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Deliverable D6.3:

Prototype of an innovative uncooked seabream processed product with high nutritional value and desired sensorial characteristics

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Table of Contents

Executive summary

In the FuturEUAqua project, protocols for the optimization of cold plasma treatment of fish products were developed to maximise the sanitation effect with minimal detrimental effect on quality. The results were used for the setup of shelf-life studies of the fish products and allowed to develop a prototype of uncooked seabream product, characterized by preserved nutritional value and increased stability compared to the untreated one. In the first part of this deliverable, the selection of the appropriate processing parameters based on the effect on the product stability is described, while in the second part the developed innovative product is presented with its physicochemical, nutritional and microbiological properties compared to the traditional untreated one.

Introduction

Seafood and seafood products are highly perishable foods due to their chemical composition (abundant moisture and poly-unsaturated fatty acids). They are regarded as highly sensitive to thermal processing technologies, as they may promote, if not correctly optimized, heat-induced changes to flavour and texture, consequent to oxidation and drip loss. These products are often subjected to various processing methods with the intent to extend their shelf-life, by maintaining quality and minimizing the loss of nutritional quality. Recently, newly developed non-thermal technologies have proven their effectiveness in inactivating of microorganisms and enzymatic activities by avoiding the use of high temperatures, which affect nutritional quality [1].

Although superchilling of the seafood is still the best non-thermal method to increase shelf-life and maintain quality of fish, the cold chain necessary to keep the product at the right low temperature is not easily guaranteed down to the consumer site. Non-thermal technologies represent a step ahead in the preservation of seafood, particularly considering that consumers are paying attention to the 'clean label foods' trend, which envisages the absence additives such as preservatives and promotes environmentally friendly processing techniques with lower energy demand and water footprint.

Plasma can be generated by a thermal process, that is, application of high temperature (>20,000 K) for ionization, termed as thermal plasma. Plasma consists of short-lived charged particles, that is, positive and negative ions, excited and ground-state molecules, atoms, radicals, electrons, and radiation. For this reason, thermal plasma is known to have a detrimental impact on food matrices due to the radical reaction that could be initiated by these reactive species [2]. However, plasma generated at atmospheric temperature (30–60◦C) is known as cold plasma (CP), a low-cost novel technology, considered as environment-friendly, due to no requirement of solvents and low energy demand [3]. Furthermore, the reactive species are very transient and therefore cannot exert any direct toxic effect, although the absence of toxic by-products generated by the reaction of highly oxidative species and fish substrate has not yet been excluded.

CP is based on the ionization of a gas mixture that provides a favourable environment initiating several chemical processes (ionisation, excitation, dissociation etc.) that generate high levels of non-equilibrium species. CP is associated with the production of several short- and long-lived species such as atoms, molecules and radicals in grounded and excited forms, electrons, positive and negative ions, free radicals, gas atoms and radiations. The mechanism of preserving food (inactivation of microorganisms, enzymes and structural modifications) varies widely due to production of different reactive species generated by different gases (O_2, N_2, CO_2, Ar) and depending on the generators used.

The application of cold plasma in food and packaging decontamination has been investigated in numerous food products, which include fresh beef pork, chicken, ready-to-eat meat, fruit and vegetables, and food packaging materials [4].

However, there are various limitations to the application of CP to foods. With specific regard to fish products, the main problems are related to the possibility of inducing the oxidation of lipid and to the formation of undesired volatile components, imparting detrimental changes [5].

The quality of fish products is described differently depending on the physicochemical and biological properties considered for its definition and has different acceptability by consumers [6]. Indeed, considering the increasing demand for healthy products by consumers, the effect of processing on the nutritional quality is of paramount importance.

When considering the nutritional value of fish, n-3 long chain polyunsaturated fatty acids (n-3 LC PUFAs) are mainly in the focus. Aquatic animal foods are a rich source of n-3 LC PUFAs compared to land living animals [7], and strong links between fish and seafood consumption and positive health effects, especially with the decreased risk of coronary heart and cardiovascular diseases, decreased inflammatory disease as arthritis and prevention of cancer have been shown by many researchers [8,9].

In addition, aquatic animal foods are a rich source of protein, have a lower caloric density than terrestrial animal foods, and contains important micronutrients.

The most vulnerable nutrients from fish are the fatty acids, as they are significantly influenced by the feed and by the processing of the fish (as selective oxidation occurs for molecules with different unsaturation), while protein and the micronutrients seem to be less affected if the fish was not starved or wrongly fed or exposed to abusive storage or processing conditions. The bio-chemical composition of fish is the vital aspect in fish processing, and different processing methods have different effect on fish chemical, physical and nutritional compositions [10]. The effect could be either chemical or physical changes, which affects digestibility due to protein denaturation and reduction in the content of mobile compounds and polyunsaturated fatty acids.

In D6.1 of this project, protocols for plasma treatment of fish fillets were developed and included in the corresponding report. These protocols were applied in further experiments to determine the effect of CP on product quality and on its shelf-life, with the aim of developing an innovative fish product characterized by increased shelf-life and high nutritional quality.

Selection of processing parameters

Based on results from D6.1, two treatments were selected and applied to verify their effects on the product shelf-life and nutritional quality, namely cold plasma under argon atmosphere and cold plasma in the presence of air.

Prototype device and parameters for plasma processing

The prototype device optimized in D6.1 was used. In detail, the prototype is composed by a generator, a plasma source and a treatment chamber (**Figure 1**). The plasma source is a Surface Barrier Discharge (SBD), powered by a high voltage pulse generator (mod. S-P300, Alintel S.r.l.). The climatic chamber allows to control treatment time and gas mixture (different combinations of O_2, N_2, CO_2, Ar, N_2O) through the connection to a quaternary gas mixer (mod. KM100-4, Witt-Gasetechnik, Witten, Germany). The aim of the FutureEUAqua project in this regard has been also to design the appropriate

geometry and filling system for the climatic chamber, considering the shape and the size of the seabream samples undergoing the processing treatment in the industrial environment.

Figure. 1 *Treatment chamber with different filling systems*.

The selected processing parameters (electrical parameters, treatment time and gas mixture) able to maximise radical species production without increasing temperature within the chamber and to maximise microbial inactivation are reported in **Table 1**.

Table 1. Processing parameters of the optimized treatment for gaseous plasma

Source Type	Surface Barrier Discharge
Frequency (kHz)	5
Output Voltage (kV)	18
Time (s)	1200
Gas mixture	80% N_2/ 20% O_2 (**Plasma-Air**)
	80% Ar/ 20% O_2 (**Plasma-Argon**)

Optical Absorption Spectroscopy (OAS) was used for the quantification of ozone (O_3) during the plasma discharge, as reported in D6.1.

Figure 2 reports the obtained results. Levels of the ozone compound, generated by the plasma treatment, are 4091±237 ppm for plasma-argon and 5018±312 ppm plasma-air. The temperature within the chamber was between 26 and 29°C for air and between 26 and 32°C for argon. Temperature between 5 (start) -14°C (end) was measured at the fillets core during the treatment.

