Rev. Bras. Saúde Prod. Anim., Salvador, v.18, n.4, p.549-559 out./dez., 2017 ISSN 1519 9940
http://mc04.manuscriptcentral.com/rbspa-scielo http://dx.doi.org/10.1590/S1519-99402017000400006

Microbiota autóctone lática, mesófila lipolítica e proteolítica em queijo colonial maturado produzido em diferentes épocas do ano

Microbiota indigenous milk, mesophilic lipolytic and proteolytic colonial cheese matured, produced at different times of the year

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SUMMARY

Cheese is the oldest form of preserving milk nutrients having nutritional, economic and cultural importance. The objective of this study was to identify the best time of the year for production, and period, in months, for maturation of traditional colonial cheese, through analysis of water activity, weight loss and counts of lactic acid, mesophilic microorganisms—proteolytic and lipolytic. Records of temperature and relative humidity (RH) were maintained. A completely randomized experimental design was used in a double factorial scheme, considering production periods and maturation times. For all production periods evaluated, there was a significant reduction in the periods for water activity values. The counts of lactic acid bacteria ranged from 10^4 to 10^9 CFU/g. There was also stability in the number of colonies for lipolytic mesophilic microorganisms, until the third month of maturation. Low counts of proteolytic mesophiles were observed for the samples produced in May and June (5.70 and 5.53 log), respectively. The production period for the months of May and June corresponding to RH of 80% and average temperatures of 15ºC were the most effective for production. Due to the presence of *Listeria,* it is recommended to respect the minimum time of 60 days of maturation for commercialization.

Keywords: dairy products, microbiology, pathogens

RESUMO

O queijo é a forma mais antiga de se conservar os nutrientes do leite possuindo importância nutricional, econômica e cultural. Objetivou-se neste estudo identificar qual a melhor época do ano para produção, e período, em meses, para maturação de queijo colonial tradicional, por meio de análise da atividade da água (AW), perda de peso e contagens de micro-organismos ácido láticos, mesófilos proteolíticos e lipolíticos. Foram mantidos os registros de temperatura e umidade relativa do ar. Foi usado delineamento experimental inteiramente casualizado em esquema fatorial duplo, considerando períodos de produção e tempos de maturação. Para todos os períodos de produção avaliados, houve uma redução significativa nos períodos para os valores de AW. As contagens de bactérias ácido-láticas oscilaram entre 10^4 a 10^9UFC/g. Houve estabilidade também no número de colônias para micro-organismos mesófilos lipolíticos, até terceiro mês de maturação. Foram constatadas baixas contagens de mesófilos proteolíticos para as amostras produzidas em maio e junho (5,70 e 5,53log), respectivamente. O período de produção referente ao mês de maio e junho correspondentes a UR de 80% e temperaturas médias de 15ºC foram os mais efetivos para produção. Em função da presença de *Listeria* recomenda-se respeitar o tempo mínimo de 60 dias de maturação para a comercialização.

Palavras-chave: derivados lácteos, microbiologia, patógenos alimentares

INTRODUCTION

Currently, artisanal cheeses have been the object of research for their economic and social importance in the regions where they are produced (MARTINS et al., 2013). Rezende et al. (2004) found that production, on an industrial scale, did not replace artisanal cheeses in the market.

Colonial cheese can be submitted to different maturation times (LAW & TAMINE, 2010). Maturation is the stage of production where bacterial enzymes slowly degrade the major components of milk, producing substances that give cheese its characteristic structure (PERRY, 2004). Enzymes are catalysts par excellence, directly influencing the speed of cheese maturation (JAY et al., 2005; COELHO et al., 2008).

The extent and type of maturation depends on temperature, maturation time, cheese composition, mainly moisture and salt contents, enzyme activities of the microorganisms present in milk or added (starter cultures), which hydrolyze protein and fat (COELHO et al., 2008) important in the development of flavor.

