Assessment of Histamine Formation During Fermentation of Sardine (*Sardina pilchardus*) with Lactic Acid Bacteria

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Abstract: In this survey, the formation of histamine during the fermentation of sardines (*Sardina pilchardus*) with lactic acid bacteria (LAB) at 22°C and 30°C were followed up during an experimental period of 25 days. The microbiological (Standard plate count, LAB, Enterobacteriaceae, Salmonella, Clostridium) and chemical (pH, titratable acidity, TVB-N and proteins) changes in fermented fish were also determined. Results showed a significant ($p < 0.05$) decrease of pH value and histamine content in fermented sardines. The pH decrease in the final product provides evidence of good acidification through LAB fermentation through starter culture. Final histamine values had not exceeded the permissible level at the end of the fermentation, except in non fermented control samples. The growth of standard plate count (SPC) and Enterobacteriaceae was significantly ($p < 0.05$) reduced in fermented sardines. Results of this study also suggest that an extension of the shelf life of fish product can result from the decreased growth of spoilage microflora.

Key words: Histamine · sardine · lactic acid bacteria · fermentation

INTRODUCTION

Fresh fish is an extremely perishable food compared to other food commodities. Sardine "*Sardina pilchardus*" is the most abundant fish species in the Atlantic coast in Morocco. A total of 120 740 tons of sardine was caught during the year 2006 [1]. Sardine is generally consumed fresh canned, salted or marinated and the fish is also utilised as fishmeal and fish oil [2]. Due to acidification and enzymes activities, lactic acid bacteria (LAB) have been playing an important role in food fermented causing flavour and texture changes together with a preservative effect resulting in increase in the shelf life of the transformed product [3, 4]. However, LAB have not been used to any great extent in fish products, with the exception of fish sauces in the South East Asia and silage fermentation [5-7].

Preservation of fermented fish products obviously depends on lactic acid and possibly bacteriocin production. However, for successful fish fermentation,

effective lactic starter cultures are necessary. Huss [8] considered that suitable starter culture must be specifically selected for application to each individual product. Other factors may also contribute to the overall keeping quality of low-salt fermented fish products [9]. The combination of low pH and organic acids (mainly lactic acid) is the main preservation factor in fermented fish products. The primary role of LAB is to ferment the available carbohydrates and thereby cause a decrease in pH.

The developments of new fish products, stable during storage, free of the undesirable odour and taste and retaining all the nutritional advantages of fish, would expand the range of applications of health-giving, fish-based foods. A promising approach to the creation of such fish products seems to be through the use of LAB [10]. Fish sauce contain about 20 g l^{-1} nitrogen; 80% of which is in the form of amino acids. Thus, fish sauce is considered as an important source of dietary proteins and amino acids and has become a

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necessity in the household in South East Asia countries [11]. Since the fermented fish products, including fish sauce and fish paste, have recently been widely utilized in a variety of processed products, their importation to theses countries continue to increase year after year [12].

Biogenic amines, including histamine, are formed through the decarboxylation of specific free amino acids by exogenous decarboxylases released from microbial populations associated with the seafood [13]. Thus mainly histamine has been proposed as a marker to evaluate fish freshness [14]. High levels of histamine in foods can have important vasoactive effects in human [15]. The FDA established a guideline for histamine of 50 mg kg^{-1} and fish with histamine above that level are prohibited from being sold for human consumption [16].

Sufficient amounts of sardine are available in Morocco; there is a possibility of developing new products. One possible alternative is the production of fermented sardines but usage is not common in the country. The objectives of the present investigation were to study the physicochemical and microbiological changes during the fermentation of raw sardines by LAB and also to assess the histamine levels in fermented fish at 22°C and 30°C during an experimental period of 25 days.

MATERIALS AND METHODS

1. Sampling: Samples of sardine *"Sardina pilchardus"* were collected aseptically in sterile poly-bags kept in an ice-box from local market of Rabat and transported to the laboratory. Fresh sardines were headed, gutted and filleted on the arrival. The fillet was then divided into two portions in sterile plastic bags and prepared for fermentation. The preparation process was carried out in a cold room at a temperature of 4°C.