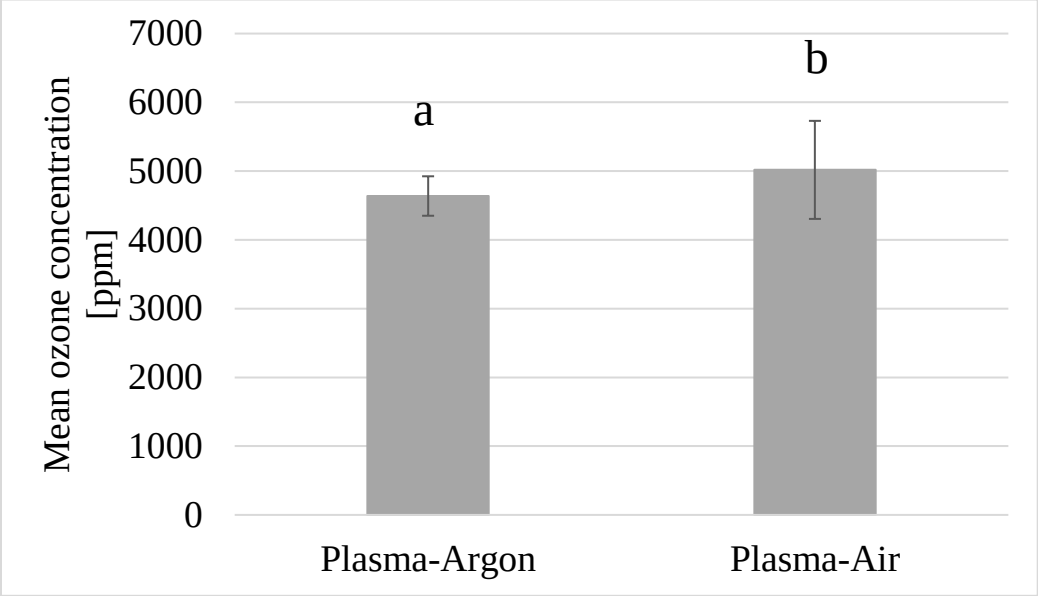

***Figure 2**. Amount of O_3 (ppm) production during plasma discharge. Data are expressed as mean ± SD and are result of **5 replicate measurements**. Statistical analysis was by the one-way ANOVA using Tukeys' as post-test and assuming p<0.05 as significant. Different letters indicate statistical significance.*

Seabream fillets (provided by the project partner Galaxidi, Greece) were received in Cesenatico at the project partner EMAR (Economia del Mare, Cesenatico, Italy) facilities, where they were gutted and filleted, and subjected to fast freezing in-45°C. Frozen samples were stored at -45°C, in the laboratories of University of Bologna, in Cesena, for 4 weeks before experiments. Before treatment, fillets were thawed overnight at 2±1°C refrigeration temperature.

Each treatment was carried out on at least 6 fillets at the same time, as shown in **Figure 3**.

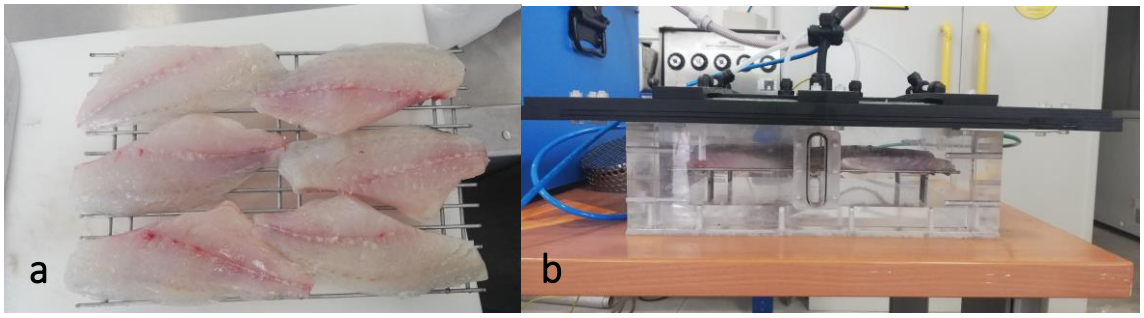

***Figure. 3** Images of fillets placed on the stainless-steel grid (a), and inside the treatment chamber (b)*

Product packaging and storage

After the CP treatment, the product was packed in polypropylene (PP) trays sealed with high barrier PP film using a packaging machine (mod. VGP, ORVED, Venezia, Italia).

According to the results from Deliverable 6.10, the products was packed using modified atmosphere packaging, specifically with the gas mixture 20% CO_2-80% N_2, that was obtained using a quaternary gas mixer (mod. KM100-4, Witt-Gasetechnik, Witten, Germany). Product volume to gas ratio was about 1:1.

Each fillet was packed individually as shown in **Figure 4**.

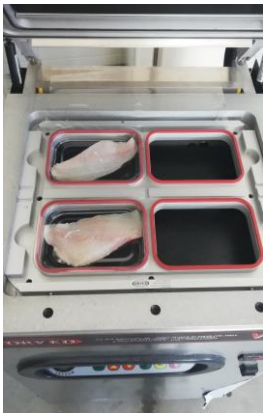

***Figure 4**. Packaging of products using a vacuum-compensated packaging machine connected to a quaternary gas mixture*

Packed samples were stored at 3 ± 1 °C while analytical determinations were carried out over two weeks for the shelf-life study.

Shelf-life study

Experimental design

Shelf-life studies are challenging in terms of sampling, distribution of samples between different laboratories, storage of samples under conditions that do not affect sample quality prior to analysis, and representativeness of samples in a real production system. These aspects are even more complicated for highly perishable food products such as fish fillets intended for consumers as raw products.

It is worth noting that the numbers and sizes of fillets were increased in these experiments, compared to those selected during the development of the protocols described in D6.1. This scale-up has been decided for two main reasons: i) matching the condition closer to a commercial size, and ii) make available a very large number of samples necessary to run the many experiments (microbiological, chemical, rheological, sensorial) in a sufficient number of replicates to provide a statistical power to the assays required by the stability studies. In fact, five sampling in triplicate were analysed over the two weeks of storage to trace the kinetic curve inspected to define the shelf-life.

The experimental design considered the number of treatments (n = 3), the days of storage (n = 5), the number of analytical methods (n = 14) and the number of replicates (n = 3). Therefore 630 fillets were subjected to analysis to characterize the shelf-life of the products under treatment compared to a traditional control product. The average weight of fillets was 53±6.5 g.

Analytical determinations

During storage, after 0, 2, 6, 9 and 14 days, at least 8 packages for each sampling time were removed from storage chamber and used for the analytical determinations related to:

- Microbiological analysis (SOP – Annex 2 to D6.1) for mesophiles, *Enterobacteriaceae* and psychrophiles
- TBARS value (according to the SOP – Annex 5 to D6.1)
- pH (according to the SOP – Annex 4 to D6.1) and water content by drying in an oven at 105°C until constant weight
- Sensory evaluation carried out according to a modified quality index method (QIM) described in detail by Stamatis & Arkoudelos [11] for sardine fillets. The attributes examined were: (1) the development of slime on the surface of the fillet; (2) muscle incision and firmness; (3) odour; and (4) overall appearance of the fish fillet. Each assessment was carried out by a minimum of six trained panellists with a long-term training and experience in fish evaluation. Four categories were ranked: highest quality or excellent (E), good quality (A), fair quality (B), and unacceptable quality (C). Samples were coded with alphanumeric random codes, each of the panellists evaluated the same 3 fillets.

