Università di Foggia

Dottorato di Ricerca in

Qualità degli Alimenti e Nutrizione umana

Coordinatore: Prof.ssa Annunziata Giangaspero

TESI DI DOTTORATO
XXVIII Ciclo

Biomolecular identification of fish species by PCR and analysis of microbiological risk linked to the consumption of ready to eat fishery products

Dottorando:
Dott. Pietro Di Taranto

Relatore:
Prof. Giovanni Normanno

ANNO ACCADEMICO 2014– 2015

ABSTRACT

The present study regarded the isolation and the characterisation of *Staphylococcus aureus* and *Listeria monocytogenes* from ready to eat (RTE) fishery products and the development and the improvement of novel PCR protocols for the identification of fish species. For the detection of *S. aureus* and *L. monocytogenes*, 99 and 135 RTE samples, respectively, were collected at local retail outlets and analysed according to ISO procedures in the laboratories of Food Microbiology of Experimental Zooprophylactic Institute of Apulia and Basilicata located in Foggia. RTE fishery products for the isolation of *S. aureus* and *L. monocytogenes* consisted of 33 and 45 samples of marinated anchovies fillets (*Engraulis encrasicolus*), 33 and 45 samples of smoked salmon (*Salmo salar*), 33 and 45 samples of seafood salad, respectively. As regards the identification of fish species, novel species-specific primers were developed by the program "Primer Express 3.0" and by the software “Primer-BLAST” to amplify fragments of 200 bp, 250 bp, 300 and 562 bp, 350 bp, 400 bp and 522 bp within COI gene for *Merluccius merluccius*, *Lates niloticus*, *Gadus morhua*, *Ruvettus pretiosus*, *Pangasius hypophthalmus*, *Epinephelus* spp., respectively. Ten samples of each fish species of interest were obtained from wholesale fishery plants. DNA was extracted from individual sample and quantified. DNA isolates were subjected to end-point PCR analysis and PCR products were sequenced. Out of 33 samples of smoked salmon, *S. aureus* was isolated from one sample (3.03%). The *S. aureus* strains carried the *icaA, seb* and *sec* genes and were resistant to ampicillin and tetracycline. *L. monocytogenes* was isolated from 2 of 45 samples of smoked salmon (4.44%). The strains of *L. monocytogenes*, isolated from both samples, resulted to belong to the serovar 1/2a and to be susceptible to all antibiotics tested. Single PCRs were performed using DNA isolates and the developed primers for each fish species of interest. After sequencing, the isolates were compared with the selected sequences of COI gene and showed a similarity ranging from 99 to 100%. Duplex and Triplex PCR protocols were developed for the simultaneous analysis of more fish species using the designed primers with several combinations. In addition, a survey on fish products was carried out to evaluate the application of labelling laws and to detect fraudulent actions using the developed PCR protocols. Forty-three fishery products were collected, in particular 18 and 25 samples at hypermarket stores, and at local fisheries and fish marketplaces, respectively. Fishery products purchased at local fisheries and fish marketplaces consisted of 20 fish fillets and 5 fish slices. After PCR analysis and sequencing, 19 (44.2%) resulted mislabelled, with 18 (41.9%) mislabelled samples from local fisheries and fish marketplaces and 1 (2.32%) from hypermarket stores. As regards fish samples purchased at local fisheries and fish marketplaces, fraudulent actions regarded more fish slices (100%) than fish fillets (65%). Regarding fish fillets,

out of four samples labelled as grouper, three (75%) resulted to be *Lates niloticus* and one (25%) *Pangasianodon hypophthalmus*. Two fillets marketed as cod (100%) were substituted with *Lates niloticus* (100%). Five samples labelled as "fillet" and two samples labelled as "perch" were identified as *P. hypophthalmus*. As regards fish slices, all samples marketed as grouper slices (*E. marginatus*) were slices of *Ruvettus pretiosus* (100%). The single case of mislabelling detected from fishery products purchased at hypermarket stores regarded a sample of "Spinycheek grouper" (*Epinephelus diacanthus*) that was indicated on label as "Grouper" (*Epinephelus marginatus*). In conclusion, our work highlights the need of a continuous surveillance on the commercialisation of fishery products, in order to reduce the food-borne risk linked to the presence of *S. aureus* and *L. monocytogenes* in RTE fishery products. Furthermore, our protocols based on PCR techniques could be useful for quality controls of fresh finfish and to strengthen controls on the most frequent fraudulent actions of marketed fishery products.

Keywords: fishery products, food safety, frauds, PCR, *Staphylococcus aureus, Listeria monocytogenes.*

TABLES OF CONTENTS

1. INTRODUCTION

Fish has always been appreciated by consumers for its particular organoleptic properties and because is easy to prepare and to cook. Eating fish is part of the cultural traditions of many people. In fact, in some populations, seafood is a major source of food and essential nutrients. Fishery products present a valid alternative to other types of animal-origin food (terrestrial animal meat, eggs, dairy products, etc.) especially for their high digestibility due to a lower presence of connective tissues and lipid component (Henderson and Tocher, 1987). Fish is a key component of a healthy diet and the consumption of about 1-2 servings of seafood per week has been associated with many benefits. From a nutritional point of view, fish is a source of energy and protein with high biological value, balanced in composition in essential amino acids, rich in methionine and lysine (EFSA, 2014). This underlines the importance of fish not only for the diet of the industrialised countries, but also for the supply of the poorest people, which is often based on the tuber or cereal consumption in which amino acids are limited. Seafood also provides important nutrients such as *n*-3 long-chain polyunsaturated fatty acids (LC *n*-3 PUFA), in particular eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) synthesized from α-linolenic acid (ALA), that is a component of dietary patterns associated with good health (Williams and Burdge, 2006). The consumption of fish, because of the beneficial effects of the LC *n*-3 PUFA on the cardiovascular system, may decrease the risk of cardiovascular disease (CVD) and may have an effect on the molecular, cellular and whole-body pathogenic processes of atherosclerosis and thrombosis (Calder, 2004). Data derived from prospective epidemiological studies and secondary prevention trials conducted in subjects at high risk of coronary heart disease (CHD) also support the conclusion that these fatty acids protect against CHD (Artham *et al*., 2008). Moreover, many studies have shown beneficial effects of increased LC *n*-3 PUFA intake on CHD mortality (Burr *et al*. 1989; GISSI-Prevenzione Investigators, 1999; Singh and Ward, 1997). Health benefits have been also observed during pregnancy and lactation. High levels of fish consumption, and of EPA and DHA, have been suggested to be responsible for the reported longer gestation rates and higher birth weights (Olsen, 2002). On the contrary, low consumption of fish can be considered as a strong risk factor for preterm delivery and low birth weight (Olsen, 2002). Adequate intakes of DHA, between 100 - 200 mg per day, have been estimated for pregnant and lactating women in order to accommodate the needs of their infants for deposition of DHA in the brain and retina (EFSA, 2010a). So, this is linked with better visual acuity in early life because of the recently-established role for DHA in GTP-dependent signal transduction pathways involved in vision (Jeffrey *et al*., 2001; Mitchell *et al.,* 2003). Moreover, a number of studies have reported the effects of consuming

