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Molecular identification of non-lactic acid bacteria isolated on MRS medium and associated to the production of biogenic amines in *adjuevan*, a fermented fish of Côte d'Ivoire

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Abstract

Biogenic amines (BAs) are important nitrogenous compounds formed mainly by decarboxylation of amino acids or by amination and transamination of aldehydes and ketones. That compounds have been detected in *adjuvant*, a fermented fish very used as condiments in Côte d'Ivoire. The aim of this study was to identify non-lactic acid bacteria (non-LAB) populations isolated in *adjuevan* on MRS agar and producing biogenic amines. Thus, non-LAB were isolated on MRS agar, then their biogenic amines production was highlighted on Niven agar. Finally, the isolates which produce biogenic amines were identified by sequencing the 16S DNA region. The results showed that non-LAB was able to grow on MRS agar. Their charges obtained in *adjuevan* were between 1.3x103 and 1.95x105 UFC/g. Those bacteria were also detected during the storage of *adjuevan* at ambient temperature and 4°C until six weeks. The prevalence of non-LAB producing biogenic amines was from 8.8 to 100% at ambient temperature and 57% at 4°C in *adjuevan* produced with *Chloroscombrus chysurus.* This prevalence was between 21 and 50% at ambient temperature and 55% at 4°C in *adjuevan* produced with Galeoides decadactylus during six weeks of storage. However, this prevalence was low in the *adjuevan* produced with *Thunnus thynnus.* The species found by molecular identification were *Staphylococcus saprophyticus, Staphylococcus kloosi, Staphylococcus gallinarum, Staphylococcus arlettae, Staphylococcus cohnii, Bacillus subtilis subsp. subtilis, Pseudomonas sp., Enterococcus faecalis, Streptococcus pyogènes and Enterobacter sichuanensis. Staphylococcus saprophyticus* was the most detected biogenic amines-producing species with over 71% of strains.

This study identified non-lactic bacteria species that produced biogenic amines in *adjuevan*, a fermented fish very consumed in Côte d'Ivoire.

Keywords: *Adjuevan*; Biogenic amines; Molecular identification; Non-lactic acid bacteria

1. Introduction

In tropical regions, the traditional procedure of drying, salting, smoking, and fermentation were developed to improve the preservation and the sanitary quality of fresh fish by increasing the lifetime, limiting the loss of nutritional elements, and improving organoleptic quality [1, 2, 3].

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In Côte d'Ivoire, fermentation technics are used for producing fermented fish called *adjuevan* which is frequently used in the food as condiments for its flavor. The production and selling of this fermented fish are important activities because of the quantity of the products on the national and international markets as well as the resulting income for the actors of the sector [1].

However, despite its socio-economic importance, Kouakou *et al*., [2] detected biogenic amines in *adjuevan*, notably histamine. Biogenic amines (BAs) are low-molecular-weight nitrogenous compounds that are formed in foods by microbial decarboxylation of the corresponding amino acids or by transamination of aldehydes and ketones by amino acid transaminases [4].

Biogenic amines have been classified regarded as potentially hazardous compounds of food that may cause disorders in consumers [5]. Ingestion of high levels of such BAs may lead to severe symptoms such as anaphylaxis, hypertensive symptoms, nervous manifestation, and death [6]. In addition, some BAs such as PUT and CAD were associated with the onset of gastric cancer as they may be converted into carcinogenic N-nitroso compounds by microorganisms in the digestive tract [7].

The important biogenic amines, both qualitatively and quantitatively in food and drinks are histamine, tyramine, putrescine, cadavérine, and b-phényléthylamine, products from the decarboxylation of histidine, tyrosine, ornithine, lysine, and b-phénylalanine, respectively [8].

Their presence in food is traditionally used as undesirable activity of microorganisms. The production of BAs in meat products has been attributed to the action of several microorganisms: *Pseudomonas* spp*., Enterobacteriaceae, Enterococci,* and *Lactobacilli* [9].

This study aims to evaluate the potential of these bacteria for biogenic amine production and to identify them by molecular method.

