Project acronym:	FutureEUAqua
Project title:	Future growth in sustainable, resilient and climate friendly organic and conventional European aquaculture
Grant number:	H2020-BG-2018-1: Project no. 817737
Coordinator:	NOFIMA, Norway
Website:	www.futureeuaqua.eu

Deliverable D6.5:

Prototype of an innovative ready-to-cook product containing flesh mince obtained by advanced recovery system

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WP/WP-leader:	WP6 Quality and safety of aquaculture products/Francesco Capozzi, UNIBO
Task/Task leader:	Task 6.1/UNIBO
Dissemination level:	PU
Deliverable type:	Dem
Approval Task/WP:	25.11.2022
Approval Project Management Board:	20.12.2022
Submission date:	21.12.2022

This project has received funding from the European Union's Horizon 2020 research and innovation programme under Societal challenge, Blue Growth, Grant Agreement No 817737.

Table of Contents

Executive summary

The FutureEUAqua project developed a novel convenient formulated fish product using farmed fish and related by-products. For this aim, trout flesh was mixed with different functional ingredients to obtain ready-to-cook fish-balls characterized by high nutritional value and desired sensory characteristics. In addition, chitosan extracted from crustacean by-products was added as edible coating to the fish-balls, allowing to increase their shelf-life and to protect from lipid oxidation during storage.

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

The seafood market is one of the food sectors that has experienced strong growth in recent years and, therefore, at the same time produces large quantities of waste and by-products. These processing by-products possess great potential for use and their valorisation could ensure the virtuous and sustainable use of resources in accordance with to the principle of the circular economy [1].

Food waste and losses (estimated as one third of food produced for human consumption) have a huge impact on food security, quality, environmental resources, and economic development [2]. Therefore, in recent years, this issue has attracted the attention of the scientific community, which has focused its efforts on the reduction, management, and valorisation of food waste and by-products [3]. To date, due to the ongoing depletion of natural resources and the increase in population and food demand, the need to limit energy consumption, minimize costs, and reduce food waste, loss, and wastage has arisen, which has led to strategies aimed at the recovery and valorisation of food waste and by-products [4]. Food loss refers to a decrease in mass (dry matter) or nutritional value (quality) of food that was originally intended for human consumption, mainly caused by inefficiencies in the food supply chains. Food waste refers to food appropriate for human consumption being discarded, often due to markets, or individual consumer habits. Food wastage refers to any food lost by deterioration or waste. Thus, the term “wastage” encompasses both food loss and food waste” [5].

To overcome these problems, the management and valorisation of food waste and by-products could play a key role. Such products are indeed characterized in most cases by a high potential for reuse and recycling but are still insufficiently recovered to obtain high added value products [3, 4].

Waste and by-products of the seafood industry

Each year, more than 20 million tons of fishery products are discarded at sea worldwide, representing 25% of the total catch. In the European Union, waste from the fish supply chain amounts to about 5.2 million tons, which includes "non-target" species, fish processing residues, and associated waste and by-products [1]. What remains of fish products after processing is commonly referred to as by-product

and, when properly handled, according to EC Reg. 1774/2002 is classified as Category 3 and can be used for human consumption (EC Reg. 1774/2002). These by-products consist of heads, viscera, skin, meat trimmings, offal, bones, fins, scales, blood, and eggs in proportions that change depending on the fish species and how it is processed [6].

Use of waste and by-products of fish production for formulating innovative foods

Approximately 70 % of fish is processed before final sale, although the percentage of waste can vary from 20 to 80 %, depending both on the type of processing and fish. In addition, a significant amount of fish is discarded from aquaculture each year [7]. The generated waste can be used as fish silage, fish meal, and fish sauce. Fish waste can also be used to produce various value-added products such as protein, oil, amino acids, minerals, enzymes, bioactive peptides, collagen, and gelatine [7, 8, 9].

In addition to recovering ingredients and additives through solid-state fermentation and enzymatic treatment, food industry by-products and waste can also be used to formulate new products to maximize the efficiency of the production process. For example, chitosan extracted from the carapace of crabs, shrimps, and mantis shrimps can be used as a thickening agent in vegetable oils, as an antioxidant in meat products, or as an antimicrobial agent in the formulation of fish burgers [10]. Products like the latter fit the global market demand for processed seafood products with higher added value, characterized by convenience of use and ease of preparation in small households [11]. Processed seafood products include refrigerated fish fillets, fish pies, fish burgers, fish sticks, frozen breaded and/or battered products, emulsified products such as fish frankfurters and fish sausages, surimi and related products as well as ready-to-cook or ready-to-eat products [11].

Fish-balls

Fish products, may not only be marketed as such or smoked, salted or cooked, but may also be used to produce ready-to-cook products. The main products in this category currently available on the national and international markets are fish burgers and fish cakes. The burgers are widely used and are mainly made from salmon (*Salmo salar*), Alaska pollock (*Gadus chalcogrammus*) and rainbow trout (*Oncorhynchus mykiss*) fillets. They are sold both frozen and refrigerated and are usually combined with Mediterranean spice blends and other ingredients such as sunflower oil, lemon juice, corn starch and potato flakes. However, scientific research is focused on producing fish burgers from species with low commercial value in order to enhance them. The most valorised species include carp [12], sand dory [13], bonefish [14], and tilapia [15].

Fish-balls represent an alternative to fish burgers that could be exploited opening new market possibilities for ready-to-cook fish products. As for fish burgers, fish-balls could be a valid alternative to enrich the diet of consumers used to eat convenience food like meatballs, that could be formulated by including spices that mask taste of fish that dissuades many consumers.

Reduction in meat consumption and increase in fish consumption is recommended for human health.

Aquatic animal foods are a rich source of protein and have a lower caloric density and have a high content of n-3 long chain polyunsaturated fatty acids (n-3 LC PUFA) compared to land living animals

[16]. 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 [17, 18].

Fish protein has since long been considered having a high nutritional value. Aquatic animal foods have higher protein content than most terrestrial meats. In addition, aquatic protein is highly digestible and rich in several peptides and essential amino acids that are limited in terrestrial meat proteins, as for example methionine and lysine [16].

Last, not least, fish is a good source of micronutrients as iodine and selenium.

Processing can strongly influence the nutritional value of fish products, the most vulnerable nutrients from fish being the fatty acids, which can easily be subjected to oxidation reactions with a consequent reduction of quality. Moreover, the processing and formulation of product might lead to changes in the digestibility of nutrients affecting their release during digestion.

For the development of innovative, ready-to-cook fish products with high added values, it is therefore important to evaluate modifications possibly occurring during processing and storage, and carefully check if innovative processing affects the nutritional value of the final product.

