Microbiological Quality of Oysters From an Estuary in Northeast Pará, Brazil, Before and After Purification Process

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Article

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Abstract: This study evaluated the microbiological quality of farm oysters, cultivated in an estuary in northeastern Pará, Brazil, before and after purification process. Water and oyster samples were collected in June, August, October, and December 2019. Oysters underwent purification for up to 36 h in a small-scale adapted debugger. Microbiological analyses were performed in oysters and water depuration collected at different times of purification (0 h pre-treatment, 6 h, 12 h, 2 4h, and 36 h), using the multiple tube technique, to determine the most probable number (MPN) of *Escherichia coli*, thermotolerant coliforms - TTC, and total coliforms - TC. Bacterial isolation was used to determine the presence of *Salmonella* spp. Before purification, oysters had *E. coli* counts above the limit allowed by Brazilian legislation (≤ 230 MPN/100g). High TTC, and TC counts were observed in all the months of the study, and *Salmonella* spp. was present in August. All water samples were contaminated by TTC beyond the level allowed (> 43 MPN/100mL) in two months. The purification process of 36 h was effective in reducing the load of *E. coli*, TTC, and TC and eliminating *Salmonella* spp. in oysters.

Keywords: Oyster; *Escherichia coli*; purification process; sanitary control

1. Introduction

Oyster farming is a widespread aquaculture practice in Brazil and, is well established in the South and Southeast regions (Castilho-Westphall, 2016). In the North region, it is still operateds as a small establishment, located mainly in the northeastern mesoregion of the state of Pará, and it has been expanding owing to the socioeconomic potential in the region. Oyster farming is authorized by the regulatory agency of the state, but the sanitary inspection of the production is not yet effective, indicating that data on the sanitary conditions of oysters are still at a primary stage (Sampaio et al. 2019; Macedo et al., 2020).

Oysters feed by filtering the surrounding water and can absorb suspended organic and inorganic matter. If the cultivation water is contaminated, oysters can accumulate natural pathogenic microorganisms from the marine environment and others of fecal origin, microplastics, heavy metal, resulting from anthropic action, which can be harmful to human health (Potasman et al., 2002; Sande et al., 2010, Senez-Mello et al., 2020, Manolaki et al., 2023).

The consumption of raw or lightly cooked oysters has been frequently reported as a cause of food-borne disease outbreaks worldwide (Robertson, 2007; Lee et al., 2013). Thus, the adoption of adequate sanitary control measures for oyster farming, in addition to the use of post-harvest processes to mitigate or eliminate possible microbiological contamination, is essential (Lee et al., 2013; Castilho-Westphall, 2016).

In Brazil, the sanitary quality of oysters is evaluated according to the guidelines of the National Program for Hygienic and Sanitary Control of Bivalves Mollusks (PNCMB), which establish

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acceptable limits for the most probable number (MPN) of *Escherichia coli* in samples of the edible part of mollusks harvested and to be marketed (Brazil, 2012).

Purification is a post-harvest process used to reduce microbiological contamination of bivalve mollusks to acceptable levels under current legislation. In this process, the animals are placed in clean, fluid water places, with environmental conditions similar to their natural habitat, so that they can carry out usual filtration to eliminate or reduce contaminants from the intestinal tract over time (Lee et al., 2013; McLeod et al., 2018; McMenemy et al., 2018).

In the North region, there are no reports of shellfish purification in crops, only a few restaurants located in local beaches purify oysters using adapted equipment. Furthermore, because oyster farms are small establishments, the farmers still do not have resources for the establishment of a purification station (Sampaio et al., 2019). Therefore, this study aimed to verify the microbiological quality of oysters before and after a purification process using adapted debugger equipment.

2. Materials and methods

2.1. Place and type of study

A descriptive and analytical study was carried out in June, August, October, and December 2019 in an oyster (*Crassostrea gasar* Adanson, 1757) farm located in the banks of the Urindeua River (0°42'07"S, 47°22'06"W) (Figure 1), in the municipality of Salinópolis, northeastern region of the state of Pará, Brazil, in Amazonian region.

2.2. Collection and conservation of water and oyster samples

In each month, 60 commercial size oysters (60 to 100 mm) were acquired to evaluate the efficiency of the *depuration* process, and 100 mL of water used for cultivation (Urindeua River). We also collected 100 mL of water used for purifying the oysters at different times: before purification (0 h), and 6 h, 12 h, 24 h, 36 h of purification. All samples were stored at 4 °C until analysis (APHA, 2005).

