mozzarella cheese factory to measure the bacterial flora during Mozzarella production and throughout its shelf life. The MBS method for TVC and for the detection and enumeration of *E. coli*, also used in this work, have already been validated (Bottini et al., 2011; Losito et al., 2012).

MATERIALS AND METHODS

Bacterial Strains Used

All bacterial strains used in this validation were available from the American Type Culture Collection (**ATCC**, Manassas, VA). Selected LAB strains were chosen from the strains most frequently isolated from milk and Mozzarella cheese and most frequently used as starter cultures in Mozzarella cheese production. These strains are *Lactobacillus casei* ssp. *casei* (ATCC 393), *Lactobacillus acidophilus* (ATCC 4356), *Lactobacillus delbrueckii* ssp. *bulgaricus* (ATCC 11842), *Lactobacillus delbrueckii* ssp. *lactis* (ATCC 12315), *Streptococcus salivarius* ssp. *thermophilus* (ATCC 19258). Non-target selected strains were *Escherichia coli* (ATCC 25992), *Enterobacter cloacae* (ATCC 13047), *Enterobacter sakazakii* (ATCC 51329), *Salmonella enterica* serovar Typhimurium (ATCC 14028), *Listeria innocua* (ATCC 33090), *Listeria monocytogenes* (ATCC 7644), *Staphylococcus aureus* (ATCC 12600), *Staphylococcus epidermidis* (ATCC 12228), *Enterococcus faecalis* (ATCC 29212), and *Clostridium perfringens* (ATCC 13124).

Preparation of Naturally Contaminated Food Samples

Naturally contaminated food samples were homogenized (2 min at high speed) by means of a stomacher (Laboratory Blender Stomacher 400; Seward Ltd., London, UK). The homogenized samples were then serially diluted in peptone water. The dilutions were used for the enumeration of bacteria using conventional microbiological methods. No sample preparation was required for the enumeration of bacteria using the MBS method. The research was carried out in collaboration with an artisanal dairy factory near Rome, Italy.

Preparation of Selected ATCC LAB Strains Suspended in Sterile Saline Solution

Selected ATCC LAB strains, suspended in sterile saline solution (0.9% NaCl), were used to achieve the number of samples required for statistical evaluation. Saline solution was contaminated with either a single ATCC LAB strain or with a mixture of the above-indicated selected ATCC LAB coming from pure cultures serially diluted in saline solution up to 10^{-9}.

Bacterial Counts Using the Reference Method

According to the reference method, TVC were determined on plate count agar (PCA; Liofilchem S.r.l., Roseto degli Abruzzi, Italy) after 72 h of incubation at 30°C according to the International Organization for Standardization (**ISO**) method 4833:2003 (ISO, 2003a); *E. coli* was counted on Tryptone Bile-X-Glucuronide Agar (TBX; Liofilchem S.r.l.) after 18 to 24 h of incubation at 44°C according to method ISO 16649-2:2001 (ISO, 2001). Lactic acid bacteria were enumerated according to standard method ISO 15214:1998 (ISO, 1998), using the plate count technique on de Man, Rogosa, and Sharpe (MRS) agar (Sigma, St. Louis, MO), after incubation at 37°C for 72 h under anaerobic conditions.

Bacterial Counts Using the MBS Method

Three different kinds of ready-to-use MBS vials for TVC, *E. coli*, and LAB, sterilized and containing the reagent, were used. To start the analysis, 10 mL of sterile distilled water was added to the vials, which were shaken until all the reagent was dissolved, and then inoculated with 1 mL or 1 g of sample. No preliminary homogenization and serial dilution were necessary for food samples. Vials were then incubated at the specific temperature required for the analysis: 30°C for TVC, 44°C for *E. coli*, and 37°C for LAB. The color was periodically controlled either by visual inspection or with a thermostated colorimeter that automatically detects the color change. For TVC, a positive result corresponds to a color change from blue to yellow, for *E. coli* and LAB, from red to yellow. The color change occurred at different times after inocula according to

the bacterial concentration. Similar to an enzymatic reaction, the time for color change was inversely related to the bacterial content of the analyzed sample: the higher the bacterial concentration, the less the time required for color change. The persistence of the starting color indicates a negative result; that is, absence of the microorganisms of interest.

