



## Quantitative risk assessment of *Listeria monocytogenes* in traditional Minas cheeses: The cases of artisanal semi-hard and fresh soft cheeses

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### ABSTRACT

This study estimated the risk of listeriosis from Brazilian cheese consumption using quantitative microbial risk assessment (QMRA). Risks associated to consumption of two cheese types were assessed: artisanal ripened semi-hard cheese (produced with raw milk) and refrigerated fresh soft cheese (produced with pasteurized milk). The semi-hard cheese model predicted *Listeria monocytogenes* growth or decline during ripening, while the soft cheese model predicted pathogen growth during refrigerated storage. Semi-hard cheese modeling scenarios considered *L. monocytogenes* starting concentration from  $-2.4$  to  $6 \log$  CFU/mL in raw milk and three ripening times (4, 22 and 60 days). Soft cheese modeling scenarios considered *L. monocytogenes* starting concentration from  $-2.4$  to  $4 \log$  CFU/mL in milk. The inclusion of anti-listerial lactic acid bacteria (LAB) in cheeses was also examined. Risk of listeriosis due to consumption of soft cheese was 6000 and 190 times greater than that of semi-hard cheese, for general and vulnerable populations, respectively. Aging semi-hard cheese reduced risk, and risk was influenced by *L. monocytogenes* starting concentration. Aging cheese with inhibitory LAB for 22 days reduced risk over 4 million-fold when *L. monocytogenes* was assumed to be  $6 \log$  CFU/mL in raw milk. The inclusion of inhibitory LAB also reduced risk of listeriosis due to soft cheese consumption, but not as much as for semi-hard cheese. QMRA results predicted that consumption of contaminated cheeses can carry a high risk of listeriosis, especially for vulnerable populations. Scenario analyses indicated that aging of semi-hard cheese and inclusion of anti-microbial LAB mix in semi-hard and soft cheeses are effective risk mitigation measures.

### 1. Introduction

*Listeria monocytogenes* is a widely distributed foodborne pathogen able to cause adverse health effects like intra-uterine infection, meningitis and septicemia. Numerous dose-response relationships for *L. monocytogenes* have been proposed (Roucourt et al., 2003), which depend on factors including the environment (food matrix), the pathogen (virulence and number of cells ingested) and the host (susceptibility and immune status). In general, the risk of a fatal listeriosis infection is 10–100 times greater for vulnerable populations (immunocompromised patients, pregnant women, newborns and elderly), compared to general population (healthy adults), resulting in a 20–30% death rate (FAO/WHO, 2004; McLauchlin, Mitchell, Smerdon, & Jewell, 2004). Consumption of ready-to-eat products has caused foodborne listeriosis and different types of cheese are involved in outbreaks worldwide (Gould, Mungai, & Behravesh, 2014; Heiman, Garalde, Gronostaj, & Jackson, 2016; Koch et al., 2010; MacDonald et al., 2005; Makino et al., 2005).

FSANZ (2009) observed that 70% of all cheese implicated in foodborne illness outbreaks were produced with raw milk.

Brazil has experienced a significant effort to improve the recognition and quality of its artisanal cheeses because of the importance of cheese made from raw milk in the economy and sustainability of Mediterranean rural regions. Minas Gerais stands out as the most important Brazilian state producing artisanal cheeses, with about 30,000 producers making more than 50,000 tons of cheese/year (Milkpoint, 2017a). Minas artisanal cheeses are defined as ripened cheeses with semi-hard consistency, yellow and smooth rind, made in the same milking property by family labor in a low-scale production, and using endogenous lactic culture denominated as “pingo” (fermented whey collected from the previous production) and typically using raw milk (Milkpoint, 2017b; Minas Gerais, 2012). Safety concerns arising from inefficient sanitary inspections, inadequate manufacturing practices and excessive handling, may result in contamination by foodborne pathogens, but this does not dissuade customers who appreciate the

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highly desirable organoleptic properties of this cheese. Surveys indicate that incidence of *L. monocytogenes* in semi-hard cheeses in Brazil has been found to vary between 1.4 and 6% (Raimundo, 2013; Silva, Hofer, & Tibana, 1998; Souza, 2002).

Another traditional and widely consumed product is a type of soft white cheese called Minas “frescal” cheese (fresh soft cheese). It is usually made with pasteurized milk (as required by the Brazilian legislation – BRAZIL, 1997). This cheese is produced by small farms and larger dairy processors and is sold refrigerated across Brazil. Fresh soft cheese is consumed by 72% of the Brazilian population (Carvalho, Venturini, & Galan, 2015). It is difficult to accurately calculate the production of this type of cheese because of the large number of small producers, but about 68,300 tons were produced in 2016 under federal inspection (ABIQ – Brazilian Association of Cheese Industry, personal communication, October 18th, 2017). This quantity may double when the informal production is considered, and those informal producers may be more likely to use raw milk for cheese production. Fresh cheeses deserve special attention because they are particularly prone to *L. monocytogenes* post-process contamination and growth due their high pH (5.0–6.3), high water activity (> 0.97), low salt content (1.4–1.6%) and chilled storage throughout shelf-life (Malheiros, Daroit, & Brandelli, 2012a). The incidence of *L. monocytogenes* in fresh soft cheese is quite uncertain as surveys indicate a prevalence ranging from 3 to 45% (Brito et al., 2008; Carvalho, 2003; Silva et al., 1998). Several studies have also demonstrated that Brazilian dairy processing environments are often contaminated by *L. monocytogenes* (Barancelli et al., 2014; Brito et al., 2008; Oxaran et al., 2017; Silva, Almeida, Alves, & Almeida, 2003).

A quantitative microbial risk assessment (QMRA) is a scientifically based method used to estimate the probability and severity of a health disturbance as a consequence of food consumption (Lindqvist & Westöö, 2000). The steps traditionally included in a QMRA are: the formulation of the problem, including the decision of which micro-organism are of concern in which products (hazard identification), determination of hazard intake resulted from food consumption (exposure assessment) and the effect on exposure on the population (hazard characterization), and, the estimation of the overall risk for a specific population (risk characterization) (Adams & Mitchell, 2002). Conducting a QMRA will often help to point out areas with insufficient information (knowledge gaps) to make reasonable decisions regarding a particular foodborne pathogen and food combination (Farber, Ross, & Harwig, 1996). In addition, QMRA may be useful in testing the impact of intervention strategies aiming to mitigate public health risks. In this sense, in a recent study, a mix of lactic acid bacteria was found to present inhibitory activity against *L. monocytogenes* in fresh and semi-hard (ripened) cheeses. Up to 4-log reduction in the counts of *L. monocytogenes* within 15–21 days of ripening of Minas semi-hard cheeses (Campagnollo et al., 2018). However, there is still no information regarding the impact of application of these lactic acid bacteria on the risk of listeriosis due to Minas-type cheeses. Fermented/ripened foods comprise an estimated 30% of the food supply, so the development of risk assessments considering this type of products, including cheeses, offers a compelling and valuable tool in successfully managing food safety hazards (Adams & Mitchell, 2002).

