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Biological silages from Tunisian shrimp and octopus by-products

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Abstract :

Biological silages were prepared from shrimp head and octopus viscera by-products recuperated from the Tunisian seafood industry. Physical and biochemical changes and microbiological profiles were determined for raw materials during fermentation and on end products. Results showed that biological silage significantly affected (P<0.05) moisture, protein, and ash contents of shrimp head (CSHS) and octopus viscera silages (COVS). CSHS and COVS were stable, and their final pH values were 4.31 ± 0.01 and 3.71 ± 0.00, respectively. Proteolysis activity was confirmed by a significant increase (P<0.05) of soluble nitrogen and low molecular weight of protein (<260 Da) found on the end products for both silages. Lipid oxidation was delayed by addition of 150 ppm ethoxyquin to the raw material prior to fermentation. Biogenic amines detected in raw shrimp and octopus samples decreased significantly (P<0.05) during the silage process. Histamine and tyramine, detected at high levels on octopus viscera, were absent in the end products. Tyramine was produced in CSHS, indicating the possibility of the bacterial decarboxylation of tyrosine. Microbiological profiles showed that both silage products were free from pathogenic and spoilage bacteria. Therefore, biological silage can be used as a conservation procedure of shrimp and octopus by-products. The storage period could be shorter than 30 days, and further analysis should be carried out to ascertain safety and nutritional value of silage products.

Keywords : Shrimp by-product, octopus by-product, biological silage, biochemical characteristics, microbiological characteristics

Introduction

Seafood products, such as shrimps (*Aristeus antennatus, Metapenaeus monoceros, Parapenaeus longirostris*, and *Penaeus kerathurus*) and cephalopods (*Eledone moschata, Loligo vulgaris, Octopus vulgaris*, and *Sepia officinalis*), are exported from Tunisian processing industries to the

European markets. Tunisia exported 21442 tones of seafood products, with a value of 285 million dinars (Anonymous, 2013). A great part of these marine resources were processed, and inedible fractions were eliminated. Because of discarding these organic wastes in public dumps, seafood processing industries contribute to environmental pollution. In recent years, great emphasis has been attributed throughout the world to the recovery and recycling of wastes, effluents, and residues resulting from processed food in order to effectively reduce post-capture and environmental impact (Laufenberg et al., 2003). According to Kim and Mendis (2006), these organic matters are rich in nutritious and bioactive compounds such as protein, unsaturated fatty acids, vitamins, antioxidants, and minerals. They could be treated to be used in diverse applications including food, feeds, cosmetic, agriculture, and medical industries (Park et al., 2001).

Production and use of fish waste silage in livestock food was first studied in Sweden in 1930, and its commercial production began in Denmark 10 years later (Edin, 1940). After that, many studies reported the use of fish silage for animal nutrition for pigs (Green et al., 1988), broilers (Santana-Delgado et al., 2008), mink (Raa and Gildberg, 1982), and fish (Goddard and Perret, 2005; Oliveira-Cavalheiro et al., 2007). In addition, preserving shrimp's by-products by silage has been reported as a successful way applied by many researchers (Torrissen et al., 1989; Shirai et al., 2001; Cira et al., 2002) to recover main bio-molecules such as chitins (Hall and De Silva, 1994; Zakaria et al., 1998; Rao et al., 2000; Bautista et al., 2001; Healy et al., 2003 ; Rao and Stevens, 2006; Xu et al., 2008), carotenoids (Torrissen et al., 1982; Hall and De Silva, 1992; Sachindra et al., 2007), and n-3 polyunsaturated fatty acids (Torrissen et al., 1982).

Silage technique has been known as a method to preserve wet or other green fodder in airtight conditions, typically in a silo, without being initially dried and used as animal feed in the winter. The same method has been applied to prepare fish silage with whole fish or parts of fish or fish offal (Raa and Gildberg, 1982). This method implies acid treatment or bacterial fermentation. Acid treatment corresponds to a direct addition of organic or inorganic acids or mixtures of both (Green et al., 1988; Cissé et al., 1995). Fermentation occurs when a fermentable carbohydrate substrate and a supply of lactic acid bacteria are added to chopped fish (Bhaskar et al., 2007; Leroi, 2010). Acid condition allows activation of enzymatic autolysis, specifically proteolysis (Raghunath and McCurdy, 1987), and protein is decomposed resulting in a solubilization of tissue into low molecular weight peptides and free amino acids. Moreover, growth of spoilage bacteria could be inhibited by acid condition and, the obtained product could be stored for a long period (Green et al., 1983). So far, marine silage is still unknown to the Tunisian industry, and only oat silage is applied by farmers (Mahouachi et al., 2003).

The main purpose of the present paper was to suggest a silage process to preserve shrimp and octopus by-products generated by seafood processing industries with minimum losses of nutrients and small changes in their chemical compositions. The originality of this work consists on the fact that this is the first work presenting silage process for up-grading Tunisian seafood by-products. Physicochemical and microbiological changes were determined on the raw materials, during the silage process and on end products. The usefulness of silage was evaluated depending on the obtained results.

Materials and Methods

Raw materials and carbon source

Penaeus kerathurus (Forskål, 1755) and *Octopus vulgaris* (Linnaeus, 1758) by-products were collected from a Tunisian freezing industry (Sfax, Tunisia). These products were fished in the gulf of Gabes (Tunisia), and the steps describing their processing are described in Figure 1. *P. Kerathurus* by-products corresponded to heads, and *O. vulgaris* by-products consisted of viscera (gonads, ink sac, digestive gland, stomach, hepato-pancreas, and gills).

Sugar (sucrose) was used as carbohydrate source for fermented silage preparation.

Chemicals and standards

Hydrochloric acid (Purity 37%, analytical degree) and concentrated sulfuric acid (98%, analytical degree) were supplied by Panreac Quimica S.L.U®. Trichloroacetic acid (20%) was purchased from Carl Roth (Karlsruhe, Germany); 2-thiobarbituric acid, ethoxyquin (Purity 75%), Butylated Hydroxy Toluene (BHT), and chloroform were supplied by Sigma-Aldrich (St. Louis, MO, USA); Absolute ethanol was purchased from Scharlab S. L® (Barcelona, Spain). Sorbate potassium was supplied by Chemiphar®. Hydroxide sodium (NaOH); 1,1,3,3-Tetramethoxypropane (purity 97%), sodium chloride (NaCl) were purchased from E. Merck (Darmstadt, Germany). All solvents used were of analytical-grade.

