

## Synergistic effect of phytochemicals *in vitro* and their antimicrobial properties against food-borne microorganisms in “coalho” cheese

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This study aimed to evaluate the synergistic effect *in vitro* of phytochemicals against pathogenic bacteria that occur in “coalho” cheese and their bioactivity on food matrix. The assay *in vitro* was performed by Minimum Inhibitory Concentration (MIC), Minimum Bactericidal Concentration (MBC) and Fractional Inhibitory Concentration (FIC) of eugenol, carvone and cinnamaldehyde phytochemicals in isolated and combined use against *Escherichia coli* (ATCC 8739) and *Staphylococcus aureus* (ATCC 6538). In the food matrix the rate of growth of *Escherichia coli* (ATCC 8739) and *Staphylococcus aureus* (ATCC 6538) inoculated in “coalho” cheese and autochthonous microbiota of “coalho” cheese was evaluated in the presence and absence of isolated and combined phytochemicals. Eugenol (0.6 mg.ml<sup>-1</sup>) and cinnamaldehyde (0.3 mg.ml<sup>-1</sup>) showed the best inhibitory effect (microstatic effect) *in vitro* against *E. coli* and *S. aureus*, respectively, and it was found synergistic effect between the phytochemicals. In food matrix the combined phytochemicals were more efficient to inhibit the growth of microorganisms.

**Keywords:** eugenol; carvone; cinnamaldehyde; autochthonous microbiota; antibacterial activity

### 1. Introduction

“Coalho” cheese is known by their intermediary moisture cheese and fat content in the range of 35-60% in dry matter [1]. Variation in chemical composition of this food has been verified due to differences in the technological process, moisture with value from 43.72% to 59.3%; dry matter between 40.68% and 56.7%; fat in dry matter from 45.13% to 50.40% and average levels of proteins and ash between 17.17% and 22.64% and from 2.88% to 3.54% respectively [2].

This variety of cheese is widely produced and consumed in the Brazilian Northeast region and its major production has been attributed to small farmers. In the state of Pernambuco, Brazil, the “coalho” cheese can be obtained from pasteurized or unpasteurized milk [3], classified as “coalho” cheese type A and B, respectively. There have been precedents for the microbiological insecurity with “coalho” cheese type B especially when it has been associated with inadequate conditions of processing and low-quality raw material. Furthermore, the absence of hygienic-sanitary practices during the production results in increased microbial growth and consequently deteriorate flavor, odor, sensory and textural properties of foods, and some microorganisms can potentially cause food-borne illness [4]. Therefore bacteria such as *Salmonella*, *Staphylococcus aureus* and *Escherichia coli* has caused food-borne illness for many decades bringing hazard and risk to the health human population.

In spite of advances in food preservation in recent years, modern methods have been applied, likely, combined with existing ones for reducing or eliminating foodborne pathogens [5]. Research efforts in plant antimicrobials have aimed to meet the demand for food products with natural additives, improvements in antimicrobial activity against pathogens mainly in relation to antibiotic resistance and contribute greatly to prolong the shelf-life, as well as, benefits for consumers and industry.

A wide range of essential oils components have been accepted by the European Commission as flavors not likely to present health risk for consumers. The FDA (The United States Food and Drug Administration) has classified the substances linalool, thymol, eugenol, carvone, cinnamaldehyde, vanillin, carvacrol, citral, and limonene as GRAS (Generally recognized as safe) have been recognized as safe for consumption, although, it clarifies that daily intakes must be previously assessed considering the regulated limit in foods [6]. Toxicity issues need to be clarified. For example, Both the Food and Agriculture Organization (FAO) and World Health Organization (WHO) have allowed an acceptable daily intake of eugenol of 2.5 mg.kg<sup>-1</sup> body weight for humans. Moreover, the U.S. Food and Drug Administration (FDA) have proclaimed eugenol as safe and it is considered non-carcinogenic and non-mutagenic [7].

The growth rate of microorganisms may be better controlled by the use of natural preservatives once their effectiveness have been well documented, among them plant extracts, essential oils, phytochemicals, organic acids and bioactive proteins [4,6]. The antimicrobial potential of natural compounds from plant source has been widely reported mainly *in vitro* conditions, where eugenol and cinnamaldehyde are considered the most effective phytochemicals in antimicrobial activity in inhibition of *E. coli* and *S. aureus* [8]. However, in face of the first reports in food matrix, it is

necessary increasing the concentration of these substances, which has been attributed to an interaction among antimicrobials and constituents of food [6,9]. Thus, the sensory qualities of the food are altered and progress in the application of spice-derived compounds as antimicrobial agents in food products has been slow. Different strategies have been used in order to make efficient antimicrobial action without affecting the organoleptic properties of food, including the associated use of antimicrobials, considering the different mechanisms of action and degree of efficiency [8,10].

