# The effect of thermal, chlorine and ozone treatments on survival of Escherichia coli and Salmonella spp. in açai berries

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**Abstract :** *Açai berries (Euterpe oleracea Mart.) have a high natural microbial load that can compromise the quality of their products. In this study, test microorganisms were inoculated on raw açai berries in drip trials, with five strains of Salmonella spp. and E. coli, separately. Açai berries sanitizing processes were: i) chlorination with 200 mg.L$^{-1}$ for a period of 15 min; ii) blanching at 80 °C for 10 s (regional legislation); iii) blanching at 90 °C for 10 s; iv) aqueous ozonation at 4 mg.L$^{-1}$ for 5 min; and v) washing with distilled water for 15 min as control treatment. Thermal treatment at 90 °C 10 s achieved the highest efficiency in reducing counts of E.coli (3.0 ± 0.8 log CFU.mL$^{-1}$). In Salmonella spp., all sanitizing treatments were similar and better than simple washing with water. Thus, blanching of açai berries at 90 °C 10 s, with immediate cooling with chlorinated water (50 mg.L$^{-1}$), is an alternative process for sanitization for açai berries, considering the application of good manufacturing practices.*

**Keywords -** *Bacteria, blanching, contamination, Euterpe oleracea; ozonation; thermal processing*

## I. INTRODUCTION

Açai (*Euterpe oleracea* Mart.) is a native palm tree of the Amazon rainforest that produces edible berries widely consumed as fresh beverages and frozen desserts in Brazil. It is also exported to many countries, mainly as pasteurized pulp. It is considered a functional and energy food, due to its high antioxidant capacity and potential health benefits, besides high nutritional value [1]. On the other hand, açai berries are typically highly contaminated in microbiological terms. The berries as well as other materials come from the field with initial microbiological contamination and this can increase during the chain until industrial processing into pulp.

The main source of açai berries contamination is water, since more than 50% of the municipalities located in the Brazilian Amazon do not use chlorinated water [2]. Raw berries traded for beverage processing can present fecal coliform concentrations > 3.0 log.g$^{-1}$ dry matter, 7.0 log.g$^{-1}$ dry matter of mesophilic bacteria and 5.2 log.g$^{-1}$ dry matter of molds and yeasts [3]. Without thermal treatment, mesophilic bacteria are also usually found in greater numbers in manually processed beverages than the limits established by current Brazilian regulations. In one study, a load of mesophilic bacterial contamination was observed of around 9.5 log CFU.kg$^{-1}$ berry [4]. There are also many reports of *Escherichia coli* and *Salmonella* spp. in açai berries, pulp and beverages [3].The regulations establish some procedures for processors, mainly blanching treatment of açai berries at 80 °C for 10 s [5], to deactivate *Trypanosoma cruzi*, the causative agent of Chagas disease [6]. Blanching for less than 10 s does not change the sensorial attributes of the beverage [7] and can be considered an efficient, easy and cheap process. In the case of cabbage, blanching in boiling water for 4 min produced a reduction of 3.8 log CFU.cm$^2$ in *Salmonella* spp. attached to the surface leaves [8].

Sodium hypochlorite is the most widely used chemical sanitizer of fresh produce, despite growing environmental and health concerns [9]. The recommendation of active chlorine concentrations for sanitizing fresh products is in the range of 50-200 mg.L$^{-1}$, with a contact time from 15 to 30 min [10]. The efficiency of chlorination can be influenced by organic matter present in water, as well as insufficient cooling, exposure to air or light, or a combination of these three factors [11]. Ozone (O$_3$) is one of the most powerful oxidizing agents (2.07 mV), effective for inactivation of Gram-negative and Gram-positive bacteria, viruses and yeasts, including sporulated forms [12], and protozoan cysts [13]. Studies have shown that treatment with ozonated water extends the shelf life of various food products, increasing quality and food safety [14; 15]. Despite ozone's rapid

decomposition in water, its antimicrobial action can be very effective for surface decontamination of fruits and vegetables [12].