2.3. Purification process

The water used in the purification came from a beach (seawater) close to the establishment responsible for the process. For the purification, the oysters were organized side-by-side in hollow plastic trays and then submerged in handcrafted purification equipment, built in a freezer-type container, containing 500 L of water, coupled to a water recirculation system, filter, temperature gauge, and ultraviolet light system with lamps of 18 to 26 W. A chlorinated solution in the proportion of 1 - 4 ppm for each liter of water was added to the purification water.

Of the total number of oysters (n = 60), 48 specimens were submitted to the purification process, and 12 were separated for pre-purification analysis. A sample comprising 12 oysters was removed from the process at different times: 6 h, 12 h, 24 h, and 36 h for analysis. At the same time, the purification waters were collected.

2.4. Microbiological analysis of the soft part of oysters and water

For microbiological analysis, the multiple tube technique in a series of three dilutions was used to determine the most probable number (100 g/100 mL) of *Escherichia coli*, thermotolerant coliforms, and total coliforms in the oyster and water samples. For the procedure, a macerate of the soft part of 12 oysters was used, from which an aliquot of 25 g was removed for analysis. Aliquots of 100 mL (each) were used to analyze the water from the cultivation area, and pre/post-purification water. The procedure were as followed: the samples were enriched in Lauryl Tryptose broth, followed by a presumptive step in specific broth for total coliforms (bright green broth) and thermotolerant coliforms (*E. coli* broth), confirmation of the presence of *E. coli* in selective culture medium (Triple Iron Sugar Agar), and biochemical tests (Indol, Methyl Red, Voges-Proskauer, and Citrate). Bacterial isolation was performed only in the oyster meat samples to verify the presence of *Salmonella* spp. in

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the selective culture medium Rappaport Vassiliadis and Selenite Cystine broth, followed by seeding on a *Salmonella-Shigella* agar plate and biochemical tests (urease, indol, malonate, citrate, and rapid agglutination serum test) (Silva et al. 2010).

2.5. Physicochemical parameters of water

Physicochemical parameters, such as pH, temperature (°C), salinity (°/°°), and dissolved oxygen (mg/L), of water from the cultivation area and from the purification water were measured using a multiparameter probe (HI9828, HANNA®). The turbidity of the water (NTU) was measured using a portable turbidimeter (model 2100P HACH® PORTUGAL) (APHA, 2005).

2.6. Statistical analysis

Data were organized in Microsoft Excel (2016) and analyzed using Bioestat software, version 5.3 (AYRES *et al*. 2005). Microorganism counts in MPN/100 g/mL were log-transformed. Data were subjected to the Shapiro-Wilk test to verify normality. General correlations between physicochemical variables and bacterial density were obtained using the Spearman's correlation coefficient. The percentage difference from the initial count to the final count of microorganisms during purification is given by the equation (Dante, 2009). Difference % (T0/TF) = (TF/T0 - 1) × 100, where T0 = initial microorganism count before purification, TF = final microorganism count after the purification process.

3. Results

Microbiological analysis of unpurified oysters showed *E. coli* counts between 200 and 9.300 MPN/100 g), thermotolerant coliforms (TTC) between 780 and 110000 MPN/100 g), and total coliforms (TC) between 200 and 110.000 MPN/100 g). The evolution of the purification process that was carried out during the study period is shown in Table 1. In the collection carried out in June 2019, the *E. coli* count progressively decreased by 97.5% after 24 h of purification. However, there was no decrease in the densities of TTC and TC. During that period, it was not possible to purify the oysters until time T36, owing to a technical problem in the purification equipment; therefore, microbiological analysis was performed until time T24.

Table 1. Effect of purification process of up to 36 h on most probable number (MPN/100 g) of microorganisms in oysters farm from Urindeua River.

