Peer-reviewed version available at *Foods* **2020**, *9*, 1186; doi:10.3390/foods9091186

Communication

Rapid microbiological assessment in raw milk. Validation of a rapid alternative method for the assessment of microbiological quality in raw milk.

N. Marri[1^], F. Losito[2^], L. Le Boffe[2], G. Giangolini[1], S. Amatiste[1], L. Murgia[2], A. Arienzo[3] and G. Antonini[*2,3]

^ These authors equally contributed to this work

[1]Istituto Zooprofilattico Sperimentale del Lazio e della Toscana *M. Aleandri* (IZSLT), Rome, Italy; nicla.marri@izslt.it; gilberto.giangolini@izslt.it; simonetta.amatiste@izslt.it
[2]Interuniversity Consortium "Istituto Nazionale Biostrutture e Biosistemi" (INBB), Rome, Italy; losito.francesca00@gmail.com ; loris.leboffe@uniroma3.it ;lorenza.murgia@uniroma3.it; giovanni.antonini@uniroma3.it
[3]Science Department, Università degli Studi Roma Tre, Rome and Italy; alyexandraarienzo@gmail.com; giovanni.antonini@uniroma3.it
* Correspondence: giovanni.antonini@uniroma3.it

Abstract: The consumption of dairy products and the dairy industry is one of the main global agro-food sectors for size, economic importance and level of technology. Microbiological quality of pasteurized milk or other milk products is dependent on microbiological quality of raw milk. A variety of microbiological count methods is available for monitoring the hygienic quality of raw milk. Among them, the pour plate method is the official essay for counting the number of colony forming units in milk samples according to ISO 4833-1:2013. The aim of the present study is the validation of the Micro Biological Survey (MBS) method, against the reference plate count method, for the assessment of the microbiological quality of raw milk. This comparative study, performed in collaboration with the "Istituto Zooprofilattico Sperimentale del Lazio e della Toscana *M. Aleandri*" (IZSLT), demonstrates the accuracy of this alternative method for the determination of total viable bacterial count in cow's raw milk. The results obtained with the MBS method highlighting its potential as a valid tool for reliable microbiological analysis in dairy industries.

Keywords: raw milk; microbiological safety; microbiological quality; food safety; dairy

MAIN TEXT

Milk has always been considered an essential food for its nutritional value, especially for children and adolescents. In fact, a significant portion of human diet is nowadays based on the consumption of dairy products, and the dairy industry is one of the main global agri-food sectors for size, economic importance and level of technology [1].

Microbial contamination can generally occur from various sources during the milking procedure: from dairy farmers to milk and cheese industry, therefore it is essential to monitor microbiological characteristics along the whole production chain to prompt preventive actions to protect human health [2].High microbial counts in raw milk are responsible for quality defects in pasteurized milk, UHT processed milk, dried skim milk, butter and cheese [3].

Current regulations define "raw milk" as milk not receiving thermal treatment above 40°C or any equivalent process [4].

The raw milk may be used for producing heat-treated milk, cheese or milk-based products or it can be directly sold from self-service vending machines. Although the Italian Health Ministry with the Decree 12 December 2012 ordered that vending machines should bear the notice ''Milk must be consumed after boiling'', some consumers may ignore this advice [5,6]. The Regulation (EC) No 178/2002, laying down the general principles and requirements of food law, establishing the European Food Safety Authority and laying down procedures in matters of food safety, sets rules, procedures and a pioneering approach regarding food safety [7].More in detail, the Regulation (EC) No 853/2004 requests routine evaluation of raw milk's microbiological quality through the assessment of the mesophilic flora upon plate culture and defines microbiological criteria for raw milk: ≤ 100,000 colony forming units (CFU)/ml upon plate count at 30°C for raw cow's milk, ≤ 1,500,000 CFU/ml for other dairy species and ≤ 500,000 CFU/ml when the final destination of milk from other species does not include heat treatment [4].

A variety of microbiological count methods is available for monitoring the hygienic quality of raw milk. Among them, the pour plate method is the official essay for counting the number of colony forming units in milk samples according to ISO 4833-1:2013 [8]. Flow cytometry technique is the most common method used for evaluating the hygienic quality of raw milk, which estimates the total number of bacteria present in milk according to ISO 21187:2004[9].