This work aimed to study the effectiveness of decontamination methods of açai berries, by reducing the microbial load of *Salmonella* spp. and *E. coli*, through thermal (blanching) and non-thermal (chlorination and ozonation) processing.

## II. MATERIAL AND METHODS

**2.1 Açai berries**

Açai berries (*Euterpe oleracea* Mart.) were collected from a stand of trees in Mazagão, Amapá state (Brazil). Açai berries were vacuum-packed (Tecmaq TM-250, Brazil) and refrigerated at 8 °C ± 2. They were weighed and washed three times in 200 mL of tap water to reduce the natural contamination.
Preliminary microbiological counts of the açai berries was performed, with 15 intact raw berries (approximately 20 g), analyzed in the three repetitions.

2.2 Test microorganisms

The microorganisms tested were a pool of five strains each of *Escherichia coli* (ATCC 8739, ATCC 10799, ATCC 11229, ATCC 25922 and ATCC 43893) and *Salmonella* spp. (*S.* Choleraesuis ATCC 10708, *S.* Enteritidis ATCC 13076, *S.* Typhimurium ATCC 13311, *S.* Typhimurium ATCC 14028 and *S.* Brazil). The cultures were maintained in Tryptic Soy Agar (TSA) at 8 ± 2 °C. All strains of each test organism were evaluated in relation to the antagonistic effect on the ability to inhibit the growth of other strains [16].

**2.3 Inoculum preparation**

The inocula of the microorganisms were prepared from stock cultures [17]. The activated strains of each test microorganism were placed in 25-mL tubes and centrifuged (2,000 g, 15 min, room temperature) three times. The supernatant was removed and the pellets were washed with 10 mL of 0.1% peptone water. The resulting cell masses were resuspended in 10 mL of 0.1% peptone water. Drops of 0.1 mL of each inoculum, in a total 1.0 mL, were applied on the surface of açai berries, which were kept in a laminar flow cabinet for 30 min to dry the inoculum.

**2.4 Treatments**

Two assays were performed separately. In the first assay, açai berries were subjected to thermal (blanching) and chemical (chlorination and ozonation) treatments separately (Figure 1). In the second assay, açai berries were blanched at 90 °C and cooled with different aqueous sources (Figure 1). The ratio between the sample mass and the volume of water used in all test solutions for açai berries treatment was 40 g.L$^{-1}$ (12). The recovered microbial enumeration from berries of two assays was performed before decontamination treatments ($N_0$).

2.4.1 Thermal and chemical treatments

All treatments were conducted according to Figure 1. Inoculated berries immersed in distilled water at room temperature for 15 min and then drained represented the control treatment.

2.4.1.1 Chlorination

Inoculated açai berries were immersed in 500 mL of a 200 mg.L$^{-1}$ chlorinated water solution for 15 min, using a commercial chlorine solution containing 2.0 to 2.5 wt of available chlorine, after which berries were drained. The initial and final residual chlorine concentrations were measured by the DPD colorimetric method [18].

2.4.1.2 Ozonation

Açai berries were immersed in 500 mL of aqueous solution that had been previously ozonated for 5 min using an ozonator (Ozone & Life 3.0 RM, Brazil) by the corona discharge method (Figure 2). The ozone was dissolved in water through a porous gas diffuser stone. The concentration of 4 mg.L$^{-1}$ was monitored by an ozone analyzer (CHEMetrics I-2019 SAM, Brazil) with a photometric analytical kit (CHEMetrics K-7423) where initial and final concentrations of $O_3$ were measured. After chlorination and ozonation treatments, a solution of 0.066 mL of 10% sodium thiosulfate (Synth) was added for ozone neutralization.

2.4.1.3 Blanching

Hot water was applied in two treatments: immersion of açai berries in 500 mL of water at 80 °C for 10 s, as determined by local regulation [5] and in 500 mL of water at 90 °C for 10 s. Açai berries were drained and cooled in 500 mL of 50 mg.L$^{-1}$ chlorinated water at room temperature for 2 min.

