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Biogenic amine producing bacteria in *adjuevan:* A fermented fish condiment produced in Côte d'Ivoire

Marina Ghislaine Abré [1, 2, *], Florent Kouadio N'guessan [1], Clémentine Amenan Kouakou-Kouamé [1], Constant Kouadio Attchelouwa [1], Corinne Teyssier [2] and Didier Montet [2]

[1] *Nangui Abrogoua University, Training and Research Unit in Food Science and Technology (UFR-STA), Laboratory of Food Biotechnology and Microbiology, 02 BP 801 Abidjan 02, Côte d'Ivoire.*
[2] *Centre for International Cooperation in Agricultural Research for Development (CIRAD), UMR 95 QualiSud, 34398 Montpellier Cedex 5, France.*

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Abstract

The traditional fermented fish *adjuevan* is widely used as a condiment in many types of flavorings in Côte d'Ivoire. However, fermentation process and storage conditions may lead to the production of biogenic amines (BAs) which an excessive consumption can induce severe toxicological effects. In order to define adapted strategies for shelf-life extension programs and ensure the safety of consumers, aminogenic enterobacteria and lactic acid bacteria (LAB) were identified during the storage of *adjuevan* in a refrigerator (4 °C) and ambient temperature (28-30 °C). To do so, *adjuevan* samples from the fish species *Chloroscombrus chrysurus, Galeoides decadactylus* and *Thunnus thynnus* were collected from local producers and stored over a period of eight weeks. BA-producing strains were isolated on Niven medium and identified by 16S rRNA gene sequencing. The results showed that BA-producing bacteria were predominantly present during storage at ambient temperature and enterobacteria were dominant, especially in *adjuevan* from *G. decadactylus*, with proportions ranging from 20% to 66.67%. A total of 14 species of BA-producing enterobacteria belonging to genus *Enterobacter, Proteus, Providencia, Klebsiella, Pectobacterium, Shigella, Mixta* and *Escherichia* were identified. The dominant species were *P. vermicola* (25.86%), *E. cloacae* (18.97%) and *E. steigerwaltii* (13.79%). Eight species of BA-producing lactic acid bacteria belonging to genus *Enterococcus* and *Pediococcus* were also identified. The dominant species varied according to the storage temperature and the fish species used. Thus, for *adjuevan* from *T. thynnus, P. vermicola* and *Shigella sonnei* were the dominant enterobacteria species at ambient temperature and refrigeration respectively.

Keywords: Ambient temperature; Biogenic amine; Enterobacteria; Lactic acid bacteria; Refrigeration; Storage

1. Introduction

Biogenic amines (BAs) are nonvolatile low-molecular weight nitrogenous organic bases. They are mainly produced from the microbial decarboxylation of free amino acids [1]. Thus, the most important BAs, histamine, tyramine, tryptamine, putrescine, and cadaverine, are formed from histidine, tyrosine, tryptophan, ornithine and lysine, respectively. BAs are likely to be present in foods containing nitrogen precursors and bacteria with decarboxylase activity, when physicochemical conditions are favourable. So, they have been detected in a variety of foods and beverages, including fish and fishery products, meat and meat products, cheese, wine, vegetables, chocolate and nuts [2-4]. Absorption of small amounts of BAs from the diet is normally not hazardous to health as they can be detoxified by amino oxidases present in the gut. However, high amounts of BAs can lower the food quality and induce several physiological symptoms, such as nausea, respiratory distress, headaches, sweating, heart palpitations and hyper- or hypotension. The main

* Corresponding author: Marina Ghislaine Abré

symptoms associated with some BAs, such as histamine and tyramine, when accompanied by alcohol and acetaldehyde, are nausea, headache and respiratory distress [5].

The amounts of BAs in foods, especially fermented foods, is influenced by several factors during the manufacturing process, including hygiene, composition and quality of the raw materials, and duration of fermentation [6]. Preconditions for the formation of BAs by microorganisms are: the availability of free amino acids, the presence of decarboxylase positive microorganisms, and conditions that allow growth, decarboxylase synthesis and decarboxylase activity [7]. According to Chong *et al.* [8], storage temperature is the most important factor contributing to BAs formation in foods. These factors can influence the formation of BAs in two ways. Firstly, they are responsible for the overall metabolism of the decarboxylating cells. Secondly, the activity of decarboxylases depends on these same parameters [9].

The aminogenic flora is very diverse in its composition. Spoilage bacteria belonging to enterobacteria and pseudomonads can accumulate histamine, putrescine and cadaverine [10, 11]. Therefore, several authors have proposed the BA content as an index of food microbial quality [12, 13]. *Photobacterium phosphoreum* and *Morganella morganii* are the main Gram-negative bacteria that produce histamine and tyramine in seafood products preserved under vacuum or modified atmosphere. In addition, *Morganella morganii, Hafnia alvei* and *Klebsiella pneumoniae* isolated from tuna and *Aeromonas hydrophila* isolated from mackerel have been identified as histamine producers [6]. Decarboxylase activity has also been described in Gram-positive microbial groups such as staphylococci, *Bacillus* spp. and, in particular, lactic acid bacteria (LAB), which are considered the most efficient tyramine producers [14]. Furthermore, the ability to produce histamine, cadaverine and putrescine by LAB has been reported [11]. In recent decades, the demand for safer food has led to more research on biogenic amines [15].