Data Analysis for Primary Validation of the MBS Method for LAB

Data analysis was carried out according to method ISO 16140:2003 (ISO, 2003b). Four parameters were analyzed: sensitivity, selectivity, linearity, and accuracy. All selected ATCC strains suspended in sterile saline solution and naturally contaminated samples were used. Sensitivity was carried out by comparing the MBS method with the reference method using different target strains. Selectivity was observed by comparing the MBS method with the reference method using target and nontarget strains. Linearity was assessed by analyzing the correlation between the 2 methods using a plot of the logarithms of the bacterial concentrations obtained with the reference method against the time occurred for color change for the MBS method. The accuracy was assessed using a plot of bacterial concentrations (log cfu/mL) obtained with the reference method against the bacterial concentrations (log cfu/mL) obtained with the alternative MBS method (ISO 16140:2003; ISO, 2003b), using the same samples.

Application of the MBS Method for Process Analysis in the Production Line of Mozzarella Cheese

Microbiological quality in the production line of Mozzarella cheese manufacture was investigated by analyzing 5 types of products: raw water buffalo milk, thermized water buffalo milk, natural whey starter, governing liquid, and water buffalo Mozzarella cheese. Twenty samples of each type were taken from an artisanal dairy factories located near Rome. Three different parameters were evaluated: LAB, TVC at 30°C, and *E. coli*. Five different samples were analyzed in duplicate with the MBS method directly in the cheese factory. Control analyses were carried out with the reference methods at the Roma Tre University laboratory.

Application of the MBS Method for the Assessment of Quality and Safety of Mozzarella Cheese

The quality and safety of the Mozzarella cheese were assessed by the evaluation of microbial dynamics in Mozzarella cheese stored for a period of 14 d at room temperature (20°C). Three different parameters were evaluated: LAB, TVC at 30°C, and *E. coli*. Three different types of Mozzarella were analyzed: cow Mozzarella cheese, industrial water buffalo Mozzarella cheese, and traditional water buffalo Mozzarella cheese. Five different samples were analyzed in duplicate with the MBS method directly in the cheese factory. Control analyses were carried out with the reference methods at the Roma Tre University laboratory.

RESULTS

Primary Validation of MBS Method for Selective and Quantitative Detection of LAB Using Selected ATCC LAB Strains Suspended in Sterile Saline Solution

The primary validation of the MBS method for selective and quantitative detection of LAB was made according to method ISO 16140:2003 (ISO, 2003b). Five different LAB ATCC strains (*Lactobacillus casei* ssp. *casei*, *Lactobacillus acidophilus*, *Lactobacillus delbrueckii* ssp. *bulgaricus*, *Lactobacillus delbrueckii* ssp. *lactis*, and *Streptococcus salivarius* ssp. *thermophilus*) were used. The performance parameters that the alternative method must demonstrate are sensitivity, selectivity, linearity, and accuracy.

Sensitivity. Preliminary experiments were carried out to determine the sensitivity of the MBS method for LAB. The estimate of sensitivity was used to ascertain whether the alternative method could detect the lowest concentration of bacteria (ISO, 2003b). For this purpose, selected ATCC LAB suspensions coming from pure precultures were previously counted and then bacteria were further diluted in sterile saline solution to reach the approximate required concentrations (ca. 10 cfu/mL). Bacterial concentrations below 10 cfu/mL were not tested because the statistical variability would be too high to give reliable values. The MBS vials for LAB were thus inoculated with 1 mL of sterile saline solution containing approximately 10 cfu/mL of the selected LAB ATCC strains indicated above. Microbiological survey vials exhibited the color change (from red to yellow) in comparable times (see Table 1), indicating good sensitivity of the MBS method and no marked differences in the response time of the MBS vials toward the selected LAB ATCC strains. Thus, the LAB concentration obtained with MBS method could be considered reliable, regardless of the type of the LAB strain presented into the sample.

