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Microbiological Quality of the Bivalve Mollusc *Saccostrea Cucullata* in the Boeny Region, Northwest of Madagascar

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Abstract:

The determination of the microbiological quality of the most exploited bivalve species (*Saccostrea cucullata*) in the Northwest part of Madagascar was conducted in the Boanamary and Katsepy areas. Six sites were selected for sampling. Three hundred and sixty (360) samples were collected for the study. The analysis consisted in the evaluation of the microbial load of the total mesophilic flora, fecal coliforms, *Escherichia coli*, coagulase-positive Staphylococci, sulphite-reducing anaerobes and the qualitative research of Salmonella and Vibrio. The results obtained show that 64.72% are satisfactory, 25% acceptable and 10.28% are qualified as unsatisfactory samples. In all the sampling sites, no Salmonella and Vibrio were detected. The anthropic activities and the animals constitute the principal factors responsible of the contamination in those sites. This bivalve is listed among the filter-feeding mollusks that can accumulate contaminants in concentrations higher than those of the ambient water. The oyster is a species of shellfish that is consumed preferably alive or raw and thus it belongs to the category of high risk foods. It has been important to carry out this study in order to improve and guarantee their quality.

Keywords: **Boeny region, *Saccostrea cucullata*, microbiological quality.**

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I. INTRODUCTION

The shoreline area is subject to multiple sources of human and animal contamination. After meat, fish and other types of seafood are the second most important source of animal protein [1]. Bivalve mollusks are aquatic invertebrates, mostly marine, well characterized by the presence of a shell with two mobile valves around a hinge, which protect their bodies in part or totally. The oyster is one of the most famous seafoods and the most consumed by the people. By settling on hard substrates and mangrove roots, natural oyster beds exist (almost) all over the coastal zone in the western coast of Madagascar [2]. The collection of this bivalve mollusc is a highly popular activity on the northwest coast of the island, most of whom are women. This mollusc allows many individuals

living in these areas to provide themselves with a food resource and a means of additional income. By filtering large volumes of water, this shellfish can accumulate pathogenic microorganisms, toxic substances and toxic phytoplankton present in the water. Likewise, it is a food that is often eaten raw or undercooked, so the bacteria it contains are poorly eliminated [3]. Thus, it will play an important role in the transmission of various diseases. In addition, numerous poisoning cases have been reported following the ingestion of shellfish [4]. This article presents the results of the microbiological quality study for oysters collected from two areas located in the Boeny Region (Mahajanga), Northwest coast of Madagascar. This study aims to analyze the flesh and the intervalvular liquid of this mollusc, to check for the presence or absence of pathogenic germs and to determine the bacteria related to fecal contamination or hygiene defects.

II. MATERIALS AND METHODS

The present research was focused on the study of a species of fixed oyster called Saccostrea cucullata. The samples were taken in two (study) areas located on the Northwest coast of Madagascar (Boeny Region).

Area n° I: On the foreshore in the rural commune of Boanamary located at 15°49'55.8" S latitude and 46°19'8.5" E longitude (Figure 1).

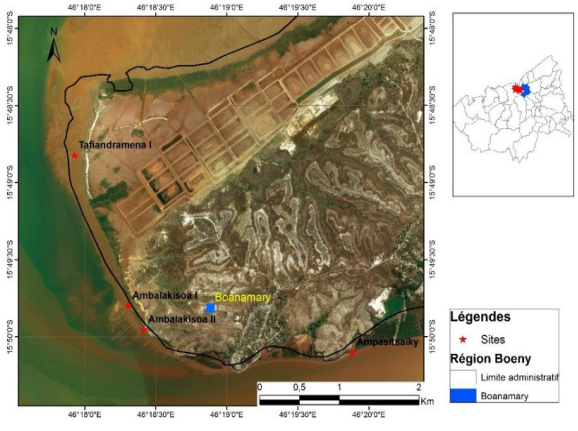

Fig 1: Collection Area n° I

Area n° II: At the foreshore in the rural commune of Katsepy located at 15°46' South latitude and 46°14' East longitude (Figure 2).