2.4.1.4 Combining thermal treatment (blanching 90 °C) and cooling

Açai berries were immersed in 500 mL of hot water at 90 °C for 10 s and then water from various sources was used for cooling. The control treatment was blanching at 90 ºC and cooling in tap water at room temperature for 2 min, followed by draining

2.4.1.5 Combining blanching with chlorination
Açai berries blanched at 90 °C for 10 s were cooled in 500 mL of chlorinated water at concentration of 50 mg.L$^{-1}$ or 200 mg.L$^{-1}$ for 2 min, using a commercial chlorine solution containing 2.0 to 2.5 wt of available chlorine, after which berries were drained.
The initial and final residual chlorine concentrations were measured by the DPD colorimetric method [18].

2.4.1.6 Combining blanching with ozonation
Açai berries blanched at 90 °C for 10 s were cooled in 500 mL of ozonated water (4 mg.L$^{-1}$ or 5 mg.L$^{-1}$) for 2 min, separately. The water was previously ozonated for 5 min according to 2.4.1.2.

**2.5 Microbiological analysis**
      The concentrations of the suspensions (CFU.mL$^{-1}$) of each strain and pool of five strains of *Salmonella* spp. and *E. coli* were determined by serial dilution (1:10) in 1.0 mL aliquots of 0.1% peptone water, followed by surface plating (0.1 mL, in duplicate) in different culture media. For enumeration of *E. coli*, we used eosin methylene blue (EMB) agar (Acumedia), while for *Salmonella* spp., we used xylose lysine deoxycholate agar (XLD) (Difco). Three isolated colonies of *E. coli* were subjected to biochemical confirmation by applying indole production, methyl red test, Voges-Proskauer reaction and citrate utilization (IMViC tests). In the recovery of test microorganisms on the açai berries processed by different treatments in both assays, it was utilized the proportional volume of water used to make thin acai beverages (14 kg açai berries: 4 L water) in processing. Açai berries were rubbed one by one on the outer layer for 1 min, to avoid removal of pulp. Then the berry was revolved for 30 seconds to homogenize the suspension. Aliquots (1 mL) of the resulting recovered suspension were serially diluted in 0.1% peptone water. The surface plating (0.1 mL) was performed in duplicate suspensions, and the counts expressed in CFU.mL$^{-1}$.

**2.6 Statistical analysis**
      The experiment consisted of two assays involving decontamination of açai berries inoculated with *Salmonella* spp. and *E. coli*. The experimental design was completely randomized, where each test was done in three replicates. The enumeration of microbial cells was expressed in log of 10 cycles. The treatments were assessed by calculating the microbial reduction (logN$_0$ - logN), where N$_0$ is recovered microbial enumeration of the sample before decontamination treatments and N is the recovered microbial load after decontamination. Data were analyzed using the Statistica software (version 8.0, Stat Soft Inc., Tulsa, USA) for analysis of variance (ANOVA). The comparison of mean values of the parameters was performed by the Tukey test at 5% significance level.

# III.      RESULTS AND DISCUSSION
The preliminary trials of untreated acai berries presented a total of aerobic count in the range of 8.6 x 10$^4$ to 1.9 x 10$^6$ CFU.mL$^{-1}$.Cross-inhibition tests showed no growth inhibition zone among all test microorganism strains evaluated. Thus, the mixing of strains that compose the respective inoculants of each test microorganism presented no problem of interaction of strains. The *Salmonella* spp. and E. coli inoculum concentrations of two assays were about 9-10 log CFU.mL$^{-1}$ (Table 1). After applying the inoculum (1.0 mL) containing the test microorganisms on the surface of the berries and subsequent drying, the recovery was about 8.2 log CFU.mL$^{-1}$ for *Salmonella* spp. and *E. coli* (Table 1). The *Salmonella* spp. recovery (8.2 log CFU.mL$^{-1}$) after inoculation of berries by drip was similar to that observed in pecans recovery (7.0 log.g$^{-1}$) after inoculation of nutmeats and unshelled nuts for 1 h by immersion in a suspension of *Salmonella* spp. (8.7 log CFU.mL$^{-1}$) [19]. The recovery values of *Salmonella* spp. (Table 1) were slightly lower than the number of mesophilic bacteria found naturally in açai berries during the off-season period (9.1 to 9.5 log CFU.g$^{-1}$dry matter), but higher than during the harvest period (6.6 log CFU.g$^{-1}$dry matter) [19]. This might indicate that the recovery test found contamination by mesophilic bacteria similar to that observed naturally on açai berries throughout the year. The recovery rate of *Salmonell*a spp. and *E. coli* from açai berries was higher than that found in whole tomatoes and green onions (6.74 log CFU.ml.g$^{-1}$) after being inoculated by drip (9.2 log CFU.mL$^{-1}$) [20].