Adjuevan, an Ivorian traditional fermented fish, is a condiment widely used as a flavoring agent in soup and sauces, giving a particular flavor and authenticity [16]. It is produced from all freshwater and marine fish in Côte d'Ivoire. Some fish species are extensively used either for their availability in all seasons of the year, or due to the good quality of the finished product while others are used because of their affordability to all social classes after fermentation. Thus, the species *Chloroscombrus chrysurus* commonly known as "Lagba lagba" is the most used species followed by *Galeoides decadactylus* (false captain), *Pseudotholithus* sp (sosso) and *Thunnus thynnus* (tuna) [17]. Like other African fermented fish, a*djuevan* is produced by spontaneous or uncontrolled fermentation that could lead sometimes to a product with variable qualities with occasional public health hazards [18]. In fact, Abré *et al.* [19] found histamine, cadaverine, putrescine and tyramine as the major BAs in samples stored at ambient temperature (28-30 °C) and in a refrigerator (4 °C) over a period of eight weeks. They also reported that for certain BAs such as histamine, their contents were over the maximum level allowed by *Codex Alimentarius*.

In order to define adapted strategies for shelf-life extension programs and ensure the safety of consumers, this work was carried to identify aminogenic bacteria (LAB and enterobacteria) in *adjuevan* produced from different fish species during their storage in a refrigerator (4 °C) and ambient temperature (28-30 °C).

2. Material and methods

2.1. Sampling procedure and storage conditions

The samples used in this work were a*djuevan* from fish species *Chloroscombrus chrysurus*, *Galeoides decadactylus* and *Thunnus thynnus*. These samples were produced by local producers located in the district of Abidjan (southern of Côte d'Ivoire) according to fermentation method 1 described by Kouakou *et al.* [17]. Samples were collected from three sites of production (Treichville, Adjamé and Vridi). At each site, samples consisting of around 2 kg of *adjuevan* produced from each fish species were collected in sterile bags and transported to the laboratory in icebox containing ice accumulator. Once at the laboratory, samples were subdivided into nine parts of 200 g each and packed in sterile Stomacher bags. Subsequently, one sample was used for initial microbiological analysis (fresh sample). The eight others were then stored, four at ambient temperature (28-30 °C) and four others in a refrigerator (4 °C) for eight weeks. Every two weeks, one sample at each storage temperature was used for microbiological analysis. A total of 162 samples were analysed (3 fish species x 3 production sites x 9 sub-samples x 2 repeats).

2.2. Bacterial count

Around 10 g of sample taken at the level of the fermented fish skin, abdomen and head were grounded, suspended in 90 ml of sterile buffered peptone water (Biokar-diagnostics, France) and homogenized for around 20 s at normal speed. Subsequently, series of decimal dilutions were performed up to 10^{-5}. The enumeration of total aerobic mesophilic flora

(TAMF) was carried out on Plate Count Agar (PCA, Biokar-diagnostics) after incubation for 24 h at 30 °C. LAB were determined on Man Rogosa and Sharpe Agar (MRS, Biokar-diagnostics). The plates were incubated at 30 °C in anaerobic jars for 48-72 h. Furthermore, enterobacteria were enumerated on Violet Red Bile Glucose Agar (VRBGA, Biokar-diagnostics) after incubation at 37 °C for 24 h. Thereafter, catalase and oxidase tests as well as Gram staining were performed on LAB isolates.

2.3. Isolation of enterobacteria and LAB strains on decarboxylase Niven's medium

The strains of enterobacteria and LAB having amino acid decarboxylase activity were revealed on modified Niven's agar as described by Fadhlaoui-Zid *et al.* [20]. This medium was constituted of 0.5% tryptone (Sigma-Aldrich, France), 0.5% yeast extract (Laboratoires Humeau, France), 0.5% NaCl (Sigma), 0.1% $CaCO_3$ (Carlo Erba Reagenti, Italy), 3% Agar (Biokar-diagnostics, France), 0.006% purple bromocresol (Sigma) supplemented with L-lysine monohydrochloride, L-histidine monohydrochloride, L-ornithine monohydrochloride at 0.25% and tyrosine disodium salt at 0.2%. All these amino acids were from Sigma (France). The pH was adjusted at 5.3 with HCl 1 N solution. Plates were incubated at 30 °C for 24-72 h and purple or slightly-purple colonies were isolated as potential biogenic amine producers.

2.4. Molecular identification of the biogenic amine-producing strains

DNA from strains that showed purple or slightly-purple colour on Niven's agar were extracted by heat shock [21]. To do so, a pure colony of each strain was inoculated into a microtube containing 100 µl of sterile Ultra-pure water (Invitrogen). The mixture was heated at 100 °C for 10 min, then immediately put at -20 °C for at least 30 min. The quality and the quantity of the extracted DNA were checked using the spectrophotometer Nanodrop (Biospec-nano, Shimadzu, Japan).