From the biochemical point of view, cheeses' microbiota can be divided into two groups: lactic acid starter bacteria (LAB) and secondary microorganisms. The former are responsible for the transformation of lactose into lactic acid and also act on maturation, being involved in the proteolysis and conversion of amino acids into volatile substances responsible for sensorial characteristics. They have a fast growth and can deteriorate the milk by acidification (BERESFORD et al., 2001).

The microbial flora characteristic of the different Brazilian artisanal cheeses, in general, is not known. There is no methodology that allows the differentiations of authenticity and origins for artisanal cheeses, which, consequently, do not have uniformity, and often present defects that could be avoided, if there was a greater control over the manufacturing practices of the same ones (NUCH, 2004).

The temperature factors (ºC) and RH have a direct influence on the microbial growth curves. In spite of negative effects, seasonality also has favorable factors of production, mainly of artisanal foods, acting as the main characterization factor of the product, being able to offer different products to the consumer market, starting from the same location and production process.

The objective of this study was to identify the best time of the year for the production and maturation time of traditional colonial cheese by analyzing water activity, weight loss and microbiological counts.

MATERIAL AND METHODS

The experiment was conducted in the municipality of Pinhão-PR, and the cheeses were produced in artisanal dairies for local trade. Ten female bovines of the Dutch breed were used, between second and fifth deliveries. The milk used in the production of cheese was milked in the morning, individually, making it possible to obtain 11 liters per animal, in order to reduce animal effect on cheese formulation. For three days of each of the five productive periods, 110 liters were coagulated raw immediately after

milking at 32°C with addition of liquid coagulant (HA-LA®).
After 50-60 minutes, the clot was cut with the help of lyre (AISI 304), the cubes having a mean diameter of 10x10 mm, with agitation and subsequent resting of the mass, were drained and shaped into Janda Plast brand (model RH-1000, with a capacity of 0.8-1.2 kg).
On the day of production, the cheeses were pressed manually until maximum serum removal and salting. The salting was done dry, adding NaCl equivalent to 2% of the weight of the cheeses. On the morning of the third day, they were washed with water at 50 ºC, packed in plastic crates, randomly distributed on the wooden shelves for drying, where they remained for 45 days and were later transferred to the maturation room for up to 100 days and capped with plastic lids to reduce dehydration.
Five productive periods were analyzed, distributed according to the production season in November, December, March, May and June, analyzing 14 samples at each time of the year, with two repetitions for each of the maturation times (months). The averages of temperature and RH were obtained by means of data logger. The percentage values for weight loss were determined from the difference in weight between the date of withdrawal of the press and the maturity of the respective maturations.
The water activity was determined in triplicate, scoring a distance of 0.8cm from the bark, with AquaLab equipment (model 4TE).
Microbiological analyses of lactic acid bacteria, lipolytic and proteolytic mesophilic and the presence of *Listeria spp*. were carried out at the Food Microbiology Laboratory of the Department of Food Engineering of the State University of the Midwest, Guarapuava - Paraná, in accordance with the recommendation and requirements of RDC no. 12 of 2 January 2001 (ANVISA, 2006; 2009). Samples, in duplicates, were weighed and homogenized in 225 ml of 0.1% buffered peptone water.
For the counting of lactic acid bacteria, MRS agar (from Mann, Rogosa and Sharpe) was used; proteolytic mesophilic bacteria, PCA agar (Plate Count Agar) plus 1 % reconstituted skimmed milk (LDR) and for proteolytic mesophiles, surface sowing on added tributyrin base agar was supplemented. Plates were incubated at 35°C/48 hours.
For the search of *Listeria spp.,* 25 g of samples were weighed into 225 ml of LEB broth (enrichment broth); after incubation at 35 °C for 24 hours, they were scored on Listeria-Aloa-Agar Chromogenic agar (Laborclin).
The data were submitted to an analysis of variance (ANOVA), applied Tukey's test, at 5% of significance and later analyzed by the statistical program *Sisvar* (FERREIRA, 2011). It was used as a completely randomized design in a double factorial scheme, considering the periods of production and maturation times.