This study aimed to evaluate the synergistic effect *in vitro* of phytochemicals against pathogenic bacteria that occur in “coalho” cheese and their bioactivity on food matrix, as well as, investigate the isolated and combined use of phytochemicals in inhibiting of autochthonous microbiota in “coalho” cheese.

## 2. Material and Methods

### 2.1 Material and bacterial suspension prepare

Eugenol, Cinnamaldehyde and Carvone phytochemicals of essential oils from clove India (*Eugenia caryophyllata*) and cinnamon (*Cinnamomum zeylanicum*), mint (*Mentha spicata*), respectively, were obtained from Sigma-Aldrich Brasil Ltda. The bacterial suspension was previously prepared in a saline solution from overnight culture of *Escherichia coli* (ATCC 8739), *Staphylococcus aureus* (ATCC 6538) and standardized ( $8 \text{ Log CFU.ml}^{-1}$ ) to 0.5 on the McFarland scale and the cell count confirmed by OD 610nm measurement.

### 2.2 Antibiogram

Sensitivity of bacterial strains to norfloxacin and chloramphenicol were performed according to CLSI (11). Discs of filter paper impregnated with antibiotics ( $5 \text{ mg.ml}^{-1}$ ) were deposited on the surface of an agar medium previously inoculated with pure cultures of strain to be studied. Upon application of the disks antibiotics diffused uniformly. After incubation (24 h at 37°C), the discs around circular inhibition zones correspond to no culture (11).

### 2.3 Determination of the Minimum Inhibitory Concentrations (MIC) and Minimum Bactericidal Concentration (MBC) and Fractional Inhibitory Concentration (FIC)

The minimum inhibitory concentrations (MIC) by macrodilution method and minimum bactericidal concentration (MBC) in plates were performed according to CLSI (11). For this analysis serial dilutions (two-fold dilution) of phytochemicals (2 a  $0.3 \text{ mg.ml}^{-1}$ , 1:2 v/v) were prepared using DMSO as solvent. After inoculated the standardized bacterial suspension and the phytochemical in its respective concentration, the tubes were incubated at 35-37°C for 24 hours. The lowest tested substance concentration that completely inhibited microbial growth (turbidity) was recorded as the MIC. Aliquots of 100  $\mu\text{L}$  from the tubes that did not present visible microbial growth and from two test tubes with higher concentrations were inoculated into test tubes containing slanted nutrient agar and incubated at 37°C for 24-48hours. The lowest concentration of the phytochemical at which the test strains did not present the capacity for growth in broth and agar was recorded as the MBC.

Synergic effect was obtained by determination of the fractional inhibitory concentration (FIC) from MIC when the phytochemical were used in combination with another agent (1:1) divided by MIC when used alone. Effects were interpreted as synergistic when FIC indexes were  $\leq 0.5$ , indifference was defined by  $\text{FIC} > 0.5$  and antagonism when FIC was  $> 4$  [12].

### 2.4 Determination of antibacterial activity of phytochemicals against standards bacterial strains in “coalho” cheese

Based on MIC from phytochemicals in use isolated and synergistic effect, the concentration of phytochemicals used in the food matrix was selected, being used at a concentration  $10 \times \text{MIC}$  *in vitro* conditions. It was produced “coalho” cheese type A and determined the survival curve analysis of bacterial strains. For the production of cheese followed the following steps: heating milk, addition of calcium chloride and coagulant, cutting and heating curd, whey drainage, pressing, package and storage at refrigeration ( $5 \text{ }^\circ\text{C} \pm 1 \text{ }^\circ\text{C}$ ) [13]. After that, cubes of “coalho” cheese (25g) were subjected to  $90^\circ \text{C}$  for five minutes autoclaving and 1 ml bacterial suspension and phytochemicals were inoculated on syringe simulation system. Sample without phytochemicals was used as control. Each cube was vacuum-packed and stored under refrigeration ( $5 \text{ }^\circ\text{C} \pm 1 \text{ }^\circ\text{C}$ ). Daily samples were plated for *E. Coli* ATCC 8739 using VRB agar and Baird-Parker agar with egg yolk tellurite supplement for counting the *Staphylococcus aureus* ATCC 6538 during five days. The inoculated plates were then incubated at  $37 \text{ }^\circ\text{C}$  for 24-48 hours and then were determined the number of Colony-Forming Units (CFUs) and the results were expressed as  $\text{log CFU.g}^{-1}$ . All assays were done in triplicate and the all data were the average values of three parallel tests.