High dietary sodium intake is associated with an increased risk of hypertension, which is a risk factor for the development of cardiovascular disease. While the intake of sodium is too high, the average global intake of potassium is below the WHO guidelines. Thus, potassium chloride represents a valuable, safe replacer for sodium chloride in food products.

In the first part of this deliverable, the optimization of fish-balls formulation was carried out and the selected one was used for the development of an innovative product prototype, that was characterized and compared to a traditional one in the second part.

Development of the fish-ball formulation

The first phase of the experimentation focused on the identification of ingredients for the formulation that could improve the chemical-physical stability of the samples. Based on the study of literature and similar commercial products, different formulations were tested and subjected to sensory analysis to assess their acceptability for consumption.

In the second part, the effect of the addition of chitosan, obtained by crustacean's by-products to the selected formulation was carried out.

The trout used for this experiment were obtained by an Italian breeding company, Allevamento Puccini (Perugia). Fish was of commercial grade, average weight 0.25 kg. It was transported under ice to EMAR, where gutting, filleting and skinning were carried out. Fillets were fast frozen at -40°C and stored at the same temperature. Before processing, fish was thawed overnight at 4°C. After manual filleting, residual flesh was mechanically separated by using a belt-drum separator (mechanical deboner model 600, Baader, Germany). In detail, flesh was forced by means of a rubber conveyor belt

through a perforated drum (holes diameter 3 mm) and collected from the inside of the drum, while fish bones were discarded on the outside. Flesh was immediately refrigerated at 2-4°C, before mixing with other ingredients.

Chitosan was extracted by *Squilla mantis* carapace provided by the partner EMAR, following the method described by Tolaimate et al. [19] and Shrinivas Rao [20].

Chitosan offers real potential for applications in the food industry; it is classified as a GRAS by the US FDA in 2001. Chitosan is a polysaccharide obtained by the deacetylation of chitin, which is the principal component of the crustacean exoskeletons. The polymeric chitosan has numerous advantages, such as biocompatibility, biodegradability and non-toxicity and has already been successfully applied in different food systems [21].

Selection of ingredients

The following ingredients were used in the formulation of the fish-balls: mechanically separated trout flesh, potato starch, sodium chloride, black pepper, garlic powder, onion powder, lemon juice, parsley powder, and nutmeg. To these basic ingredients, whose proportion in the total dough remained constant throughout, breadcrumbs, citrus fibre, wheat fibre, potassium chloride, xanthan gum, and whey protein were added in different concentrations.

In particular, in this first screening phase, 6 independent variables (breadcrumbs, citrus fibre, wheat fibre, whey protein, xanthan gum, and whey) were considered by varying them in the concentration range of 0%-6.5%.

In **Table 1**, the different tested formulations are reported with the composition in terms of ingredients shown.

Table 1. Composition in terms of variable amounts of thickeners and structuring agents within different formulations tested for best acceptance

	A	PG-A	F-A	FF-A	WP-A	SL-A	GX-A
Trout flesh	87,5%	81%	81%	81%	81%	81%	81%
Breadcrumbs	-	6,5%	-	-	-	-	-
Citrus fiber	-	-	6,5%	-	-	-	-
Wheat fiber	-	-	-	6,5%	-	-	-
Whey protein	-	-	-	-	6,5%	-	-
Milk whey	-	-	-	-	-	6,5%	-
Xanthan gum	-	-	-	-	-	-	6,5%
Potato starch	8%	8%	8%	8%	8%	8%	8%
Sodium chloride	1,5%	1,5%	1,5%	1,5%	1,5%	1,5%	1,5%
Black pepper	0,5%	0,5%	0,5%	0,5%	0,5%	0,5%	0,5%
Garlic powdre	0,8%	0,8%	0,8%	0,8%	0,8%	0,8%	0,8%
Onion powder	0,8%	0,8%	0,8%	0,8%	0,8%	0,8%	0,8%
Lemon juice	0,2%	0,2%	0,2%	0,2%	0,2%	0,2%	0,2%
Parsley	0,5%	0,5%	0,5%	0,5%	0,5%	0,5%	0,5%
Nutmeg	0,2%	0,2%	0,2%	0,2%	0,2%	0,2%	0,2%

Furthermore, to the formulations previously described the effect of replacing part of sodium chloride with potassium chloride, in different concentrations (ranging from 0.3% to 1.5%) was also evaluated. Table 2 reports the different formulations containing a lower amount of sodium chloride (1.25 instead of 1.5%) substituted by increasing amount of potassium chloride. Potassium chloride (KCl), unlike sodium chloride (NaCl) is not a sweet salt, but a bitter salt that, if added in too high concentrations, can negatively affect the taste of food products.

Table 2: Composition in terms of variable amounts of potassium chloride within different formulations tested for best acceptance, also containing two different structuring agents (either citrus fibre or xanthan gum).

	F-PG	F-PG 0,8	F-PG 1	F-PG 1,5	GX-PG	GX-PG 0,8	GX-PG 1	GX-PG 1,5
Trout flesh	81%	80,2%	80%	79,5%	81%	80,2%	80%	79,5%
Breadcrumbs	6,5%	6,5%	6,5%	6,5%	6,5%	6,5%	6,5%	6,5%
Citrus fibre	2%	2%	2%	2%	-	-	-	-
Xanthan gum	-	-	-	-	2%	2%	2%	2%
Potato starch	8%	8%	8%	8%	8%	8%	8%	8%
Sodium chloride	1,25%	1,25%	1,25%	1,25%	1,25%	1,25%	1,25%	1,25%
Potassium chloride	-	0,8%	1%	1,5%	-	0,8%	1%	1,5%
Black pepper	0,05%	0,05%	0,05%	0,05%	0,05%	0,05%	0,05%	0,05%
Garlic powder	0,2%	0,2%	0,2%	0,2%	0,2%	0,2%	0,2%	0,2%
Onion powder	0,2%	0,2%	0,2%	0,2%	0,2%	0,2%	0,2%	0,2%
Lemon juice	0,3%	0,3%	0,3%	0,3%	0,3%	0,3%	0,3%	0,3%
Parsley	0,4%	0,4%	0,4%	0,4%	0,4%	0,4%	0,4%	0,4%
Nutmeg	0,1%	0,1%	0,1%	0,1%	0,1%	0,1%	0,1%	0,1%

The acceptability tests were performed internally in the laboratory and consisted in evaluating the overall acceptability of the fish-balls after cooking in an oven hedonic scale of 1 to 9, where 1 corresponded to "extremely low" and 9 to "extremely high" and 5 was the threshold for acceptability. According to the test results, formulation GX-PG 0,8 has been selected for further tests including chitosan.