RESULTS AND DISCUSSION

The mean values obtained for temperature and RH are shown in Table 1. Emphasis is on the relevance of time, temperature and RH control during storage, contributing to the final quality of the cheeses (EMATER-MG, 2011).
The importance of the study of temperature and humidity is due to greater uniformity of the product during the months and also the effects of different times on the cheeses'

microbiota, with respect to the counts of bacteria technological, pathogenic and deteriorating (MAXIMILIANO et al., 2011).

The cheeses lose a certain amount of water during the process of elaboration and maturation, greater losses occurring during the desorption and pressing. For all the samples evaluated over time, significant losses occurred (Table 2). The greatest weight loss in the first month of maturation occurred for cheeses produced in November, although statistically similar to those produced in March and June.

At six months of maturation, the largest loss occurred for the cheeses produced in March, although statistically similar to the others; this fact may be probably due to different temperature averages, 20.5ºC and 16.5 ºC respectively, for November and March (Table 2).

Table 1. Averages of temperature and relative humidity for the production periods and subsequent maturation times

Production	TºC/RH	MT0	MT 1	MT 2	MT 3	MT 4	MT 6
November	Average	19.37	20.65	20.46	20.53	19.14	17.03
	Average	87.48	78.07	85.36	74.36	87.33	79.24
March	Average	17.49	17.45	16.48	13.71	14.08	14.69
	Average	86.50	77.06	80.13	89.26	78.36	73.56
May	Average	15.45	14.35	13.18	14.35	16.11	20.81
	Average	95.09	87.14	85.34	72.13	72.90	73.24
June	Average	10.58	13.11	14.99	16.12	18.43	20.74
	Average	89.94	85.00	70.91	74.12	76.87	79.63

MT 0 to 6= maturation times in months.

Table 2. Percentage weight loss rate of cheeses produced in November, March, May and June for maturation times (months)

	Production Periods			
Maturation Times	November	March	May	June
1	30.3 $^{b\ A}$	27. 2 $^{c\ AB}$	22.5 $^{c\ B}$	23.4 $^{c\ AB}$
2	37.6 $^{ab\ A}$	29.1 $^{bc\ B}$	31.6 $^{b\ AB}$	32.1 $^{b\ AB}$
3	37.2 $^{ab\ A}$	36.7 $^{ab\ A}$	32.5 $^{ab\ A}$	35.1 $^{ab\ A}$
4	38.2 $^{a\ A}$	38.3 $^{bc\ A}$	36.5 $^{ab\ A}$	39.0 $^{a\ A}$
6	40.5 $^{a\ A}$	41.9 $^{a\ A}$	39.3 $^{a\ A}$	39.62 $^{a\ A}$

*Lowercase letters in the column, when equal indicate statistical equality. Capital letters in the line, when equal, indicate statistical equality at the 5% level (Tuckey). CV = 7.12%.

It can be attributed that difference between the cheeses produced in November at high ambient temperature is associated with low levels of RH (78.07%), which due to osmotic difference, justifies a greater dehydration of these cheeses. Differently, the cheeses produced in June, which were exposed to the averages of 13.11ºC and 85% RH, provided an environment unfavorable to dehydration.

Figueiredo et al. (2015), evaluating samples of cheese Minas in the region of Serro MG, verified temperature incidence for November of 25.2 ºC and RH of 90.2% and also verified that there was no interaction between the chemical physical parameters of the samples and the periods studied (January, March, May, June, July, September and November).

Regarding the AW values, there are several factors affecting the AW content of a food, with a close correlation between these values and the environment's equilibrium RH. Food preserved in an environment with RH higher than its AW tends to absorb moisture from the environment, causing increase in its AW. On the other hand, they will lose water if environmental humidity is inferior to their AW, interfering in the multiplication capacity of the microorganisms present (FRANCO & LANDGRAF, 2008).

