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## PAPER

# Factors influencing total bacterial count in tanks: an application of linear mixed-effect models

Mirela Gurgel Guerra,<sup>1</sup>

Adriano H. do Nascimento Rangel,<sup>2</sup> Maria

H. Constantino Spyrides,<sup>3</sup>

Idemauro A. Rodrigues de Lara,<sup>4</sup>

Viviane Maia de Araújo,<sup>5</sup>

Emerson Moreira de Aguiar<sup>2</sup>
<sup>1</sup>Centro de Desenvolvimento Sustentável do Semiárido, Universidade Federal de Campina Grande, Brazil

<sup>2</sup>Unidade Acadêmica Especializada em Ciências Agrárias, Universidade Federal do Rio Grande do Norte, Brazil

<sup>3</sup>Departamento de Estatística, Universidade Federal do Rio Grande do Norte, Brazil

<sup>4</sup>Escola Superior de Agricultura Luiz de Queiroz, Universidade de São Paulo, Brazil

<sup>5</sup>Universidade Federal Rural de Pernambuco, Brazil

## Abstract

The objective of this research was to evaluate the influence of procedures for hygienic milking and cleaning of dairy equipment and milk cooling tanks on the levels of total bacterial count (TBC). Mixed-effect models were used in order to model the structure of dependence between and within dairy farms. To this end, four bulk samples of milk were collected from each tank in eight properties for TBC analysis, employing the flow cytometry method. A questionnaire was applied in each property to assess the current situation of milking procedures on each production system that took part on this research, followed by training of employees in good agricultural practices in the production of milk and monitoring of the TBC measurements. Analysis of longitudinal data was the method employed, which focused on random effects models. The results showed that the handling procedures for milking and the cleanliness of the cooling tanks contributed to a further reduction in the levels of total bacterial count in raw milk cooling tanks.

## Introduction

Milk is made of protein, carbohydrates, lipids, mineral salts, vitamins and water, being a food of high nutritional value for men and as well as an excellent medium for the development of several desirable and undesirable microorganisms. The presence and multiplication of these microorganisms cause physico-chemical changes to the milk that limit its durability (Pereira *et al.*, 2010).

The microbiological quality of raw milk depends on the health of the mammary glands, the management conditions of the herd, the hygienic conditions of milking and of the milking parlors, equipment and tools, the health conditions of the milking workers and the conditions of storage and transportation of the milk to the industry (Cerqueira *et al.*, 1999; Santos and Fonseca, 2000; Holm *et al.*, 2004). Due to these factors, in order to monitor and improve the microbiological quality of raw milk, it is possible to analyze the milk from the expansion tank as an important tool, since, during the interpretation process of results, there were an association between laboratory results and information on management practices performed at the dairy farm.

Total bacterial count (TBC) is the count of the number of bacterial colony-forming units present in the milk sample, giving a quantitative evaluation of the total number of bacterial colony-forming units per milk milliliter (Ministério da Agricultura, Pecuária e Abastecimento, 2003). TBC is an indicator of hygienic and cleaning conditions in milk production and handling in the farm, as well as of its adequate refrigeration. It is, therefore, of extreme importance to control and follow the procedures of dairy systems. In this context, we focused on evaluating the influence of hygienic dairy procedures, of cleansing of the dairy equipment and of the milk cooling tank on TBC levels, and even, to use mix-effect models as to model the dependency structure between and into dairy farms.

## Materials and methods

### Characterization of the farms

The farms are placed in the counties of Ielmo Marinho, São Pedro, Monte Alegre, Macaíba, Brejinho, São José de Mipibú, Goianinha and Extremoz, all of them in the State of Rio Grande do Norte. The predominant feeding system is semi-confined, with herds composed by animals

Corresponding author: Prof. Mirela Gurgel Guerra, Centro de Desenvolvimento Sustentável do Semiárido (CDSA), Universidade Federal de Campina Grande, Paraíba, Brazil.  
Tel. +55.84.99825118.  
E-mail: mirelaguerra@yahoo.com.br

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belonging to the Girolando, Dutch, Jersey and Brown Swiss breeds, varying in number from 48 to 281 lactating animals, and producing 720 to 5026 liters/day.

## Procedures for milk sampling

The monthly collect was conducted with four samples of raw milk from cooling tanks from eight farms located in the rural region of Rio Grande do Norte, during the period of January 2010 to July 2011. A number of 443 milk samples were collected in a total of 59; 55; 53; 59; 45; 57; 56 and 59 for Ielmo Marinho; São Pedro; Monte Alegre; Macaíba; Brejinho; São José de Mipibú; Goianinha and Extremoz, respectively. The milk was firstly homogenized by activating the tank stirrer during five minutes. After this, the milk was transferred, with the help of a stainless steel scoop, to sterile 40 mL plastic vials were four drops of azidol<sup>®</sup> preservative were added. The vials were subsequently placed in isothermic boxes filled in with recyclable ice and sent to the laboratory of the Milk Clinic (ESALQ/USP) in Piracicaba (SP, Brazil).