Identification of the aminogenic strains was done by amplification of the 16S rRNA gene using the universal primers 27f (5'-GTGCTGCAGAGAGTTTGATCCTGGCTCAG-3') and 1492r (5'-CACGGATCCTACGGGTACCTTGTTACGACTT-3') [21]. PCR was performed in 25 µl of reaction mixture containing 6.5 µl of molecular water, 0.5 µl of each forward and reverse primers at 10 µM (Sigma, USA), 12.5 µl of Phusion Master Mix 2X (Biolabs, France) and 5 µl of DNA extract. The reactions were conducted using a thermal cycler Mastercycler X50s (Eppendorf AG 22331 Hamburg, Germany). The PCR reaction was performed under the following conditions: initial denaturation at 94 °C for 3 min followed by 35 cycles consisting of denaturation at 94 °C for 30 s, annealing at 55 °C for 30 s, extension at 72 °C for 1 min and a final extension step at 72 °C for 5 min. The amplified products were controlled in a 2% agarose gel in Tris-Acetate-EDTA 0,5X and the expected band size was about 1500 bp [21]. The PCR products were further sent to the GenSeq platform (University of Montpellier, France) for sequencing. The sequences were analysed using the BLAST program on NCBI. Then, the strains were identified by comparing the obtained sequences with the sequences deposited in NCBI. Strains with at least 98% homology were considered to the same species.

2.5. Statistical analysis of the data

The statistical analysis was performed using analysis of variance (ANOVA) and Tukey HSD tests. These tests were carried out with XLSTAT software 2021 (Addinsoft, New York, USA) for Microsoft Excel 2019, to compare the microbial loads of the samples analyzed as well as the evolution of biogenic amine-producing strains during *adjuevan* storage. Differences were considered significant at $p < 0.05$.

3. Results

3.1. Influence of storage temperature on the microbial load

The TAMF was detected at both storage temperatures, regardless of the fish species used. At ambient temperature, the load of this flora varied from 3.79±0.32 log (cfu/g) to 6.66±0.31 log (cfu/g) in *adjuevan* from *C. chrysurus* (Table 1). A decrease was observed after two weeks of storage before an increase until the end of storage. In *adjuevan* from *G. decadactylus*, an increase was observed until the fourth week of storage with values ranging from 6.28±0.12 log (cfu/g) to 6.63±0.20 log (cfu/g) followed by a decrease at the sixth to the eighth week. In contrast, in *adjuevan* from *T. thynnus*, a fluctuation was observed and the highest loads were determined at the beginning of storage and after six weeks. During the storage in the refrigerator, the load decreased in *adjuevan* from *C. chrysurus* and *T. thynnus*. In the samples from *G. decadactylus*, the load decreased first to 5.78±0.21 log (cfu/g) after two weeks and increased to the fourth week and decreased again at the end of storage.

Table 1 Evolution of total aerobic mesophilic bacteria load (log cfu/g) during *adjuevan* storage at different temperatures

Storage temperature	Fish species	Storage period (week)				
		0	2	4	6	8
28-30 °C	*Chloroscombrus chrysurus*	6.66±0.31^{a}	3.79±0.32^{c}	4.47±0.46^{bc}	4.87±0.27^{b}	4.85±0.20^{b}
	Galeoides decadactylus	6.28±0.12^{a}	6.58±0.14^{a}	6.63±0.20^{a}	4.71±0.18^{a}	5.71±1.89^{a}
	Thunnus thynnus	5.97±0.08^{a}	4.89±0.08^{b}	4.89±0.12^{b}	5.79±0.09^{a}	4.47±0.14^{c}
4 °C	*Chloroscombrus chrysurus*	6.66±0.31^{a}	4.19±0.08^{c}	4.94±0.19^{b}	3.75±0.38^{c}	2.93±0.22^{d}
	Galeoides decadactylus	6.28±0.12^{a}	5.78±0.21^{b}	6.68±0.23^{a}	5.45±0.11^{b}	4.10±0.09^{c}
	Thunnus thynnus	5.97±0.08^{a}	5.43±0.11^{b}	4.26±0.14^{e}	4.87±0.07^{c}	4.58±0.06^{d}

Values are expressed as means±SD for three independent trials. Different letters (a, b, c, d, e) in the same line indicate significant differences (P <0.05).

At ambient temperature, LAB were only detected at the beginning of storage in the samples from *C. chrysurus* and *T. thynnus* with loads of 1.72±0.19 log (cfu/g) and 5.56±0.17 log (cfu/g), respectively (Table 2). In *adjuevan* from *G. decadactylus*, they were not detected after two weeks of storage. The highest load was observed at the beginning of storage (4.56±0.20 log cfu/g). In the refrigerator, LAB were present throughout storage in samples from *G. decadactylus* and *T. thynnus* with a fluctuation of loads from 4.56±0.20 log (cfu/g) to 2.43±0.13 log (cfu/g) and from 5.56±0.24 log (cfu/g) to 2.33±0.06 log (cfu/g), respectively. In *adjuevan* from *C. chrysurus*, an increase was observed at the beginning of storage to the second week. However, LAB were not detected after two weeks of storage.

