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Metabarcoding for the authentication of complex seafood products: The fish burger case

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ABSTRACT

The species composition of fish burgers - declared as composed by European seabass (*Dicentrarchus labrax*) - collected in the context of a seafood company self-control was assessed using metabarcoding. Positive/negative controls, replicates, samples processed in dirty laboratory environment were also included as quality measures. A ≈ 200 bp of the *16S rRNA* gene was selected as molecular target. The sequencing was performed on Illumina platform, and the data were analysed using DADA2 R package. The species taxonomic assignment was performed using Blastn against GenBank (identity value ≥ 99 %). The sequences assigned to *D. labrax* were highly predominant in all the products, with percentages ≥ 99.34 %, except for one, where also a high number of sequences assigned to Atlantic salmon (*Salmo salar*) were found (12.41 %). Sequences identified with other species (seafood, mammals, insects) were in percentage ≤ 0.57 %, and in the 14 % of the cases they even did not achieve the 0.001 %. A threshold value of 3.3 % to remove false positives was fixed based on the results of the positive controls. Overall, metabarcoding was proved as effective technique to assess the ingredients contained in complex seafood products. However, further investigation including a higher sample number and inter-laboratory validation should be performed to validate the procedure.

1. Introduction

Food fraud is any suspected intentional action carried out by businesses or individuals for the purpose of deceiving purchasers and gaining undue advantage therefrom (European Commission, 2018) and it has increasingly diffused due to the globalization and complexity of modern supply chains. In the European Union (EU), mislabeling - meaning false claims or distortion of the information reported on the label (European Commission, 2018) is currently the preponderant form of food fraud (Brooks et al., 2021; Visciano & Schirone, 2021).

Seafood is among the commodities at high risk of mislabelling (Kroetz et al., 2020). The most common is the substitution of high-value with less expensive species or farmed versus wild sourcing, the selling of fish from illegal fishing, and the recycling of by-catches or fish waste (Kroetz et al., 2020; Reilly, 2018). Potential consequences include economic losses, ecological impact, undermining of sustainability efforts, and breach of religious or ethical reasons. In addition, the illicit presence of toxic species or the omission of ingredients potentially causing allergies (e. g. crustaceans or molluscs) may lead to human health risks (Giusti et al., 2023).

Food business operators (FBOs) hold the primary legal responsibility for ensuring compliance with food law within the activities under their control (Regulation EC No 852/2004). The FBOs must implement a personal self-control system, which prevents and reduces food-borne hazards, and which is based on the internationally recognized Hazard Analysis and Critical Control Point (HACCP) principles (Regulation EC No 852/2004;). Beyond the laws and regulations, commercial and safety standards have expanded to address food fraud and FBOs are increasingly focusing their approach on VACCP (Vulnerability Assessment and Critical Control Point), that is used to assess and mitigate vulnerabilities from food fraud/authenticity and adulteration. The challenge for FBOs is to implement a monitoring program, which could be in part augmented by analytical testing to identify potentially fraudulent activity. In fact, sampling and analysis is one of the main mitigation

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measures and actions for fraudulent activity (Butler et al., 2021). The global impact of food frauds is encouraging the food companies to increasingly invest in means and tools, which include innovative molecular technologies, to prevent, manage and reduce this phenomenon.

Molecular methods, based on DNA analysis, are currently the most applied to assess seafood products species composition, and their use is also encouraged at the regulatory level (Regulation EU No, 1379/2013). Among them, DNA barcoding (Hebert et al., 2003) is the most applied (Fernandes et al., 2021; Giusti et al., 2023;). However, its efficiency is limited by the number of target species that can be simultaneously identified. This aspect may represent a concrete issue when more complex products, presumably composed of several species (e. g. minced matrices composing burger, surimi, fillings, etc.) are analysed (Haynes et al., 2019). These products have captured a growing market share, reflecting the diet habits of consumers that are increasingly addressed to ready-to-eat/ready-to-cook products (Giusti et al., 2017a).

The Next Generation Sequencing (NGS) technologies are high-throughput methods able to simultaneously sequence all DNA molecules present within one sample, including those in trace amounts (Goodwin et al., 2016). They represent the most promising analytical tool for the analysis of processed multispecies seafood products.

The use of universal PCR primers to amplify massively one or more taxonomically informative targets, combining the NGS with DNA barcoding, has been termed metabarcoding (Fernandes et al., 2021). The application of this technique for the analysis of food composition is still limited differently from other investigation fields, such as the analysis of biodiversity in environmental samples or the trophic interactions, where it is well developed and widely used. The reason for its still marginal use in this field can be related to the lack of standardized protocols which may render the analysis complex, time-consuming, and expensive and discourage its routinely application by laboratories (Dobrovolny et al., 2019; Haynes et al., 2019; Pan et al., 2020).

In 2021, the FishLab (Department of the Veterinary Sciences of the University of Pisa) was consulted by an Italian seafood company that, under self-control, requested the authentication of two fish burgers that, when analyzed by both DNA barcoding and metabarcoding, showed different results. In particular, the DNA barcoding identified one fish burger as European seabass (*Dicentrarchus labrax*) using the *16S rRna* gene and as Gilthead seabream (*Sparus aurata*) using the *COI* gene; both the samples, analyzed with metabarcoding (using a shorter *16S rRNA* region) were found as containing *D. labrax*, even though sequences of other species, including *S. aurata*, were detected. Therefore, in this study, other fish burgers, provided by the same company, were analysed using metabarcoding, giving particular emphasis to the quality control of the entire process, from the DNA extraction to the final results interpretation. This case -study represents a preliminary step to define an efficient internal protocol, also potentially transferable to other labs after a proper validation, to combat fraud in the seafood chain.

