

## ***Allium sativum* antimicrobial potential in fresh cheese**

Potencial antimicrobiano de *Allium sativum* em queijos minas frescal

Potencial antimicrobiano de *Allium sativum* em queijos frescos

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### **Abstract**

Cheese consumption has shown an important increase throughout the Brazilian territory, being the second most imported product since 2015. Among the different types of cheese, minas frescal represents one of the most popular, due to its simple processing, low cost and high yield. However, its perishability requires refrigeration and relatively fast consumption, thus representing an important limiting factor in commercialization. Aspects such as having a high moisture content, not undergoing maturation, and containing no preservatives, provide favorable conditions for microbiological contamination. Thus, it was aimed to assess the antimicrobial potential of the aqueous suspension of garlic (SAA), in fresh cheese before and after contamination *Staphylococcus aureus* (ATCC 25923)  $10^5$  CFU mL<sup>-1</sup>. The minimum inhibitory concentration (MIC) was determined by microdilution, and the following treatments were evaluated in quintuplicate: SAA in freeze-dried and fresh forms (1.5; 2.5; 5; and 10%), negative control treatment (cheese absent contamination), and positive (cheese absent from SAA). After superficial inoculation in Agar Baird Parker, enriched with egg yolk and potassium tellurite, the incubation was performed at 35°C for 48 hours. Although SAA in lyophilized and fresh form applied before contamination did not show antibacterial activity, the fresh form applied after contamination showed a MIC equivalent to 2.5%. So, based on the results obtained by the present study, it was possible to conclude that SAA above 2.5% had antimicrobial properties, thus favoring the conservation of fresh cheese.

**Keywords:** Natural food preservatives; Garlic; Medicinal plants; Natural antimicrobial.

## Resumo

O consumo de queijos vem apresentando importante aumento em todo território brasileiro, sendo o segundo produto mais importado desde 2015. Dentre os diversos tipos de queijos, o minas frescal representa um dos mais populares, devido ao seu processamento simples, baixo custo e alto rendimento. Porém, sua perecibilidade exige armazenamento sob refrigeração e consumo relativamente rápido, representando assim importante fator limitador na comercialização. Aspectos como possuir alto teor de umidade, não sofrer maturação, e não conter conservantes, propiciam condições favoráveis à contaminação microbiológica. Dessa forma, objetivou-se avaliar o potencial antimicrobiano da suspensão aquosa de alho (SAa), em queijo minas frescal antes e após contaminação por *Staphylococcus aureus* (ATCC 25923)  $10^5$  UFC mL<sup>-1</sup>. A concentração inibitória mínima (MIC) foi determinada por microdiluição, e os seguintes tratamentos foram avaliados em quintuplicada: SAa nas formas liofilizada e *in natura* (1,5; 2,5; 5; e 10%), tratamento controle negativo (queijo ausente de contaminação), e positivo (queijo ausente de SAa). Após inoculação superficial em meio Agar *Baird Parker*, enriquecido com de gema de ovo e telurito de potássio, a incubação foi realizada a 35°C por 48 horas. Embora a SAa na forma liofilizada e *in natura* aplicada antes da contaminação não tenham apresentado atividade antibacteriana, a forma *in natura* aplicada após contaminação apresentou MIC equivalente a 2,5%. Dessa forma, a partir dos resultados obtidos pelo presente estudo, foi possível concluir que SAa a partir de 2,5%, apresentou propriedade antimicrobiana, podendo favorecer assim a conservação do queijo frescal.