New methods for the detection and enumeration of microorganisms have been developed to provide accurate, rapid tools to flank standard plate culture protocols. In this context, the application of Micro Biological Survey (MBS) method should be considered as an additional safety measure, which cannot replace reference methods. The MBS method, developed by MBS Srl and Roma Tre University (Rome, Italy), is a colorimetric, culture-based system that allows to perform quantitative and qualitative microbiological analyses in the absence of either dedicated facilities or specialized personnel. Analyses are performed into ready-to-use, sterile, disposable vials, designed for *in situ* use, following a simple and straightforward protocol in order to reduce human error associated with sample manipulation [10, 11].

The aim of the present study is the validation of the MBS method, against the reference plate count method, for the assessment of the microbiological quality of raw milk on naturally contaminated samples. All validation experiments were carried out in partnership with IZSLT, which provided raw milk samples and logistic resources, and in collaboration with MBS Srl, which provided the MBS analytical kit.

Total viable mesophilic bacteria were evaluated in 173 samples of cow's raw milk following the reference method, according to ISO 4833-1: 2013, and the MBS method, in parallel. All samples for both methods were analyzed in duplicate.

According to the reference method, for each raw milk sample, 1 ml of the undiluted sample and selected serial decimal dilutions were plated on Skim Milk agar using the pour plate method; plates were then incubated at 30°C for 72 hours. Only plates displaying results falling within the 10-324 colonies were considered for the count according to ISO 7218:2013, then results underwent verification by G^2 factor test for the proportionality of the counts [12].

Concomitantly, the MBS method was performed using the Total Viable Count (TVC) vials for the quantification of total viable mesophilic bacteria. All vials were produced by MBS Srl (Rome, Italy). According to the proprietary user's manual, TVC vials were inoculated with 1 ml of the undiluted raw milk samples. After inoculation, vials were incubated for up to 30 hours at 30 °C in the MBS Multireader, a thermostatic optical reader that automatically detects the color change of the MBS vials and calculates the bacterial concentration in the analyzed sample. The absence of color change after 30 hours indicates the absence of the microorganisms of interest. MBS results were expressed as time (hours).

Results obtained using the reference method were grouped into four classes according to the detected bacterial concentration, arranged in ascending order: ≤ 100,000 CFU/ml (102 samples, 57.3%), 101,000 – 500,000 CFU/ml (43 samples, 26.5%), 501,000 – 1,000,000 CFU/ml (13 samples, 8.1%), and > 1,000,000 CFU/ml (15 samples, 8.1%). The lowest recorded value was 2,500 CFU/ml, while the highest was 4,700,000 CFU/ml. Results were then converted into $\log_{10}$. The statistical analysis was

Preprints (www.preprints.org) | NOT PEER-REVIEWED | Posted: 3 August 2020 doi:10.20944/preprints202008.0063.v1

Peer-reviewed version available at Foods 2020, 9, 1186; doi:10.3390/foods9091186

performed using MedCalc for Windows (MedCalc 12, MedCalc Software Ltd, Ostend, Belgium). The repeatability of the MBS method was verified on two levels (first level mean: 0.80 log10; second level mean 8.62 $\log_{10}$), performing 10 replicates for each level. Repeatability's standard deviation was Sr = 0.03 $\log_{10}$ and Sr = 0.02 $\log_{10}$ for first and second level respectively.

The average values for the colony count at 30°C obtained with the plate count reference method was compared with the average values obtained with the MBS method for each of the 173 samples of raw cow's milk analyzed. The regression line obtained plotting the log of bacterial concentration values obtained with the reference method and the log of results obtained with the MBS method (time required for the MBS vials to change color), shown in Figure 1, confirms the linearity of the MBS method, and provides a correlation line defined by the equation:

$$\frac{log_{10}CFU}{ml} = -5.7889 * log_{10}MBStime + 10.4419 \quad (1)$$

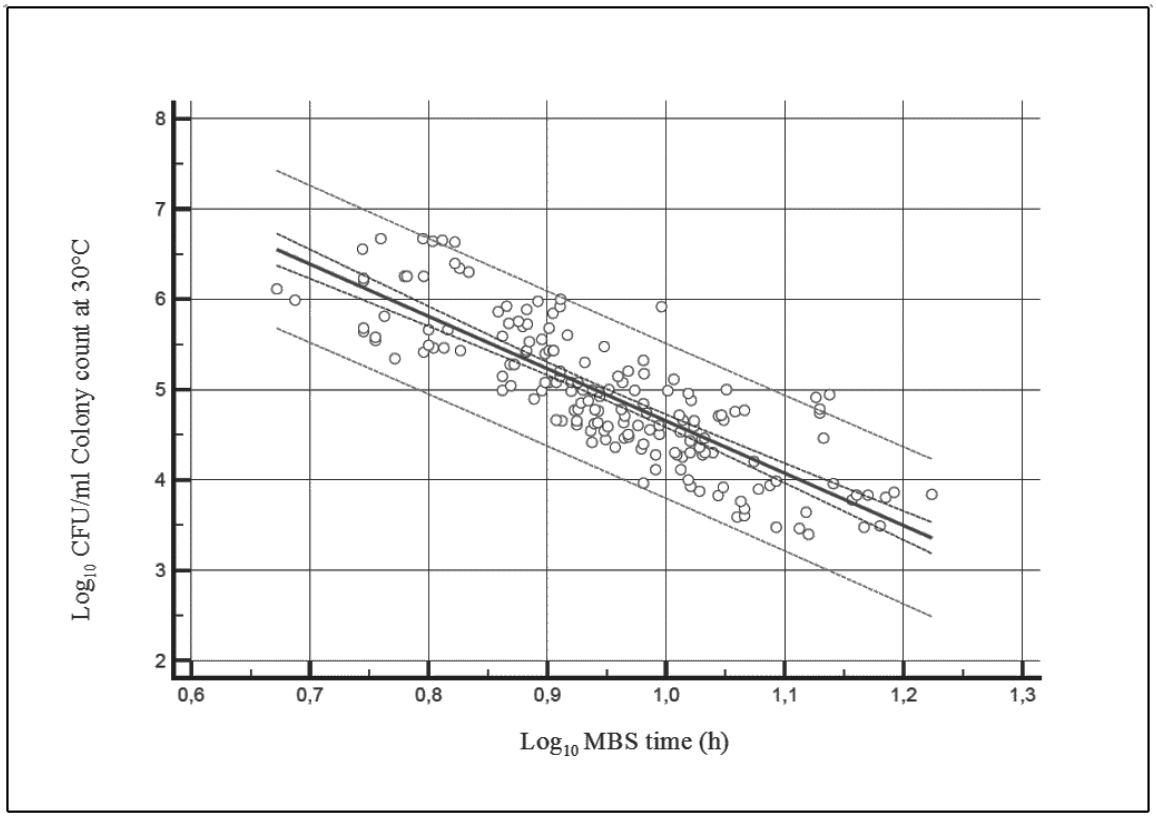

Figure 1. Regression line (R^2 = 0,69) obtained plotting the decimal logarithms of the bacterial concentration values yielded with the reference method (Log CFU/mL) and the decimal logarithm of the time required for MBS vials color change yielded with the MBS method (Log h).

Table 1. summarizes regression main parameters.

Table 1. Main parameters defining the regression line obtained plotting reference method and MBS results.

N samples	Pearson (r)	R^2	Sy:x	Intercept (Standard Error)	Slope (Standard Error)

173	0.83	0.69	0.43	0.29	0.30

After that, the determination of the residuals of the previously calculated linear regression was carried out, demonstrating that the distribution of residues obtained through the use of the above-mentioned equation displays a normal pattern (Figure 2). The results obtained with the two methods were also graphically compared by the Bland-Altman test. The confidence interval (CI) at 95% of the mean difference was identified using the following equation:

$$CI = Mean \pm 1.96 * s \quad (2)$$

Mean = Mean of differences of reference and alternative method

s = standard deviation of differences.