Fish-balls preparation

Fish pulp obtained by mechanical separation was added with the selected ingredients. The powder ingredients were added first and then the liquids. Once all the ingredients were added, the kneading phase was carried out, which was conducted by using the food processor until homogeneous doughs were obtained. The doughs were then subjected to a cooling phase at a temperature close to 0 °C and for a time of about an hour, in order to allow for more compacting of the structure. After the rest-time was over, the forming phase of the fish-balls was carried out.

The fish-balls were formed manually by taking about 15 g of dough and then giving it the desired round shape. Once formed, the fish-balls were cooled to a temperature below zero degrees centigrade (about -18 °C) for a time of 20 minutes. This compacted the structure of the fish-balls and

decreased its brittleness (which could have resulted in deformation because of the handling operation).

Fish-balls appearance after formulation is shown in **Figure 1**.

Figure 1. *Raw fish-balls after preparation*

Fish-balls cooking

Once formed, the fish-balls were baked in a static oven for 15 minutes at a temperature of 180°C. Cooking was carried out only for the aim of delivering an edible product to the sensorial panel.

Figure 2 shows fish-balls appearance after baking.

Figure 2. *Fish-balls after baking at 180 °C for 15 minutes in a static oven*

Sensorial analysis

Sensory analysis was conducted by a panel of 20 untrained male and female panellists, aged 20-65 years. Samples (n=3 for each formulation, to allow multiple attributes evaluation) were presented to the panellists as randomized alphanumerical coded samples, cooked and at warm temperature as for the habitual consumption.

For the screening tests, only overall acceptability was tested, while for the selection of the chitosan addiction, a more complex sensorial test was carried out. The test included visual, gustatory, olfactory, texture and overall liking evaluation of the fish-ball samples. The evaluation of the different fish-ball formulations was conducted by the panel using the evaluation form shown in **Table 3**, through which the following attributes were evaluated: i) surface homogeneity; ii) surface appearance (evaluation of any defects); iii) odour intensity; iv) odour liking; v) flavour intensity; vi) flavour liking; vii) juiciness; viii) toughness; ix) chewability; and x) overall liking.

Attributes were rated based on a hedonic scale of 1 to 9, where 1 corresponded to "extremely low" and 9 to "extremely high" and 5 was the threshold for acceptability.

Table 3: sensory evaluation scheme for fish-balls assessment

ATTRIBUTE	SCORE*								
	1	2	3	4	5	6	7	8	9
Surface homogeneity									
Surface appearance									
Odour intensity									
Odour acceptability									
Taste intensity									
Taste acceptability									
Juiciness									
Hardness									
Chewiness									
Overall acceptability									

* 1= extremely low; 9= extremely high

Statistical analysis

Statistical analysis was carried out by the one-way ANOVA using Tukeys' as post-test and assuming $p<0.05$ as significant, with the software STATISTICA (StatSoft, Tulsa, Oklahoma), version 8.0.

Results of the sensorial analysis

Table 4. Results of the overall acceptability of fish-balls formulated with variable amounts of thickeners and structuring agents

	A	PG-A	F-A	FF-A	WP-A	SL-A	GX-A
Overall acceptability	7.02 ± 0.51^{c}	8.33 ± 0.17^{a}	7.63 ± 0.25^{b}	6.34 ± 1.13^{c}	6.43 ± 1.18^{c}	6.91 ± 1.07^{c}	7.65 ± 0.31^{b}

From the results, the presence of breadcrumbs in the formulation was particularly appreciated, both in terms of texture and flavour. The addition of citrus fibre and xanthan gum was also appreciated although resulted in a flourier texture.

Loss of liquid (syneresis) within a few hours of moulding and in some cases the presence of unpleasant aftertastes from a sensory point of view were observed and considered for the selection of the optimal formulation.

Figure 1. *Examples of the appearance of different formulated fish-balls after cooking*

Table 5 reports the overall acceptability of the fish-balls formulated with the partial replacement of sodium chloride with increasing concentrations (0.8-1.5%) of potassium chloride.

Table 5: Composition in terms of variable amounts of potassium chloride within different formulations tested for best acceptance, also containing two different structuring agents (either citrus fibre or xanthan gum).

	F-PG	F-PG 0.8	F-PG 1	F-PG 1.5	GX-PG	GX-PG 0.8	GX-PG 1	GX-PG 1.5
Overall acceptability	6.93 ± 0.11^{b}	6.51 ± 0.42^{b}	5.93 ± 0.15^{b}	5.01 ± 1.43^{c}	6.33 ± 0.21^{b}	7.53 ± 0.23^{a}	6.05 ± 0.21^{b}	5.25 ± 0.34^{c}

Results showed that increasing concentration of potassium chloride above 0.8% promoted a decrease of the overall acceptability, probably because of a bitter after-taste. Therefore, considering the combination that allowed to maximise the overall acceptability of the fish-balls, the first basic formulation was selected as reported in Table 6.

Table 6: Basic formulation

Ingredient	Relative content
Mechanically separated trout flesh	80.2%
Breadcrumbs	6.5%
Xanthan gum	2%
Potato starch	8%
Sodium chloride	1.25%
Potassium chloride	0.8%
Black pepper	0.05%
Garlic powder	0.2%
Onion powder	0.2%
Lemon juice	0.3%
Parsley powder	0.4%
Nutmeg	0.1%

Evaluation of the addition of chitosan

Once the optimal formulation was selected for obtaining fish-balls based on the higher overall acceptability, the effect of adding chitosan was also evaluated. Chitosan was added to the base formulation in concentrations of 0.5, 1 and 1.5 % by replacing the same amount of trout flesh; in addition, the effect of applying chitosan in the form of edible coating (CC) (1% w/v) was also evaluated.

The edible coating was formulated according to the method of Previdi et al. [10]. Once the chitosan solution was obtained, raw fish-balls were immersed in it for 30 sec (**Figure 4**), then kept at room temperature for 30 sec and immersed again for additional 30 sec in the chitosan solution. After being removed from the chitosan solution, the fish-balls were allowed to drain and dry at a temperature of 4°C in order to allow solidification and coating formation.