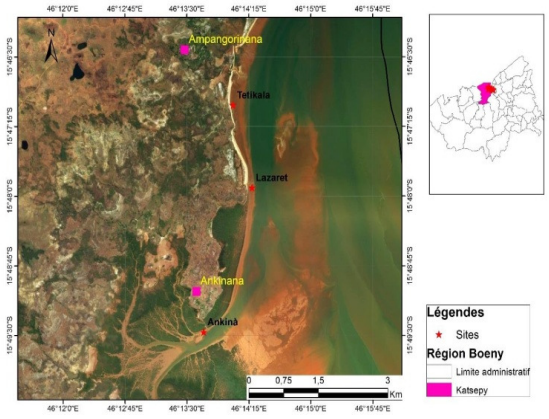

Fig 2: Collection Area n° II

The choice of sampling sites is based on their proximity and accessibility which leads to intensive exploitation. For this study, six sampling sites were selected and three hundred and sixty (360) oysters were collected. The analyses have been focused on the determination of Total Aerobic Mesophilic Flora (TAMF), Anaerobic Sulfite Reducing (ASR), Fecal Coliforms (FC), Staphylococcus (SP), *Escherichia coli* (EC) with particular attention in the search for Salmonella and Vibrio. The collection of the samples was carried out at the time of low spring tide at the level of the mediolittoral stage. The samples are placed in a basin and washed under seawater to remove the external dirt of the shell. After washing, they are placed in a cooler and quickly transported to the laboratory.

A. Preparation of the initial suspension and decimal dilutions

The uniformity and the representativity of the sampling are respected. The preparation of the initial suspension and the different dilutions are done by following the French standard NF EN ISO 6887-1 [5]. Under the hood after flaming, the shell shucking was done near the Bunsen burner with a sterile knife. After aseptic opening, the entire

contents (flesh and inter-valvular fluid) are retrieved in sterile boxes.

B. Inoculation and incubation mode

The inoculation of countable microorganisms was carried out either at depth or at the surface based on the appropriate standard. The culture media and incubation modes are respectively shown in Table I.

Table I: Culture media and incubation mode

Germes	Inoculation	Culture media (about 15 ml)	Incubation mode	
ASR	5 ml	Tryptone Sulfite with Cyclosérine (TSC)	46 °C	20 h
FAMT	1 ml	Plate Count Agar	30 °C	72 h
Staphylocoques	0,1 ml	Baird Parker	37 °C	48 h
CF	1 ml	Violet Red Bile Lactose	44 °C	24 h
Escherichia coli	2,5 ml (x 2)	Tryptone Bile X-β-D glucuronide	44 °C	24 h

Salmonella was tested according to ISO 6579 [6]. It requires the four classical procedures, a pre-enrichment EPT in 37±1°C for 18 to 24 hours, an enrichment in liquid selective medium Rappaport Vassiliadis Soja (RVS) at 41.5±1 °C for 18 to 24 hours followed by the isolation on Hecktoen. If suspected colonies are present, further biochemical and serological analyses are essential for identification and confirmation.

Vibrio was tested in 10g of sample according to ISO 6579 [6]. It requires the standard procedures, a supplementation of Alkaline Salt Peptone Water (ASPW) at 37°C for 10 h followed by isolation on TCBS agar for 18 to 24 h at 37°C. The results of the latter two analyses are expressed in terms of presence or absence.

C. Enumeration of the different germs

1) Total Aerobic Mesophilic Flora (TAMF)

The characteristic colonies are white or yellowish white in color. The standard NF ISO 4833-1 [7] was used as a reference technique for the enumeration of Total Aerobic Mesophilic Flora (TAMF). The horizontal method was adopted in a solid medium consisting of determining the colony forming unit(s) expressed in gram or milliliter of sample using the following formula:

$$\mathbf{N} = \frac{\sum a}{V(n_1 + 0{,}1n_2)d}$$

With**:**

N: Number of colonies per gram (CFU/g) or per milliliter (CFU/ml)

∑a: Sum of CFU counted on all retained plates of two successive dilutions, at least one of which contains a minimum of 15 blue CFU;

V: Volume of inoculum inoculated;

$\mathbf{n_1}$: Number of plates retained at first dilution;

$\mathbf{n_2}$: Number of plates retained at second dilution;

d: Dilution factor corresponding to the first dilution.