Two fillets were dedicated to microbiological assays, three of them were analysed by *in vitro* digestion and physicochemical assays (sensory analyses, pH and water content), and three of them were selected at each time point and frozen at -80°C for the subsequent chemical analyses (TBARS, fatty acids profile, protein content).

Moreover, to define the effect of the processing on nutritional quality, the fatty acid profile and content (as methyl esters) of the products was determined at the beginning of the storage and at the end of the microbiological shelf-life according to SOP-Annex11 to D6.1.

In addition, the seabream fillets underwent *in vitro* digestion according to the method described in the Annex 12 to D6.1. Notably, the chemical composition of food reflects only partly their nutritional value, since not all components are released from the food matrix to become available for absorption. The relative release of food components during digestion strongly depends on the food matrix, and processing can modify it. In this light, the composition of digested food is of paramount importance to understand their actual nutritional value.

The digested products were evaluated for:

- Fatty acid composition and content (as methyl esters)
- Protein content using two different assays (a) and (b) as described below:

  Determination of protein concentration in digested samples

  Samples were centrifuged at 50.000 *g* for 20 minutes at 4 °C and then filtered on 0.22 μm syringe filter. Protein concentration was assessed spectrophotometrically by o-phthaldialdehyde (OPA) assay and measuring the absorbance at 280 nm using L-glutamic acid and non-fat dry milk as standard, respectively. The protein content from the enzymes added

during in vitro digestion was subtracted, and values were standardized for the dilution factor due to the addition of digestive fluids.

(a) OPA assay

The o-phthaldialdehyde (OPA) assay is based on the reaction of free amino acid NH_2 groups or small peptides (< 5 amino acid) with OPA solution [12]. The OPA solution was prepared by combining the following chemicals and diluting to a final volume of 25 ml with distilled water: 12.5 ml of 0.1 mol/L sodium tetra borate; 2.5 ml of 10% (w/w) sodium-dodecylsulfate (SDS); 0.5 ml of 49g/L o-phthaldialdehyde-solution (OPA), 0.5 ml of 200g/L Na-Mes-solution; 1.25 ml of 100g/L of Triton X-100 solution. Standard curve was performed using 100 mmol/L of L-glutamic acid. 8 µl of diluted samples or L-glutamic acid and 232 µl of OPA solution was added in white multiwell and the reading was taken at 335 nm, after 10 minutes of incubation in dark.

(b) Absorbance at 280 nm

Absorbance at 280 nm is based on the detection of the concentration of aromatic amino acids (phenylalanine, threonine, and tryptophan) in both free and bound form, which exhibit the maximum absorbance at 280 nm [13]. Standard curve was performed using 2mg/mL of non-fat dry milk. The absorbance of the standard and diluted samples was read at 280 nm in quartz cuvettes.

- 1H-NMR evaluation of fish proteins digestibility according to SOP-Annex13 to D6.1 (only after treatment)

Statistical analysis

Statistical analysis was carried out by the Students' t test or by the one-way ANOVA using Tukeys' as post-test and assuming $p<0.05$ as significant, with the software Statistica for Windows (StatSoft, Tulsa, Oklahoma), version 8.0.

Results

Microbial loads

Table 2 reports the result of microbial loads of mesophiles, *Enterobacteriaceae* and psychrophiles measured in the seabream fillets along storage.

Table 2. Mesophiles, *Enterobacteriaceae* and Psychrophiles loads measured in the seabream fillets during refrigerated storage

Storage day	Control	Plasma Air	Plasma Argon	ANOVA (*p* value)
Mesophiles				
0	3.08±1.91 ^{aC}	3.05±1.88 ^{abB}	2.81±1.89 ^{bB}	0.000007
2	3.11±2.09 ^{aC}	3.09±2.29 ^{aB}	3.06±2.03 ^{aB}	0.422850
6	3.88±2.24 ^{aC}	3.91±2.62 ^{aB}	3.73±2.59 ^{aB}	0.000003
9	5.10±4.21 ^{cB}	5.56±4.12 ^{bA}	5.90±4.53 ^{aA}	<0.000001
13	7.29±6.25 ^{aA}	6.61±4.92 ^{cA}	7.08±6.05 ^{bA}	0.000004
p value	<0.000001	0.000001	<0.000001	
Enterobacteriaceae				
0	2.03±1.37 ^{aC}	1.71±1.49 ^{bB}	2.02±1.30 ^{aB}	0.029549
2	3.27±2.49 ^{aB}	2.00±1.51 ^{bB}	1.82±1.21 ^{bB}	0.321316
6	4.41±3.67 ^{aB}	3.43±2.59 ^{bAB}	3.10±2.32 ^{bA}	0.000001
9	4.42±3.59 ^{aB}	4.42±3.64 ^{aA}	3.14±2.41 ^{bA}	0.000011
13	5.54±4.29 ^{aA}	4.25±3.40 ^{bA}	3.23±2.23 ^{cA}	0.002928
p value	0.000160	0.000001	0.000001	
Psychrophiles				
0	2.89±2.27 ^{aC}	2.73±2.02 ^{aC}	2.86±2.06 ^{aC}	0.207807
2	4.94±3.71 ^{aB}	4.76±4.07 ^{aB}	4.83±3.40 ^{aB}	0.443364
6	6.58±5.75 ^{aA}	5.52±4.82 ^{bAB}	5.04±4.16 ^{bB}	<0.000001
9	7.08±6.21 ^{aA}	5.99±5.38 ^{bA}	5.33±4.42 ^{bB}	<0.000001
13	7.66±6.79 ^{aA}	6.74±5.57 ^{bA}	6.73±5.81 ^{bA}	0.000001
p value	0.001331	<0.000001	<0.000001	

*Data are expressed as log CFU/g sample and are mean ± SD of **2 biological replicates and 2 technical replicates** (n =4) in each condition. Statistical analysis was done by the one-way ANOVA using Tukeys' as post-hoc test and assuming p<0.05 as significant. Different lowercase letters indicate statistical significance within a row of values; Different uppercase letters indicate statistical significance within a column of a microbial group.*

The initial reduction was almost negligible for all microbial groups investigated. The starting microbial load, in fact, is not significantly influenced by the CP treatment, perhaps due to a lower inactivation of microorganisms in the conditions of larger sample dimensions and chamber volumes adopted to produce the prototypes. However, during storage, an inhibition of the growth of spoilage bacteria was

observed in the treated samples, suggesting that CP-related inactivation mechanisms begin mainly during the lag phase, postponing the onset of the exponential phase. The end of the shelf-life can be considered as the time necessary to reach the microbiological load of mesophiles at 6 log CFU/g (Colony Forming Unit/g) and of *Enterobacteriaceae* at 4 Log10 CFU/g according to standard references [14, 15]. Mesophiles load threshold was reached after 9 days for the control and the Plasma Argon samples, and after 13 days for the Plasma Air one. Therefore, considering mesophiles load, the application of Plasma Air allowed to increase the shelf-life by around 40% of the seabream fillets, with respect to products without treatment, an improvement not replicated by the Plasma Argon application. This result is consistent with the generation of oxygen-related species that could be the main inactivating factors, thus raising attention to possible degradation phenomena that could affect the overall quality of the products.