Preparation of lactic acid bacteria used as inoculants for biological silage

The strain selected for biological silage was *Lactobacillus plantarum* RF06 CIP103151 (30 °C, MRS), which belongs to IFREMER collection (STBM Laboratory, Nantes). This strain was inoculated in 100 ml of MRS broth (70.3 g/L; Biokar, diagnostics) and cultured at 30 °C for 24 h to achieve optimal growth. After checking the good growth of strain, the volume of MRS broth was centrifuged at 3000 g/10 min (Baskar et al., 2007). The bacterial cell pellet was washed twice with a sterile physiological saline solution (8.5% NaCl). Then, the washed pellet was re-suspended in 100 ml of sterile saline solution (8.5% NaCl). Series of six decimal dilutions were prepared from the mother solution. *Lactobacillus plantarum* count was determined on MRS medium (70.3 g/L, Biokar; diagnostics) in anaerobic jars (Anaerocult A., Merck) at 30°C for 48 h.

Preparation of caramote shrimp head silage (CSHS) and common octopus viscera silage (COVS)

Preparation of CSHS and COVS was conducted according to Vidotti et al. (2003). *P. kerathurus* and *O. vulgaris* by-products were ground in a blender (Moulinex 400 W) to obtain a paste and passed through a 3 mm diameter sieve. Then, 15 % (w/w) of sucrose was added to ground materials, which were inoculated by 10 % (v/w) of *Lactobacilus plantarum* at concentration of 10^8 cfu. In order to slow down lipid oxidation, 150 ppm of ethoxyquin (purity 75 %) was added.

Fungal activity was inhibited by adding 0.2 % (w/w) sorbate potassium. The mixture was stored in glass jar and incubated at 30°C for 30 days (Figure 2).

Physicochemical analysis

The physical and chemical analyses were performed on raw materials during the silage process and on both end products (COVS and CSHS). Analyses included determination of quality indexes: pH, total titratable acidity (TTA), soluble nitrogen (SN), molecular weight of peptides and proteins (MWPP), thiobarbituric acid reactive substance (TBARs), and biogenic amines (BA) values. Determination of moisture, protein, lipid, chitin, and ash was carried out on shrimp head, octopus viscera, and CSHS and COVS obtained at the end of storage (AFNOR, 2001). All analyses were achieved in triplicate.

Dry matter: Moisture was determined after oven-drying at 110°C a sample of 10 ± 0.1 g to constant weight (Memmert HORO D.r Ing.A. Hofnam). Weights were determined using an analytical balance (Sartorius d=0.1 mg Laboratoire HUMEAU).

Ash: Ash was measured by incinerating the dried residue in porcelain crucible kept for at least 5 h at 550°C in a muffle furnace (Barnstead Thermolyne 72700).

Total nitrogen (TN): Total nitrogen (TN) was determined according to Kjeldahl (1883) method, and protein was estimated as TN corrected x 6.25. Correction consisted of subtraction of total nitrogen from chitin's nitrogen. Acid destruction was conducted by Kjeldahl digestion bloc (behr-labor-Technic GmbH) and distillation by semi-automatic distiller (behr).

Total lipid: Total lipid was determined according to the method of Folch et al. (1957). Extraction of lipids was carried out from pre-weighed samples with a blend of chloroform and methanol (2: 1, v/v) as solvent, containing 0.025 % (w/v) BHT as antioxidant. Solvent was evaporated using a rotary evaporator (BÜCHI, Germany), and evaporation was achieved under a stream of nitrogen. Lipid mass was gravimetrically determined.

Chitin: Chitin content was determined according to Welinder's (1994) method. Defatted sample was demineralized with 20 ml of hydrochloric solution (1 N) and deproteinized with sodium hydroxide solution (2 N). After discoloration with acetone, a sample was dried, and its mass was determined. The dried sample was incinerated, and the mass was gravimetrically measured.

Microbiological analysis

Shrimp and octopus by-products were defrosted at 4°C to avoid their spoilage. Samples were ground with a sterile Warning Blender (New Hartford, CO, USA), and aliquots of 30 g were aseptically weighed and put in a sterile plastic bag with 120 ml of sterile chilled physiological tryptone salted solution (NaCl: 8.5 g l^{-1} and tryptone: 1 g l^{-1}) (five-fold dilution). Plastic bags were homogenized with a stomacher 400 (PT1200) for 2 min. Analysis was done after 20 min at room temperature for bacterial revivification. Total aerobic mesophile floras (TAMF) were determined on marine agar (MA. Difco, Le Pont de St Claix, France). Plates were incubated for 72 h at 30°C. Lactic acid bacteria (LABs) were enumerated on spread plates of Elliker agar (48.5 g/L, Biokar Diagnostics). Plates were incubated for 48 h at 30°C for mesophilic counts in anaerobic jars (Anaerocult A, Merck) and for 7 days at 15 °C for psychrotrophic counts. *Enterobacteriaceae* were counted on pour plates of Casein Soya (CASO, Merck) overlaid by

Violet Red Bile Glucose agar (VRBG, Biokar diagnostics) after 24 h at 37°C. *Vibrionaceae* were enumerated on spread plates of Thiosulfate Citrate Bile Saccharose agar (TCBS, Biokar Diagnostics) and incubated for 24 h at 30°C. *Pseudomonas* were counted on Cephalosporin Fucidin Cetrimid agar (CFC, Biokar diagnostics) supplemented with CFC selective supplement and incubated at 25°C for 48-72 h. The *E Coli* was searched on Tryptone Bile X-Glucuronide (TBX, Oxoid) and incubation was carried out for 24 h at 44°C. The enumeration of sulfite-reducing anaerobic bacteria was performed on Tryptone Sulfite Neomycin agar (TSN, Biokar Diagnostics). Incubation was carried out at 37 °C for 24 h. *Staphylococcus aureus* was counted on Baird Parker agar (BP, Biokar diagnostics) supplemented with egg yolk and tellurite and incubated for 24-48 hours at 37°C. The detection of *Salmonella* involves four steps: pre-enrichment of 25 g of sample in buffered peptone (Biokar diagnostics) broth during 18-20 h at 37°C, followed by enrichment in both broths: Rappaport–Vassiliadis (RV) incubated for 24 hours at 42°C and Selenite Cystine broth (SC, Biokar diagnostics) incubated for 24 h at 37°C. The isolation was carried out on selective media. From each enrichment broth, inoculums were spread on Salmonella-Shigella agar (SS, AES) and Brilliant Green Phenol Red agar (BGPR, Biokar diagnostics). These media were incubated for 24 h at 37°C. Identification of respective colonies was done on striped lines on Kligler media incubated for 24 h at 37°C. All floras were counted on *P. kerathurus* and *O. vulgaris* by-products and on end product.