2) Fecal coliforms (FC)

The enumeration of fecal coliforms or thermo-tolerants was carried out according to the NF ISO 4832 standard [8] and expressed according to the following equation:

$$\mathbf{N} = \sum C / 1.1 \times D$$

With:

∑C : Sum of the characteristic colonies counted on the two selected plates

D: Dilution rate corresponding to the first dilution counted.

3) *Escherichia coli* (EC)

Escherichia coli (*E. coli*) is a Gram-negative bacterium belonging to the Enterobactericeae family. It has been enumerated according to the NF V08-053 standard [16] and expressed according to the formula:

$$\mathbf{N} = \frac{\sum a}{V(n_1 + 0{,}1n_2)d}$$

When both plates contained less than 15 characteristic CFU, the estimation of small

numbers has been performed according to the same principle as the previous TAMF.

4) Sulfite-reducing anaerobes (SRA)

The enumeration of Sulfite-Reducing Anaerobes was performed according to the NF V08-061 standard [10] and expressed according to the formula:

$$N = \sum C / V\,(n_1 + 0{,}1n_2)\,d$$

With:

ΣC: Sum of the colonies counted on the two retained plates

$\mathbf{n_1}$: Number of plates retained at first dilution

$\mathbf{n_2}$: Number of plates retained at second dilution

d: Dilution rate corresponding to the first dilution retained

V: Volume of inoculum added to each plate, in milliliter.

5) Coagulase positive presumptive pathogenic staphylococcus (PS)

The presumed pathogenic coagulase-positive Staphylococcus germ was enumerated according to the NF V08-057-1 standard [11] expressed according to the formula:

$$N = \frac{\sum a}{V.1{,}1\,.F} \qquad a = \frac{b^c}{A^c} \cdot c^c + \frac{b^{nc}}{A^{nc}} \cdot c^{nc}$$

With:

$\mathbf{A^c}$: Number of characteristic colonies transplanted

$\mathbf{A^{nc}}$: Number of characteristic colonies transplanted;

$\mathbf{b^c}$: Number of characteristic coagulase-positive Staphylococcus colonies;

$\mathbf{b^{nc}}$: Number of characteristic coagulase-positive Staphylococcus colonies;

$\mathbf{C^c}$: Total number of characteristic coagulase-positive Staphylococcus colonies per plate;

$\mathbf{C^{nc}}$: Total number of characteristic coagulase-positive Staphylococcus colonies per dish;

$\sum a$: Sum of coagulase positive Staphylococcus colonies identified for two retained plates;

F: Dilution rate corresponding to the first dilution retained;

V: Volume spread on each plate.

D. ***Interpretation of results***

The interpretation of the analytical results is done according to a three-class plan following the standards of the general product requirements NF ISO 7218 [12].

Satisfactory	Acceptable	Unsatisfactory
N ≤ m	**m < N ≤ M**	**N > M**

N: number of germs per gram;

m: threshold below which all results are considered "Satisfactory";

M: threshold below which all results are considered "Acceptable" and above which results are considered "Not Satisfactory".

III. RESULTS

A. ***Evaluation of the microbiological quality***

The results of microbiological analyses of the hollow oyster *Saccostrea cucullata* in the Boeny Region recorded in Table n°II show the average values of bacteriological germs searched in the six sites studied in CFU per gram ($CFUg^{-1}$).

No Salmonella or Vibrio were reported in any of the samples. The concentration of germs detected varies from site to site; coagulase-positive Staphylococcus presents low values in all sites.