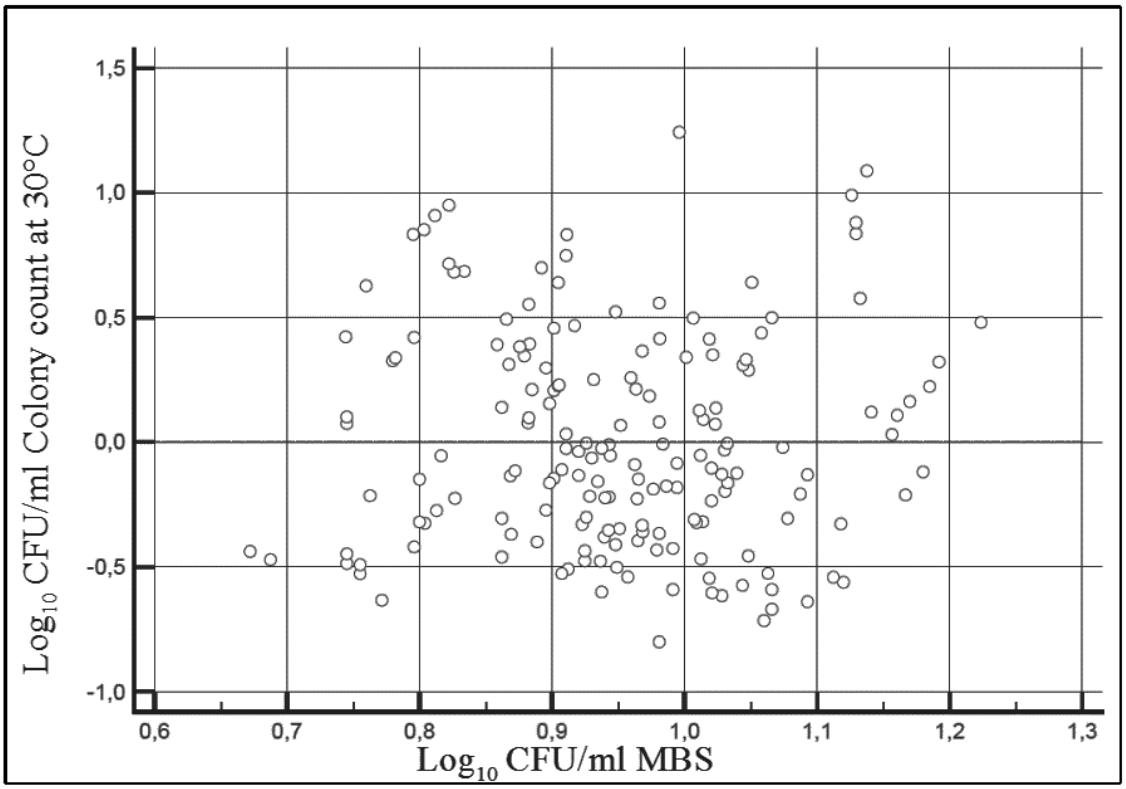

Figure 2. Determination of residues of linear regression obtained comparing results yielded with the two methods.

The upper and lower limits were defined as CI = ± 0.85. The results obtained with the Bland-Altman test (Figure 3) show a substantial reliability with respect to the interchangeability of the two methods.

