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Antimicrobial and antioxidant activities of cactus polyphenols extract on seafood preservation

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ABSTRACT
The present work investigated the antimicrobial and antioxidant activities of polyphenols extracted from cactus (*Opuntia ficus indica*) fruit-peels on sardine fillets during refrigerated storage. Biochemical, microbiological and sensorial indicators of treated sardine fillets; were studied comparatively to control lot. Microbial communities were characterized using phenotyping and molecular identification of bacterial isolates; and culture-independent method (PCR-TTGE) for fingerprinting of bacterial DNA extracted from fillets.
A principal component analysis (PCA) of all the studied descriptors and variables revealed that discrimination along first principal component (PC1) was mainly correlated positively with peroxidation level and storage time but negatively with polyphenol treatment while along second component (PC2) it was mainly positively correlated to polyphenols treatment and polyene index; confirming the effect of treatment on preservation of sardine fillets. Sensory data studied by the correspondence factorial analysis (CFA) revealed that the addition polyphenols extract extended the shelf life of sardines without altering their sensorial properties. Cactus fruit-peels appeared a promising source of natural bio-preservative agent for aquatic food processing.
Keywords: *Cactus polyphenols, PCR-TTGE, Biochemical indicators, PCA analysis, Sardine fillets, Sensorial analysis.*

1. INTRODUCTION
The sardine (*Sardina pilchardus*) has high commercial importance across most of its distribution area i.e. throughout most of the Mediterranean Sea and the north eastern Atlantic from the North Sea to Senegal [1]. The total landing of this species reported to FAO is around 1M metric tonnes/year, Morocco and Spain being the countries with the largest catches [2]. The benefit of eating sardine is related to its well established nutritional and sensorial attributes. In addition, as a low trophic level feeder with a short life cycle, the species do not suffer from over exploitation in most fisheries, and do not pose problems of heavy accumulation of toxics compounds, such as heavy metals and pesticides, despite its high lipid contents [3] and therefore it is a highly recommendable food source for humans.

Fresh sardine fillets represent an appealing ready to use product, which could be distributed directly to restaurants, fish markets, and seafood stores targeting consumers who prefer minimally processed, nutritional and healthy products. However, as small pelagic fish, sardines are highly perishable seafood and filleting makes the fish product more susceptible to degradation. This is due to the particular features of sardines muscle, including the high lipid content with a high rate of unsaturated fatty acids, water activity, neutral pH, large quantities of free amino acids and the presences of autolytic enzymes [4]. Hydrolytic and oxidative reactions are the main causes in the alterations associated with the loss of seafood freshness including the loss of shine and color intensity of skin, as well as oxidation of lipids and polyunsaturated fatty acids [4] giving rise to undesirable flavour and substances of health concern. The susceptibility of fish to degradation is also due to specific micro-organisms involved in sensory quality deterioration [5] mainly through the production of biogenic amines, including histamine the most important biogenic amine which has been implicated as the causative agent in several outbreaks of food poisoning [6-7]. The characterisation of these communities oh micro-organisms is of prime importance in order to develop suitable control methods. The applications of culture-independent methods are being steadily used in parallel with classical microbial methods to develop a complete overview of the bacterial community characteristics. Fingerprinting molecular methods, such as DGGE and TTGE, are powerful tools to compare structural changes in microbial communities as well as for monitoring bacterial population dynamics. As in most seafood products, microbial spoilage in sardine fillet often depends on the proliferation level reached by the bacterial community developing during storage. Minimising this level may help to fight against seafood product spoilage.

A large variety of techniques such as refrigerated storage, chilling or freezing [5, 8]; salting, drying or smoking [9-11]; vacuum, bioactive or modified atmosphere packaging [9, 12] and less frequently gamma irradiation [13] are used during fish processing or storage to extend shelf life of products. If additives are used, consumers are increasingly anxious about synthetic compounds in food, opting preferentially towards the use of natural bio-preservative agents. The use of spices and herbs or essential oils with antioxidant properties related among others to their polyphenols content is receiving increasing attention [5, 9, 10, 12, 14]. There is also a growing interest in identifying bioactive compounds with antioxidative/antimicrobial properties from cheaper natural sources such as under-exploited biological resources or by-products.

Opuntias, and in particular the cactus pear (*Opuntia ficus indica*), are widely spread in many countries of America, Africa, Asia and Europe. They are mainly cultivated for the production of forage (900 000 ha) or fruit (100 000 ha) and with lesser importance for vegetable, cochineal or extraction of bioactive compounds as well as soil and water conservation [15]. The nutritional, industrial and pharmacological valorisation of the different parts (fruits, flowers and cladodes) is recognised and documented since long [15-16]. The fruit is considered a good source of bioactive compounds including flavonoids, vitamins and minerals and large amounts of biomasses are under-exploited. Moreover, due to the succulent texture and long lasting permanence on the plant, the fruit is available throughout most of the year [17]. In addition, the thick peels are discarded in most of the uses such as consumption of fresh fruit, juices or processed beverages as well as jellies sweeteners or jams [15, 18]. *Opuntia* thus are considered an endless source of products and functions, as a crop for both subsistence and market-oriented agriculture, providing resources and contributing to the food security of populations in agriculturally marginalized areas [15].

The objective of present work was to evaluate the potential preservative effect of polyphenols extracted from cactus pears peels on preservation of sardine fillets. The biochemical, bacterial and sensorial changes of sardine fillets soaked in polyphenols aqueous solution and wrapped in polyethylene film was studied during a long refrigerated storage comparatively to a control treatment.