2. Preparation of LAB Cultures: Strains of LAB were preserved in MRS broth (Difco, USA) with 25% (v/v) glycerol at -18°C. The LAB strains used as starter culture were from either frozen or freshly prepared cultures. Both the frozen and the fresh cultures were prepared by incubating (at 1%) 200 ml of MRS broth with a freshly prepared MRS broth culture of and incubating it at 30°C for 18 h. Cells were harvested from the broth by centrifugation for 10 min at 6000x g, followed by filtration of the supernatant, through a Millipore membrane filter (0.45 μm).

3. Characterization of LAB strains: The LAB strains were initially characterized on the basis of Gram-reaction, morphology (phase contrast microscopy), motility, catalase (10% H_2O_2) and Oxidase production. Production of acetoin from glucose was determined by the Voges-Proskauer test (MR-VP medium), hydrolysis of arginine was tested as Leisner [17]. The Growth at pH 3.9 and 6.5, the ability to grow at 10, 15 and 45°C and the growth in MRS-broth containing 5%, 6.5% and 10% NaCl (w/v) were also cheeked. Glucose fermentation and gas production were tested in MRS-broth supplemented with 1% glucose containing inverted Durham tubes at 30°C for 48 h, as described by Dykes *et al.* [18]. Carbohydrates fermentation (acid production) was determined on MRS broth containing bromocresol purple (0.04 g l^{-1}) as a pH indicator. The carbon sources were added to the medium to give a final concentration of 1% (w/v). To ensure anaerobic conditions, each tube was supplemented with liquid paraffin after inoculation [19]. Strains of LAB were finally identified with API 50 CH system (Bio-Merieux, France).

4. Inoculation of Raw Material: Fresh sardines were inoculated with a starter culture of a LAB strain (*Lactobacillus delbrueckii subsp. delbrueckii*) with approximately 10^6 to 10^7 cfu g^{-1}. The inoculated minced fish fillets (1st batch) was thoroughly mixed, packed in plastic bags and left to ferment. After several assays, we have concluded that a carbohydrate source (glucose, 4% w/w) supplemented with salt (NaCl, 5% w/w) (data unpublished) is suitable for fermentation of sardines by LAB. Samples were incubated at different temperatures (22°C and 30°C) for 25 days. The non-inoculated portion (2nd batch) added only with salt served as control. Samples were withdrawn for analysis at predetermined intervals with the pH measurements, chemical and microbiological analysis.

5. Chemical Analysis: The pH values of the fermented fish were measured using a digital pH meter (766 ion analyser, Knick Climatic). 5 g of sample were homogenized in 10 ml of purified water for 1 min in a stomacher and the pH measured with a glass electrode. Appropriate weights were analyzed for protein (total Nitrogen x 6.25), according to AOAC procedures [20]. Total volatile basic nitrogen (TVB-N, mg N/100 g fish) was determined in a distillatory system (UDK 130 A). The distillate was collected in a solution of excess boric acid in the presence of indicators (methyl red mixed with bromocresol green) titrated with sulfuric acid.

Titratable acidity was calculated by titrating the filtrates with NaOH (0.1 N) using phenolphtalein as a pH indicator [20].

Histamine content of fish was determined by the standard AOAC. [20] fluorometric method. For fish samples, 10 g portion were mixed and homogenized by Ultra-turrax T25 (Janke and Kunkel, IKA-Labortechnic, Staufen, Germany) in 90 ml of TCA (10%) and centrifuged, the extract was filtered through whatman paper N°1. The filtrate was fractionated in an ion exchange column packed with amberlite G 50 type 1, 70 to 150 micron (100-200 mesh); then eluted with HCl 0.2 N (20 mL). The column eluant was derivatized with *o*-phtaldehyde (OPA) and fluorescence intensity was determined using a spectrophotofluorometer (spectrometer LS 30) at an excitation and emission wavelength of 350 nm and 450 nm, respectively.