2. Materials and methods

2.1. Sampling, total DNA extraction and evaluation

Overall, nine fish burger products (2–3 samples each, for a total of 24 samples), sent to the FishLab by an Italian seafood company for molecular authentication, were analysed (Table 1). Total DNA was extracted from all the 24 samples with the protocol proposed by Armani et al. (2012). This phase was entirely performed under hood, trying to avoid external contamination and/or cross contamination among samples (clean lab environment – CLE). Total DNA concentration and purity were evaluated with Nanodrop ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE, US) by two subsequent measurements of the absorbance value at 260 nm and calculation of A260/A280 and of A260/A230 ratio according to manufacturer's indications. Ratios A260/A280 and A260/A230 around 2.0 were considered as satisfactory values of DNA purity. Each DNA sample was stored at − 20 °C until further analysis.

Table 1
Products collected in this study (n = 9) with the corresponding analyzed samples (n = 24).

Product	Sample
P-1	P-1(A)
	P-1(B)
	P-1(C)
P-2	P-2(A)
	P-2(B)
	P-2(C)
P-3	P-3(A)
	P-3(B)
	P-3(C)
P-4	P-4(A)
	P-4(B)
	P-4(C)
P-5	P-5(A)
	P-5(B)
	P-5(C)
P-6	P-6(A)
	P-6(B)
	P-6(C)
P-7	P-7(A)
	P-7(B)
P-8	P-8(A)
	P-8(B)
P-9	P-9(A)
	P-9(B)
9	24

2.2. DNA barcoding analysis

One replicate of one sample from each product (n = 9) was preliminary submitted to DNA barcoding analysis targeting *COI* and *16S rRNA* genes. Also, two samples used as positive control and four used as analytical blanks during the DNA extraction (described below in Section 2.3.1) were included in this analysis. The *COI* region (~ 650 bp) was amplified using the primer pair FISH_COILBC (5'- TCAACYAATCAYAAAGATATYGGCAC-3') e FISH_COIHBC (5'-ACTTCYGGGTGRCCRAARAATCA-3') (Handy et al., 2011) with the following PCR protocol: 20 µl reaction volume containing 2 µl of a 10X buffer (BiotechRabbit GmbH, Berlin, Germany), 100 mM of each dNTP (Euroclone Spa, Milano, Italy), 200 nM of forward primer, 200 nM of reverse primer, 1.0 U PerfectTaq DNA Polymerase (BiotechRabbit GmbH, Berlin, Germany), 100 ng of DNA, and DNase free water (Euroclone Spa, Milano, Italy). The following cycling program was applied: 95 °C for 30 s; 40 cycles at 95 °C for 30 s, 55 °C for 30 s, 72 °C for 30 s and final extension at 72 C for 5 min. The *16 S rRNA* region (~600 bp) was amplified using the primer pair 16Sar_L (5'-CGCCTGTTTATCAAAAACAT-3') e 16Sbr_H (5'-CCGGTCTGAACTCAGATCACGT-3') (Palumbi, 1996) with the same PCR protocol of *COI* and the following cycling program: 95 °C for 30 s; 40 cycles at 95 °C for 30 s, 57 °C for 15 s, 72 °C for 15 s and final extension at 72 C for 5 min. Five microliters of each PCR product were checked by gel electrophoresis on a 2 % agarose gel. The amplification of fragments of the expected length was assessed by making a comparison with the standard marker SharpMass™ 50-DNA ladder (Euroclone Spa, Milano, Italy), and the concentration of PCR products by making a comparison with the intensity of the bands of the DNA ladder. Positive reactions (PCR products with concentration >5 ng/µl) were sent to sequencing. The obtained sequences were visualized, aligned, and edited using BioEdit version 7.0.9 (Hall, 1999). All the sequences were submitted to a BLAST analysis against GenBank (https://www.ncbi.nlm.nih.gov/genbank/) and, in the case of *COI* gene, also to IDs analysis on BOLD system (https://www.boldsystems.org/). Values ≥ 98 % and ≥ 99 % were selected as threshold for species identification using *COI* and *16S rRNA* genes, respectively (Ardura et al.,

2012; Barbuto et al., 2010).

2.3. Metabarcoding analysis

2.3.1. Quality control of the process

Sixteen samples were used to assess eventual environmental and/or cross-contamination that might affect metabarcoding outcomes. These samples, shown in Fig. 1, were represented by: 1) six samples belonging to the products P-1 and P-3 that, during the total DNA extraction, were also processed outside the hood (dirty lab environment – DLE); 2) Two samples to be used as positive control of the analysis (K+), prepared by mixing tissue of Gilthead seabream (*Sparus aurata*) and Blunthead puffer (*Sphoeroides pachygaster*), using 50 mg for each species).; 3) Eight analytical blank samples (K-): 4 consisting of no tissue samples, that were included in the total DNA extraction phase, and 4 consisting of no DNA, included in the amplification phase. Thus, the metabarcoding analysis was performed on 24 samples belonging to the nine fish burger, and on the 16 samples used as measures to assess contaminations, for a total of 40 samples (Fig. 1).