Table 2 Evolution of the lactic acid bacteria load (log cfu/g) during *adjuevan* storage at different temperatures

Storage temperature	Fish species	Storage period (week)				
		0	2	4	6	8
28-30 °C	*Chloroscombrus chrysurus*	1.72±0.19	< 1	< 1	< 1	< 1
	Galeoides decadactylus	4.56±0.20^{a}	4.34±0.28^{a}	< 1	< 1	< 1
	Thunnus thynnus	5.56±0.08	< 1	< 1	< 1	< 1
4 °C	*Chloroscombrus chrysurus*	1.72±0.27^{b}	2.38±0.08^{a}	< 1	< 1	< 1
	Galeoides decadactylus	4.56±0.20^{a}	2.61±0.16^{c}	3.86±0.20^{b}	3.58±0.14^{b}	2.43±0.13^{c}
	Thunnus thynnus	5.56±0.24^{a}	3.51±0.11^{b}	2.80±0.22^{c}	3.55±0.18^{b}	2.33±0.06^{c}

Values are expressed as means±SD for three independent trials. Different letters (a, b, c) in the same line indicate significant differences (P <0.05).

Enterobacteria were more present at ambient temperature than in the refrigerator (Table 3). In *adjuevan* from *C. chrysurus* and *G. decadactylus*, the load decreased until the fourth week and then increased to reach respectively 4.66±0.18 log (cfu/g) and 5.63±0.02 log (cfu/g) at the end of storage at ambient temperature. In contrast, in *adjuevan* from *T. thynnus*, an increase was observed until the fourth week of storage. During the storage in refrigerator, these enterobacteria were only detected at the beginning of storage in *adjuevan* from *T. thynnus* with a load of 2.92±0.21 log (cfu/g), as well as during the first two weeks of storage in *adjuevan* from *C. chrysurus*.

Table 3 Evolution of the enterobacteria load (log cfu/g) during *adjuevan* storage at different temperatures

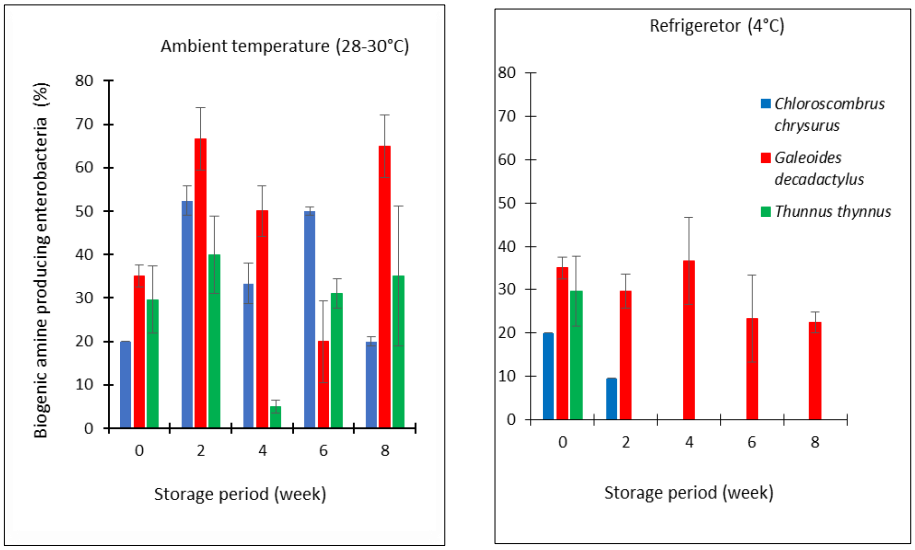
Storage temperature	Fish species	Storage period (week)				
		0	**2**	**4**	**6**	**8**
28-30 °C	*Chloroscombrus chrysurus*	4.66±0.29^{a}	2.86±0.18^{b}	2.79±0.28^{b}	4.72±0.18^{a}	4.66±0.18^{a}
	Galeoides decadactylus	5.55±0.22^{a}	3.41±0.15^{a}	3.81±0.15^{a}	4.44±0.02^{a}	5.63±0.02^{a}
	Thunnus thynnus	2.92±0.28^{d}	3.82±0.22^{bc}	5.57±0.33^{a}	4.49±0.28^{b}	3.27±0.13^{cd}
4 °C	*Chloroscombrus chrysurus*	4.66±0.29^{a}	1.03±0.13^{b}	< 1	< 1	< 1
	Galeoides decadactylus	5.55±0.22^{a}	5.54±0.36^{a}	2.46±0.10^{c}	3.40±0.01^{b}	1.46±0.06^{d}
	Thunnus thynnus	2.92±0.21	< 1	< 1	< 1	< 1

Values are expressed as means±SD for three independent trials. Different letters (a, b, c, d) in the same line indicate significant differences (P <0.05).

3.2. Prevalence of biogenic amine-producing strains in *adjuevan*

Enterobacteria and LAB with positive decarboxylase activity on Niven medium were mostly detected at ambient temperature. Biogenic amine-producing enterobacteria were present throughout storage at ambient temperature, regardless of the fish species (Figure 1a). Indeed, these strains were mostly found in *adjuevan* from *G. decadactylus* with proportions ranging from 20% to 66.67% followed by *C. chrysurus* (20% to 52.38% of isolated strains). Furthermore, the proportion of biogenic amine-producing strains increased from the beginning to the second week of storage and then decreased by the fourth week for all fish species used. But after four weeks of storage, these proportions varied according to fish species. When the samples were stored in the refrigerator, biogenic amine-producing strains were less detected. They were mainly found in the *adjuevan* from *G. decadactylus* with a proportion varying from 22.5% to 36.67% throughout the storage period. For samples from *C. chrysurus* and *T. thynnus*, biogenic amine-producing strains disappeared after four and two weeks of storage, respectively.