**3.1 Thermal and chemical treatments**
Treatment of açai berries by blanching at 90 °C for 10 s showed the highest efficiency in reducing the microbial load of açai berries contaminated with strains of *E.coli* (3.0 ± 0.8 log CFU.mL$^{-1}$) than all other treatments (Table

2). However, when the berries were infected with *Salmonella* spp., blanching at 90 °C for 10 s (2.6 ± 0.8 log CFU.mL$^{-1}$) obtained greater cell reduction only in relation to single washing with distilled water (0.8 ± 0.7 log CFU.mL$^{-1}$). Reductions in microbial load of *Salmonella* spp. when the berries were blanched at 90 °C (2.6 ± 0.8 log CFU.mL$^{-1}$) and 80 °C (2.0 ± 0.2 log CFU.mL$^{-1}$) for 10 s were statistically similar. The use of blanching at 80 °C for 10 s (2.6 ± 0.8 log CFU.mL$^{-1}$) on berries inoculated with *Salmonella* spp. and *E. coli* showed the same efficacy in microbial reductions after washing with ozonated water at 4 mg mL$^{-1}$ for 5 min, washing with chlorinated water at 200 mg.mL$^{-1}$ and single washing with distilled water (Table 2).

Raising the blanching temperature (80 °C to 90 °C) (Table 2) should cause a greater reduction of the number of microorganisms. Indeed, this would theoretically increase the lethal ratio (L) 10-fold [21]. In testing *E. coli*, there was a significant reduction ($p < 0.05$) of microbial load when the blanching temperature increased (Table 2). In one study testing several blanching temperatures for the period of 10 s, the authors observed a discrete logarithmic reduction, but not significant, in mesophilic bacteria when raising the blanching temperature from 80 °C to 90 °C and 100 °C [19]. In another study, blanching of bean sprouts was more effective in reducing *Salmonella* spp., and *E. coli* compared to washing with distilled and ozonated water [9].

The ozonated washing at concentrations of 4 mg mL$^{-1}$O$_3$ for 5 min did not result in a significant reduction in the cell counts of *E. coli* (0.6 ± 0.1 log CFU.mL$^{-1}$) when compared to blanching at 80 °C for 10 s (1.1 ± 0.6 log CFU.mL$^{-1}$) or washing in 200 mg.mL$^{-1}$ chlorinated water (0.9 ± 0.1 log CFU.mL$^{-1}$) or distilled water (0.5 ± 0.2 log CFU.mL$^{-1}$) (Table 2). Treatment of blueberries with aqueous ozone at a concentration of 3.7 mg.mL$^{-1}$ for 8 min caused a reduction of 0.1 ± 1.50 log CFU.g$^{-1}$ in *E. coli* O157: H7 and 2.40 ± 0.10 log UFC.g$^{-1}$ in *Salmonella* spp. [22]. These different results for the efficacy of ozonation from artificially inoculated microorganisms can be explained by various factors, such as the different plants studied, the test organism used, the physiological state of microbial cells and the ozone release method [14].

Chlorination of berries at a concentration of 200 mg.L$^{-1}$ did not perform better than any other treatment, including mere washing with water, for all test microorganisms (Table 2). The reductions of the microbial loads of *Salmonella* spp. (1.5 ± 0.3 log CFU.mL$^{-1}$) and *E. coli* (0.9 ± 0.1 log CFU.mL$^{-1}$) when washed with chlorinated water (200 mg.L$^{-1}$) were similar to those found on tomatoes treated with the same concentration of chlorine for 5 min [17]. The variety of results from these and other published studies of the effectiveness of chlorination for vegetable and fruit sanitizing is related to factors such as chlorine concentration applied, pH and immersion time. Some authors have found typical microbial reductions of < 2 log [23] and depending on the test microorganism combined with these factors, reductions can be achieved of < 1 to 3.15 log CFU.g$^{-1}$ [24].

