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Decline in *Mycobacterium bovis* and *Brucella abortus* populations during the maturation of experimentally contaminated parmesan-type cheese

Decaimento do *Mycobacterium bovis* e da *Brucella abortus* durante a cura de queijo tipo parmesão experimentalmente contaminado

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Abstract

Brazilian legislation allows the manufacture of raw milk cheese with a maturation exceeding 60 days at room temperature above 5°C, but there is a lack of solid scientific evidence on the efficacy of this maturation process in inactivating important pathogens that may be present in milk, such as *Mycobacterium bovis* and *Brucella abortus*. Thus, the objectives of this study were to produce parmesan-type cheese experimentally contaminated with *M. bovis* and *B. abortus* and evaluate the survival of these pathogens along 2-month maturation. Parmesan-type cheese was manufactured in the laboratory using whole pasteurized milk with or without inoculation with *M. bovis* (SB1033) or *B. abortus* (1119-3) and matured at 18°C for up to 63 days. *M. bovis* was inoculated in Stonebrink-Leslie medium supplemented with antibiotics and incubated at 37°C for 45 days, and *B. abortus* was incubated in Farrel medium at 36°C for 3 days. The average $D_{18°C}$ value, weighted by variance, was 37.5 ± 5.3 days for *M. bovis* and 5.9 ± 0.7 days for *B. abortus*. The average physicochemical parameters in the cheese at the end of the study were as follows: pH = 4.89, water activity = 0.976, and moisture percentage = 43.1%. The pH might have contributed to the reduction in the population of *B. abortus* but seems not to have significantly influenced the population of *M. bovis*. We conclude that the duration of the maturation process influences the size of the surviving populations of *M. bovis* and *B. abortus*, and that the shortening of the maturation duration might not ensure a decline in pathogen levels to safe levels. Thus, complementary studies considering the effect of several other technological aspects on the survival of these pathogens are required, including the effect of the lactic acid bacterial population, salt content, and temperature of maturation.

Key words: Brucellosis. Cheese maturation. D-value. Microbial death. Mycobacteria.

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Resumo

A legislação brasileira permite a fabricação queijos de leite cru com maturação superior a 60 dias em temperatura acima de 5°C, mas falta evidência científica sólida sobre a eficácia do processo de maturação na inativação de importantes patógenos que podem estar presentes no leite, como o *Mycobacterium bovis* e a *Brucella abortus*. Assim, os objetivos desse estudo foram produzir o queijo tipo parmesão experimentalmente contaminado com *M. bovis* e *B. abortus* e avaliar a sobrevivência desses patógenos ao longo de 2 meses de maturação. Queijo do tipo parmesão foi fabricado em laboratório com leite pasteurizado integral com ou sem inoculo de *M. bovis* (SB1033) ou *B. abortus* (1119-3) e maturado a 18°C por até 63 dias. *M. bovis* foi semeado em meio Stonebrink-Leslie, acrescido de antibióticos, e incubado a 37°C por 45 dias e a *B. abortus* foi semeada em meio Farrel a 36°C por 3 dias. O valor $D_{18°C}$ médio, ponderado pela variância, foi de 37,5 ± 5,3 dias para o *M. bovis* e de 5,9 ± 0,7 dias para a *B. abortus*. Os parâmetros físico-químicos médios do queijo no final do estudo foram como se segue: pH = 4,89, atividade de água = 0,976 e umidade percentual = 43,1%. O pH pode ter contribuído para redução da população de *B. abortus* mas parece não ter influenciado significativamente a população de *M. bovis*. Concluiu-se que a duração do processo de maturação influencia o tamanho da população sobrevivente de *M. bovis* e de *B. abortus* e que o encurtamento do período de maturação pode não garantir um declínio desses patógenos para níveis seguros. Assim, estudos complementares que considerem o efeito de vários outros aspectos tecnológicos na sobrevivência desses patógenos são necessários, incluindo o efeito da população de bactérias ácido láticas, teor de sal e temperatura de maturação.

Palavras-chave: Brucelose. Maturação. Micobactéria. Morte microbiana. Valor D.

Introduction

Mycobacterium bovis and *Brucella abortus* cause tuberculosis and brucellosis, respectively, in cattle. Milk is one of the main transmission routes of these zoonotic diseases to humans. The extensive and systematic employment of milk pasteurization has significantly reduced the incidence of these diseases in the human population (SINHA, 1994). However, in countries such as Brazil, where these diseases are still currently widespread in herds (BRASIL, 2006a) and the consumption of dairy products prepared with raw milk persists (SOUSA, 2005; ROCHA et al., 2014), it is reasonable to accept the occurrence of human cases by this route of transmission, despite the lack of Brazilian epidemiological data corroborating this hypothesis.

