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ANALYSIS OF TOTAL, THERMOTOLERANT AND *Salmonella* spp. IN ARTISANAL CHEESES WITH THE ARTE SEAL SOLD IN RIBEIRÃO PRETO – SP

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Abstract: Artisanal cheese is seen as an important source of income and is sold throughout the country. It is a handmade product with raw milk, therefore, a vehicle for microorganisms of food origin. However, it was only in June 2018 that Law 13,680 was published, which created the Art Seal for products of animal origin produced by hand to be marketed nationally.

INTRODUCTION

The milk production chain is one of the main economic activities in Brazil that stand out with a strong effect on job and income generation. In 2019, the gross value of primary milk production reached almost BRL 35 billion, the seventh highest among national agricultural products (BRAZIL, 2019a). Brazil is the third largest milk producer in the world, second only to the United States and India, according to data from the Food and Agriculture Organization of the United Nations (FAO, 2019). Cheese, as it is a means of preserving milk, a food known worldwide for its high nutritional value, under inadequate conditions for obtaining and storing that do not respect its natural qualities and properties, is capable of transmitting numerous microorganisms (FERREIRA et al, 2011).

In order to avoid contamination during the cheese manufacturing process, care must be taken to obtain milk in a hygienic way, pasteurization, adoption of Good Manufacturing Practices (GMP) and adequate refrigeration until the final consumer (SERIDAN et al. al., 2009).

From the need to the taste of its consumers, cheese has acquired, throughout its historical process, a wide typological range, much of it artisanal in character, maintained by the tradition that seeks to preserve, exactly, as a regional identity and, also, catering to the taste of food. Currently, people have handicraft products were sought and food with traditional, cultural or regional characteristics was valued. For this market, there is a need for quality assurance, with the assurance that production is artisanal and, at the same time, respects good agricultural and sanitary manufacturing practices (MENESES, 2006).

Seeking to value and regulate artisanal cheese, Law No. 13,680 of Selo Arte was approved on June 14, 2018, which modified legislation from 1950, which dealt with the industrial and sanitary inspection of products of animal origin. With the change, the interstate commercialization of food products produced in an artisanal way is allowed, with characteristics and traditional or regional methods, following good agricultural and manufacturing practices and, subject to inspection by public health agencies of the states and the Federal District. (CNA, 2019).

In 2019, Decree Nº 9.918, of July 18, 2019, was published, regulating the Selo Arte, aiming to contribute to increasing the quality of artisanal cheeses. The Ministry of Agriculture, Livestock and Supply – MAPA proposed Law No. 13,860, of 07/18/2019, which provides for the preparation and commercialization of these products and other measures (BRAZIL, 2019b).

Subsequently, Normative Instruction No. 28, of 07/23/2019, was published, which instituted the Art Seal Construction and Application Manual. In the same year, the guiding documents were established, Normative Instruction No. 67, of 12/10/2019, which establishes the requirements for the States and the Federal District to grant the Art Seal and Normative Instruction No. 73, of 23 /12/2019, which establishes the Technical Regulation of Good Agricultural Practices for rural producers who supply milk for the manufacture of artisanal dairy products (BRAZIL, 2019c).

Since artisanal cheese and honey, so far,

are the only products of animal origin with legislation published in the Official Gazette of the Union, through Law 13,860 of July 18 of 2019, which deals with the elaboration and commercialization of artisanal cheeses and also provides other measures (BRAZIL, 2019b), there is a need to research the microbiological quality of these products available on the market for consumers.

In the production of artisanal cheese, sanitary care is required in all processes, from the selection of raw materials from sanitary controlled animals, through the control of production processes to distribution for consumption. The lack of control in these steps, associated with the characteristics of the product, confer great susceptibility to contamination, mainly microbiological and physical hazards, increasing the risk of occurrence of Foodborne Diseases - DTA (OPAS, 2009). The evaluation of the microbiological quality of food is essential to find out if the product was produced following the necessary hygiene standards, because microbiological contamination poses risks to the health of the consumer, causing various pathologies. This contamination can happen during the various stages of the production process, in addition to the process of obtaining the raw material or even in the stage of use by the consumer (LIMA et al., 2015).

The coliform group has a wide variety of terms and genera. In Resolution nº 518/2004, the Health Surveillance Agency subdivided the group into total coliforms and thermotolerant coliforms. Thermotolerants differ from the total ones due to their ability to ferment lactose at 44.5 ± 0.2 ºC in 24 hours, with Escherichia coli as the main representative, considered the most accurate indicator of fecal contamination of exclusively fecal origin (RECHE; PITTOL; FIUZA, 2010; BRAZIL, 2004).