Table II: Global results of microbiological analysis of *Saccostrea cucullata*

Sites	Germes (UFC.g^{-1})						
	FAMT	CF	EC	SP	ASR	Salmo	Vibrio
A	6,73.10^2	1,91.10^2	1,17.10^1	<50	7,67	Abs	Abs
B	9,83.10^2	3,31.10^2	1,70.10^1	<50	1,37.10^1	Abs	Abs
C	2,27.10^2	2	1,33	<50	3	Abs	Abs
D	2,58.10^5	2,18.10^2	2,92.10^2	<50	1,4.10^1	Abs	Abs
E	4,73.10^2	1,93.10^1	8,67	<50	1,67.10^1	Abs	Abs
F	5,03.10^2	5	1,67	<50	4,67	Abs	Abs
Normes	< 10^5. g^{-1}	< 3. g^{-1}	<2. g^{-1}	< 10^2. g^{-1}	< 10. g^{-1}	Abs/25g	Abs/25g

A:Lazaret ;B:Tetikala ;C:Ankinana;D:Ambalakiso ;E:Tafiandramena;F: Ampasitsaiky; FAMT: Total Mesophilic Aerobic Flora; FC:Fecal Coliforms; EC: *Escherichia coli;* SP: Presumptive Coagulase-positive Staphylococcus; ASR: Anaerobic Sulphite Reducing; Germs; Salmo: *Salmonella sp*; Vibrio: *Vibrio sp*; Abs: Absent

As for the level of germ contamination, Figure 3 illustrates the microbiological quality of the oysters studied from each site and the frequency of their related germ.

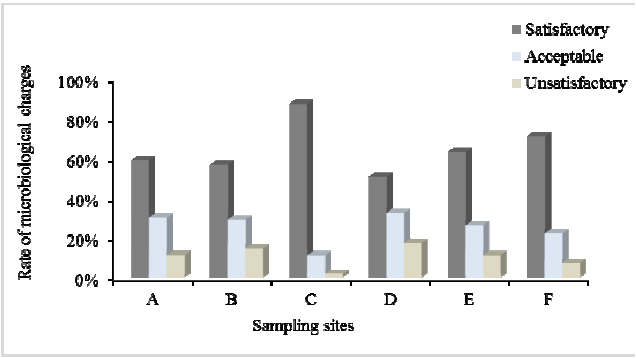

Fig 3: Rate of salubrity by Site

Samples from Site C were more sanitary compared to the other sites with frequencies ranging from 87.22% (satisfactory) to 1.67% (unsatisfactory). Besides, a high germ load was recorded in site D with a rate of 17.22% judged as unsatisfactory.

B. *Evaluation of contamination based on germs*

The following figure 4 illustrates the levels of germ contamination.

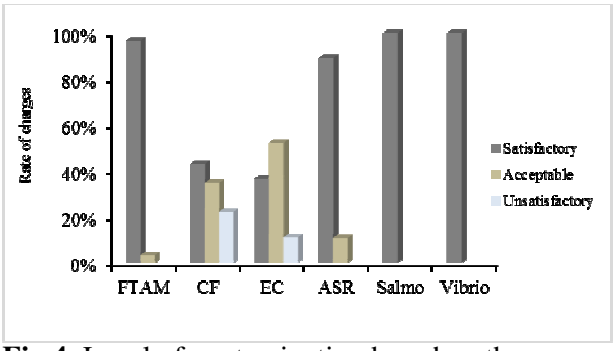

Fig 4: Level of contamination based on the germs

1) *TAMF contamination*

TAMF was present in all samples, 3.33% of the samples tested had loads greater than 10^5 germs per gram and 96.64% had contamination levels less than 10^5 germs per gram. The analyses recorded a significant peak of TAMF at site D respectively 2.58.10^5 (Table II).

2) *Fecal Coliform (FC) and Escherichia coli (E. coli) contamination*

The indicator flora of fecal pollution contains in particular fecal coliforms and *E. coli*. The load of fecal coliforms varies according to the sites with values ranging from 1 to 7.70.10^2 CFUg^{-1}; 43.06% of the samples containing fecal coliforms are considered satisfactory and 22.22% unsatisfactory. Le site B présente la charge maximale 2,18.10^2 UFCg^{-1} tandis que la minimale provient des échantillons du site C respectivement 2 UFCg^{-1}. Site B has the maximum load of 2.18.10^2 CFUg^{-1} while the minimum is from site C samples, respectively 2 CFUg^{-1}.