A recent study carried out in 13 Brazilian states, which hold 75% of the country cattle population, showed the prevalence of tuberculosis infected herds ranging from 0.36% in the Federal District to 9.0% in São Paulo (BAHIENSE et al., 2016; BARBIERI et al., 2016; DIAS et al., 2016b; GALVIS et al., 2016; GUEDES et al., 2016; LIMA et al., 2016; NÉSPOLI et al., 2016; QUEIROZ et al., 2016; RIBEIRO et al., 2016; ROCHA et al., 2016; SILVA et al., 2016a; VELOSO et al., 2016; VENDRAME et al., 2016). In relation to bovine brucellosis, 18 Brazilian states were recently studied, comprising 85% of the country cattle population, and the prevalence ranged from 0.91% in Santa Catarina to 30.6% in Mato Grosso do Sul (ALMEIDA et al., 2016; ALVES et al., 2009; ANZAI et al., 2016; BARDDAL et al., 2016; BAUMGARTEN et al., 2016; BORBA et al., 2013; CLEMENTINO et al., 2016; DIAS et al., 2009; DIAS et al., 2016a; INLAMEA et al., 2016; KLEIN-GUNNEWIEK et al, 2009; LEAL FILHO et al., 2016; OGATA et al., 2009; OLIVEIRA et al., 2016; ROCHA et al., 2009; SILVA et al., 2016b; SILVA et al., 2009).

The informal milk market in 2014 represented 29.6% of the milk produced in Brazil, i.e., 10,412 billion out of total of 35,174 billion liters of milk (MILKPOINT, 2015). A bulk of this produce goes into the production of derivatives, mainly cheeses, besides a small portion that is retained for personal consumption in the farms. This is attributed to the ease of manufacturing associated with high-value products that are in huge demand. Usually, these

products are prepared with raw milk and without hygiene and sanitary control measures. This scenario illustrates the potential extent of exposure of the population to products that may be contaminated with *M. bovis* or *B. abortus*. The exposure is possibly even larger, as Brazilian legislation allows the use of raw milk for the manufacture of certain cheeses, e.g., for cheese with maturation periods exceeding 60 days (BRASIL, 1996) and for the production of so-called artisanal cheeses, which have short maturation or no maturation at all, i.e. in the states of Minas Gerais and Pernambuco (ESTADO DE MINAS GERAIS, 2011; ESTADO DE PERNAMBUCO, 2007).

The survival kinetics of pathogens during the manufacture and maturation of cheese is not well established scientifically, and it is probably influenced by the characteristics of the manufacturing process and the milk matrix, such as pH, water activity (Aw), redox potential (Eh), interaction with accompanying microbiota, presence or absence of a heating step of the milk or curd, and addition of acid.

There have been few studies on the behavior of *M. bovis* or *B. abortus* in cheese (FORGRAVE et al., 2014; KARA; AKKAYA, 2015). The occurrence of disease outbreaks caused by *M. bovis* and *Brucella melitensis* contamination in cheese made with raw milk in the United States and the United Kingdom (HARRIS et al., 2007; BROUGH et al., 2011) has aroused the interest of the scientific community on the subject. One of the challenges for this type of study is the lack of a specific isolation technique directly from the dairy product for detecting low levels of contamination by these pathogens in the presence of a high load of companion microorganisms, particularly when cells are injured.

The lack of scientific knowledge on the subject highlights the need for pertinent research, especially because there is strong pressure in Brazil to change the federal law to reduce the minimum time of maturation for cheese manufactured from raw milk. The evaluation of microbiological risk associated with length of time of maturation was the technical-scientific parameter set by the government for future decision making on the subject (BRASIL, 2013).

Studies investigating the decline of these pathogens in cheese are complex, expensive, and highly risky for the operator and the environment. Given that many variables can influence the behavior of the microorganisms in cheese, and that the Brazilian legislation has defined the need for studies on the effect of maturation time to ensure the safety of cheese made from raw milk, the objective of this study was to produce parmesan-type cheese experimentally infected with *M. bovis* and *B. abortus* and evaluate the survival of these pathogens according to the cheese maturation time.