2. EXPERIMENTAL SECTION

2.1. Biological materials. Fresh sardines (*Sardina pilchardus*) were purchased directly from the port of Bizerte (North of Tunisia) and rapidly transferred to the laboratory where fish were de-headed, gutted and filleted within 24 h after capture. The fruits of (*Opuntia ficus-indica*) were equally bought from local market. They were hand-peeled and the peel parts were cut into thin slices and frozen at -20 °C until used.

2.2. Polyphenols extraction from *Opuntia* fruit peels. Based on previous study (unpublished data) on the optimisation of polyphenol recovery from the cactus peel part, 10g of frozen sample were thoroughly mixed with 40 mL of cold ethanol/water (80:20v/v). The mixture was sonicated for 20 min and centrifuged at 10000*g* for 15 min at room temperature. The supernatants were collected, pooled, and concentrated to dryness at 40 °C under reduced pressure. To prevent oxidation of the polyphenols, the extract was conserved refrigerated (4 °C) in the dark.

2.2.1. Total phenolic content determination. Phenolic compounds were assayed using the Folin-Ciocalteu reagent, following Singleton's method which was slightly modified [19]. Briefly, 0.125 ml of a 10-fold diluted extract was mixed with an equal volume of the Folin-Ciocalteu reagent. The mixture was shaken and allowed to stand for 6 min, before adding 1.25 ml of 7% sodium carbonate (Na_2CO_3) solution. The mixture was then incubated at room temperature for 45 min and the absorbance measured at 725nm. Total phenolic content (three replicates) was expressed as mg catechin equivalents (CATE)/100 g of fruit peel.

2.1.1. Radical Scavenging Activity. The antioxidant activity of the polyphenolic extract or standard pure phenolic compounds was determined using 2,2-diphenyl-1-picrylhydrazyl (DPPH) as a free radical. The reaction for scavenging DPPH radicals was performed in polypropylene tubes by adding 2 ml of DPPH (4×10^{-5} M) in methanolic solution to 50 μL of sample. The mixture was shaken vigorously and left for 60 min at room temperature. The absorbance of the resulting solution was measured at 517 nm. Methanol was used as a blank solution, and DPPH solution without any sample extract served as control. The Trolox equivalent antioxidant capacity (TEAC) values were calculated from the equation determined from linear regression after plotting known solutions of Trolox with different concentrations (0.02-0.8 mM). The antiradical activity was also expressed as the inhibition percentage and was calculated using the following formula: % radical scavenging activity = (control OD - sample OD/control OD) ×100.

2.2. Fish fillets treatment. Fish fillets were divided in two lots: the first lot was treated by soaking for 5 min in pre-chilled (2°C) distilled/sterilized water and was considered the control (S); while the second lot was soaked in pre-chilled distilled/sterilized water containing diluted (1⁄10) cactus phenolic solution (SP). The ratio of fish to soaking solution was 1⁄10 (w⁄v). Optimisation of the extract concentration was performed previously (unpublished data). After draining, fish fillets were divided in 6 in small polystyrene containers (100g of fillets/container) sealed with food polystyrene film. All lots were stored at 2°C for 11 days. Sampling was undertaken after 0, 3, 5, 7, 9 and 11 days of storage and analysis was performed separately on each fish fillet (n = 6 fillets from each sampled container for each lot). For evaluating sensory attributes of ready to eat sardine, both treated fillets soaked in diluted (1⁄10) cactus polyphenolic extract and control (water washed) fillets were cooked in microwave (4 min x 800 watt) at each sampling time.

2.3. Lipid extraction and fatty acids analysis. Total lipids were extracted according to the method of [20]. Sample (1g) was homogenized 5 min with a mixture of 5 ml of a chloroform/methanol (2:1) and 50 ppm of butylated hydroxytoluene (BHT). Lipid extract was solubilised in Vorbeck solution (4/1 toluene/ethanol) and stored at -40 °C until analysis. Fatty acids Methyl Esters (FAMEs) were obtained according to the method described by Metcalfe et al. [21]. The methyl esters were analysed by gas chromatography (GC) using an Agilent Technologies chromatograph 6890N (Agilent Technologies, Palo Alto, CA, USA) equipped with a flame ionization detector (FID), a split-less injector and a polar INNOWAX 30 M silica capillary column (0.25 mm i.d. & 0.25 µm film thickness). The temperature of the injector and detector were 220°C and 275°C, respectively. Helium was used as a carrier gas with a flow rate of 1.5 ml/mn. Peaks were identified by comparison of their retention times with FAMEs standards (SUPELCO). The polyene index (PI) was used

for the monitoring of polyunsaturated fatty acids degradation during storage [22].

2.4. Assessment of quality indicators. Thiobarbituric acid reactive substances (TBARS) in extracted lipid fraction, as well as trimethylamine (TMA), total volatile base nitrogen (TVB-N) and total free amino acids measured as ninhydrin positive substances (NPS) in aqueous homogenates were used as chemical indicators for the assessment of quality.

The TBARS was determined in oils and fats without preliminary isolation of secondary oxidation products as described in [14]. Lipid extract (50-200 mg) was solubilized in 10 ml of 1-butanol, mixed with 10 ml of 0.2% TBA in 1-butanol, incubated 2 h in a 95°C water bath and cooled for 10 min under tap water. Absorbance was measured at 532 nm with a standard curve determined by the TBARS reaction of a series of aliquots (0.1-1 ml) of 0.2 mM TMP (1,1,3,3-tetramethoxypropane) prepared in 1-butanol. The results were expressed as mg malonaldehyde (MA)/kg of fish.