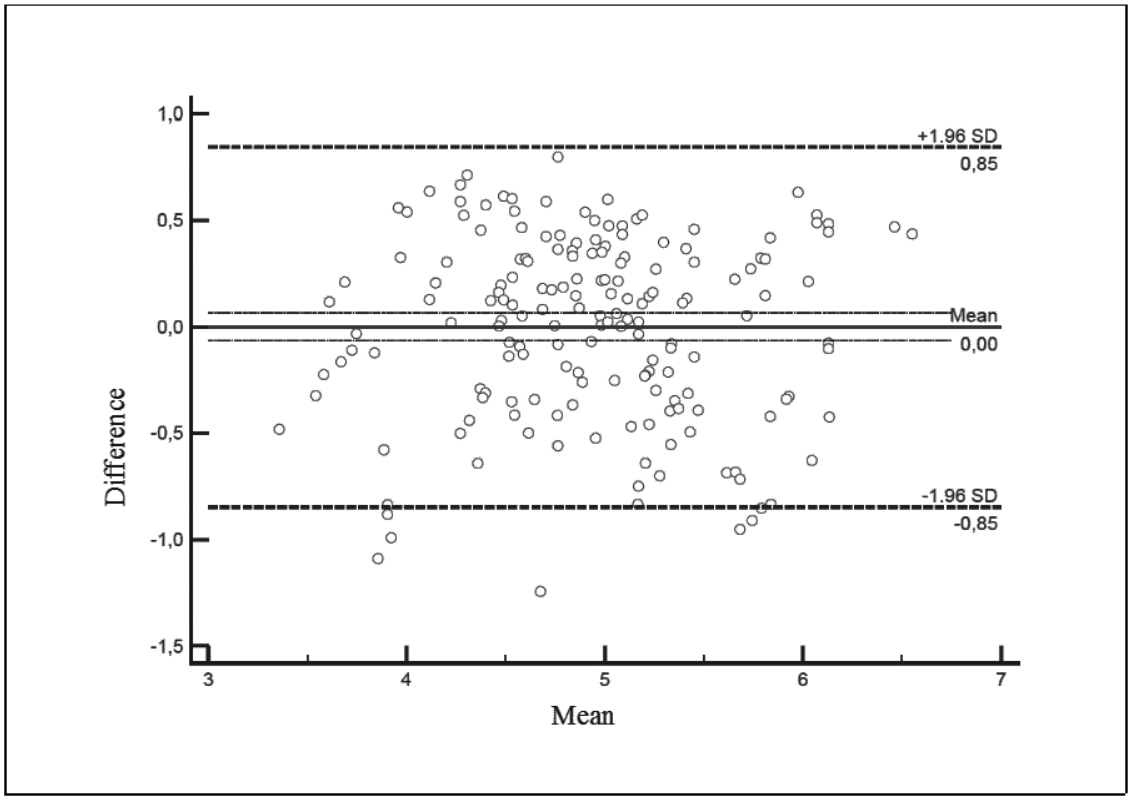

Figure 3. Bland-Altman test. The mean values of the two methods are shown on the X axis. The difference of the two values is shown on the Y axis.

Reference results were plotted against alternative method results, converted to the same unit of measurement of reference method (Figure 4). The MBS method's accuracy was evaluated according to ISO 16297:2013, calculating 95% CI for the difference observed between results obtained with the two methods within 6 categories identified by a 0.5 $\log_{10}$ interval. The CI acceptance limit, as specified by ISO 16297:2013, was CI ± 0.8 $\log_{10}$ [13].