According to the enumeration values, the samples from site D are the most contaminated with *E. coli*, with a maximum value 4.98.10^{-2} CFUg^{-1}, the minimum value was recorded in site C and F respectively 0 and 1 CFUg^{-1}. 11.11% of the samples showed a level above the reference criteria (Figure 3).

3) *Contamination with Staphylococcus presumed pathogenic*

Analysis of all samples revealed the presence of presumed pathogenic Staphylococcus with loads largely below the required standards.

4) *Contamination by Sulfite-Reducing Anaerobes*

The SRA germs are strict anaerobic bacteria that also indicate fecal contamination. In this analysis, ASR was present in almost all samples with average loads of 9.63 $CFUg^{-1}$.

5) *Salmonella and Vibrio contamination*

This analysis showed the absence of salmonella and vibrio in all samples.

C. *Evaluation of the global contamination level*

According to the three-class design we adopted (satisfactory, acceptable and unsatisfactory), 64.72% of the samples studied are satisfactory, 25% are acceptable and 10.28% are non-compliant or unsatisfactory (Figure 5).

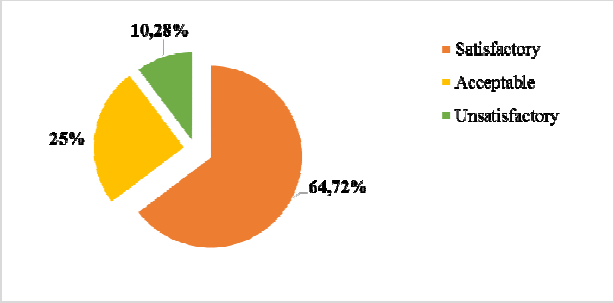

Fig 5: Compliance level of the samples

IV. DISCUSSION

The oyster *Saccostrea cucullata* is one of the target species of Bivalves for foot fishing in the Boeny Region, especially in Mahajanga. Because of their system of filtration of the water in which they live, like all the bivalve molluscs, oysters concentrate in their organisms particles and microorganisms. They are often consumed raw or undercooked, so it plays an important role in the transmission of pathogens.

The microbiological characteristics of oysters have provided information on the hygienic quality of these products. Indeed, the adoption of various methods (qualitative and quantitative), allowed to identify the presence or absence of pathogens and to determine the related concentrations.

The results obtained from these microbiological analyses show the presence of fecal coliforms, *Escherichia coli*, total aerobic mesophilic flora, sulphite-reducing anaerobes (SRA), Staphylococcus presumed pathogenic and the absence of Vibrio and Salmonella.

The prevalence of contamination of the analyzed samples was 86.67%. The dominant pathogenic bacteria in the analyzed samples are total coliforms and *Escherichia coli* with prevalence of 86.11% and 80.56% respectively. These results are almost similar to the results obtained by [13] that evoked that total and fecal coliforms are dominant bacteria in seafood. Among the 10.28% of non-compliant samples, fecal coliforms were identified in 75.68% of the samples or 28lots and 24.32% for *Escherichia coli* or 9lots. These proportions are lower compared to the results obtained in a similar study in Senegal with 100% of samples contained Escherichia coli above the standard and that obtained in Tunisia on 539 samples of clams analyzed 36% have presented a contamination rate by *E coli* at the limit set by the standard [14].

Fecal coliforms are a subgroup of total coliforms capable of fermenting lactose at a temperature of 44.5°C. The species most frequently associated with this bacterial group is *Escherichia coli* [15]. Apart from fecal contamination, fecal coliforms could come from waters rather enriched in organic matter, such as industrial effluents from the pasta and paper sector or from food processing [16]. In addition, fecal coliforms can also allow the detection of fecal contamination resulting from the infiltration of polluted water into the pipes [14]. The high load of these germs in site B ($7.70.10^{-2}CFU.g^{-1}$) could be due to the presence of the pipe just near the village discharging towards the exploitation site.