A different consideration deserves the case of *Enterobacteriaceae*, for which the last time point of acceptability was reached between day 2 and day 6 for the Control and at day 6 for both the treated samples. In conclusion, no initial bacterial inactivation was observed for all investigated microbial species upon application of cold plasma, both using air and Argon. However, CP promoted a slower growth of *Enterobacteriaceae* and *psychrophiles* starting from the 2nd and the 6th day, respectively, compared to the untreated sample.

Finally, since the product is refrigerated, the growth of psychrophiles can be considered a good indicator a microbiological quality. Considering the threshold for shelf life 6 log CFU/g, control samples reach it after 6 days, while both plasma treated samples, only after 13 days.

In the experiments dedicated to the technical set up of the cold plasma treatment, described in D6.1, we found that a lower amount of ozone was generated from oxygen in presence of nitrogen compared to the gas mixture containing argon. We could explain it by considering a competitive action of oxygen and nitrogen towards ionization reactions. In absence of nitrogen, argon will not compete, leaving the ionization energy transfer only at the oxygen level. When scaling up to a larger chamber, as the one used for this deliverable, the effect of argon was mitigated, and the amount of ozone was even higher in presence of nitrogen. The chemistry of cold plasma is still difficult to be predicted when different chamber geometry and sizes are tested, thus we decided to apply both conditions for cold plasma to investigate the sensorial and nutritional quality at larger scale of production.

Oxidation and sensory aspects

Cold plasma is indeed known to promote oxidation, and special attention must be dedicated to evaluating the chemical and sensorial quality of the product. Purposely, a lipid oxidation index is measured, and a sensorial analysis is conducted after the treatment and at the end of the shelf-life. The results are presented in Table 2 and 3, respectively.

Table 3. TBARS value measured in the seabream fillets during refrigerated storage

Storage day	Control	Plasma Air	Plasma Argon
0	1.56±0.52 [a]	2.09±0.14 [a]	1.97±0.69 [a]
2	1.12±0.44 [b]	3.49±0.65 [a]	3.39±0.46 [a]
6	1.15±0.59 [b]	2.38±0.24 [a]	3.68±0.41 [a]
9	1.63±0.19 [b]	4.29±0.34 [a]	5.20±1.06 [a]
13	3.30±0.64 [b]	6.76±0.62 [a]	5.39±0.46 [a]

Data are expressed as log mg MDA/kg *sample and are mean ± SD of* ***3 biological replicates*** *in each condition. Statistical analysis was by the one-way ANOVA using Tukeys' as post-test and assuming p<0.05 as significant. Different letters indicate statistical significance.*

According to the TBARS assay, treatment with CP causes some oxidation of lipids, either in the presence of air or Argon. However, as explained below in the description of the quality traits of the prototypes, TBARS values below 5 are still considered acceptable in terms of rancidity. Nevertheless, as sensory appreciation is primarily considered for consumer acceptance, flavours has been evaluated by qualified panellists, considering that there is no direct correlation between sensory quality and lipid oxidation. In fact, other factors, such as reduced microbial development, might contribute to the appreciation of the fish fillets by compensating for some unpleasant off-flavours linked to very slight rancidness. The classification method based on flavour as attribute, is not only related to rancidity, but to an overall evaluation, with unacceptable level (C) indicating 'sour and putrid' flavour, generally consequent to microbial contamination.

Table 4. Sensorial score measured in the seabream fillets during refrigerated storage

Storage day	Control	Plasma Air	Plasma Argon
0	E	E	E
2	E	E	E
6	A	A	A
9	B	A	A
13	C	B	B

Excellent quality (E), good quality (A), fair quality (B), and unacceptable quality (C). Results are obtained by the evaluation of **3 fillets, by 6 panellists**.

pH values did not show any significant difference among samples and were in the range of 6.41-6.52 throughout the shelf life study.

Sensorial analysis (**Table 4**) showed that just after the treatment, seabream fillets were considered as excellent quality, as the control one. As expected, acceptability decreased during storage, but on day 13 both air and Argon plasma-treated samples were still considered acceptable while control samples reached the unacceptability score. This finding points out that the rancid level reached during 13 days of storage is not the main origin of detrimental sensory properties, thus confirming that TBARS up to 8 mg/kg is still acceptable in sea bream fillets. However, to have a clearer picture, other oxidative parameters, such as peroxide value, para-anisidine and a full sensorial evaluation, should be assessed.

Fish lipids are considered good quality nutrients due to the large proportion of polyunsaturated fatty acid chains, particularly those of the n-3 series, which are associated to the prevention of cardiovascular disease. However, unsaturation is the target of oxygen-mediated radical reactions, thus making the fish susceptible to unwanted loss of nutritional quality as well. For this reason, the lipid profile of fish fillets is normally considered a routine evaluation for quality attribution. It is worth noting that fatty acids are substrates not readily accessible to oxygen when incorporated as droplets into cell or tissues as triglycerides but become more prone to oxidation when released into tissues as free fatty acids. Release can be accelerated by CP treatment as oxidation can also occur at the fillets' membranes and not, as required, just at the level of the microbial membranes. Furthermore, for the same reason, CP treatment can positively influence gastrointestinal digestion by human lipases as the substrate could be made more accessible to enzymatic attack. However, the digestion processes, occurring under exposure to air, could also favour the oxidation of lipids, thus making positive and negative phenomena (positive for nutritional aspects and negative for oxidative aspects), compensating differently depending on the exposure rate. For this reason, the lipid profile was characterized on both undigested and digested fillets.

The plasma treatment, carried out with both the gas mixtures, did not significantly affect the fatty acid composition (as methyl esters - FAMEs) of undigested fillets, thus accounting for a neutral effect in terms of preferential pauperization of some specific lipid categories. However, it should be noted that there is a large variance in the total content of lipids in all treatments, especially in the fillets undergoing argon CP processing.

The fatty acid composition of plasma-treated and untreated fillets was analysed again after 9 days of storage, and results were compared to the composition at T0 in the corresponding treatment condition (Tables 5, 6 and 7).