Selectivity. Further tests were carried out to determine the selectivity of the MBS method. Selectivity is defined as a measure of the degree of noninterference in the presence of nontarget analytes (ISO 2003b). Table 2 shows the selectivity tests, indicating the minimum detection limit (expressed as cfu/mL) of the MBS

Table 1. Time needed for color change in the microbiological survey (MBS) lactic acid bacteria (LAB) vials inoculated with an approximately 10 cfu/mL dilution of 5 different LAB American Type Culture Collection (ATCC, Manassas, VA) strains suspended in sterile saline solution[1]

Bacterial strain (ca. 10 cfu/mL)	Time (h) for color change
Lactobacillus acidophilus	88.2 ± 5.6
Lactococcus lactis	72.7 ± 5.5
Lactobacillus casei	73.4 ± 4.9
Lactobacillus bulgaricus	70.1 ± 5.1
Streptococcus thermophilus	85.9 ± 5.3

[1]Five different dilutions of each bacterial strain were tested in duplicate with the MBS method. The color of the MBS vials changed from red to yellow. The values are mean ± SD of 10 experiments carried out in triplicate.

method for LAB toward selected ATCC strains. The lowest detection limit represents the minimal bacterial concentration of strains other than LAB required to induce the color change from red to yellow in MBS LAB vials after 96 h, which was the maximum time of the analysis (no color change observed) when no LAB were present.

Linearity. Linearity is the ability of a method to give results that are in proportion to the amount of analyte present in the sample when used with a given matrix; that is, an increase in analyte should correspond to a linear or proportional increase in results, as indicated by method ISO 16140:2003 (ISO, 2003b). The estimate of linearity can be achieved graphically, as illustrated in Figure 1, by plotting bacterial concentrations (log cfu/mL) obtained with the reference method in artificially contaminated samples against the time occurred for color change of the MBS vials for the same samples. Five ATCC LAB strains (*Lactobacillus casei* ssp. *casei, Lactobacillus acidophilus*, *Lactobacillus delbrueckii* ssp. *bulgaricus*, *Lactobacillus delbrueckii* ssp. *lactis*, and *Streptococcus salivarius* ssp. *thermophilus*) were used. Bacterial suspensions coming from pure precultures were diluted in sterile saline solution and then analyzed simultaneously with both the reference and the MBS methods. A linear inverse relationship between the time needed for the MBS vials to change color and the bacterial concentrations was observed. Linear regression analysis parameters (including all the points) were log cfu/mL = $-0.095 \times$ time (h) + 8.85, with a correlation factor (R^2) of 0.98. In Figure 1, the dispersion of points could be due either to the metabolic differences between the analyzed strains or to the intrinsic statistical variability of both the reference method and the MBS method. The linear regression analysis allowed transforming the times needed for color change to the corresponding bacterial concentrations [i.e., a color change occurring after 60 h corresponds to ($-0.095 \times 60 + 8.85$) 3.15 log cfu/mL or 400 cfu/mL].

Accuracy. Accuracy is the degree of correspondence between the response obtained with the reference method and the response obtained with the alternative method on the identical samples (ISO, 2003b). Figure 2 allows an immediate visualization of the correspondence between the bacteria number (log cfu/mL) obtained with the traditional counting method and the alternative MBS method for the identical dilutions in sterile saline solution of the 5 different selected LAB ATCC strains. Even if a slight dispersion of the experimental

Table 2. Results of the selectivity tests[1]

LAB	ATCC[2] strain	Lowest detection limits of the MBS method for LAB (cfu/mL)
Enterobacter cloacae	ATCC 13047	$>10^6$
Enterobacter sakazakii	ATCC 31329	$>10^6$
Pseudomonas aeruginosa	ATCC 27853	$>10^5$
Salmonella enterica serovar Typhimurium	ATCC 14028	$>10^5$
Escherichia coli	ATCC 25922	$>10^6$
Enterococcus faecalis	ATCC 29212	$>10^4$
Listeria innocua	ATCC 33090	$>10^6$
Listeria monocytogenes	ATCC 7644	$>10^6$
Staphylococcus aureus	ATCC 12600	$>10^6$
Staphylococcus epidermidis	ATCC 12228	$>10^6$
Clostridium perfringens	ATCC 13124	$>10^6$
Lactobacillus delbrueckii ssp. *lactis*	ATCC 12315	1
Lactobacillus delbrueckii ssp. *bulgaricus*	ATCC 11842	1
Lactobacillus casei ssp. *casei*	ATCC 393	1
Lactobacillus acidophilus	ATCC 4356	1
Streptococcus salivarius ssp. *thermophilus*	ATCC 19258	1