Biogenic amine-producing LAB were not detected after the fourth week of storage at both ambient temperature and refrigerator (Figure 1b). At ambient temperature, these strains were preponderant in *adjuevan* from *T. thynnus* followed by *G. decadactylus* where the proportions ranged between 7.69% and 75% and between 21.05% and 42.86% respectively. The maximum values were observed at the fourth week. In the refrigerator, they were dominant at the second week of storage in *adjuevan* samples from *C. chrysurus* where the proportion was 60%. In the samples of *G. decadactylus* and *T. thynnus*, their proportion was between 10% and 18% and between 5% and 10%, respectively.



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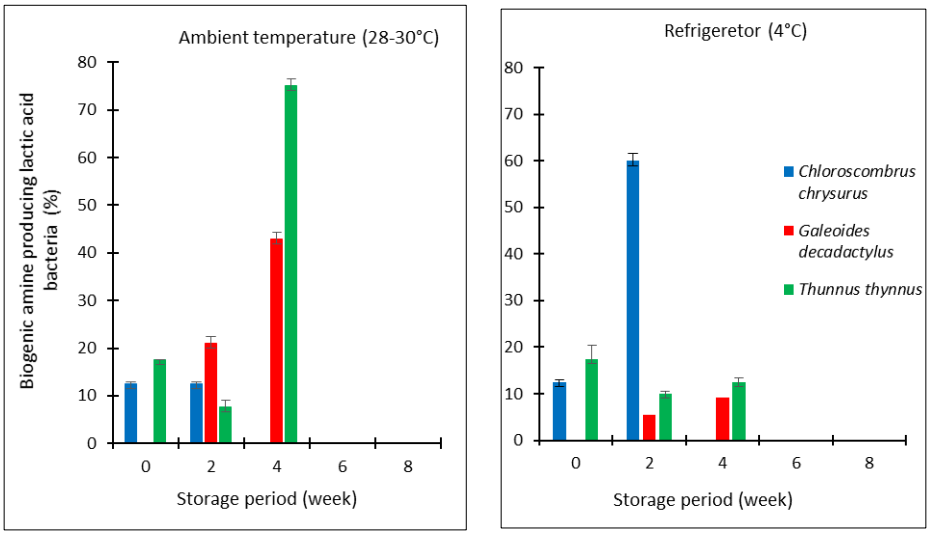

b

Figure 1 Proportion of biogenic amines producing enterobacteria (a) and lactic acid bacteria (b) strains during the fermented fish *adjuevan* storage at different temperatures

3.3. Diversity of biogenic amine-producing strains

In total, 58 strains of biogenic amine-producing enterobacteria and 30 strains of biogenic amine-producing LAB isolated during *adjuevan* storage were further identified using PCR-based methods. When the nucleotide sequences were compared to those available in the Genbank database, 14 species of enterobacteria were identified with a dominance of species belonging to the genus *Enterobacter* (Table 4). These species were *Enterobacter steigerwaltii, E. bugandensis, E. bugandensis-like, Proteus mirabilis, Providencia vermicola, Klebsiella pneumoniae, K. aerogenes, E. cloacae, E. cloacae-like, Pectobacterium aroidearum, P. rettgeri, Shigella sonnei, Mixta calida* and *Escherichia fergusonii*. When we considered the biogenic amine-producing LAB, a dominance of species belonging to the genus *Enterococcus* was observed (Table 5). These biogenic amine-producing LAB were grouped into 8 species which are *E. faecalis, E. faecalis-like*, *Pediococcus pentosaceus, P. pentosaceus-like, E. faecium, E. faecium-like, E. durans* and *E. durans-like*.

Table 4 Homology between the nucleotide sequences of biogenic amine-producing enterobacteria isolated during *adjuevan* storage and the sequences of bacteria in the NCBI database

Sequenced strains	Corresponding species in Genbank	Number of nucleotides compared (pb)	Number of different nucleotides (pb)	Gap	Percentage of homology
ET8, ET19	*Enterobacter hormaechei subsp. steigerwaltii EN-562*	747	0	0	100
ET1, ET11, ET52, ET63, ET64, ET70, ET84, ET98	*Enterobacter hormaechei subsp. steigerwaltii EN-562*	658-765	3-4	0	99
ET2, ET86, ET95	*Enterobacter bugandensis 247BMC*	686-713	3-4	0	99
ET47, ET74	*Enterobacter bugandensis 247BMC*	708-715	18	0	< 98
ET5	*Proteus mirabilis JCM 1669*	556	0	0	100
ET34, ET62	*Proteus mirabilis JCM 1669*	485-717	4-5	0	98-99