The TVB-N, NPS and TMA were determined by flow injection analysis according to the method of Ruiz-Capillas et al. [23] and Sadok et al. [24-25] respectively. Samples of 1g of tissue from each fish fillet were homogenized with 1 ml ultra pure water for 1 min and then 0.5ml of 6% perchloric acid was added. The homogenates were then centrifuged (14,000g for 20 min) and supernatants were used for the measurements using flow injection analysis.

2.5. Enumeration of total aerobic viable bacteria. For each sardine sample, 3 packages were used to produce the homogenized analysis solution. A 30-g portion of each package was aseptically weighed into 120 ml of sterile physiological saline solution (0.85% NaCl) with 0.1% peptone in a sterile plastic bag and blended with a stomacher 400 (Seward Medical, London, UK) for 2 min. After 30 min at room temperature for bacterial resuscitation, 30 ml of each blend were pooled into a sterile vial and thoroughly mixed to constitute the homogenized analysis solution. Several appropriate 10-fold dilutions of the analysis solution were carried out in sterile physiological saline solution and 0.1 ml of each was spread on a plates (n = 3) containing Long and Hammer (LH) agar medium with 1% NaCl. The total aerobic viable counts (TAVC) were determined after each 2 days of incubation at 2°C.

2.6. Identification of bacterial isolates: PCR and TTGE. When sardine samples were considered as spoiled, approximately 25 to 30 isolates were randomly selected by picking colonies from LH plates of the highest dilution showing growth. Isolates were collected and purified twice on brain heart infusion agar (BHI, Biokar Diagnostic, Beauvais, France). Each isolate was examined for motility, Gram reaction with KOH, catalase activity by the 3% H_2O_2 method and cytochrome oxidase production by Bactident Oxidase reagent (Merck, Darmstadt, Germany).

In order to characterize more precisely the isolates at species or genus level, molecular tests based on Polymerase Chain Reactions (PCR) were used. The chromosomal DNA of the isolated strains was extracted using the Qiagen DNeasy Tissue Kit (Qiagen, S.A., Courtaboeuf, France). All the oligonucleotide PCR primers used were obtained from Invitrogen (Invitrogen, Cergy Pontoise, France). Finally, for each strain or group of strains identified as belonging to a bacterial species or genus, the 16S rDNA gene was partially sequenced (about 700 bp) for one or several representative strains of each group, depending on the group size. The 16S rDNA was amplified by PCR as described by Macé et al. [26]. The nucleotide sequence of the amplified 16S rDNA was partially determined with an automated sequencer (Beckman Coulter Genomics, Takeley, UK). The sequences were submitted to the National Center for Biotechnology Information (NCBI, Bethesda, USA, http://www.ncbi.nlm.nih.gov/). The computer program CLUSTAL W (20) was used for sequence alignment and the Basic Local Alignment Search Tool 2 program (BLAST) for representation of sequence and similarity searches in the GenBank database.

TTGE analysis was performed also as described by Macé et al. [26] using the homogenized analysis solution prepared for enumeration of bacteria. The chromosomal DNA of isolates and bacterial DNA of the sardine matrix were used to amplify 16 S rRNA V3 region (194bp) with primers V3P2 and V3P3 GC-Clamp (Table 1) as described previously [27].

2.7. Sensory analysis. Sensory analysis was conducted by a taste panel consisting of 80 Tunisian consumers gathered during a National Seafood Exhibition in Tunisia. For sample evaluation, pre-treated and control fillets were individually coded and randomly presented to the panellists. The panel members shared samples tested. A four-point hedonic scale was used for rating: 1 = low, 2 = lightly, 3 = moderate and 4 = high quality [28] according to its spoilage level (SS: strongly spoiled, LS: lightly spoiled, MS: moderately spoiled, NS: non-spoiled). Panellists were instructed to consume the whole sample and to rinse their mouths with water between samples to minimize any residual effect. Sensory assessment included the following parameters: astringency, colour, odour and firmness. Sour odour and firmness loss were chosen as being directly related to rancidity and autolysis development. Astringency indicates a marked polyphenolic character.

2.8. Statistical analysis. For each lot and at each sampling time, the results were presented as mean ± standard deviation (SD) of n = 6 fillets. After verification of homogeneity of variances and normality of data, the results were analyzed using two-way analysis of variance (ANOVA) the analyzed factors being time and treatments along with the interaction effects of factors. The fisher least significant difference LSD was applied for post hoc comparisons of the data, and was used to determine the possible significant differences among mean values at the 5% level. When a significant interaction between factors was detected (time and treatments), homogenous groups were determined with the interaction factor effect. Levels of spoilage (NS, LS, MS, SS), frequencies were treated by a correspondence factorial analysis (CFA). Multivariate data processing was performed with Xlstat software.

Data were also explored by principal component analysis (PCA) using multivariate statistical software (The Unscrambler version 9.8, CAMO Software AS, Oslo, Norway). Leverage

correction was applied to all the data. The variables were weighted with the inverse of the standard deviation of all the objects in order to compensate for the different scales of the variables. Martens Uncertainty test was used to examine the influence of considered variables on the treatment with polyphenol.

3. RESULTS SECTION

3.1. Total polyphenolic content and antioxidant activity of cactus fruit peel extract. The total polyphenolic content of the cactus pear peel extract was 1472 ± 7 mg per 100 g of fruit peel. The cactus fruit peel extracts showed a much higher polyphenol proportions compared to the extracts of *Opuntia* cactus fruit pulp (909.47 ± 29.34 mg/l and 15.34 ± 0.73 mg/kg respectively) found in others studies [29]. As expected and in agreement with the strong correlation reported between antioxidant activity and phenolic content in cactus fruit extracts [30], the peel extract also showed a strong antioxidant activity. It had an average value of 100 µmol ± 0.015 of TEAC/g fw which represent a 4 to 20 fold stronger source of antioxidants when compared to the pulp, for which values ranging from 4.20 to 26.3 µmol TEAC/g of fresh weight are reported [30].