2.3.2. Amplification, sequencing

The primer pair 16sf-var (5'-CAAATTACGCTGTTATCCCTATGG-3') and 16sr-var (5'-GACGAGAAGACCCTAATGAGCTTT-3') (Chapela et al., 2002), amplifying a fragment of about 200 bp of the *16S rRNA* region with high inter-species variability (Giusti et al., 2017a), were added to Illumina overhang adapter sequences: Forward overhang (5'-TCGTCGGCAGCGTCAGATGTGTATAAGAGACAG-[16sfvar] and Reverse overhang: 5'-GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAG-[16sr-var]. The *16S rRNA* region (~210 bp without primers) was amplified with the same PCR protocol reported in section 2.4, with the following cycling program: 95 °C for 30 s; 40 cycles at 95 °C for 30 s, 53 °C for 30 s, 72 °C for 15 s and final extension at 72 C for 5 min. PCR products were checked as describe in section 2.4. Positive reactions (PCR products with concentration >10 ng/μl) were sent to the external sequencing company IGA Technology Services Srl (Udine, Italy), where the amplicons were purified and indexed (NexteraXT Index Kit, FC-131–1001/FC-131–1002) and libraries were sequenced on NovaSeq instrument (Illumina, San Diego, CA) using 150-bp paired-end mode.

2.3.3. Data analysis and species assignment

Folder containing files with raw reads (fastq raw files, forward and reverse reads separated) and FastQC reports (files with parameters that give insight into overall processing and sequencing quality) were provided by the company. Once received, the fastq raw files were processed to generate amplicon sequence variants (ASVs) using DADA2 R package (Callahan et al., 2016). Briefly, primers were removed, forward and reverse reads were trimmed based on the Quality Score (QS). The filtered reads were used to train the error model using machine learning approach. Then forward and reverse reads were dereplicated to generate unique sequences and denoised (collapsed) in amplicon sequence variants (ASVs) applying the trained error model. Finally, forward and reverse reads were merged and checked for chimera sequences. Representative sequences for each ASV were assigned using Blastn against GenBank database (https://blast.ncbi.nlm.nih.gov/Blast.cgi). The species with ≥ 99 % similarity will be merged and considered at species level (Ardura et al., 2012). The percentage of each species (for each sample and for each product) was calculated based on the read numbers. Moreover, the taxonomic assignments were used to calculate the diversity index for each product (n = 9). In particular, Simpson's diversity index (SDI), a measure of diversity which considers the number of species present, as well as the relative abundance of each species was calculated. The following formula was used to calculate the SDI, with n = number of reads of a single species and N = number of total reads in the product:

$$SDI = 1 - \left(\frac{\sum n(n-1)}{N(N-1)}\right)$$

The value of SDI ranges between 0 and 1. With this index, 1 represents infinite diversity and 0, no diversity.

Also, the Shannon-Weiner Diversity Index (SWDI) was calculated using the following formula, with p_i= proportion of reads of *i* species in the product.

$$SWDI = -\sum[(pi_i) \quad x\log(pi_i)]$$

The minimum value the SWDI can take is 0, intended as there's no diversity - only one species is found in that product.

3. Results and discussion

3.1. Total DNA extraction and evaluation

Total DNA concentration extracted from the 24 analysed samples and from the six DLE samples ranged from 116 ng/μl to 1354 ng/μl (mean 556 ng/μl), with quality ratios A260/A280 and A260/230 of about 2. Higher total DNA concentration was observed for the 2 K+ samples (mean 1397 ng/μl; range 1045–1750 ng/μl), with similar quality ratios. As expectable, the 4 K- showed a low total DNA concentration (mean 2.7 ng/μl; range 1.5–3.9 ng/μl) with quality ratios < 1. All the DNA samples, including K-, were used for the amplification, since it was decided that also these samples would be sequenced to evaluate the eventual presence of DNA in trace.

3.2. Preliminary DNA barcoding analysis

The target *16S rRNA* region was successfully amplified (presence of specific amplification band with concentration >5 ng/μl) from all the nine analyzed samples and the two K+ samples. Contrariwise, the target *COI* region was successfully amplified from four out of the nine analyzed samples and the two K+ samples (Table 2). No amplification was obtained from the four K- samples, included in both *16S rRNA* and *COI* genes PCR. Therefore, 17 PCR products (11 from *16S rRNA* and six from *COI*) were sent to sequencing, and the same number of usable sequences were obtained. All the nine analyzed samples, from P-1(A) to P-9(A), were assigned to the species *D. labrax* (European seabass) using *16S rRNA,* while the four *COI* sequences were identified as *S. aurata* (Table 2). This occurrence was also observed in one of the two fish burgers analysed using both DNA barcoding and metabarcoding in 2021, while *D. labrax* was found as the predominant species in both of them using metabarcoding (Table SM-1). In this context, targeted PCR assays showed that the *COI* primer pair had a high affinity to *S. aurata* respect to other species; contrariwise, the pair showed a markedly low affinity for *D. labrax*, which was found to be amplified only in the event that it was the only species in the sample (data not shown). The 2K+ samples were instead assigned to the species *S. aurata* (factually contained in these samples) using both the genes (Table 2). In the light of the obtained results, it seems that the PCR generates a well-known bias, which is related to primer–template mismatches and to purely stochastic effects (Piñol et al. 2015). In the presence of multispecies matrices, some species increase their relative abundance, others decrease it, while others are not amplified and so remain hidden (Piñol et al., 2015; Zhang et al., 2020). Therefore, preliminary PCR tests for evaluating primer amplification efficiency are pivotal for the selection of universal primers with a wide species coverage (Giusti et al., 2017b). In this respect, the *16 S rRNA* primer pair projected by Chapela et al. (2002) and used in this study were assessed (*in silico* and in vitro) on a wide range of fish and cephalopods (Giusti et al., 2017b).