The reduced effectiveness in lowering the number of microbial cells observed in trials involving thermal and non-thermal treatments applied to açai berries may be due to microbial cell infiltration into deeper fruit layers. Human pathogenic microorganisms can infiltrate the fruit through injuries and cracks of the cuticle and epidermal tissues, preventing the action of sanitizers applied to the surface [25]. The açai berry has a pulpy epicarp that is about 1 mm thick, enveloping a voluminous and hard endocarp and seed, and an abundant marbled endosperm [3]. This thin pulp is only 5-15% of the total fruit volume [19] and is very fragile, so it can be broken by simple friction, allowing entry of pathogens during harvest, transport and processing. The infiltration process in fruits and vegetables depends on temperature, time and atmospheric pressure. It is promoted when the water pressure on the product surface becomes higher than the internal pressure of the tissues. The pathogens can establish colonies that are extremely difficult to reach by aqueous chemical solutions [25]. Infiltration studies have been performed on tomatoes [26] and apples [27] involving *Salmonella* spp. and *E. coli*, reporting reduced efficacy of sanitizer treatments. The effectiveness of decontamination can be reduced due to infiltration, as in the case of lower *E. coli* elimination in apples treated with chlorinated solutions [27].

### 3.2 Thermal treatments (90 °C) and cooling

Cooling of the açai berries with water after heat treatment may be a source of microbiological recontamination [28], mainly if the water used is unclean. Moreover, during the cooling process, pathogenic microorganisms can withdraw into the fruit [25]. Several sources of water were investigated for berry cooling after blanching at 90 °C to observe the effects on sanitizing performance (Figure 3). Heat treatment at 90 °C combined with cooling in previously sanitized water produced a more pronounced average logarithmic reduction of *Salmonella* spp. load in relation to simple blanching at 90 °C, although not in relation to the combination of blanching and ozonation (Figure 3). Cooling with chlorinated water at concentrations of 50 mg.L$^{-1}$ and 200 mg.L$^{-1}$ after led to greater reduction of *Salmonella* spp., but did not differ from cooling with tap water. Cooling with water with the two ozone concentrations (3 and 4 mg.L$^{-1}$) performed worse and did not differ from each other in the logarithmic reduction of *Salmonella* spp. Regarding the berries contaminated with *E. coli* and heat treated at 90 °C, the different sources of water used for cooling the berries showed no significant difference in reducing the initial population (Figure 4). *E. coli* load reductions observed in the combined treatments were lower than those found when the blanching at 90 °C was applied with distilled water (Table 2).In one study, combining technologies (hurdle method) was found to improve log reduction [23], while in another the treatments had