E. coli is a versatile bacteria that includes commensal bacteria of the digestive tube, pathogenic bacteria and bacteria adapted to the environment belonging to the Enterobactericeae family.

It is part of the intestinal microflora of humans and some animal species that include birds [17]. Like all warm-blooded animals, zebus and small ruminants can harbor enterobacteria in their intestines. These animals will gather in the

mangrove forest right next to the harvest site to enjoy their fruits during low tide.

Due to the movement of this tide, their feces will contaminate the sea water, as well as marine food products. Moreover, in some places where they do not have mangrove forests, these animals feed right next to the oyster bed. The excess of *Escherichia coli* in some of the samples analyzed can be explained by the presence of these animals and also indicates the presence of pollution of fecal origin. Furthermore, the risk of human and animal fecal contamination in bivalve mollusks is estimated by determining the concentration of *E. coli* in samples taken from production areas [18]. Then, like all poultry, ducks and wild ducks that feed in the drain may host this bacterium. The high contamination reported can be explained by the presence of this pipe that drains into the seawater. Gourmelon and al reported the presence of non-O157 (Shiga-toxin producing) *E. coli* in shellfish from the French coast [19]. However, microbiological loads vary from one site to another. The sulfito-reducing anaerobes include different bacteria that multiply in the absence of air and have a remarkable resistance.

Some of them can contaminate seafood as the genus Clostridium [20].

As for the results obtained, 64.72% are satisfactory and 10.28% acceptable. This result is comparable to those obtained by [21] who revealed that 100% of the analyzed seafood is satisfactory.

Mesophiles contain the majority of microorganisms capable of growing in a temperature range of 15-45°C [22]. Our results revealed that all samples were in conformity (satisfactory + acceptable). These results are better than the one obtained by [23] who found $2.9.10^6$ CFU.g-1 against 1.10^5 $CFU.g^{-1}$ on average for all flesh and inter-valvular liquid.

Staphylococci are ubiquitous and pathogenic bacteria, they are widespread in nature (air, water and soil), and can survive in the marine environment [24]. *S. aureus* belongs to the commensal flora of humans and can be found on the skin, pharynx and nasal mucosa [25]. Our results show a very low contamination (< 50 CFU.g-1). Based on the microbiological criterion, the samples are satisfactory. These low numbers can be explained by the application of hygienic rules during samples preparation. These charges are higher than those obtained by [26] in Algeria who showed the presence of *Staphylococcus aureus* in the limpet and sea urchin samples.

Salmonella are enterobacteria of the intestinal flora of vertebrates. They are found in the digestive tract of many food animals (poultry, cattle) and in some pets such as dogs, cats, birds and reptiles [27]. They are responsible for many infections [28].The main mode of contamination in humans is ingestion from water or contaminated food. In our samples, at the six-targeted sites, we did not detect this germ. In this sense, our results agree with those obtained by [29] in the three bivalve species in Morocco.

Vibrios are bacteria belonging to the family Vibrionaceae living in the marine environment. They can be found in seafood as well as in mollusks [30]. For all sites, no Vibrio was recorded in all samples analyzed. Our results agree with those found by [29], that they did not detect *Vibrio cholerae* in the three species of bivalves in Morocco. On the other hand, [31] recorded a *Vibrio parahaemolyticus* species in 1.4% of *Ruditapes decussatus* samples in Tunisia. The density of vibrio varies according to the season and it reaches the maximum during the warm season [32].

V.CONCLUSION

This study has highlighted the microbiological quality of the bivalve *Saccostrea cucullata* populating the Northwest part of Madagascar (Boeny Region Mahajanga). The analyses were focused on the enumeration of hygiene indicator microorganisms and the identification of safety indicator germs. The results have shown a 10.28% contamination rate of non-standard charges related to bacteria of fecal pollution along with the absence of salmonella and vibrio in all samples. With the absence of these two germs and according to the results of surveys conducted among local people living in those areas, the

consumption of this species - in a microbiological sense - is not harmful to human health. And yet, it would be wise to respect the rules of hygiene and to complete this study by other researches, which would aim at the distribution of trace metals in this species.

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