increased amounts of dietary α-LNA on the fatty acid composition of plasma or cell lipids (Burdge and Calder, 2005). Fish presents high levels of minerals (calcium, iron, iodine, selenium, phosphorus and fluorine), vitamin A, vitamin E and vitamins of B group (Elvevoll and Osterud, 2003). An iodine-deficient diet can cause a wide spectrum of illnesses, including goitre and mental retardation (Haldinmann *et al*., 2005). In fact, maternal iodine deficiency during pregnancy can cause foetal iodine deficiency, which impairs early brain development with consequent physical and mental retardation and lower cognitive and motor performance in later life (Zimmermann, 2012). Despite of countless benefits, fish consumption may address safety aspects, for example hazards related to contamination with chemicals, mainly heavy metals, pesticides, dioxins, furans, polychlorinated biphenyls and brominated flame retardants (EFSA, 2015). In fact, attempts to increase consumption of fish appear to be undermined by the risk to expose consumers to contamination with mercury and methyl mercury. High mercury levels are directly associated with the risk of myocardial infarction and may reduce the cardio-protective effect of fish intake (Guallar *et al.,* 2002). Mercury, cadmium, and lead concentrations were determined in various fishery products (fishes, cephalopod molluscs, and crustaceans) imported into Italy from many European and non-European coastal countries (Storelli *et al*., 2012). Moreover, the consumption of fishery products is not free from biological health-care risks. Fish, molluscs and crustaceans are susceptible to a wide variety of viruses (Haepatitis A virus (HAV), Calicivirus and Norovirus), parasites (*Anisakis*, *Diphyllobothrium latu, Opisthorchis felineus*) and bacteria (*Vibrio* spp., *Clostridium botulinum*, *Aeromonas* spp.). Bacterial load of raw fish depends on the environmental conditions and microbial quality of the water where fish is caught or farmed, temperature of the water, salt content of the water, distance of harvesting area from areas contaminated with human and animal pollutions, fishing method and cooling conditions (Feldhusen, 2000; Saito *et al*., 2011). Most of bacteria which affect fish are considered to be saprophytic in nature, meanwhile, others as *Vibrio*, *Aeromonas*, *Salmonella* species, are potentially pathogenic bacteria (Hassanien *et al*., 2014). The bacterial pathogens associated with fish are classified as indigenous and non-indigenous. *Vibrio cholerae, V. parahaemolyticus, V. vulnificus, Clostridium botulinum* and *Aeromonas hydrophila* are indigenous pathogenic bacteria that are found naturally in the sea and rivers and may infect humans after consumption (Çakli and Kişla, 2003; Da Silva *et al*., 2010; Eklund *et al*., 2004). Bacteria like *Salmonella* spp., *Escherichia coli, Shigella* spp., *Campylobacter* spp., and *Yersinia enterocolitica* can be found in fish due to faecal contamination of water (Onmaz *et al*., 2015). Toxigenic strains of *Bacillus cereus, Listeria monocytogenes, Staphylococcus aureus* and *Clostridium perfringens* are non-indigenous bacteria and may contaminate food during the handling and processing (Da Silva *et al*., 2010; Grigoryan *et al.*, 2010; Huss *et al.,* 2003; Latorre *et al*., 2007; Normanno *et al*., 2005;

Simon and Sanjeev, 2007). Further, food poisoning due to consumption of toxic fishery products belonging to Tetradontidae, Molidae, Diodontidae, Canthigasteridae and Gempylidae families may occur (Lawrence *et al*., 2007; Noguch and Arakawa, 2008), despite their marketing is forbidden by European Regulations (EC Reg. 853/2004; EC Reg. 854/2004; EC Reg. 2074/2005). For these reasons, a careful risk analysis is required in order to protect consumer's health, in conformity with EU food hygiene regulations (EC Reg. 178/2002). So, the only way to guarantee an acceptable level of safety and quality of these products is to optimise and control production procedures, also because consumers have become more exigent in terms of quality. Quality in fish sector is a complex concept involving a whole range of factors which for the consumer include for example: safety, nutritional quality, availability, convenience and integrity, freshness, eating quality and the obvious physical attributes of the species, size and product type (Bremner, 2000).

2. FISH AND FISHERY PRODUCTS CONSUMPTION

2.1. World fisheries and aquaculture production and utilisation

In the last five decades, world fish production has steadily grown. This growth proceeds at the same time with food fish supply increasing at an average annual rate of 3.2 per cent, outpacing world population growth at 1.6 per cent. World *per capita* apparent fish consumption increased from an average of 9.9 kg in the 1960s to 19.2 kg in 2012 (FAO, 2014). This impressive development has been driven by a combination of population growth, rising incomes and urbanisation, and allowed by the strong expansion and development of fish production and distribution channels.

In 2012, global fish production confirmed the huge volumes involved in its sector with 158 million tonnes, of which 136.2 (86.2%) was directly used for human consumption, while the remaining part was used for the production of fish meal and fish oil. Moreover, global fish production consisted of 91.3 million tonnes captured by commercial fishing in wild fisheries, plus 66.6 million tonnes produced by fish farms (table 1) (FAO, 2014).

Table 1. World fisheries and aquaculture production and utilisation (FAO, 2014).

	2007	2008	2009	2010	2011	2012
			million tonnes			
PRODUCTION						
Capture						
Inland	10.1	10.3	10.5	11.3	11.1	11.6
Marine	80.7	79.9	79.6	77.8	82.6	79.7
Total Capture	90.8	90.1	90.1	89.1	93.7	91.3
Aquaculture						
Inland	29.9	32.4	34.3	36.8	38.7	41.9
Marine	20.0	20.5	21.4	22.3	23.3	24.7
Total Aquaculture	49.9	52.9	55.7	59.0	62.0	66.6
Total World Fisheries	**140.7**	**143.1**	**145.8**	**148.1**	**155.7**	**158.0**
UTILISATION						
Human consumption	117.3	120.9	123.7	128.2	131.2	136.2
Non-food uses	23.4	22.2	22.1	19.9	24.5	21.7
Population (billions)	6.7	6.8	6.8	6.9	7.0	7.1
Per capita food fish supply (kg)	17.6	17.9	18.1	18.5	18.7	19.2

2.2. Capture fisheries production

Global capture production has increase in the last years. In fact, in 2011 and 2012, global fishery production in marine waters was 82.6 million tonnes and 79.7 million tonnes, respectively. Moreover, in 2011 global capture production was about 93.7 million tonnes confirming as the second-highest ever, slightly below the 93.8 million tonnes of 1996 (FAO, 2014). In these two years, 18 countries caught more than an average of one million tonnes per year, accounting for more than 76 per cent of global marine catches. Eleven of these countries are in Asia (including also the Russian Federation, which fishes much more in the Pacific than in the Atlantic).

Although Asian countries, mainly Philippines and the Republic of Korea, have shown considerable increases in marine catches in the last 10 years, Japan, Russian Federation, India, Malaysia and Thailand have registered decreases. However, marine catches submitted to FAO by Myanmar, Viet

Nam, Indonesia and China have shown continuous growth with astonishing decadal increases (e.g. Myanmar up 121 per cent, and Viet Nam up 47 per cent).
China is, by far, the largest exporter of fish and fishery products. However, since 2011, it has become the world's third-largest importing country, after the United States of America and Japan. As regards the European Union (Member Organisation), it may be considered as the largest market for imported fish and fishery products, also because of its decreasing capture production. The decline in catches in the Northern Atlantic areas and in the Mediterranean and Black Sea seemed to have ended at the beginning of the 2010s, but data for 2011and 2012 again showed shrinking catches.