antagonistic effects and consequently a hurdle effect was not observed [12]. In our study, the combination of blanching at 90 °C with subsequent cooling with chlorinated water resulted in synergistic effect for the logarithmic reduction of *Salmonella* spp., because the values were higher than those found when the berries were blanched and cooled with distilled water. But cooling with ozonated water at any concentration did not show this increased effect for *Salmonella* spp. and could even be considered to have an antagonistic effect because the values were below the average for blanching to 90 °C with cooling in distilled water. The indiscriminate action of ozone on bacteria and organic matter can explain the reduced effectiveness of ozone treatments in both assays compared to simple washing with water, because cuts on fruit surface promote the lixiviation of organic matter, which can react with ozone before acting on the bacteria adhered on the fruit surface [23]. Other authors considered that aqueous ozone when applied in açai fruit did not affect the sensory quality of its beverage enough to be discriminated by consumers [29]. Moreover, some studies show that ozone was significantly effective in reducing dislodged microorganisms in wash water, but had limited impact on bacteria attached to produce surfaces [20, 30].
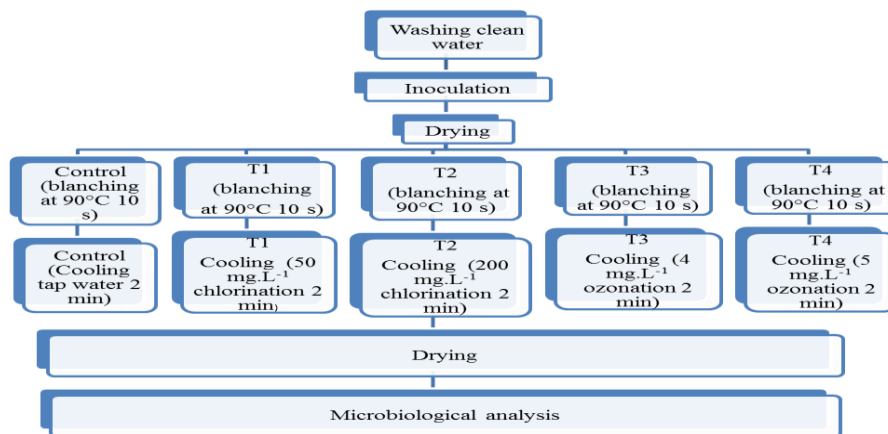
## IV.        FIGURES AND TABLES



Figure 1. Flow diagram with thermal (blanchings at 80 °C and 90 °C) and non-thermal (chlorination and ozonation)) treatments used to sanitization of açai berries
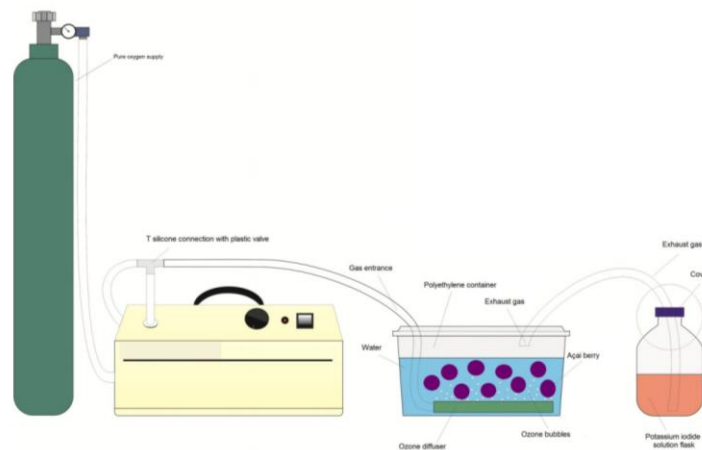


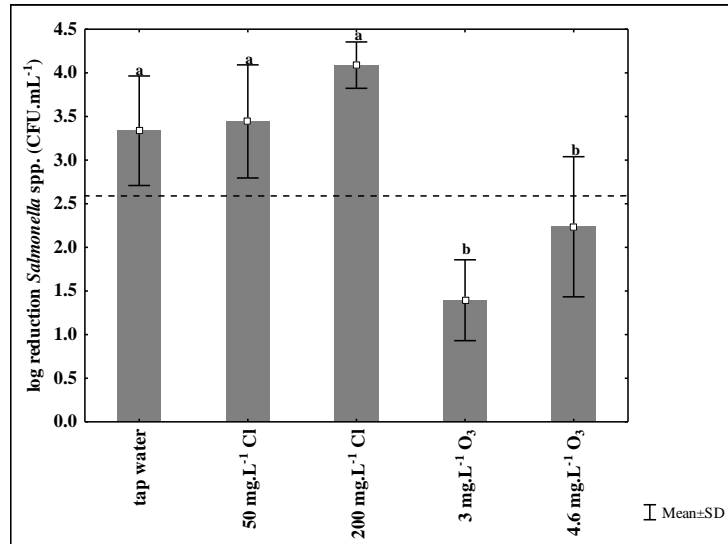Figure 2.  Aqueous ozonation scheme of açai berries

