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# A study in Ecuador of the calibration curve for total bacterial count by flow cytometry of raw bovine milk

## Estudio en el Ecuador de la curva de calibración para el conteo total de bacterias por citometría de flujo de leche cruda bovina

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**Resumen**

El análisis del conteo total de bacterias (CBT) como parámetro de la calidad higiénica en leche cruda es uno de los más requeridos mundialmente por las industrias y entidades de vigilancia de la inocuidad de alimentos. Este análisis se realiza tradicionalmente por el método referencial del conteo en placas expresado en UFC/mL, sin embargo, el método alternativo por citometría de flujo actualmente, se muestra como un método confiable y más rápido, que permite determinar las poblaciones bacterianas a través del Conteo Individual de Bacterias (IBC/ml). El objetivo del presente estudio fue determinar la ecuación de regresión y correlación entre ambos métodos debido a que éstos pueden variar en cada país en función de las actividades de manejo del ganado durante el ordeño. Para su evaluación en Ecuador, fueron utilizadas 357 muestras de leche con rangos entre 100000 a 1,5 millones de UFC/mL provenientes de dos pisos altitudinales: Zona 1 (*Z*1) (> 3000 msnm) y Zona 2 (*Z*2) (1050 msnm). Los análisis fueron realizados bajo ambos métodos simultáneamente, (máximo de desviación de $\pm$ 1 h) y siguiendo las recomendaciones de la IDF/ISO-196 y la norma AOAC 986,33. Los resultados mostraron una alta correlación entre los métodos ($r = 0,91$) y al no encontrar diferencias estadísticas significativas para el efecto de las zonas (*Z*1 y *Z*2), el mismo no fue considerado, sugiriendo el uso de una única ecuación de regresión lineal.
***Palabras clave***: CBT, IBC, Bactoscan, citometría, calidad de leche.

**Abstract**

The total bacteria count (TBC) analysis, as a parameter of hygienic quality in raw milk, is one of the most requested analysis by dairy industries and food safety organizations. This analysis is done by the reference method of plate count

expressed as colony-forming unit per milliliter (CFU/mL). However, the alternative method by flow cytometry currently is a reliable and faster method than the reference method of Individual Bacteria Count per milliliter (IBC/mL). The aim of the study was to determine the linear regression equation and correlation between the reference method and the alternative method by flow cytometry. Because the activities of livestock management during milking are different in each country, a total of 357 milk samples with a range from 100000 to 1,5 millions of UFC/mL from two altitudinal floors: Z1 ($>$ 3000 masl) and Z2 (1050 masl) were done in Ecuador. Both methods were analyzed at the same time, (maximum deviation of $\pm$ 1 h), following the recommendations of the $IDF/ISO-196$ and the AOAC 986.33 standard. The results showed a high correlation between the two methods ($r = 0{,}91$) and since there were no statistic differences for the effect of Zones 1 and 2, it was not considered, suggesting the use of a unique linear regression equation.
***Keywords***: CBT, IBC, Bactoscan, cytometry, quality milk.

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# 1 Introduction

The production of bovine milk has an important role in the economic and social field of Ecuador. The increase in production in the last 15 years has been favored by the establishment of industries with new technologies, formation of producers' associations and different financing possibilities, among other factors (Cuenca, 2015; CEPAL, 2017). Currently, the total production of the country is estimated at 5.5 million liters of milk per day, with 73% from the central region of the country (INEC, 2014) and its main destination is the supply of the domestic market.

Milk, because of its nutritional composition, stands out as a complete food containing proteins, vitamins, lipids and minerals with important biological benefits in the growth stage and in the bone maintenance of the body (Micinski, 2013; Pereira, 2014). Globalization presents itself as the main responsible for the increase of milk and the consumption dairy products, influenced by changes in food patterns, population growth and the priority of today's society of having nutritious and healthy foods (for Primary Industries, 2015; CEPAL, 2017). In Latin American countries there is a forecast for increased consumption of 1,6% for liquid milk and 2,8% for other dairy products (Hoddinott et al., 2015; CEPAL, 2017)

Ecuador offers great potential to expand the supply of livestock food such as milk and its derivatives due to their natural resources (Requelme and Bonifaz, 2012; Cuenca, 2015) and quality control, being essential to guarantee the safety of the food marketed. Following the recommendation of international organizations, entities linked to the control of public health in Ecuador have established surveillance regulations at all stages of milk production, from production, milking, processing until it reaches the consumer (FAO and IDF., 2011; INEN, 2012; MAGAP, 2008, 2013).