3.2. Biochemical assessment of spoilage.

3.2.1. Lipid content.

The lipid content in fresh sardine fillets had an average value of 3.60 ± 0.09 g/100g. This value which seems low was in the normal seasonal range of lipid content found in the muscle of sardine caught in September in the northern zone of Tunisia (unpublished data). It is well known that catching season is among factors that significantly affect the sardine lipid content. During refrigerated storage, a progressive decrease in lipid contents was observed in the fillets of both, control (S) and treated (SP), lots (Fig.1). Nevertheless, the two lots distinguished as this decrease was more precocious and more important in the control lot (S) comparatively to the treated lot (SP). It was already significant ($p < 0.05$) from 3 days of storage at 2ºC and reached the minimal value of 1.90 g of lipids/100g of fillet at the last sampling in the first while it became significant from the 9th day of storage and reached a minimal value of 2.91 g of lipids/100g of fillet in the second lot.

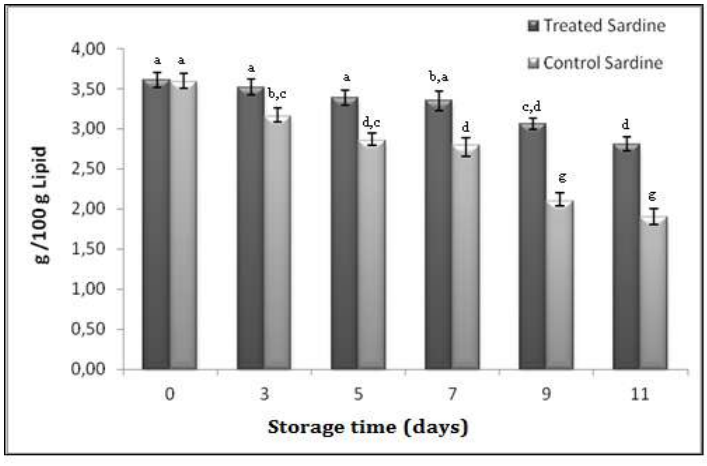

Figura 1. Flesh total lipid content in control and polyphenol- treated sardine during refrigerated storage. Data are mean ± standard deviation, (n = 6; in each case). Means within the same row with different superscript are significantly different ($p < 0.05$).

Thus polyphenols treatment showed an effective action on preservation of muscle lipid content during storage. Such effect could be due to the inhibition of enzymatic and bacterial degradation of fish lipids by cactus fruit peel extract. Indeed, the high level of polyphenols may act as free radical acceptors and metal chelators [31], thus reducing lipid oxidation. Moreover, it is well known that polyphenol may react with protein to give complex [32]; and such complex formed at the surface of the fish fillets may act as a barrier to inhibit the loss of liquid, including water and lipid from the flesh, as reported in an earlier study of sea bream fillets treated with powdered thyme [14].

3.2.2. Changes in fatty acids composition and polyene index.

Similarly to lipid contents, the fatty acids profiles showed significant changes in both control and polyphenols treated lots during the 11 days of refrigerated storage (Table 1). Initially, in fillets of both lots, saturated fatty acids (SFA) represented the majority of the fatty acids followed by polyunsaturated (PUFA) and monounsaturated fatty acids (MUFA). Within SFA, the major fatty acid was the palmitic acid C16:0 and within the MUFA, the main fatty acids were the palmitoleic acid C16:1 n-7 and the oleic acid C18:1 n-9. The high levels of C18:1 and C16:0 were in accordance with results of earlier studies on pelagic fish including sardines [33]. As reported in other studies, docosahexaenoic acid C22:6(n-3) (DHA) was the most abundant among the identified PUFA (Table 1), followed by eicosapentaenoic acid C20:5(n-3) (EPA). After 7 days of refrigerated storage, significant changes ($p<0.05$) consisting in a marked decrease in PUFA levels with a concomitant increase in SFA levels was observed in control lot (S). Such changes were observed after 11 days of refrigerated storage in the polyphenol-treated lot (SP). The relatively high levels of PUFA with important numbers of double-bounds in the lipid fraction determine the major susceptibility of the flesh oxidation [4]. According to our results, it seems that oxidation of PUFA was delayed by the addition of polyphenols to sardine fillets. We may suppose that by using an aqueous solution of cactus fruit peel polyphenolics extract, the bioactive compounds migrate from the water to the flesh surface and are adsorbed within the fish muscle to act as free radical scavengers, as suggested for other antioxidant.

Due to their high degree of un-saturation, EPA and DHA are readily oxidized. Such characteristic has suggested the use of the polyene index [(EPA/DHA)/16:0] to evaluate oxidative deterioration of polyunsaturated fatty acid in fish lipids. It is well known that polyene index (PI) is a good index in monitoring degradation of polyunsaturated fatty acids during storage [22]. In this study, the PI showed a significant decrease after 7 and 11 days of refrigerated-storage (2°C) in both control and polyphenol treated fillets. However, the PI remained higher in the polyphenol treated lot throughout storage time, which indicates higher n-3 fatty acids content probably related to their better preservation by the action of polyphenols.

Table 1. Fatty acids composition of control and polyphenols treated sardine fillets during refrigerated storage (+2°C).