2.3. Inland aquaculture and mariculture

World aquaculture production continues to grow, although at a slowing rate. According to the latest available statistics collected by FAO, in 2012 world aquaculture production recorded another all-time high of 90.4 million tonnes (live weight equivalent), including 66.6 million tonnes of food fish and 23.8 million tonnes of aquatic algae (FAO, 2014).
Global food fish productions from inland aquaculture and from mariculture registered different growths, with average annual rates of 9.2 and 7.6 per cent, respectively. As a result, inland aquaculture steadily increased its contribution to total farmed food fish production from 50 per cent in 1980 to 63 per cent in 2012. Of the 66.6 million tonnes of farmed food fish produced in 2012, two-thirds (44.2 million tonnes) were finfish species grown from inland aquaculture (38.6 million tonnes) and mariculture (5.6 million tonnes) (FAO, 2014). Aquaculture development and production distribution are imbalanced, in fact, about 88 per cent of world aquaculture production by volume come from Asia. Worldwide, 15 countries produced 92.7 per cent of all farmed food fish in 2012 (FAO, 2014). The development of aquaculture has made a great contribution to the supply of food fish for consumption, mainly in the world's most populous countries such as China, India, Indonesia, Pakistan, Bangladesh and Japan.

2.4. In Italy

Italy, home of the Mediterranean diet, has always set his eating habits on fish consumption. It is important to say that Italy has two fishery products registered with PDO and PGI protection mark, such as "*acciughe sotto sale del Mar Ligure*" (PDO) and "*tinca gobba dorata del pianalto di Poirino*" (PGI).
In the last year, fish consumption at national level has recorded positive trends compared to 2014 (+ 4.3%). Mainly, this increase regards north-western and central regions of Italy, though the highest

consumption has been recorded in South of Italy (33%) (www.ismea.it). This rise has showed a difference in sale between modern and traditional distribution channels. In fact, consumers have mainly purchased fishery products at hypermarkets and at discount supermarkets (79.8%) rather than at traditional fisheries (20.2%) (www.ismea.it). This is also due to the rising share of supermarkets in the retail of seafood products which increases their availability, leading to increase the consumption.

Taking a look at table 2, it is easily noticeable that the consumption shifts away from traditional fresh fish towards other products. In fact, processed products (fresh and defrosted both packaged and unpackaged) seem to be preferred by consumers in line with a more and more frenetic lifestyle. This increase in consumption of convenience products is possible because people have less and less time to spare for meal preparation. The category of dry, salted and smoked products have grown more, also thanks to smoked salmon, a product appreciated by a growing number of consumers in every period of the year.

An increase in demand for fish has been added to the growth of consumption. Foreign demand has appeared more dynamic than in 2013. In fact, exports have recorded an overall amount of 135,000 tonnes, with an increase of 6.2% and an export value grown by 7.3% (www.ismea.it). The growth was mainly derived from increased supplies to the European countries (116,000 tonnes) such as Spain, France and Germany, with an export volume grown by 9.6%. Fishery products that have been mainly exported are canned tuna, bivalve molluscs and fresh and chilled sardine (www.ismea.it). At the same time, imports have increased both in volume (+ 5.7%) and value (+ 6.2%). Of 975,000 tonnes of imported fish, 56.4% (550,000 tonnes) come from European countries; mainly canned tuna, frozen squids and octopus, bivalve molluscs from Spain, Netherlands and Greece (www.ismea.it). In conclusion, in the last years national fish trade balance has shown a clear difference between imports and exports confirming that Italy is within the first ten positions of the world as importation.

Table 2. Fish consumption in Italy: annual dynamics in both volume and value (www.ismea.it).

Fishery Products	Var% volume				Var% value			
	2012/ 2011	2013/ 2012	2014/ 2013	2015/ 2014	2012/ 2011	2013/ 2012	2014/ 2013	2015/ 2014
Fresh and defrosted – packaged ad unpackaged	**-1,1**	**-0,5**	**-2,1**	**-1,1**	**-2,6**	**-0,8**	**-0,2**	**4,0**
Raw	-1,1	-0,3	-2,8	-1,9	-2,6	-0,5	-1,0	2,7
Processed	-1,9	-3,4	10,6	12,0	-2,4	-4,9	9,2	18,1
Frozen and unpackaged	**-4,7**	**6,1**	**-1,8**	**0,2**	**-2,7**	**5,7**	**-1,3**	**4,9**
Raw	-4,0	7,7	-4,9	1,7	-2,2	7,1	-3,6	6,9
Processed	-8,3	-3,1	17,4	-7,0	-6,4	-4,3	18,3	-8,6
Frozen and packaged	**1,2**	**-2,4**	**3,7**	**0,3**	**2,9**	**-1,8**	**2,4**	**-1,1**
Raw	0,0	-7,7	-4,8	0,9	-0,1	-5,9	-5,4	-0,4
Processed	3,0	5,1	14,0	-0,4	7,6	4,3	12,8	-1,9
Preservers and semi-preservers	**-1,0**	**-2,2**	**2,8**	**0,7**	**2,3**	**3,6**	**2,7**	**2,9**
Dried, salted and smoked	**1,9**	**-1,7**	**10,0**	**15,5**	**2,1**	**0,9**	**11,1**	**19,4**
TOTAL FISHERY PRODUCTS	**-0,8**	**-0,9**	**0,5**	**0,4**	**-0,4**	**0,5**	**1,7**	**4,3**

3. TRACEABILITY

Traceability is synonymous with total transparency and it is the knowledge of every stage and treatment performed for the food production. The fishing industry, in particular, has aspects of considerable complexity about the food supply chain that makes it even more necessary, though more difficult, the creation of full transparency. It is important to protect and to inform the consumer giving right information about fish and fishery products. For example, if fish was caught or farmed, when and where it was caught (country of origin) or, especially for fishery products, whether it was fresh or frozen and thawed. So, in fishery sector the traceability and labelling are an imperative and urgent needs of the market. Traceability, in fact, is increasingly becoming a requirement in major fish importing countries. It can safeguard public health and demonstrate that fish has been caught legally from a sustainably managed fishery or produced in an approved aquaculture facility. European laws on food safety and traceability of fish and fishery products are:

- Council Regulation (EC) No. 178/2002 of the European Parliament and of the Council of 28 January 2002 laying down the general principles and requirements of food law, establishing the European Food Safety Authority and laying down procedures in matters of food safety.

- Regulation (EU) No. 1379/2013 of the European Parliament and of the Council of 11 December 2013 on the common organisation of the markets in fishery and aquaculture products.

- Regulation (EU) No. 1169/2011 of the European Parliament and of the Council of 25 October 2011 on the provision of food information to consumers.
- Regulation (EU) No. 1420/2013 of 17 December 2013 informing consumers about fishery and aquaculture products.
- Council Regulation (EC) No. 1224/2009 of 20 November 2009 establishing a Community control system for ensuring compliance with the rules of the common fisheries policy.
- Regulation (EU) No. 404/2011 of 8 April 2011 laying down detailed rules for the implementation of Council Regulation (EC) No. 1224/2009.

Moreover, it is important to include the principles of Community Regulations regarding the hygiene of foodstuffs such as EC Reg. 852/2004, the hygiene for food of animal origin such as EC Reg. 853/2004 and organisation of official controls on products of animal origin intended for human such as EC Reg. 854/2004.

4. LABELLING

In food sector, traceability is guaranteed by the label. Labelling ensures the consumer about the right correspondence of the product and about its hygienic and sanitary features. It has not to attribute to the product effects or properties which it does not possess. So, label is the only real tool which the manufacturer and retailer uses to communicate food information to consumers. Since 13 December 2014, a new food labelling European Regulation has been come into force. It is EU Reg. 1169/2011. The Regulation defines the term 'labelling' as follows: "any words, particulars, trademarks, brand name, pictorial matter or symbol relating to a food and placed on any packaging, document, notice, label, ring or collar accompanying or referring to such food". EU Reg. 1169/2011 brings general and nutritional information together in order to simplify and consolidate existing labelling legislation. It applies to food business operators at all stages of the food chain. Thanks to the new law, consumers can receive clearer and more comprehensive information on food content, and they can make a more accurate choice about what they purchase. For these reasons, food and beverage manufacturers and retailers have updated their labels to comply with the new Regulation. In accordance with EU Reg. 1169/2011, food information that must be provided on label are the following:

- The name of the food.
- The list of ingredients.