The salt also has an effect on AW, as it decreases AW due to its low molecular weight and high solubility, and in maturated cheeses without packaging or any other protective film, there is a reduction in moisture loss through evaporation and consequent reduction of AW (BERESFORD et al., 2001). In the present study, the samples remained uncovered for 100 days and were subsequently capped to reduce dehydration.

For Law and Tamine (2010), foods with high AW values (above 0.90) have solubility of diluted nutrients that serve as a substrate for the growth of microorganisms. Based on this principle, all cheeses, except for those produced in November, were maintained with AW higher than 0.9 even after four months of maturation (Table 3), providing a more favorable environment for undesirable microorganisms.

Table 3. Values of water activity of the cheese samples during maturation

	Production Periods			
Maturation Times (months)	November	March	May	June
0	0.996 $^{a\,B}$	0.997 $^{a\,B}$	0.999 $^{a\,A}$	0.993 $^{a\,B}$
1	0.935 $^{b\,C}$	0.955 $^{b\,B}$	0.963 $^{b\,AB}$	0.974 $^{b\,A}$
2	0.897 $^{c\,C}$	0.948 $^{b\,B}$	0.952 $^{bc\,AB}$	0.961 $^{b\,A}$
3	0.872 $^{d\,C}$	0.909 $^{c\,B}$	0.944 $^{cb\,A}$	0.940 $^{c\,A}$
4	0.847 $^{e\,C}$	0.920 $^{c\,B}$	0.935 $^{d\,A}$	0.911 $^{d\,B}$
6	0.860 $^{de\,C}$	0.876 $^{d\,B}$	0.894 $^{e\,A}$	0.889 $^{e\,A}$

*Averages followed by the same letter are statistically the same by the Tukey test at the 5% level. Lowercase letters in the column and uppercase letters in the row. CV = 0.81 %.

For all the production periods evaluated, there was a significant reduction in the periods for AW values ($p < 0.05$), with cheeses produced in November and March, at six months of maturation, with the lowest values of AW, therefore being more stable. Sobral et al. (2013) also found a decrease in AW values in artisanal cheeses produced in different regions during maturation.

For cheeses produced in May and June, the low temperature in the first month of maturation may have delayed the curd desorption process, followed by rapid dehydration and crust. The low average temperatures and higher daily

thermal amplitudes may have retarded enzymatic reactions, determinants of the reduction of water activity (LAW & TAMINE, 2010), being verified in these samples, with higher AW values at six months of maturation.

According to Souza et al. (2003), the most pronounced decrease in AW in summer is probably due to higher evaporation of water and protein hydrolysis during maturation, as a function of higher temperatures (mean 18-26ºC), different from the averages of 4-13ºC in winter.

According to the authors in their study, in the summer cheese dehydration occurred, because the maturation room maintained low RH, confirmed by the significant variation of the moisture of the cheeses between the seasons. At the end of the 60 days of maturation, AW was not limited to controlling the growth of most groups of microorganisms analyzed, and there was no significant variation between the production times, with AW values at 60 days of maturity being 0.93 for summer cheeses and 0.95 for winter cheeses.

Silveira Junior et al. (2012) evaluated samples of colonial cheeses at 30 days of maturation in different municipalities of the state of Paraná, obtained AW values of 0.82 to 0.85 for the four climatic seasons, the latter being favorable for the development of *Staphylococcus aureus*. Similar values of AW were also observed in the present study for the month of November (0.86).

The main characteristics of maturation involve two main organic constituents: proteins and lipids. Lactic bacteria are mainly responsible for the formation of amino acids and small peptides, due to proteolytic enzymes found in these microorganisms (FOX et al., 2000).

The number of lactic acid bacteria was significantly higher at 30 days of maturation when compared to the amounts of curd on the day of production (Table 4). Counts ranged from 10^4 to 10^9 CFU/g.