Month	Microorganism	Purification time (hour)					Difference
		T0	T6	T12	T24	T36	(T0-TF)
June	***E. coli***[a]	9300	9300	3000	230	-	-97.5%
	TTC[b]	110000	110000	110000	110000	-	0%
	TC[c]	110000	110000	110000	110000	-	0%
	Salmonella spp.	Abs[d]	Abs	Abs	Abs	-	-
August	*E. coli*[a]	1700	<1.8	<1.8	<1.8	<1.8	-99.9%
	TTC[b]	2700	2300	1300	<1.8	220	-92.0%
	TC[c]	1700	1700	200	<1.8	<1.8	-99.9%
	Salmonella spp.	**Pres**[e]	Abs	Abs	Abs	Abs	-
October	*E. coli*[a]	200	<1.8	<1.8	<1.8	<1.8	-99.9%
	TTC[b]	780	<1.8	<1.8	<1.8	<1.8	-99.9%
	TC[c]	200	<1.8	<1.8	<1.8	<1.8	-99.9%
	Salmonella spp.	Abs	Abs	Abs	Abs	Abs	-
December	*E. coli*[a]	610	220	<1.8	220	<1.8	-99.9%
	TTC[b]	930	200	400	400	780	-16.1%

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	TC[c]	930	200	<1.8	400	200	-78.5%
	Salmonella spp.	Abs	Abs	Abs	Abs	Abs	-

[a]*Escherichia coli* (MPN/100 g). [b]TTC: Thermotolerant coliforms (MPN/100 g). [c]TC: Total coliformes (MPN/100 g). [d]Absence. [e]Presence.

In August 2019, the counts of *E. coli*, TTC, and TC at T0 were 1.700, 2.700, and 1.700 MPN/100 g, respectively. These values decreased from time T6 onwards by 99.9% for *E. coli* and TC and 92% for TTC at the end of 36 h of purification.

In the third collection (October 2019), all initial bacterial counts (*E. coli*, TTC, and TC) decreased (99.9%) after the time T6, and remained the same (<1.8 MPN/100 g) until the end of 36 h of purification.

The analysis carried out in December 2019 showed that at the initial time (T0), 610 MPN/100 g of *E. coli* progressively decreased until time T36, with a final reduction of 99.9 %. However, the count of TTC decreased at T6, with a subsequent increase at times T12, T24, and T36, and with a decrease of only 16.1 % of the initial load at the end of the purification. At T6 and T12, total coliform count decreased and increased again at T24, with a 78% reduction in the bacterial count at T36.

The presence of *Salmonella* spp. was observed in the pre-purified oyster samples in August and was no longer detected between the purification intervals.

The analysis of the variation in the density of microorganisms showed that the greatest differences during the purification intervals were T0 - T12 and T0 - T36 for *E. coli*; T0 - T24 for TTC; and T0 - T12 and T0 - T24 for TC.

In cultive water samples, *E. coli* counts, TTC, and TC were between 2 and 43 MPN/100 mL, 4.5 and 460 MPN/100 mL), and (2 and 460 MPN/100 mL, respectively. The highest number of microorganisms was observed in June and December. The count of *E. coli,* TTC, and TC in water purification was between 2 and 360 MPN/100 mL), 2 and 460 MPN/100 mL), and 2 and 240 MPN/100 mL, respectively. Table 2.

Table 2. Microorganism count (MPN/100 mL) in water samples from oyster cultivation in the Urindeua River and water (T0) used for purification process.

Month	Microorganism (MPN/100 mL)	Farming water	Water purification (T0)
June	*E. coli*	43	360
	TTC[a]	460	460
	TC[b]	460	240
August	*E. coli*	2	2
	TTC[a]	4.5	2
	TC[b]	2	2
October	*E. coli*	13	33
	TTC[a]	33	33
	TC[b]	11	33
December	*E. coli*	33	33
	TTC[a]	79	33
	TC[b]	79	33

[a]TTC: Thermotolerant coliforms. [b]TC: Total coliformes.

Throughout the purification process, there was variation in the physical-chemical parameters of the water. Table 3.

Table 3. Physicochemical factors of the water used to purify the oysters in different times.

Month	Physicochemical factors

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	Temperature (°C)	pH	Salinity (°/°°)	Turbidity (NTU)	OD[a] (mg/L)
June					
T0	27.9	8.8	25.3	58.9	6.7
T6	28.4	8.0	23.6	29.8	6.9
T12	30.9	7.6	22.6	21.3	6.5
T24	40.1	7.9	25.0	3.7	5.2
August					
T0	29.2	8.1	30.0	30.9	4.2
T6	27.2	8.1	29.4	31.3	4.5
T12	27.0	8.0	29.3	30.8	4.2
T24	27.8	7.8	29.5	3.5	4.4
T36	27.6	7.8	29.1	3.5	4.0
October					
T0	29.4	8.0	20.6	18.7	6.4
T6	26.1	7.6	20.4	8.8	6.9
T12	26.3	7.4	20.4	8.9	6.0
T24	26.2	7.3	20.5	4.9	7.0
T36	25.4	7.6	20.7	8.2	6.5
December					
T0	29.4	8.1	37.4	18.7	3.9
T6	26.7	7.7	36.7	10.5	3.8
T12	25.6	7.7	36.8	8.5	4.4
T24	24.4	7.8	36.8	8.7	4.8
T36	24.3	7.6	36.9	4.4	4.7

[a]OD: dissolved oxygen.