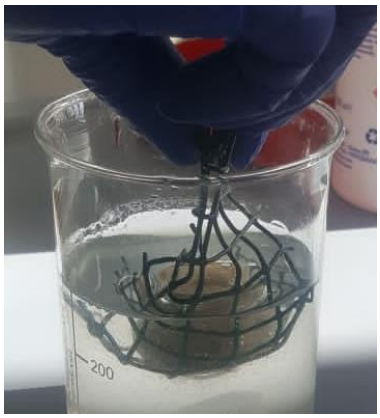


Figure 4. *Application of chitosan coating by immersion (dipping) in 1% (w/v) solution*

Five formulations were obtained and tested for sensorial score. Samples were coded as B for the basic formulation, C0,5, C1 and C1,5 for the formulations in which chitosan was added at concentration of 0.5, 1 and 1.5%, respectively, and CC for the formulation in which chitosan was added as coating.

Table 7 shows the results of the sensorial test on the 5 formulations obtained by adding chitosan in powder at different concentrations (C0.5, C1 and C1.5) and coating form (CC).

Table 7: sensory analysis results

	B*	C 0.5*	C 1*	C 1.5*	CC*
Surface homogeneity	7.00 ± 1.02^{a}	7.50 ± 0.55^{a}	5.83 ± 1.47^{b}	6.83^{a} ± 1.33^{a}	6.33 ± 1.25^{a}
Surface appearance	7.17 ± 0.98^{a}	6.83 ± 1.17^{b}	7.17 ± 0.98^{a}	7.00 ± 1.03^{a}	6.17 ± 1.34^{b}
Odour intensity	7.33 ± 1.11^{a}	6.40 ± 1.41^{b}	5.67 ± 1.05^{c}	6.17 ± 1.15^{b}	5.67 ± 1.07^{c}
Odour acceptability	6.33 ± 1.21^{a}	6.83 ± 0.25^{a}	6.50 ± 1.27^{a}	6.50 ± 1.32^{a}	6.33 ± 1.16^{a}
Taste intensity	6.83 ± 1.17^{a}	5.67 ± 1.51^{b}	5.99 ± 0.43^{b}	6.00 ± 0.89^{b}	6.83 ± 0.75^{a}
Taste acceptability	7.17 ± 0.98^{a}	6.00 ± 1.55^{b}	5.83 ± 1.47^{b}	5.33 ± 1.37^{b}	6.33 ± 1.75^{b}
Juiciness	6.67 ± 0.82^{a}	4.50 ± 1.22^{c}	4.83 ± 1.17^{c}	5.17 ± 1.33^{b}	6.83 ± 1.17^{a}
Hardness	2.83 ± 1.17^{a}	4.33 ± 1.51^{b}	4.50 ± 1.21^{b}	4.50 ± 1.23^{b}	2.83 ± 1.72^{a}
Chewiness	6.50 ± 1.22^{a}	5.50 ± 1.76^{b}	5.50 ± 1.32^{b}	5.00 ± 1.54^{c}	6.33 ± 1.25^{a}
Overall acceptability	7.54 ± 0.32^{a}	6.17 ± 0.96^{b}	6.00 ± 1.55^{b}	5.67 ± 1.16^{b}	6.87 ± 0.78^{b}

**1= extremely unpleasant; 9= extremely pleasant*

On the basis of the results obtained, it can therefore be stated that the formulation most appreciated from a sensory point of view was the B formulation (Traditional formulation). The addition of chitosan in powder form resulted in a change in the texture of the fish-balls, with an increase of hardness, a reduction of juiciness, odour and taste acceptability, resulting in a lower overall acceptability. By contrast, the use of chitosan in coating form resulted in similar values in terms of hardness, juiciness and odour acceptability. So, even if the taste acceptability was slightly reduced, the 'overall acceptability' was close to those of the control sample B.

Selected formulations

Based on the obtained results, the selected formulation with and without the addition of chitosan in the form of edible coating are reported in Table 8 and were considered as 'traditional' and 'innovative' formulations.

Table 8 Composition of the 2 selected formulations

	Traditional	Innovative
Mechanically separated trout flesh	80.2%	80.2%
breadcrumbs	6.5%	6.5%
Xanthan gum	2%	2%
Potato starch	8%	8%
Sodium chloride	1.25%	1.25%
Potassium chloride	0.8%	0.8%

Black pepper	0.05%	0.05%
Garlic powder	0.2%	0.2%
Onion powder	0.2%	0.2%
Lemon juice	0.3%	0.3%
Parsley powder	0.4%	0.4%
nutmeg	0.1%	0.1%
chitosan	-	*coating* 1% (w/v)

Product packaging and storage

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

According to the results of Deliverable 6.10, the products has been packed using modified atmosphere packaging, in particular 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).

For each package, six fish-balls of 15g each (gas to product ratio was about 4:1) were packed as shown in **Figure 6**. Packed samples were stored at 3 ± 1 °C while analytical determinations were carried out for the shelf-life study.

***Figure 6**. Fish-balls packed in 20% CO_2-80% N_2 using a vacuum-compensated packaging machine*

Shelf-life study

Analytical determinations

During storage, after 0, 2, 5, 8 and 14, 16 and 21 days, 3 packages for each sample were removed from storage and used for the analytical determinations related to:

- Microbiological analysis (SOP – Annex 2 to D6.1) for mesophiles, *Enterobacteriaceae* and psychrophiles
- Peroxide value as lipid oxidation index (according to the method described by Chapman & McKay [22])

Moreover, in order to define the effect of the formulation on the nutritional quality, the fatty acid profile composition 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.

To evaluate the nutritional value of the innovative fish-balls compared to the traditional ones, just after the preparation and after 14 days of storage, the following analyses were performed as first step:

- Fatty acid composition and content (as methyl esters) according to SOP-Annex11 to D6.1

Then, the fish-balls underwent *in vitro* digestion according to the method described in the Annex 12 to D6.1, and 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 [23].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 [24]. 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 formulation)

Statistical analysis

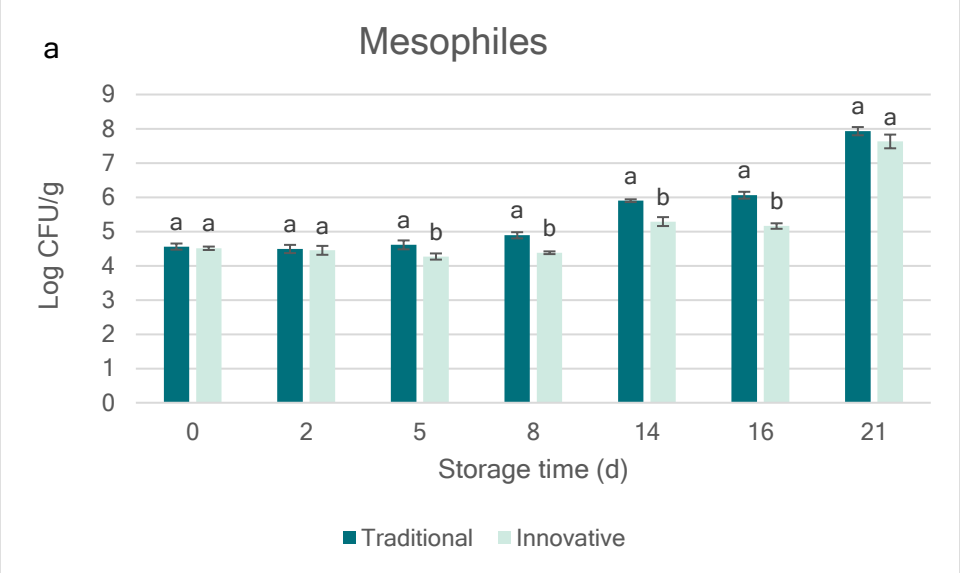
Statistical analysis was carried out by the Students' t test assuming $p<0.05$ as significant, with the software STATISTICA (StatSoft, Tulsa, Oklahoma), version 8.0.