Table 4. Lactic acid bacteria count (expressed in log) between production periods of November, December, May and June at maturation times 0, 1, 2 and 6 months

	Production Periods			
Maturation Times	November	December	May	June
0	$5.33^{b\,AB}$	4.30^{cB}	5.65^{bA}	5.88^{bA}
1	$6.48^{\,aB}$	8.34^{aA}	9.07^{aA}	8.94^{aA}
2	5.04^{bB}	8.53^{aA}	5.91^{bB}	8.38^{aA}
6	4.56^{bB}	5.78^{bA}	5.72^{bA}	5.57^{bAB}

*Averages followed by the same letter are statistically the same by the Tukey test at the 5% level. Lowercase letters in the column and uppercase letters in the row. Values expressed in log; CV1 = 13.69% (production period); CV2 = 6.05% (maturation time), zero represents the day of production.

In the Portuguese cheese Serra Estrela, Dahl et al. (2000) found that lactic acid bacteria were dominant throughout the maturation period, ranging from 10^7 to 10^9 CFU/g. Lactic bacteria produce various glycolytic, lipolytic and proteolytic enzymes that transform the essential nutrients of milk and cheese into compounds with desirable sensory properties (VILJOEN, 2001).

Lactic bacteria have as products of their metabolism various antimicrobial substances such as: organic acids, hydrogen peroxide, carbon dioxide, diacetyl, acetaldehyde and bacteriocins, which act favorably on the product as

they have a broad spectrum of actions against pathogenic and deteriorating microorganisms (ALEXANDRE et al., 2002). At six months of maturation, there was stability in the counts for all samples produced, except those produced in December (5.78 log), where there was a significant increase in counts compared to the day of production (maturation time 0).

There was also stability in the number of colonies for lipolytic mesophilic microorganisms until the end of the third month of maturation (Table 5). At the sixth maturation month, those obtained in May and June obtained the lowest counts (2.36 and 2.73 log); a fact associated with the temperature variable, since they were periods of temperatures below average.

Table 5. Counting of lipolytic mesophilic bacteria (log values) between periods

	Production Periods			
Maturation Time (months)	November	March	May	June
0	4.54	NC	5.15	5.20
1	2.80^{bC}	6.33^{aA}	5.42^{aAB}	4.06^{abBC}
2	3.34^{bB}	4.18^{bcAB}	6.15^{aA}	4.98^{aA}
3	4.24^{abA}	6.62^{aA}	5.77^{aAB}	4.86^{aAB}
4	5.68^{abA}	3.56^{cAB}	2.61^{bB}	3.61^{abAB}
6	3.15^{bA}	5.74^{abA}	2.36^{bB}	2.73^{bB}

*Averages followed by the same letter are statistically the same by the Tukey test at the 5% level. Lowercase letters in the column and uppercase letters in the row. Values expressed in log; CV1 = 34.03% (production period); CV2 = 23.17% (maturation time). NC = not counted. Zero represents the day of production.

According to Furtado (2005), the deterioration caused by lipolytic bacteria is aggravated by long periods of exposure to temperatures of 5 to 10ºC, and the slower the refrigeration process the greater the deterioration.

Souza et al. (2003) evaluating Serrano cheeses produced in summer and winter and later submitted to two months of maturation, did not verify significant variation for counts of lipolytic bacteria. Counts 5.18 (summer) and 6.15 log (winter) were quantified in the curd with one day. According to Furtado (2005), the deterioration caused by lipolytic bacteria is aggravated by long periods of exposure to temperatures of 5 to 10ºC, and the slower the refrigeration process the greater the deterioration.

The proteolytic mesophilic bacteria are characterized by a strong proteolytic activity and the microorganisms that may be responsible for the late stunting belong to the genus *Clostridium* (LAFARGE et al., 2004).

The low counts of this microbial group are desirable, a fact found for the samples produced in May and June (5.70 and 5.53log) (Table 6).

The proteolytic microorganisms cause changes in the aroma, taste and physicochemical characteristics of milk and its derivatives. Although typically proteolytic microorganisms are undesirable, certain lactic acid bacteria have proteolytic activity, which is very important in the maturation of cheeses. Proteolytic bacteria include *Pseudomonas*, *Achromobacter*, *Flavobacterium* and *Bacillus* (PERRY, 2004).