The Brazilian consumption of cheese has increased 8.3% yearly between 2006 and 2013 (Carvalho et al., 2015). The growing popularity of cheese highlights the importance of understanding the behavior of *L. monocytogenes* in these foods. QMRA can help determine the degree of *L. monocytogenes* occurrence, as well as how its probability of growth or decline influences risks from these cheeses. This study estimated the risk of infection by *L. monocytogenes* due to consumption of two Minas traditional cheeses, an artisanal ripened semi-hard type produced with raw milk or a refrigerated fresh soft type typically produced with pasteurized milk.

## 2. Material and methods

### 2.1. Model overview

QMRA were developed for both cheese types starting with milk at bulk tank just before beginning cheese making and ending at cheese consumption. For semi-hard Minas cheese, it was assumed that there was no milk pasteurization and that *L. monocytogenes* contamination occurred during milking, transport to or storage in the bulk tank, if the cheese was produced on the same farm where milk was collected. It was assumed that fresh soft cheese was made from pasteurized milk and that the presence of *L. monocytogenes* was caused by post-contamination at or before reaching the milk bulk tank, whether that cheese was produced by artisanal farms or larger dairy processors. Another supposition was that milk contaminated by *L. monocytogenes* was the only source of pathogen and changes in its concentration due to cross-contamination after this step was negligible.

The presence of *L. monocytogenes* in raw milk may occur due to contact with environmental sources such as feces, sewage, water, animal feeds, vegetation and soil. Additionally, bovine mastitis caused by this same pathogen can serve as another relevant source of raw milk contamination. Since *L. monocytogenes* is cold tolerant (i.e., it can grow at refrigeration temperatures as low as  $-1.5^{\circ}\text{C}$ ) and can form environmentally stable biofilms resistant to sanitation (McIntyre, Wilcott, & Naus, 2015), sources of contamination for pasteurized milk include the environment and the equipment used for milk storage or cheese production.

The risk assessment model is composed of three modules: (a) cheese making, (b) ripening for semi-hard cheese or refrigerated shelf-life for fresh soft cheese, and (c) consumption and risk characterization, considering the estimate of exposure to *L. monocytogenes* (pathogen concentration per serving) and a dose-response function to predict risk of listeriosis. Baseline and alternative scenarios were constructed to evaluate the effect of changing the starting concentration of *L. monocytogenes* and the presence of anti-listerial lactic acid bacteria. The model was built in Excel spreadsheet (Microsoft, Redmond/WA, version 2010) and the simulations were carried out using @Risk software version 7.5 (Palisade Corporation, Ithaca/NY). A total of 100,000 iterations using Monte Carlo sampling were run for each scenario with the random generator seed fixed at 1 to guarantee that results were repeatable and different scenarios could be compared.

### 2.2. Cheese-making module

Table 1 lists the steps of cheese-making module starting from *L. monocytogenes* contaminated milk at the bulk tank. For semi-hard cheese, baseline scenario considered an initial *L. monocytogenes* concentration of 6 log CFU/mL of milk, on the other hand, for fresh soft cheese the starting concentration was 1 log CFU/mL of milk. Milk coagulation is the first step that influences *L. monocytogenes* count in this module. Data from Yousef and Marth (1988), who concluded that 2.4% of *L. monocytogenes* cells are lost during milk coagulation of Colby cheese (a semi-hard type), and from Papageorgiou and Marth (1989), who found that this loss is 3.2% for Feta cheese (a soft type), were used since there are not published data for Minas-style Brazilian cheeses.

Cheese yield changes with cheese type, final moisture content, composition and quality of milk and manufacturing process (Cipolat-Gotet, Cecchinato, De Marchi, & Bittante, 2013). Since no single value represents cheese yield, the values used in this QMRA were gathered from the literature. For semi-hard cheese yield, a Uniform distribution was chosen and the minimum and maximum values of 0.084 and 0.133 kg cheese/L of milk, respectively, were used according to data from Furtado (1980), Araújo (2004) and Silva (2007), who established, cheese yields for Serro, Araxá and Canastra cheeses (all Minas semi-hard cheese types) respectively. Data from Neves-Souza and Silva (2005) and Cardoso (2006) was used for fresh soft cheese yield. Since

**Table 1**Summary of variables for cheese making module of risk assessment model for *L. monocytogenes* in artisanal semi-hard and fresh soft Minas cheeses.

Cheese Making Module				
Notation	Description	Value	Units	Source
LMC <sub>tank</sub>	<i>L. monocytogenes</i> concentration in the milk tank	Semi-hard cheese: 6 Soft cheese: 1	log CFU/mL	Assumption
F <sub>curd</sub>	Fraction of <i>L. monocytogenes</i> cells entrapped in the curd	Semi-hard cheese: 97.6 Soft cheese: 96.8	%	Yousef and Marth (1988) Papageorgiou and Marth (1989)
LMC <sub>coag</sub>	<i>L. monocytogenes</i> concentration after milk coagulation	= LMC <sub>tank</sub> * F <sub>curd</sub>	log CFU/mL	Calculated
CY	Cheese yield	Semi-hard cheese = RiskUniform (0.084–0.133)  Soft cheese = RiskNormal (0.152,0.011,RiskTruncate (0.141,0.167))	Kg cheese/L milk	Furtado (1980); Araújo (2004); Silva (2007) Neves-Souza and Silva (2005); Cardoso (2006)
CW <sub>form</sub>	Weight of a cheese package	Semi-hard cheese = RiskLoglogistic (-274.08,1042,11.069; RiskTruncate (326,1504.2)) Soft cheese = RiskGeneral (250,3000,{250,500,1000},{1,2,1})	g	Campagnollo et al. (2018)  BRAZIL (1997); Silva (2005); ABIQ (2017)
VM <sub>form</sub>	Milk volume necessary for a cheese package	= CW <sub>form</sub> /CY	mL	Calculated
LMC <sub>form</sub>	<i>L. monocytogenes</i> concentration in a cheese package	= RiskPoisson (LMC <sub>coag</sub> *VM <sub>form</sub> )	log CFU/package	Sanaa et al. (2004)
LMC <sub>cheese</sub>	<i>L. monocytogenes</i> concentration per gram of cheese	= LMC <sub>form</sub> /CW <sub>form</sub>	log CFU/g	Calculated

the variation between values was smaller than that found for semi-hard cheese, a Normal distribution was used considering the mean value of  $0.152 \pm 0.011$  kg cheese/L of milk, truncated in the minimum and maximum values (0.141 and 0.167 kg cheese/L milk, respectively).