The highest temperature was 40.1 ºC in T24 due to a failure in the refrigeration system, which led us to interrupt the experiment. Over the months of the study, the temperature value at T0 (pre-depuration) varied between 27.9 - 29.4 ºC.

Salinity had little variation in relation to T0. The lowest salinity observed in T0 was in October. However, no influence of this low temperature was observed on the efficiency of purification, which managed to reduce 99.9% of the number of E. coli and coliforms. Table 1.

The turbidity decreased considerably throughout the process, probably due to the removal of particles by the filter attached to the system. The highest turbidity was observed in the T0 (58.9 NTU) of the water collected in June. That same month, we also observed the highest counts of E. coli and coliforms. It is likely, in the experiment carried out in June, the combination of physicochemical factors in the water (high turbidity and pH before purification; increase in temperature throughout the process) influenced the elimination of TTC, and TC, which were in high concentration in the oyster submitted to purification. Table 4.

Table 4. Correlation between physicochemical factors of water and microorganism count in oysters submitted to purification process.

Physicochemical factors	Microorganism					
	E. coli		TTC[a]		TC[b]	
	rs	*p*	*rs*	*p*	*rs*	*P*
Temperature (°C)	0.64	0,002*	0.54	0,016*	0.61	0,004*
pH	0.51	0,023*	0.60	0,006*	0.62	0,004*
Salinity (°/°°)	0.11	0,642	0.19	0,425	0.19	0,433
Turbidity (NTU)	0.51	0,022*	0.53	0,016*	0.60	0,006*
OD[c] (mg/L)	0.10	0,475	0.03	0,899	0.06	0,784

[a]TTC: Thermotolerant coliforms. [b]TC: Total coliformes. [c]OD: dissolved oxygen.

4. Discussion

Oyster samples from the Urindeua River estuary showed *E. coli* counts above 230 MPN/100 g in June, August and December 2019, inacceptable limits for direct marketing. High TTC and TC counts, as well as *Salmonella* spp. were also found. Thus, post-harvest treatment is necessary for further purification of oysters before consumption (Brazil, 2012; Brazil, 2020).

In a study on the sanitary quality of oyster culture by Oliveira et al. (2020) in the northeastern mesoregion of Pará, the density of *E. coli* was more than that allowed by the Brazilian legislation for oysters from the same estuary investigated in this study. They analyzed oysters cultivated in three other cultivation areas located in the municipalities of Curuçá, Augusto Corrêa, and São Caetano de Odivelas, in same region, and observed that the *E. coli* counts were within acceptable limits.

Silva et al. (2020) observed the presence of *Salmonella* spp. in oyster meat from estuaries of the municipalities of Augusto Correa and Curuçá, and 38.33% of the samples were contaminated. The presence of this pathogen in oysters from this region was previously described by Veríssimo (2018) in an analysis of oysters sold in a beach in northeastern Pará, and 33.3% of the samples showed growth of *Salmonella* spp. Many reports in the literature have associated this bacterium to be one of the main etiological agents involved in food-borne disease outbreaks in several countries; thus, its presence in raw foods, such as oysters, can pose a potential risk to human health (Maijala et al. 2005; Luvsansharav et al. 2020; Marshall et al. 2020).

Brazilian legislation does not include criteria for evaluating the amounts of coliforms or microorganisms such as protozoa or viruses in raw bivalve mollusks. However, the concentration of coliforms and *E. coli* in this food indicates contamination by fecal material, emphasizing poor sanitary conditions. Furthermore, the presence of *Salmonella* spp. in food makes the food inadequate for consumption (Jeamsripong et al. 2018; Brazil, 2020).

Several studies have reported contamination of cultured water and oysters by various enteric pathogens, and in most cases, these areas are adjacent to urbanized areas with great human impact, boat movement, and ballast water and garbage disposal in river areas (Potasman et al. 2002; Ramos et al. 2010; Figueiredo et al. 2016; Santos et al. 2016). Oliveira et al. (2017) reported that the region where the cultivation area of the present study is located suffers from environmental problems such as poor access to basic sanitation and irregular disposal of waste. This may contribute to the increase in the risk of contamination of soil and surface water by pathogenic microorganisms (Matos, 2010).