[1]Each bacterial strain was tested in quintuplicate with microbiological survey (MBS) vials for lactic acid bacteria (LAB). The lowest detection limit represents the minimal bacterial concentration required to induce the color change from red to yellow in MBS LAB vials after 96 h.

[2]American Type Culture Collection (ATCC), Manassas, VA.

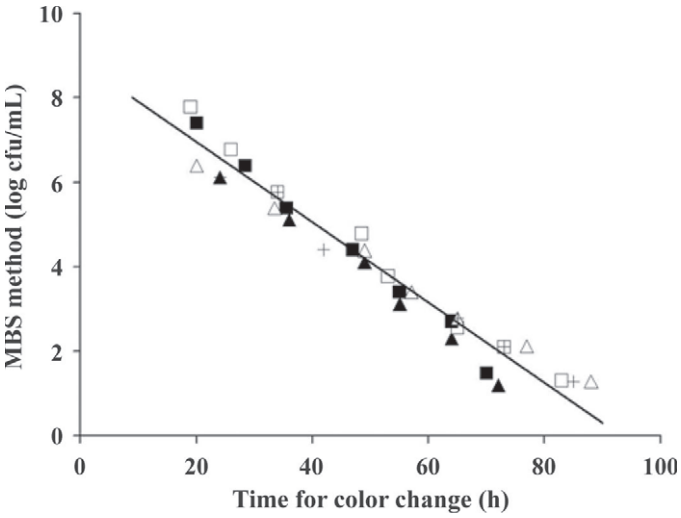

Figure 1. Linearity: correlation line between analyte concentrations with the time needed for color change in the microbiological survey (MBS) vials using sterile saline solution suspensions of selected American Type Culture Collection (ATCC, Manassas, VA) lactic acid bacteria (LAB) strains. Bacterial concentrations (log cfu/mL) obtained with the reference method on sterile saline solution suspensions of selected ATCC LAB strains are plotted against the time needed for color change of the identical samples analyzed with the MBS method. The straight line represents the linear regression analysis, including all the points. The correlation factor (R^2) is 0.98. Each point is the mean of 3 different samples. □ = *Lactobacillus casei* ssp. *casei*, Δ = *Lactobacillus acidophilus*, ■ = *Lactobacillus delbrueckii* ssp. *bulgaricus*, ▲ = *Lactobacillus delbrueckii* ssp. *lactis*, + = *Streptococcus salivarius* ssp. *thermophilus*.

points was observed, the slope of the linear regression analysis (0.991) was close to the theoretical value x = y (i.e., slope = 1.00 and the results appeared to be almost independent of the LAB strain used). The correlation factor (R^2) was 0.98.

The accuracy plot was repeated on naturally contaminated samples, comparing the LAB counts obtained with the reference method with the LAB counts obtained with the MBS method (see Figure 3). Three different types of samples were used: cow industrial Mozzarella, cow artisanal Mozzarella, and water buffalo Mozzarella. To obtain a wide range of LAB concentrations, aliquots of the samples were diluted in sterile saline solution after homogenization to obtain low LAB concentrations. Also in this case, a slight dispersion of the experimental points was observed, although the slope of the linear regression analysis (0.99) was close to the theoretical value x = y (i.e., slope = 1.00 and the results appeared to be almost independent of the type of Mozzarella used). The correlation factor (R^2) was 0.94 and the dotted lines in the figure represent the 95% confidence limits. These results indicate that the MBS method for LAB can be used with reliability on all the Mozzarella samples, independently of their origin.