Changes in physicochemical properties of CSHS and COVS during fermentation

Changes in chemical composition of CSHS and COVS were controlled by taking samples at different times for a series of analysis.

Determination of pH and TTA values

Evaluation of pH and TTA was determined every two days.

pH: pH value was measured with a pH-meter (Mettler DELTA320, AES Laboratoire) in a sample of 1 ± 0.1 g homogenized with distilled water according to the method described by AOAC (2000).

Total Titratable Acidity: TTA, expressed as percentage of lactic acid (g of lactic acid/ 100 g of sample), was determined by titrating 1 ± 0.1 g diluted in 5 ml of distilled water with NaOH solution (0.1 N) in presence of three drops of phenolphthalein indicator (1%). When pink color appeared (endpoint varied from 8.4 to 8.6), volume of NaOH solution was noted. Results were expressed as % of lactic acid (AOAC, 2000).

Changes in protein during fermentation

Changes in protein during fermentation were followed by evaluation of soluble nitrogen content every week and determination of molecular weight of peptides and proteins every two weeks.

Soluble nitrogen: Protein was hydrolyzed during the silage process, and the nitrogen became more soluble. SN content was estimated according to the method described by Lo et al. (1993). A sample of 5 g was homogenized with 10 ml of trichloroacetic acid 20% for 2 min in order to precipitate protein, then the blend was filtered and filtrate was analyzed according to Kjeldahl (1983) method.

Molecular weight of peptides and proteins: A solution of 2 mg/ml buffer was prepared by diluting a sample of 20 ± 0.5 g in 10 ml of elution buffer (ACN 5%, TFA 0.1%, NaCl 50 Mm) in ultrasound bath for 30 min. Poorly soluble samples were centrifuged for 10 min at 10000 g, and a soluble fraction was filtrated on 0.45 µm filter paper. The molecular weight of the extract obtained was estimated using a Superdex® Peptide 10/300 GL column (Sigma Aldrich, debit: 0.5 ml; detection at 214 nm). The amount of protein in the different fractions (80 µL) was appreciated by the absorbance at 214 nm.

Changes in lipid during fermentation

Lipid oxidation was controlled by determination of the 2-thiobarbituric acid reactive substances values every week.

TBARs values: Lipid oxidation was evaluated by the 2-thiobarbituric acid method as described by Vincke (1975). The result is expressed in mg malonaldialdehyde/kg sample. The standard range was prepared from a solution of 1.1.3.3-Tetraethoxypropane ($2x10^{-5}$ M). Absorbance was read at wavelength of 532 nm using a spectrophotometer (Shimadzu model UV-1800).

Evolution of microbial flora

LAB and *enterobacteriaceae* counts were enumerated (according to previously described microbiological analysis) every week during fermentation in order to follow their respective growth.

Determination of biogenic amines contents

Biogenic amines were considered as indicators of the degree of freshness or spoilage of the product. Its contents were determined every two weeks. Levels of monoamines: histamine, tyramine, and diamines: putrescine, cadaverine, and polyamines (spermidine and spermine) were expressed in ppm. The detection limits were approximately 4 mg/kg for putrescine, histamine, tyramine, and spermine, and 6 mg/kg for cadaverine and spermidine. Stock solutions of putrescine, cadaverine, spermidine, and spermine were prepared at a concentration of 25 ppm, 50 ppm, 100 ppm, and 250ppm. BA was quantified using high-performance liquid chromatography (HPLC, Kontron, detector UV 535) according to the ANSES method (Duflos et al., 1999).

Extraction of Biogenic amines

Biogenic amines were extracted from raw materials, CSHS, and COVS by homogenizing a 5 g of each sample with 10 ml of 0.2 N perchloric acid solution; then, the mixture was centrifuged at 3000 x g/10 min at 4 °C. The supernatant was collected, and the residue was extracted again with an equal volume of perchloric acid solution. Both supernatants were combined, and the final

volume was adjusted to 25 ml with perchloric acid. The extract was filtered through Whatman paper No. 1 (~0.45 µm).

Derivatization of the extract

One milliliter of each extract was mixed with 200 ml of a solution NaOH (2 M) and 300 ml of saturated sodium bicarbonate. Next, 2 mL of a dansyl chloride solution (10 mg/ml) prepared in acetone were added to the mixture and then incubated at 40 °C for 45 min safe from the light. Residual dansyl chloride was removed by adding 100 ml of 25% ammonium hydroxide. After 30 min incubation at room temperature, the extract was adjusted to 5 ml with acetonitrile. Finally, the mixture was centrifuged at 2500 g for 5 min, and the supernatant was filtered. BA standard solutions were derivatized using the same method in the sample extracts to obtain a calibration curve. Separation of biogenic amines was conducted with a HPLC apparatus (Kontron, detector UV 535).

Statistical analysis

Results are given as the means values ± standard deviation for triplicate analysis. All data were analyzed using STATISTICA 8. 550 Software (StatSoft, Inc. 1984-2004).

For each by-product (shrimp head or octopus viscera), the impact of biological treatment on biochemical components (moisture, protein, lipid, ash, and chitin) and on microbial floras counts was determined using t- test for dependent samples. Differences in means between each raw material and its end product were evaluated. Different letters were used to distinguish results of each comparison.

For each silage (CSHS or COVS), significant differences between means by comparing variances of quality parameters values (pH, TTA, SN, MWPP, TBARs and BA) and microbial floras (LAB and *enterobacteriaceae*) were evaluated using repeated measures analysis of variance (ANOVA) .

In order to compare nutritional value of CSHS and COVS, physical and biochemical component values were evaluated using Student T-test; independent samples were considered statistically significant when $T > t_{(\alpha,v)}$.