Cheese weight also varies greatly depending on the producing region and also the producer and consumer preferences. Semi-hard cheese weights from 274 artisanal cheeses collected by our research group previously (Campagnollo et al., 2018) were integrated using the distribution fitting tool in @Risk, to create a log-logistic distribution. Cheese weights ranged from 326.0 to 1504.2 g, so the log-logistic distribution of variability was truncated considering these values as minimum and maximum, and using parameters of 780.74 for central tendency and 168.04 for variability. Fresh soft cheese weights were gathered from legislation (BRAZIL, 1997), from the Brazilian Agricultural Research Corporation – EMBRAPA (Silva, 2005) and from ABIQ (2017), resulting in weights ranging from 250 to 3000 g. Since it was uncommon to find cheeses weighing 3000 g at retail, an online survey was performed to determine the most common weights for this type of cheese available in the market, and weights of 250, 500 and 1000 g were determined to be the most popular. Fresh soft cheese weights were modeled by a General distribution including the minimum, the maximum and the most popular weights, with 500 g receiving a double load because it was the most prevalent one.

The volume of milk necessary to produce cheese package was calculated, and the *L. monocytogenes* concentration expected for this mass of cheese was estimated using a Poisson distribution derived from Sanaa, Coroller, and Cerf (2004). This information was used to determine pathogen concentration per weight of cheese.

### 2.3. Ripening module for artisanal semi-hard Minas cheese

Artisanal semi-hard cheeses are traditionally ripened for 17–22 days. According to Brazilian federal legislation, however, commercial sale of raw milk cheeses is only allowed for cheeses ripened for 60 days above 5 °C (MAPA, 2000). New Brazilian legislation was drafted in 2011 reconcile this situation, and allow the commercial sale of cheeses with ripening times of < 60 days if scientific studies reviewed by the Ministry of Agriculture could show the cheese to be safe (MAPA, 2011). We decided to assume a 22-day ripening period at room temperature ( $22 \pm 2$  °C) for the baseline scenario (Table 2), after obtaining relevant expert opinion (Élcio, manager of APROCAME (Association of Canastra Cheese Producers of Medeiros) – Medeiros/MG/Brazil, personal

communication, May 3rd, 2016).

Campagnollo et al. (2018) performed a challenge test to determine *L. monocytogenes* behavior during ripening of artisanal semi-hard cheese. Data from this publication were used to estimate parameters of a Normal distribution for predicting *L. monocytogenes* behavior from an initial concentration of 6 log CFU/mL of milk. At the end of ripening period, pathogen concentration was calculated by modifying its initial concentration in the cheese based on subsequent changes during ripening.

### 2.4. Refrigerated shelf-life module for fresh soft cheese

Time and temperature during fresh soft cheese distribution from industry to retail were modeled based on data of Koutsoumanis, Pavlis, Nychas, and Xanthiakos (2010) for pasteurized milk since no fresh soft cheese data for transportation in Brazil were available (Table 3). These authors recorded time and temperature in trucks during transportation for five working days in 12–15 different trucks for each day ( $n = 83$ ), finding temperature ranging from 3.6 to 10.9 °C (mean of  $6.7 \pm 1.6$  °C) and transport times varying between 0.2 and 10.2 h (mean of  $3.7 \pm 2.0$  h). Normal distributions truncated in the minimum and maximum values were used to model each variable.

Rocha, Buriti, and Saad (2006) reported that Minas-style fresh soft cheese shelf-life varied from 21 to 30 days for commonly available commercial brands and that samples with less than seven-day shelf life were seldom recovered at retail. Carvalho, Viotto, and Kuaye (2007) collected fresh soft cheese samples from retail and observed that the average age varied from 10 to 15 days, and the manufacturer reported minimum and maximum shelf-life ranged from 15 to 25 days and 45–63 days, respectively. Time of retail storage was modeled using a Triangular distribution considering 1, 7 and 20 days as the minimum, most likely and maximum values, respectively. Temperature at retail storage was gathered from the “Product Analysis Program” for fresh soft cheese conducted by the Brazilian National Institute of Metrology, Quality and Technology (INMETRO, 2006). Samples were collected at 21 retail markets and temperatures were recorded at the time of collection, varying from 6.4 to 15.5 °C. Observed temperatures were integrated using the distribution fitting tool of @Risk resulting in a Triangular distribution, which was truncated at these minimum and maximum values.

A Triangular distribution was used to model domestic storage time. The minimum time of consumption was assumed to be zero (i.e., the

**Table 2**  
Summary of variables for ripening module of risk assessment model for *L. monocytogenes* in artisanal semi-hard Minas cheese.

Ripening module for semi-hard Minas cheese				
Notation	Description	Value	Unit	Source
$R_{\text{time}}$	Ripening time	22	days	Assumption
$LMR_{\text{growth}}$	<i>Listeria monocytogenes</i> growth IF $LMC_{\text{tank}} = 6 \log \text{CFU/mL}$	= RiskNormal (0.74,0.32)	log CFU/g	Campagnollo et al. (2018)
$LMR_{\text{inact,LAB}}$	<i>Listeria monocytogenes</i> inactivation IF $LMC_{\text{tank}} = 6 \log \text{CFU/mL}$ with lactic acid bacteria added	= RiskNormal (-3.7,0.05) IF $R_{\text{time}} = 4$ days = RiskNormal (-3.7,0.05) IF $R_{\text{time}} = 12$ days = RiskNormal (-5.81,0.05) IF $R_{\text{time}} > 19$ days	log CFU/g	Campagnollo et al. (2018)
LMIR	<i>Listeria monocytogenes</i> inactivation rate IF $LMC_{\text{tank}} < 6 \log \text{CFU/mL}$	= 0.0352 IF $LMC_{\text{tank}} = 3.5 \log_{10} \text{CFU/mL}$ = 0.0204 IF $LMC_{\text{tank}} = 1.0 \log_{10} \text{CFU/mL}$ = 0.00244 IF $LMC_{\text{tank}} = -2.35 \log_{10} \text{CFU/mL}$	log CFU/day	Pinto et al. (2009)
$LMR_{\text{inact}}$	<i>Listeria monocytogenes</i> inactivation IF $LMC_{\text{tank}} < 6 \log \text{CFU/mL}$	= $LMIR * R_{\text{time}}$	log CFU/g	Calculated
$LMC_{\text{ripening}}$	<i>Listeria monocytogenes</i> concentration in cheese after ripening time	= $LMC_{\text{cheese}} + LMR$ ( $LMR = LMR_{\text{growth}}$ or $LMR_{\text{inact,LAB}}$ or $LMR_{\text{inact}}$ )	log CFU/g	Calculated

cheese was consumed on the day of purchase), the maximum time was set to 10 days and the most likely time of consumption was assumed to be three days after purchasing. Data from Silva, Celidonio, and Oliveira (2008) analyzed the temperatures of 25 Brazilian domestic refrigerators for one week and observed mean maximum and minimum values of 10.82 °C and 3.04 °C, respectively. These data were used to model domestic storage temperatures and were described using the Uniform distribution.