Application of the MBS Method for the Assessment of the Microbiological Safety and Quality of Mozzarella Cheese

The MBS method was applied to assess the microbiological safety and quality of Mozzarella cheese in an artisanal dairy factory located near Rome, Italy. The TVC at 30°C and *E. coli* count were determined to assess the microbiological safety, as their concentrations should be kept as low as possible. In contrast, LAB were counted to assess the microbiological quality, as the greater the LAB concentration, the greater the cheese quality. Analyses carried out directly in the artisanal dairy factory, in the absence of a dedicated microbiological laboratory, and control analyses, carried out with the reference method at the Roma Tre University laboratory, gave almost identical results.

Figure 4 shows a summary bar graph of the bacterial concentrations (mean ± SD) obtained for each parameter (LAB, TVC, and *E. coli*) and each type of product with the MBS method. The microbiological quality of milk and dairy products is influenced by the initial flora of raw milk, the processing conditions, and post-heat treatment contamination. The data demonstrate that the raw materials used by the dairy factory complied

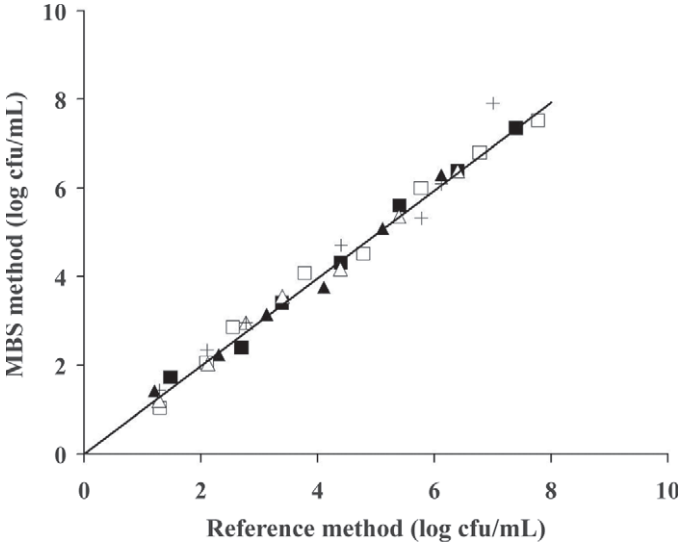

Figure 2. Accuracy: correlation line between alternative microbiological survey (MBS) methods and the reference method using sterile saline solution suspensions of selected American Type Culture Collection (ATCC, Manassas, VA) lactic acid bacteria (LAB) strains. Bacteria concentrations (log cfu/mL) obtained with the MBS method are plotted against bacteria concentrations (log cfu/mL) obtained with the reference method on the identical artificially contaminated samples. Continuous line: linear regression analysis slope = 0.99 (R^2 = 0.98). Each point is the mean of 3 different samples. □ = *Lactobacillus casei* ssp. *casei*, Δ = *Lactobacillus acidophilus*, ■ = *Lactobacillus delbrueckii* ssp. *bulgaricus*, ▲ = *Lactobacillus delbrueckii* ssp. *lactis*, + = *Streptococcus salivarius* ssp. *thermophilus*.