- Any ingredient or processing aid listed in Annex II or derived from a substance or product listed in Annex II causing allergies or intolerances used in the manufacture or preparation of a food and still present in the finished product, even if in an altered form.
- The quantity of certain ingredients or categories of ingredients.
- The net quantity of the food.
- The date of minimum durability or the 'use by' date.
- Any special storage conditions and/or conditions of use.
- The name or business name and address of the food business operator.
- The country of origin or place of provenance.
- Instructions for use where it would be difficult to make appropriate use of the food in the absence of such instructions.
- With respect to beverages containing more than 1.2% by volume of alcohol, the actual alcoholic strength by volume.
- A nutrition declaration (it becomes mandatory for packaged foods from 13 December 2016).

5. LABELLING OF FISH AND FISHERY PRODUCTS

The mandatory information about labelling of fish and fishery products have to comply with the new European Regulation: EU Reg. 1379/2013. The information on label have to be updated considering the requirements of EU Reg. 1169/2011 and the lists of denominations of the fish species of commercial interest in Italian D.M. Mi.P.A.F. 31/12/2010. Regulations specify that labels of different fishery products, such as prepacked and non-prepacked products, have to indicate appropriated information (European Commission, 2014). The mandatory information to be reported on label of non-prepacked products are the following:

a) the commercial designation of the species and its scientific name. These names must match those on the official list drawn up and published by each EU country.
(b) the production method, in particular by the following words "… caught …" or "… caught in freshwater …" or "… farmed …".
(c) the area where the product was caught or farmed, and the category of fishing gear used in capture of fisheries. The catch area for fish caught at sea is the FAO area, sub-area or division where the fish were caught. Fish caught in freshwater must display both the name of the body of water (river, lake, etc.) and the country where the product was caught. Farmed fish (aquaculture) must display the country of production. Wild fish must display one of the following fishing gear

categories used to catch the fish: ‘seines’, ‘trawls’, ‘gillnets and similar nets’, ‘surrounding nets and lift nets’, ‘hooks and lines’, ‘dredges’, and ‘pots and traps’.
(d) whether the product has been defrosted. This information need not accompany the name of the food, although it must be shown on billboards or posters. This information is not necessary if fishery and aquaculture products: are ingredients present in the final product; or have been previously frozen for health safety purposes; or have been defrosted before smoking, salting, cooking, pickling, drying or a combination of these processes; or are foods for which freezing is a technologically necessary step.
(e) the date of minimum durability, where appropriate. It corresponds to the ‘best before’ date or ‘best before end’ date. For all non-prepacked products, products prepacked for direct sale or on sales premises at the consumer’s request, EU countries can decide whether to adopt national rules stipulating that the ‘best before’ or the ‘use by’ date should be displayed.

As regards allergens, for non-prepacked products, information is also mandatory, as required by EU Reg. 1169/2011. However, European countries can adopt national measures about the ‘means’ by which this information is provided. Moreover, where no list of ingredients exists, the presence of allergens must be indicated as follows: ‘contains…’. This information is not required when the food name clearly refers to allergen(s).
As regards the prepacked products, different information must be reported on label, in particular:

- whether the product has been defrosted, this information must accompany the commercial name.
- For the date of minimum durability, all pre-packed products which are not highly perishable must display the ‘best before’ date. By contrast, highly perishable products should display the ‘use by’ date.
- For allergens, a clear reference to the name of any allergens should be included in the list of ingredients. This should appear in a typeset (e.g. font style, or background colour) which clearly distinguishes it from the rest of the list of ingredients.

Additional requirements for prepacked products must be provided, in addition to that listed above. For example:

- List of ingredients: a list of all ingredients in descending order of weight should be displayed next to ‘Ingredients’. This is not necessary for single-ingredient foods that have the same name as the ingredient.

- Quantity of ingredients: this must be expressed as a percentage. This must be shown when the ingredient appears in the name of the food, is emphasised on the labelling, and is essential to characterise a food.
- Net quantity: this must be expressed in grams or kilograms. The drained net weight of the food must also be shown where a solid food is in a liquid medium (also frozen or quick-frozen). If the food has been glazed, the declared net weight of the food must exclude the glaze.
- Conditions for storage and use: any special storage conditions and/or conditions of use must be shown.
- Name or business name and address of the food business operator: the name and address of the food operator responsible for the food information, and under whose name the food is marketed, should be displayed.
- Instructions for use: only if needed.
- Nutrition declaration: only from 13 December 2016.
- 'Date of freezing' or 'Date of first freezing': this requirement only applies to unprocessed products. The date must be indicated as follows: 'Frozen on day/month/year'.
- Identification mark: the name of the country, the approval number of the establishment where production takes place and the abbreviation EC, or its translation in other EU languages, must be shown when the product is produced in Europe (EC Reg. 853/04). For imported products, only the name of the country and the approval number of the establishment are mandatory.
- Date of packaging: this date must be shown for live bivalve molluscs. This date must comprise at least the day and the month.

The mandatory information must be available and easily accessible. In contrary case, an increase of commercial (*aliud pro alio*) and sanitary (commercialisation of toxic organisms) frauds could occur.

6. READY TO EAT FISHERY PRODUCTS

Ready to Eat (RTE) products are processed foods which are normally packed and served or consumed when required. They are prepared or cooked in advance, with no further cooking or preparation required before being eaten. The changes in the socio economic pattern of the society like the changing life style, increasing number of working women, increasing in the family income which makes the RTE foods affordable, awareness about healthy foods, changes in the meal pattern and existing food habits have all contributed to the growth of RTE industry.

Moreover, RTE products are very appreciated by consumers because they are tasty, practical, and undemanding. They are made by mild technologies. These technologies allow to minimise thermal, mechanical and oxidative damage and to reduce chemical and bacteriological contaminations.

In this way, different processing and preservation methods are mainly used to improve shelf life and to obtain desirable sensory characteristics of the products. Often, these gastronomic preparations are vacuum packaged or packed in modified atmosphere (MAP). The anaerobic conditions could reduce microbial deterioration supported by Specific Spoilage Organism (SSO) such as *Pseudomonas*, *Photobacterium* and *Shewanella*. On the contrary, this packaging may allow the multiplication of microorganisms responsible of foodborne diseases such as *Listeria monocytogenes*, *Clostridium perfringens, C. botulinum* and enteroxigenic *Staphylococcus aureus*.

Italian RTE food scenario is exhibiting tremendous growth rate in the recent years. In Italy, 70% of fish consumption is composed of new products such as slices, fillets, breaded products, precooked, ready to cook and RTE fishery products. So, RTE fishery products can be considered as a current phenomenon with an important impact on traceability, food safety and quality.

7. AIMS OF THE THESIS

This thesis is included in the field of food safety and food control and it focused on two important aspects about fishery products: the microbiological safety of RTE fishery products and the issues on the fraudulent marketing of fishery products. The last issue could have implications on the marketing of fishery products and on the public health.

The study on the prevalence of *Staphylococcus aureus* and *Listeria monocytogenes* in RTE fishery products, represents the first and the second aim of the thesis. The choice to investigate these two important bacterial food-borne pathogens arises from the study of the literature about the emerging food-borne infectious diseases and of the recent EFSA reports on the monitoring of food-borne outbreaks in EU. In detail, the investigated microorganisms, will be isolated and characterised using phenotypic and molecular techniques in order to define their pathogenic profile.