ET10, ET15, ET16, ET23, ET29, ET38, ET42, ET48, ET53, ET54, ET75, ET91, ET100, ET101, ET102	*Providencia vermicola DSM 17385*	428-768	2	0-1	99
ET14	*Klebsiella pneumoniae*	688	10	4	98
ET27, ET46, ET61	*Klebsiella aerogenes KCTC 2190*	686-763	0-1	0	99
ET21, ET22, ET26, ET31, ET36, ET39, ET40, ET81, ET82, ET93, ET99	*Enterobacter cloacae DSM 30054*	652-788	1-3	0-1	99
ET18, ET45	*Enterobacter cloacae DSM 30054*	680-770	0-20	0	< 98
ET25, ET77	*Pectobacterium aroidearum SCRI 109*	693	3	0	99
ET65	*Providencia rettgeri DSM 4542*	522	10	1	98
ET50, ET67	*Shigella sonnei ATCC 29930*	686-715	1-8	0	98-99
ET71	*Mixta calida DSM 22759*	781	2	0	99
ET72, ET76	*Escherichia fergusonii ATCC -35469*	671-731	4-6	0	99

Table 5 Homology between the nucleotide sequences of biogenic amine-producing lactic acid bacteria isolated during *adjuevan* storage and the sequences of bacteria in the NCBI database

Sequenced strains	**Corresponding species in Genbank**	**Number of nucleotides compared (pb)**	**Number of different nucleotides (pb)**	**Gap**	**Percentage of homology**
BL2, BL16, BL35, BL36, BL37, BL46, BL52, BL53, BL66, BL86	*Enterococcus faecalis ATCC 19433*	788	0	0	100
BL19, BL45, BL70, BL71, BL76, BL81	*Enterococcus faecalis ATCC 19433*	556-733	0-4	0-2	98-99
BL48, BL63	*Enterococcus faecalis ATCC 19433*	637-640	16-82	3-16	< 98
BL22, BL55	*Pediococcus pentosaceus DSM 20336*	719	0	0	100
BL31, BL64, BL75, BL80	*Pediococcus pentosaceus DSM 20336*	699-739	1 à 2	0-2	99
BL62	*Pediococcus pentosaceus DSM 20336*	664	70	10	< 98
BL17, BL25	*Enterococcus faecium JCM 8727*	727	12	2	98

BL59	*Enterococcus faecium ATCC 19434*	600	83	1	< 98
BL74	*Enterococcus durans JCM 8725*	827	0	0	100
BL3	*Enterococcus durans ATCC 19432*	774	38	2	< 98

3.4. Distribution of biogenic amine-producing strains according to fish species

Table 6 shows the frequency of detection of biogenic amine-producing enterobacteria in the different samples stored at ambient temperature and in refrigerator. At ambient temperature, the main detected species in *adjuevan* from *C. chrysurus* were *E. steigerwaltii* and *E. cloacae* with a proportion of 25%, followed by *Proteus mirabilis* and *P. vermicola* (17%). The species least present in these samples were *E. cloacae-like* and *Pectobacterium aroidearum* with a detection frequency of 8% each. In *adjuevan* from *G. decadactylus, P. vermicola* was the most abundant (32%) followed by *E. steigerwaltii* and *E. cloacae* (23%). *Pectobacterium aroidearum* and *Mixta calida* were detected in a minority (4%). Furthermore, *P. vermicola* was the dominant species in *adjuevan* from *T. thynnus* (35%). *E. steigerwaltii, E. cloacae* and *Shigella sonnei* were more or less detected with proportions varying from 18% to 11%. During the storage in refrigerator, only *P. vermicola* was detected in the samples from *C. chrysurus* (100%). In *adjuevan* from *G. decadactylus*, *Klebsiella aerogenes* and *Escherichia fergusonii* were the most detected with frequencies of 34% and 22%, respectively. For *adjuevan* from *T. thynnus*, the species present were *P. vermicola, E. cloacae, E. cloacae-like* and *Shigella sonnei*.

Concerning biogenic amine-producing LAB, only *E. faecalis* was detected at both ambient temperature and refrigerator in *adjuevan* from *C. chrysurus* (100%) (Table 7). In *adjuevan* from *G. decadactylus, E. faecium* was the most detected species at ambient temperature (40%) while in a refrigerator, *E. faecalis* and *Pediococcus pentosaceus* were the most abundant (33%). In *T. thynnus* samples, *E. faecalis* was the most detected at both temperatures at 50% and 83% respectively.

Table 6 Frequency (%) of biogenic amine-producing enterobacteria during *adjuevan* storage according to fish species

Strains identified	***Chloroscombrus chrysurus***		***Galeoides decadactylus***		***Thunnus thynnus***	
	AT (28-30 °C)	**REF (4 °C)**	**AT (28-30 °C)**	**REF (4 °C)**	**AT (28-30 °C)**	**REF (4 °C)**
Enterobacter steigerwaltii	25	-	23	-	18	-
Enterobacter bugandensis	-	-	9	-	6	-
Enterobacter bugandensis-like	-	-	5	-	6	-
Proteus mirabilis	17	-	-	11	-	-
Providencia vermicola	17	-	32	-	35	20
Klebsiella pneumoniae	-	-	-	11	-	-
Klebsiella aerogenes	-	-	-	34	-	-
Enterobacter cloacae	25	-	23	11	18	20
Enterobacter cloacae-like	8	-	-	-	6	20
Pectobacterium aroidearum	8	-	4	-	-	-
Providencia rettgeri	-	100	-	-	-	-
Shigella sonnei	-	-	-	-	11	40
Mixta calida	-	-	4	11	-	-
Escherichia fergusonii	-	-	-	22	-	-