Results and Discussion

Approximate biochemical composition of CSHS and COVS

Approximate biochemical composition of raw materials and silages prepared from *P. kerathurus* head and *O. vulgaris* viscera is illustrated in Table 1. Acidification of both by-products in anaerobic conditions during 30 days caused significant decrease ($P<0.05$) in moisture, protein, and ash contents for both COVS and CSHS compared to shrimp head and octopus viscera. However, no significant difference ($P=0.26$) was observed on lipid content for both COVS and CSHS and on chitin content for CSHS. Losses of ash (from 36 to 40%) and protein (from 25 to 33 %) contents were recorded for CSHS and COVS, respectively. In the case of moisture, loss was significantly ($P<0.05$) minimal and ranged from 4 to 5 % for CSHS and COVS, respectively. Student T-test proved that COVS was significantly richer in moisture ($P=0.03$) and protein ($P=0.01$) than CSHS, which contained significantly higher values in ash contents

(P=0.01). Loss of ash was the result of the demineralization process, which started when fermentation occurred and acids were produced in the medium. This result was confirmed by the work of Xu et al. (2008), who showed that demineralization efficiencies were achieved by lactic-acid-producing bacteria and with glucose as a carbon source. Regarding the decease of protein values, it was proved that protein was hydrolyzed due to enzymatic autolysis when acid condition occurred (Faid et al., 1997).

According to Mousavi et al. (2013), fish silage composition is very similar to the initial material from which the silage is produced. Espe and Lied (1999) reported that raw material composition, storage temperature, and storage time affected the chemical composition of fish silage. Hassan and Health (1986) reported differences in the proximate composition when comparing fresh and fermented fish silage of white perch, viscera, and heads (using *Lactobacillus plantarum* and 5% lactose) stored after 35 days at ambient temperature and at 37°C.

Microbiological characterization of CSHS and COVS

The microbial profiles of raw materials and silage products reported in Table 2 showed a significant impact of biological silage on the initial flora. The results of microbiological analyses of CSHS and COVS showed the absence of *Enterobacteriacea, vibrionaceae*, *Pseudomonas*, *E. coli*, sulfite-reducing anaerobic bacteria, *Staphylococcus*, and *Salmonella*. Similar results were achieved in the work of Faid et al. (1997), who observed a rapid decrease of coliforms, *Clostridium*, lipolytic, and proteolytic microorganisms notably during the fermentation and reached a minimum after 5 to 8 days. The reported biological fermentation was performed by using chopped pilchard wastes (viscera, heads and tails) mixed with 25% molasses and

inoculated with a starter culture composed of *Saccharomyces* sp. and *Lactobacillus plantarum*, which was incubated at 22°C for 15 days.

Changes in physicochemical properties of CSHS and COVS during fermentation

pH and TTA values

P. kerathurus and *O. vulgaris* by-products had initial pH mean values of 8.33 ± 0.06 and 5.81 ± 0.06, respectively. Addition of 15% sucrose and 10% *Lactobacillus plantarum* as inoculums resulted in significant decrease ($P<0.05$) of pH (Fig. 3), and means values reached were 4.79 ± 0.01 and 3.90 ± 0.01, respectively, for CSHS and COVS after 48 hours of storage. After that, pH mean values remained stable and at the end of storage period, values were about 4.31 ± 0.01 and 3.71 ± 0.00, respectively, for CSHS and COVS. These results are within the range of acceptability according to Raa and Gildberg (1982), who showed that at least 10 % of carbohydrates should be added to produce stable silage. The significant fall of pH ($P<0.05$) for both types of silages resulted by the action of acids that neutralized basic compounds and contributed to favorite acidic medium. This result was approved by several authors (Roberts, 1995; Murphy et al, 2007). In the work of Ennouali et al. (2006), a decrease of pH was noticed in the third day for the silage, prepared from fish waste (viscera, backbones, heads, and tails) composed mainly of 50% of sardines and incubated at the ambient temperature varying between 30 and 33°C during 30 days. Ten kg of molasses and stumps of LAB and yeasts were used by the authors to inoculate the mixture. The difference between the decrease in the pH and lactic acid

production in the various studied silage wastes was due to the different buffer capacity presented by the wastes, provided by the input of soluble nitrogen in the raw materials (Faid et al., 1994; Gutiérrez et al., 2003). In the study conducted by Javeed and Ahmed (1995), the time to reach a stable pH (4.40) was 15 days more than the present study. The ensiling of crustacean processing waste led to a drop in pH from 7.5 – 8.0 to 4.0 – 4.5 depending on the nature of the waste, intensity of liquefaction, protein hydrolysis, and the release of calcium from the prawn shell (Hall, 1995).

Decrease of pH was correlated with significant increase ($P<0.05$) of lactic acid (Fig.3), which raised continuously over 10 days for CSHS, reaching 5.95 % of acidity expressed as lactic acid. However, for COVS, lactic acid was produced progressively over 21 days reaching 6.02 % of acidity expressed as lactic acid. In the work of Martinez et al. (2012), lactic acid still produced over six days, reaching 1.4% of acidity expressed as lactic acid, which resulted in a pH value of 4.0, indicating the end of the fermentative process. Student T-test showed that COVS was more acidic ($P=0.005$) than CSHS, but no difference ($P=0.66$) in amount of lactic acid produced in both silages was detected in the present study.

Changes in protein during fermentation

Changes in protein properties in CSHS and COVS during storage were evaluated by determination of SN values and MWPP profiles.