Peer-reviewed version available at Foods 2020, 9, 1186; doi:10.3390/foods9091186

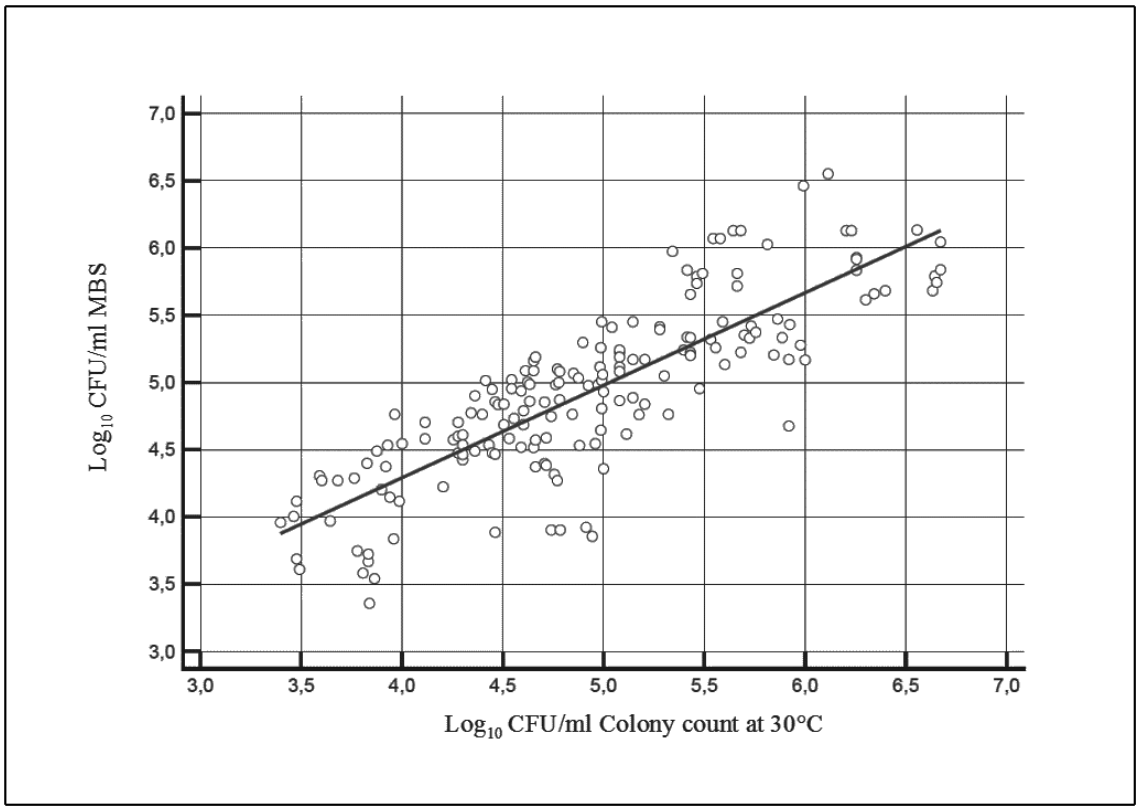

Figure 4. Relation between the results (Log CFU/ml) of the alternative MBS method and the reference method for total bacterial count at 30°C in bovine raw milk.

Table 2 shows CI results for each category. Results obtained in the $8.10*10^3$ – $776*10^3$ CFU/ml range comply with the required CI; CIs from the $2.5*10^3$ – $8.0*10^3$ CFU/ml range and the $832*10^3$ – $4.677*10^3$ CFU/ml range display higher values (1.05 $\log_{10}$ CFU/ml and 1.37 $\log_{10}$ CFU/ml respectively) not comply with the required CI. This suggests an overestimation of results obtained with the MBS method for the first group results, compared to the reference method, and an underestimation for the last group. However, the threshold value (100,000 CFU/ml) for colony count at 30°C specified by regulations (Regulation (EC) No 853/2004) falls within the $8.10*10^3$ – $776*10^3$ CFU/ml range, which displays satisfactory CI [4].

Table 2. Confidence Interval (CI) results for the 6 categories identified in the study.

Group range ($\log_{10}$ CFU/ml)	**3.40-3.90**	**3.92-4.40**	**4.41-4.90**	**4.91-5.40**	**5.41-5.89**	**5.92-6.67**
Group range (CFU/ml x 1000)	2.5 – 8.0	8.1 - 25	26 - 80	81 - 251	257 - 776	832 – 4.677
N samples	19	22	47	34	30	21
CI upper	**1.05**	0.77	0.77	0.59	0.57	0.23

Peer-reviewed version available at Foods 2020, 9, 1186; doi:10.3390/foods9091186

CI lower	-0.37	-0.04	-0.54	-0.78	-0.77	**-1.37**

In conclusion, this comparative study performed between the MBS method and the reference method demonstrates the accuracy of the alternative method for the determination of total viable bacterial count in raw cow's milk. These data highlight the potential of the MBS method as a valid tool for reliable microbiological analysis for the dairy industries; its features, including accuracy and rapidity, make it particularly suitable for use in small and medium-sized dairy companies.

The application of MBS method should, however, be considered as an additional tool to increase safety and quality of dairy products, not replacing officially approved analytical methods.

Author Contributions: Conceptualization, G.A., S.A., L.L., N.M. and F.L.; methodology, L.L., N.M. and F.L.; investigation, N.M., G.G. and F.L.; resources, G.A. and S.A.; writing—original draft preparation and editing, N.M., G.G., F.L. and L.M..; review, G.A. and S.A.; supervision, G.A. and S.A.. All authors have read and agreed to the published version of the manuscript.

Funding: This work has been partially supported by two Italian National Grants: "Development of a rapid, fast and low-cost analytical kit to evaluate quality and safety of milk and dairy products" (Italian Ministry of Health, GR-2011-02348227), and the National Funding for Centers of Excellence (Science Department, Roma Tre University - MIUR, Articolo 1, Commi 314–337 Legge 232/2016) and by Lazio Regional Grant LazioInnova, MBSMART Project, - POR FESR LAZIO 2014-2020 CUP F85F18000120007

Acknowledgments: Dr.ssa Ottavia Stalio, Dr. Carlo Boselli and Daniela Patriarca are gratefully acknowledged for helpful.