AT = Ambient Temperature; REF = Refrigerator

Table 7 Frequency (%) of biogenic amine-producing lactic acid bacteria during *adjuevan* storage according to fish species

Strains identified	*Chloroscombrus chrysurus*		*Galeoides decadactylus*		*Thunnus thynnus*	
	AT (28-30 °C)	**REF (4 °C)**	**AT (28-30 °C)**	**REF (4 °C)**	**AT (28-30 °C)**	**REF (4 °C)**
Enterococcus faecalis	100	100	20	33	50	83
Enterococcus faecalis-like	-	-	-	17	10	-
Pediococcus pentosaceus	-	-	20	33	30	-
Pediococcus pentosaceus-like	-	-	-	-	10	-
Enterococcus faecium	-	-	40	-	-	-
Enterococcus faecium-like	-	-	-	17	-	-
Enterococcus durans	-	-	-	-	-	17
Enterococcus durans-like	-	-	20	-	-	-

AT = Ambient Temperature; REF = Refrigerator

4. Discussion

The traditional fermentation process and storage conditions of *adjuevan* may contribute to high microbial contamination and lead to the production of BAs through the presence of strains with decarboxylase activity. The aim of this study was to identify strains of enterobacteria and LAB that are potentially BA-producers. For this purpose, the biochemical detection method based on the modified Niven medium was used. This rapid and inexpensive method of detecting BA-producing bacteria has been used in several recent studies. For example, Soliman *et al.* [22] used it to identify BA-producing bacteria in fish and fish products in Egypt. They obtained a proportion of 44.4% of BA-producing bacteria among which 46.7%, 39.1% and 14.2% were cadaverine, putrescine and histamine producers, respectively. Furthermore, enterobacteria and LAB are the most studied microorganisms with regard to the detection of BA-producing strains using differential media such as modified Niven's medium.

In the present study, the proportions of BA-producing enterobacteria at the beginning of storage were 20%, 35% and 29.63% for *adjuevan* from *C. chrysurus, G. decadactlus* and *T. thynnus* respectively. They were more abundant than LAB and were found throughout the storage period at ambient temperature, regardless of the fish species. However, in the refrigerator, they disappeared after two weeks of storage except in *G. decadactylus*. The storage temperature therefore has an influence on the presence of BA-producing strains. These results are in line with previous studies, according to which the content of BAs in food and beverages increases with time and storage temperature [23]. Thus, 20-37°C would be the optimal temperature for the production of BAs by microorganisms, while a temperature below 5°C or above 40°C would favor a decrease [24]. The increase in temperature would have two main effects: an effect on proteolysis due to increased microbial growth and a direct effect on decarboxylase activity. In contrast to refrigerated storage, the proportions of histamine, cadaverine, putrescine and tyramine producing isolates were higher during storage of *adjuevan* at ambient temperature for all fish species. These results confirm once again the higher impact of temperature on the formation of BAs in food. Furthermore, according to Fadhlaoui-Zid *et al.* [20], enterobacteria are described as strong producers of histamine and the diamines putrescine and cadaverine in fish.

Although diamine production is generally attributed to Gram-negative bacteria, such as enterobacteria and *Pseudomonas*, LAB are considered to be mainly responsible for the production of BAs in fermented foods [25]. So, BA-producing LAB were detected in *adjuevan*. These bacteria were present both at ambient temperature and in the refrigerator. However, their significant decrease at the fourth week of storage in a refrigerator (4 °C) showed their sensitivity to this temperature. The proportions of tyramine-producers were the highest among the tested LAB isolates, regardless of storage temperature and fish species used. These results corroborate those of Pons-Sánchez-Cascado *et al.* [26] who indicated that, in general, LAB are mainly tyramine producers. The presence of these BA-producing bacteria

could be due to the composition, pH, processing and storage conditions [9] of *adjuevan*, which would favor the growth of these bacteria and the activity of decarboxylases.