Table 5. Fatty acid composition (as FAME) of undigested, not treated (control) fillets at T0 and T95

	T0	T9
14:0	121.04±14.01 [a]	218.09±92.37 [a]
16:0	586.00±61.00 [a]	966.69±397.94 [a]
16:1 n-7	191.05±18.12 [a]	347.62±160.49 [a]
18:0	117.74±11.77 [a]	190.76±76.90 [a]
18:1 n-9	1046.92±120.48 [a]	1786.96±823.53 [a]
18:2 n-6	382.51±41.61 [a]	680.22±292.66 [a]
18:3 n-3	92.02±10.09 [a]	169.82±76.43 [a]
20:1	149.86±16.72 [a]	256.71±111.54 [a]
20:4	28.68±2.55 [a]	51.94±20.36 [a]
20:5 n-3	170.80±13.96 [a]	278.51±110.69 [a]
22:5 n-3	85.42±8.12 [a]	151.10±63.49 [a]
22:6 n-3	364.45±33.17 [a]	609.68±199.21 [a]
ΣSFA	824.78±86.36 [a]	1375.54±567.08 [a]
ΣMUFA	1387.83±155.11 [a]	2391.30±1095.47 [a]
ΣPUFA	1123.87±107.39 [a]	1941.28±762.72 [a]
ΣPUFA n-3	712.69±63.81 [a]	1209.12±449.73 [a]
ΣPUFA n-6	411.19±44.14 [a]	732.16±313.00 [a]
Σ n-6/Σ n-3	0.57±0.02 [a]	0.60±0.04 [a]

Total	3336.49±348.00 [a]	5708.11±2425.0 [a] 1

*Data are expressed as mg FAME/100 g sample and are mean ± SD of **3 biological replicates** in each condition. Statistical analysis was by the Students' t test, assuming p<0.05 as significant.*

Table 6. Fatty acid composition (as FAME) of undigested, Plasma-Air treated fillets at T0 and T9

	T0	T9
14:0	90.74 ±8.49 [a]	212.74±184.88 [a]
16:0	446.92± 32.18 [a]	924.81±722.29 [a]
16:1 n-7	146.52 ±27.14 [a]	339.88±292.51 [a]
18:0	86.35 ±3.17 [a]	184.94±135.98 [a]
18:1 n-9	784.42± 109.14 [a]	1735.10±1428.21 [a]
18:2 n-6	295.09± 32.43 [a]	638.01±517.99 [a]
18:3 n-3	71.11± 9.93 [a]	160.05±133.31 [a]
20:1	112.67± 11.29 [a]	245.86±211.88 [a]
20:4	24.40±2.89 [a]	49.69±34.54 [a]
20:5 n-3	132.19±13.20 [a]	256.42±189.16 [a]
22:5 n-3	69.60±8.88 [a]	142.62±101.93 [a]
22:6 n-3	303.96± 26.21 [a]	580.71±367.08 [a]
ΣSFA	624.01± 42.93 [a]	1322.49±1043.12 [a]
ΣMUFA	1043.61±146.40 [a]	2320.84±1932.59 [a]
ΣPUFA	896.36±92.26 [a]	1827.52±1343.56 [a]
ΣPUFA n-3	576.87± 57.35 [a]	1139.81±791.04 [a]
ΣPUFA n-6	319.49 ±35.30 [a]	687.70±552.52 [a]
Σ n-6/Σ n-3	0.55±0.01 [a]	0.57±0.07 [a]
Total	2563.98± 280.73 [a]	5470.85±4318.95 [a]

*Data are expressed as mg FAME/100 g sample and are mean ± SD of **3 biological replicates** in each condition. Statistical analysis was by the Students' t test, assuming p<0.05 as significant.*

Table 7. Fatty acid composition (as FAME) of undigested, plasma-Argon treated fillets at T0 and T9

	Plasma-argon T0	Plasma-argon T9
14:0	158.25±124.32 [a]	206.72±114.88 [a]
16:0	729.84±516.09 [a]	865.66±443.38 [a]
16:1 n-7	239.99±183.78 [a]	311.29±173.76 [a]
18:0	143.87±98.21 [a]	177.55±86.41 [a]
18:1 n-9	1358.82±1026.06 [a]	1660.55±911.50 [a]
18:2 n-6	495.96±361.31 [a]	644.28±328.15 [a]
18:3 n-3	121.77±91.35 [a]	161.71±85.94 [a]
20:1	192.79±147.31 [a]	238.73±124.06 [a]
20:4	36.07±20.95 [a]	49.41±21.01 [a]
20:5 n-3	205.19±127.09 [a]	235.53±117.33 [a]
22:5 n-3	108.46±70.40 [a]	211.84±132.08 [a]
22:6 n-3	441.62±245.80 [a]	484.65±283.43 [a]
ΣSFA	1031.96±738.43 [a]	1249.93±644.61 [a]
ΣMUFA	1791.60±1357.12 [a]	2210.57±1209.18 [a]

ΣPUFA	1409.06±916.85 [a]	1787.42±837.81 [a]
ΣPUFA n-3	877.03±534.59 [a]	1093.73±490.96 [a]
ΣPUFA n-6	532.02±382.26 [a]	693.70±349.10 [a]
Σ n-6/Σ n-3	0.57±0.11 [a]	0.62±0.08 [a]
Total	4232.61±3011.88 [a]	5247.92±2690.48 [a]

*Data are expressed as mg FAME/100 g sample and are mean ± SD of **3 biological replicates** in each condition. Statistical analysis was by the Students' t test, assuming p<0.05 as significant.*

Regardless the treatment, no modification in the fatty acid composition was observed after 9-day storage, except an increase with time of the content of palmitoleic acid (C16:1) in not treated fillets. It is worth noting that a huge variability was detected among samples of the same treatment group. However, beyond the statistical significance, an average increase of the overall quantity of lipids over the time seems occurring in all samples, regardless the processing. This may be explained by considering the water loss associated to the storage, making the total weight of fillets decreasing over the time, at the expenses of water but not of the solid matter. To explore the occurrence of this possible phenomenon, we measured the water content for all treatment at the beginning and at the end of the shelf-life, and the results are reported in Table 8.

Table 8. Water content (%) of seabream fillets at T0 and T9

	T0	T9
Control	73.04 ± 1.01[a]	72.36 ± 0.50[a]
Plasma-Air	73.65 ± 1.69[a]	73.98 ± 0.26[a]
Plasma-Argon	74.92 ± 0.55[a]	72.07 ± 1.36[a]

*Data are expressed as g/100 g sample and are mean ± SD of **3 biological replicates** in each condition. Statistical analysis was by the Students' t test, assuming p<0.05 as significant.*

As reported, there is no significant difference in water loss during storage, therefore making any explanation for a hypothetical change in lipid content only speculative, as the large standard deviation masks any eventual variation.

After *in vitro* digestion, the fatty acid composition was examined again in the digested products. Fatty acids derived from the blank digestion, i.e., enzymes added to digestion fluids in absence of food, were subtracted. Indeed, blank digestion contains fatty acids from added bile. Results are reported in **Table 9**.