Soluble Nitrogen

P. kerathurus and *O. vulgaris* by-products were characterized by high percentages of SN content (53% and 76%, respectively) with significant ($P<0.05$) high value recorded in *O. vulgaris* viscera. The rate of SN increased significantly ($P<0.05$) from the first week in CSHS (Fig.4), and maximal value was noticed on the 21st day (87.34±1.99 %). In the case of COVS, SN content increased slightly with significant difference ($P<0.05$) from the 14th week, and a maximal value (81.37±3.02 %) was recorded at the 21^{tst} day (Fig.4). Student T-test proved that decomposition of protein into short chain peptides and free amino acids was similar ($P=0.08$) for both type of silages. According to Faid et al. (1997), about 80% of the protein in fish silage product becomes liquefied, and release of amino acids and other metabolites originating from protein increased significantly during storage. Generally, liquefaction occurred rapidly at 20-30°C within a day, but the process becomes progressively slower as temperature decreased (Tatterson, 1982). It was reported by various researchers (Tatterson and Windsor, 1974; Wood et al., 1985) that the degree of hydrolysis of protein into amino acids and short chain of peptides was measured by determination of non-protein nitrogen (NPN). They showed that high percentage of NPN in the fish silage was normal, since values are in the range of 60-70 %. Proteolysis activity revealed during incubation of CSHS might be explained by the fact that shrimp head contained hepatopancreas organ, which synthesized digestive enzymes involved in the physiology of most crustaceans (Lehnert and Johnson, 2002). Enzymes corresponded to amylase, trypsin, carboxypeptidase A and B, leucine, aminopeptidase (Villasante-Vega et al., 1995), cellulase, chitinase, cathepsin L, and other enzymes (Hu and Leung, 2007; Li et al, 2008). However, pepsin

and chymotrypsin were not detected as well as lipases, which appeared with weak activities in penaeid species (Munilla-Moran and Stark, 1989). Concerning *O. vulgaris*, Monique et al. (2014) showed that a high content of total proteases was measured, in agreement with its carnivorous diet. A large range of digestive enzymes (Trypsin, chymotrypsin, amylase, lipase) in the salivary gland, in caecum, and in the digestive gland was found. The increase of soluble nitrogen might be due also to the hydrolyzing capacity of the *Lactobacillus*, which took part in the degradation of the proteins by the proteolytic enzymes (Ottati et al., 1990).

Molecular weight of peptides and proteins profiles

The molecular weight of protein in *P. kerathurus* head and *O. vulgaris* viscera was greater than 7.2 kDa (about 72 amino-acids) for 14 % and 34 % of protein, respectively (Table 3 and Figures 5 and 6). Protein having molecular weight inferior to 260 Da corresponded to 45 % and 19 % for *P. kerathurus* head and *O. vulgaris* viscera, respectively. After 30 days of fermentation process, molecular weight of protein lower than 260 Da (about 2 amino-acids) increased significantly ($P<0.05$) and reached 65% and 46% for CSHS and COVS, respectively. Thus, getting lower MWPP confirmed a significant autolysis of protein ($P<0.05$). In their work, Le Bihan et al. (2006) observed that about 89% of protein in cuttlefish viscera silage stored at 25°C for 50 days has a molecular weight lower than 6.5 KDa. The molecular weight of protein in animals at death was greater than 20 kDa for 80 % of protein. So, we could conclude that shrimp and viscera by-products have been exposed to protein autolysis from the operation of catching to processing.

Changes in lipid during fermentation

P. kerathurus and *O. vulgaris* by-products were considered as lean products (2.51±0.01 % and 1.57±0.03 fat, respectively on wet matter). In case of "lean fish", lipids are stored in the liver or other parts of the body (Muhamed and Mohamed, 2012).

Determination of lipid oxidation during fermentation process

Analysis of TBARs is a very important quality index characteristic of lipid oxidation, and malondialdehyde (MDA) is the result of auto-oxidation of polyunsaturated fatty acids (Pegg et al., 1992). Mean values of TBARs determined in shrimp and octopus by-products were 2.74 ± 0.22 mg MDA/kg and 2.39 ± 0.27 mg MDA/kg, respectively (Figure 7). During incubation, TBARs values decreased significantly ($P<0.05$) and attained minimal values after three weeks of storage. After that, values increased slightly. The fall of TBARs values could be the result of reaction of malondialdehyde with proteins and amino acids in silage during storage (Buttkus and Bose, 1972). Another reason is that during the analysis method of TBARs value, addition of 1% BHT in the sample during fat extraction method resulted in lower value of TBARs (Salih et al., 1987). According to Schormüller (1969), TBARs value should be less than 3 mg MDA equivalent/kg for high quality materials and should not exceed more than 5 mg MDA equivalent/kg for modest quality materials. The acceptable limit varied from 7 to 8 mg MDA equivalents/kg. In our study, the raw materials and fermented silages contained lower

concentration of oxidized compounds. Fagbenro and Jauncey (1998) obtained in the biological silage preserved by 250 ppm ethoxyquin a TBARs value of 6 meq/kg fat however the initial value in raw materials was 15 meq/kg. According to Mach and Nortvedt (2009), it is not necessary to add the antioxidant silage prepared from low-fat materials.

Evolution of microbial flora

LAB and *enterobacteriaceae* counts were enumerated every week during fermentation in order to follow their respective growth. High levels of LAB counts were maintained, as shown in Figure 8. For CSHS, charge of mesophile LAB increased until the fourth day, and maximal number was 9.23 log (cfu/g). About enterobacteriaceae, charge number was eliminated on the fourth day of incubation. Similar results were obtained for COVS and mesophile LAB load was maintained elevated (7.39 log (cfu/g) at 30 days). *Lactobacillus plantarum* used as acidifying agent not only produced acids but also some antimicrobial metabolites, which may contribute to the preservation of silage products against pathogens (Faid et al., 1994).

Evolution of biogenic amines contents during fermentation process

Determination of BA values is presented in Table 4. Putrescine, cadaverine, histamine, and tyramine were detected at high levels in *O. vulgaris* samples, and values dropped significantly ($P<0.05$) during the fermentation process. So, histamine and tyramine were absent at the end of COVS storage. Putrescine and cadaverine were detected in *P. kerathurus* samples, and silage process affected only values of putrescine, which decreased significantly ($P<0.05$) and reached low values at the end of storage. Tyramine was produced during fermentation in CSHS,