The presence of coliforms in cheese is directly related to the quality of milk, and the presence of microorganisms causes changes in milk, including fat degradation. In addition, it makes the food unfit for consumption, being able to carry diseases, and being possible contamination at any stage of the artisanal cheese manufacturing process (OLIVER et al., 2008). The group of total coliforms is a subgroup of the family Enterobacteriaceae, Gram Negative bacilli, aerobic or facultative anaerobes, which do not produce spores. In the second edition of Bergey's Manuals of Systematic Bacteriology (BRENNER; FARMER III, 2005), 44 genera and 176 species were included. In this group, there are only enterobacteria capable of fermenting lactose with gas and/or acid production in a period of 24-48 hours at 35oC in culture media.

More than 20 species fit this definition, among which are both bacteria originating from the gastrointestinal tract of humans and other warm-blooded animals (Escherichia coli), as well as non-enteric bacteria (species of Citrobacter, Enterobacter, klebisiella and Serratia, among others). These characteristics are used in traditional methods of counting total coliforms (APHA, 2015).

Thermotolerant coliforms are classified as a subgroup of total coliforms that provide concrete information about contamination and hygienic conditions of the product and better indication of the possible presence of enterobacteria originating in the intestinal tract, such as E. coli (SILVA, 2007). In the past, this definition included only enterobacteria of fecal origin (E. coli), but it is now known that this group includes members of non-fecal origin such as strains of Klebsiella pneumoniae, Pantoea agglomerans, Enterobacter cloacae and Citrobacter freundii (SILVA et al., 2017).

Consequently, the presence of thermotolerant coliforms does not necessarily mean that there is fecal contamination. However, the presence of total and/or thermotolerant coliforms in food may indicate

failures in the hygiene of manufacturing processes and post-process contamination in pasteurized foods (SILVA et al., 2017).

Salmonella is a genus of gram-negative, rod-shaped bacteria that belongs to the Enterobacteriaceae family. They are capable of forming acid and, most often, gas from glucose. As they are facultative aerobic microorganisms, they tend to grow with or without the presence of oxygen and can survive at temperatures between 5 and 47 °C, being completely eliminated at cooking temperatures above 70 °C (PUI et al., 2011; POPOFF; LE MINOR, 2015).

For many years, the nomenclature of Salmonella was a subject of debate. In 2005, the international community made this nomenclature official, with the division of the genus into two different species:

S. bongori and S. enterica. In the same year, a new species, S. subterranea, was proposed, but later phylogenetic studies reclassified this species as Atlantibacter subterranea (HATA et al., 2016). S. bongori has no subspecies, only 257 serotypes, while the species S. enterica has six different subspecies: enterica, salamae, arizonae, diarizona, houtenae and indica (TINDALL et al., 2005; LAMAS et al., 2018).

The main route of transmission of salmonella occurs through the food chain, as the bacteria is present in animals raised for commercial purposes. Infections caused by Salmonella spp. they occur through the consumption of contaminated water or food, especially products of animal origin, such as eggs, milk, meat or even vegetables that have been irrigated with contaminated water or fertilized with manure containing the pathogen. In addition, poor hygiene in food handling can also carry the bacteria (BRAZIL, 2011; WHO, 2021).

Commonly in dairy products, Salmonella spp. can be transmitted through the consumption of raw milk in particular or insufficient pasteurization of cheeses made with contaminated milk (AHMED; SHIMAMOTO 2014; CANCINO-PADILLA et al., 2017). A Contamination of raw milk is derived from various sources such as the skin or feces of the producing animal, insects, infected udders or even equipment used in milking (OMAR et al., 2018). According to Normative Instruction No. 60, of December 2019 of the National Health Surveillance Agency (BRAZIL, 2019d), microbiological standards for dairy products advocate the absence of any serotype of Salmonella spp. in 25 g of food. Even so, it is not uncommon to see the presence of this bacteria in dairy products sold and consumed in Brazil.

GOALS

To evaluate the microbiological quality of artisanal cheeses with the ARTE Seal sold in the city of Ribeirão Preto - SP. By means of total and thermotolerant coliforms and Salmonella spp.

MATERIALS AND METHODS

For analysis, 16 samples of artisanal cheeses with Selo Arte sold in only one commercial establishment in the city of Ribeirão Preto - SP, produced in the states of Minas Gerais and Santa Catarina were used. The samples were identified (time/day/place), packed in isothermal boxes and sent to the Microbiology Laboratory of Centro Universitário Barão de Mauá. For the preparation of samples and serial dilutions, the recommendations of the Manual of Methods and Microbiological Analysis of Food and Water (SILVA et al., 2021) were followed.