The third aim of the thesis was to develop a set of original primers for the molecular identification of commercial fish species, using Polymerase Chain Reaction (PCR) technique. The need to develop new primers for this purpose derives from the need to set up duplex and triplex-PCR protocols for the simultaneous detection of more fish species. The final objective of the study was to apply the developed protocols for quality and safety purposes in the field of food control.

8. *Staphylococcus aureus*

Staphylococcus aureus belongs to the family *Micrococcaceae* and is part of the genus *Staphylococcus*, which contains more than 30 species. *S. aureus* is a 1 μm, gram positive bacterium that, under microscope, appears as single cells, in pairs or as grape-like irregular clusters. It is characterised as coagulase and catalase positive, non-motile, non-sporeforming and as facultative anaerobic bacterium (Winn *et al.*, 2006). It can grow over a relatively wide range of pH (4–10, with the *optimum* being 6–7) and temperature (7–48°C) (Bianchi *et al*., 2014). *S. aureus* has the capacity to grow in the presence of high concentrations of salt (Parfentjev and Catelli, 1964). Some strains can grow in NaCl concentrations as high as 3.5 M, equivalent to a water activity of 0.86 (Scott, 1953). *S. aureus* strains carry a pattern of toxin genes and genetic background which encode for different virulence factors. In fact, *S. aureus* has numerous mechanisms to produce disease and to evade host defences. In the last years, studies have highlighted the presence of numerous genes implicated in virulence. Precisely, *S. aureus* harbours staphylococcal enterotoxin (*se*) genes located both on stable regions of the chromosome and mobile genetic elements (MGEs), which can encode staphylococcal enterotoxins (SEs) (Bianchi *et al*., 2013). SEs are potent heat-stable pepsin-resistant exoproteins synthesized by *S. aureus* throughout the logarithmic phase of growth or during the transition from the exponential to the stationary phase (Argudín *et al*., 2010; Normanno *et al*., 2005). The presence of *se* genes (*sea, seb, sec, sed, see, seg, seh, sei, sej, sek, sel, sem, sen, seo, sep, seq, ser, seu*) is linked to the production of the corresponding SEs. In fact, in addition to the classical SEA, SEB, SEC (with the SEC1, SEC2 and SEC3, SEC ovine and SEC bovine variants), SED, and SEE, new variants of SEs have been identified (SEG to SER, and SEU), in the order that they were discovered (Ortega *et al*., 2010).

The spread of these genes among *S. aureus* isolates can modify their ability in virulence. Moreover, *S. aureus* strains produce an extracellular thermostable nuclease (thermonuclease - TNase), a protein with a molecular mass of 17,000 Da, which can degrade both DNA and RNA, and the enzymatic activity can resist 100°C for at least 1 hour (Brakstad *et al*., 1992). Furthermore, in *S. aureus*, the presence of *ica*A gene, encoding the polysaccharide intracellular adhesin (PIA) or polymeric N-acetyl-glucosamine (PNAG), appears to have an important role in staphylococcal biofilm development (Brooks and Jefferson, 2014).

Some *S. aureus* strains can show resistance to antibiotics, essentially to methicillin and all β-lactams. The resistance to methicillin in *S. aureus* is mediated by the *mec*A gene that encodes a modified penicillin-binding protein (PBP), the PBP2a (Bystroń *et al*., 2009). This gene is located on staphylococcal cassette chromosome *mec* (SCC*mec*). An isolate with this type of resistance is referred to as methicillin-resistant *S. aureus* (MRSA). MRSA was initially reported as a nosocomial

pathogen in human hospitals (hospital-associated MRSA) (Deurenberg and Stobberingh, 2008). During the last 45 years, various HA-MRSA clones disseminated worldwide. In addition, since the 1990s, MRSA strains were isolated from affected people with no epidemiological connection to hospitals; strains that cause such infections are referred to as community-associated MRSA (CA-MRSA) (EFSA, 2009). Moreover, in the last years a zoonotic MRSA clade associated with farmed animals (Livestock-Associated, LA-MRSA) has been isolated and identified as a cause of human infections, also among workers in contact with animals, especially abattoirs, veterinarians and farmers (Normanno *et al*., 2015; van Cleef *et al*., 2014).

In Italy the incidence of human MRSA infections, among staphylococcal infections, ranges from 30.3 to 34.4% and is one of the highest in Europe (Normanno *et al*., 2007). Some *S. aureus* strains, mainly MRSA, often harbour genes encoding for Panton–Valentine leukocidin (PVL), which is one of the major exotoxins of *S. aureus* (Fetsch *et al.*, 2014). PVL is a pore-forming toxin that targets cells of the immune system such as polymorphonuclear neutrophils (PMNs).

8.1. FOOD CONTAMINATION BY *Staphylococcus aureus* AND PATHOLOGY

Staphylococcus aureus is commonly present on the skin and mucosal surfaces of humans and animals as well as in the environment (Rola *et al*., 2015). Approximately 20–30% of humans persistently carry *S. aureus* as a commensal bacterium (Kluytmans and Wertheim, 2005).

Beyond asymptomatic carriage, *S. aureus* causes a wide range of infections, such as skin and soft tissue infections (SSTI), bone, joint and implant infections, pneumonia, septicaemia and food poisoning (Monecke *et al*., 2011).

S. aureus is one of the main bacterial agents causing foodborne diseases in humans worldwide (EFSA, 2010; Le Loir *et al.,* 2003). Its pathogenicity is mainly related to a combination of toxin-mediated virulence, invasive capacity, and antibiotic resistance (Argudín *et al.,* 2010).

Cases of staphylococcal food poisoning (SFP) are often underestimated. Contributing factors for the low incidence of SFP include misdiagnosis, improper sample collection and laboratory examination, lack of seeking medical attention by the affected persons complicating the laboratory confirmation, and lack of routine surveillance of clinical stool specimens for *S. aureus* or its enterotoxins (Kadariya *et al.,* 2014).

The presence of staphylococci on food is an indication of *post*-harvest contamination due to poor personnel hygiene. Foods can be contaminated with *S. aureus* by improper handling and subsequent storage at elevated temperatures. People colonised with *S. aureus* asymptomatically, who handle food, can introduce the bacteria into the food chain during processing, cooking or distributing the food product (Argudín *et al*., 2010; Asao *et al*., 2003; Hennekinne *et al*., 2012).

Moreover, *S. aureus* can attach to food contact surfaces and form biofilms, where they survive even after cleaning and disinfection. The ability to form biofilms allows *S. aureus* to survive in hostile environments such as food industry surfaces, and this enhances the recurrence of food contamination (Gutiérrez *et al*., 2012).

SFP results from the ingestion of food containing staphylococcal enterotoxins (SEs) produced by coagulase-positive staphylococci (mainly *S. aureus*) while coagulase negative staphylococci have never been reported as cause foodborne outbreaks (Kerouanton *et al*., 2007; Le Loir *et al*., 2003). One-half of the isolates found among humans proved to be enterotoxigenic (Becker *et al*., 2003).

SFP is characterised by gastrointestinal symptoms and occurs after ingestion of SEs in food (Bianchi *et al.,* 2013). The symptoms of this illness include nausea, vomiting, abdominal cramps, and diarrhoea occurring 1 to 8 hours after consumption of contaminated food (Rola *et al*., 2015).