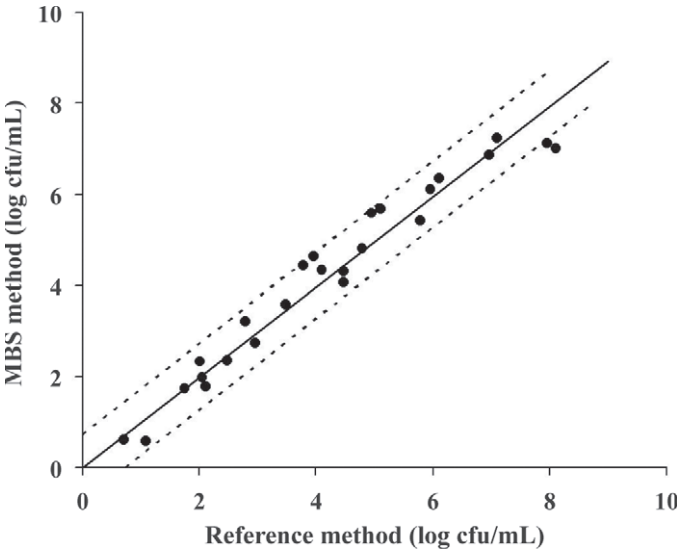

Figure 3. Accuracy: correlation line between alternative microbiological survey (MBS) methods and the reference method on naturally contaminated Mozzarella samples. Bacterial concentrations (log cfu/mL) obtained with the MBS method are plotted against bacteria concentrations (log cfu/mL) obtained with the reference method on identical naturally contaminated Mozzarella samples. Three different types of samples were used: cow industrial Mozzarella, cow artisanal Mozzarella, and water buffalo Mozzarella. Continuous line: linear regression analysis slope = 0.991; dotted lines = 95% confidence limits; correlation factors (R^2) = 0.99. Each point is the mean of 3 different samples.

with the health requirements set by law with regard to *E. coli*, which is considered a good indicator of hygiene. Furthermore, the high quality of raw materials was demonstrated by the high concentration of LAB. For the finished product, it should be noted that the processes of thermization and acidification, necessary to transform water buffalo milk into Mozzarella cheese, significantly affected the TVC and *E. coli* count (safety microbiological parameters), causing a decrease in their concentration without affecting LAB concentrations (quality microbiological parameter).

The evaluation of microbial dynamics during a period of 14 d was also observed using the MBS method directly in the cheese factory. As a general rule, Mozzarella cheese is stored at room temperature to maintain its peculiar organoleptic characteristics (Farkye et al., 1991). To evaluate the safety and quality of Mozzarella, it is therefore important to study the evolution of the microbial population at 20°C. Three different types of Mozzarella were analyzed: cow Mozzarella cheese, industrial water buffalo Mozzarella cheese, and traditional water buffalo Mozzarella cheese.

Figure 5 shows the microbiological profiles of cow Mozzarella cheese (panel A), industrial water buffalo Mozzarella cheese (panel B), and traditional water buffalo Mozzarella cheese (panel C) at different times and demonstrates that the growth of spoiling bacteria was inversely related to the content of LAB and that Mozzarella cheese maintained its organoleptic characteristics until 7 d from production when stored at 18 to 20°C. In fact, TVC and *E. coli* concentrations displayed an opposite trend compared with LAB. These data support the hypothesis that the reduction of LAB would correspond to worsening of the microbiological safety and quality, deterioration of the product, and increased growth of the spoilage bacteria.

DISCUSSION

The quality and safety of fresh cheese such as Mozzarella are strictly related to their microbial content (Ruegg, 2003). In particular, some microbiological parameters have to be checked for safety, such as TVC and *E. coli*. Their concentrations are indicators of the hygienic state of the products. The Regulation (EC) No. 2073/2005 on microbiological criteria for foodstuffs requires *E. coli* quantification as an indicator of the level of hygiene. In cheeses made from milk or whey that has undergone heat treatment, *E. coli* content is unsatisfactory at >1,000 cfu/g.

For the evaluation of the quality of Mozzarella cheese, it is also important to evaluate the concentration of LAB. These bacteria are widely distributed in nature and occur in the indigenous microflora of raw milk (Coeuret et al., 2003; Luquet and Corrieu, 2005). They play an important role in many food and feed

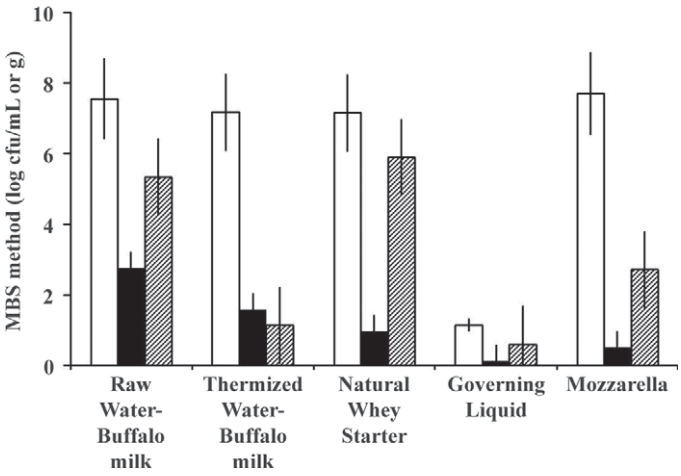

Figure 4. Evaluation of the microbial population in the production line of Mozzarella cheese manufacture as observed with the microbiological survey (MBS) method. The analyzed parameters were lactic acid bacteria (LAB) as white bars, total viable count (TVC) at 30°C as striped bars, and *Escherichia coli* as black bars. The different products analyzed were (1) raw water buffalo milk, (2) thermized water buffalo milk, (3) natural whey starter, (4) governing liquid, and (5) water buffalo Mozzarella cheese (final product). The values are the means ± SD of 5 different samples analyzed in duplicate.