indicating the possibility of the bacterial decarboxylation of tyrosine (Halasz et al., 1994). Production of BA was influenced by several factors such as storage conditions (Komprda et al., 2001), manufacturing process (Rivas et al., 2008), charge of the microbial population with decarboxylase activity (Santos, 1996), raw materials quality (Maijala et al., 1995b), and the availability of free amino acids (Maijala et al., 1995a). However, BA could be endogenous organic bases at low concentrations in non-fermented food (Lima and Glória, 1999). Elevated values of BA measured in raw materials could be explained by a high level of total mesophilic aerobic flora (TMAF), LAB, and *Enterobacteriaceae* (Table 2). As the number of bacterial charges decreased significantly ($P<0.05$) during the fermentation process, BA amounts were reduced. Production of histamine, cadaverine, and putrescine can be attributed to Gram negative spoilage flora (*Photobacterium phosphoreum* or *enterobacteria* (Emborg et al., 2002). Tyramine is mainly the consequence of the presence and growth of *Photobacterium phosphoreum* and also lactic acid bacteria from the genus *Carnobacterium* and *Lactobacillus* (Jørgensen et al., 2000). Different methodologies were applied to prevent BA formation or to reduce their levels once formed in foods were achieved using existing methods and emerging methods (Naila et al., 2010). The latter include the addition of starter cultures that degrade BA, application of hydrostatic pressures, irradiation, packaging, using food additives and preservatives. On the other hand, it was proved that the addition of sugar may also slightly reduce BA formation (Bover-Cid et al., 2001). Starter cultures, such as *Lactobacillus* spp, used in fermentation can also delay the formation of BA (Dapkevicius et al., 2000; Mah and Hwang, 2009). Significantly high values of BA concentration were observed in *O. vulgaris* viscera according to Student T-test. This could be explained by high charge of *Enterobacteriaceae* with desamination or

decarboxylation activity in *O. vulgaris* viscera ($1.25x10^4$ cfu/g) compared to fresh shrimp head (3.5×10^2 cfu/g). Samples of CSHS and COVS examined at 14^{th} and 30^{th} days of storage contained lower BA concentrations. According to Nout (1994), maximal acceptance level of histamine and tyramine in foods should be in the range of 50–100 mg/kg and 100–800 mg/kg, respectively. Furthermore, oral toxicity levels of putrescine, spermine, and spermidine are 2000, 600, and 600 ppm, respectively (Til et al., 1997). These results confirmed the safety of both end products (CSHS and COVS).

Conclusion

This work shows the possibility to develop biological silages from caramote shrimp (*Penaeus kerathurus*) and common octopus (*Octopus vulgaris*) by-products generated by seafood processing industries and to keep them stable for a period of 30 days at 30°C. Investigated ensiling conditions allowed a slight change of some nutrients (protein, ash and moisture) and eliminated spoilage and pathogenic bacteria. However, both silages (shrimp head silage and octopus viscera) are a rich source of protein, lipid, minerals, and chitin. Further investigation of the physico-chemical characteristics of biological silages are needed to determine the safety (absence of heavy metals) of the end products and their nutritional values (such as essential amino-acids and fatty acids profiles).

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Fig.1: Processing steps of seafood products in a Tunisian industry

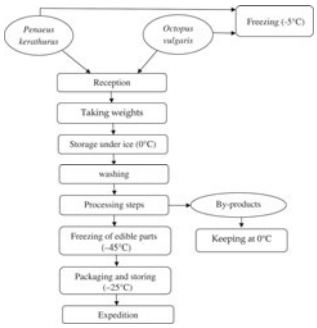

Fig.2: CSHS and COVS incubated at 30°C

Fig. 3: Evolution of pH and TTA means values during fermentation processes

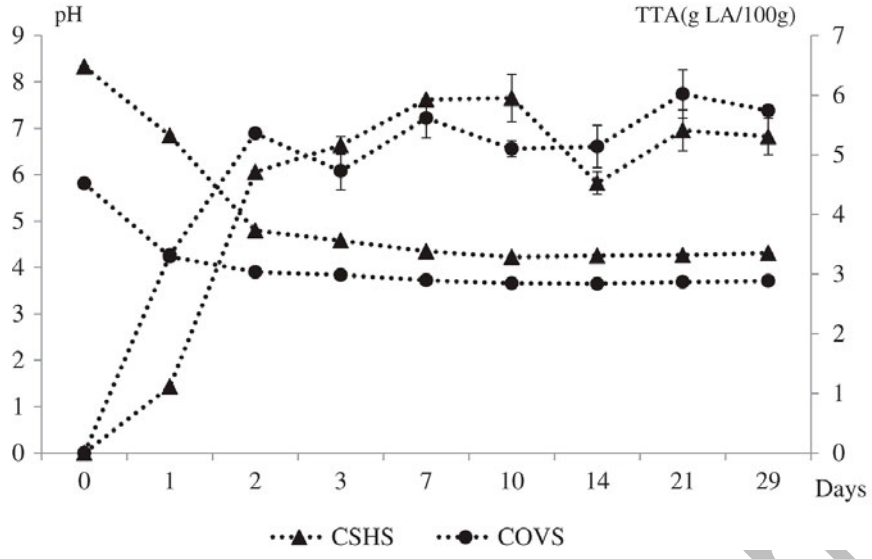

Fig. 4: Evolution of SN/NT (%) means values of CSHS and COVS during fermentation process

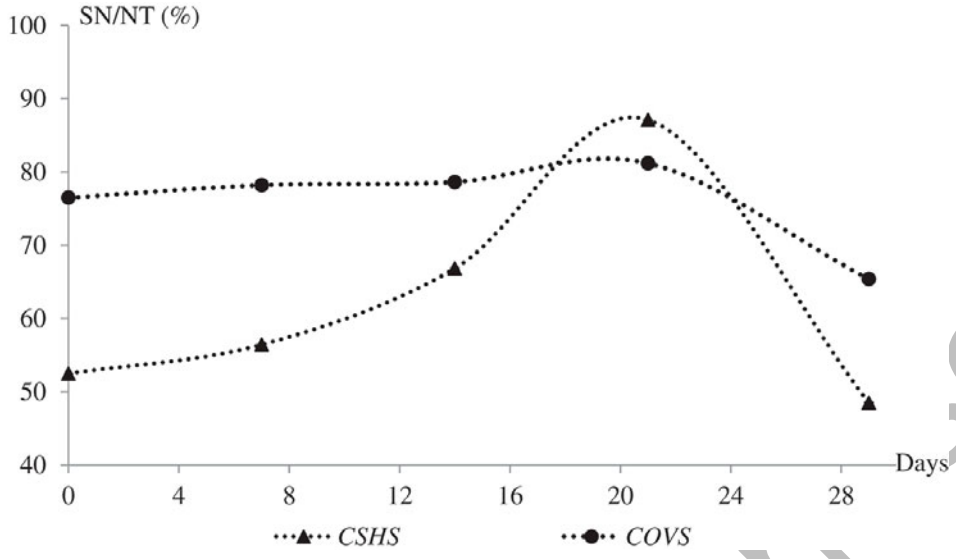

Fig. 5: Protein molecular weight distribution using gel filtration in shrimp head and CSHS during storage at 30°C.