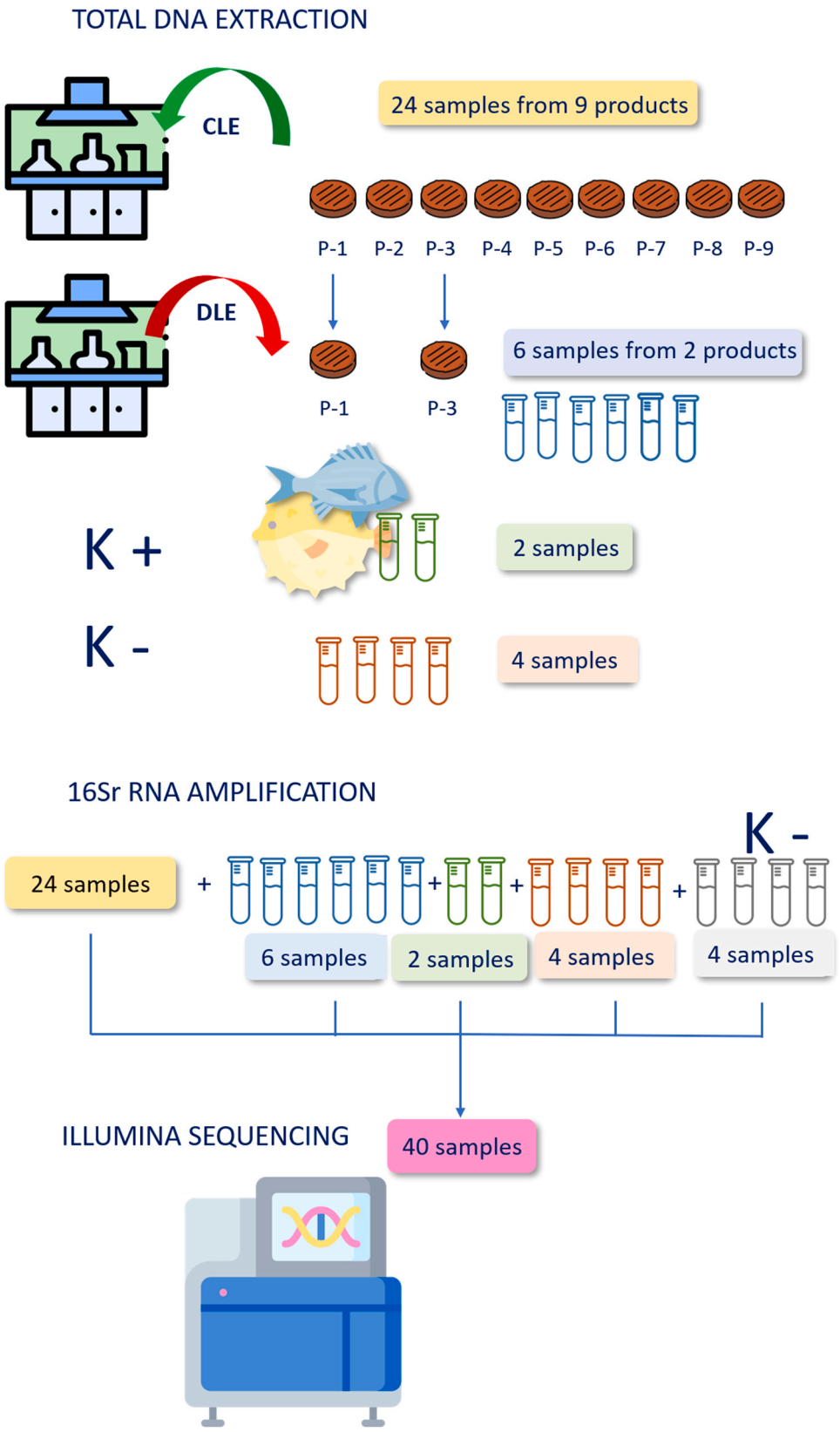

Fig. 1. Samples analyzed using metabarcoding, included samples used for the process quality control. CLE: clean laboratory environment; DLE: dirty laboratory environment; K+ : positive control samples; K-: Analytical blanks.

Table 2
DNA barcoding (*16S rRNA* and *COI*) outcomes: successful amplifications and samples identity; K+ : positive control sample. The two samples already identified by DNA-barcoding and metabarcoding (FB-1, MM-1) are included.