The packages were sanitized, before opening, with cotton soaked in 70% alcohol. Then, close to the Bunsen burner, 25 g of cheese were removed using tweezers and previously sterilized scalpels, transferred to Erlenmeyer flasks containing 225 mL of 0.1%

peptone saline solution, corresponding to a 10-1 dilution.

The 10-2 dilution was achieved by transferring 1 mL of the 10-1 dilution to a tube of assay containing 9 mL of the diluent. Subsequent dilutions were obtained in the same way, until reaching the dilution: 10^{-3}.

DETERMINATION OF THE MOST PROBABLE NUMBER (MPN) OF TOTAL AND THERMOTOLERANT COLIFORMS

The samples were analyzed for MPN of total coliforms and thermotolerant coliforms, following the recommendations of the Manual of Methods and Microbiological Analysis of Food and Water (SILVA et al., 2021) and the protocol of the American Public Health Association (APHA, 2015).

An aliquot of 25 g of cheese was added to 225 mL of 0.1% peptone water (Laborclin), and homogenized for 60 seconds, where a 10-1 dilution was obtained and from this, the other dilutions were obtained in 0.1% peptone water. 1%, which were analyzed. For the presumptive analysis of coliform MPN, 1 mL of dilutions 10-1 to 10-3 were taken and inoculated, respectively, in three tubes containing 10 mL of Lauryl Sulfate Tryptose Broth (LST), with inverted Durham tubes, in a series of three tubes. The tubes were incubated in an oven at 37oC for 24 to 48 hours. The positive tubes, which showed turbidity and gas production, were duly separated and analyzed for coliforms at 35oC (total) and coliforms at 45oC (thermotolerant).

For coliforms at 35oC (total), tubes containing 10 mL of Caldo Verde Brilhante Bile 2% (CVBB) were used, with inverted Durham tubes, incubated at 37oC, in a bacteriological oven for 24 to 48 hours, tubes with turbidity of the medium and gas production in the Durham tubes, in a maximum period of 48 hours.

For coliforms at 45oC (thermotolerant) tubes containing 10 mL of E. coli Broth (EC) were used, with inverted Durham tubes, incubated at 45oC in a water bath, for 24 to 48 hours, those with turbidity of the coliform being considered positive. medium and gas formation in the inverted Durham Tubes, in a maximum period of 48 hours.

Finally, for the quantification of the analysis,

positive tubes of CVBB broth and EC broth were taken, the presence of turbidity in the culture medium and gas production were checked, using the Most Probable Number table for counting coliforms at 35oC and 45oC per gram of analyzed cheese.

RESEARCH OF *Salmonella* spp

The samples were analyzed for the presence or absence of Salmonella spp, following the recommendations of the Manual of Methods and Microbiological Analysis of Food and Water (SILVA et al, 2021) and the protocol of American Public Health Association (APHA, 2015).

An aliquot of 25 g of cheese was added to 225 mL of 0.1% peptone water (Laborclin®), and homogenized for 60 seconds, obtaining a 10-1 dilution. With that, the flask containing the dilution was taken to the oven at 36±1 ºC for 24 hours, characterizing the first stage of the methodology called pre-enrichment. After 24 hours, the stage for selective enrichment began, where the samples were removed from the oven and the flask was homogenized by 60 seconds. The tubes were duly identified with the selective broths Rappaport Vassiliadis (RV) and Selenito-Cistine (SC), where both contained 10 mL of the respective culture medium. Then, with the aid of a graduated pipette, an aliquot of 0.1 mL was transferred from the pre-enrichment flask to the RV tube and 1 mL to the SC tube, both then taken to an oven at 36 ± 1 ºC for 24 hours. Subsequently,

the RV and SC tubes were removed from the oven and homogenized, and the selective isolation stage began. With the aid of a loop, an aliquot was transferred from the RV tube and inoculated on the surface of the plate containing Modified Brilliant Green Agar (BPLS), streaking in order to obtain isolated colonies, repeating the same procedure with the SC tube. Then another loop of the RV tube was transferred and inoculated on the surface of the plate containing Xylose, Lysine, Deoxycholate Agar (XLD) streaking in order to obtain isolated colonies, repeating the procedure with the SC tube, and incubating the plates at 35± 1oC for 24 hours.