2. Material and methods

2.1. Samples collection and storage

Samples of *adjuevan* for the study were collected from three sellers in three markets of Abidjan (Treichville, Adjamé, and Vridi). The samples of *adjuevan* collected were produced with three species of fish (*Chloroscombrus chysurus*, *Galeoides decadactylus,* and *Thunnus thynnus*). For each seller, 2 kg of *adjuevan* produced with each species of fish were collected. The samples collected were brought to the laboratory in a cooler with dry ice. Once at the laboratory, each sample of *adjuevan* was divided into 9 samples of 110 g each in sterile Stomacher bags. One sample of each species of fish was immediately used for the microbiology analyses. Then four (4) samples were put at 4°C in a refrigerator, and the other four samples were stored at room temperature (28-30°C). Every two weeks, one sample at each storage temperature was used for microbiological analysis. The experiences were carried from each seller in duplicate.

2.2. Enumeration of non-LAB on MRS agar

Ten grams of samples were diluted with 100 ml of distilled water. Then 0.1 ml of the dilutions were spread on MRS agar. Plates were incubated at 30°C for 48 h under anaerobic conditions. Two types of colonies were observed: round, small size, white colonies with characteristics of lactic acid bacteria, and round, small orange colonies. The dishes containing small, round, and orange colonies were selected for counting.

2.3. Isolation of non-LAB

A total of 264 isolated colonies were picked from MRS agar plates and streaked on MRS agar plates for purification. The purity of strains is revealed by homogeneous colonies having the same external appearance (color, size, and shape). Each purified colony was suspended in 1.5 ml of nutrient broth and incubated at 37ºC for 24 h. Then, isolates were maintained in 20% of glycerol at -20°C.

2.4. Highlighting the production of biogenic amines by the isolated bacteria

The strains producing biogenic amines were detected on Niven agar modified as described by Fadhlaoui-Zid *et al*. [10]. The Niven medium (0.5% tryptone, 0.5% yeast extract, 0.5% NaCl, 0.1% $CaCO_3$, 3% agar, 0.006% bromcresol purple, pH 5.3) was supplemented with L-lysine, L-histidine monohydrochloride, L-ornithine monohydrochloride, L-phenylalanine, 0.25% L-arginine, and 0.2% tyrosine disodium salt. The strains were spread plated on the surface of the

medium and the inoculated plates were incubated at 30ºC during 1-3 days. After the incubation time, the color around the colonies was reported. Presumptively, the appearance of a purple or slight-purple color indicated biogenic amine production.

2.5. Molecular identification of bacteria producing biogenic amines

2.5.1. DNA extraction

DNA extraction was carried out according to the method of Hassaïne *et al*. [11]. Bacteria strains stored at −20° C were inoculated into nutriment broth (1.5 mL) for overnight incubation. The Culture was centrifugated (13000 rpm, 10 min), the supernatant was removed and the pellet was resuspended in 200 µL of lysis buffer (2% triton 100X; 1% SDS; 10 mM NaCl; 10 mM Tris-HCl, pH 8, and 1 mM EDTA, pH 8), 0.30 g of microbead 0.5 mm and 200 µl of chloroform/isoamyl alcohol. The mixture was homogenized using a vortex and then centrifuged at 13000 rpm for 5 min. Subsequently, the supernatant was collected and then added with 20 µL of sodium acetate and 600 µL of ethanol. The mixture was centrifuged at 12,000 rpm for 10 min at room temperature. After the elimination of the supernatant, the pellet was washed with 500 µL of 70% ethanol. The DNA is finally obtained after final centrifugation at 13000 rpm for 2 min and drying at 37°C for 30 min. The DNA was resuspended in 100 µl of TE buffer (Tris, 10 mM; EDTA, 1 mM, pH 8) and stored at -20 C.

2.5.2. Amplification of 16S rDNA

The 16S rDNA gene of bacteria producing biogenic amines was amplified with the primers fD1 (5'-AGAGTTTGATCCTGGCTCAG-3') and rDB1 (5'-CCAAGCTTGAGGTTTACAACCCGAA-3') (Eurofins genomic) described by Devi *et al*. [12]. PCR mixtures contained 25 µl of 2X master mix, 0.2 µM of each, primer, and 5 µL of genomic DNA. The cycling program was started with an initial denaturation step at 94°C for 5 min followed by 40 cycles of denaturation at 94°C for 1 min, annealing at 56°C for 1 min, and elongation at 72°C for 1 min. The PCR was ended with a final extension at 72°C for 10 min. The amplicons were visualized using a UV transilluminator (Cleaver Scientific, England) after electrophoresis on 1% agarose gel.