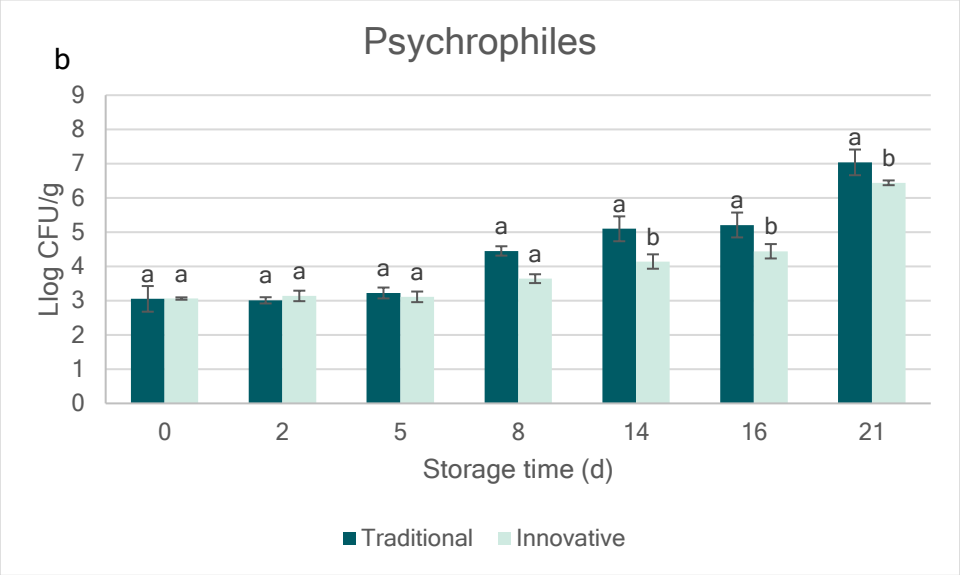
Shelf-life results

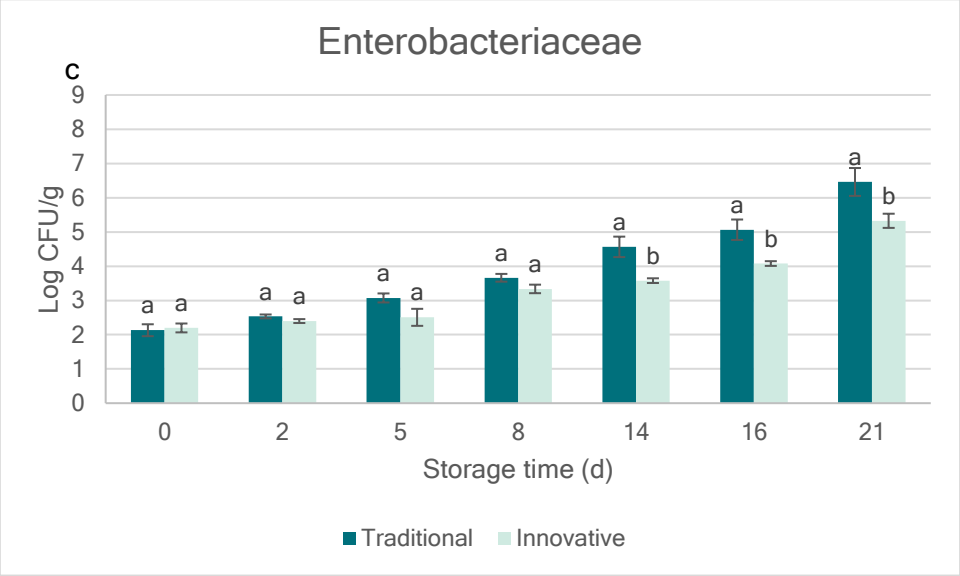
Microbial loads

Figure 7 reports the microbial loads (log CFU/g) of the mesophile bacteria in fish-balls during storage. Initial mesophiles loads were relatively high (approximately 4.5 log CFU/g), probably due to the intense handling of the fish flesh and to the addition of various ingredients.

Figure 7. Mesophiles (a), Enterobacteriaceae (b) and Psychrophiles (c) loads measured in the fish-balls during refrigerated storage.







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

The end of the shelf-life was considered as the time necessary to reach the microbiological load of 6 log CFU/g (Colony Forming Unit/g) of mesophiles according to standard references [25, 26, 27].

Therefore, the shelf-life of the traditional product was considered of 14 days, while that of the innovative one as 16 days, with an increase of 14%.

Oxidation indexes

Table 9. Peroxide value measured in the fish-balls during refrigerated storage

Storage day	Traditional	Innovative
0	5.06±0.10 [a]	3.70±0.40 [b]
2	8.03±0.22 [a]	6.75±0.12 [b]
5	8.87±0.31 [a]	8.38±0.08 [a]
8	8.92±0.01 [a]	8.48±0.08 [a]
14	10.79±0.38 [a]	9.89±0.03 [b]
21	8.05±0.07 [a]	7.54±0.13 [a]

Data are expressed as log mg meq O_2/kg *sample and are mean ± SD of* ***3 biological replicates*** *in each condition. Different letters indicate significant difference (p<0.05) by the Students' t test, assuming p<0.05 as significant between the products for each parameter.*

Table 9 shows the peroxide value recorder in fish-balls during storage. A significantly lower values was observed in the innovative product after 0, 2 and 14 days of storage.

The antimicrobial and antioxidant activity of chitosan used as coating on seafood products has been showed previously by other authors [10, 28], showing its high potentiality as a natural preservative.

Fatty acids

The fatty acid (as methyl esters) qualitative and quantitative composition of not-digested traditional and innovative products at T0 is reported in **Table 10**.