Material and Methods

Eight batches of parmesan-type cheese were manufactured with type-A whole pasteurized milk, as described below, at the Laboratory of Food Microbiology, Faculty of Veterinary Medicine and Zootechny, University of São Paulo. Two batches of cheese were produced with milk contaminated with *M. bovis* (6 log CFU mL^{-1}), 3 with *B. abortus* (6 log CFU mL^{-1}), and 3 others without inoculum, to determine pH, moisture content (M%), and Aw and quantify the indicator microorganisms. The cheeses were maintained at 18°C and analyzed at different time points during the maturation.

Inoculum of Mycobacterium bovis

M. bovis spoligotype SB1033, isolated from cattle slaughtered in the state of São Paulo, was grown at 36°C for 15 days in Stonebrink-Leslie medium (CENTRO PANAMERICANO DE ZOONOSIS, 1985). The inoculum was prepared using 0.300 g of culture, first suspended in 0.5 mL of 0.85% saline solution containing 0.05% Tween 80, and later made up to 12 mL with 0.85% saline solution. Five milliliters of inoculum was used to contaminate 12 L of milk.

Inoculum of Brucella abortus

B. abortus strain 1119-3, a variant of biovar 1, provided by the Biological Institute of São Paulo, was cultivated at 36°C for 3 days in tryptose agar and then suspended with 0.85% saline solution until a turbidity equivalent to the 0.5 standard of the McFarland scale was attained. Forty-eight milliliters of inoculum was used to contaminate 12 L of milk.

Production of cheese experimentally contaminated with B. abortus or M. bovis

Twelve liters of milk inoculated with *B. abortus* or *M. bovis* was heated in a water bath to 32-35°C. Previously prepared thermophilic culture blend freeze-dried lactic culture (TCC20; 1 sachet contains 50 IU of lyophilized yeast in 500 mL of ultra-heat-treated whole milk) was used for cheese production. Twelve milliliters of the culture was used for Direct Vat Set (DVS, Chr Hansen, Brazil), followed by homogenization. After setting this aside for 30 min, 6 mL of calcium chloride, 6 mL of sodium nitrate, 2.4 mL of lactic acid, and 3 mL of liquid coagulant (Três Coroas, Chr Hansen) were added, followed by a further 30 min of rest for coagulation. The curd was then cut, heated to 50°C under homogenization, placed in molds, pressed with a 5-kg weight, maintained at 25°C for 24 h, and then salted. The salting process was performed by immersion of the cheese in 2 L of sterile 20% saline solution for 2 h, followed by immersion in a bath with natamycin 0.075% solution (1.5 g of Natamax® in 1000 mL of water). The cheese was transferred to a closed-lid glass container, sealed with Parafilm®, and incubated in an oven at 18°C for maturation. Daily tumbles were performed for the first 3 days, and subsequently, tumbles were performed on the days of sampling for analysis.

Sample analysis

Milk

The milk used in the manufacture of the contaminated cheese was subjected to assessment for *Mycobacterium* spp. and *Brucella* spp. before and after inoculation to quantify the initial load of the bacteria according to the method described below. The milk used in the manufacture of uncontaminated cheese was analyzed for total and fecal coliforms and coagulase-positive *Staphylococcus* as indicator agents (BRASIL, 2003).

Cheese

Frequency of analysis

The enumeration of the indicators in the uninoculated cheeses was performed on days 1, 8, 15, 22, 29, 36, 43, 50, 57, and 64. Colonies in the cheese inoculated with *M. bovis* were counted on days 1, 7, 14, 21, 28, 35, 42, 49, 56, and 63, while in cheese inoculated with *B. abortus*, counting was performed twice a week, with successive intervals of 3 and 4 days, until completing 63 days or until 2 consecutive analyses resulted negative results.

Sampling

A slice of cheese was removed for analysis, and the exposed surface of the cheese was treated with a solution of 0.075% natamycin, after which the cheese was returned to the aging oven. Our preliminary tests have proven that the natamycin solution employed does not alter the counts of *Mycobacterium* spp. nor of *Brucella* spp. (unpublished data).

Physicochemical analyses and enumeration of indicator microorganisms in the uncontaminated samples

The analyses of M%, pH, and indicators were performed according to the Brazilian Official Method (BRASIL, 2006b, 2003), while the Aw was measured

using an Aqualab® series 3TE Decagon (Pullman, Washington, USA) apparatus according to the manufacturer's instructions. The physicochemical analyses were performed in triplicate for each sample, and the average was recorded.