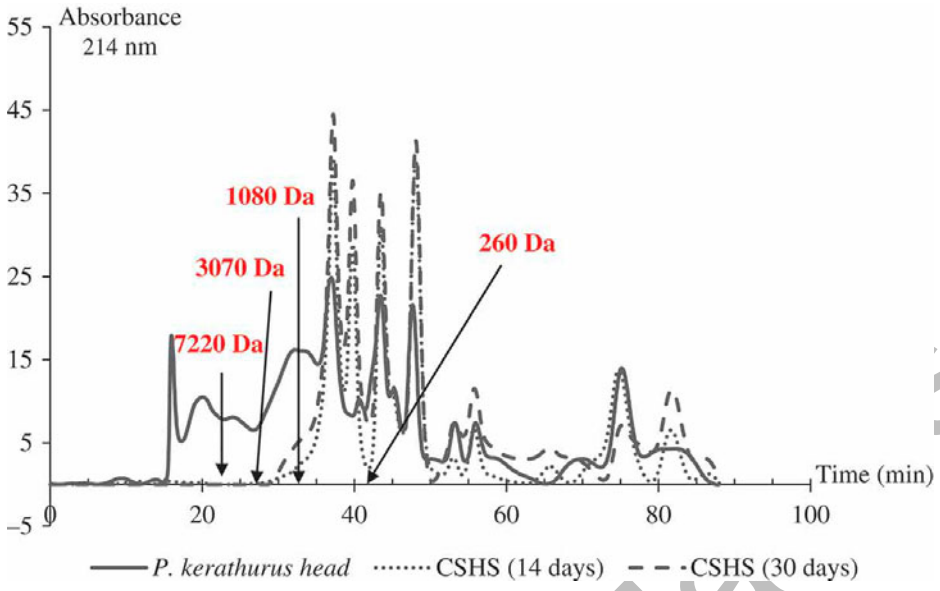

Fig. 6: Protein molecular weight distribution using gel filtration in octopus viscera and COVS during storage at 30°C.

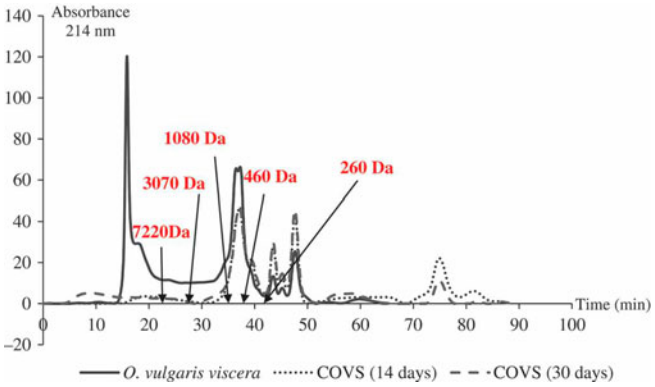

Fig.7: Evolution of TBARs means values of CSHS and COVS during fermentation processes

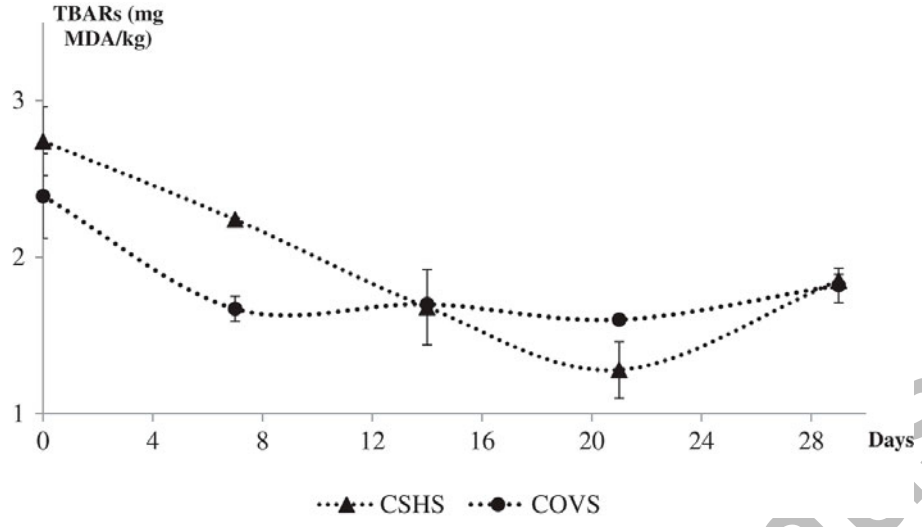

Fig. 8: Microflora evolution of CSHS and COVS during fermentation processes

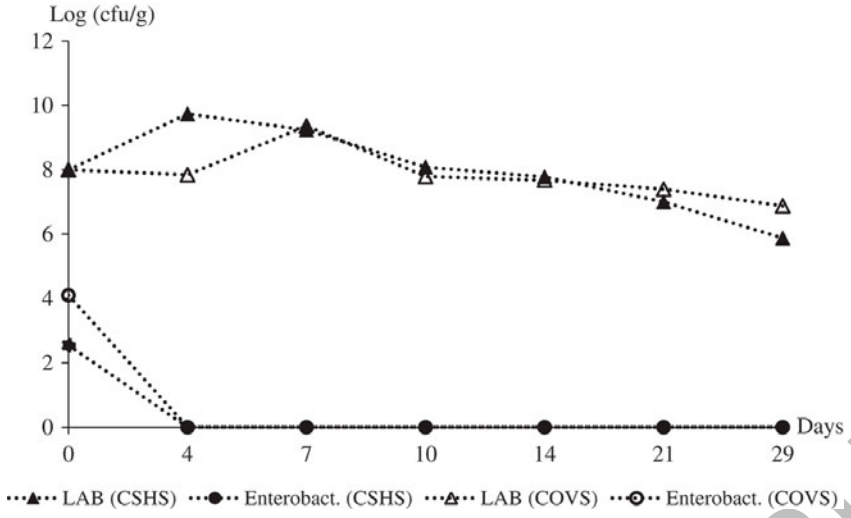

Table 1: Approximate biochemical composition of raw *P. kerathurus* and *O. vulgaris* by-products; CSHS and COVS (n=3)