Also according to Perry (2004), associated with proteolytic

microorganisms and generally as undesirable as they are, the lipolytic ones are responsible for the breakdown of the fatty fraction and that entails rancidity as the main problem. The majority of these microorganisms have characteristics of psychotrophic bacteria. Among the lipolytic microorganisms are bacteria such as *Pseudomonas* and *Alcaligenes*.

Table 6. Count of proteolytic mesophilic bacteria (log values) between periods of production

Production Periods			
Maturation time (months)	March	May	June
0	NC	4.36 ^{c}	5.64 ^{c}
1	6.26 ^{bcB}	6.69 ^{aB}	8.89 ^{aAB}
2	6.10^{cC}	7.41 ^{aB}	8.59 ^{aA}
3	7.58 ^{aA}	6.85 ^{aA}	7.23 ^{bA}
6	7.11 ^{abA}	5.70^{bB}	5.53 ^{cB}

*Averages followed by the same letter are statistically the same by the Tukey test at the 5% level. Lowercase letters in the column and uppercase letters in the line. Values expressed in log; CV1 = 10.08% (production period); CV2 = 7.07% (maturation time). NC = Not counted. Zero represents the day of production.

According to Souza et al. (2003), the higher counts of proteolytic bacteria than lipolytic bacteria can be attributed to the fact that proteolytic activity is higher than lipolytic activity in Serano cheese, which according to the authors is common in this type of cheese maturation period. The present study also found higher values for proteolytic bacteria.

The production of cheese with raw milk requires control over pathogens and other undesirable microorganisms in animals, in milk, during milking, storage and transport (ICMSF, 1996).

Regarding *Listeria*, the presence of this microorganism indicates an environmental contamination (SCHOCKEN-ITURRINO et al., 2005; RIBEIRO et al., 2006), which is very frequent in silages. Of the 10 samples evaluated at 30 days of maturation, three were positive for *Listeria*. Normative Instruction nº57 MAPA allowed the reduction of the maturation period for artisanal cheeses (less than 60 days), when technical-scientific studies proved that there will be no compromise of quality and safety (BRASIL, 2011). According to these results, the cheeses evaluated are suitable for consumption because they do not offer a risk of exposure to listeriosis at 60 days of maturation, since they were evaluated at 60 days and there was no microorganism.

As the presence of *Listeria spp*. was identified in a sample of cheese from the period where the mean ambient temperatures were higher and also in two samples of cheeses produced in winter, the climatic conditions cannot be considered responsible for such occurrence. However, according to Zaffari et al. (2007), in general, the seasons of the year influenced the isolation of *Listeria spp*., being the spring with the largest number of isolates and the summer with the lowest, confirming that sites or times with milder temperatures are more

propitious for multiplication or survival of this genus.

According to Jay et al. (2005) the optimum growth temperature of *Listeria* is 30 to 37°C. Based on this characteristic, it can be deduced that other factors such as water activity and longer time for dehydration can maintain the most favorable medium for its development, in order to compensate for the adversity of lower temperatures. In milder temperatures (10 to 25ºC) such microorganisms present mobility, causing the dispersion of the cells, in a facilitated way in humid environments that following the elevation to ideal temperature has its contaminating effect potentiated.

For cheeses, low temperatures favor the selection of deteriorating microorganisms, disadvantage the desirable acidophilic lactic acid bacteria, and reduce the physical effect of reducing water activity by dehydration (FURTADO, 2005). For Martins et al. (2013), artisanal cheeses should not be stored for longer than three months, due to enzymatic reactions and decreased humidity.

For the months studied, the cheeses produced in May/June with temperatures of 15ºC, RH of 80% and matured for six months presented better results, although they had higher counts of lipolytic bacteria. Due to the presence of *Listeria,* it is recommended to respect the minimum time of 60 days of maturation for commercialization.

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Data de recebimento: 02/06/2016
Data de aprovação: 17/10/2017