fermentations, contributing to the development of the characteristic taste and texture of cheese. In addition, LAB increase the shelf life of fresh cheese due to the production of antimicrobial compounds (Oberg et al., 1991; Osman Mohamed Abdalla and Nourein Mohammed Ibrahim, 2010). Evaluating the microbiological quality of the final Mozzarella cheese product as well as the intermediates throughout the production line is an important contribution to control the quality of the product.

Dairy industries usually cannot monitor autonomously the microbiological parameters of their raw milk, intermediates, and final products and usually rely on external analysis laboratories that provide results in times that are usually not compatible with the need for a rapid surveillance program throughout the production process. To overcome this problem, in recent years, a remarkable development of alternative methods for the microbiological analysis of food has occurred, with the purpose of shortening the time required for analytical responses. These rapid alternative methods, however, usually require fully equipped laboratories to carry out the analysis. In this context, a rapid colorimetric method, such as the MBS method, may play an important role. This is a fast colorimetric system for the detection and selective count of bacteria in agro-food samples that can be used without a microbiological laboratory. Colorimetric methods currently available are mainly based upon the measurement of microorganisms' secondary metabolism. In contrast, the MBS method measures the catalytic activity of redox enzymes of the main metabolic pathways of bacteria, 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 logarithm of bacterial concentration. Similar to an enzymatic reaction, the greater the number of bacteria, the faster the color change (Antonini et al., 2007; Bottini et al., 2011; Losito et al., 2012). The MBS method allows analyses to be carried out by untrained personnel and wherever analyses are necessary, without the need for any instrumentation other than MBS vials and a thermostat provided on request. Thus, MBS method is particularly suitable for the Agro-food small and medium enterprises not having an internal analysis laboratory. The time required for microbiological analysis with the MBS method is shorter than that required for traditional methods based on plate counting on selective media. For TVC in the range 10^3 to 10^5 cfu/g, the MBS analytical time is 16 h (48 h for plate counting); for *E. coli* in the range 1 to 10^3 cfu/g, the MBS analytical time is 21 h (48 h for plate counting); for LAB in the range 10^5 to 10^8 cfu/g, the MBS analytical time is 44 h (72 h for plate counting). In addition,