The growth of *L. monocytogenes* during refrigerated shelf-life of fresh soft cheese was described by the relationship between growth rate and temperature represented by the linear regression model proposed by Ratkowsky, Olley, McMeekin, and Ball (1982) and shown in Equation (1):

$$\sqrt{r} = b(T - T_0) \quad (1)$$

Where:  $\sqrt{r}$  is the square root of maximum growth rate ( $\mu$ ),  $b$  is the slope of the regression line,  $T$  is the temperature (°C) and  $T_0$  is the theoretical minimum temperature for microbial growth. Data from Campagnollo et al. (2018), Malheiros, Sant'Anna, Barbosa, Brandelli, and Franco (2012b), Naldini, Viotto, and Kuaye (2009) and Pingitore, Todorov, Sesma, and Franco (2012) were described the square root model with parameters as shown in Equation (2):

$$\sqrt{\mu} = 0.068(T + 2.765) \quad (2)$$

*L. monocytogenes* growth during transportation, storage at retail and domestic storage was calculated using Equation (2). Final pathogen concentration was the sum of its initial concentration in cheese and the subsequent growth during the refrigerated shelf-life.

### 2.5. Consumption, dose-response and risk of infection module

The description of consumption, dose-response and risk of infection module is common to fresh soft cheese and semi-hard cheese (Table 4). Carlos, Rolim, Bueno, and Fisber (2008) observed that, in the city of São Paulo/Brazil, a small size serving of fresh soft/semi-hard cheese varied from 20 to 30 g, the mean serving was 20–40 g, and the biggest serving ranged from 30 to 60 g depending on the consumer (men or women, adult or elderly). Therefore, we assumed that 20, 30 and 60 g were the minimum, most likely and maximum values, respectively, of a Triangular distribution. Final *L. monocytogenes* concentration in a serving of cheese was calculated from serving size and final pathogen concentration at the end of shelf-life/ripening using a Poisson distribution based on Condoleo et al. (2017), who performed a QMRA for listeriosis from consumption of raw sheep's milk semi-soft cheese.

The dose-response function to estimate the risk of invasive listeriosis from cells consumed from a single cheese serving was calculated using the exponential model of Buchanan, Damert, Whiting, and von Schothorst (1997). The “ $r$ ” parameter for general ( $2.37 \times 10^{-14}$ ) and

vulnerable ( $1.06 \times 10^{-12}$ ) populations represented in the model was extracted from FAO/WHO (2004), which interprets “ $r$ ” as the probability that a single cell will cause invasive listeriosis in an individual. While vulnerable population may represent 20% of the total population with a higher risk for developing listeriosis (Buchanan et al., 1997; Lindqvist & Westöo, 2000), we chose to estimate risk to each of these populations separately. The outputs of this QMRA model, both for general and vulnerable populations, were the risk of listeriosis per serving (probability of infection due to consumption of one serving) and the number of listeriosis cases in a population of 10,000. We also calculated relative risk to better comparing the baseline with the alternative scenarios proposed.

### 2.6. Evaluation of alternative scenarios

Alternative scenarios which considered different *L. monocytogenes* initial concentrations and the addition of lactic acid bacteria (LAB) with anti-listerial activity to cheeses were used to explore the effect of different risk mitigation procedures. LAB are known to produce various antimicrobial metabolites (in addition to lactic acid) which can inhibit *L. monocytogenes* even during cold storage, and these include hydrogen peroxide, diacetyl, reuterin and bacteriocins (Guillier, Stahl, Hezard, Notz, & Briandet, 2008). Several studies have shown that LAB isolated from different types of cheese have clear anti-listerial properties (Alexandre, Silva, Souza, & Santos, 2002; Campagnollo et al., 2018; González et al., 2007; Guedes Neto, Souza, Nunes, Nicoli, & Santos, 2005; Ortolani et al., 2010; Ribeiro et al., 2014; Rivas, Castro, Vallejo, Marguet, & Campos, 2012; Sip, Wieckowicz, Olejnik-Schmidt, & Włodzimierz, 2012).

The baseline scenario for artisanal semi-hard cheese assumed an initial pathogen concentration of 6 log CFU/mL of milk and a 22-day ripening period. Alternative scenarios evaluated were: (a) *L. monocytogenes* initial concentration of 6 log CFU/mL plus the addition of 6 log CFU/mL of LAB with anti-listerial activity (based on data of Campagnollo et al., 2018) and considering ripening periods of 4 or 22 days; and (b) lower *L. monocytogenes* initial concentrations of 3.5, 1, 0 or  $-2.4 \log \text{CFU/mL}$  of milk and considering ripening times of 4, 22 and 60 days. The concentration of  $-2.4 \log \text{CFU/mL}$  was chosen based on Brazilian legislation for the presence of *L. monocytogenes* in cheese (ANVISA, 2001), which states the absence of this pathogen in 25 g of product, using the following logic. If we assume the presence of 1 CFU in 25 g of cheese (i.e.,  $1.4 \log \text{CFU/g}$  of cheese ready for ripening, just after finishing the production), this concentration would have resulted from bulk tank milk with a concentration of  $-2.4 \log \text{CFU/mL}$  *L. monocytogenes*. The baseline and LAB scenarios used a ripening period of 22 days since this is what is traditionally used by artisanal producers and because the LAB experiments that form this model showed that the

**Table 3**  
Summary of variables for refrigerated shelf-life module of risk assessment model for *L. monocytogenes* in fresh soft Minas cheese.