Individual susceptibility to SEs and the amount of SEs ingested influence the onset and severity of the symptoms (Hennekinne *et al*., 2012; Tranter, 1990). As less as 100 - 200 ng of enterotoxin A (SEA) can lead to a disease (Evenson *et al*., 1988). Environmental factors and a conspicuous combinations of parameters of the food can influence and contribute to the production of SEs, such as water activity, pH, redox potential and temperature; besides, bacterial antagonism is known to play an important role (Genigeorgis, 1989; Hennekinne *et al*. 2012; Schelin *et al*. 2011). Food poisoning caused by staphylococcal enterotoxins is among the leading causes of food-borne outbreaks in the European Union (EFSA, 2013). Staphylococcal foodborne intoxication is reported to be one of the most common bacterial foodborne diseases in several countries (Bean *et al*., 1990; Baird-Parker, 2000; European Commission and Health, 2003). *S. aureus* was detected in many kind of food (Normanno *et al*., 2005; Normanno *et al*., 2006; Normanno *et al.*, 2007). Fishery products are often subjected to contamination by *S. aureus* because of their handling. Different kind of fishery products are contaminated by *S. aureus*, both fresh and frozen. Thanks to its halophilic characteristics and its resistance in the environment, high levels of contamination by *S. aureus* have been found in many categories of semi-processed and processed foods, such as salted fish, smoked fish and ready-to-cook products (Basti *et al*., 2006; Vázquez-Sánchez *et al*., 2012; Zarei *et al*., 2012). Ready to eat (RTE) fishery products are often involved in SFP. This regards both traditional fishery products and also new products, such as sushi and sashimi, nowadays more and more popular in Italy. In fact, an examination of *S. aureus* prevalence among RTE raw fishery products revealed the contamination of 174 on 180 sashimi samples (87%) (Hammad *et al*. 2012). In many cases, Coagulase positive staphylococci can be present on food at high levels of concentration and so, it is possible that a significant proportion of fishery products do not comply with legal limits in enforced by EC Reg. 2073/05 (Vázquez-Sánchez *et al*., 2012). In addition to fishery products, *S.*

aureus has been also isolated from water samples from food preparation plants, which had repeated hand contact during food preparation (Sokari, 1991).

Despite of the hygiene of the surfaces is important for the overall quality and safety of the food product, *S. aureus* has been often found both on work surfaces and on food handlers in fishery plants (Adetunji *et al*., 2014; Simon and Sanjeev, 2007). There are other serious human infections caused by MRSA, which are becoming a new possible risk of food safety concern (Normanno *et al*., 2007). Recently, contamination of RTE fishery products occurred in Japan: MRSA were isolated from sushi and sashimi samples collected from supermarkets and restaurants (Hammad *et al*., 2012; Puah et al., 2016).

9. *Listeria monocytogenes*

Listeria monocytogenes is a gram-positive, non-sporeforming, facultative anaerobic, small (0.4–0.5 × 1–2 μm) coccoid rod which grows between -0.4 and 50°C, optimum 30-37°C (Farber and Peterkin, 1991; Juntilla *et al*., 1988; Walker *et al*., 1990). Thanks to peritrichous flagella, *L. monocytogenes* is motile at 20-25°C, but not at 37°C (Gründling *et al*., 2004).

L. monocytogenes is catalase positive, oxidase negative, expresses *β*-hemolysis on blood agar and produces acid from rhamnose, but not from xylose (McLauchlin and Rees, 2009). It is CAMP (Christine, Atkins, Munch- Petersen test) positive with *Staphylococcus aureus* and negative with *Rhodococcus equi* (McLauchlin and Rees, 2009). The optimum pH range for *L. monocytogenes* is 6 to 8, but it can grow in pH range from 4.0 to 9.6 (Farber and Peterkin, 1991; Lado and Yousef, 2007). *L. monocytogenes* is able to grow at an activity water ($a_{w)}$ level that is usually lethal to other bacteria, such as a_w 0.90, and survive even at lower values, a_w 0.88 (Lado and Yousef, 2007; Nolan *et al*., 1992). *L. monocytogenes* is included among moderately halophilic microorganisms which tolerate 5-20% salt; in fact it can grow at 10% NaCl concentration and survive at 16% salt for one year (McClure *et al.,* 1989). According to phenotypic characteristics, which are a result of gene expression, *L. monocytogenes* is divide into 13 serotypes, based on O and H antigens: 1/2a, 1/2b, 1/2c, 3a, 3b, 3c, 4a, 4ab, 4b, 4c, 4d, 4e and 7 (Seeliger and Höhne, 1979).

9.1. FOOD CONTAMINATION BY *Listeria monocytogenes* AND PATHOLOGY

Listeria monocytogenes is the causative agent of listeriosis, a food-borne disease affecting susceptible sections of the population such as young, old, pregnant, immune compromised (Y.O.P.I.) people. In Y.O.P.I. people listeriosis can occur with low morbility and high lethality (30%) in serious different forms: neuromeningeal (meningitis, encephalitis), maternal-neonatal

(intra-uterine infections, spontaneous abortions) and febrile gastroenteritis; in severe cases it can lead to septicaemia and death (Goulet *et al*., 2012). Healthy adults are generally unaffected by *L. monocytogenes*. Listeriosis is mainly reported from industrialised countries, and the reported incidence in Africa, Asia and South America is low (Rocourt *et al*., 2003). It is estimated that 99% of listeriosis cases are due to contaminated food (Mead *et al*., 1999). Out of 13 known serotypes, three (1/2a, 1/2b, and 4b) are known to be responsible for 95% of human listeriosis cases (Parisi *et al*., 2010). Contamination of food occurs in food-processing plants where *L. monocytogenes* may persist for years (Rørvik *et al*., 1995; Miettinen *et al*., 1999; Keto-Timonen *et al*., 2007). Because of its environmental distribution, *L. monocytogenes* can enter food plants thanks to a variety of sources, including soil (the key reservoir), vegetation, feed (mainly silage), pests, insects, personnel, faecal material and water (Botzler *et al*., 1974; Driehuis and Elferink, 2000; Iida *et al.*, 1991; El-Shenawy, 1998; Pava-Ripoll *et al.*, 2012; Sauders *et al.*, 2005; Schoder *et al.*, 2011). It is also present on raw ingredients of both vegetable and animal origin, and in intestines of domestic and wild animals (Yoshida *et al.,* 2000). In addition, equipment, contact surfaces, drains, workers and protecting clothing of employees in various food-processing plants have been proven to be contaminated with *L. monocytogenes* (Autio *et al.*, 1999; Bērziņš *et al.*, 2010; Chasseignaux *et al.*, 2001; Giovannacci *et al.*, 1999; Keto-Timonen *et al.*, 2007; Miettinen *et al.*, 1999). In fact, complex machineries that are in contact with large production lots and are often difficult to clean, such as coolers, conveyors, cutting, slicing, brining and packaging machines, are the most heavily contaminated. They can maintain the contamination in factories despite regular cleaning and disinfecting (Autio *et al.*, 1999; Bērziņš *et al.*, 2010; Keto-Timonen *et al.*, 2007; Lundén *et al.,* 2003; Miettinen *et al.*, 1999). It is due to persistent strains that represent a continuous source of contamination of large amounts of products over long periods of time (Orsi *et al.,* 2008). The persistence in food plants is due to the capacity of *L. monocytogenes* to form biofilms and to adhere to surfaces (Lundén *et al.,* 2003). In biofilms, microorganisms are enclosed in a matrix made up of polysaccharide material, thereby gaining enhanced resistance to sanitizers, disinfectants and antimicrobial agents (Robbins *et al*., 2005). *L. monocytogenes* forms biofilms on stainless steel, plastic, and polycarbonate surfaces and many other food contact surface materials (Chmielewski and Frank, 2003). In addition*, L. monocytogenes* can survive and grow over a wide range of temperature, pH and a_w limits, under aerobic and anaerobic atmosphere as well as in modified atmosphere packages (MAP) and under adverse conditions (Lado and Yousef, 2007). These characteristics enable the pathogen to survive and multiply to high numbers during a product's shelf-life. The prevalence of *L. monocytogenes* is often high in products that are minimally processed or have potential of contamination after heat treatment. Other criteria for risk include

support of the growth of *L. monocytogenes* in product, extended storage in chilled temperature and lack of heat treatment before consumption (Keto-Timonen *et al.*, 2011). So, RTE products can be considered as an ideal source of infection, in which *L. monocytogenes* can survive and grow to high numbers exceeding the legal safety limits (EC Reg. 2073/2005). Epidemiological investigations of all large outbreaks that have occurred since 1981 demonstrate that nearly all kinds of RTE foods can transmit the infection and that the outbreaks were associated with industrially manufactured foods (Rocourt *et al*., 2003).