Table 9. Fatty acid composition (as FAME) of not-treated and Plasma-Argon treated fillets (T0) after in vitro digestion

	Control	Plasma Air	Plasma Argon
14:0	167.65±70.41[a]	107.2221±22.47[a]	163.23± 90.06[a]
16:0	754.70±291.95[a]	468.81±103.12[a]	728.97±365.72[a]
16:1 n-7	270.07±101.05[a]	185.39±43.47[a]	254.50±139.02[a]
18:0	125.09±52.30[a]	50.55±20.88[a]	121.20±60.44[a]
18:1 n-9	1463.86±540.90[a]	972.17±193.97[a]	1383.99±729.49[a]
18:2 n-6	514.45±190.92[a]	355.50±68.99[a]	490.74±256.48[a]
18:3 n-3	129.61± 47.02[a]	85.73±16.49[a]	121.31±64.88[a]
20:1 n-9	212.80±79.29[a]	148.41±27.10[a]	203.76±106.18[a]

20:4	33.52±10.58[a]	24.17±4.14[a]	31.69±14.47[a]
20:5 n-3	194.34±60.93[a]	134.04±20.38[a]	179.43±88.81[a]
22:5 n-3	114.49±40.59[a]	84.67±17.21[a]	110.07±49.28[a]
22:6 n-3	418.85±127.46[a]	330.87±53.53[a]	410.37±176.43[a]
ΣSFA	1047.44±414.60[a]	626.58±145.15[a]	1013.40±515.76[a]
ΣMUFA	1946.72±721.23[a]	1305.96±263.85[a]	1842.26±974.67[a]
ΣPUFA	1405.25±476.94[a]	1014.97±179.90[a]	1343.62±649.94[a]
ΣPUFA n-3	857.29±275.61[a]	635.31±106.84[a]	821.18±379.26[a]
ΣPUFA n-6	547.97±201.45[a]	379.67±73.14[a]	522.43±270.95[a]
Σ n-6/Σ n-3	0.63±0.03[a]	0.60±0.02[a]	0.62±0.06[a]
Total	4399.42±1611.76[a]	2947.52±584.63[a]	4199.28±2139.53[a]

*Data are expressed as mg FAME/100 g sample and are mean ± SD of **3 biological replicates** in each condition. Statistical analysis was by the one-way ANOVA using Tukeys' as post-test and assuming p<0.05 as significant. Different letters indicate statistical significance.*

As in undigested samples, regardless the treatment, no statistical differences in the fatty acid composition were detected in digested samples, largely depending on the broad range of compositions found in the raw material.

The Relative Release (RR) of fatty acid at the end of *in vitro* digestion was calculated as mg FAMEs in digested/mg FAMEs in non-digested samples *100, and it is reported in Table 10.

Table 10. Relative release of fatty acids after in vitro digestion.

	Control	Plasma Air	Plasma Argon
14:0	136.24±46.84[a]	117.35±15.68[a]	132.18±60.09[a]
16:0	126.88±39.77[a]	104.32±18.14[a]	116.07±36.90[a]
16:1 n-7	139.30±42.09[a]	125.77±10.78[a]	130.40±51.11[a]
18:0	104.22±34.43[a]	58.67±24.99[a]	95.46±27.44[a]
18:1 n-9	137.35±37.12[a]	123.57±15.05[a]	124.34±47.85[a]
18:2 n-6	132.19±36.92[a]	119.94±14.85[a]	117.77±40.69[a]
18:3 n-3	138.41±36.71[a]	120.21±13.08[a]	122.32±48.32[a]
20:1 n-9	139.61±39.45[a]	131.21±15.73[a]	132.74±57.12[a]
20:4	115.48±28.30[a]	98.87±11.31[a]	93.29±14.03[a]
20:5 n-3	112.51±28.03[a]	101.32±11.33[a]	94.31±17.40[a]
22:5 n-3	132.02±35.52[a]	120.93±12.41[a]	116.25±35.85[a]
22:6 n-3	113.93±28.67[a]	108.90±16.21[a]	98.88±16.70[a]
ΣSFA	125.01±40.00[a]	99.86±19.02[a]	114.87±37.67[a]
ΣMUFA	137.85±38.04[a]	124.68±14.56[a]	126.01±49.19[a]
ΣPUFA	123.39±32.74[a]	112.97±14.40[a]	106.35±26.41[a]
ΣPUFA n-3	118.95±30.52[a]	109.99±14.29[a]	102.03±21.59[a]
ΣPUFA n-6	131.03±36.34[a]	118.34±14.58[a]	115.49±37.77[a]
Total	129.81±36.78[a]	114.50±15.64[a]	115.89±37.32[a]

*Data are mean ± SD of **3 biological replicates** in each condition. Statistical analysis was by the one-way ANOVA using Tukeys' as post-test and assuming p<0.05 as significant. Different letters indicate statistical significance.*

As expected, the RR of single and total fatty acids was similar in all conditions. To observe RR > 100 is not surprising, since it was already reported that *in vitro* digestion is more exhaustive than the chemical

extraction adopted for the undigested fillets [16]. Of note, in preliminary experiments lipid extraction was performed using two different techniques, and the Bligh and Dyer method appeared the most suitable one. Our results indicate that the use of chemical extraction for evaluation of the lipid content of food could lead to underestimation. For this reason, a possible suggestion emerging from our specific application to sea bream fillets is to consider the in vitro digestion as a mandatory step in the nutritional evaluation of food products, especially when bioaccessibility is a relevant issue.

Digestibility of proteins

The hydrolysis of proteins during *in vitro* digestion was also determined, using two different standard methods. Differently treated fillets were compared, and results evidenced that plasma treatment did not modify the release of proteins during *in vitro* digestion (**Table 11**).

Table 11. Protein release after in vitro digestion

Method	Control	Plasma Air	Plasma Argon
A 280nm	13.32±2.25[a]	12.33±1.73[a]	14.16±1.97[a]
OPA	15.32±1.77[a]	15.28± 2.15[a]	14.73±2.34[a]

Data are mean ± SD of 3 biological replicates in each condition and are expressed as g protein in digested sample/100 g of corresponding not-digested sample. Statistical analysis was by the one-way ANOVA using Tukeys' as post-test and assuming p<0.05 as significant. Different letters indicate statistical significance.

The methods here applied in the evaluation of protein digestibility are the most diffused among the nutritional laboratories, and the complementarity of spectrophotometric absorbance at 280 nm and the OPA assays has been evidenced in the methods' section. The spectrophotometric reading at 280 nm accounts for the fraction of soluble amino acids, peptides and short proteins containing aromatic side chains. The method is reliable provided that the amino acid compositions of fillet proteins and the standard protein are similar. During digestion, this condition is not always granted. On the other side, OPA assay is sensitive to the free amino end of amino acids and peptides. Thus, the method is strongly dependent on the level of protein hydrolysis: keeping constant the quantity of hydrolysed proteins, smaller the fragments higher the response. Although both techniques have some drawbacks, the comparative application on similar substrate still provide useful information, as under- or over-estimation are parallel in all samples.

To further investigate the impact of CP treatment on fillets digestion, the nuclear magnetic resonance (NMR) spectroscopy has been applied to the same samples analysed with the spectrophotometric and the OPA assays. The advantage of using this further technique is associated to its universal detection capability, without the requirement of an external standard to calculate an instrumental response factor. Provided that the molecules under investigation contain at least one atom of hydrogen and are soluble in the solvent of the sample, all molecules released by digestion satisfy these requirements, including amino acids, peptides, and larger soluble fragments of proteins. Thus, the area of diagnostic signals in specific regions of the NMR spectrum is directly proportional to the concentration of hydrogen atoms belonging to the molecule to be quantified (either single amino acids, short peptides, small or

large protein fragments). As only the soluble molecules are detected, the NMR technique provides the condition necessary to evaluate the accessibility of nutrients upon digestion. Table 12 reports the results of the NMR spectroscopy analysis carried out on the in vitro digested samples deriving from air and argon CP treatments, compared to the control.