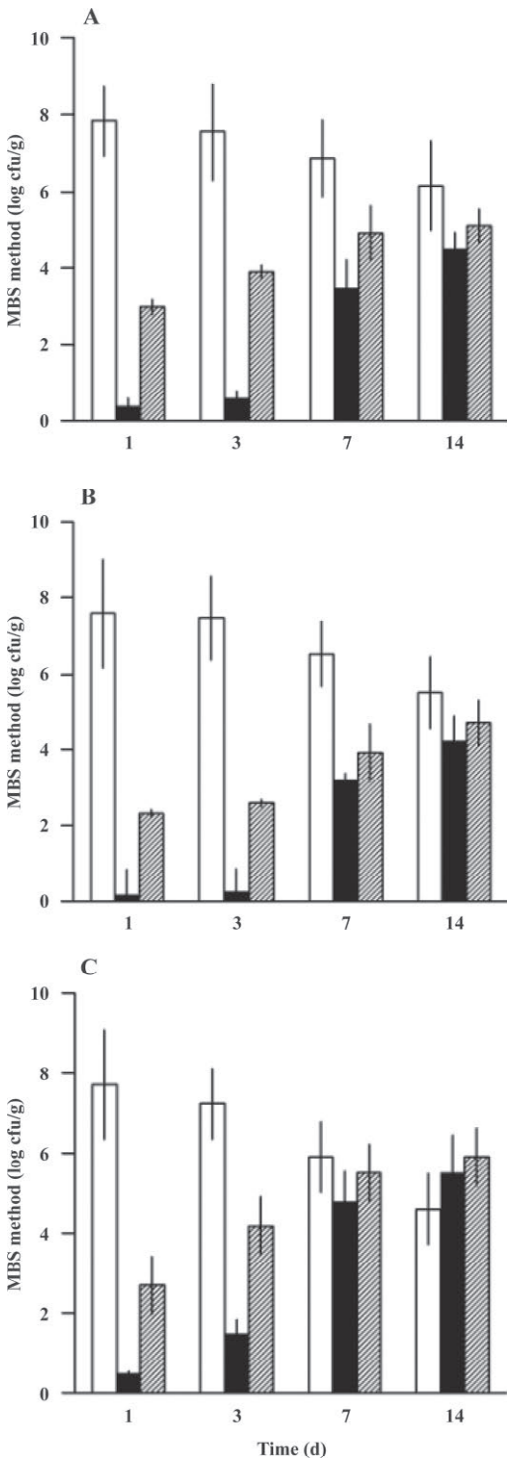

Figure 5. Evolution of the microflora of Mozzarella cheese at 20°C as observed with the microbiological survey (MBS) method. The parameters analyzed with the MBS method were lactic acid bacteria (LAB) as white bars, total viable count (TVC) at 30°C as striped bars, and *Escherichia coli* as black bars. Samples of Mozzarella cheese stored at 20°C were collected at different times (1, 3, 7, and 14 d after production). The values are the means ± SD of 5 different samples analyzed in duplicate. Panel A: cow Mozzarella cheese; panel B: industrial water buffalo Mozzarella cheese; panel C: artisanal water buffalo Mozzarella cheese.

the overall cost of the analysis carried out with the MBS method is much lower compared with external analysis laboratories.

CONCLUSIONS

The purpose of the present study was to demonstrate that the MBS method can be successfully applied to analyze the safety and quality of Mozzarella cheese. The primary validations of MBS method for selective and quantitative detection of TVC and *E. coli* have been already published (Bottini et al., 2011). In the current paper, we carried out the primary validation of the MBS method for selective and quantitative detection of LAB, which showed high reliability and correlation with traditional plate count methods. The MBS method was thus applied for process and product analysis of Mozzarella cheese. Afterward, LAB, TVC, and *E. coli* concentrations were evaluated throughout the production line of typical Mozzarella cheese in an artisanal dairy factory located near Rome (Italy) using the MBS method. It should be recalled that the analyses carried out directly in the artisanal dairy factory, which did not have a dedicated microbiological laboratory, and in a control analysis carried out at the Roma Tre University laboratory gave almost identical results. The results obtained confirmed the correlation between the LAB concentration and process quality: a high concentration of LAB in the raw materials and in the whey starter ensured safe and good final products. Lactic acid bacteria, TVC, and *E. coli* concentrations were also evaluated using the MBS method in 3 different kinds of final product (cow Mozzarella cheese, industrial water buffalo Mozzarella cheese, and traditional water buffalo Mozzarella cheese) at different times during 14 d of storage at 20°C to monitor the evolution of the bacterial population and its consequence on the shelf life of the products, which are traditionally stored at room temperature to avoid loss of taste and texture. The results obtained showed that the growth of spoiling bacteria was inversely related to the content of LAB and that Mozzarella cheese maintained its microbiological characteristics until 7 d from production when stored at 18 to 20°C. In addition, these results confirm the hypothesis that LAB concentration is related not only to quality but also to safety. The TVC and *E. coli* parameters give information only on the hygienic state of the product. A high LAB concentration ($\sim 10^7$), instead, ensures high quality of the product in terms of organoleptic characteristics and shelf life and also hygienic safety, with LAB concentration being inversely related to the concentration of spoilage and pathogenic bacteria. In conclusion, the data obtained show that the MBS method can give reliable results for the analysis of LAB, TVC, and *E. coli* in fresh cheese, allowing small- to medium-size cheese factories to monitor autonomously the safety and quality of Mozzarella cheese.

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