Sample	Successful amplification		*16S rRNA* ID	COI
	16S rRNA	*COI*		
P-1(A)	yes	yes	*D. labrax*	*S. aurata*
P-2(A)	yes	yes	*D. labrax*	*S. aurata*
P-3(A)	yes	-	*D. labrax*	-
P-4(A)	yes	-	*D. labrax*	-
P-5(A)	yes	-	*D. labrax*	-
P-6(A)	yes	-	*D. labrax*	-
P-7(A)	yes	-	*D. labrax*	-
P-8(A)	yes	yes	*D. labrax*	*S. aurata*
P-9(A)	yes	yes	*D. labrax*	*S. aurata*
FB-1	yes	yes	*D. labrax*	*S. aurata*
MM-1	yes	yes	*D. labrax*	*D. labrax*
K+ (1)	yes	yes	*S. aurata*	*S. aurata*
K+ (2)	yes	yes	*S. aurata*	*S. aurata*

3.3. Metabarcoding analysis

3.3.1. Quality control of the process

To the best of our knowledge, a very exiguous number of studies applying metabarcoding to seafood authentication relied on the inclusion of replicates, positive and negative controls. Taking replicates for DNA extractions was suggested to increase reliability of species detection (Lanzen et al., 2018). Considering that a limited number of species was expected to be found in the products given the nature of the fish burgers, three replicates for each sample (two in case of scares tissue amount) were used. Given the high sensitivity characterizing the metabarcoding technique the detection of false positives - contaminant DNA from taxa that was not likely to be present in the sample - that can be introduced at any stage of the metabarcoding process, is frequent. (Alberdi et al. 2019; Drake et al., 2022; Yang et al., 2021). The certain identification of false positive in a sample is challenging (Drake et al., 2022; Sepulveda et al., 2020), and the inclusion of positive and negative controls is essential to verify the effectiveness of the process, the impact of the contamination and the adequacy of bioinformatics filtering procedures (Zinger et al., 2019). Theoretically, negative controls (extraction blanks and PCR blanks) should contain no DNA, and positive controls should only contain DNA from selected taxa (Drake et al., 2022). Negative and positive controls should be sequenced concurrently with biological samples (Zinger et al. 2019).

In other research fields (e. g. investigation of biodiversity in environmental samples and trophic interactions), the use of experimental samples to evaluate contamination at various workflow levels and to establish a reads threshold below which a species may be considered a false positive has become a common practice (Sepulveda et al., 2020; van der Loos & Nijland, 2020). Thus, in this study both K+ and K- were included. The K+ were especially produced using on the one hand a common fish species (*S. aurata*) since, based on our lab experience, we were sure that the used primer pair would be able in amplifying it. On the other hand, *S. pachygaster* was chosen as we considered highly improbable to found it in commercial fish products. In fact, species from Tetraodontidae must not be placed on the EU market (Commission Regulation EC No, 1020/2008); thus, in the hypothesis we found sequences from this species in the analysed samples, we would have been sure that it was related to a cross-contamination during the analytical workflow.

3.3.2. Amplification outcomes

PCR products suitable for sequencing (were obtained from all the 24 samples (Table 1), the six DLE samples and the two K+. As expectable, no amplification bands were obtained from the eight K-.

3.3.3. Sequences quality

The FastQC reports were observed for all the 40 sequenced samples (Table 3). In this first sequencing, a QS $>$ 30 was observed in 17 out of the 24 analysed samples (70.8 %), with reads number ranging from 25,006 to 80,403 (forward & reverse separated) (Table 3). With respect to the 16 samples used as quality control of the process, a QS $>$ 30 was observed only in five out of the six DLE samples (reads number 42,678–66,505). A low sequence quality was observed for all the 8 K-sand also for both K+ samples (Table 3).

Therefore, we re-examined each phase of the workflow to investigate the potential cause/s that could have determined the lower quality of some samples respect to others. We noticed that the low QS was associated to the samples that showed a non-specific amplification band of about 120 bp after the electrophoretic run. The occurrence and the extent of this event was casual, and we supposed that this could be linked to the presence of hairpins loops and dimers. In fact, an unexpected small peak at 120–170 bp during quality checks of sequencing libraries indicates the presence of adapter dimers according to Illumina guidelines (https://knowledge.illumina.com/library-preparation/general/library-preparation-general-troubleshooting-list/ 000001911).

Therefore, we amplified a second time the samples with low QS, together with a selection of samples with good QS. Two parallel PCR were performed, one using the same amplification protocol (section 2.4.2) and one using ½ primer concentration. The electrophoretic run of the PCR with ½ primer concentration did not show non-specific bands. Thus, the samples that showed low QS in the previous analysis, including K+ , were sent to sequencing again (second sequencing). Good QS (>30) were obtained from all the samples, with a reads number ranging from 101,723 to 264,841 (Table 3).

3.3.4. Bioinformatic analysis

The ASV-based approach using DADA2 (Callahan et al., 2016), is especially recognized as optimal bioinformatic tool to analyze metabarcoding data (Drake et al., 2022). In fact, only identical sequences are clustered together, whereas traditional percentage identity-based clustering methods may generate different taxonomic diversities which could impact the perception of contamination (e. g. by obscuring some instances of contamination where these are taxonomically close to sample taxa) (Drake et al., 2022). In this study, the outcomes of the bioinformatic analysis showed that the percentage of maintained reads for each sample ranged from 73.8 % to 96.8 %, being over 91.3 % in all the samples except for four (P-1A; P-6 C; P-7B, P-3(C)DLE), for which it ranged from 73.8 % to 78.4 % (Table 3).