After 24 hours, the plates were removed from the oven and checked for the growth of suspected colonies (black colonies and/or pink colonies with a black center, smooth and rounded with regular edges) of Salmonella spp. With the aid of a needle, they were removed from 5 to 7 colonies (selected separately) from the BPLS plates, and seeded in pits and grooves on the ramp in a tube containing "Triple Sugar Iron Agar" (TSI) agars, the same procedure being performed in a tube containing "Lisine Iron Agar" (LIA). Then, the same procedure was performed with the XLD Agar plate, also sowing in tubes containing TSI and LIA. After sowing, all tubes were incubated for 24 hours at 35ºC ±1.

Finally, after 24 hours, the tubes were removed from the oven for reading. According to the literature used, they are considered positive for Salmonella spp. the tubes with growth in TSI that present a yellowish base due to the production of acid, blackening at the site of the bite due to the production of H2S (Hydrogen Sulfide) and apex without color change. In LIA, tubes with Salmonella spp. present with the color of the apex and base unchanged due to the non-production of acid and blackening of the bite site due to the production of H2S (SILVA *et al*., 2021).

RESULTS AND DISCUSSION

In this study, in July and August 2021, 16 samples of artisanal cheeses with the ARTE Seal sold in Ribeirão Preto - SP were analyzed. With a view to determining the MPN/g for total and thermotolerant coliforms, it can be seen in Table 1 that the values range from < 3.0 to > 1,100 MPN/g, for both groups of coliforms

Samples	Total coliforms * NMP/g	Thermotolerant coliforms * NMP/g
1	< 3,0	< 3,0
2	72	< 3,0
3	> 1.100	> 1.100
4	240	240
5	> 1.100	6
6	> 1.100	> 1.100
7	23	< 3
8	23	< 3
9	9,2	< 3
10	23	< 3
11	9,2	< 3
12	460	< 3
13	< 3,0	< 3
14	460	< 3
15	460	9,4
16	23	< 3

* The results were analyzed using the Most Probable Number table (APHA:8, 2015).

Table 1 - Determination of the Most Probable Number per gram (NMP/g) for total and thermotolerant coliforms in samples of cheeses with the ARTE Seal sold in the city of Ribeirão Preto - SP, in July and August 2021

Source: prepared by the author (2021).

Based on the data obtained, the presence of total and thermotolerant coliforms was detected in all samples of the analyzed cheeses, however artisanal cheeses with the ARTE Seal still do not have a specific technical standard established on quality and identity. To this

end, the Ministry of Agriculture, Livestock and Food Supply, through Normative Instruction IN n0 60, of 2019, established as a microbiological standard for medium-humidity cheeses, in which artisanal cheeses with the ARTE Seal fit, maximum limits only for thermotolerant coliforms, which must be up to 102 CFU/g. Thus, the results of this study obtained by MPN/g were compared to other results obtained in studies published in scientific literature referring to medium-moisture artisanal cheeses and even high-moisture cheeses.

According to ANVISA Resolution RDC No. 12 of January 2, 2001 (BRAZIL, 2001), values are established for each microorganism, with a maximum count limit of 1.0 x 103 MPN/g for total coliforms and 5.0 x 102 MPN/g for thermotolerant coliforms. According to these values, samples 3 and 6 (12.5%) are above the allowed limit, in which the count was >1,000 MPN/g for both analyzes and sample 5 (6.25%) above the limit allowed for counting only total coliforms.

Sá and Bandeira (2020), in microbiological analyzes carried out on artisanal cheeses sold in three municipalities in the Tocantina Region of Maranhão, of the ten samples surveyed, 7 (70%) samples showed the presence of total coliforms and 3 (30%) samples showed the presence of thermotolerant coliforms, ranging from 4 to 240 MPN/g, according to the microbiological standard established by RDC No. 12 of ANVISA.

Mendes et al. (2011) found levels above the legal limit for total and thermotolerant coliforms. Of the six analyzed samples of Minas Artesanal Cheese marketed in Rio Parnaíba - MG, all presented counts greater than 5.0 x 103 MPN/g for total coliforms and four presented counts greater than 5.0 x 102 MPN/g for thermotolerant coliforms.

When evaluating the quality of Minas cheeses Handcrafted commercialized in Montes Claros - MG, Cruz et al. (2010) found that 30% of the analyzed samples showed contamination by coliforms at 45oC, and about 12% had counts higher than the maximum limit established by Brazilian legislation.

In research carried out with 40 samples of Minas Artesanal Cheese produced in the Serro region - MG, Brant, Fonseca and Silva (2007) observed that 32 samples (80%) had total coliform counts greater than 5.0 x 103 MPN/g. The authors state that, even though the total coliform count at 35oC is not required by current legislation for products collected in the market, these microorganisms are generally environmental contaminants, and their high count indicates a deficiency in hygienic-sanitary quality.