2.5.3. Sequencing of PCR products and identification of bacterial species

The amplified fragments were sent to Eurofins (Germany) for sequencing of the 16S rDNA region. Sequence data were formatted and analyzed using the BioEdit software. The sequences obtained were subjected to the basic local alignment search tool (BLAST) using the 16S ribosomal RNA sequence database to determine the identity of the isolates.

2.6. Statistical analysis

Analysis of variance (ANOVA) is the technique used for the statistical processing of data using R software (R i386 3.1.2). The principle of the analysis of variance test is based on the assumption that the total variability observed in the results is due to a random fluctuation. Tukey's test was also applied considering that the variabilities are significantly different for probabilities less than 0.05.

3. Results

3.1. Charge of non-LAB on MRS agar during storage of *adjuevan* at ambient temperature

Non-LAB on MRS agar were colored in orange. They were present in all *adjuevan* samples with charges between 1.30 x 10^3 and 6.85 x 10^5 CFU/g (Table 1). During storage at room temperature, charges of orange bacteria drop and disappear after two or six weeks depending on the sampling site. Thus, orange bacteria are regularly detected during the first six weeks of storage in *adjuevan* from Vridi. The charges decreased significantly from 1.30 x 10^3 to 1.70 x 10^2 CFU/g for the *adjuevan* produced with *Chloroscombrus chysurus*, and from 1.95 x 10^5 to 1.10 × 10^3 CFU/g for the charges obtained in the *adjuevan* produced with *Galeoides decadactylus*.

On the contrary, for *adjuevan* from Adjamé, the orange bacteria disappeared from the third week of storage. In addition, a decrease in the charge was observed in the second week from 6.75×10^3 to 2.25×10^2 CFU/g and from 4.00×10^3 to 3.0^5×10^2 CFU/g respectively for the *adjuevan* produced with *Chloroscombrus chysurus, and Thunnus thynnus*. As for the *adjuevan* sampled at Treichville, the orange bacteria were detected only in the second week of storage in the samples produced with *Thunnus thynnus* whereas these bacteria were regularly present in the samples produced with *Galeoides decadactylus* until the sixth week of storage. The charges obtained decreased significantly at the 5% threshold from 6.50x10^4 to 1.5x10^2 CFU/g for *Galeoides decadactylus* and from 6.85x10^5 and 1.5x10^2 CFU/g for *Thunnus thynnus*. In all the samples the orange bacteria were not detected at the eighth week of storage.

Table 1 Non-LAB content (UFC/g) of *adjuevan* during the storage at ambient temperature

Sampling site	Fish species used for production	Storage time (week)				
		0	2	4	6	8
Vridi	*Chloroscombrus chysurus*	(1,30±1,84) $\times 10^{3a}$	(9,05±11,4) $\times 10^{3a}$	(2,50±7,78) $\times 10^{2b}$	(1,70±2,40) $\times 10^{2b}$	0
	Galeoides decadactylus	(1,95±0,35) $\times 10^{5a}$	(1,00±1,41) $\times 10^{4b}$	(1,30±1,84) $\times 10^{3b}$	(1,10±1,56) $\times 10^{3b}$	0
Adjamé	*Chloroscombrus chysurus*	(6,75±4,45) $\times 10^{3a}$	(2,25±3,40) $\times 10^{2b}$	0	0	0
	Thunnus thynnus	(4,00±1,41) $\times 10^{3a}$	(3,05±2,47) $\times 10^{2b}$	0	0	0
Treichville	*Galeoides decadactylus*	(6,50±9,19) $\times 10^{4a}$	(1,18±1,44) $\times 10^{4a}$	(1,50±2,12) $\times 10^{2b}$	(1,00±9,3) $\times 10^{2b}$	0
	Thunnus thynnus	(6,85±9,69) $\times 10^{5a}$	(1,45±3,75) $\times 10^{2b}$	0	0	0

On the same line, the values with the same letters are statistically identical at the threshold α= 0,05

3.2. Charge of non-LAB bacteria during storage on MRS agar during storage of *adjuevan* at 4 °C

During storage at 4°C, lactic acid bacteria were regularly detected during the eight weeks of storage in the *adjuevan* samples taken at Treichville (Table 2). Charges decreased significantly from 7.80×10^4 to 6.05×10^2 CFU/g in the *adjuevan* produced with *Galeoides decadactylus* and from 8.01×10^5 to 4.30×10^2 CFU/g in the sample produced with *Thunnus thynnus*. At the Vridi site, these bacteria disappeared after two weeks of storage in the *adjuevan* produced with *Chloroscombrus chysurus* and after six weeks of storage in that produced with *Galeoides decadactylus* (Table 2). In the *adjuevan* samples taken at Adjamé, lactic acid bacteria were not detected during storage at 4°C in the samples produced with *Chloroscombrus chysurus*. On the other hand, in the *adjuevan* produced with *Thunnus thynnus* these bacteria disappear after four weeks of storage (Table 2).