Fatty Acids	Storage time (0 days)		Storage time after 7 days		Storage time after 11 days	
	S J0	SP J0	S J7	SP J7	S J11	SP J11
(C14:0)	8,01[a]	8,41[a]	7,56[b]	6,35[c]	5,07[d]	6,92[c]
(C16:0)	37,08[a]	37,18[a]	39,85[a,b]	37,84[a]	42,19[b]	38,72[a,b]
(C18:0)	7,92[a]	7,48[a]	9,71[b]	8,61[a,b]	11,81[c]	9,90[b]
(C16:1 n-3)	4,50[a]	4,74[a]	3,98[a,b]	3,80[b,c]	3,13[c,b]	3,62[b]
(C16:2 n-4)	1,51[a]	1,43[a]	1,39[a]	1,20[a]	1,04[a]	0,83[b]
(C18:1 n-9)	5,26[a]	5,58[a]	5,29[a]	5,31[a]	5,45[a]	5,29[a]
(C18:1 n-7)	1,57[a]	1,57[a]	1,58[a]	1,64[a]	1,47[a]	1,51[a]
(C18:2 n-6)	2,18[a]	2,23[a]	1,97[a]	2,10[a]	1,70[a]	1,98[a]
(C18:3 n-3)	0,83[a,b]	0,92[a]	0,67[b]	0,81[a,b]	0,49[c]	0,74[b]
(C18:4 n-3)	1,51[a]	1,68[a]	1,20[b,c,c]	1,38[c,c]	0,75[d]	1,33[c]
(C20:4 n-6)	1,06[a]	1,05[a]	0,86[b]	0,97[a,b]	0,85[b]	0,86[b]
(C20:5 n-3)EPA	5,29[a,c]	5,78[a]	4,05[b,d]	4,74[c,b]	3,45[d]	4,27[b,d]
(C22:5 n-3)	0,00[a]	0,00[a]	0,00[a]	0,00[a]	0,00[a]	0,00[a]
(C22:6 n-3)DHA	14,07[a]	13,83[a]	10,28[b]	13,66[a]	10,96[b]	12,37[a]
SFA	53,01[a]	53,42[a]	57,12[b]	54,04[a]	59,07[b]	55,54[a]
MUFA	11,32[a]	11,89[a]	10,85[a]	10,56[a]	10,05[b]	10,60[a]
PUFA	26,12[a]	26,86[a]	20,41[b]	24,88[c,a]	19, 63[b]	22,37[c]
n-3	21,70	22,21	16,19	21,65	15,49	18,70
n-6	3,23	3,28	2,82	3,08	2,82	2,84
(EPA+DHA/C16:0) PI	52[a]	52[a]	35[b]	48[a]	34[b]	42[a]

S: control lot sardine. SP: treated lot sardine. J0: day 0, J7: day 7, J11: day 11, SFA: saturated fatty acid. MUFA: monounsaturated fatty acid. PUFA: polyunsaturated fatty acid. Others SFA: C15:0 C17:0 C19:0. Others MUFA: C18:1 n-5 C20:1 n-7 C22:1 n-11 C22:1 n-9. Others PUFA: C16:3 n-3C18:3 n-6 C18:3 n-3 C20:4 n-6 C20:4 n-3 C22:1 n-11 C22:2 n-6. Data are mean ± standard deviation, (n=3).

[a–e] Means within the same row with different superscripts indicate significant differences ($p<0.05$).

3.2.3. ***Changes in quality indicators.*** The initial values of TBARS in control and treated sardine lipid were 0.8 mg MA/kg muscle. These levels are lower than those reported for sardine in previous studies [22, 33]; indicating the good quality of the starting material. The four studied quality indicators (TBARS, TMA, NPS and TVB-N) showed similar changes (Fig. 2). During storage, a progressive but significant increase ($p<0.05$) is observed for each indicator in each lot. Nevertheless, the two lots distinguished systematically, the increase being lower and delayed in the treated lot (SP) compared to the control lot (S).

The pattern of changes of TBARS (Fig. 2a) clearly showed that cactus fruit peel polyphenols exhibited significant antioxidant properties and were able to retard lipid oxidation in sardine fillets during the 11 days of refrigerated-storage. Indeed, TBARS are secondary products of oxidation which are formed and accumulated with time.

By the end of storage, TMA levels (Fig. 2b) in the treated lot remained < 10mg $100g^{-1}$ whereas control lot TMA level was higher than the established threshold for seafood (12mg $100g^{-1}$, 30). TVB-N levels in all sardine lots (Fig. 2c) did not reach the established limit for fishery products (35 mg-N TVB-N 100 g^{-1}), although sardine fillets were considered strongly spoiled after 5 and 12 days of storage in control and polyphenols-treated samples respectively. The changes in free amino acids levels (FAAs) also followed a similar pattern (Fig. 2d). Globally, the results showed that cactus fruit-peel polyphenols were able to retard lipid oxidation, to delay and inhibit formation of TMA, to reduce release of TVB-N and diminish muscle proteolysis during the refrigerated-storage of the sardine fillets. Thus they are showing the obvious preservative effect of cactus fruit peel extract probably thanks to the activity of the contained polyphenols against development of spoiling bacteria as reported.