## 2.5 Determination of antibacterial activity of phytochemicals against autochthonous microorganisms in “coalho” cheese.

“Coalho” cheese type B samples were obtained from shops in Vitória de Santo Antão/PE and taken to the laboratory under refrigeration on the package itself for antimicrobial activity analysis of phytochemical in food matrix against the autochthonous microbiota. Cubes of “coalho” cheese (25g) were inoculated with Cinnamaldehyde (3.0 mg.g<sup>-1</sup>), Eugenol and Cinnamaldehyde (1:1, 3.0 mg.g<sup>-1</sup>), on syringe simulation system. Sample without phytochemicals was used as control. A concentration of phytochemicals 10 x MIC was used. Each cube was vacuum-packed and stored under refrigeration (5 ° C ± 1 ° C). During 16 days and every four days, each sample was subjected to analysis for *Staphylococcus aureus*, total coliforms, *Escherichia coli* and mesophiles count according to standard methods [14] and the results were expressed as log CFU.g<sup>-1</sup>. All assays were done in triplicate and the all data were the average values of three parallel tests.

## 2.6 Statistics analysis

The data were subjected to analysis of variance (ANOVA) followed by the Duncan's test to determine significant differences (p<0.05), by software “statistic for Windows” (STATSOFT, 2002).

## 3. Results and Discussion

The results of the MIC, MBC and the FIC of phytochemicals for each microorganism are given in Table 1. The isolated use of cinnamaldehyde showed the best inhibitory effect against *S. aureus* at a concentration of 0.3 mg.ml<sup>-1</sup> (microstatics effects) and 0.6 mg.ml<sup>-1</sup> (microbicidal effects), and eugenol showed the best inhibitory against *E. coli* at a concentration of 0.6 mg.ml<sup>-1</sup> for both microstatics and microbicidal effects. Bacteria such as *S. aureus* and *E. coli* showed susceptibility to the antibiotics, most notably chloramphenicol and Norfloxacin, with inhibition zones ranging from 20 to 29 mm, as recommended by CLSI [11].

There was a synergistic effect when the antibacterial effect of the phytochemical in double and triple combinations including carvone was compared to its isolated effect for both microorganisms (Table 1). The best efficacy of eugenol and cinnamaldehyde in inhibiting of *E. coli* and *S. aureus* were confirmed by the results of this study conform mentioned in previous studies [8]. Therefore, it was found the lower MIC for *S. aureus* to double (eugenol and cinnamaldehyde) and triple combinations of phytochemicals, synergistic effect occurring only when compared to eugenol and carvone, however, the use of cinnamaldehyde was sufficient to control the bacterial growth (Table 1).

To inhibit *E. Coli* the combined use of cinnamaldehyde and eugenol (1:1) was the best alternative (Table 1). The phytochemicals cinnamaldehyde and eugenol in 1:4 or 1:8 ratio inhibited *E. coli* this is probably due to interaction between these compounds with proteins and enzymes of the bacterial cell [15]. Differently from results obtained in this study, the MIC values was 1.6 mg.ml<sup>-1</sup> for eugenol and 0.4 mg.ml<sup>-1</sup> for cinnamaldehyde and by means of combination, MICs of eugenol and cinnamaldehyde decreased to 0,4 mg.ml<sup>-1</sup>. Consequently, the negative impacts of unpleasant smell of these components could be minimized, making it possible to add them to foods as preservatives [15]. Still Eugenol induced cell lysis of Gram-negative and Gram-positive bacteria by damaging the cell wall and membrane caused leakage of protein and lipid contents [16].

**Table 1** The minimum inhibitory concentrations (MIC), minimum bactericidal concentration (MBC) and the fractional inhibitory concentration (FIC) of phytochemicals in inhibition of *S. aureus* and *E. coli*.