The total bacteria count (TBC) is the most used method to characterize the microbiological quality or bacteria population in the food. The method does not differentiate the types of bacteria, but it is useful to obtain a general information of the hygiene of the products and the conditions of the process (Silva, 2010; Sampaio, 2015).
For its production, the industry requires milk with low bacterial counts of approximately 100 000 to 300 000 UFC/ml (Belli, 2013; Botaro et al., 2013; MAGAP, 2013), since some spores, enzymes and bacterial metabolites can survive thermal processes, affecting the sensory characteristics of foodstuffs such as flavor, texture or the reduction of the useful life (Barbano et al., 2006; Silva, 2010; Gopal, 2015). In addition, the high counts of bacteria in raw milk can be alerts of deficiencies in the hygiene and sanitation in the production and procedure areas of milking, in materials or equipment, as well as errors in the control of the temperature of storage and the microbiological quality of water used, among others (Matsubara, 2011; Ruiz-Cortés et al., 2012; Almeida, 2016; Reyes, 2017).
Microbiological plaque count is the reference method for the analysis of TBC. Its result is expressed as colony forming unit (UFC/ml) and it determines the amount of mesophilic bacteria present in raw milk. Although the importance of the analysis for the dairy industry is not practical, taking into account that the results are obtained at 48 hours of incubated the sample (Silva, 2010; Cassoli, 2016)..

The search for alternative analytical methods that simplify laboratory procedures and accelerate the delivery of results has demanded the development of reliable technologies such as flow cytometry, which allows the counting of bacterial populations through the Individual bacteria count (IBC/ML) based on the scattering of light and fluorescence of a bacterial DNA marker. Flow Cytometry also has the advantage that it does not require the preparation of culture media, reducing the manipulation of milk samples and the delivery time of the results (Evangelista, 2008; Araujo, 2009; Silva, 2010; Jatobá, 2014; Numthuam, 2017). This electronic method is used in the Bactoscan equipment (Foss Analytical Instruments).

The use of alternative analytical methods requires the comparative study with referential methods, and the definition of a linear regression equation. In countries of the European Union, a single conversion equation by convention has been adopted for the application of this method, but in other countries, such as those in South America, an equation is used for each nation (Botaro et al., 2013; Cassoli, 2016).

Additionally, the regulations for the permissible maximum limits of TBC in raw milk are different in each country. Thus, in the European Union, the values range from 100 000 to 500 000 UFC/ml, imposing economic penalties for milk with higher counts (Botaro et al., 2013). In Ecuador, since the year 2008, the government determined the technical requirements for the payment of quality raw milk, establishing the need to carry out laboratory analyses of the raw material of the total bacteria count, before it can be marketed and processed in the industry (INEN, 2012; MAGAP, 2013).

The objective of this study was to determine the linear regression equation and correlation between the reference method by counting on plaques expressed in UFC/ml and the flow cytometry technique expressed in IBC/ml, for the determination of the total bacteria count (TBC) in raw milk, which allows to establish the calibration curve between the two methods for the evaluation of the microbiological quality of bovine raw milk in Ecuador.

# 2 Materials and methods

## 2.1 Sample Collection

357 milk samples were collected for this study between the years 2013 and 2014, in the months of January, February, March, May, September, October, November and December. Samples were collected from refrigeration tanks of organized producers (49 %) and refrigeration tanks of individual producers (51 %).

All milk producers were located in the central region of the country, in the Province of Pichincha ($0°5'14''$ N and $78°6'12''$ W); two floors altitudinal were considered in this region, one in Cayambe (Z1), located at $>$ 3000 masl, and another in San Miguel de los Bancos (Z2), at 1050 masl. The Z1 presented an annual average temperature between $8-15°C$ and a relative humidity of 65 %. The Z2 presented an average temperature of $18-24°C$ and a relative humidity of 85 % (Geographic Information System Laboratory-SIG-Universidad Salesiana Ecuador, 2018).

Reference procedures were followed during the collection of milk samples, using sterile 50*ml* vials and Azidiol preservative. Samples were transported in refrigeration at $4-7°C$ to the Milk Quality Laboratory of Universidad Politécnica Salesiana.