## Research outline

The study was performed from January 2010 to July 2011, with monthly visits to the farms, divided into three periods: diagnostics, training and monitoring. Period 1 was the diagnostic phase, where a questionnaire was applied to each farm in order to evaluate the present situation of dairy procedures in each production system participating in the research. Training of collaborators on good farming and milk production practices was given during period 2.

This time, a plan of activities developed in the farm was elaborated in order to guarantee quality and safety for milk production, followed by a re-evaluation of several factors related to dairy procedures. The main areas were mastitis control and milking hygiene. Period 3 corresponded to monitoring milk collecting for TBC analysis. Among many factors related to dairy herd management applied in the farms we considered: i) Tamis test; ii) pre-dipping; iii) drying with towel paper; iv) alkaline cleaning of dairy equipment; v) acid cleaning of dairy equipment; vi) sanitation of dairy equipment; vii) cooling of the tank; viii) cleaning of the tank; ix) mechanical cleaning of the tank; x) sanitation of the tank. For analysis purposes, these factors were grouped into three different categories: dairy herd management, covering items 1 to 3; cleaning 1, corresponding to items 4 to 6, and cleaning 2, covering the remaining items related to several aspects of cooling tank cleaning. For this work, dairy herd management means the sum of procedures for milking and refer to 1 to 3; cleaning 1 were procedures 4 to 6 and cleaning 2 all the other procedures.

### Laboratory analysis

Each sample was analyzed in terms of TBC through the method of flow cytometry using the Bactocount® 150 equipment (Bentley Instruments, Inc., Chasca, MN, USA) and the results were expressed in number of colony-forming units/mL of milk (CFU/mL).

### Statistical modeling - Linear mixed-effect models

During the study and monitoring of TBC in the three periods mentioned above, repeated measures of the same variable are observed for the same property in the three moments. In this case, the observations of the same property tend to be correlated among them. Correlations among time-close observations are high and, generally, decrease as time among them increase. In the analysis of repeated measures, it is important that models incorporate possible tendencies among observations taken in the same farm, with the intention of improving the precision of estimates. Being that way, it is necessary to adjust a model taking into consideration, simultaneously, the mean general structure as well as its variability as much among farms as among the references of the same farm. Mixed-effect models allow to consider the intercept and/or the coefficients of the regression as random or fixed effects. This model assumes the vector with repeated measures along observed periods as following a model of linear regression, considering that

some of the regression parameters are specific for the studied population, that is, they are the same for all of the farms, while other parameters are specific for each farm. This is important, because it is not always possible to control every variation source involved in the process, as those derived from biological factors, which are difficult to measure or require an elevated cost in order to get information from it. The behaviour of TBC in each farm might be adjusted with different regression intercepts and coefficients, corresponding to initial indexes and to the increase or decrease rate along the periods, respectively. Namely, each farm presents a growth rate that is different from the behaviour of the population-average, so in terms of TBC as in terms of increase or decrease along the periods. The random effects model therefore allows to estimate the variation of individual deviations in terms of the population-average for each parameter of the model, turning guesses more precise. The random effects model is given by equation 1 (Laird and Ware, 1982; Liang and Zeger, 1986):

$$Y_i = X_i\beta + Z_i b_i + \epsilon_i \quad (1)$$

with fixed effects  $\beta$  and specific effects by year  $b_i$ , with  $b_i \sim N(0, D)$  and  $\epsilon_i \sim N(0, S^2 I)$ . In the eq. (1),  $Y_i$  expresses the answer vector (TBC) for the farm  $i$ , of dimension  $n_i$ , with  $1 \leq i \leq N$ ;  $N$  corresponding to the total number of farms;  $X_i$  and  $Z_i$  are matrices of known covariates.

Considering that TBC is a counting variable and, therefore, does not follow a normal distribution, the Box-Cox transformation, suggested

by the neperian logarithm of the TBC variable, was applied. Thereupon, an adjustment of the mixed-effects model was performed, including the factors of dairy herd management, cleaning 1 and cleaning 2. The method of variable selection was used with the help of backward procedure in order to select the main effects. A comparison of the AIC (Akaike Information Criterion) with the likelihood function logarithm among the adjusted models was taken into account in order to choose the best model. Two stochastic components were considered in the modeling: one on random effects and another on serial correlation among repeated measures. Several structures of the matrix of variants and covariants were tested in order to model the variant structure of the random effects. The R Development 2.14 statistical program (R Development Core Team, 2011) was used for statistical analyses.