Notably in this study, the bacterial density in oysters was higher than that in water, corroborating the results of other studies, which showed bioaccumulation of residues by bivalve mollusks (Potasman et al. 2002; Ramos et al. 2010; Sande et al. 2010; McLeod et al. 2017).

In oyster purification, it was observed that the purification process for up to 36 h was effective in reducing the load of *E. coli* (to ≤ 230 MPN/100 g) and *Salmonella* spp. Corroborating other results reported in the literature, bacteria such as *E. coli* and *Salmonella* spp. can be eliminated with short purification intervals between 6 and 48 h, but other pathogens such as vibrios, viruses, and protozoa may require more time and ideal environmental conditions for their elimination (Love et al. 2010; Corales et al. 2013; Ballesteros et al. 2016; Sutthikornchai et al. 2016; Sorio & Peralta, 2017; Trombeta et al. 2017; McLeod et al. 2017; Torkasky et al. 2018). Dispite this, the effects of purification on the concentrations of thermotolerant coliforms and total coliforms were heterogeneous, with decreases and increases during the process. It was observed that purification was not as effective in reducing the number of these microorganisms with initial high densities (month of June).

Santos et al. (2016) observed that purification over a 24 h interval also did not significantly decrease the number of thermotolerant coliforms in *Crassostrea* spp. The authors attributed the result to the delay in acclimating the mollusks to the purification conditions. In our study, only in December did we observe an increase in thermotolerant coliforms at the end of the experiment.

Forcelini (2009) analyzed the purification time for eliminating thermotolerant coliforms in mangrove oysters and found fluctuations of reduction/increase/reduction in the count of microorganisms during purification, corroborating the result of the present study. According to the author, counts decreased in the first 48 h and increased again with the elimination of pathogens only after 168 h of purification.

Another aspect related to fluctuations in the number of microorganisms during purification would be that bacterial pathogens survived during the process in pseudo feces excreted by bivalve mollusks, which are deposited at the bottom of the purification plant. Thus, depending on recirculation or any other agitation of the water in the container, the bottom sediments could be resuspended, increasing the number of microorganisms in the surface water, which could re-contaminate the mollusks when they feed, before other sanitization such as chlorination and use of ultraviolet light that eliminate the contamination (Lee et al., 2013; McMenemy et al. 2018).

In this study, we observed an inefficiency in the elimination of large amounts of fecal and thermotolerant coliforms in the presence of physicochemical factors in the water, such as high turbidity at the beginning of purification and increase in water temperature throughout this process. Studies have shown that physicochemical parameters of water can interfere with purification dynamics, as they influence the filtration rate of oysters and the survival of pathogens (Efiuvwevwere & Amadi, 2015; McMenemy et al., 2018). The stability of pH, temperature, salinity, and dissolved oxygen helped oysters to adapt to the environment, inhibiting stress, increasing the filtration rate of the oysters, favoring the assimilation of substances present in the water, and favoring digestion and excretion. However, low dissolved oxygen levels can lead to oyster mortality (Mignani et al. 2013; Forcelini et al. 2013; Efiuvwevwere & Amadi, 2015; Freitas et al. 2017).

5. Conclusion

Oysters and water, collected almost every month, during oyster farming in the Urindeua River showed contamination above what is allowed by the Brazilian legislation. The results obtained in this study showed the effectiveness of purification period of up to 36 h in eliminating microorganisms, emphasizing that the adoption of this post-harvest treatment could be an alternative for oyster farmers and/or local traders as a way of improving the sanitary quality of the product intended for consumption. Monitoring these bivalves throughout the year can determine the best period for their harvesting and direct marketing, without the need for a purification process, since most oyster farmers cannot afford to purchase purification equipment, even an adapted handmade one. Thus, the effective inspection of the oyster production in Pará state should be prioritized, including cyanotoxins, as stated in the current legislation. Inspections and control measures need to take into account the scale of production of oysters, which is still small and cannot be evaluated based on the logistical criteria used for farms operating on a large scale. Our study showed even in places little impacted by man, such as the Amazonian region, the cultivation of oysters must be monitored in order to improve the sanitary quality of the product intended for consumption.

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