CFU count of M. bovis and B. abortus in contaminated samples

Twenty-five grams of cheese was used for the quantification of the pathogens. For counting *M. bovis* colonies, serial tenfold dilutions were made in 0.1% peptone water, and 100 µL of each dilution in duplicate was seeded in Stonebrink-Leslie medium (CENTRO PANAMERICANO DE ZOONOSIS, 1985) containing antibiotics and incubated at 36°C for 45 days. To each 120 mL of Stonebrink-Leslie medium, 3.865 mg of 6,000 IU polymyxin B, 0.6 mg of amphotericin B, 2.4 mg of nalidixic acid, and 0.6 mg of trimethoprim were added, as adapted from Donaghy et al. (2003).

For the quantification of *B. abortus*, serial ten-fold dilution was made in tryptose broth, and 100 µL of each dilution was plated in duplicate in Farrel medium and incubated at 36°C for 3 days.

Colonies were counted in the dilutions that showed between 10 and 150 colonies, and the results were expressed in log CFU per milliliter (milk) or gram (cheese).

Calculation of the D-value

The D-value is a unit internationally recognized by microbiologists, and in this case, it represents the time required for 1 log decrease in the count of the microbe for a given condition of cheese ripening (FORGRAVE, 2014). A linear regression equation was used to fit the number of surviving bacteria in the cheese as a function of the time of maturation, and the regression line was used to calculate the $D_{18°C}$ value of each microorganism for each sample of cheese. The average value of $D_{18°C}$ was expressed for each microorganism, weighted by the variance.

Biosecurity measures in the manufacture and analysis of contaminated cheese

During the manufacture of cheese, only authorized people remained in the laboratory, and no personnel entered or exited the facility until the manufacturing process was over. Individual protection equipment (IPE) used were long-sleeved cotton laboratory coats, long cotton pants, socks, and rubber boots barreled into the pants, all of which were reusable. Over these items, a disposable long-sleeved laboratory coat with a closed back and another tied around the waist to protect the legs were worn. In addition, a disposable bathing cap, safety glasses (Rimpac, model A.C. 8126), disposable protection mask (Descarpack, N 95), and 2 disposable rubber gloves on each hand were used. The external pair of gloves was changed frequently to reduce the chance of spread of contamination to other utensils or the environment. The utensils used in manufacturing were immersed in a disinfectant solution immediately after use. For the aluminum or stainless steel materials, formol solution (Lysoform® 1:10 v/v) was used, and for plastic or glass materials, bleach was used (Candida® 1:2 v/v).

After the manufacture, the aluminum containers with the waste material, such as water in the water bath, whey, and cheese leftovers, were packaged in autoclavable bags. Lysoform® was added in sufficient quantity for a final concentration of 1:2 v/v or 1:3 v/v, and the bags were sealed. The floor of the laboratory was wiped a disposable cloth soaked in Lysoform®. This cloth and the IPE were packaged in autoclavable bags, and the disposable materials were separated from the reusable materials. Formalin tablets (Ricie®) were added to the bags and sealed. The laboratory was fumigated using 20 mL of formaldehyde added to 10 g of potassium permanganate per m^3 of space (SOBESTIANSKY et al., 1998) and kept closed for 24 h. All the material remained in the laboratory in this period and was later autoclaved at 121°C/15 min (except the boots and glasses, which were washed

with 1:10 v/v Lysoform®). The entire manipulation of cheese and samples was performed in a laminar flow fume hood (VECO, mod. VLFS-18FL Series 7090; Campinas, São Paulo, Brazil).

Results and Discussion

No detectable levels of the indicator microorganisms of *M. bovis* or *B. abortus* (total and fecal coliforms and coagulase-positive *Staphylococcus*) were detected in the uninoculated milk, which is as expected for pasteurized milk (BRASIL, 2011). Similarly, there was no growth of the indicator microorganisms in cheese produced with uninoculated milk until the end of the study. This can be partly explained by the absence of detectable levels of these agents in milk and partly by the aseptic conditions during the cheese production in the laboratory. This reduces the chance of pathogen decline in the study from being directly influenced by these agents or their metabolites. Some of these contaminants are prolific producers of acids and, according to Macuamule et al. (2016), the quantity and type of acid produced can affect the kinetics of *M. bovis* death.