Table 2 Non-lab content (UFC/g) of *adjuevan* during storage at 4°C

Sampling site	Fish species used for the production	Storage time (week)				
		0	2	4	6	8
Vridi	*Chloroscombrus chysurus*	(1,30±1,84) $\times 10^{3a}$	(9,76±7,42) $\times 10^{4a}$	(2,42±3,37) $\times 10^{4a}$	(5,50±7,78) $\times 10^{3a}$	(1,55±1,20) $\times 10^{3a}$
	Galeoides decadactylus	(1,95±0,35) $\times 10^{5a}$	(8,80±9,91) $\times 10^{4ab}$	(4,05±5,59) $\times 10^{3b}$	(2,25±3,04) $\times 10^{2c}$	0
Adjamé	*Chloroscombrus chysurus*	(6,75±4,45) $\times 10^{3a}$	(2,65±2,33) $\times 10^{3a}$	(2,55±3,46) $\times 10^{2b}$	0	0
	Thunnus thynnus	(4,00±1,41) $\times 10^{3a}$	(2,50±2,12) $\times 10^{2b}$	0	0	0
Trechville	*Galeoides decadactylus*	(6,50±9,19) $\times 10^{4a}$	(2,50±4,12) $\times 10^{2b}$	0	0	0
	Thunnus thynnus	(6,85±9,69) $\times 10^{5a}$	(2,90±4,10) $\times 10^{3b}$	(1,50±2,12) $\times 10^{2c}$	0	0

On the same line, the values with the same letters are statistically identical at the threshold α= 0,05

3.3. Prevalence of isolates of non-lactic bacteria producing biogenic amines

A total of 61 strains appeared as presumptively biogenic amine-producer in the differential Niven´s medium. The results of the prevalence of non-LAB producing biogenic amines showed the presence of these bacteria in all the *adjuevan* samples produced with *Chloroscombrus chysurus*, *Galeoides decadactylus* and *Thunnus thynnus* (Table 3). The lowest prevalence was observed in *Chloroscombrus chysurus* 8.8%. At ambient temperature, the percentage increased from 8.8% at the start of storage to 100% at the sixth-week in *Chloroscombrus chysurus*, from 21% to 50% in *Galeoides decadactylus*, and from 11.1% to 37.5% in *Thunnus thynnus* (Table 3).

Concerning storage at 4°C, the prevalence increased to 57% in the second week and then dropped to 25% in the sixth week in *Chloroscombrus chysurus*. In *Galeoides decadactylus*, the prevalence increased from 20% to 55.5% in the sixth week.

Table 3 Prevalence of non-LAB isolates producing biogenic amines during storage of *adjuevan* at ambient temperature and 4°C

Storage Temperature	Fish species used for production	Storage time (week)				
		0	2	4	6	8
Ambient temperature	*Chloroscombrus chysurus*	3/34(8, 8%)	2/14(14, 3%)	3/9(33, 33%)	3/3(100%)	0
	Galeoides decadactylus	4/19(21%)	4/8(50%)	3/14(21, 4%)	0/12	0
	Thunnus thynnus	1/9(11, 11%)	3/8(37, 5%)	0	0	0
Refrigerator (4°C)	*Chloroscombrus chysurus*	3/34(8, 8%)	8/14(57, 1%)	9/25(36%)	4/16(25%)	0/14
	Galeoides decadactylus	4/19(21%)	4/20(20%)	2/9(22, 2%)	5/9(55, 5%)	0/0
	Thunnus thynnus	1/9(11, 1%)	3/13(23, 1%)	0/14	0	0

3.4. Species of bacteria producing biogenic amines identified by sequencing of the 16S rDNA

The length of the amplification products of the 16S rDNA of non-LAB producing biogenic amines during storage of *adjuevan* was 1500 pb (Figure 1). After sequencing the amplified region, species of bacteria were identified with a percentage of homology ranging from 80 to 99%. *Staphylococcus saprophyticus* is the main species with 43 strains identified, followed by *Staphylococcus kloosi* with 6 strains (Table 4). The other species are *Staphylococcus gallinarum* (2 strains), *Staphylococcus arlettae* (2 strains), *Staphylococcus cohnii* (2 strains), and *Bacillus subtilis* subsp. *Subtilis* (2 strains), *Pseudomonas* sp. (2 strains), *Enterococcus faecalis* (2 strains), *Streptococcus pyogenes* (1 strain), and *Enterobacter sichuanensis* (1 strain).