Table 10. Fatty acid composition (as FAME) of the traditional and innovative products at T0 and T14

	Traditional – T0	Innovative T0	Traditional – T14	Innovative T14
14:0	35.40±4.81 [a]	27.32±3.07 [b]	35.17±5.94 [a]	39.86±0.55 [a]
16:0	333.29±31.14 [a]	271.82±19.24 [b]	343.52±45.23 [a]	380.26±16.32 [a]
16:1 n-7	53.51±10.13 [a]	41.19±6.63 [b]	54.08±10.83 [a]	59.74±2.71 [a]
18:0	90.63±8.92 [a]	74.61±3.62 [b]	94.56±12.02 [a]	105.67±6.46 [a]
18:1 n-9	1138.04±115.42 [a]	885.86±90.67 [b]	1139.49±191.27 [a]	1286.17±5.17 [a]
18:2 n-6	590.41±69.51 [a]	468.56±57.36 [b]	602.99±85.29 [a]	658.20±14.40 [a]
18:3 n-3	119.72±11.84 [a]	92.94±9.35 [b]	122.26±16.06 [a]	133.93±5.20 [a]
20:1	55.47±3.97 [a]	42.60±4.50 [b]	55.86±8.66 [a]	63.60±2.98 [a]
20:4	24.20±1.97 [a]	20.10±1.16 [b]	26.47±2.19 [a]	28.30±1.60 [a]
20:5 n-3	69.68±7.04 [a]	58.30±3.26 [b]	76.22±6.92 [a]	76.91±2.51 [a]
22:5 n-3	22.71±1.92 [a]	18.68±1.21 [b]	24.73±3.97 [a]	25.77±1.67 [a]
22:6 n-3	221.55±17.56 [a]	185.73±7.38 [b]	239.16±25.05 [a]	241.97±21.82 [a]
ΣSFA	459.32±44.77 [a]	373.75±25.80 [b]	473.25±63.04 [a]	525.78±23.14 [a]
ΣMUFA	1247.02±129.48 [a]	969.65±101.64 [b]	1230.36±208.73 [a]	1409.52±52.71 [a]
ΣPUFA	1048.28±107.84 [a]	844.31±75.66 [b]	1110.90±102.37 [a]	1165.07±46.81 [a]
ΣPUFA n-3	433.67±37.56 [a]	355.65±18.21 [b]	462.36±38.11 [a]	478.58±30.91 [a]
ΣPUFA n-6	614.61±71.48 [a]	488.67±58.37 [b]	629.47±86.74 [a]	686.50±15.99 [a]
Σ n-6/Σ n-3	1.42±0.06 [a]	1.37±0.10 [b]	1.36±0.19 [a]	1.44±0.06 [a]
Total	2754.62±282.00 [a]	2187.71±202.24 [b]	2814.50±373.78 [a]	3100.37±121.79 [a]

Data are expressed as mg FAME/100 g sample and are mean ± SD of ***3 biological replicates*** *in each condition. Different letters indicate significant difference (p<0.05) by the Students' t test, assuming p<0.05 as significant between the products for each parameter.*

As expected, we observed that the fatty acid composition of the fish-balls mirrored their main ingredient composition (trout). The predominant fatty acids were docosahexaenoic acid among long chain n-3 PUFAs, palmitic acid among saturated fatty acids (SFAs), and oleic acid among monounsaturated fatty acids (MUFAs).

The total FAME content was significantly lower in the innovative than traditional product. This reduction involved almost all fatty acids, which were present in lower amount in the innovative food. Particularly, the total content of SFAs and MUFAs was lower in the innovative product, while the total content of PUFAs was similar. Among PUFAs, n-3 ones decreased in the innovative products; notwithstanding, the n-6/n-3 ratio was similar in the traditional and innovative fish-balls. A possible explanation is in the coating contribution to the total weight, chitosan being a fat-free ingredient replacing fat-containing flesh in the total weight constitution. However, the proportion of chitosan in the formulation does not explain the discrepancy between traditional and innovative fish-balls, unless the highly hygroscopic nature of this compound is considered. Therefore, at T0 the water retention holding capacity of chitosan reflects the lower lipid content in the innovative products. At the same time, since chitosan facilitates the transfer of water from the fish-balls core to their surface, it is expected that after storage, the total amount of water will decrease by evaporation from the product surface, and the lipid content may be higher in innovative rather than in traditional fish-balls

The fatty acid composition of not-digested products was analysed again after 14 days of storage and compared to the corresponding product at T0. Results are also reported in **Table 10.**

After 14 days of storage, the fatty acid composition of the traditional fish-balls was not modified. As expected, at T14 the content of total and single fatty acids of innovative fish-balls was higher than at T0.

The storage did not influence the fatty acid composition on traditional fish-balls. The increase in the fatty acid total content was observed since the FAME content is expressed on fresh weight. As explained above, their increase is consequent to a decrease in residual humidity facilitated by water-mass transfer from core to surface exserted by chitosan.

In-vitro digestion

The chemical composition of food reflects only in part their nutritional value, since not all components are released from the food matrix and became 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, to verify the composition of digested food is of paramount importance to understand their actual nutritional value. To this aim, both traditional and innovative fish-balls (at T0) underwent *in vitro* digestion, and the fatty acid composition was examined again in the digested products. Fatty acids derived from the blank digestion, i.e., without food, were subtracted. Indeed, blank digestion contains fatty acids from added bile. Results are reported in **Table 11**.

Table 11. Fatty acid composition (as FAME) of the traditional and innovative products (T0) after in vitro digestion

	Traditional	Innovative
14:0	25.60±2.17 [a]	19.82±2.17 [a]
16:0	214.28±32.24 [a]	166.94±27.59 [b]
16:1 n-7	41.51±2.63 [a]	32.41±3.07 [a]
18:0	31.18±15.65 [a]	19.32±13.13 [a]
18:1 n-9	851.01±47.84 [a]	706.13±70.14 [b]
18:2 n-6	432.26±22.64 [a]	357.47±31.48 [b]
18:3 n-3	82.90±4.08 [a]	69.30±5.48 [b]
20:1	45.05±3.40 [a]	36.45±3.62 [b]
20:4	13.60±1.13 [a]	18.29±5.98 [a]
20:5 n-3	43.99±2.26 [a]	37.52±3.81 [a]
22:5 n-3	16.03±0.59 [a]	9.62±1.46 [b]
22:6 n-3	136.64±5.88 [a]	116.76±14.24 [a]
ΣSFA	271.05±49.97 [a]	206.09±41.89 [a]
ΣMUFA	937.56±53.41 [a]	776.00±76.77 [b]
ΣPUFA	725.41±36.28 [a]	599.36±60.70 [b]
ΣPUFA n-3	279.56±12.69 [a]	235.99±24.55 [a]
ΣPUFA n-6	445.85±23.59 [a]	363.37±37.11 [b]
Σ n-6/Σ n-3	1.59±0.01 [a]	1.54±0.06 [a]
Total	1934.03±134.27 [a]	1581.45±178.19 [a]