A total of 14 species of potentially BA-producing enterobacteria belonging to eight genera (*Enterobacter, Proteus, Providencia, Klebsiella, Pectobacterium, Shigella, Mixta, Escherichia*) were identified during *adjuevan* storage. This result is in agreement with those of Nunez *et al.* [27]. Indeed, these authors reported that the main microorganisms associated with the production of histamine and other BAs in fish and seafood are Gram-negative spoilage bacteria belonging to genera *Citrobacter, Klebsiella, Escherichia, Proteus, Shigella, Enterobacter* and *Aeromonas*. In addition, in *suan yu*, a traditional fermented fish from China, Meng *et al.* [28] identified BA-producing enterobacteria belonging to genera *Enterobacter, Klebsiella, Pantoea, Morganella* and *Citrobacter*. Furthermore, the genera *Klebsiella* and *Shigella* are reported to be associated with the production of large amounts of putrescine and cadaverine in food [24]. The dominant enterobacteria species in *adjuevan* were *Providencia vermicola* (25.86%), *E. cloacae* (18.97%) and *E. steigerwaltii* (13.79%). The species of the genus *Providencia* are mainly known as opportunistic human pathogens but have been isolated from a wide range of natural environments. The species *Prov. vermicola* has been reported as a fish pathogen [29]. Therefore, its presence in *adjuevan* is not fortuitous. Moreover, this species belongs to the family Morganellaceae of which several genera and species have been identified as BA producers in various food products [30,28]. This observation therefore confirms the potential of *Prov. vermicola* strains isolated from *adjuevan* as BA producers. This species was particularly dominant in *G. decadactylus* and *T. thynnus* samples stored at ambient temperature. Similarly to our results, in salted and boiled *pindang* fish produced from Indonesia, Rachmawati [31] isolated *P. rettgerii* and *P. rustigianii* as BA producers. The presence of *Enterobacter* species as BA producers has been widely reported in fish or fish products. Thus, *Enterobacter cloacae* has been reported to be dominant among the BA-producing species in sardine and mackerel fish sold in markets in Egypt [30]. This species is found to simultaneously produce putrescine, cadaverine and histamine [26]. It was particularly co-dominant with *E. steigerwaltii* in *C. chrysurus* samples stored at ambient temperature. During refrigerated storage, the dominant species capable of producing BAs were *P. rettgeriii, Klebsiella aerogenes* and *Shigella sonnei*. This variation in dominant species according to fish species and storage temperature may be related to the influence of storage temperature on the bacterial species but also to variable contamination according to the fish species used. *Klebsiella pneumoniae*, a species reported to be a strong histamine producer [24] was also identified in *adjuevan* from *G. decadactylus* stored in a refrigerator.

Although LAB are considered GRAS (Generally Regarded As Safe) organisms, they may have the ability to produce toxic compounds such as BAs in fermented foods and strains of *Lactobacillus, Enterococcus, Lactococcus, Pediococcus, Streptococcus* and *Leuconostoc* have been associated with high levels of these compounds. In this study, BA-producing LAB were mainly represented by species belonging to genera *Enterococcus* and *Pediococcus* with *E. faecalis* (53.33%) and *P. pentosaceus* (20%) as dominant species. The genus *Enterococcus* has not yet been classified as non-pathogenic for human consumption, as most species harbor a range of virulence and antibiotic resistance factors and have been associated with several infections. *Enterococcus* species also have the ability to mediate gene transfer with different genetic elements, including plasmids, phages and conjugative transposons [25]. The role of enterococci in fermented foods remains controversial. They show remarkable ecological adaptability and ability to thrive in adverse conditions. Due to their tolerance to salt and low pH, they are highly adapted to several food systems and they are also involved in the traditional fermentation process of fish and other fishery products [32, 30]. This observation is consistent with our results which showed a strong dominance of species of the genus *Enterococcus* with *E. faecalis* as the dominant species in *adjuevan* from *C. chrysurus* and *T. thynnus*, regardless of storage temperature. In *adjuevan* from *G. decadactylus*, *E. faecium* was dominant at ambient temperature while in a refrigerator *E. faecalis* was codominant with *P. pentosaceus*. These results are in line with those of Küley *et al*. [33] who pointed out that the production of BAs depended on two criteria: the LAB strains and the fish species. *Enterococcus faecium* and *E. faecalis* were furthermore considered to be responsible for BA production in various fermented foods such as soybean [34] and tofu [35]. The presence of enterococci capable of producing BAs is a relevant food issue also in meat products [36]. Komprda *et al.* [37] showed that *E. faecium* and *E. faecalis* possess a tyrosine decarboxylase gene. The results of this study are also in line with Özogul and Hamed [32] who showed that *E. faecalis, E. faecium* and *E. durans* strains are very high tyramine producers. *Pediococcus pentosaceus* as BA producer has also been reported by several authors in fermented foods such as fermented seafood (pickled and smoked mackerel, smoked salmon), meat products (chicken, turkey and beef salami; chicken and beef sausage), and dairy products (traditional Turkish cheese, butter, yoghurt and kefir) [38]. According to Barbieri *et al.* [25], species belonging to this genus are producers of tyramine and cadaverine in beer.

5. Conclusion

Providencia vermicola, Enterobacter cloacae and *Enterobacter steigerwaltii* were found to be the dominant species of BA-producing enterobacteria while *Enterococcus faecalis* and *Pediococcus pentosaceus* were the dominant species of BA-

producing LAB. Compared to enterobacteria, LAB were weakly involved in BA production in *adjuevan* and were fully absent after 4 weeks of storage. Taking into account storage temperature, ambient temperature led to a greater involvement of enterobacteria in the synthesis of BAs, especially in *adjuevan* produced from *G. decadactylus*. On contrary, storage in a refrigerator led to a decrease or even disappearance of BA-producing enterobacteria and LAB, particularly in *adjuevan* produced from *T. thynnus*. Thus, the storage of *adjuevan* at ambient temperature may conduct to a higher risk of BA synthesis, especially during the first two weeks regardless of the fish species used. However, some bacteria, mainly LAB, have been shown to be non-BA producers. These could be used to degrade or reduce the synthesis of these toxic compounds during fermentation and storage at ambient temperature of *adjuevan*.

Compliance with ethical standards

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Disclosure of conflict of interest

The authors declare that they have no competing interests.

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