In inoculated milk, the initial average loads of *M. bovis* and *B. abortus* were, respectively, 6.1 log CFU mL^{-1} and 5.8 log CFU mL^{-1}, while on day 1 of the cheese processing, the average loads were 5.5 log CFU g^{-1} and 5.4 log CFU g^{-1}, respectively (Table 1). Note that the processing of milk into cheese reduced the population of the inoculated bacteria, which is probably associated with the loss of microorganisms in the serum fraction, in addition to a relative increase in remnant population, due to the concentration of solids. This phenomenon was also observed in the experimental production of cheddar and Caerphilly cheese inoculated with *M. bovis* (FORGRAVE et al., 2014), as well as in cheddar cheese inoculated with *M. paratuberculosis* (DONAGHY et al., 2004).

Table 1. Counts of *Mycobacterium bovis* SB1033 and *Brucella abortus* 1119-3 in milk and during the maturation (18°C) of parmesan-type cheese.

agent	cheese sample	agent count										
		milk (log CFU mL^{-1})	cheese (log CFU g^{-1})									
			day 1	day 7	day 14	day 21	day 28	day 35	day 42	day 49	day 56	day 63
Mycobacterium bovis	1	6.0	*	5.3	5.0	4.9	4.6	5.0	3.9	4.3	4.1	4.3
	2	6.1	5.5	5.7	5.0	4.5	4.3	4.3	4.1	4.3	3.7	3.9
	average	6.1	5.5	5.5	5.0	4.7	4.5	4.7	4.0	4.3	3.9	4.1
	standard deviation	0.07		0.28	0.00	0.28	0.21	0.49	0.14	0.00	0.28	0.28
			day 1	day 4	day 8	day 11	day 15	day 18	day 22	day 25	day 29	day 32
Brucella abortus	1	6.2	5.7	5.4	3.9	4.3	4.8	4.1	4.0	2.2	0.0	0.0
	2	5.8	5.8	6.0	4.0	4.8	2.5	2.5	0.0	0.0	***	***
	3	5.5	4.8	4.6	3.9	3.0	0.0***	1.7	0.0	1.7	0.0	0.0
	average	5.8	5.4	5.3	3.9	4.0	2.4	2.8	1.3	1.3	0.0	0.0
	standard deviation	0.4	0.6	0.7	0.1	0.9	2.4	1.2	2.3	1.2	0.0	0.0

* Lost dat

** No growth at the lowest dilution (< 50 CFU g^{-1})

*** Sample not analyzed because the 2 preceding ones were negative.

The affinity of mycobacteria for fat (MCFADDEN et al., 1992) might result in a smaller reduction in *M. bovis* numbers during the processing of milk to cheese compared to *B. abortus*, but the difference between these bacteria was minor. This can be attributed to the tendency for lump formation by *M. bovis* (MCFADDEN et al., 1992), which could result in a lower number of CFU g^{-1}.

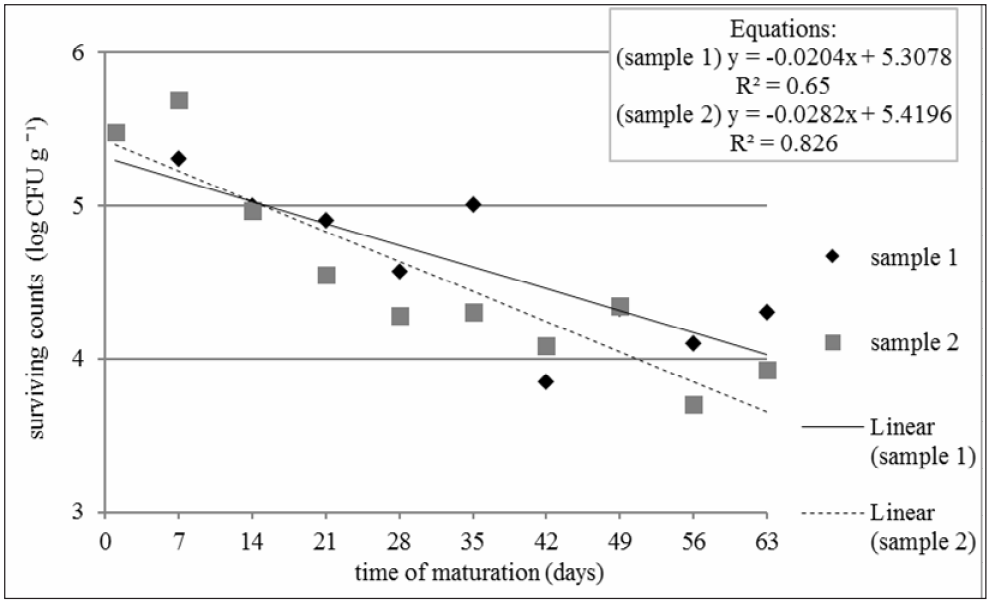
The maturation time of the cheese at 18°C negatively affected the survival of both the pathogens, but the decay rate was more accentuated for *B. abortus* than for *M. bovis*. While the population of *B. abortus* was reduced to undetectable levels by our technique (<50 CFU g^{-1} or <1.7 log CFU g^{-1}) in a maximum of 29 days of maturation, *M. bovis* remained in quantifiable levels until the end of the study, with an average reduction of 1.4 log CFU g^{-1} (Table 1).