## Results and discussion

Considering the dairy herd management procedures, like Tamis test, disinfection during pre-dipping and drying the teat ends with towel paper in the farms evaluated, there was a reduction of 1465.0 to 613.3 and 3167.0 to 499.0 CFU/mL in TBC for Tamis test and pre-dipping application with a reduction of 58% and 85%, respectively. Namely, when these procedures were not applied, the mean TBC was much higher than when applying good procedures (Table 1). According to Galton *et al.* (1986),

**Table 1. Values (x 1000) for total bacterial count (CFU/mL), related to dairy herd management and clean milking along the analyzed period, in function of its application or not.**

	Applying		Not applying		P value
	Mean	SD	Mean	SD	
Dairy herd management					
Tamis test	1465	776	613.3	1660.1	0.016
Pre-dipping	3167	3244	499.0	1373.8	0.000
Paper-drying	-	-	644.2	1643.7	-
Cleaning 1					
Alkaline dilution	4558	3856	534.8	1397.8	0.001
Alkaline contact	4558	3856	534.8	1397.8	0.001
Alkaline temperature	1375	2283	522.4	1480.6	0.001
Acid dilution	3836	4038	427.8	1038.0	0.001
Acid contact	4103	3798	409.7	1035.2	0.000
Acid temperature	1961	2949	391.6	1083.7	0.000
Sanitation dilution	1961	2949	391.6	1083.7	0.000
Sanitation time	1961	2949	391.6	1083.7	0.000
Sanitation temperature	1961	29490	391.6	1083.7	0.000

SD, standard deviation.

disinfecting teats before milking (pre-dipping) followed by a complete drying of the teats, reduces TBC in 54%. Furthermore, according to Bramley (1992), just washing the teats, without drying, does not improve the bacteriological quality of the milk. Therefore, the importance of manual drying of the teats must be emphasized as a way to reduce contamination and, by consequence, to reduce the mean bacterial count of the milk.

For cleaning 1, the mean TBC followed the tendencies of dairy herd management, considerably diminishing when the procedures were applied in an adequate way (Table 1), reducing TBC, in average, approximately 82% when procedures were applied. Brito *et al.* (2004) analyzed the effect of sanitation of dairy tools entering into contact with the milk (buckets, cans, milking equipment and cooling tanks) and verified a reduction in microorganism count from 1,900,000 CFU/mL to 3600 CFU/mL when such tools were sanitized, responding the value found with the present study, in which there was a reduction of 1961.0 for 391.6 CFU/mL in TBC. This fact shows that even better results can be reached by adopting good practices in milk production and cleaning of milking equipment and tanks. Alkaline cleaning resulted in a decreasing mean TBC when applied (Table 1). According to Alvares (2008), alkaline cleaning is good for removing organic compounds from milk that provide nutrients that accelerate bacterial metabolism and which, if not properly removed, become layers hard to be removed. Acid cleaning also resulted in decreasing mean TBC when applied (Table 1). Acid detergents are intended to remove mineral fillings acting as shelters for bacteria. Daily use of acid detergent in the final stage of cleaning prevents accumulation of those fillings and promote a hostile environment for bacterial reproduction (Alvares, 2008). Santos and Fonseca (2001) found contamination indexes of 2,200,000 CFU/mL when evaluating samples of milking machines without mentioning the hygienic conditions used for cleaning the equipment. In studying some dairy farms in the South Eastern region, Brito *et al.* (2004) observed that sanitation procedures in milking parlors, tools and equipment are made in a deficient way or are ignored in several farms. These fails may partly explain, the high bacterial counts found in several herds and the reduced shelf life of milk and dairy products, as well as affecting the health of the lactating cow. For cleaning 2, the mean TBC dropped in about 69% when the procedures were applied to the cooling tank, thus showing the importance of cooling, cleaning and sanitation applied in a proper way in order to diminish

TBC (Table 2). Milk cooling prevents the multiplication of acidificant bacteria and of the production of thermostable toxins and/or enzymes that might deteriorate the final product. Quick refrigeration, to a maximum temperature of 4°C, in expansion tanks is considered as the most adequate procedure for conservation of raw milk (Robbs and Campelo, 2002). Nevertheless, according to Guerreiro *et al.* (2005), milk refrigeration becomes a problem when milk with a high initial bacterial count is stored. The process of refrigerating milk does not prevent multiplication of some bacteria, the psychrotrophic bacteria. These

bacteria are able to double their population each 20 or 30 min and, because of this, milk must be properly handled, avoiding contamination from the moment of milking to its arrival to dairy industries and to its arrival to the final consumer, as low efficiency in milk refrigeration is evident in a short time if the microbial load is already high. Still about cleaning of the cooling tanks, Santos (2010) emphasizes the importance of sanitizing with a chlorine-based disinfectant, in order to reduce contamination, a fact corroborated in this study, as the mean TBC dropped considerably with this procedure, as well as with the mechanical cleaning per-