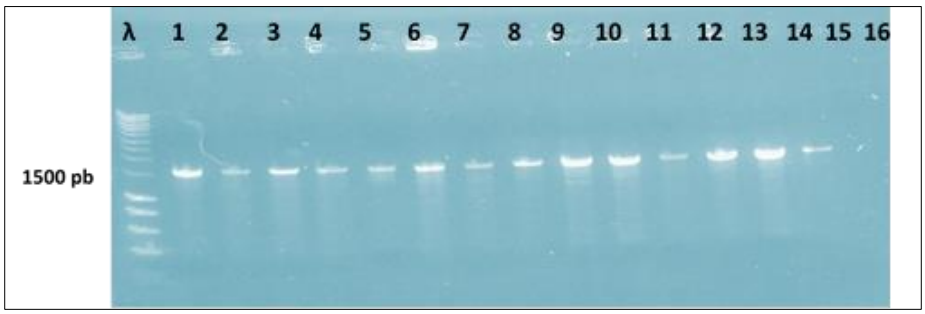

Figure 1. PCR profile of bacteria isolated during storage of *adjuevan* λ: marker "200 bp DNA Ladder"; Lines 1 to 15: non-LAB isolates; Line 16: negative control

Table 4 Homology between the nucleotide sequences of the 16S rDNA of bacteria producing biogenic amines and the sequences of bacteria in the NCBI database

Number of strains sequenced	Matching species in Genbank	Number of nucleotides compared	Number of nucleotides differents	Percent homology
43	*Staphylococcus saprophyticus* subsp *saprophyticus* ATCC 15305	606	6	99
6	*Staphylococcus kloosi* ATCC 43959	520	13	98
2	*Staphylococcus gallinarum* DSM20610	423	14	97
2	*Staphylococcus arlettae* ATCC 43957	192	25	87
2	*Staphylococcus cohnii* subsp *urealyticus* CK27	519	11	98
2	*Bacillus subtilis subsp. subtilis* ATCC 6051	385	21	95
2	*Pseudomonas* sp.	292	57	80
2	*Enterococcus faecalis* ATCC 19433	351	44	87
1	*Streptococcus pyogènes* FDAARGOS	543	13	84
1	*Enterobacter sichuanensis* wchecl 1597	424	27	94

3.5. Distribution of identified species during the storage of *adjuevan*

Species producing biogenic amines were not detected in the samples provided to Treichville and stored at ambient temperature. Only the species *Bacillus subtilis* was isolated in the samples stored at 4°C (Table 5). *Staphylococcus saprophyticus* (43 strains) were the species more frequently present in *adjuevan* samples. Other species were sporadically detected during the storage of *adjuevan* (Table 5). The distribution of the strains producing biogenic amines showed that most of the strains were isolated from the samples provided to Vridi.

Table 5 Distribution of biogenic amine-producing species during storage of *adjuevan*

		Species identified	Storage time (week)				
			0	2	4	6	**Total**
Ambient temperature	Vridi	*Staphylococcus saprophyticus*	5	3	4	5	17
		Staphylococcus kloosi	2				2
		Staphylococcus arlettae				2	2
		Staphylococcus cohnii			2		2
		Enterococcus faecalis			2		2
	Adjamé	*Staphylococcus saprophyticus*	3				3
Refrigerator (4°C)	Vridi	*Staphylococcus saprophyticus*	5	4	5	5	19
		Staphylococcus kloosi	2			2	4
		Pseudomonas sp.		2			2
		Staphylococcus gallinarum		2			2
		Streptococcus pyogènes			1		1
	Adjamé	*Staphylococcus saprophyticus*			4		4
		Enterobacter sichuanensis		1			1
	Treichville	*Bacillus subtilis*		2			2

4. Discussion

This study aimed to identify non-LAB isolated on MRS agar during storage of *adjuevan* and producing biogenic amines. These bacteria were detected in most of the *adjuevan* samples with loadings between $1.95x10^5$ and $1.3x10^3$ CFU/g. The presence of these bacteria in *adjuevan*, could be due to the uncontrolled conditions of *adjuevan* production. Moreover, these production conditions, particularly hygiene during production, vary from one producer to another [3, 13]. This could be the cause of the variation in the load of non-LAB observed on the different production sites. According to Kouakou *et al*. [2], *adjuevan* would be a source of contamination because these authors detected the presence of *Clostridium*, which is a pathogenic germ, and thermotolerant coliforms.