## 2.2 Laboratory analysis

The plaque count was carried out according to the reference procedure of the Ecuadorian National Institute of Standardization and the International Standardization Organization (INEN y ISO, 2014), using 1 mL of milk sample (after homogenization) diluted in 9 ml of sterile peptonated water to 0,1 % with serial dilutions ($10-1$ to $10-5$). Finally, 1000 mL were sown in Petrifilm plates for aerobic counts (3 M, Saint Paul, MN, USA) in duplicate and incubated at $32°C$ ($\pm 1°C$) $\times$ 48 h ($\pm$ 3 h), according to AOAC 986.33 reference for dairy products. For the total result, plaques that had between 25 to 250 colonies of bacteria were selected. The results are expressed as CFU/mL (Cassoli, 2007; Silva, 2010). All samples were analyzed within 48 h after the collection (INEN, 2006; Cassoli, 2016) and by both methods analyzed at the same time (maximum deviation of $\pm$ 1 h), according to the norm IDF/ISO-196.

The alternative method by analysis of IBC was performed using flow cytometry, with Bactoscan FC (Foss Analytical Instruments) with analysis capacity of 50 samples/hour. Its analytical principle is based on the addition of ethidium bromide (a dye substance) that is put in DNA and bacterial RNA. The injected specimen passes through a flow chamber where the optical system detects particles dyed by fluorescence emission. In addition to the coloring substance, a buffer solution with proteolytic enzymes is required. The intensity and height of the fluorescence emission are the selective parameters for the identification of the bacteria of interest. The results were expressed in IBC/ml (Jatobá, 2014; Numthuam, 2017).

## 2.3 Statistical analysis

In the statistical analysis, the SAS programs version 9.3 was used (Statistical Analysis System). For the verification of the normal data distribution, all the results, both of CFU/ml and IBC/ml, were transformed into 10 base logarithms. Linear regression was evaluated to verify the correlation between the referential method and the alternative method. The reference method was considered as a dependent variable and the alternative method by flow cytometry as an independent variable. The proposed linear model was $Y = ax + b$. Where $Y$ = is the dependent variable in log (UFC), $x$ = Independent variable in log (IBC), $a$= slope of the curve, $b$= intercept or linear coefficient. Additionally, due to differences in environmental temperature and relative humidity conditions, the results of the Z1 and Z2 zones were analyzed separately.

**Table 1.** Distribution of milk samples according to the total levels of bacteria count. n represents the number of samples.

| Classification | Total Bacteria Count (UFC/ml) | n | % |
|---|---|---|---|
| Low | $<$ 100000 | 233 | 65.3 |
| Medium | 100000 – 500000 | 68 | 19.1 |
| High | 500000 – 1′000000 | 35 | 9.8 |
| Very High | $>$ 1′000000 | 21 | 5.9 |

# 3 Results and Discussion

For this study, the sample distribution of aerobic mesophylls is presented in Table 1. From a total of 357 samples of milk analyzed, 34,7 % indicated the presence of bacteria between the classification ranks from medium to very high. 65,3 % corresponded to lower ranks than 100 000 UFC/ml.

The samples came from cattle with a higher percentage of Holstein, Brown Swiss and Jersey cows present in the evaluated areas (Requelme and Bonifaz, 2012) and for each of them the correlation and linear regression equation were

analyzed.

$$\text{Zone 1:} log(UFC) = log(IBC) * 1{,}08135 + 1{,}2798 \quad (1)$$

$$\text{Zone 2:} log(UFC) = log(IBC) * 0{,}9764 + 0{,}7836 \quad (2)$$

Figure 1 shows the regression analysis for the alternative method expressed in $log(UFC)$ versus the alternative method $log(IBC)$ for each area investigated; 80% ($n = 286$) of the analyzed samples corresponded to Z1 and 20% ($n = 71$) to Z2.