L. monocytogenes has been found in many types of foods, including dairy, fishery and meat products (Latorre *et al*., 2007; Parisi *et al*., 2013). In the last years, numerous studies have reported the contamination of different kinds of RTE fishery products with *L. monocytogenes,* such as raw, gravad, minced, hot and cold smoked, partially cooked, dried, vacuum-packaged fishery products, fish roe products and seafood salad (Lianu and Sofos, 2007; Loncarevic *et al*., 1995; Miya *et al*., 2010; Nakamura *et al*., 2004; Uyttendaele *et al*. 2009). For this reason, due to high frequency of contamination with *L. monocytogenes* of RTE fishery products, Food Business Operators (FBOs) are obliged to demonstrate and to guarantee, to the satisfaction of the Competent Authority, that products will not exceed the limit 100 CFU/g at the end of the shelf-life, as referenced under EC Reg. 2073/2005.

10. IDENTIFICATION OF FISH SPECIES

Identification of species represents a key aspect of biodiversity studies, both for food control and food safety. As regards fishery products, identification of species is an important tool to ascertain frauds. In fact, fishery products, with olive oil, are subjected to fraud more than any other food (Moore *et al*., 2012). In the last years, there has been an increase in fish and seafood consumption at a national level, also thanks to growing amount of fishery products given by zootechnical sector and international trade. This growth regards both the acquisition of fishery products in a traditional way and new products, for example fillets, slices, fish burger, “ready to cook” breaded products or RTE products. In this situation, fish is not easily identifiable from a phenotypic point of view with the increase of commercial and sanitary frauds. Victims of these kind of frauds can be both consumers and fishery industries. Traditionally, fish species identification is based on body external features. Morphological characters that are mainly considered are number and position of fins, morphology of scales and other dermal structures, body shape, various measurements of body parts, pigmentation and colour patterns (Strauss and Bond, 1990). In some cases these morphological features are not useful for identification and differentiation purposes, even with whole specimens,

because fish can show both considerable intraspecific variations and small differences between species. So, the necessity of an excellent authentication of fish and seafood species has led to develop new methods. The new techniques for an efficient identification of fish species are based on separation and characterisation of specific proteins and also on deoxyribonucleic acid (DNA) analysis. The methods based on protein analysis use electrophoretic techniques, such as isoelectric focusing (IEF), capillary electrophoresis (CE), high performance liquid chromatography (HPLC) or immunoassay systems, such as Enzyme-Linked Immuno Sorbent Assay (ELISA) (Asensio *et al.,* 2008; Hubalkova *et al*., 2007; Kvasnička, 2005; Rehbein, 1990). These methods are generally reliable for use with fresh and frozen tissue but not with other conditions. In fact, proteins lose their biological activity after animal deaths (Asensio, 2007). Moreover, proteins are heat-labile and become irreversibly denatured when the flesh is cooked. In fact, heat processing or drying can destroy the biochemical properties and structural integrity of proteins, making analysis impossible (Rasmussen and Morrisey, 2008). DNA-based identification methods present several advantages over protein analysis, including increased specificity, sensitivity, and reliable performance with processed samples (Lenstra and Lees, 2003). In fact, DNA molecules are more resistant and thermo-stable than proteins. Furthermore, DNA can provide much more information than proteins; it is due to the degeneracy of the genetic code and the existence of noncoding regions (Lockley and Bardsley, 2000). Besides, DNA is present in all cells of an organism, so it could be retrieved from many substrates. The main DNA-based methods applied to the fish species identification in the last decade are eleven. Some of them, namely PCR-SSCP (Single Strand Conformation Polymorphism), PCR-RAPD (Random Amplified Polymorphic DNA), PCR-DGGE (Denaturing Gradient Gel Electrophoresis), PCR-ALFP (Amplified Fragment Length Polymorphism), and cloning and sequencing, seem to be abandoned today (Teletchea, 2009). On the contrary, six techniques are more and more used nowadays; they are PCR-RFLP, PCR-sequencing, PCR-specific primers, Real-time PCR, microarray technology and DNA-barcoding (Teletchea, 2009).

10.1 PCR-specific primers

Primers are strands of short nucleic acid sequences (generally about 20 base pairs) that serve as a starting point for DNA synthesis. In order to generate target DNA amplicons, visualised by agarose gel electrophoresis, primers need to match the beginning and the end of the DNA fragment to be amplified. Reaction occurs under suitable stringent conditions, involving DNA polymerase, specific *Tm* (melting temperature) and DNA extracted from a given species. Besides, to preclude the possibility of false positive or negative results, appropriate controls should be included (the lack of amplified fragment on the gel may be due to technical problems rather than due to the absence of

the target DNA). For the simultaneous amplification of many targets of interest, Multiplex-PCR is often performed using more than one pair of primers in one reaction tube. Multiplex-PCR has the potential to produce considerable savings of time and efforts within the laboratory.

This DNA-based technique has been applied to the identification of numerous species of fish and seafood, including gadoids (Akasaki *et al*., 2006; Moran and Garcia-Vazquez, 2006), flatfish (Comesaña *et al.,* 2003; Sanjuan and Comesaña, 2002), salmonids (Dooley *et al.,* 2005; Zhang and Cai, 2006), swordfish (Hsieh *et al.,* 2007), scombroids (Lin and Hwang, 2008), sardines and anchovies (Jérôme *et al.,* 2003; Santaclara *et al.,* 2006), eels (Lin *et al.,* 2002), molluscs (Klinbunga *et al.,* 2003; Rego *et al.,* 2002), tuna (Michelini *et al.,* 2007) and many more.

10.2. COI gene

Animal mitochondrial DNA (mtDNA) contains one major noncoding region, thirteen protein-coding genes, twenty-two genes coding for transfer ribonucleic acid (tRNA), and two genes coding for ribosomal RNA (rRNA) (Céspedes *et al.,* 2000). This presents many advantages. Mainly, mtDNA is small, easy to extract and does not undergo genetic recombination (Aranishi *et al.,* 2005; Céspedes *et al.,* 2000; Civera, 2003). The mtDNA is much expanded for using as a marker in species identification. The genes such as D-loop, cytochrome *b* and cytochrome *c* oxidase are more usual for this aim (Tobe *et al.*, 2010). Cytochrome *c* oxidase subunit 1 (COI) DNA size is around 1548 bp which has 70.2% total T content (Shaikevich and Zakharov, 1993). In addition, COI encodes Cytochrome *c* oxidase (COX), the terminal complex of the mitochondrial respiratory chain, which catalyses the electron transfer from reduced cytochrome *c* to oxygen (Valnot *et al*., 2000). There are three different subunits of cytochrome *c* oxidase, COI, COII and COIII. COI is the largest one and the most conserved among them (Beard *et al*., 1993). For these reasons, COI is one of the most used gene for species identification on some of the search engines such as GenBank BLAST and BOLD (Dawnay *et al*., 2007). COI gene has been used for the identification of many fish species, such as Alaska skates (*Amblyraja, Bathyraja* and *Raja*: Rajidae) (Spies *et al.,* 2006), Indian sciaenids (*Otolithes cuvieri, Otolithes ruber, Johnius dussumieri, Johnius elongatus, Johnieops vogleri, Otolithoides biauritus* and *Protonibea diacanthus*) (Lakra *et al*., 2009), 17 members of the family *Scombridae* common to the western Atlantic Ocean (Paine *et al.,* 2007), 58 seafood samples in Italy (Cutarelli *et al.,* 2014), scombrid larvae in an area off the Kona Coast of Hawaii Island (Paine *et al.,* 2008) and tuna species (genus *Thunnus*) (Viňas and Tudela, 2009).