Therefore, it is believed that contamination of samples 3, 5 and 6 may have occurred during manipulation, analyzed in the present work. Samples 3 and 6 were the ones that showed values that exceeded the amounts of MPN/g of total coliforms (> 1,100 MPN/g) and thermotolerant (> 1,100 MPN/g), that is, they are in disagreement with the legislation. These results may indicate greater contamination, probably due to a lack of technical guidance on hygiene and Good Manufacturing Practices (GMP) in the preparation, transport or even conservation at the time of marketing, since the cheeses collected were sold in average of 20°C.

Thus, the presence of values higher than those allowed by legislation for total coliforms is indicated as an environmental indicator, whereas thermotolerant ones may indicate contamination of fecal origin, and are used as indicators in all ready-to-eat products from the dairy chain (BELOTI et al., 2015).

With regard to salmonella research, IN n0 60, of 2019, recommends the absence of Salmonella spp. in food for sale and consumption (BRAZIL, 2019d). In the present study, it appears that the pathogen was not

detected in the 16 analyzed cheese samples, that is, they did not offer risks to the health of consumers in terms of the presence of this microorganism.

In microbiological analyzes carried out on artisanal cheeses sold in three municipalities in the Tocantina Region of Maranhão, of the 10 samples (100%) surveyed, 7 samples (70%) showed the presence of salmonella (SÁ; BANDEIRA, 2020).

In a study carried out to verify the microbiological quality control of matured artisanal cheeses sold at the Feira do Pequeno Produtor in the city of Cascavel - PR, of the 5 samples (100%) analyzed, Eckert and Webber (2016) found the presence of salmonella in all samples. In the study by Pinto et al. (2011), the results were satisfactory in the salmonella research carried out in artisanal cheeses and cheeses inspected in the city of Santa Helena - PR, where 100% of the samples showed absence of this microorganism, as occurred in the present work.

In a study by Prattes (2016), Salmonella spp. was positive in 63% of the samples, making the cheese unfeasible for human consumption. High counts of these microorganisms characterize inadequate hygienic-sanitary conditions during product processing and the need to implement safety and quality assurance systems at all stages of production.

Lima et al. (2019), in the analysis to determine the presence or absence of Salmonella spp., pointed to contamination in almost all of the units tested, with 19 samples (95%) showing unsatisfactory results and only 1 (5%) being within the limits.

Standards established for Minas Frescal Cheese.Different results were found by Silva and Belo (2018) in artisanal Minas fresh cheeses, when observing the absence of Salmonella spp. in all research samples. Batistella et al. (2019), when studying the same type of cheese, observed that the 18 analyzed samples were free of salmonella. So, for the same product, quality variation may occur according to good handling practices. And, in this case, as it is an enteric bacterium responsible for serious food infections, the presence of this bacterium in the samples qualifies the cheeses as unfit for consumption (FAVA et al., 2012).

The absence of Salmonella spp. in the samples, in addition to being related to the absence of contamination, can be explained by the fact that the bacteria is not a good competitor, suffering injuries in acidic media or with the presence of other bacteria, such as lactic and coliforms, especially if the initial contamination for a small number of cells. Under these conditions, microorganisms may disappear or remain in undetectable numbers in acidic or highly contaminated foods (BRAZIL, 2003).

Even if there is no contamination by Salmonella spp., the consumer must be made aware that the quality of the product begins with the raw material and needs to be maintained at all stages of the production chain, emphasizing that Good Manufacturing Practices (GMP) are essential in sanitary control in order to prevent contamination (FERNANDEZ; MARICATO, 2010).

CONCLUSION

Based on the results of the microbiological analysis, it was verified that 18.75% of the samples of artisanal cheese with the ARTE Seal analyzed in this study were considered unsuitable for consumption in relation to the coliform count. With a view to the presence of Salmonella spp., its absence was identified in all samples. In view of this, it is believed that in the production of some cheeses, the necessary hygienic-sanitary measures were not adopted, from obtaining raw milk to cheese manufacturing, as well as care may not have been taken in transport and storage at the

establishment. commercial, which reinforces the presence of a veterinarian throughout the production chain. Finally, it is suggested that new research be carried out to verify whether the artisanal cheeses sold with the ARTE Seal meet the quality parameters of current legislation, so that they do not pose risks to the health of the consumer and even serve as specific parameters for production. artisanal cheeses with the aforementioned Seal.

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