**Palavras-chave:** Conservantes naturais de alimentos; Alho; Plantas medicinais; Antimicrobiano natural.

## Resumen

El consumo de queso ha mostrado un aumento importante en todo el territorio brasileño, siendo el segundo producto más importado desde 2015. Entre los diversos tipos de queso, las minas frescal representa uno de los más populares, por su simple elaboración, bajo costo y alto rendimiento. Sin embargo, su perecibilidad requiere almacenamiento refrigerado y un consumo relativamente rápido, lo que representa un importante factor limitante en la comercialización. Aspectos como tener un alto contenido de humedad, no madurar y no contener conservantes, proporcionan condiciones favorables para la contaminación microbiológica. Así, el objetivo fue evaluar el potencial antimicrobiano de la suspensión acuosa de ajo (SAa), en queso minas frescal antes y después de la contaminación por *Staphylococcus aureus* (ATCC 25923)  $10^5$  UFC mL<sup>-1</sup>. La concentración mínima inhibitoria (CMI) se determinó por microdilución y se evaluaron los siguientes tratamientos por quintuplicado: SAa en forma liofilizada y fresca (1,5; 2,5; 5; y 10%), tratamiento control negativo (queso ausente contaminación) y positivo (queso ausente de SAa). Después de la inoculación superficial en medio Agar *Baird Parker*, enriquecido con yema de huevo y telurito potásico, la incubación se realizó a 35°C durante 48 horas. Aunque el SAa en forma fresca y liofilizada aplicada antes de la contaminación no mostró actividad antibacteriana, la forma fresca aplicada después de la contaminación mostró una CIM equivalente al 2,5%. Así, SAa del 2,5%, presentó propiedades antimicrobianas, favoreciendo así la conservación del queso fresco.

**Palabras clave:** Conservantes alimentarios naturales; Ajo; Plantas medicinales; Antimicrobiano natural.

## 1. Introduction

Cheese consumption has shown an important increase throughout the Brazilian territory, being the second most imported product since 2015. Among the different types of cheese, minas frescal represents one of the most popular, due to its simple processing, low cost and high yield (Gouvea et al., 2017). Nowadays it is highly valued mainly for its nutritional aspects. Its fast digestibility, low fat and sodium content, make the fresh cheese often recommended for restrictive diets. However, its perishability requires storage under refrigeration and relatively fast consumption, thus representing an important limiting factor in commercialization.

Usually fresh cheese is produced using pasteurized milk added to the rennet, resulting in enzymatic coagulation and dough formation. This cheese, because it does not undergo maturation, preserves a high moisture content. Moreover, its marketing *in natura* does not make use of preservatives, compromising the product as to its validity to consumption, which should be fast. Thus, these aspects provide favourable conditions for microbiological contamination (Bastos et al., 2020); which in turn can occur even when preserved and manipulated correctly (Silva et al., 2019). However, factors related to inadequate forms of production and storage of the product at temperatures that favour bacterial growth should be considered (Zocche et al., 2012).

Contamination by microorganisms can determine changes in sensory characteristics (taste and odor), affecting commercialization, besides to being a risk to public health, the possibility of food poisoning and toxification. Thus, cheese that is unfit for consumption is a recurring problem, and these contaminated products continue to be found in commerce.

The presence of microorganisms such as coagulase positive Staphylococci, fecal coliforms, *Salmonella typhimurium*, *Staphylococcus aureus*, and aerobic mesophilic, molds, yeasts, among others, is still often registered (Oliveira et al., 2017; Queiroz et al., 2017; Boas et al., 2020; Camargo et al., 2020; Motta & Farias, 2020). On the other hand, consumers are increasingly aware of food safety, looking for natural, healthy foods that are free of medicinal residues (Sampaio & Junior, 2018). Thus, research on natural preservatives has gained prominence in recent years (Barros et al., 2020; Coutinho et al., 2020; Mendonça et al., 2020; Souza et al., 2020).

Some condiment plants, by also having medicinal properties, may represent important natural alternative to chemical preservatives for food preservation, because even in high concentrations usually do not determine health risk (Freire et al., 2011; Galo et al., 2018; Coutinho et al., 2020). The increase in the use of condiment plants in recent decades, is mainly due to factors such as the appreciation of the use of natural products (Galo et al., 2018), among which *Allium sativum* (Horita, 2016) stands out.

*Allium sativum* L. is a condiment consumed and appreciated worldwide. Popularly known as garlic it is also considered nutraceutical by the National Health Surveillance Agency (ANVISA) - RDC n° 26 of may 13 of 2014 (Brasil, 2014). Its benefit and its wide use are mainly due to the presence of phenolic and organosulfur compounds, responsible for its antioxidant action, aroma and flavor (El-Sayed et al., 2017; Lanzotti et al., 2014). Its antimicrobial activity is mainly associated with the presence of allicin, which possibly acts by the cellular breakdown of microorganisms (Li et al., 2016) and oxidative stress (Rabinkov et al., 1998). The essential oil of garlic showed antibacterial action against *Staphylococcus aureus*, *Escherichia coli*, *Bacillus subtilis* (Guo, 2014), *Staphylococcus choleraesuis* (Coutinho et al., 2020), besides the antifungal action on *Penicillium funiculosum* (Li et al., 2014). Raw garlic inhibited *Helicobacter pylori* (Zardast et al., 2016), and in the form of nanovesicles it was effective against *Listeria monocytogenes* (Pinilla et al., 2017). Thus, the present work aimed to evaluate the antimicrobial activity of *Allium sativum*, as aqueous suspension (SAa) and *in natura* (Ain), on fresh cheeses contaminated with *S. aureus* (ATCC 25923).