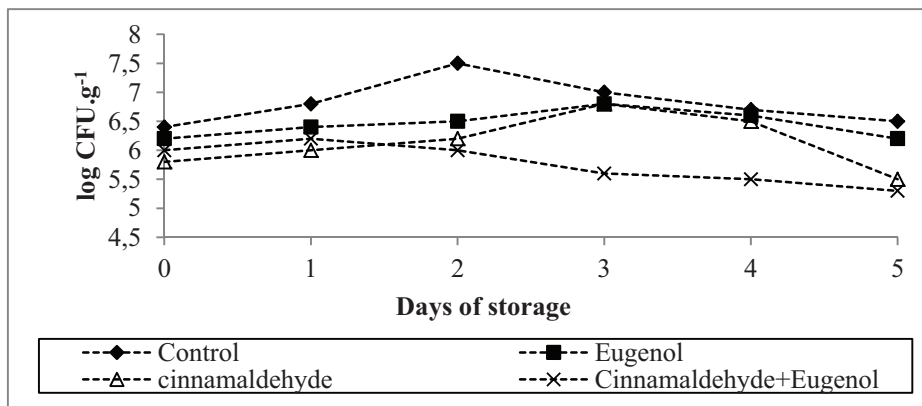
Phytochemicals	<i>S. aureus</i>		<i>E. coli</i>			
	MIC mg.ml <sup>-1</sup>	MBC mg.ml <sup>-1</sup>	MIC mg.ml <sup>-1</sup>	MBC mg.ml <sup>-1</sup>		
Isolated Use	Cinnamaldehyde	0.3c	0.6b	1.2b	1.2b	
	Eugenol	0.6b	1.2a	0.6c	0.6c	
	Carvone	1.2a	1.2a	2.4a	2.4a	
Combinated Use: 3 phytochemicals	Cinnamaldehyde: Eugenol: Carvone	1	0.25	0.25	0.25	
		0.5	0.6b	0.3d	0.5	1.2b
		0.25			0.12	
Combinated Use: 2 phytochemicals	Carvone: Cinnamaldehyde	0.5	1.2a	0.6c	0.5	1.2b
		2			1	
		0.6b				
Combinated Use: 2 phytochemicals	Eugenol: Carvone	1	1.2a	0.6c	1	1.2b
		0.5			0.25	
		0.6b				
Combinated Use: 2 phytochemicals	Eugenol: Cinnamaldehyde	0.5	1.2a	0.3d	0.5	1.2b
		1			0.25	

<sup>a-d</sup>Means followed by the same letter vertically do not differ significantly by Duncan's test (p<0.05).

Effect of cinnamaldehyde on *E. coli* and *S. aureus* membrane was evaluated [17]. Studies of scanning electron microscopy and transmission electron microscopy prove that bacteria such as *S. aureus* and *E. coli* treated with cinnamaldehyde showed numerous changes and abnormalities, as rough cell membranes with particulate matter, cytoplasmic membrane separation from the cell wall, lysing the cell membrane, cytoplasmic content leakage and cytoplasmic content condensation. These results indicate that bacterial cell morphology, membrane integrity, and permeability are damaged when the microorganisms cells are exposed to the minimum inhibitory concentrations of cinnamaldehyde ( $0.31 \text{ mg.ml}^{-1}$ ). In addition, the higher the cinnamaldehyde concentration, the more serious the bacterial membrane damage is [17].