The decay curves of *M. bovis* according to the time of maturation are represented in Figure 1 and those of *B. abortus* in Figure 2. The average $D_{18°C}$ value, weighted by the uncertainties, was 37.5 ± 5.3 days for *M. bovis*, indicating that, with 95% confidence, the $D_{18°C}$ value lies between 27.1 and 47.9 days. For *B. abortus*, the average value was 5.9 ± 0.7 days, i.e., the $D_{18°C}$ value of this agent lies between 4.6 and 7.1 days with 95% confidence.

Studies on the persistence of *M. bovis* and *B. abortus* in cheeses have been limited, but quantitative assessments of survival of these agents after cheese maturation are even scarcer. In the only study found, which used a methodology similar to the one used in this study, the average $D_{12°C}$ values obtained for *M. bovis* were 50.75 and 57.59 days, respectively, in cheddar and Caerphilly cheese produced with milk containing a high initial load (5-6 log CFU mL^{-1}), and 30.49 days and 21.48 days, respectively, when produced with a low initial load (2-3 log CFU mL^{-1}) (FORGRAVE et al., 2014). In another study, viable *M. bovis* was detected after 3-4 months of maturation of artificially contaminated blue cheeses (4 log CFU mL^{-1}), despite a rapid reduction in the population in the first 14 days after the manufacture (LAFONT; LAFONT, 1981). Certain studies have determined D-values for *M. paratuberculosis* in a variety of cheeses. The D-value curve of temperature (12°C-22°C-12°C) was 27.8 days in semi-hard Swiss cheese (Tilsiter), while in medium-hard Swiss cheese (Emmental), the $D_{15°C}$ was 45.5 days (SPAHR; SCHAFROTH, 2001). In white soft cheese (Queso Fresco), the $D_{4°C}$ value was 59.9 days (SUNG; COLLINS, 2000). In cheddar-type cheese, the $D_{10°C}$ values were 90, 96, and 107 days, respectively, for a reference strain (NCTC) and two isolates from milk, reinforcing the theory that the strains cultivated in the laboratory for long periods are less resistant (DONAGHY et al., 2004).