## 2. Methodology

### Production of the aqueous suspension of garlic

For the production of the 10% (m / v) *Allium sativum* (SAa) aqueous suspension, 87.5 g of peeled and macerated garlic bulbs were added to 875 mL of ultrapure Milli-Q water. Subsequently, the suspension was ground in a blender for approximately three minutes, filtered on household filter paper, and immediately used for evaluation of the different treatments. The mean pH of the suspension was 5.6 (Rodríguez García & Peraza Echeverría, 2019).

### Preparation of the *Staphylococcus aureus* suspension (ATCC 25923)

For the production of the *S. aureus* solution used in the minimum inhibitory concentration (MIC) test, the bacteria were grown in *Baird Parker* Agar. Subsequently, the colonies were diluted in 0.85% peptone saline to the concentration of 10<sup>8</sup> UFC/mL (McFarland Scale 1) (Garcia, 2010). Subsequently, 10 µL of this solution was added to 990 µL of *Mueller Hinton* broth up to 10<sup>5</sup> CFU/mL.

### Minimum Inhibitory Concentration (MIC)

The determination of the minimum inhibitory concentration (MIC) against *Staphylococcus aureus* (ATCC 25923) was evaluated in triplicate using a microdilution method according to Clinical and Laboratory Standards Institute (CLSI, 2015). For this, polystyrene plates with 96 flats bottom wells were used. 50 µL of Mueller Hinton broth, and 50 µL of SAa were added. This solution was then successively diluted and subsequently 50 µL of the *S. aureus* solution was added.

After incubation in a bacteriological oven for 24 hours at 35°C, the presence of turbidity of the medium and formation of precipitate, corresponding to bacterial growth, were evaluated.

### Preparation of cheeses

The cheeses were produced and inoculated according to the protocols of Soares et al. (2008) and Campos et al. (2019). The milk was pasteurized in a water bath until heating to 65°C and held for 30 minutes, then cooled to 38°C and added with 0.5 mL of calcium chloride per liter along with one ml of rennet per liter. After 50 minutes rest, the mass of the cubes was cut with a cm<sup>3</sup>, homogenizing the mass were performed for five minutes and rest for five minutes. Then the dough was placed in the forms for further cooling at 4°C.

After 24 hours in refrigeration the pieces of cheese were removed from the forms and the surface contamination by immersion was carried out. For this, the bacteria were cultured in 25 mL of Brain Heart Infusion (BHI) liquid medium, incubated at 37°C for 24 hours. The culture was seeded in *Baird Parker* Agar and again incubated at 37°C for 24 hours. Colonies were then removed from the plate and diluted in peptone water, and then compared to the McFarland scale at 1.0x10<sup>8</sup> CFU/mL. Then this solution was used for the contamination of the cheeses, which were immersed in it for approximately two minutes each piece, with the exception of the negative control.

Fresh cheeses were individually made by the addition of 2 liters of pasteurized milk at 125 mL (SAa125), 250 mL (SAa250) and 500 mL (SAa500) from SAa, totaling three pieces of fresh cheese, with SAa concentrations of 0.58% 1.11% and 2%, respectively. The following treatments were evaluated: SAa125, SAa250, SAa500, cheese without SAa that corresponded to the negative control (CN), and cheese added to *S. aureus* ATCC 25923, which corresponded to the control (CT). Three runs were performed in order to obtain three experimental units of each treatment.

For the treatments with garlic *in natura* (Ain), 50 grams of peeled and chopped garlic were added to each liter of milk, totaling three treatments: fresh garlic cheese, garlic-free cheese corresponding to the negative control (CN) and cheese contaminated with *S. aureus* that corresponded to the control (CT).