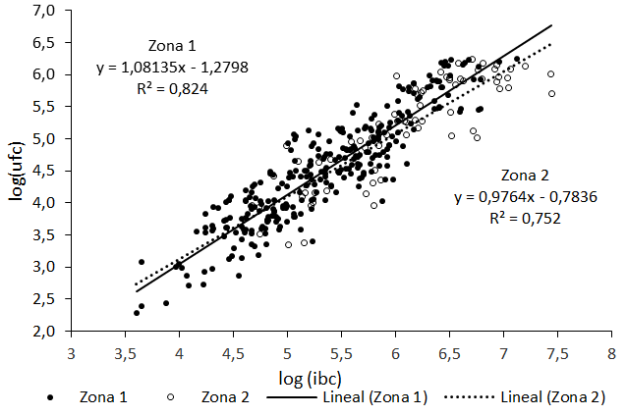


**Figure 1.** Distribution and regression line in $log(IBC/ml)$ and $log(UFC/ml)$ for zone 1 and zone 2.

In the analysis of residues, samples considered atypical in the study population were not found or patterns that indicate heterogeneity difference. In relation to the variation coefficient that suggests the quality of the data, the results obtained in this study were less than 10%, suggesting that the methods are reproducible and reliable for the microbiological analysis performed (Sampaio, 2015). The probability that the counts in each of the techniques have a significance level of 95% was determined with the P value. It was concluded that there were no statistically significant differences ($p > 0{,}05$) among the techniques under study.

The effect of the Z1 and Z2 zones was not considered in the method of analysis because the observed differences were not statistically significant (Figure 2). Thus, a single equation was defined: $log(UFC) = log(IBC) * 1{,}03466 - 1{,}05124$.

The studies carried out by Cassoli (2007) indicated the importance of considering flow cytometry as an alternative method for the counting of bacteria in milk and the determination of hygienic quality raw milk prior to its commercialization. A high count of aerobic mesophylls microorganisms may be related to different sources of contamination at the time of milking and transporting the dairy, as well as with variations in storage temperature (Matsubara, 2011; Martins, 2017). The Ecuadorian regulations for aerobic mesophylls define a maximum limit of 1.5 million of UFC/mL. However, industrial regulations penalize the producer in the payment per liter of milk for counts exceeding 300 000 UFC/mL. Counts exceeding 700 000 UFC/mL were observed in 6,1% to 10% of milk samples rom refrigeration tanks of producer associations (Almeida, 2014; Neppas, 2014), demonstrating the need for greater control in hygiene during milking. Hence, the importance of defining a correlation equation with ranges between 100 000 and 1.5 million UFC/mL.

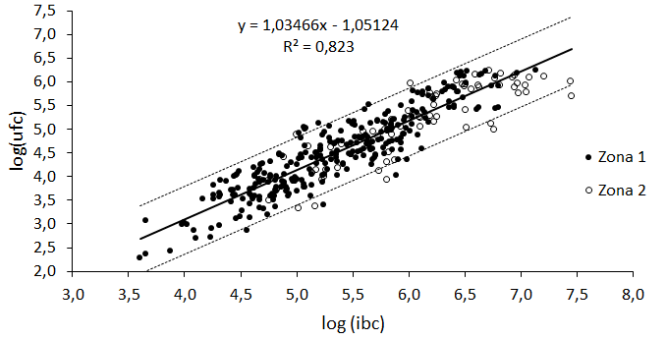


**Figure 2.** Distribution, regression line and prediction interval (95 %) $log(IBC/ml)$ and $log(UFC/ml)$.

Although in countries such as Norway, Canada and the United Kingdom the expression of results such as IBC/mL directly (without conversion) to CFU/ml has been adopted, the reference method in international and national regulations requires its expression as UFC/mL (ISO, 2006; Cassoli, 2007). The transformation of the analytical results obtained by electronic counting equipment based on flow cytometry is also used in other countries with the calibration curve and linear regression, such as Brazil, Uruguay and Colombia (INEN, 2012; Ruiz-Cortés et al., 2012; Cassoli, 2016).

In this study, statistical analysis showed a significant correlation between the standard technique and the flow cytometry technique ($r = 0{,}91$), a similar result to that of previous works reporting a value between 0.80 to 0.93 (Cassoli, 2007; Jatobá, 2014). Sampaio (2015) indicated that the correlation between the two methods can be affected by the size, shape of the bacterial cells or their form of grouping.

The implementation of management systems and quality control between laboratories allows to compare equations and verify differences. In addition, another important advantage of flow cytometry is the price of this analysis: in Ecuador it represents a difference of 3 to 4 times less compared to the price of the referential method, positioning as a useful tool in the monitoring programs and control of high demand quality.

# 4 Conclusiones

Since the demand for the analysis of milk samples in Ecuador is becoming frequent and no significant differences were found for the zones (> 2900 masl and 1050 masl) as those evaluated in this study, it is possible to use a single linear regression equation for the determination of TBC by the alternative flow cytometry method using the Bactoscan FC equipment.

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