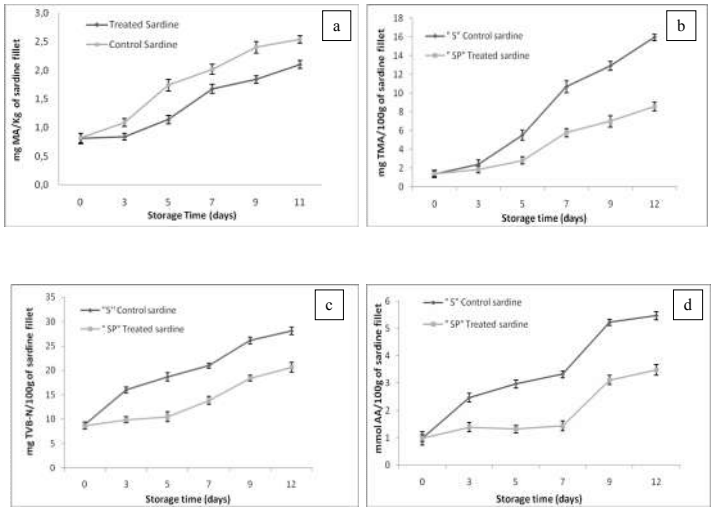

Figura 2. (a) Changes in TBARS (mg MA/kg muscle) of control and polyphenol treated sardines stored at 4°C, MA: malonaldehyde. Data are mean ± standard deviation (n = 6 in each case). **(b)** Trimethylamine (TMA) production (mg TMA/100g muscle) in 2 batches of Control S and treated sardine fillet SP, stored at 2°C. **(c)** Total volatile base nitrogen (TVBN) production (mg TVBN/100g muscle) in both batches of Control (S) and treated sardine fillet (SP), stored at 2°C. **(d)** Changes in ninhydrin-positive substances (NPS) of 2 batches of Control S and treated sardine fillet SP, during the storage period at 2°C Means within the same row with different superscript are significantly different ($p < 0.05$).

3.3. Microbiological assessment of spoilage

3.3.1. ***Enumeration of total aerobic viable microbiota.*** Initially, control (S) and polyphenols treated (SP) lots showed total aerobic viable counts (TAVC) of 3 Log CFU g^{-1} and 2.8 Log CFU g^{-1} respectively. Considering the proposed upper limit for aerobic plate count of 5.10^5 CFU g^{-1} for salubrity of fresh fish [34], the microbiota showed satisfactory initial values in both lots, indicating the good quality of the used sardines.

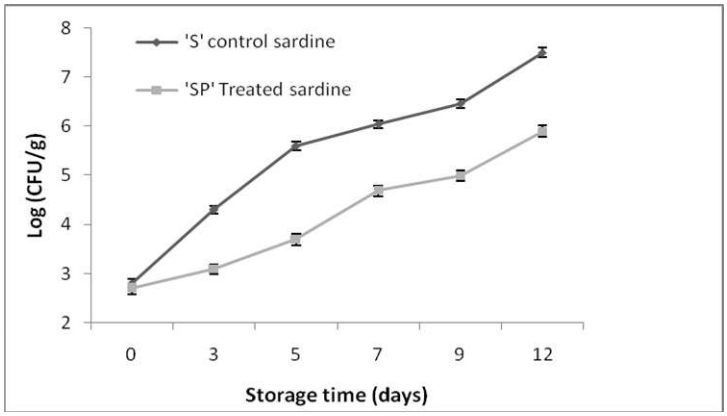

Figure 3. Bacterial growth evolution in total aerobic viable count (TAVC), of control (S) and treated sardine (SP), stored at 2°C.

During refrigerated storage, control lot - microbiota increased rapidly to reach value of 6 Log CFU g^{-1} after 5 days (Fig.3 over passing the salubrity limit. Such level was reached after 9 days of refrigerated storage in the polyphenol-treated lot (5 Log CFU g^{-1}). These results revealed the inhibitory effect of the polyphenols on the development of the natural microflora and thus the bacteriostatic activity of these bioactive compounds.

3.3.2. Identification of bacterial isolates. A total of 37 bacterial isolates were obtained from both control and polyphenols treated lots. Their pheno-typing (Gram reaction, oxidase, catalase) revealed that the dominating bacterial community evolving during

storage belongs to Gram negative/oxidase positive group. The strains isolates were confirmed and were identified using total 16S rRNA gene sequencing. The sequences were compared to those available in the GenBank using the BLAST application. All the reference bacteria identities were confirmed with 98% to 100% similarity with corresponding species. The results revealed the presence of *Psychrobacter* and *Pseudomonas*.

Table 2. Identification of the bacterial strains isolated from the sardine batches by 16S rRNA gene sequencing during refrigerated storage (2°C).

Sample product	Strain	Sequence size	IDENTIFICATION
Control sardine	SJ3	709 bp	Psychrobacter
Control sardine	SJ5	717 bp	Psychrobacter
Control sardine	SJ9	709 bp	Psychrobacter
Control sardine	SJ11a	716 bp	Psychrobacter
Control sardine	SJ11b	704 bp	Pseudomonas
Treated sardine	SPJ5	722 bp	Psychrobacter
Treated sardine	SPJ9	704 bp	Psychrobacter
Treated sardine	SPJ11	704 bp	Psychrobacter

The TTGE profiles of sardine samples revealed 3 different major bands (H, G, F) among which 2 could be potentially assigned (H, G) by comparing the band migration position to that of the strain profiles (Fig. 4a). The (F) bands were unassigned and were considered as unknown bands. These assignments were then confirmed by sequencing in order to identify the bacterial species to which they corresponded (Table 2). During storage, two major bands were assigned and confirmed by sequencing as *Psychrobacter* (band H), *Pseudomonas* (band G) for the control lot, whereas for the treated lot, only one band was displayed, corresponding to *Psychrobacter* (band H).

TTGE analysis revealed that *Psychrobacter* (H band) and *Pseudomonas* were the major microflora detected in control lot from the beginning of storage (Fig. 4b), whereas in treated lot only one strain of *Psychrobacter* was detected (Fig. 4c). During refrigerated storage, *Psychrobacter* density increased in control and treated lots from the 3 and 7 days respectively. Thus the antimicrobial activity of the extract was the most effective on *Pseudomonas*.