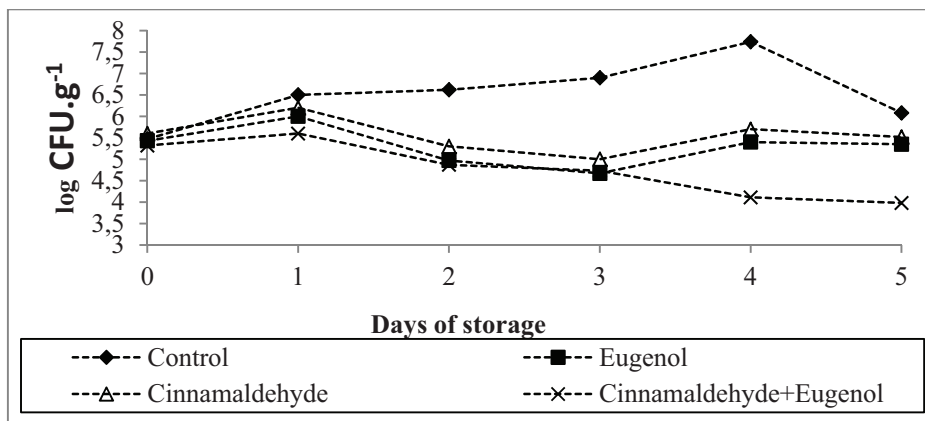
Based on results cinnamaldehyde and eugenol applied together (1:1) and isolated cinnamaldehyde were selected for the study with food matrix at a concentration  $10 \times \text{MIC}$  *in vitro* condition ( $3.0 \text{ mg.g}^{-1}$ ). Lower growth rate of *E. coli* and *S. aureus* in food matrix was provided by the combined use of phytochemicals, when compared with the control, after five days (Figs. 1, 2). There was no significant difference ( $p > 0.05$ ) in reducing *S. aureus* ATCC 6538 provided by the effect of the combined use ( $1.2 \text{ log CFU.g}^{-1}$ ) and cinnamaldehyde in isolated use ( $1.0 \text{ log CFU.g}^{-1}$ ) (Fig. 1). Nevertheless, there was significant reduction ( $p < 0.05$ ) in *E. coli* ATCC 8739 from  $2.1 \text{ log CFU.g}^{-1}$  in comparison to the best inhibitory effect (combined use), followed by eugenol ( $0.72 \text{ log CFU.g}^{-1}$ ) and cinnamaldehyde ( $0.38 \text{ log CFU.g}^{-1}$ ), showing a significant difference ( $p < 0.05$ ) between them during the evaluation period (Fig. 2).

The results performed at the food matrix were consistent with *in vitro* analysis about bacterial growth rate regarding to target microorganism. Some studies have recorded the antimicrobial efficacy of natural compounds, alone or in combination with other preservation methods *in vitro* and when directly applied to milk or to cheese by spraying, immersing, or dusting the products. It was identified in this studies antimicrobial effect of bioactive proteins; cellulose, chitosan and galactomannans with antimicrobials as edible coatings; microbial sachet, and essential oils or plant extracts in inhibition of bacteria, and therefore at different conditions performed in this study. The efficacy of films incorporating nisin and natamycin was first evaluated *in vitro* and then on sliced mozzarella cheese, and best effects were found when the two compounds were applied together on cheese. Mozzarella was packaged in a brine that contained lysozyme ( $0.25 \text{ mg.ml}^{-1}$ ) and different amounts of EDTA (10, 20, and  $50 \text{ mmol.l}^{-1}$ ), and stored at  $4 \pm 1^\circ\text{C}$  for 8 days. The packaging system significantly inhibited growth of coliforms and *Pseudomonadaceae*. Eleven essential oils were evaluated *in vitro* for their antibacterial properties against *Vancomycin-resistant Enterococci* and *E. coli* O157:H7 and the most active essential oils against bacteria were thyme oil, eucalyptus, juniper, and clove oils. However, the addition of thyme oil at concentrations of 0.5 and 1% caused best significant reduction in microbial growth effects against the same microbial groups experimentally inoculated in Feta soft cheese and stored at  $7^\circ\text{C}$  for 14 days [4].



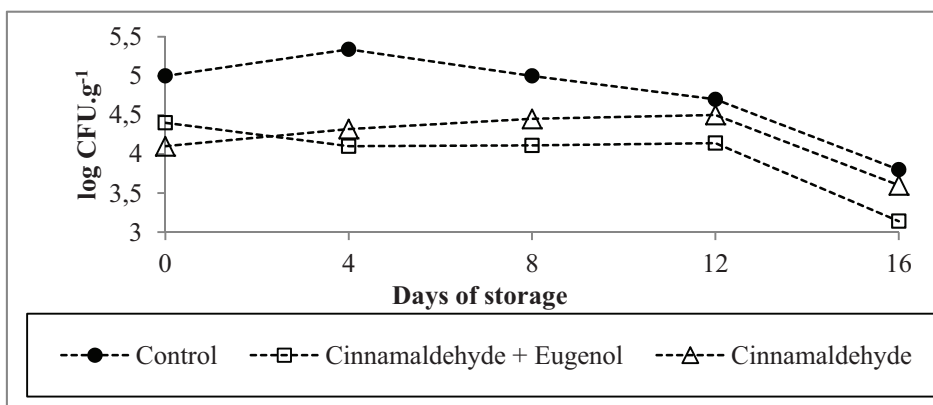
**Fig. 1** Antimicrobial effect of phytochemicals in *S. aureus* ATCC 6538 survival curve in “coalho” cheese vacuum-packed and stored under refrigeration ( $5 \pm 1^\circ\text{C}$ ).