Components	***P. kerathurus* head**		***O. vulgaris* viscera**	
	Raw material	CSHS (30 days)	Raw material	COVS (30 days)
Moisture*	74.90 ± 0.06^{a}	71.91 ± 0.18^{b}	$78.35 \pm 0.01^{a'}$	$74.43 \pm 0.07^{b'}$
Protein**	38.30 ± 1.41^{a}	28.58 ± 1.36^{b}	$67.60 \pm 4.88^{a'}$	$44.79 \pm 2.23^{b'}$
Lipid**	9.73 ± 0.40^{a}	10.53 ± 0.10^{a}	$6.83 \pm 0.63^{a'}$	$8.04 \pm 1.63^{a'}$
Ash**	29.92 ± 0.79^{a}	19.21 ± 0.03^{b}	$16.66 \pm 0.00^{a'}$	$10.07 \pm 0.12^{b'}$
Chitin**	8.40 ± 0.45^{a}	7.70 ± 2.21^{a}	-	-

**g/100 g of wet weight matter*

***g/100 g of dry weight matter*

Within a row, values with different letters are significantly different (P<0.05);

*Letters **a** and **b** were used to compare data of shrimp head and CSHS;*

*Letters **a'** and **b'** were used to compare data of octopus viscera and COVS;*

Table 2: Changes in total microbial viable counts (Log (cfu/g of product of CSHS and COVS)

Microbial flora	Raw shrimp head	CSHS (30 days)	Raw octopus viscera	COVS (30 days)
Total aerobic mesophile flora	7.08±1.25[a]	8.11±1.56[b]	5.04±0.74[a']	7.77±1.50[b']
Mesophile lactic flora	6.65±0.98[a]	7.34±0.65[a]	4.64±0.32[a']	6.88±0.56[b']
Psychrotrophile lactic flora	6.88±0.86[a]	7.82±0.87[b]	4.61±0.42[a']	6.24±0.10[b']
Enterobacteriaceae	2.54±0.53	Absence	4.10±0.30	Absence
Vibrionaceae	4.52±0.75	Absence	4.03±0.60	Absence
Pseudomonas	2.90±0.23	Absence	Absence	Absence
Sulfite-reducing anaerobic bacteria	Absence	Absence	Absence	Absence
E. coli	Absence	Absence	Absence	Absence
Staphylococcus	Absence	Absence	Absence	Absence

Salmonella	Absence	Absence	Absence	Absence

*Within a row, values with different letters are significantly different (P<0.05); Letters **a** and **b** were used to compare data of shrimp head and CSHS;Letters **a'** and **b'** were used to compare data of octopus viscera and COVS;*

Table 3: MWPP of raw and silage products during storage at 30°C

Samples	PM>7200 D (%)	7200 D5400 D (%)	5400 D3000 D (%)	3000 D2100 D (%)	2100 D1000 D (%)	1000 D460 D (%)	460 D260 D (%)	PM<260 D (%)
Raw *P. kerathurus head*	14.09±0.90^{a}	2.19±0.08^{a}	3.9±0.31^{a}	3.82±0.11^{a}	9.93±0.19^{a}	15.16±0.40^{a}	5.33±0.30^{a}	44.98±0.86^{a}
CSHS (14 days)	0.66±0.18^{b}	0^{b}	0.05±0.00^{b}	0.25±0.09^{b}	1.65±0.54^{b}	19.5±0.28^{b}	13.84±0.07^{b}	63.86±1.29^{b}
CSHS (30 days)	0^{b}	0^{b}	0^{b}	0.15±0.00^{c}	2.83±0.26^{c}	17.92±1.28^{c}	14.07±0.45^{b}	64.83±1.86^{b}
Raw *O. vulgaris viscera*	34.45±2.18$^{a'}$	2.28±0.11$^{a'}$	4.16±0.06$^{a'}$	2.75±0.00$^{a'}$	5.5±0.80$^{a'}$	25.18±1.70$^{a'}$	6.03±0.88$^{a'}$	19.18±0.75$^{a'}$
COVS (14 days)	5.08±0.40$^{b'}$	0.64±0.35$^{b'}$	0.94±0.03$^{b'}$	0.24±0.08$^{b'}$	0.58±0.12$^{b'}$	23.19±3.50$^{b'}$	10.75±0.09$^{b'}$	58.32±2.18$^{b'}$

COVS (30 days)	13.95±1.88$^{c'}$	0.73±0.23$^{b'}$	0.87±0.18$^{b'}$	0.10±0.00$^{b'}$	2.23±0.10$^{c'}$	23.97±1.14$^{b'}$	12.00±1.13$^{c'}$	45.86±0.98$^{c'}$

Within a colon, values with different letters are significantly different (P<0.05);

*Letters **a**, **b** and **c** were used to compare data of shrimp head and CSHS during storage at 30°C;*

*Letters **a'**, **b'** and **c'** were used to compare data of octopus viscera and COVS during storage at 30°C.*

Table 4: Biogenic amine contents (ppm) in shrimp and octopus by-products and CSHS and COVS

Amines (ppm)	***P. kerathurus* head**			***O. vulgaris* viscera**		
	Raw material	CSHS (14^{th} day)	CSHS (30^{th} days)	Raw material	COVS (14^{th} days)	COVS (30^{th} days)
Putrescine	35.44 ± 0.01^{a}	0.77 ± 0.30^{b}	0.68 ± 0.13^{b}	$104.01\pm0.01^{a'}$	$6.06\pm0.01^{b'}$	$9.09\pm0.01^{c'}$
Cadaverine	0.53 ± 0.01^{a}	0.49 ± 0.24^{a}	0.53 ± 0.00^{a}	$33.38\pm0.01^{a'}$	$4.93\pm0.01^{b'}$	$8.87\pm0.01^{c'}$
Histamine	Not detected	Not detected	Not detected	$379.68\pm0.01^{a'}$	$0.45\pm0.01^{b'}$	Not detected
Tyramine	Not detected	2.62 ± 0.61^{a}	2.91 ± 0.01^{a}	26.30 ± 0.01	Not detected	Not detected

Within a row, values with different letters are significantly different ($P<0.05$);

*Letters **a**, **b** and **c** were used to compare data of shrimp head and CSHS during storage at 30°C;*

*Letters **a'**, **b'** and **c'** were used to compare data of octopus viscera and COVS during storage at 30°C*