6. Microbiological Analysis: 25 g of fish samples were aseptically weighed into 225 ml of peptone-physiological saline solution (0.1% peptone; NaCl, 0.85% w/v) in a sterile plastic bag and then blended in Stomacher Lab-blender (400, Seward Medical, London, UK) for 30 s prior to measuring the pH. Samples (0.1 ml) of serial dilutions (10^{-1} to 10^{-8}) of fish homogenates were spread on the surface of the appropriate media in petri dishes for determination of:

- The standard plate count (SPC) was determined by pour-plating appropriate dilutions on standard count agar (Biokar, France), the plate were incubated at 30°C for 48h.
- LAB counts were determined by plating appropriate dilutions on MRS-agar (Difco, USA). Plates were incubated for 48 h at 30°C.
- Enterobacteriaceae counts were determined by plating dilutions from 10^{-1} to 10^{-6} on Violet Red Bile Glucose agar (VRBGA; Oxoid CM 458, Basingstoke, Hampshire, England). The plates were incubated at 37°C, for 24 h.
- *Salmonella* were determined on 25g of the sample added to 125 mL of sterile buffered peptone water and incubated at 37°C for 18 h. For enrichment in buffered peptone water overnight, tubes of Rappaport-Vassiliadis broth (Oxoid CM 669) and selenite-cysteine broth (Merck, Germany) were inoculated with 1mL from cultures in buffered peptone water overnight and incubated at 37°C for 24 h. The positive tubes of broth media were streaked on Hektoen agar (Merck, Germany) and Salmonella-

Shigella agar medium, plates were incubated at 37°C for 24 h. Non colored colonies with and without a dark center were purified and streaked on trypticase soya agar (Biokar, France) slants and stored at 4°C.

- Sulfite polymyxin sulfadiazine agar (Merck, Germany) was used for clostridia counts, incubated at 37°C for 24 h.

7. Sensory Evaluation: Oganoleptic analysis was performed by evaluating three characteristics (odour, texture and taste) according to 10 point-scale. Panel of ten persons trained in seafood evaluation were used for sensory assessment. A three-class evaluation scheme was used according to Paleologos *et al.* [14] as follows: class 1: no off-flavours; class 2: slight off-flavors but not spoiled; class 3: unacceptable class, clearly recognizable off-flavors.

8. Statistical Analysis: Results of the microbiological and chemical analyses were reported as means±standard deviation. Data were analyzed with ANOVA test using the General Linear Models Procedure of the statistical analysis system software of SAS Institute [21]. The least significant difference (LSD) procedure was used to test for differences between means at the 0.05 significance level.

RESULTS AND DISCUSSION

1. Characterization and Selection of LAB Strains: Further characterization of LAB strains included criteria essential for selection of strains suitable for fish fermentation under the conditions of our experiment. The identification of strains was based on phenotypic characterisation (biochemical tests and carbohydrates fermentation). Strains isolated were characterized as LAB, based on positive Gram reaction, rod-shaped, no motile, oxidase negative and absence of catalase activity. LAB showed a positive reaction for the Voges-Proskauer test and did not produce the CO_2 from and NH_3, LAB grows at 5%, 6.5% and 10% NaCl (w/v) and at pH values from 3.9 to 6.5. The growth of strains at different temperature values was also tested in MRS broth after incubation for 5-7 days at 10, 15 and 30°C.

Although LAB strains differed in the qualitative changes they induced in fermented sardines, probably because of the differing fermentation capacities of strains under the experimental conditions. Some organoleptic changes in sardines were observed after only one week of fermentation. Among several LAB strains isolated

Table 1: Chemical changes of raw material and fermented sardines

Chemical Parameters	Raw material	Fermented sardines			
		22°C		30°C	
		I	F	I	F
PH	5.87±0,08	6,14±0.05	3.97±0.06	6.10±0.11	4.11±0.06
Titratable Acidity (%)	0.43±0.02	0.83±0.05	2.05±0.10	1.05±0.05	2.17±0.22
Protein (%)	14.16±1.12	13.77±0.10	15.83±0.63	14.18±0.38	15.77±0.46
TVB -N	13.32 ±0,90	14.08±0.27	31.22±0.80	14.46±0.80	35.51±0.77

Values represent mean score (n=3) with standard deviations. I: initial, F: final.

Table 2: Microbial load of fermented fish samples

Assays (T°C)		Log_{10} (cfu g^{-1})		
		SPC	LAB	ENT
22°C	I	4.12±1.11	4.36±1.13	1.69±0.21
	F	3.20±1.41	9.65±0.98	1.30±4.24
30°C	I	5.23±2.00	4.65±3.18	1.35±3.04
	F	3.66±1.48	9.28±5.66	Nd

* Mean value±RSD (Relative standard deviation), n=3; I: initial, F: final;

Nd: not detected   SPC: standard plate count

LAB: Lactic acid bacteria   ENT: Enterobacteriaceae

from sardines, only one strain of LAB (*Lactobacillus delbrueckii subsp. delbrueckii*) was selected for the use in the different experimental assays. Indeed, sensory evaluation showed that this strain gives a good acidification through lactic acid production and was responsible for the disappearance of bad odours and the appearance of positive organoleptic proprieties that were appreciated during the sensory evaluation and sardines fermented with *L. delbrueckii subsp. delbrueckii* achieved a maximum score of 10; the fermented sardines acquired a distinctly meaty taste and scent.