The growth rate inhibition of standards strains of *S. aureus* and *E. coli* in ricotta cheese by essential oils from oregano and black pepper (1% and 5%) over 21-days period, with samples stored at  $7^\circ\text{C}$  was analyzed [18]. Among the best results was observed a decrease of  $3.36$  and  $1.0 \text{ log CFU.g}^{-1}$  for *S. aureus* and *E. coli*, respectively, at concentration of 1% of essential oil of oregano. When were used essential oil of black pepper (5%) was noted a dropped by  $1.39$  and  $1.17 \text{ log CFU.g}^{-1}$  for *S. aureus* and *E. coli*, respectively [18]. Differences in results may be related to the methodology as the study was conducted in relation to the type and the addition method of the antimicrobial, which was mixed the product, as well as, to factors compared to the type of cheese.



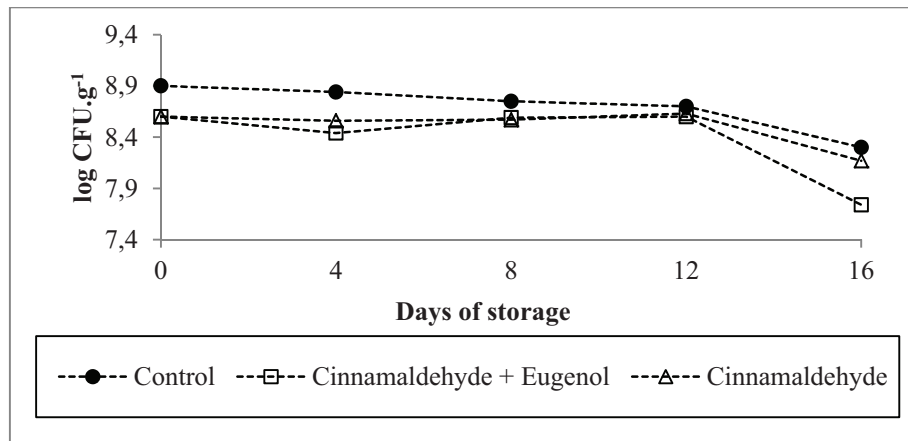
**Fig. 2** Antimicrobial effect of phytochemicals in *Escherichia coli* ATCC 8739 survival curve in “coalho” cheese vacuum-packed and stored under refrigeration (5±1 °C).

The effect of phytochemicals on the count of *S. aureus* and mesophilic, total coliforms and *E. coli* for 16 days in “coalho” cheese vacuum-packed and stored under refrigeration are shown in Figs. 3, 4, 5 and 6, respectively. When initial and final bacterial count were compared in each treatment and bacterial growth rates compared to the control group over 16 days, there was a significant difference ( $p < 0.05$ ) among treatments for both microorganisms evaluated, and it was found the best antibacterial effect for the combined use of cinnamaldehyde and eugenol (3.0mg.g<sup>-1</sup>). When initial and final bacterial count were compared there was reduction in the growth rate for all microorganisms with a reduction of 1.26 log CFU.g<sup>-1</sup> of *S. aureus* (Fig. 3), 0.86 log CFU.g<sup>-1</sup> of mesophiles (Fig. 4), 1.32 log CFU.g<sup>-1</sup> of total coliforms (Fig. 5), and 0.41 log CFU.g<sup>-1</sup> of *E. coli* (Fig. 6) at the end of 16 days of storage, however, when compared to the control group the reduction of the growth rate was 0.66 log CFU.g<sup>-1</sup> of *S. aureus* (Fig. 3), 0.56 log CFU.g<sup>-1</sup> of mesophiles (Fig. 4), 1.13 log CFU.g<sup>-1</sup> of total coliforms (Fig. 5) and 0.37 log CFU.g<sup>-1</sup> of *E. coli* (Fig. 6).