3.3.5. Reads taxonomic assignment

D. labrax, the species declared by the dealer, was found as major component in term of reads number in all the 24 samples (Table SM-2).

In fact, the percentage of reads identified with *D. labrax* was $\geq$ 98.89 % (range 98.89–99.99 %) in all the samples except for the two replicates of P-7 (the unique minced product), with 87.36 % (P-7A) and 88.53 % (P-7B). In these samples, 12.59 % and 11.36 % of reads identified as *S. salar* (Atlantic salmon), respectively, were also found (Table 4). The reads identified with other species were in percentage $\leq$ 0.57 %, and in 14 % of the cases they even did not achieve the 0.001 %.

The choice of the threshold below which a species can be linked to a contamination should be carefully considered as it can considerably impact the data; low thresholds will be unsuccessful at removing false positives whereas high thresholds may remove too much data, resulting in false negatives (Hänfling et al., 2016). Drake et al., 2022 demonstrated that thresholds around 1 % eliminate a remarkably high proportion of contamination in the field of eDNA. Respect to food authentication, a threshold of 1 % (w/w) for undeclared meat species in meat products was set by the UK Food Safety Authority and Department for Environment Food and Rural Affairs (Defra) in Europe (Chaora et al., 2022). In studies applying metabarcoding to food authentication, the 1 % threshold value was adopted by Cottenet et al. (2020), Xing et al.

Table 3
Reads number obtained from the samples in the first and second sequencing (input) and outcomes of the bioinformatic analysis using DADA2 R package (Callahan et al. 2016). *Samples unusable in the first sequencing (low Quality Score) and submitted do the second sequencing.

Sample	Input	filtered	denoisedF	denoisedR	merged	nonchim	maintained (%)
P-1(A)	25,006	19,268	19,261	19,263	19,207	19,207	76.8
P-1(B)	48,467	47,082	47,073	47,075	46,935	46,926	96.8
P-1(C)	45,761	44,151	44,136	44,143	44,035	44,035	96.2
P-2(A)	30,237	28,693	28,676	28,686	28,609	28,609	94.6
P-2(B)	80,403	75,635	75,555	75,608	75,323	75,278	93.6
P-2(C)	48,237	46,543	46,528	46,489	46,317	46,296	96.0
P-3(A)*	231,198	222,786	222,722	222,316	217,025	215,293	93.1
P-3(B)*	247,583	239,258	239,095	239,199	236,084	233,543	94.3
P-3(C)	52,559	49,193	49,169	49,179	49,065	49,065	93.4
P-4(A)*	180,853	174,893	174,322	174,624	171,999	171,283	94.7
P-4(B)*	158,672	152,784	152,624	152,710	151,486	149,759	94.4
P-4(C)	30,103	28,070	28,053	28,051	27,986	27,986	93.0
P-5(A)*	209,185	201,346	201,303	199,938	197,798	197,261	94.3
P-5(B)*	170,446	164,803	164,417	164,732	163,543	162,815	95.5
P-5(C)	42,490	40,362	40,347	40,347	40,268	40,268	94.8
P-6(A)	57,132	52,574	52,559	52,558	52,417	52,413	91.7
P-6(B)	44,011	41,457	41,435	41,435	41,329	41,329	93.9
P-6(C)	51,321	39,932	39,917	39,911	39,770	39,770	77.5
P-7(A)*	239,027	231,035	230,557	230,917	223,841	220,881	92.4
P-7(B)	50,105	38,284	38,268	38,263	37,907	36,964	73.8
P-8(A)	56,471	52,149	52,128	52,132	51,955	51,896	91.9
P-8(B)	70,559	67,517	67,479	67,473	67,153	67,124	95.1
P-9(A)	34,885	33,516	33,508	33,510	33,366	33,362	95.6
P-9(B)	59,342	56,921	56,910	56,740	56,553	56,553	95.3
P-1(A)DLE	66,505	63,964	63,935	63,951	63,729	63,716	95.8
P-1(B) DLE	62,267	57,223	57,206	57,207	57,057	57,057	91.6
P-1(C) DLE	55,416	52,364	52,351	52,253	52,103	52,097	94.0
P-3(A) DLE	42,678	39,492	39,488	39,487	39,402	39,402	92.3
P-3(B) DLE	62,110	57,129	56,986	57,079	56,807	56,807	91.5
P-3(C) DLE*	199,787	187,411	187,304	187,300	181,711	176,577	88.4
K+ (1) *	264,841	250,642	250,599	250,546	243,305	241,682	91.3
K+ (2) *	101,723	96,316	96,268	96,310	94,503	94,350	92.8

(2019) and Kappel et al. (2017). Higher values (2 %) were used by Chaora et al. (2022), Wang et al. (2021) and Xing et al. (2021). Also, lower values (<0.1 %) were reported (Dobrovolny et al., 2019; Pan et al., 2020; Ribani et al., 2018a, 2018b).