### Microbiological analyzes

Aliquots of 10 g of each sample (cut into a cuticle) containing 90 mL of 0.9% NaCl solution as the diluent were used. The sample was then transferred to sterile plastic bags made of Nasco WHIRL-PAK® low density polyethylene and homogenized in Stomacher 400 for 60 seconds. From the initial dilution at 10<sup>-1</sup>, a series of decimal dilutions were prepared up to the dilution of 10<sup>-4</sup>, using test tubes with 9.0 mL of 0.9% NaCl solution. From each of the dilutions of the examined cheese samples, 0.1 mL was transferred to *Baird Parker* Agar surface enriched with egg yolk and potassium tellurite in Petri dishes. The inoculum was then spread over the entire surface of the medium with the aid of a Drigalsky loop and the plates incubated at 36°C for 48 hours in a bacteriological oven.

After incubation, we counted the number of colonies that presented characteristics typical of *S. aureus* as a bright black color, with a white precipitation zone around it and surrounded by transparent halo. The counted colonies were multiplied by factor 10, and by the reciprocal of the dilution corresponding to the counting plate, thus obtaining the value of the *S. aureus* count per gram of cheese. The result was expressed in CFU/g.

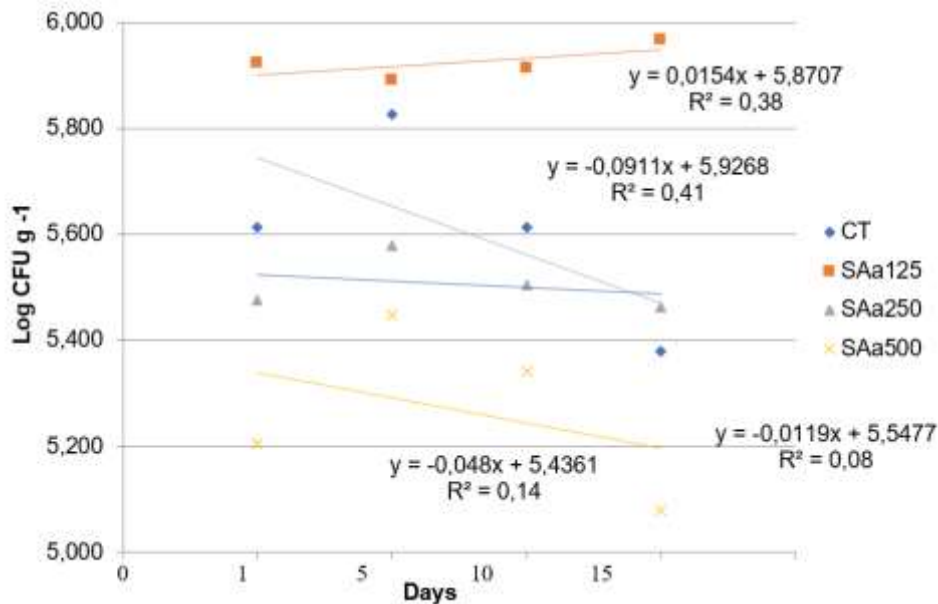
The microbiological analyzes described above were performed one day after cheesemaking (D1) and after days D5, D10 and D15 in order to evaluate the effect of SAa since the expiration date of a fresh cheese usually corresponds to 7 to 10 days after the date of manufacture. At the same time, color, odor, softness and texture characteristics were also evaluated using a descriptive method (Teixeira, 2009).

### 3. Results and Discussion

For the MIC test, the minimum concentration of SAa *in vitro* capable of inhibiting any bacterial growth was 1.25%. SAa demonstrated antimicrobial capacity against *S. aureus*. Saha et al. (2016) evaluated the MIC of aqueous extract of *Allium sativum* against *S. aureus* and *E. coli* observing the concentrations of 400 µg/ mL and 700 µg/mL as MIC, respectively.

For the first cheese processing, treatments SAa250 and SAa500 demonstrated a decrease in the bacterial count of 0.26% and 2.40% respectively (Figure 1). However, the treatment of SAa125 showed an increase of 0.7%.

**Figure 1.** *Staphylococcus aureus* count (log CFU g<sup>-1</sup>) in fresh cheese during 15 days of storage.



Source: Authors.