The inhibition of *Pseudomonas* development may contribute to explain the extension of sensory shelf-life of the polyphenols treated sardine fillets lot. Indeed, in addition to reducing the activity of endogenous enzymes, the polyphenols treatment slows down bacterial development and thus the rate of deterioration during refrigerated storage. It is known that proteolysis is strongly influenced by the strain of microbial group present in fish [35].

3.4. Sensory evaluation. The utilization of polyphenols in seafood preservation may be limited by sensory considerations; consequently a sensory evaluation was conducted on control and polyphenols-treated sardine fillets. Using the four-point hedonic scale, nearly similar patterns of percentages were observed in each sensorial attribute for treated and non- treated samples. To gain better understanding of the different quality parameters of control and polyphenolic extracts treated-sardines, the systematic structure in the sensory data was studied by the correspondence factorial analysis (CFA).

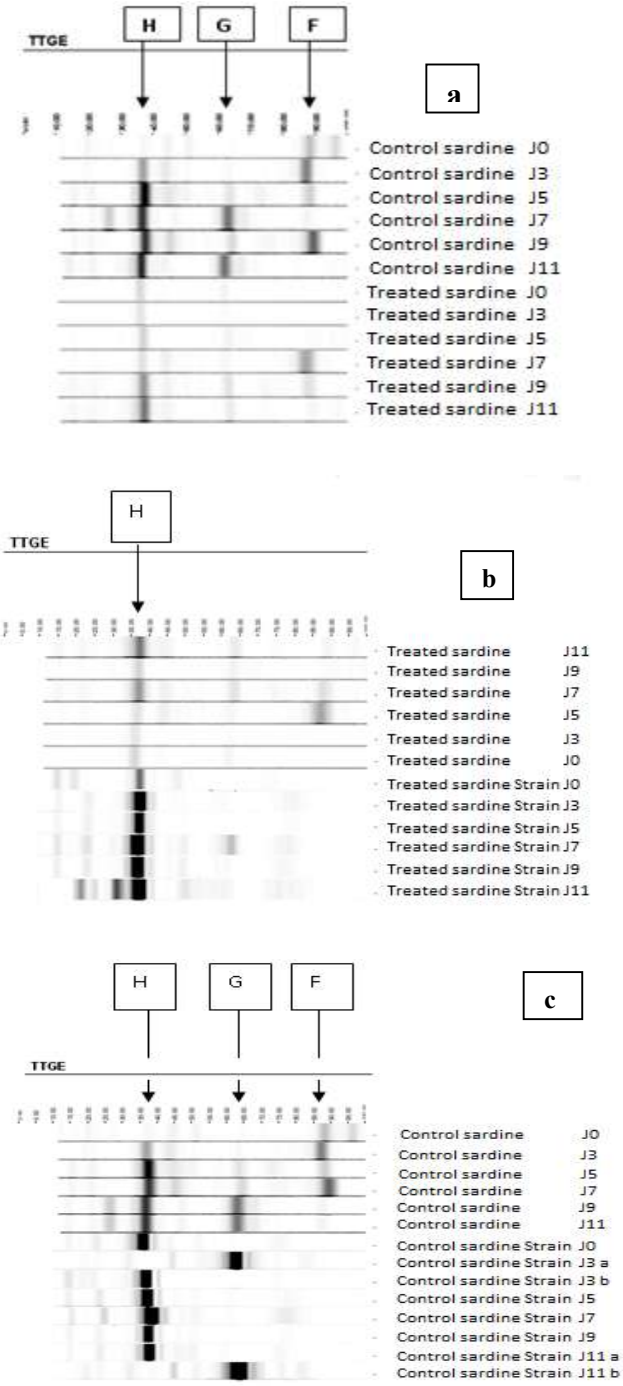

Figure 4. Fingerprints and bacterial population dynamics of (a) sardine fillet samples, (b) control sample and strains; and (c) treated sample and strains during storage. The digitized TTGE profiles of 16S rRNA gene V3 regions were obtained by PCR amplification from bacterial DNA of six samples (day 0, day 3, day 5, day 7, day 9, day 11) from 2 lots (S: control sardine; SP: treated sardine) stored at 2°C. Assignment of PCR-TTGE bands obtained from sardine matrix to strain profiles: H, *Psychrobacter*; G, *Pseudomonas*; F: unassigned bands with the strain profiles, considered as unknown bands.

Before any storage, the sensory attributes showed similarities between polyphenol-treated (SP0) and control (S0) fresh fillets, both exhibiting high scores quality. In the correspondence factorial analysis (CFA) performed on the frequencies of the four levels of spoilage, the first two principal components allowed restoration of around 82.83% of the total variation. The first axis, which restored

72.57% of the total variation, allowed the separation of strongly-spoiled samples (left part of Fig. 5) from non-spoiled samples (right part of Fig. 5). For instance, control samples (S0) and polyphenol-treated samples (SP3) were located in the non-spoiled area after 1 and 3 days of storage respectively; whereas control samples (S7) and treated-sardine samples (SP11) were located in the strongly-spoiled area after 7 and 11 days of refrigerated storage respectively.

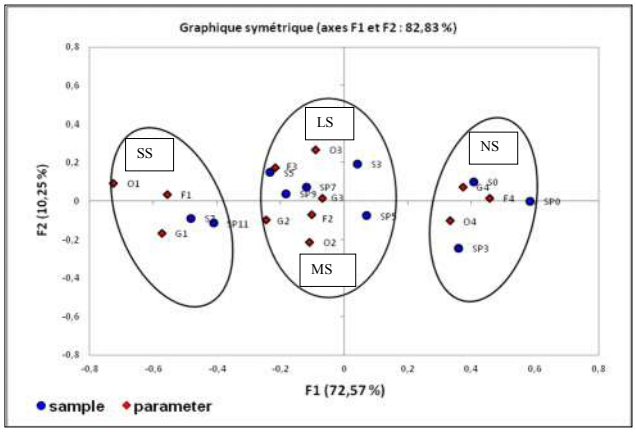

Figure 5. Simultaneous representation of samples and spoilage levels of sardine samples stored at 2°C on plane 1-2 of correspondence factorial analysis (CFA). NS: non-spoiled; LS: lightly spoiled; MS: moderately spoiled; SS: strongly spoiled. Sample nomenclature: S, control samples (non-treated); SP, Treated sample with polyphenol. Number express duration of storage (days).