In this study, reads identified as *D. labrax* in percentage of 3.33 % and 1.3 % were also found in the two experimental K+ specially prepared using tissue from *S. pachygaster* and *S. aurata* (section 2.4.1) (Table SM-2). Assuming that they were related to contamination occurred during the lab procedures, we hypothesized that our threshold could be 3.33 %. We can also suppose that all the species found in percentages below 1.3 % (the minimum percentage of contamination of our K+) may originate from contaminations occurred before the samples receiving, at various level of the production chain. This aspect was also confirmed by the fact that no substantial differences were observed among CLE and DLE samples (Table SM-2). In fact, respect to the results of the two fish burger analysed in 2021, we observed that the percentage of reads that could be attributed to false positives was overall higher. Thus, overall, we confirm that the choice of thresholds must depend on the individual study as reported by Drake et al. (2022).

With respect to the two experimental K+ samples, although the same tissue amount of *S. pachygaster* and *S. aurata* were used for their preparation, the percentage of reads identified as *S. pachygaster* was much higher respect to *S. aurata* (91.82 % vs 4.85 % in K+1; 94.96 % vs 4.01 % in K+2). This may highlight a different affinity of the used primer pair respect to the two species. To note that, analyzing the same two K+ with DNA barcoding, only *S. aurata* was identified (Section 3.3). Also, *S. aurata*, over-estimated using *COI* primers, seemed instead under-estimated using the *16 S rRNA* primer of Chapela et al. (2002). For such cases, a multi-genic approach can be considered (Carvalho et al., 2017; Ribani et al., 2018a; Ribani et al., 2018b) or more primer pairs for each gene could be used (Bertolini et al., 2015). In addition, the simultaneous amplification of long and short fragment from the same regions are reported for seafood identification in commercial products (Kappel et al. 2017; Ho et al., 2020; Gense et al., 2021; Noh et al., 2021).

3.3.6. *False positives assessment*

The other species found in the samples and considered as false positives (contaminants) according to the established threshold belonged to fish, cephalopods, mammals, and insects. Also, cases of reads showing no significative similarity with sequences deposited on GenBank (NSSF) were observed.

Among fish, reads of *S. pachygaster* and *S. aurata,* the species used to prepare the experimental K+ , were found in 54.2 % (n = 13) and 41.7 % (n = 10) of the 24 samples, respectively (Table SM-2) probably, as already mentioned, due to contamination occurred at various level of the workflow. Contrariwise, DNA from several other seafood species, - randomly found in most of the samples (Table SM-2) – are presumably related to contamination occurred before the samples receiving. This evidence is not surprisingly, as fish companies usually process an extremely wide range of seafood species.

Cephalopod contaminations, linked to the species *Octopus vulgaris* (Common octopus) and *Amphioctopus membranaceus* (Webfoot octopus) was found in four samples (16.7 %). In this respect, the presence of undeclared molluscs poses a potential health threat for allergic consumers. This hazard was also encountered in a previous work analysed surimi-based products (Giusti et al., 2017a).

Among mammals, DNA from human (*Homo sapiens*) was found in nine out of the 24 samples (37.5 %) (Table SM-2). The detection of human DNA with metabarcoding is important to define the quality of the laboratory procedures (Tillmar et al., 2013). We can assume that human contamination in our lab was almost absent considering the range in which we supposed that a contamination may be related to the laboratory environment (above 1.3 %). Contrariwise, it is highly probable that this contamination occurred at the seafood company level, where the product manipulation is higher. The presence of other mammalian or avian species in seafood products was already reported (Ho et al., 2020;

Table 4
Product final authentication using metabarcoding.

Product	Species	ASV (n)	%	Final ID
P-1	*Dicentrarcus labrax*	109,881	99.87	*D. labrax*
	Sparus aurata	92	0.08	
	Argyrosomus spp.	30	0.03	
	Sphoeroides pachygaster	12	0.01	
	NSSF	5	0.005	
P-2	*Dicentrarcus labrax*	148,900	99.86	*D. labrax*
	Sparus aurata	149	0.10	
	Argyrosomus spp.	46	0.03	
	Gadus morhua/Gadus macrocephalus	14	0.01	
	Sphoeroindes pachygaster	5	0.003	
P-3	*Dicentrarchus labrax*	497,792	99.96	*D. labrax*
	Homo sapiens	75	0.015	
	NSSF	66	0.01	
	Tribolium confusum	53	0.01	
	Sphoeroindes pachygaster	19	0.004	
	Pleuronectes platessa/Platichthys spp./*Lepidosetta* spp./*Psettichthys melanosticus/Limanda ferruginea*	7	0.001	
P-4	*Dicentrarchus labrax*	346,724	99.34	*D. labrax*
	NSSF	682	0.19	
	Homo sapiens	678	0.19	
	Sitophilus oryzae	588	0.17	
	Callosobruchus maculatus/Bruchus pisorum	332	0.09	
	Sphoeroides pachygaster	13	0.004	
	P. platessa/Platichthys spp./ Lepidosetta spp./P. melanosticus/L. ferruginea	8	0.002	
P-5	*Dicentrarchus labrax*	398,695	99.59	*D. labrax*
	Katsuwonus pelamis	604	0.15	
	Homo sapiens	599	0.15	
	Callosobruchus maculatus/Bruchus pisorum	358	0.09	
	NSSF	67	0.02	
	Sitophilus oryzae	12	0.003	
	Sphoeroides pachygaster	9	0.002	
P-6	*Dicentrarchus labrax*	133,028	99.92	*D. labrax*
	NSSF	66	0.05	
	Callosobruchus maculatus/Bruchus pisorum	12	0.01	
	Dicentrarcus punctatus	11	0.01	
	Sitophilus oryzae	6	0.005	
	Katsuwonus pelamis	7	0.005	
	Sphoeroides pachygaster	2	0.002	
	Octopus vulgaris	3	0.002	
	Tribolium confusum	2	0.002	
P-7	*Dicentrarcus labrax*	225,580	87.53	*D. labrax*+ *S. salar*
	Salmo salar	31,982	12.41	
	Octopus vulgaris	95	0.04	
	Homo sapiens	39	0.01	
	Sphoeroides pachygaster	15	0.01	
	P. platessa/Platichthys spp./ Lepidosetta spp./P. melanosticus/L. ferruginea	6	0.002	
P-8	*Dicentrarcus labrax*	117,924	99.41	*D. labrax*
	Sparus aurata	175	0.15	
	Argyrosomus spp.	146	0.12	
	Homo sapiens	132	0.11	
	Salmo salar	76	0.06	
	Dicentrarcus punctatus	56	0.04	
	Xiphias gladius	50	0.04	
	Chelon labrosus/Mugil cephalus/ Liza aurata/Oedalechilus labeo	31	0.03	
	Paralichthys olivaceus	19	0.02	
	NSSF	16	0.01	
	Scomber scombrus	2	0.002	
P-9	*Dicentrarcus labrax*	89,426	99.42	*D. labrax*
	Homo sapiens	239	0.27	
	Sparus aurata	93	0.10	
	Argyrosomus spp.	53	0.06	
	Salmo salar	51	0.06	
	NSSF	33	0.04	
	Capreolus capreolus	18	0.02	
	Paralichthys olivaceus	15	0.02	
	Amphioctopus membranaceus	8	0.01	
	Xiphias gladius	5	0.01	
	Scomber scombrus	3	0.003	
	Lates niloticus	3	0.003	