Conflicts of Interest: The authors declare that they have no conflict of interest.

References

1. Griffiths, M.W. Improving the safety and quality of milk. Woodhead Publishing Limited, Cambridge, UK. **2010**.
2. Giacometti, F.;Bonilauri, P.;Albonetti, S.;Amatiste, S.; Arrigoni, N.; Bianchi, M.;Bertasi, B.;Bilei, S.;Bolzoni, G.;Cascone, G.; Comin, D.;Daminelli, P.;Decastelli, L.;Merialdi, G.;Mioni, R.;Peli, A.;Petruzzelli, A.;Tonucci, F.;Bonerba, E.;Serraino, A. Quantitative Risk Assessment of Human Salmonellosis andListeriosis Related to the Consumption of Raw Milk in Italy. *J. of Food Prot.*, **2015**, 78(1), 13–21. http://dx.doi:10.4315/0362-028X.JFP-14-171.
3. Barbano, D. M.; Ma,Y.; Santos,M. V. Influence of raw milk quality on fluid milk shelf life. J. Dairy Sci., **2006**, 89(E Suppl.): E15–19; http://dx.doi: 10.3168/jds.S0022-0302(06)72360-8.
4. Regulation (EC) n. 853/2004 of the EuropeanParliament and of the Council of 29 April 2004 laying down specific hygiene rules for food of animal origin.
5. Ministero della Salute – Decreto 12 dicembre 2012. Informazioni obbligatorie e misure a tutela del consumatore di latte crudo o crema cruda, in attuazione dell'art. 8, commi 6 e 9, del decreto-legge 13 settembre 2012, n. 158, recante "Disposizioni urgenti per promuovere lo sviluppo del Paese mediante un più alto livello di tutela della salute" convertito, con modificazioni, dalla legge 8 novembre 2012, n. 189.
6. Giacometti, F.;Bonilauri, P., Serraino, A.;Peli, A.;Amatiste S.; Arrigoni N.; Bianchi M.;Bilei S.;Cascone, G.; Comin, D.;Daminelli, P.;Decastelli, L.;Fustini, M.;Mioni, R.;Petruzzelli, A.;Rosmini, R.;Rugna, G.;Tamba, M.;Tonucci F.;Bolzoni, G.Four-year monitoring of foodborne pathogens in raw milk sold by vending machines in Italy.*J. Food Prot.*, **2013**, 76(11), 1902-7; http://dx.doi:10.4315/0362-028X.JFP-13-213.
7. European Commission, 2002. Commission Regulation of the European Parliament and of the Council of 28 January 2002 laying down the general principles and requirements of food law, establishing the European Food Safety Authority and laying down procedures in matters of food safety, 178/2002/EC.
8. International Standard Organization ISO 4833-1:2013. Microbiology of the food chain — Horizontal method for the enumeration of microorganisms — Part 1: Colony count at 30 °C by the pour plate technique.

9. International Standard Organization ISO 21187:2004 (IDF 196:2004) Milk -- Quantitative determination of bacteriological quality -- Guidance for establishing and verifying a conversion relationship between routine method results and anchor method results.
10. Losito, F., Arienzo, A., Bottini, G., Priolisi, F. R., Mari, A., Antonini, G.Microbiological safety and quality of Mozzarella cheese assessed by the microbiological survey method. *J. Dairy Science*,**2014**,97 :46–55.http://dx.doi: 10.3168/jds.2013-7026.
11. Arienzo, A.; LositoF., Stalio, O.; Antonini,G.Comparison of uncertainty between traditional and alternative methods for food microbiological analysis. *Am. J. Food Technol.***2015**, 11, 29-36; http://dx.doi.org/10.3923/ajft.2016.29.36.
12. International Standard Organization ISO 7218:2007/AMD 1:2013 Microbiology of food and animal feeding stuffs — General requirements and guidance for microbiological examinations.
13. International Standard Organization ISO 16297:2013 – Milk – Bacterial count – Protocol for the evaluation of alternative methods.