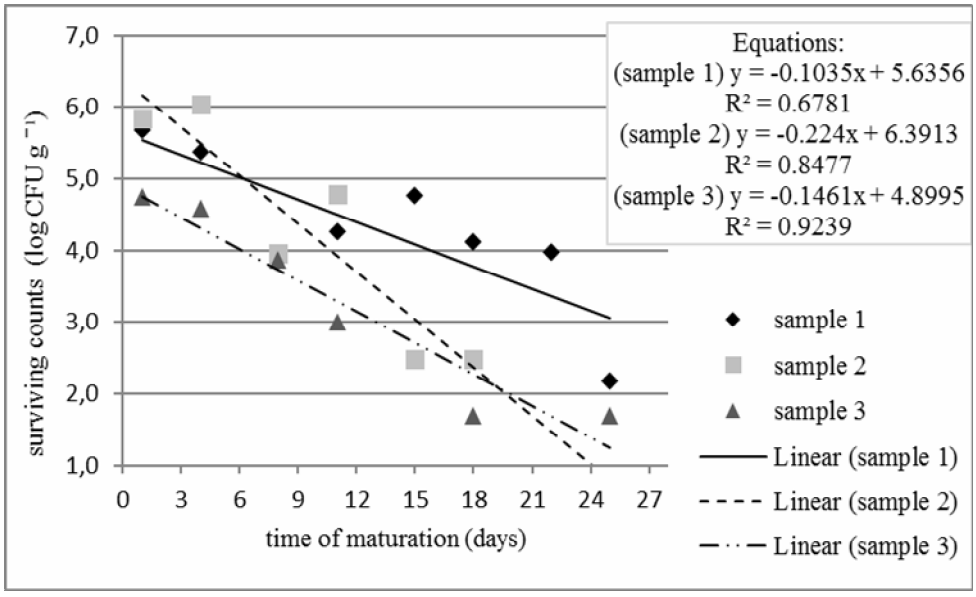
No data for D-values are available for *B. abortus* in cheeses, but studies have reported the survival of this bacterium in cheese. Gilman et al. (1946) observed *B. abortus* in samples of cheddar cheese with 6 months of storage. Plommet et al. (1988) assessed *B. abortus* in camembert cheese manufactured from cows' milk infected experimentally and reported that the initial load in the cheese (3-4 log CFU g^{-1}) was reduced to undetectable levels after 18 days at 12°C. Méndez-González et al. (2011) reported the influence of maturation temperature of cheese on the survival of *B. melitensis*, which was recovered up to 50 days after maturation at 4°C and up to 20 days after maturation at 24°C. Kara and Akkaya (2015) studied the survival of *Brucella* spp. during the maturation of Afyon Tulum cheese and reported that the survival of *B. abortus* was influenced by the initial load: it was detected up to the 15th day of maturation when the inoculum was 4 log CFU g^{-1} and until the 45th day when the inoculum was 6 log CFU g^{-1}; however, *B. melitensis* was inhibited on the 30th day of maturation with either of the initial loads. The variability in the survival of the pathogens during the maturation of various cheeses found by different studies can be explained by differences in the manufacturing technology, maturation temperature, physicochemical characteristics of the milk matrix, companion microbiota, and other aspects related to the methodology used in the study.

Figure 1. Decay curve of *Mycobacterium bovis* spoligotype SB 1033 in parmesan-type cheese during maturation at 18°C.



Obs. day 1 of cut 1: lost data.

Figure 2. Decay curve of *Brucella abortus* 1119-3 in parmesan-type cheese during maturation at 18°C.



Obs. We considered only the time points at which it was possible to quantify *Brucella*: until day 25 in cuts 1 and 3 and until day 18 in cut 2.

Regarding the effect of maturation at 18°C on the physicochemical parameters studied, the pH of the cheese decreased from 5.57 to 4.89, while the Aw decreased from 0.980 to 0.976 and the M% from 44.7 to 43.1% (Table 2). M% is the legal parameter used to establish the microbiological criteria in cheeses. The M% obtained in this study (<35.9%) did not meet the legal standard for parmesan type-cheese (BRASIL, 1996), which was expected since the maturation time in this study was lower than that required for the commercial product (180 days) (BRASIL, 1997).

Table 2. Results of the physicochemical analysis of parmesan-type cheese matured at 18°C.

parameter	sample	sampling									
		day 1	day 7	day 14	day 21	day 28	day 35	day 42	day 49	day 56	day 63
Aw	1	0.983	0.997	0.977	0.981	0.982	0.981	0.978	0.977	0.978	0.98
	2	0.989	0.98	0.982	0.976	0.983	0.978	0.973	0.978	0.977	0.964
	3	0.968	0.98	0.976	0.978	0.977	0.975	0.978	0.971	0.97	0.983
	average	0.980	0.986	0.978	0.978	0.981	0.978	0.976	0.975	0.975	0.976
M%	1	47.3	*	44.0	40.7	43.3	44.7	42.7	44.0	44.7	44.0
	2	*	42.0	42.0	45.3	44.0	43.3	44.7	43.3	42.7	41.3
	3	42.0	34.7	42.0	44.0	42.7	41.3	40.0	42.7	42.7	44.0
	average	44.7	38.4	42.7	43.3	43.3	43.1	42.5	43.3	43.4	43.1
pH	1	5.51	5.24	5.21	5.28	5.14	4.64	4.58	4.61	4.59	4.79
	2	5.54	5.3	5.34	5.19	4.89	4.89	4.73	4.61	4.8	4.82
	3	5.66	5.24	5.27	4.86	4.91	4.92	4.93	4.72	4.97	5.07
	average	5.57	5.26	5.27	5.11	4.98	4.82	4.75	4.65	4.79	4.89

* Lost data.