The changes of the microbial flora of fermented sardines are shown in Table 2. Standard plate counts in the raw sardine (fresh material) was $3.0x10^5$ cfu g^{-1}. SPC gives a general information on the microbiological quality of the analyzed food. The general recommended value for the total viable bacteria in fish is $5x10^5$ cfu g^{-1} [22].

Enterobacteriaceae counts in fermented sardine samples reached $2.8x10^3$ and $3.9x10^3$ cfu g^{-1} after 7 days at 15°C, 22°C and 30°C, respectively. The initial count of Enterobacteriaceae is significantly (p < 0.05) reduced and eached inal levels of $1.2x10^1$ and $1.8x10^1$ cfu g^{-1} at the end of the experimental period, respectively. *Salmonella* spp. And *Clostridium* spp. were not detected during the

experiment. Enterobacteriaceae were found to be part of the spoilage microflora of fermented fish. During LAB fermentation, Enterobacteriaceae disappear due to the microbial competition, acidity and may be by the production of inhibitory compounds. Preservation of fermented fish products obviously depends on lactic acid and possibly bacteriocin production [23]. *Lactobacillus sake* strains have been reported in lightly preserved fish products and representatives of these genera are known to produce bacteriocins [24]. The constant low level of Enterobacteriaceae reached during processing may indicate a regular stability and the success of the fermentation process of the fish fillets against the undesirable biochemical breakdown of the organic matter leading to putrefaction of the product during storage. LAB strain inoculating minced sardine were also counted at selected intervals throughout storage period. LAB counts increased rapidly during the first 7 days; thereafter, the growth continued slowly and after three weeks reached maximum values which ranged of $6.4x10^9$ and $3.8x10^9$ cfu g^{-1} in the two samples fermented at 22 and 30°C, respectively [25], using a minced fish-salt-glucose model, suggested that a pH of 4.5 should be reached within the fist 48 h of fermentation. LAB strain used for the inoculation of sardine samples would constitute a suitable starter culture for the fermentation. The high population of LAB found at the end of the fermentation pointed out the suitable conditions for growth of the culture and also the efficiency of the used strain.

Changes in pH values are shown in Table 1. The pH of raw sardine (fresh fish) used in this study was 5.85±0.08. A significant decrease (p < 0.05) in the pH values to relatively low levels were noticed during experiment process. At the end of the fermentation period, the pH was decreased and reached 3.79±0.06 and 4.11±0.06, respectively for sardine stored at 22°C and 30°C (Table 1). The pH decrease in the final product provides a good acidification through LAB fermentation. Similar results were reported in fish waste fermentation and inoculated with a starter culture of *Lactobacillus plantarum* for 20 days [6]. Thus, our results with fermented fish are in agreement with previous reports of Gelman *et al*. [10] and Milton *et al*. [24]. For titratable acidity, values increased from 0.83±0.05 to 2.05±0.10 and from 1.05±0.05 to 2.17±0.22, respectively for sardine fermented at 22 and 30°C (Table 1). Similar results showing a net decrease of pH value and an increase of the titratable acidity in fermented fish were reported by Milton *et al*. [24].
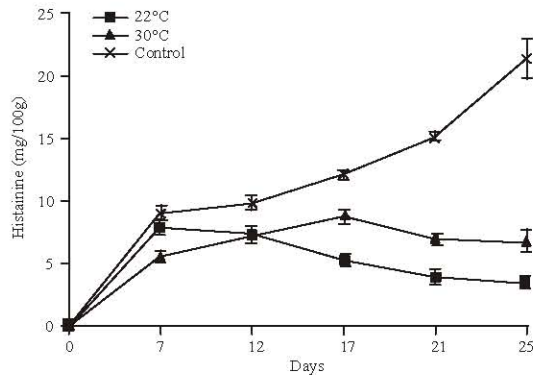
Fig. 1: Histamine levels in sardines during the experimental period