This increase may be the result of a possible contamination of the SAa125 aqueous solution with *S. aureus* prior to addition to the cheese or of inefficiency of the aqueous suspension at that concentration. For garlic *in natura*, there was a decrease of 4.98% in the bacterial count, which can be verified in Table 1.

**Table 1.** Count of colony forming units in log per gram of cheese analyzed during storage days.

|        | CT    | Ain   |
|--------|-------|-------|
| Day 1  | 4,929 | 5,389 |
| Day 5  | 5,008 | 5,217 |
| Day 10 | 4,770 | 5,120 |

CT: control

Ain: cheese with garlic *in natura* (50g/liter of milk used)

Source: Authors.

Fresh garlic had better results compared to SAa, possibly due to the concentration of alicina, the main antimicrobial component (Corzo-Martínez et al., 2007) that is diluted in aqueous solution.

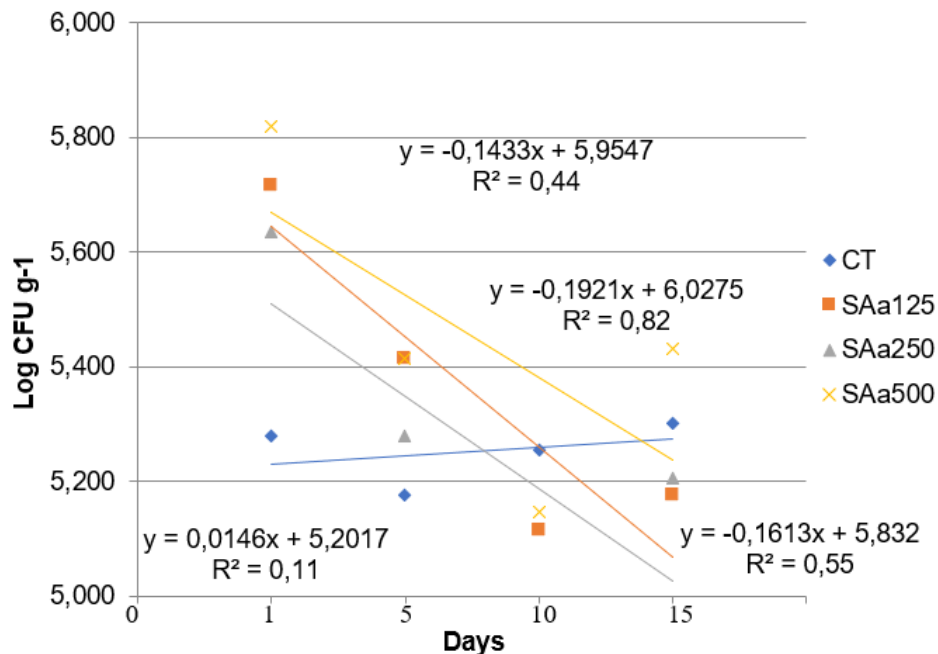
Yadav et al. (2015) found that 50% fresh garlic juice has antibacterial properties against Gram-positive and Gram-negative organisms such as *Escherichia coli*, *Staphylococcus aureus*, *Klebsiella pneumonia*, *Proteus mirabilis* and *Pseudomonas aeruginosa* each of 4-8% with the exception of *P. aeruginosa* which is 4-16% (v/v).

However, Dantas et al. (2018) evaluating the hydroalcoholic and glycolic extract of *A. sativum*, both at 30% and 70%, in the *in natura* and dry forms, did not record *S. aureus* antimicrobial action, evidencing that the solvent choice is important for the desired results. Differently, Alli et al. (2011) and Daka (2011) verified antibacterial action of garlic extract at 12.5% in *S. aureus* clinical isolates.

Deresse (2010) evaluated the antimicrobial activity of the aqueous extract of garlic on 30 clinical isolates of *S. aureus*, checking the concentration of 7.5 mg/mL as MIC. The same work revealed that the activity of the extract is sensitive to heat due to the results presented after autoclaving the extract at a temperature of 121°C for 15 minutes where the antimicrobial capacity was lost, for the material at room temperature or refrigerated, the antimicrobial capacity remained. However, when using fresh garlic this capacity is higher. This may be due to the greater presence of allicin, which totally inhibits RNA synthesis and partially the synthesis of DNA and bacterial proteins (Feldberg et al., 1988).