Refrigerated shelf-life module for soft Minas cheese				
Notation	Description	Value	Unit	Source
$ST_{distribution}$	Transport time during distribution	= RiskNormal (3.7,2,RiskTruncate (0.2,10.2))	hours	Koutsoumanis et al. (2010)
$T_{distribution}$	Temperature during distribution	= RiskNormal (6.7,1.6,RiskTruncate (3.6,10.9))	°C	Koutsoumanis et al. (2010)
$ST_{retail}$	Storage time at retail	= RiskTriang (1.7,20)	days	Rocha et al. (2006); Carvalho et al. (2007)
$T_{retail}$	Temperature during storage at retail	= RiskTriang (6.4,6.4,1.6,979,RiskTruncate (6.4,15.5))	°C	INMETRO (2006)
$ST_{domestic}$	Storage time at home	= RiskTriang (0.3,10)	days	Assumption
$T_{domestic}$	Temperature at domestic storage	= RiskUniform (3.04,10.82)	°C	Silva et al. (2008)
$LM\mu_{growth}$	<i>L. monocytogenes</i> growth rate as a function of temperature	Only LM: $(0.068 * T + 0.188)^2$	log CFU/day	Campagnollo et al. (2018); Malheiros et al. (2012b); Naldini et al. (2009); Pingitore et al. (2012)
	<i>L. monocytogenes</i> growth rate as a function of temperature with added lactic acid bacteria	LM + LAB <sup>2</sup> : $(-0.1244 * T + 0.717)^2$	log CFU/day	Campagnollo et al. (2018); Nascimento et al. (2008); Pingitore et al. (2012)
$LM\mu_{distribution}$	<i>L. monocytogenes</i> growth rate as a function of temperature in distribution	Only LM: $(0.068 * T_{distribution} + 0.188)^2$	log CFU/day	Calculated
	<i>L. monocytogenes</i> growth rate as a function of temperature with added lactic acid bacteria	LM + LAB: $(-0.1244 * T_{distribution} + 0.717)^2$	log CFU/g	Calculated
$LMG_{distribution}$	<i>L. monocytogenes</i> growth during distribution	= $LM\mu_{distribution} * (ST_{distribution}/24)$	log CFU/g	Calculated
$LM\mu_{retail}$	<i>L. monocytogenes</i> growth rate as a function of temperature at retail	Only LM: $(0.068 * T_{retail} + 0.188)^2$	log CFU/day	Calculated
	<i>L. monocytogenes</i> growth rate as a function of temperature at retail with added lactic acid bacteria	LM + LAB: $(-0.1244 * T_{retail} + 0.717)^2$	log CFU/g	Calculated
$LMG_{retail}$	<i>L. monocytogenes</i> growth during retail storage	= $LM\mu_{retail} * ST_{retail}$	log CFU/g	Calculated
$LM\mu_{domestic}$	<i>L. monocytogenes</i> growth rate as a function of temperature in homes	Only LM: $(0.068 * T_{domestic} + 0.188)^2$	log CFU/day	Calculated
	<i>L. monocytogenes</i> growth rate as a function of temperature in homes with added lactic acid bacteria	LM + LAB: $(-0.1244 * T_{domestic} + 0.717)^2$	log CFU/g	Calculated
$LMG_{domestic}$	<i>L. monocytogenes</i> growth in homes	= $LM\mu_{domestic} * ST_{domestic}$	log CFU/g	Calculated
$LMC_{shelflife}$	<i>L. monocytogenes</i> concentration at the end of shelflife	= $LMG_{cheese} + LMG_{distribution} + LMG_{retail} + LMG_{domestic}$	log CFU/g	Calculated

**Table 4**

Summary of variables for consumption, dose-response and risk of infection module of risk assessment model for *L. monocytogenes* in artisanal semi-hard and fresh soft Minas cheeses.

Consumption, dose-response and risk of infection module				
Notation	Description	Value	Unit	Source
$S_{\text{servicing}}$	Serving size	RiskTriang (20; 30; 60)	grams	Carlos et al. (2008)
$LMC_{\text{servicing}}$	<i>L. monocytogenes</i> concentration in a serving	RiskPoisson ( $S_{\text{servicing}} * LMC_{\text{ripening}}$ )	log CFU/serving	Condoleo et al. (2017)
$r_{\text{general}}$	Parameter “r” for dose response for general population	$2.37 \times 10^{-14}$	–	FAO/WHO (2004)
$r_{\text{vulnerable}}$	Parameter “r” for dose response for vulnerable population	$1.06 \times 10^{-12}$	–	FAO/WHO (2004)
$Risk_{\text{general}}$	Risk of listeriosis per serving for general population	$1 - \text{Exp}(-r_{\text{general}} * LMC_{\text{servicing}})$	–	Buchanan et al. (1997), Calculated
$Risk_{\text{vulnerable}}$	Risk of listeriosis per serving for vulnerable population	$1 - \text{Exp}(-r_{\text{vulnerable}} * LMC_{\text{servicing}})$	–	Buchanan et al. (1997), Calculated
$NL_{\text{general}}$	Number of listeriosis in 10,000 for general population	RiskBinomial (10000, $Risk_{\text{general}}$ )	cases of listeriosis/ 10,000	Calculated
$NL_{\text{vulnerable}}$	Number of listeriosis in 10,000 for vulnerable population	RiskBinomial (10000, $Risk_{\text{vulnerable}}$ )	cases of listeriosis/ 10,000	Calculated
$NS_{\text{general}}$	Number of servings for 1 case of listeriosis for general population	$1/Risk_{\text{general}}$	number of servings/case	Calculated
$NS_{\text{vulnerable}}$	Number of servings for 1 case of listeriosis for vulnerable population	$1/Risk_{\text{vulnerable}}$	number of servings/case	Calculated

pathogen was inactivated below the limit of detection after 19 days of ripening (Campagnollo et al., 2018).

Models for *L. monocytogenes* behavior during ripening for lower initial concentrations used in scenario (b) were based on data of Pinto et al. (2009). These authors studied *Listeria innocua* survival in Serro cheese, an artisanal semi-hard cheese, during a 60-day ripening period with starting inoculation levels of 1, 2 and 3 log CFU/mL in milk. *L. innocua* is a non-pathogenic surrogate of *L. monocytogenes*, which can be also isolated from raw milk cheeses (Carvalho et al., 2007), and is physiologically similar to the pathogen (Bermúdez-Aguirre & Barbosa-Cánovas, 2008), differentiated only by the inability of producing listeriolysin (an enzyme capable of lysing red blood cells) (FAO/WHO, 2004). The inactivation rate per day (IR) for each *L. monocytogenes* concentration (LM) was determined, generating a linear regression described by Equation (3), which was used to calculate the inactivation rates per day for *L. monocytogenes* concentrations used for scenario (b). The inactivation rate was multiplied by the number of days of the ripening period considered in each alternative scenario and summed to pathogen concentration in cheese just before ripening. Descriptions of the calculations used in scenarios (a) and (b) of semi-hard cheese are listed in Table 2.