Figure 3. Log reduction of inoculated *Salmonella* spp. in açai berries treated by blanching at 90 °C and cooled at different sources of water. Dotted line represents log reduction of blanching 90 °C
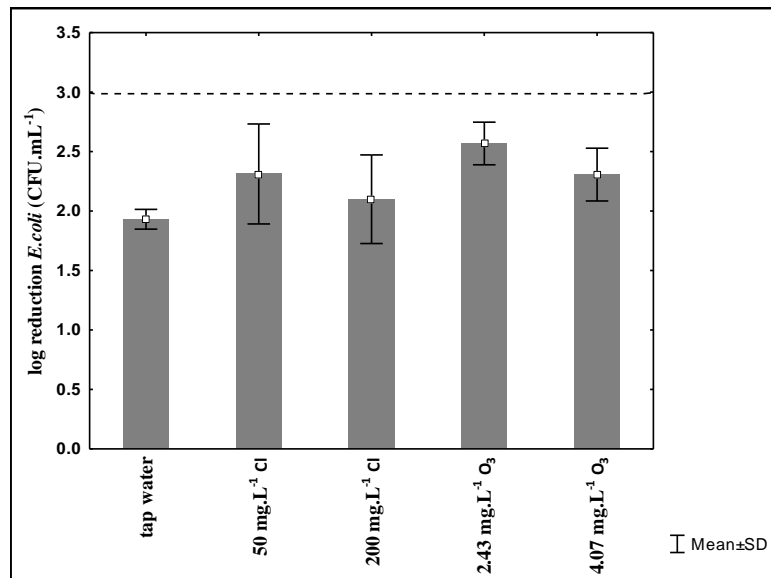


Figure 4. Log reduction of inoculated *E. coli* in açai berries treated by blanching at 90 °C and cooled at different sources of water. Dotted line represents log reduction of blanching 90 °C

Table 1 - Inoculum suspension concentration (log CFU.mL$^{-1}$) and enumeration of test microorganisms recovered from açai berries inoculated by drip at assay #1 (thermal and chemical treatments) and assay #2 (blanching at 90°C and cooling)

| Test microorganism | Inoculum suspension (log CFU.mL$^{-1}$)[a] | | Recovery in açai berries (log CFU.mL$^{-1}$)[a] | |
|---|---|---|---|---|
| | Assay #1 | Assay #2 | Assay #1 | Assay #2 |
| *Salmonella* spp. | 9.4 | 9.3 | 8.2 | 7.2 |
| *E.coli* | 9.6 | 9.3 | 8.2 | 7.7 |

[a] Values represent the mean of three replicates.

Table 2. Logarithmic reduction (log CFU.mL$^{-1}$) relative to thermal and non-thermal treatments in açai berries inoculated with test microorganisms

| Treatment | *Salmonella* spp. *p=0.04012* | *E. coli* *p=0.00009* |
|---|---|---|
| Blanching at 90 °C 10 s | 2.6 ± 0.8 a[1] | 3.0 ± 0.8 a |
| Blanching at 80 °C 10 s | 2.0 ± 0.2 ab | 1.1 ± 0.6  b |
| Ozonated water (4 mg.L$^{-1}$) 5 min | 1.6 ± 0.1 ab | 0.6 ± 0.1  b |
| Chlorinated water (200 mg.L$^{-1}$) 15 min | 1.5 ± 0.3 ab | 0.9 ± 0.1  b |
| Distilled water 15 min | 1.2 ± 0.1  b | 0.5 ± 0.2  b |

[`] Values in the same column with different letter are significantly different ($p < 0.05$).

## V.      CONCLUSION

The thermal treatment of açai berries in water at 90 °C for a period of 10 s is was most effective in reducing contamination of the pathogenic microorganisms studied, mainly *E. coli*, but it should be combined with cooling with water containing some type of sanitizer capable of destroying any pathogenic microorganisms present, and consequently preventing infiltration of the berry. It is important to consider that the pathogens studied showed a high rate of survival, regardless of the treatment. Thus, the microbiological safety of açai berries depends fundamentally on the prevention of contamination. Future studies will be conducted of combining blanching treatment with cooling in chlorinated and ozonated water to sanitize açai, as well as the nutritional value and sensory properties of the açai berries.

## VI.      Acknowledgements

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