In the second cheese processing, all treatments were efficient to decrease the bacterial count, decreasing by 9.44%, 7.62% and 6.67% respectively (Figure 2); corroborating with Nejad et al. (2014).

**Figure 2.** *Staphylococcus aureus* count (log CFU g<sup>-1</sup>) in fresh cheese during 15 days of storage.



Source: Authors.

They evaluated the antimicrobial effect of aqueous extract of garlic (500g to 200mL of saline solution) on hamburger with three different amounts of extract: 1mL, 2mL and 3mL, noting that in all amounts during the storage period the extract was able to inhibit *S. aureus* growth. For garlic *in natura*, there was a decrease of 10.01% of the microorganisms, represented in Table 2.



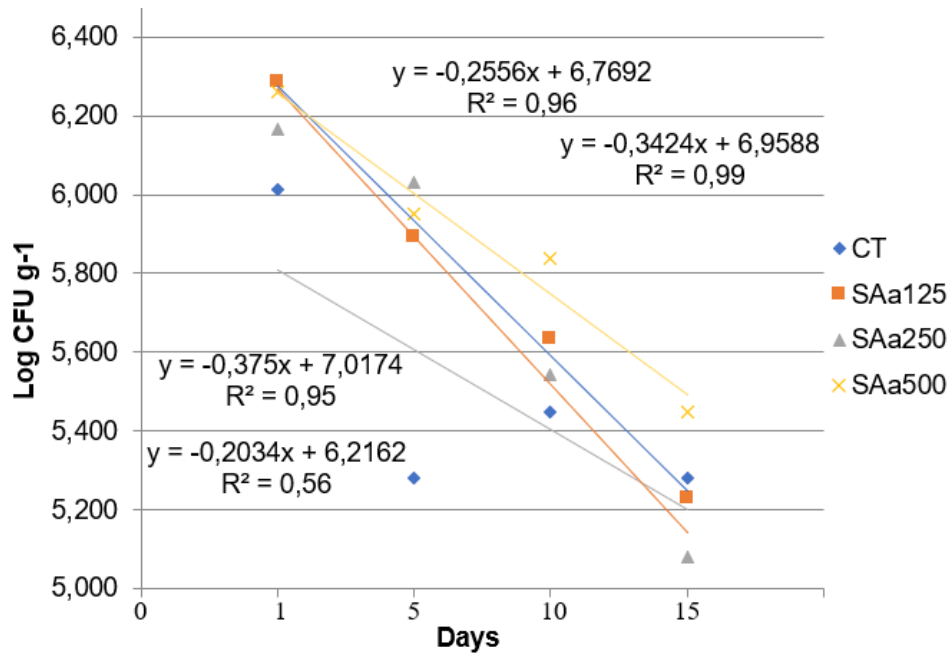
**Table 2.** Count of colony forming units in log per gram of cheese analyzed during storage days.

|        | CT    | Ain   |
|--------|-------|-------|
| Day-1  | 4,995 | 5,181 |
| Day-5  | 4,491 | 4,826 |
| Day-10 | 4,414 | 4,662 |

CT: control  
 Ain: cheese with garlic in natura (50g/liter of milk used)  
 Source: Authors.

In the third cheese processing, the decrease in the bacterial count was 16.78%, 17.64% and 13.01% for the treatments SAa125, SAa250 and SAa500 respectively (Figure 3).

**Figure 3.** *Staphylococcus aureus* count (log CFU g<sup>-1</sup>) in fresh cheese during 15 days of storage.



Source: Authors.

However, it is verified that the control cheese also had a reduction in the microbial count. Because it does not contain aqueous solution of garlic, it can be reported that the storage temperature (less than 5°C) can be a contributing factor for the reduction of the microbial load. For garlic *in natura*, there was a decrease of 3.72% in the bacterial count, and it can be observed in Table 3.

**Table 3.** Count of colony forming units in log per gram of cheese analyzed during storage days.

|        | CT    | Ain   |
|--------|-------|-------|
| Day 1  | 4,770 | 5,045 |
| Day 5  | 4,568 | 4,869 |
| Day 10 | 4,447 | 4,857 |

CT: control

Ain: cheese with garlic in natura (50g/liter of milk used)

Source: Authors.

It is observed that the best treatment is SAa250 in which there was a mean decrease in the *S. aureus* count of 8.48%, followed by the SAa500 treatment in which the mean count decrease was 7.36%.