*Data are expressed as mg FAME/100 g sample and are mean ± SD of **3 biological replicates** in each condition. Different letters indicate significant difference (p<0.05) by the Students' t test, assuming p<0.05 as significant between the products for each parameter.*

The total content of fatty acids was similar in digested traditional and innovative products, although some differences were observed in the content of single fatty acids. The concentration of palmitic (16:0), oleic (18:1 n-9), linoleic (18:2 n-6), linolenic (18.3 n-3), gondoic (20:1 n-9), and docosapentaenoic acid (22:5 n-3) was lower in innovative fish-balls than traditional ones, leading to a decreased concentration of total MUFAs and PUFAs, with no modification in n-3 PUFA concentration.

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 12**. The only significant difference is related to the content of docosapentaenoic acid (22:5 n-3).

Table 12. Relative release of fatty acids after in vitro digestion.

	Traditional	Innovative
14:0	73.68±15.19 [a]	72.66±3.65 [a]
16:0	65.22±14.94 [a]	61.16±6.38 [a]
16:1 n-7	79.88±18.39 [a]	79.23±5.08 [a]
18:0	35.68±19.66 [a]	25.37±16.85 [a]
18:1 n-9	75.54±11.27 [a]	79.73±1.15 [a]
18:2 n-6	74.14±12.03 [a]	76.50±2.65 [a]
18:3 n-3	69.89±9.86 [a]	74.68±2.37 [a]
20:1	81.75±11.42 [a]	85.62±2.90 [a]
20:4	56.56±8.12 [a]	90.40±26.05 [a]
20:5 n-3	63.67±8.48 [a]	64.25±3.07 [a]
22:5 n-3	71.00±7.79 [a]	51.28±4.78 [b]
22:6 n-3	62.01±6.67 [a]	62.75±5.71 [a]
ΣSFA	60.03±15.81 [a]	54.82±7.95 [a]
ΣMUFA	75.98±11.55 [a]	80.06±1.33 [a]
ΣPUFA	69.87±9.98 [a]	70.94±1.01 [a]
ΣPUFA n-3	64.90±7.75 [a]	66.23±3.60 [a]
ΣPUFA n-6	73.44±11.84 [a]	74.46±1.29 [a]
Total	71.00±11.54 [a]	72.20±2.05 [a]

*Data are mean ± SD of **3 biological replicates** in each condition. RR = mg FAMEs in digested/mg FAMEs in corresponding non-digested sample *100. Different letters indicate significant difference (p<0.05) by the Students' t test, assuming p<0.05 as significant between the products for each parameter.*

As evidenced in table 11, no significant differences were observed in the RR between the traditional and innovative products, except for docosapentaenoic acid. Therefore, the observed differences in the fatty acid composition of digested samples could not be ascribed to a different lipid digestibility in traditional and innovative products, but to the different fatty acid composition of not digested fish-balls. As far the fatty acid component concerns, the chitosan coating did not affect the triglycerides hydrolysis and therefore lipid digestibility of fish-balls.

The release of proteins during *in vitro* digestion was also determined using two different methods. As reported in **Table 13**, it was similar in the traditional and innovative products indicating that the chitosan coating had no effect also on protein hydrolysis during digestion.

Table 13. Protein release after in vitro digestion

Method	Traditional	Innovative
A 280nm	9.61±0.80 [a]	11.26±0.90 [a]
OPA	10.48±0.74 [a]	12.37±1.54 [a]

*Data are mean ± SD of **3 biological replicates** in each condition and are expressed as mg protein in digested sample/100 g of corresponding not-digested sample. Different letters indicate significant difference (p<0.05) by the Students' t test, assuming p<0.05 as significant between the products for each parameter.*

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 fish-balls 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 fish-balls 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 14 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 14. Relative concentrations, assessed by NMR spectroscopy, of molecular species released by in vitro digestion of fish-balls proteins, classified according to the spectral regions where signals resonate

	Traditional	Innovative
Hydrophobic Amino Acids Region (0.20-2.00 ppm)	87.64 ± 4.16 [a]	87.64 ± 4.16 [a]
Hydrophilic Amino Acids Region (2.00-3.00 ppm)	29.21 ± 1.35 [a]	28.85 ± 2.67 [a]
Total Amino Acids (α-CH) Region (3.20-4.70 ppm)	95.09 ± 6.82 [a]	96.46 ± 9.01 [a]
Aromatic Amino Acids Region (6.40-7.70 ppm)	8.15 ± 0.70 [a]	8.80 ± 0.92 [a]
Total Soluble Proteins Region (7.70-9.60 ppm)	3.43 ± 0.37[a]	3.77 ± 0.47 [a]

*Data are mean ± SD of **3 biological replicates** in each condition and are expressed as arbitrary integral units/5 g of digested sample. Different letters indicate significant difference ($p<0.05$) by the Students' t test, assuming $p<0.05$ as significant between the products for each parameter.*

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 traditional and innovative fish-balls. By inspection of Table 11, no differences emerge from NMR spectral data in the protein digestion of the two different food products.

Conclusion

Based on reported results, it is possible to state that the chitosan applied as coating to the fish-balls allowed to obtain a product with good sensorial properties, with a lower acceptability of taste but no negative effect on sensorial parameters of texture, juiciness, odour and overall acceptability.

At the same time, the innovative formulation containing chitosan edible coating was characterized by a longer shelf-life. Indeed, chitosan coating allowed to slightly reduce microbial growth and inhibit lipid oxidation, with an overall increase in quality of the product.

In addition, no detrimental effect was observed on the examined nutritional parameters. Particularly, the release of fatty acids and proteins/peptides from the food matrix during digestion was not negatively affected by the coating, indicating no modification of the actual nutritional value of the innovative product, at least for macronutrients.

Prototype characterization

Physicochemical properties

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

Water content was determined gravimetrically on the chopped-up fish-ball after drying in an oven at 105°C until constant weight.

Texture was measured with a Texture Analyser mod. TA.HDi 500 (Stable Micro Systems, Godalming, UK) equipped with a 50 kg load cell. The fillet was subjected to a double compression with a cylindrical probe (TPA-Texture Profile Analysis) that is also defined as a chewing test, since, through the two compression-decompression phases, it simulates the chewing action. Test speed was set at 1.0 mm/s with a total strain of 40% and a relaxation time of 5 s.