Brazilian legislation does not establish acceptance criteria for the other physicochemical parameters studied, although they play a relevant role in the control of microbial development in food. Acidity is an important factor for ensuring the development of lactic microbiota and for maintaining the different enzymatic reactions during cheese maturation (SALÄUN et al., 2005), while Aw is widely regarded as a major factor determining microbial growth (JAY, 2000).

Although the borderline values specific to *M. bovis* are not defined, this bacterium is renowned for its high resistance to acidic and alkaline environments (ROWE; DONAGHY, 2008). However, the growth of *B. abortus* is limited by pH values below 4.5-5.1 and above 8.2-8.8, temperatures below 6°C and above 42°C, and salt concentrations above 4% (ICMSF, 1996). The pH of the cheese obtained in this study was close to the minimum limit allocated to the growth of *B. abortus*; therefore, it is possible that this factor had an important effect on the decrease in this population, as has been observed by Falenski (2011). The salt content in the curd was not studied here, and therefore, we could not evaluate the participation of this factor in the decay of *B. abortus*.

Méndez-González et al. (2011) evaluated the survival of *B. melitensis* in goat cheese subjected to two maturation temperatures for demonstrating the interrelationship of technological parameters with the physicochemical characteristics of the final product and the survival of pathogens. Their results showed that when the maturation was performed at 4°C, the pH of the cheese was 5.0 and the bacteria were detected up to the 50th day, and when performed at 24°C, the pH of the cheese was 4.0 and the bacteria were detected up to the 20th day.

It is important to highlight that the D-values of strains circulating in the country may be greater than those obtained in this study because circulating strains tend to be more resistant than those maintained for long periods in the laboratory. However, in actual conditions, it is likely that the lactic acid bacteria (LAB) in raw milk and "starter" cultures can interfere negatively with the survival of these pathogens. Macuamule et al. (2016) studied the effect of milk fermentation on the inactivation of *M. bovis* BCG and observed that the phenomenon was influenced by both the quantity and variability of the LAB population and metabolites produced, such as acids and bacteriocins.

Assuming the worst case scenario, in which milk presents the maximum natural contamination, i.e., 4 log CFU mL^{-1} *M. bovis* (BALL, 1943) and *B. abortus* (CAPPARELLI et al., 2009), and that

cheese maturation results in a lower rate of decline envisaged by the study (which is the upper value of the confidence interval), we simulated microbial load survival in the product ready for consumption, taking 4 periods of maturation: 10, 20, 30, and 60 days (Table 3). The significance of these results is difficult to interpret, given that the dose-response in humans by oral route is not well established for any of these agents, but it is suggested that the infectious doses for *M. bovis* is on the order of ten to millions of cells and fewer than 500 organisms for *Brucella* spp. (FDA, 2012). In the particular case, the consumption of 10 g of cheese could largely surpass the more conservative infectious dose suggested for *M. bovis*, at any stage of simulated maturation; the same could be noted for *Brucella* until the 20th day of maturation.

We demonstrated the production of cheese experimentally infected with strains of *B. abortus* and *M. bovis*. We also showed that the extension of the maturation negatively influenced the size of the surviving population, and that the shortening of the maturation time cannot ensure the reduction of these pathogens to safe levels. However, due to the uncertainties and variability related to the phenomena measured in this study, other studies are necessary to evaluate the degree of safety obtained by the maturation of cheese prepared from raw milk.

Table 3 Simulation of surviving levels of *M. bovis* or *B. abortus* in parmesan-type cheese subjected to maturation at 18°C for different periods in the worst case scenario.

Agent	Initial load in the milk (log CFU mL $^{-1}$)*	Initial load in the cheese (log CFU g $^{-1}$)**	D18°C value (days)***	Level of surviving counts (log CFU g $^{-1}$) after a maturation period of:			
				10 days	20 days	30 days	60 days
M. bovis (SB1033)	4	3.4	48	3.2	3.0	2.8	2.2
B. abortus (1119-3)	4	3.6	7	2.2	0.7	-0.7	-5.0

* Maximum natural contamination of milk reported in the literature, according to Ball (1943) (*M. bovis*) and Capparelli et al. (2009) (*B. abortus*).

** Estimated population in the cheese considering the effect of the processing of the milk into cheese observed in this study: reduction of 0.6 log CFU g^{-1} for *M. bovis* and 0.4 log CFU g^{-1} for *B. abortus.*

*** Time required to reduce the agent by 1 log (upper value of the 95% confidence interval), as obtained in the present study.

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