Lightly-spoiled and moderately-spoiled were grouped in the same area with no real discrimination as the second axis gave information accounting for only 10.25% of the total variation. In this case, S3, SP5 and SP7 were lightly spoiled samples and S5 and SP9 were at the moderately spoiled level. These observations were in close agreement with the results of biochemical and bacteriological spoilage indicators.

To resume, sardines treated with polyphenolic extract, maintained good quality up to day 3 (SP3) and were acceptable up to day 7 (SP7), however control sardine fillets presenting rancid odour, faded colour, sour and dislike taste; were considered spoiled at day 7 (S7). These observations are in perfect agreement with previously discussed descriptors. Overall, they demonstrated that the use of polyphenols extract allowed the extension the shelf life of sardine fillets without altering their sensorial properties.

Finally, all the descriptors (storage time - TPS, TVB-N, TMA, NPS, TBARS, PI, Lipid content, microbiological analysis, sensory analysis and treatment) were submitted to a principal component analysis (PCA) in order to assess the effect of the biochemical, microbiological and sensory quality indicators on the discrimination between control and the polyphenols treated lots (Fig. 6). The first principal component (PC1) accounted for 82% of total variability and along which discrimination was mainly correlated positively with peroxidation level and storage time but negatively with polyphenol treatment. Along second component (PC2) discrimination was mainly positively correlated the lipid contents, polyene index (PI), sensory evaluation and polyphenols treatment Thus, results of this multifactorial analysis confirmed the effective protective effect of polyphenols treatment on processed sardine during refrigerated (2°C) storage.

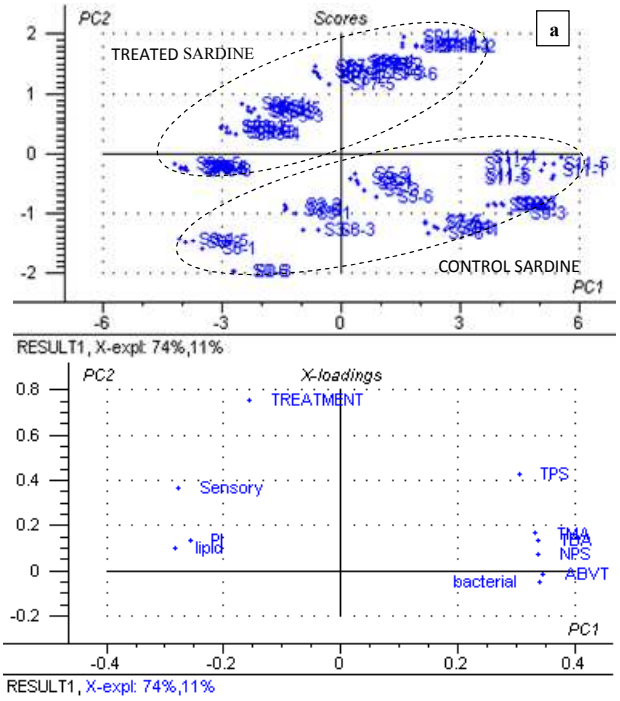

Figure 6. Score (a) and loading (b) plots from to principal component analysis (PCA) carried out on all X-variables (TPS: storage time, TVB-N, TMA-N, NPS, TBA, PI, Lipid, microbiological analysis, Sensory analysis and the Polyphenol treatment). Polyphenol treated (SP) and control (S) groups are encircled.

Present study demonstrated for the first time that polyphenols from cactus peel fruits may have promising applications in fish product and maybe more generally in aquatic food processing. Their use, either in the frame of sound application in local contexts or in the frame of industrially produced extracts, may also be regarded as a new opportunity for the valorisation of an under-exploited agro-resource. In addition, like most cactacea, The *Opuntias* may be produced in arid regions without competing for use of water and fertile land, and thus may become a source of incomes in usually underdeveloped regions where job opportunities are particularly scarce [36]. Nevertheless, the subject merits further research efforts for a precise identification of the bioactive compounds as well as the biological mechanisms involved in the observed preservative and antibacterial activities. Efficient collaborations with pharmacologists, pathologists and microbiologists are crucial to see the complete development of an interesting lead compound into an exploitable product [37].

4. CONCLUSIONS

The soaking of sardine fillets in solution containing cactus fruit-peel extract improved the chemical quality attributes during refrigerated storage without affecting the initial sensorial aspect, which thus resulted in a significant extension of the shelf life of the product. The effect seemed related to the particularly high level of total polyphenolic content and antioxidant activity of the

extract. The treatment showed an effective action on preservation of lipid and polyunsaturated fatty acids contents during storage by the inhibition of enzymatic and bacterial degradation of fish lipids. It also retarded lipid oxidation, delayed and inhibited formation of TMA, reduced release of TVB-N and diminished muscle proteolysis during the refrigerated-storage of the sardine fillets. The bacterio-static activity of the extract was the most effective on *Pseudomonas* bacteria. The present study provides promising results for the seafood industry to prevent rancidity and sensorial deterioration of fish fillets during refrigerated storage.

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