$$IR = -0.0056 * LM - 0.0156 \quad (3)$$

The fresh soft cheese baseline scenario was built assuming an initial concentration of 1 log CFU/mL *L. monocytogenes* in milk. Alternative scenarios for this cheese included: (a) other pathogen initial concentrations (–2,4; –1; 0; 2; 3 and 4 log CFU/mL); and (b) different *L. monocytogenes* initial concentrations (1; 2; 3, 4 and 5 log CFU/mL) associated with the addition of LAB with anti-listerial activity. For scenarios in (a), calculations were performed using Equation (2). Data from Campagnollo et al. (2018), Nascimento, Moreno, and Kuaye (2008) and Pingitore et al. (2012) were used to build a new relationship between *L. monocytogenes* growth rate and the temperature represented by the linear regression model of Ratkowsky et al. (1982) in Equation (4) for scenarios with LAB addition:

$$\sqrt{\mu} = -0.124(T - 5.764) \quad (4)$$

Information regarding the details of the additional scenarios for fresh soft cheese is summarized in Table 3 above.

### 3. Results

The simulated concentration of *L. monocytogenes* at the end of semi-hard cheese ripening process ranged from 6.2 to 9.2 log CFU/g of

cheese with a mean of  $7.7 \pm 0.3$  log CFU/g. *L. monocytogenes* concentration at the beginning of shelf-life of refrigerated fresh soft cheese showed a mean of  $1.8 \pm 0.02$  log CFU/g of cheese and was  $10.9 \pm 4.4$  log CFU/g just before consumption (after simulating the effects of transportation, retail sale and domestic storage). A mean typical cheese serving for both types of cheese was  $36.7 \pm 8.5$  g since the same distribution and values for cheese consumption were used.

The main outputs of the QMRA model for baseline scenarios regarding the risk of listeriosis and the number of cases in a population of 10,000 are described in Table 5. As expected, vulnerable populations had a higher risk of listeriosis for both types of cheese compared to general population. Risk of listeriosis for fresh soft cheese was 6000 and 190 times greater than that found for semi-hard cheese, for general and vulnerable populations, respectively. Our simulation predicts that consumption of semi-hard cheese contaminated with assumed levels of *L. monocytogenes* would cause a mean of 26 cases of listeriosis in a vulnerable population of 10,000. Fresh soft cheese consumption would result in 3443 and 4897 mean illnesses in general and vulnerable

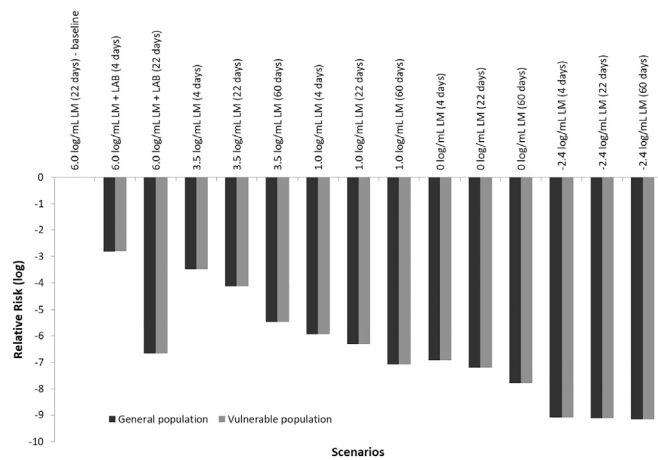
**Table 5**

Output of QMRA model<sup>a</sup> regarding the baseline scenarios<sup>b</sup> of artisanal semi-hard and fresh soft cheeses.

	Artisanal semi-hard cheese		Fresh soft cheese	
	General population	Vulnerable population	General population	Vulnerable population
Risk of listeriosis per contaminated serving				
Mean	5.73E-05	2.56E-03	3.44E-01	4.90E-01
SD	5.30E-05	2.35E-03	4.49E-01	4.69E-01
Minimum	1.19E-06	5.33E-05	3.77E-10	1.68E-08
5 <sup>th</sup> percentile	1.16E-05	5.20E-04	1.88E-07	8.39E-06
Median	4.20E-05	1.88E-03	9.53E-03	3.48E-01
95 <sup>th</sup> percentile	1.53E-04	6.83E-03	1.00E+00	1.00E+00
Maximum	1.07E-03	4.68E-02	1.00E+00	1.00E+00
Number of listeriosis cases in a population of 10,000				
Mean	0.6	26	3443	4897
SD	0.9	24	4494	4688
Minimum	0	0	0	0
5 <sup>th</sup> percentile	0	4	0	0
Median	0	19	95	3484
95 <sup>th</sup> percentile	2	69	10,000	10,000
Maximum	14	488	10,000	10,000

<sup>a</sup> Based on a total of 100,000 iterations using Monte Carlo sampling.

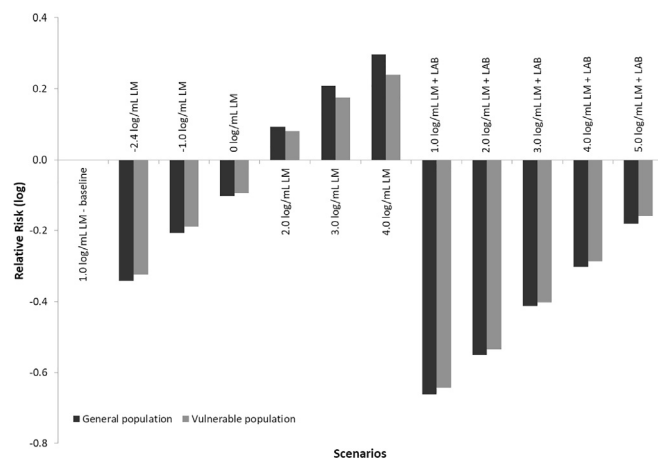
<sup>b</sup> Baseline scenarios: Artisanal semi-hard cheese: 6 log CFU/mL *L. monocytogenes* in milk and 22-day ripening period; Fresh soft cheese: 1 log CFU/mL *L. monocytogenes* in milk.



**Fig. 1.** Mean relative risk of consumption of artisanal semi-hard cheese contaminated with *L. monocytogenes* (LM). Horizontal axis represents the baseline and alternative scenarios studied considering different LM concentrations with/without the addition of lactic acid bacteria (LAB) and ripening for 4, 22 or 60 days.

populations respectively, and there were some iterations of the simulation where all the people in both populations were predicted to fall ill.