Table 12. Relative concentrations, assessed by NMR spectroscopy, of molecular species released by in vitro digestion of fillets proteins, classified according to the spectral regions where signals resonate

	Control	Plasma Air	Plasma Argon
Hydrophobic Amino Acids Region (0.20-2.00 ppm)	104.45 ± 2.60 [a]	94.62 ± 5.15 [b]	106.18 ± 6.48 [a]
Hydrophilic Amino Acids Region (2.00-3.00 ppm)	36.90 ± 0.76 [a]	33.13 ± 1.85 [b]	37.41 ± 0.71 [a]
Total Amino Acids (α-CH) Region (3.20-4.70 ppm)	76.70 ± 2.04 [a]	69.41 ± 3.72 [b]	77.39 ± 1.95 [a]
Aromatic Amino Acids Region (6.40-7.70 ppm)	9.82 ± 0.18 [a]	9.15 ± 0.37 [b]	9.74 ± 0.31 [a]
Total Soluble Proteins Region (7.70-9.60 ppm)	4.40 ± 0.16 [a]	3.89 ± 0.21 [b]	4.31 ± 0.33 [a]

Data are mean ± SD of 3 biological replicates in each condition and are expressed as arbitrary integral units/5 g of digested sample. Statistical analysis was by the one-way ANOVA using Tukeys' as post-test and assuming $p<0.05$ as significant. Different letters indicate statistical significance.

Five diagnostic regions are examined in the NMR spectra of digestates, each of which represents a specific category of hydrolysis products: i) the hydrophobic amino acid region collects the signals generated by the hydrogen atoms belonging to alanine, valine, leucine and isoleucine; ii) the hydrophilic amino acid region collects the signals generated by serine, cysteine and threonine; iii) the spectral region collecting the hydrogen atoms in the alpha position is directly related to the total amount of amino acids in the digestion fluid, as they all contain this specific atom; iv) the region of aromatic amino acids collects signals from phenylalanine, tyrosine, histidine and tryptophan. These four regions provide information on the amino acid composition of the oligopeptides and small fragments released during digestion. The fifth region collects signals belonging to peptide hydrogen atoms that are not accessible to water because hindered in a larger protein fragment made soluble by detachment from the insoluble myofibrillar protein. The comparison of the integral areas in all these different spectral regions can highlight different protein digestion profiles between untreated and treated fillets. By inspection of Table 12, the only difference emerging from NMR spectral data is an overall 10% reduction in protein digestion of fillets treated with air-plasma compared to argon-plasma and control. Since the lowering is of the same extent in all NMR spectral regions, it is argued that the digestion profile is not affected by the treatment, although a slight decrease in the overall digestibility of the proteins emerged. Since this phenomenon does not reproduce in Argon-plasma treated samples, further investigations are needed to exclude that the underestimation of digestibility is notably due to a different fillet composition which is reflected in a lower amount of protein to be digested.

Conclusion

Fish is a very perishable products due to microbial spoilage. The application of cold plasma has shown potential in delaying the microbial growth thus increasing the products shelf-life.

The treatments applied in this experiment were able to inhibit the growth of some spoilage microbial groups during refrigerated storage. In particular, considering the end of the shelf-life as the time necessary to reach the microbiological load of mesophiles at 6 log CFU/g (Colony Forming Unit/g), air-plasma allowed to increase the shelf-life of the seabream fillets with around 40%.However, since the products is expected to be refrigerated, a better indicator could be the psychrophile load; in this case, the increase of the shelf life is over 50% for both the plasma treated samples.

The use of Argon atmosphere, as alternative to nitrogen in the gas mixture used for plasma generation, did not bring any additional improvement compared to the use of air. Therefore, also considering its higher cost, it does not seem a promising treatment for fish fillets.

At the same time, lipid oxidation measured as TBARS value was only marginally increased, without significantly affecting the sensorial perception of the products, in terms of flavour.

Overall, our results indicate that plasma treatment is a good strategy to increase the shelf-life of seabream fillets, preserving their nutritional value. Indeed, no modifications of the fatty acid composition were observed neither at the beginning nor at the end of the storage. In addition, plasma treatment did not reduce the relative release of fatty acids and the extent of protein hydrolysis.

Therefore, the product obtained by the application of plasma air treatment was selected as prototype of innovative uncooked seabream product, and, according to the research plan, it has been subjected to a deeper characterization in comparison with the traditional untreated product.

Prototype presentation

The product prototype that will be presented in this section was developed consequently to the processing parameters optimization carried on before. Therefore, the innovative product obtained with the optimized parameters was compared to the untreated one, considered as he traditional product.

The product was characterized in terms of physicochemical characteristics (water content, pH, colour and textural parameters), oxidation status (lipid and protein) and sensorial acceptability, and for nutritional composition (nutritional fact labels) compared to the traditional untreated product.

Figure 6 shows images of the seabream fillets after packaging.

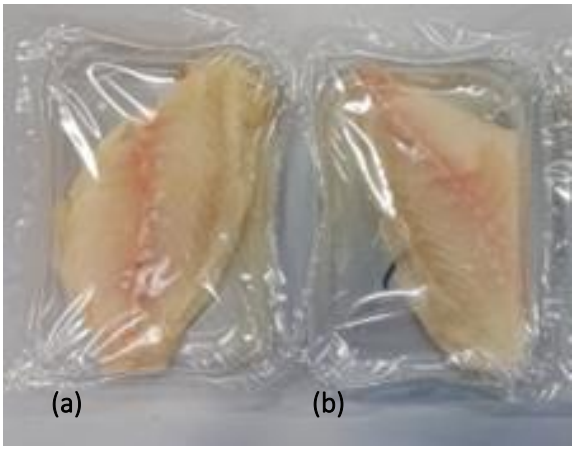

Figure 6. *Images of traditional (a) and innovative (b) seabream fillets individually packed in MAP using PP trays*

Physicochemical parameters

pH and colour were determined according to the SOPs attached in D6.1.

Water content was determined gravimetrically after drying in an oven at 70°C until constant weight.

Texture was measured with a Texture Analyser mod. TA.HDi 500 (Stable Micro Systems, Godalming, UK) equipped with a 25 kg load cell. The fillet was subjected to a compression with a cylindrical probe with a flat head, setting a descent rate of 3 mm/s, and using a cell load of 5 kg. The stress applied to the sample was measured through a stress-strain curve and the maximum force expressed in Newton (corresponding to the maximum peak reached during the compression of the sample) and normalized by the sample weight was considered.

Physicochemical properties are reported in **Table 13** compared to the traditional untreated product just after treatment and at the end of the shelf-life (considered as 9 days for the traditional product and 13 days for the innovative one).