Physicochemical properties are reported in **Table 15** compared to the traditional uncoated product just after treatment and at the end of the shelf-life (considered as 14 days for the traditional product and 16 days for the innovative one based on microbial growth and PV).

For each determination, three replicates were carried out, each sample was obtained from 2 different fish-balls.

Table 15. Physico-chemical characteristics of the innovative fish-balls compared to the traditional ones

	Traditional formulation	Innovative coating	Traditional formulation	Innovative coating
	T0		Tf	
Water content (%)	73.04 ± 1.08^{a}	73.65 ± 1.68^{a}	63.91 ± 0.22^{b}	66.52 ± 0.98^{a}
pH	6.70 ± 0.04^{b}	6.56 ± 0.02^{a}	6.52 ± 0.01^{a}	6.35 ± 0.03^{b}
L*	51.83 ± 0.06^{b}	53.68 ± 1.36^{a}	53.04 ± 0.73^{b}	57.58 ± 0.50^{a}
a*	-1,57 ± 0.45^{a}	-1.52 ± 0.51^{a}	-1.76 ± 0.49^{a}	-2.14 ± 0.09^{a}
b*	14.81 ± 0.58^{a}	13.69 ± 0.49^{a}	14.27 ± 1.03^{a}	11.52 ± 0.27^{b}
Firmness (N)	2.73 ± 0.10^{a}	3.96 ± 0.47^{a}	4.81 ± 0.24^{a}	3.83 ± 0.09^{b}
Gumminess (N)	0.98 ± 0.07^{b}	1.52 ± 0.22^{a}	1.08 ± 0.9^{a}	1.16 ± 0.14^{a}

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

Some significant differences were observed between the samples. The lower pH observed in innovative sample was probably related to the chitosan coating, that, according to the method of Previdi et al [8], was obtained by dispersing chitosan in a 0.5% solution of acetic acid. The application of the coating was also probably responsible for the higher L* value in the innovative sample. These differences were maintained throughout the storage period. However, at the end of the shelf-life, also a higher water content and a lower firmness value were detected in the innovative sample compared to the traditional one. On the other, side, while gumminess was higher for the innovative product at

the beginning, similar values were observed at the end of the shelf life. Other textural parameters (cohesiveness and adhesiveness) did not show any difference between the samples (data not reported). The higher water quantity in the innovative products at end of shelf-life (T14) is in contrast with what expected from the lipid analysis described above. Indeed, the higher amount of lipids in the T14 innovative product was explained by a lower amount of water, a result not replicated in the prototypes. Depending on the layer depth of the edible coating and on the porosity of the matrix, one of two mechanisms may prevail: i) reduced drip loss during storage, thus maintaining a softer texture (firmness value like the beginning of the shelf-life), ii) water recall from the fish-ball core to its surface with consequent larger evaporation on the long storage times. At this stage, without further chemical and structural details, any other hypothesis is speculative, also considering the analytical method for water quantification, based on drying at 105°C, which may underestimate water content in presence of hygroscopic chitosan.

Oxidation status and sensorial acceptability

Since for fish products parameters such as lipid oxidation and sensorial perception are important quality indexes, they were also evaluated just after formulation and until the end of the microbiological shelf-life.

As lipid oxidation index PV value was measured, and a sensorial analysis was conducted at the beginning and at the end of the shelf-life. Sensory evaluation was carried out according to the method described previously in this deliverable, considering only overall acceptability as parameter.

Table 16. TBARS values and sensory score determined in trout fishballs (traditional and innovative one) after the treatment (T0) and at the end of the shelf-life (Tf).

	Traditional	Innovative
	T0	
PV (m_{eq} O_2/kg)	5.06 ± 0.14^b	3.70 ± 0.56^a
Sensory score	7,5 ± 0.3^a	6,9 ± 0.8^b
	Tf	
PV (m_{eq} O_2/kg)	8.05 ± 0.1^b	7.54 ± 0.19^a
Sensory score	5.8 ± 0.8^a	6.0 ± 0.4^a

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

As reported in **Table 16**, the use of chitosan allowed to reduce the lipid oxidation index both at the beginning and at the end of the storage, with only a slight reduction of the sensorial acceptability. Indeed, samples were perceived in a similar way, above the threshold for acceptability (score of 5) by the panel until the end of the microbiological shelf-life.

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 and innovative products is reported in **Table 17**.

Table 17. Nutrition facts of traditional and innovative products

	Traditional (n=3)	Innovative (n=3)
Energy (kcal/100 g)	168.33±3.21 [a]	175.67±4.93 [a]
Total lipids (g/100 g)	3.67±0.25 [a]	3.60±0.26 [a]
Carbohydrates (g/100g)	17.28±0.93 [b]	19.67±0.86 [a]
Sugars (g/100g)	0.38±0.13 [a]	0.43±0.15 [a]
Proteins (g/100g)	16.54±0.32 [a]	16.08±0.27 [a]
Salt (g/100g)	1.00±0.09 [a]	0.86±0.06 [a]
Humidity (g/100g)	59.17±0.96 [a]	57.40±0.89 [a]
Ash (g/100g)	3.35±0.22 [a]	3.25±0.15 [a]

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

The composition and energy content of the traditional and innovative products were similar, except a higher content of total carbohydrates in the innovative one. This might be explained by the presence of chitosan that being a dietary fibre is included in the total carbohydrate count and by the presence of glycerol acting as plasticizer in the coating solution [8]. In fact, for the conventional nutrition facts label, carbohydrates content is defined as the complement to 100 g of water, ashes and all nutrients present in the food (except for sugars, which are part of carbohydrates).

Final remarks

The formulation developed in this project can be exploited for the production of an added value formulated fish products based on trout flesh allowing to obtain an added value ready-to-cook product with high quality and nutritional value. The combination with chitosan obtained by crustaceans' carapaces allowed to increase the shelf-life by 14% maintaining the nutritional profile,

the sensorial properties and limiting the lipid oxidation during storage and at the same time to increase the overall value of the fish processing sector by valorising a by-product.

The approach used in this research, combining sensorial acceptability test followed by the evaluation of different stability indexes and nutritional quality proved to be efficient for maximizing quality of the final product and could be applied also to develop formulations using different raw materials (different fish flesh and/or secondary ingredients).

New formulations that combine fish flesh and various ingredients, including those deriving from by-products can be also obtained based on different tastes and habits in different European regions to meet a larger sector of consumers and further encourage fish consumption.

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