The mean relative risk of listeriosis due to consumption of semi-hard cheese or fresh soft cheese contaminated with *L. monocytogenes* for general and vulnerable populations and considering alternative scenarios relative to the baseline is represented in Figs. 1 and 2, respectively. Each figure compares the risk of listeriosis in the baseline scenario (expressed as 1, or as 0 log relative risk in the figures) to alternative scenarios. All the alternative scenarios evaluated for semi-hard cheese (Fig. 1) led to lower risks of listeriosis. This is not surprising since each of the scenarios used either a lower *L. monocytogenes* starting concentration in milk used to produce cheese, or intervention (i.e., the addition of LAB) that reduced *L. monocytogenes* concentration. Fig. 1 also shows that longer ripening periods (4, 22 or 60 days) progressively reduce risk, e.g., consumption of cheese produced with a *L. monocytogenes* starting concentration of  $-2.4$  log CFU/mL in milk and ripened for 60 days is  $> 9$  log ( $\sim 1.5$  billion times) less risky than consumption of cheese produced in the baseline scenario. The simulation also showed that the addition of LAB with anti-listerial properties also reduced the risk of listeriosis, even when assuming the same very high starting concentration of *L. monocytogenes* used in the baseline



**Fig. 2.** Mean relative risk of consumption of fresh soft cheese contaminated with *L. monocytogenes* (LM). Horizontal axis represents the baseline and alternative scenarios studied considering different LM concentrations with/without the addition of lactic acid bacteria (LAB).

scenario (6 log CFU/mL milk). Anti-listerial LAB was able to reduce *L. monocytogenes* concentration from 7.7 log CFU/g of cheese in the baseline scenario to 1.1 log CFU/g of cheese in the alternative scenario after 22 days of ripening (data not shown), reducing risk by  $> 6$  log (Fig. 1).

The use of lower *L. monocytogenes* concentrations in milk used to produce fresh soft cheese in alternative scenarios also resulted in lower relative risks of listeriosis (Fig. 2) although the effect was much less dramatic than with semi-hard cheese (note the difference in the relative risk scales between Figs. 1 and 2). When the addition of LAB with anti-listerial properties was considered, even increasing the pathogen concentration in milk by up to 10,000 times compared to the baseline scenario (1 log to 5 log), the risk of listeriosis was reduced  $\sim 1.5$  fold ( $-0.18$  log) in the general population, and almost the same amount in the vulnerable population. The simulation predicts that the addition of anti-listerial LAB to milk contaminated with *L. monocytogenes* at 1 log CFU/mL (the same concentration used in the baseline), reduced the risk of listeriosis by 4.6 fold ( $-0.66$  log) in the general population, and almost the same amount in the vulnerable population.

#### 4. Discussion

This QMRA assumed that *L. monocytogenes* contamination of semi-hard cheese arose solely from the raw milk itself, and cross-contamination during cheese making or subsequent steps were not considered. Although studies in Brazil pointed out that contamination of raw milk with *L. monocytogenes* is quite low (Costa Sobrinho et al., 2012; Nero et al., 2008; Silva et al., 2003), raw milk can serve as a primary vehicle for transmission of this pathogen. *L. monocytogenes* is widespread on farms, and milk contamination can come from environmental sources such as feces and soil and may occur during milking, storage or transport. Sanaa, Poutrel, Menard, and Serieys (1993) concluded poor quality silage, inadequate animals housing and lack of hygiene during milking were all associated with milk contamination by *L. monocytogenes*. Fox, Hunt, O'Brien, and Jordan (2011) observed that 6.3% of raw milk samples and 12.3% of environmental (silage, bedding and pooled water) samples were contaminated by *L. monocytogenes* on Irish farms. Van Kessel, Karns, Gorski, McCluskey, and Perdue (2004) found that 6.5% of raw milk in US dairy bulk tanks was contaminated by *L. monocytogenes*. Bovine *L. monocytogenes* mastitis may also represent another important source of contamination, as milk produced by cows with mastitis may contain up to  $10^6$  *L. monocytogenes* cells/mL (Sanaa et al., 1993). Our choice of a high *L. monocytogenes* starting concentration for artisanal semi-hard cheese produced with raw milk is consistent with this mastitis caused contamination.

We assumed fresh soft cheese was produced using pasteurized milk, as required by Brazilian legislation, so any contamination by *L. monocytogenes* would arise post-pasteurization. Normal pasteurization conditions can eliminate typical *L. monocytogenes* concentration from raw milk, however, despite this, *L. monocytogenes* can be isolated from fresh soft cheese made from pasteurized milk (Brito et al., 2008; Silva et al., 1998, 2011). Although we made the simplifying assumption that milk was re-contaminated before cheese making, post-pasteurization contamination can occur at different stages during cheese production, as dairy processing plants can harbor *L. monocytogenes* in the environment and equipment (Barancelli et al., 2014; Oxaran et al., 2017; Silva et al., 2003). Brito et al. (2008) recovered *L. monocytogenes* from coolers/refrigeration units, sinks, cheese molds and from the excess liquid found on trays, while Barancelli et al. (2011) found that the main sources of this pathogen were non-food contact surfaces including drains, floors and platforms. Alessandria, Rantsiou, Dolci, and Coccolin (2010) concluded that molecular methods were able to detect more *L. monocytogenes* contaminated samples in dairy plants compared to more traditional methods. In some cases, molecular methods detected up to 60% positive samples while traditional methods detected none. This means

that contamination of cheese samples may be higher than reported. We analyzed a variety of different levels of *L. monocytogenes* contamination in fresh soft cheese to consider this fact.

Our simulation results indicate that fresh soft cheese may present a much greater risk of listeriosis compared to semi-hard cheese, even when the latter is produced with raw milk. Intrinsic characteristics of soft cheeses (Malheiros et al., 2012a) include a high pH (5.0–6.3), high water activity (> 0.97) and percent moisture (55–58%) and low percent salt (1.4–1.6%), all of which contribute to the possibility of *L. monocytogenes* growth. While favorable intrinsic properties of fresh soft cheese contribute to *L. monocytogenes* risk, the fact that some non-inspected producers of this type of cheese may still use raw milk (in violation of Brazilian legislation – BRAZIL, 1997), further increases risks from these cheeses. Although a low frequency of *L. monocytogenes* contamination is observed in artisanal and non-inspected Minas fresh soft cheese, Peresi et al. (2001) and Vinha (2009) have reported that 76.7% and 95% of cheese samples were not in compliance with Brazilian microbiological standards (ANVISA, 2001). Vinha (2009) also isolated *L. monocytogenes* at the production plant of a non-inspected agribusiness. Our simulation results showed that even when higher contamination levels of *L. monocytogenes* are assumed in fresh soft cheese, the application of LAB with anti-listerial activity may help to reduce the risk of listeriosis.