Shan et al. (2011) evaluated the antimicrobial effects of five hydroalcoholic extracts (cinnamon, oregano, clove, pomegranate peel and grape seed) against *Listeria monocytogenes*, *Staphylococcus aureus* and *Salmonella enterica* in cheddar cheese. The clove extract showed the highest antibacterial and antioxidant activity, showing the potential of the use of spices to favor the stability and conservation of the cheese during the storage period.

The concentration levels of the extracts may vary according to the species of *Allium* sp. Their secondary metabolites produced in the different countries, different ways of processing the raw material, as well as the innocuous concentrations for the test (Deresse, 2010).

The content of lipids and proteins in foods is a factor that affects the efficiency of natural antimicrobial agents, due to the possibility of a physical protection barrier, preventing the contact of the compound with the microorganism, thus reducing the effectiveness of the extract or essential oil (Gutierrez et al., 2008). Cava et al. (2007) verified that the antimicrobial activity of essential oils of clove and cinnamon was impaired by the presence of fat in the milk in comparison to the skim milk, evidencing that it is necessary to study the interaction of oils and extracts with food composition.

Hassanien et al. (2014) demonstrated that, in culture medium, 0.1% cumin oil was able to inhibit the growth of *L. monocytogenes*, *S. aureus*, *S. enteritidis* and *E. coli*. Cheeses at this concentration were not efficient in inhibiting *S. aureus* and *L. monocytogenes*. Emphasizing that the concentrations of extracts and essential oils vary according to the microorganism and its particular sensitivity.

Cheese is a nutritionally rich food, because it has high levels of proteins and lipids. Due to this feature, to achieve an efficient degree of microbiological inhibition, the concentrations of natural compounds to be added should be higher than the concentrations found in *in vitro* tests (Moro et al., 2015).

Mahajan et al. (2016) studied the use of pine needle extract (*Cedrus deodara* (Roxb.) Loud.) in kalari cheese (a variety of hard and dry cheese typical of India). They found a decrease in the degradation of proteins and fats, improving the conservation during the storage period under refrigeration.

Gouvea et al. (2017) in a study on the addition of plant extracts and essential oils as antimicrobials in cheeses show that extracts and oils from medicinal plants such as oregano, cumin, pepper, rosemary and thyme have satisfactory *in vitro* antimicrobial activity against contaminating cheese pathogens (Caleja et al., 2015), representing an important alternative for the conservation of these products. However, *in vitro* results should not be considered as strictly true for food, since there is a great complexity of the food matrix and also external and internal interference factors of the food itself (Calo et al., 2015).

Regarding the characteristics of the cheeses, the coloring from the fifth day after application of the treatments, ceased to be white, becoming gradually yellowish, and over time the yellow coloration was predominant in all treatments, which may



have been favored by *S. aureus* contamination. The yellowish coloration ends up harming the acceptance of cheese consumption, even when the product is still in the shelf life (Dias et al., 2012).

Initially, the cheeses presented characteristic garlic odor in a weak and subtle way. This aspect is of extreme relevance, since it does not strongly compromise the taste of the food. In general, flavors and odors predominant in food, are not desired in the gastronomy, as they compromise the balance of the delicacy. When using aromatic and flavoring plants, it is intended to associate aromas in order to enhance the flavor of the food produced in a harmonious way. Flavors and flavors excessively preponderant, in a way, are undesired by jumping and thus compromising consumer acceptance (Nascimento, 2016).

Regarding texture and softness, all cheeses submitted to different treatments over time were gradually becoming parched and hardened, with cracks on their surface.

#### 4. Conclusion

*Allium sativum* presented antimicrobial activity against *Staphylococcus aureus*, both in aqueous and *in natura* suspension form. Therefore, it was possible to verify the antimicrobial activity of garlic in fresh cheese, contributing not only to its storage but also to its flavor, representing a differential for the product and thus favoring its commercialization. Regarding future perspectives, the next studies should take into account the need to promote the greatest possible control of the antimicrobial action of garlic on cheese. However, at the same time, it is necessary to consider the palatability of the product, so as not to compromise the consumer acceptance.

Further studies are needed to confirm the antimicrobial activity of the aqueous extract of *Allium sativum in natura*, in order to better verify its effects against other contaminating microorganisms in cheese.

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