The protein levels in fermented fish were higher (p<0.05) in comparison with protein value (14.16±1.12%) of the raw control sample (Table 1). During the fermentation period, a significant increase (p<0.05) in the TVB-N values to relatively high levels of 31.22±0.80 and 35.51±0.77 mg N/100 g was observed in fermented fish at 22°C and 30°C respectively (Table 1). The control assay (43.26±0.50 mg N/100 g fish flesh, data not shown) exceeded the maximal permissible level (25 to 35 mg N/100 g) proposed by Ababouch *et al.* [26] for Moroccan sardines and 35 mg TVB-N/100g fish flesh, specified by the EC guidelines [27]. El Marrakchi *et al.* [28] reported that the TVB-N value was more useful for assessing the degree of sardine deterioration than for evaluating the changes occurring during the first storage stages; the TVB-N value is affected by fish species, age and sex; the catching season and the region of fishing.

Generally, it is difficult to establish the limits of acceptability especially for the TVB-N, because of the big variability between species and regions, particularity for fat fish [29]. The slight increase in TVB-N during the first stage of fermentation may be generally caused by autolytic enzymes and desamination. Such increases in TVB-N can be explained easily by the volatile basis production (NH3, TMA, DMA, hypoxanthine) and non volatiles (histamine) and those compensatory of free fatty acids resulting from lipids deterioration [30]. Similar results of the increase in TVB-N were reported by Gelman *et al.* [10] in fermented fish (Yellowfin tuna "*Thunnus albacores*"), by Goulas and Kontominas [31] in brined chub mackerel, by Karaçam *et al.*[32] in brined anchovies and by Sallam *et al.* [33] in brined or marinated fillets of Pacific saury (*Cololabis saira*). Conversely, a much higher TVB-N level (60.5 mg N/100 g) was reported by Chouliara *et al.* [34] in vaccum-packaged, salted sea bream (*Sparus aurata*).

The evolution of histamine formation during sardine fermentation is represented in Figure 1. As shown, a slight increase in the histamine value was noticed by the days, 12 and 17 for sardine fermented at 30°C. However, a significant decrease (p < 0.05) in the histamine value to relatively low levels was observed for sardine fermented at 22°C at the end of the experimental period. However in the control sample, the amount of histamine reached the maximum permissible level. High levels of histamine in foods can have important vasoactive effects in humans [35]. Histamine values found in fermented sardines are below the regulatory or toxicological limit of 100 mg kg^{-1} [15]. A histamine level of 50 mg kg^{-1} is an indicator of decomposition, this value is the allowable limit set by the Food and Drug Administration (FDA) for scombroid fish and/or products [16] in the USA and several values have been set as legal limits for histamine concentrations that are regarded as safe for human consumption such as 100 mg kg^{-1} in Europe [36] and 200 mg kg^{-1} in Australia [37].

Biogenic amines, including histamine, are formed through the decarboxylation of specific free amino acids by exogenous decarboxylases released from microbial populations associated with the seafood [13]. It has been reported that bacteria of this group are not very active in forming amines. However, according to Kim *et al.* [38], Enterobacteriaceae are involved in formation of histamine from the decarboxylation of the amino acid histidine. The data obtained in the present study showed that histamine values decreased gradually to reach a low concentration at the end of fermentation. These results are in agreement with the findings of Gelman *et al.* [10] which reported that starter cultures of LAB decreased the histamine level in the fish product. Thus, our results with fermented sardines are also in agreement with previous reports on the use of LAB (*Lactobacillus sakei 2a*) as effective starter cultures for the fermentation of *Sardinella brasiliensis* [24]. Paleologos *et al.* [14] reported that the presence of histamine only in filleted Sea bass *"Dicentrarchus labrax"* samples may be related to the specific bacterial microflora and the number of bacteria originating from cross-contamination of fish during the gutting and filleting procedures.

CONCLUSIONS

It could be pointed of from this study that the process used for the fermentation of sardines allows histamine levels in fermented sardines below the legal limit comparing to the non fermented control assay. LAB counts had reached maximal values and the pH decreased

to minimal values with a net decrease of spoilage bacteria count at the end of the fermentation period. Theses results validate the feasibility of selecting new LAB starter for fish fermentation and suggest their use in the preservation of sardines highly produced for the production of new fish products.

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