The intrinsic properties of ripened semi-hard cheese, like pH reduction due to lactic acid production, increase of sodium chloride concentration and decrease of water activity, associated to the interaction with natural and/or intentionally added LAB (which can produce antimicrobial compounds such as hydrogen peroxide and bacteriocins) all work to reduce *L. monocytogenes* risk. This is true despite the ability of this pathogen to survive over a wide pH (4.0–9.5) and temperature (1–45 °C) ranges, and down to water activities as low as 0.92 (Melo, Andrew, & Faleiro, 2015). Pinto et al. (2009) investigated the survival of *L. innocua* during a 60-day ripening of Serro cheese (a type of Minas semi-hard cheese) in the presence of natural microflora and observed reductions of 1.66, 1.51 and 1.07 log CFU/g for initial concentrations of 3, 2 and 1 log CFU/mL of milk, respectively. High initial concentrations of *L. monocytogenes* may not be controlled by the presence of natural microflora, so the additional LAB with known anti-listerial activity or the use of other antimicrobial components may be necessary. Campagnollo et al. (2018) concluded that natural microflora could not adequately control high concentrations of *L. monocytogenes* (6 log CFU/mL) during a 22-day ripening of semi-hard cheese, but when LAB with anti-listerial capacity was added, *L. monocytogenes* concentration was reduced by 4 log CFU/g in pasteurized milk cheese and > 5.8 log CFU/g (below the limit of detection) in raw milk cheese.

We assumed that the serving size was similar between general and vulnerable populations and between both cheese types. We believe this assumption is reasonable for fresh soft cheese since has a mild taste and is widely consumed across Brazil. This assumption may be more suspect in the case of artisanal semi-hard cheese because it is more expensive, has a stronger characteristic flavor, and its distribution is not as widespread. We also suspect that vulnerable population (e.g., newborns, elderly and the hospitalized) may not consume semi-hard cheese at all or may do so only to a limited degree. Using a triangular distribution to describe the variability and uncertainty regarding cheese serving size may reduce the estimated risk of listeriosis for the general population and increase the estimated risks for vulnerable populations. Finally, not all individuals in a given population are equally susceptible, which may result in illnesses of different severity (Lammerding & Fazil, 2000), which is not considered in our analysis.

Numerous factors may influence the results obtained in a QMRA, including the choice of distributions used, the availability of specific data regarding some aspects of the process (e.g. fraction of *L. monocytogenes* entrapped in the curd, cheese yield and weight), distribution and storage (e.g. *L. monocytogenes* growth/inactivation rates) and consumption (e.g. serving size and dose-response model). Probability

distributions were selected based on fits of empirical data or derived from expert opinion if no information was accessible. Several studies have evaluated the fate of *L. monocytogenes* in Minas fresh soft cheese (Campagnollo et al., 2018; Malheiros et al., 2012b; Naldini et al., 2009; Nascimento et al., 2008; Pimentel-Filho, Mantovani, Carvalho, Dias, & Vanetti, 2014; Pingitore et al., 2012), but only one analyzed in Minas semi-hard cheese (Campagnollo et al., 2018), which may influence the results obtained. If more detailed information was available for artisanal semi-hard cheese, QMRA results could be modified and lead to improved (or at least different) decisions made by risk managers.

Different studies have estimated the risk of listeriosis due to consumption of cheese in different regions around the world; however, ours is the first study to estimate the risk of infection caused by *L. monocytogenes* due to consumption of Minas traditional cheeses using QMRA. Condoleo et al. (2017) used the same dose-response model to calculate the risk of listeriosis per contaminated serving of semi-soft cheese made from raw sheep's milk and observed values of  $3.53 \times 10^{-10}$  for healthy adults and  $1.58 \times 10^{-8}$  for the vulnerable population. These authors also reported that 56% of *L. monocytogenes* contaminated servings would exceed 1 log CFU/g, 15.1% would exceed 2 log CFU/g and 5.2% would exceed 3 log CFU/g. Bemrah, Sanaa, Cassin, Griffiths, and Cerf (1998) estimated that the median risk of listeriosis with the consumption of soft cheese made from raw milk was  $1.86 \times 10^{-8}$  for high-risk population and  $9.74 \times 10^{-13}$  for low-risk healthy population, and the probability of consuming a contaminated cheese serving was 65.3%. Soto-Beltran et al. (2013) performed a QMRA of *L. monocytogenes* in queso fresco in Culiacan, Mexico and observed that the average probability of illness with the consumption of one cheese serving was  $9.03 \times 10^{-9}$  for the healthy population and  $1.72 \times 10^{-4}$  for the immunocompromised and elderly population. All these studies found a lower risk of listeriosis compared to the estimates of our study; however, care must be taken in making such comparisons. Each study evaluated different types of cheese consumed in different places and used different assumptions that could have a strong influence on the results. Since the purpose of our study was to compare different risk mitigation measures, we evaluated very high starting concentrations of *L. monocytogenes* in raw milk, and we assumed 100% prevalence. If more realistic values for prevalence and starting concentration of *L. monocytogenes* in raw milk were used or if we had realistic values for prevalence and concentration of *L. monocytogenes* in semi-hard cheese or fresh soft cheese, the results of our analysis would be much different.

## 5. Conclusion

The results of the QMRAs predicted that consumption of contaminated Minas traditional cheeses can present a high risk of listeriosis, especially for vulnerable populations given the assumptions regarding starting prevalence and concentration mentioned above. The risk assessments support the idea that refrigerated fresh soft cheese presents higher risk of listeriosis compared to ripened semi-hard cheese and methods to reduce the starting concentration of *L. monocytogenes* and either control its growth or enhance its inactivation (e.g. use of anti-listerial LAB) will reduce risk. While we did not evaluate improved sanitation practices or regular inspections in order to reduce informal production, if these reduce the starting concentration of *L. monocytogenes* in these products they should also reduce risk. Scenario analyses indicated that aging of semi-hard cheese and inclusion of LAB with anti-listerial activity in semi-hard cheese and fresh soft cheese are effective risk mitigation measures. This is the first attempt to estimate the risk of listeriosis due to consumption of artisanal semi-hard cheese produced with raw milk and fresh soft cheese made from pasteurized milk, and we hope these models will help Brazilian risk managers improve food safety and facilitate communication regarding risk management with Brazilian health authorities. Future research findings may change and improve the results of the QMRAs, especially the

availability of more data regarding the fate of *L. monocytogenes* during ripening of semi-hard cheese, refrigerated shelf-life and distribution of fresh soft cheese and consumption of both types of cheese.

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