**Table 2. Values (x 1000) for total bacterial count (CFU/mL) related to cleaning of the cooling tank along the analyzed period, in function of its application or not.**

	Applying		Not applying		P value
	Mean	SD	Mean	SD	
Cleaning 2					
Cooling	730	1887	614.2	1552.1	0.000
Cleaning dilution	2994	3551	532.6	1408.7	0.000
Cleaning contact time	2994	3551	532.6	1408.7	0.000
Cleaning temperature	1294	2170	519.6	1493.9	0.003
Mechanical cleaning	2994	3551	532.6	1408.7	0.000
Sanitation dilution	1662	2744	394.2	1102.4	0.001
Sanitation contact time	1662	2744	394.2	1102.4	0.001
Sanitation temperature	1662	2744	394.2	1102.4	0.001

SD, standard deviation.

**Table 3. Estimated coefficients and confidence intervals of the linear mix-effects model for the logarithm of the total bacterial count (CFU/mL) in milk from tanks in farms of Rio Grande do Norte, along the period from January 2010 to July 2011.**

	Estimate	Standard error	P value
Mix-effects			
Intercept	10.92	0.85	<0.001
Periods	1.01	0.28	<0.001
Milking	-1.23	0.22	<0.001
Cleaning 1	-0.11	0.08	0.192
Cleaning 2	-0.39	0.08	<0.001
Confidence intervals 95%			
	Lower limit	Estimate	Upper limit
Mix-effects			
Intercept	9.25	10.92	12.60
Period	0.47	1.01	1.55
Milking	-1.66	-1.23	-0.81
Cleaning 1	-0.28	-0.11	0.06
Cleaning 2	-0.55	-0.39	-0.22
Random effects			
σ intercept	1.04	1.79	3.09
σ period	0.40	0.72	1.29
σ residual	1.02	1.09	1.17
-2 Log-likelihood	-687.66		
Akaike information criterion	1393.33		



formed with appropriate brushes to this end (76% and 82% of reduction, respectively). Considering the number of procedures used in each stage of the process (dairy herd management; cleaning 1 and 2), the mixed-effect model was adjusted in order to verify the influence on TBC. TBC levels were affected by the particular period (diagnostic, training and monitoring) and the dairy herd management (Tamis test, pre-dipping and drying of the teat with towel paper), showing the importance of using as many dairy procedures as possible in order to reduce total bacterial count, as well as cleaning the cooling tank, represented by the following factors: cooling, cleaning, mechanical cleaning of the tank and sanitation (Table 3). The sign of the coefficients allows to verify that the more procedures of dairy herd management procedures and cleaning 2 were applied by the farms for milk production, the less was the value of TBC, implying an improvement of milk quality. Table 3 shows the estimates of the parameters and the confidence intervals for fix and random parameters of the model, with their respective p-values associated to Wald tests for individual coefficients. Despite being of no lesser importance, cleaning of milking equipment did not represent a significant difference in TBC reduction ( $P=0.192$ ). Probably those differences are to be detected in studies with larger samples. According to Germano and Germano (2001), cleaning of the equipment diminished the microbial load though the mechanical action of water and by the germicide action of detergents or of rinsing, when made with hot water. Nevertheless, the number of surviving microorganisms might be still high, turning sanitation into an obligatory procedure. Anyway, improper cleaning of milking equipment may lead to a TBC rise in the tank, causing quality loss and even discarding of milk (National Mastitis Council, 1996), showing the importance of cleaning procedures in reducing TBC.

This study allowed to show that little adjustments in procedures are quickly reflected in better conditions and benefits to farms. It also shows that, from the training period, most of the farms started to adopt the procedures recommended by the Normative No. 51 (Ministério da Agricultura, Pecuária e Abastecimento, 2002), favoring a reduction in the mean bacterial count in milk from farms, demonstrating the importance of training and advise of professionals directly involved in milk production. In spite of the existence of efficient producers, correctly applying adequate management practices and correctly using the available technology, some others

need better training about management, milking hygiene and cleaning procedures for the equipment used in producing milk with a lower TBC.

## Conclusions

The effects of milking management and cleaning of storage tanks contributed to the significant reduction in the total bacterial count of raw milk in cooling tanks. The milking cleaning did not affect the levels of TBC. It was found that the higher the number of appropriate procedures, the higher the effectiveness in reducing the TBC.

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