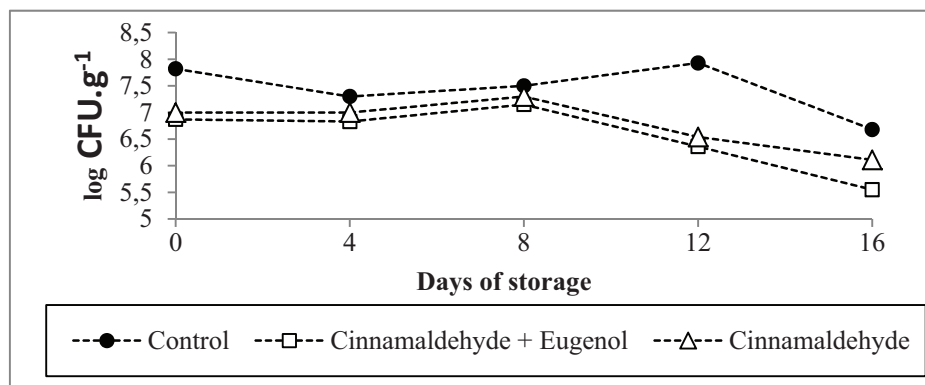
**Fig. 3** Effect of isolated and combined phytochemicals against autochthonous *S. aureus* in “coalho” cheese vacuum-packed and stored under refrigeration (5±1 °C).

In fact, data regarding antimicrobial effect in food aiming inhibit or decrease contaminating microorganism in food show that would be required an increased concentration of antimicrobial when compared to concentration normally used for *in vitro*. On the other hand the antimicrobial efficacy of essential oil obtained from *Eugenia caryophyllata* leaves with eugenol as major component (79%) against autochthonous microorganisms in “coalho” cheese samples vacuum packed and refrigerated over 15 days when applied in the curd was verified comparing to microbial growth rate of mesophiles in the presence and absence (control) of the same antimicrobial concentrations showed microstatic effect *in vitro* at 5, 10 e 20 µg.g<sup>-1</sup>, being demonstrated a dose-dependent reduction [19], however, there was not decreasing on initial microbial growth rates.

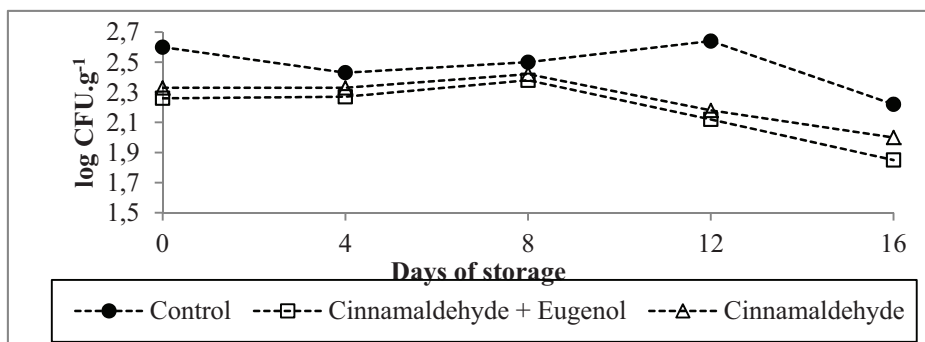


**Fig. 4** Effect of isolated and combined phytochemicals on mesophilic count in “coalho” cheese vacuum-packed and stored under refrigeration (5±1 °C).

The effective microbial control in foods is very challenging and may occurs by clarification the mechanism of action of antimicrobial agents considering physical and chemical complexity of food. Current research on food warn possible factors that influence: lipids, proteins, peptides, humidity and water activity, in addition to pH and temperature (20, 21, 22). Still, the hidrofobic components of essential oils are absorbed in the lipid fraction of the food thus avoiding contact of phytochemicals with the bacterial cells to the hydrophilic phase and consequently decreasing the antimicrobial effect [23]. The presence of different microorganisms in a competition should be considered in this context, being important to point out.



**Fig. 5** Effect of isolated and combined phytochemicals on total coliforms count in “coalho” cheese vacuum-packed and stored under refrigeration (5±1 °C).



**Fig. 6** Effect of isolated and combined phytochemicals on autochthonou *Escherichia colic* count in “coalho” cheese vacuum-packed and stored under refrigeration (5±1 °C).

## 4. Conclusion

It was concluded that the tested phytochemicals showed significant antibacterial activity *in vitro* and in the food matrix, the synergistic effect is phytochemicals in combination and target microorganism dependent, and a higher concentration of isolated or combined phytochemicals than 10 x MIC concentration *in vitro* should be used to reduce further the bacterial growth rate on cheese, being important to consider the regulated limit of phytochemicals in food regarding acceptable daily intake.

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