Wang et al., 2021; Mottola et al. 2022; Piredda et al., 2022). As highlighted, this is a critical aspect for consumer protection based on ethical issues (Piredda et al., 2022). In this study, the species *Capreolus capreolus* (roe deer) was found in two sample (4.1 %) from the same product (P-9). Also in this case, since in our lab this species was never treated, its presence was assumed to result from previous contaminations.

Finally, *Callosobruchus maculatus/Bruchus pisorum, Sitophilus oryzae, Tribolium confusum* are reported as insect pests recorded on stored food commodities (Barwal & Devi, 1993; Stejskal et al., 2015). This minimum degree of contamination is not surprisingly as breaded product are involved. This outcome proved that the metabarcoding analysis could also represent a way to evaluate the FBOs management respect to the food hygiene criteria during processing.

3.3.7. Final products authentication

No differences were observed among the two-three sample replicates, so that data were aggregated for each analyzed product. Outcomes of the final product authentication are reported in Table 4. The contaminant DNA supposed as originating from laboratory procedures (*S. pachygaster; S. aurata*) did not achieve the 0.01 % by aggregating data in many cases (Table 4).

Respect to the products' SDI, the values ranged from 0.0006 to 0.2184. As expectable, the higher SDI (0.2184) was observed in P-7, found as produced using two fish species (*D. labrax* and *S. salar*). All the other products showed SDIs very close to 0 (0–006–0.0115). In fact, they were found as composed by only *D. labrax*, while all the other found species can be assumed as contaminants. It is interesting to note that, by including data from P-1(DLE) and P-3 (DLE) to P-1 and P-3 (thus achieving 6 replicates each), the SDIs remained very similar, with values of 0.0024 and 0.0005, respectively. Similarly, the SWDI ranged between 0.0041 and 0.0482 in all the products except for P-7, where it was 0.3804, corroborating the hypothesis we made during the selection of the number of replicates. At the end of the analysis, we can state that according to the declared main ingredient (*D. labrax*) all the analyzed products except for one (P-7), were compliant. In fact, only in this sample a consistent percentage of sequences assigned to *S. salar* were found (Table 4).

4. Conclusions

This study was developed to address the need of a private seafood company to authenticate multispecies seafood products using metabarcoding. For the setting up of the internal protocol, quality control measures (including positive and negative controls, replicates, samples processed in dirty laboratory environment, threshold value to remove false positives) was especially used. Metabarcoding was confirmed as efficient tool to authenticate multispecies seafood products, and the selected primer pair was proved as performant in amplifying all the species included in the analysed products. Nonetheless, this can only be regaded as a preliminary study, since a higher sample number should be used for the protocol validation, which should also include inter-laboratory trials. The availability of a validated protocol would allow a more extensive use of metabarcoding in the context of both official controls by Competent Authorities and FBOs self-control and might contribute to increase the capability to reduce food frauds.

CRediT authorship contribution statement

Alice Giusti: Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Supervision, Writing – review & editing. **Andrea Armani:** Conceptualization, Data curation, Formal analysis, Funding acquisition, Methodology, Project administration, Supervision, Writing – original draft, Writing – review & editing. **Chiara Malloggi:** Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Writing – review & editing. **Riccardo Forzano:** Conceptualization, Writing – review & editing. **Viola Lonzi:** Investigation. **Antonio Solimeo:** Investigation. **Beatrice Meneghetti:** Investigation. **Lara Tinacci:** Writing – review & editing.

Declaration of Competing Interest

The authors have no conflict of interests.

Data Availability

Data will be made available on request.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.jfca.2023.105559.

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