Table 13. Physicochemical characteristics measured at the production day (T0) and at the end of shelf-life (Tf) set to 9 days and at 13 days for the traditional and innovative seabream product, respectively

	Traditional (untreated)	Innovative (Plasma treated)	Traditional (untreated)	Innovative (Plasma treated)
	T0		Tf	
Water content (%)	73.04 ± 1.08[a]	73.65 ± 1.68[a]	74.13 ± 0.38[a]	73.98 ± 0.23[a]
pH	6.57 ± 0.02[b]	6.45 ± 0.03[a]	6.51 ± 0.01[a]	6.46 ± 0.09[a]
L*	48.36 ± 2.26[b]	51.93 ± 2.42[a]	46.02 ± 2.07[a]	44.23 ± 4.87[a]
a*	2.63 ± 1.44[a]	-0.05 ± 1.07[b]	0.01 ± 0.59[a]	-0.93 ± 1.01[a]
b*	17.58 ± 1.19[a]	18.29 ± 1.54[a]	19.55 ± 0.29[a]	19.93 ± 1.79[a]
Shear force (N)	1.58 ± 0.42[a]	1.76 ± 0.39[a]	2.69 ± 0.81[a]	2.90 ± 0.36[a]

*Data are mean ± SD of **3 biological replicates** in each condition. Different letters indicate significant difference (p<0.05) between the products for each parameter, by the Students' t test, assuming p<0.05 as significant.*

A slightly lower pH was observed in treated compared to untreated fillets at T0, and small variations in the colour parameters L* and a*, consistent with the findings reported in D6.1. Moreover, there was a decrease of pH in untreated samples during the storage, not observed in the treated samples.

However, at the end of the shelf-life no differences were observed for any of the considered parameters. Therefore, it is possible to state that the much-extended shelf-life do not affect the physicochemical quality when the innovative treatment is applied to seabream fillets.

Oxidation status and sensorial acceptability

- TBARS value (according to the SOP – Annex 5 to D6.1)
- Protein oxidation status according to the method described by Soglia et al [17]
- Sensory evaluation carried out according to a modified quality index method (QIM) described in detail by Stamatis & Arkoudelos. [9] for sardine fillets.

As shown in **Table 14**, during the storage of the innovative sea bream fillets an increase in lipid oxidation was observed, while not observed for the traditional ones as documented by the different values at the end of the shelf-life. This finding was not surprising, because the same species generated in the cold plasma gas that are responsible for inhibiting microbial growth are expected to initiate the radical reaction underlying lipid oxidation. The optimization of the new processing treatment is mainly dedicated to maintaining the balance of the process with the negative oxidation well below the positive effect on the prolongation of the shelf-life. Therefore, the goal of optimization is to extend the shelf life without compromising nutritional and sensory properties. The limit of acceptability related to the TBARS value is unclear, as some authors state that the perception of rancid occurs when this value is greater than 4 mg MDA / kg [18], while others report that the limit of acceptability is 8 mg MDA / kg. This probably depends on the specific type of fish considered and the sensory analysis is decisive for the sea bream fillet.

As regard the protein oxidation levels, no differences were observed just after the treatment, although a slightly higher value was found at the end of the shelf-life compared to the untreated product. However, the value remained quite low and very similar to the beginning of the shelf-life.

Table 14. TBARS values and sensory score determined in seabream samples (traditional one – NT and innovative one – CP) after the treatment (T0) and at the end of the shelf-life (Tf).

	Traditional (untreated)	Innovative (Plasma treated)
	T0	
TBARS (mg MDA/kg)	2.09 ± 0.14[a]	1.56 ± 0.52[a]
Carbonyl content (nmol/g)	3.37 ± 0.82[a]	3.60 ± 0.53[a]
Sensory score	E	E
	Tf	
TBARS (mg MDA/kg)	1.63 ± 0.18[a]	6.76 ± 0.72[b]
Carbonyl content (nmol/g)	2.85 ± 0.27[a]	3.82 ± 0.54[b]
Sensory score	B	B

*Data are mean ± SD of **3 biological replicates** in each condition. Different letters indicate significant difference (p<0.05) between the products for each parameter at the same sampling time, by the Students' t test, assuming p<0.05 as significant.*

No differences were detected between control and treated samples regarding the sensory characteristics. At the end of the microbial shelf-life, both samples were found to be of fair quality (B) by the panel, as shown in **Table 14**.

This indicates that, although induction of lipid and protein oxidation was observed, it remained modest and below the consumer detection limit.

Nutritional characteristics

Nutrition facts label

Analyses were performed using accredited methods:

- Total lipids: UNI ISO 1443:1991
- Total carbohydrates: MIC 039 Rev.00 2013
- Total sugars: MIC 041 Rev.00 2013
- Proteins: UNI ISO 937:1991
- Salt: ISTISAN 96/34 page 124
- Humidity: ISTISAN 96/34 page 124
- Ashes: UNI 10590:1997

Energy was calculated according to EU Regulation N. 1169/2011.

The "nutrition facts" label of traditional (untreated) and Innovative (plasma-treated) fillets, obtained at T0, is reported in **Table 15**.

The composition and energy content of the untreated and plasma-treated fillets were similar, except for a lower ash content in the treated ones.

Table 15. Nutrition facts of traditional and innovative products

	Traditional (Untreated)	Innovative (Plasma treated)
ENERGY (kcal/100g)	121.33±2.52 [a]	123.67±3.06 [a]
TOTAL LIPIDS (g/100g)	4.03±0.21 [a]	4.23±0.25 [a]
TOTAL CARBOHYDRATES (g/100g)	0.72±0.08 [a]	0.88±0.14 [a]
SUGARS(g/100g)	0.67±0.06 [a]	0.77±0.15 [a]
PROTEINS (g/100g)	20.58±0.28 [a]	20.46±0.19 [a]
SALT (g/100g)	0.20±0.02 [a]	0.18±0.02 [a]
HUMIDITY (g/100g)	72.73±0.46 [a]	72.87±0.32 [a]
ASH (g/100g)	1.93±0.06 [a]	1.56 ±0.14 [b]

*Data are mean ± SD of **3 biological replicates** in each condition. Different letters indicate significant difference ($p<0.05$) between the products for each parameter by the Students' t test, assuming $p<0.05$ as significant.*

Final remarks

The combination of the plasma treatment and of the packaging conditions were fundamental to obtain a product characterized by good nutritional quality and extended shelf-life.

The innovative raw sea bream product has a shelf-life increased by 40% compared to the raw product, with minimal variations in terms of quality and nutritional value and without the use of additives, allowing a "clean label" to the final product.

As discussed in D6.1, many aspects still need further investigation in relation to the application of cold plasma technology in food manufacturing. Considering that cold plasma is not yet approved for utilization in food processing, these results represent useful knowledge that can increase the existing one about the effect of these types of treatments of fish product quality, safety and nutritional properties, thus promoting the future industrial exploitation of this technology. Useless to say that application of cold plasma must undergo the safety assessment according to the novel food legislation in EU, before releasing the fish products to human consumption.

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