During storage at room temperature, loads of non-LAB decreased then these bacteria disappeared after two or six weeks of storage. The disappearance of non-LAB during storage at room temperature and in the refrigerator could be due to the presence of microorganisms capable of inhibiting the growth of these bacteria. The inhibitory action of microorganisms results from the production of several natural antimicrobial compounds [14]. This inhibitory action could be driven by lactic acid bacteria or yeasts present in *adjuevan*. Several authors have shown that the presence of yeasts and lactic acid bacteria in food could play a beneficial or inhibiting role on certain undesirable bacteria [15, 16]. Yeasts and lactic acid bacteria were also isolated in *adjuevan*. Kouakou *et al*. [13] isolated five main species of lactic acid bacteria there, namely *Lactobacillus fermentum*, *Lactobacillus delbrekii* subsp *bulgaricus*, *Lactobacillus helveticus*, *Leuconostoc lactis* subsp *lactis*, and *Pediococcus pentosaceus*. The yeast species identified are *Saccharomyces cerevisiae*, *Candida tropicalis, Kluyveromyces marxianus*, *Hansenula anomala*. Among the non-LAB detected during storage of *adjuevan*, some were detected to produce biogenic amines. The production of biogenic amines by these bacteria could be due to the presence of proteins whose content is 49.3% in *adjuevan* [1, 17]. These microorganisms would therefore be able to transform free amino acids from proteins into biogenic amines such as putrescine, cadaverine, spermidine,

spermine, aliphatic amines and histamine, tryptamine, tyramine, aromatic amines as shown by Tsai *et al*. [18] and Kerr *et al*. [19]. However, only the histamine content has been regulated by European legislation (EC Regulation No. 2073/2005 of the commission of November 15, 2005). For fishery products, the content is fixed between 100 and 200 mg/Kg. Histamine has been detected in *adjuevan* [2] and in similar products such as *lanhouin* and *guedj* [3]. In humans, biogenic amines are involved in brain activity, body temperature regulation, and gastric acid secretion, immune response, and blood pressure variations [20]. However, biogenic amines can cause adverse effects ranging from irritation to death if consumed in high doses [19]. Thus, the presence of biogenic amine-producing bacteria in *adjuevan* can be detrimental to the health of consumers. Moreover, the thermal stability of histamine does not reduce the risks during cooking [21]. It is not destroyed by freezing, salting, and sterilization [22].

The species of non-LAB producing biogenic amines in *adjuevan* were *Staphylococcus saprophyticus*, *Staphylococcus Kloosi*, *Staphylococcus gallinarum*, *Staphylococcus arlettae*, *Staphylococcus cohnii*, *Bacillus subtilis* subsp. *Subtilis*, *Pseudomonas* sp, *Enterococcus faecalis*, *Streptococcus pyogenes,* and *Enterobacter sichuanensis*. *Staphylococcus saprophyticus* was the dominant species with over 71% of non-LAB producing biogenic amine in *adjuevan*. Species of the genera *Staphylococcus* have also been detected in *adjuevan* [2] and in similar products [23, 24]. Moreover, species of the genera *Staphylococcus*, *Enterobacter,* and *Pseudomonas* have also been identified as producers of biogenic amines [25].

5. Conclusion

The characterization of undesirable germs in foods is necessary in order to determine effective means to ensure their safety. So, this study aimed to identify non-LAB isolated on MRS agar and producing biogenic amines during the storage of *adjuevan*. It appears that non-LAB producing biogenic amines in the *adjuevan* are able to grow on MRS agar. *Adjuevan* obtained with the fish *Thunnus thynnus* was less favorable to the growth of non-LAB producing biogenic amines. *Staphylococcus saprophyticus* was predominantly detected as the biogenic amine-producing species. Due to the presence of these biogenic amine-producing bacteria, the consumption of *adjuevan* could constitute a danger for the consumer.

Compliance with ethical standards

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Disclosure of conflict of interest

The authors of this study declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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