10.3. Frauds

Fraud is a false representation of a matter of fact - whether by words or by conduct, by false or misleading allegations, or by concealment of what should have been disclosed - that deceives and is intended to deceive another so that the individual will act upon it to her or his legal injury. Frauds are characterised by voluntariness, aimed directly or indirectly to subtract a value to a business and are mainly perpetrated for profit to the benefit of those who commit them. In food safety, fraud is considered an act or deception that is configured in a decrease in the value of the goods, economic or nutritional. It is performed by the manufacturer or seller with the modification of the characteristics of food, making it different from the one agreed. Food frauds are practiced with several illegal conduct in order to adulterate, counterfeit, substitute and alter food products with the ultimate goal to benefit.
Food frauds can be divided in two types:

- "Sanitary fraud", also called toxic fraud, they are a threat to consumers' health causing harm.
- "Commercial fraud", they damage the economic interests of the consumer without causing, necessarily, harm (Semeraro, 2011).

In addition, according to the effects on composition and/or the external aspects, frauds are distinguished as: fraud inherent product quality and fraud regarding the marketing of foods.
Frauds inherent product quality are:

- Alterations: are changes in the composition and organoleptic characteristics of food caused by degenerative phenomena for bad or prolonged storage.
- Adulterations: are changes in the natural composition of a food product, due to voluntary and unreported addiction or subtraction of some components, in order to obtain an economic profit. These frauds have both negative commercial and nutritional impact. Moreover, adulterations may expose the consumer to health risks such as allergic reactions.
- Sophistications: are voluntary changes in natural composition of a food product by the addition of foreign substances, or the substitution of one or more of its elements with substances of lower quality and value, or by the addition of chemical substances not allowed by the laws. These frauds are practiced in order to improve its appearance or to cover its defects.

Frauds regarding the marketing of foods are:

- Falsifications: are fraudulent operations which consist in the replacement of a food with another.

- Counterfeiting: is a fraudulent action which consists in labelling products using a name different from the real one, usually of a high-value product.

The most common frauds which involve fishery products are:

1) substitution of a high-value fish species with a less expensive or lower quality alternative ("*aliud pro alio*"). Some examples are exchange of flying squid (*Todarodes sagittatus*) for common squid (*Loligo vulgaris*), blue whiting (*Micromesistius potassou*) for hake (*Merluccius merluccius)*, scaldfish (*Arnoglossus* spp.) for sole (*Solea vulgaris*), but mostly takes place on slice and on fillets, where the recognition becomes more difficult.
2) Mislabelling or fraudulent substitution of fishery products with toxic puffer fish. An example is the exchange of puffer fish (fam. Tetraodontidae) for angler (*Lophius piscatorius*).
3) The marketing of defrosted fishery products as fresh ones. European Regulations oblige to declare on label if fish is "defrosted"; otherwise the product is intended sold as fresh.
4) The marketing of farmed fish as wild caught ones (EC Reg. 1224/2009).
5) False *rigor mortis* actually obtained with the cold, in the refrigerator a few hours before of sale.
6) The marketing of fishery products with histamine content in excess of what is permitted (EC Reg. 2073/2005).
7) Bad state of preservation of fish.
8) Use of additives permitted beyond the set limit or not permitted by law (EC Reg. 1129/2011).
9) Import of fishery products subject to specific sanitary prohibitions (EC Reg. 853/04; EC Reg. 854/04).

10.4. Frauds about fish species of interest

Fish frauds can involve many species and can happen anywhere. They may occur in the wholesale fish markets, but more frequently in the retail sale of fish markets, from street vendors, and in supermarkets or in restaurants. Mislabelling and fraudulent substitution for certain fish species is rampant and widespread. Recently, Oceana's report showed findings from one of the largest fish fraud investigations in the world, performed over a two-year period to determine the prevalence of mislabelled fish sold by 674 retailers in the U.S. such as sushi venues, grocery stores and restaurants. DNA analysis of 1215 fish samples from 21 States revealed that one-third were mislabelled. Forty-four per cent of the retail outlets visited sold mislabelled fish (Warner *et al.*, 2013). However, mislabelling rates varied greatly depending on the type of fish purchased. Among finfish categories, grouper (*Epinephelus* spp.) and Atlantic cod (*Gadus morhua*) were chosen as

species of interest and 26% and 30%, respectively, were found to be mislabelled (Warner *et al*, 2013).

As regards Atlantic cod, food products from *Gadidae* fish species are often subjected to frauds also because they are sold commercially in many forms, including fresh/frozen fillets, frozen fillet blocks, surimi blocks, salt-cured or smoked, fish sticks, canned fish, and roe. Increases in the international trade of these processed fishery products have also increased the feasibility of fish species substitution, especially due to the similar appearance of many gadoids. According to data from Oceana's report, fish species marketed or mislabelled as Atlantic cod were mainly Pacific cod (*Gadus macrocephalus*), tilapia (*Oreochromis aureus*), Asian catfish (*P. hypophthalmus*), white hake (*Urophycis tenuis*), red drum (*Sciaenops ocellatus*), pacific halibut (*Hippoglossus stenolepis*) and haddock (*Melanogrammus aeglefinus*) (Warner *et al*., 2013). Asian catfish (*P. hypophthalmus*) is widely exported due to great acceptability, affordable cost, and the white colour of the meat, which can replace expensive white fishes such as cod and grouper. Currently, catfish fillets have been exported to over 80 countries worldwide including Netherlands, Germany, and United States, which demand mainly frozen fillets without skin and bone (Karl *et al*. 2010; Phan *et al*. 2009). In this situation, the value of frozen catfish, when sold as grouper, quadruples, as does the loss to consumers (Jacquet and Pauly, 2007). In addition, typical cases of mislabelling that involve catfish and frequently occur at retailers are the marketing of frozen fillets as fresh. Beyond fraudulent actions, the consumption of catfish fillets may represent a serious health risk because of its possibility to contain chemical contaminants (Guimarães *et al*., 2015). In fact, catfish is primarily farmed in Vietnam along the Mekong River, a body of water that has become polluted in many areas due to increases in unregulated mining activity and anthropogenic run-off (Fu *et al*., 2012). Moreover, Vietnamese fish farming regulations are often less stringent than European laws. Although this situation, many authors report that chemical quality parameters regarding frozen catfish fillets are below the recommended limits established by regulations (Van Leeuwen *et al.,* 2009). Even if, in a study carried on in Brazil, 50% of imported frozen catfish fillets have demonstrated methyl mercury concentrations over 0.5 mg kg^{-1} (FAO limit) (Guimarães *et al.*, 2015).

Growing problems of fraudulent substitution for grouper and cod products in the production and distribution chain are involving oilfish (*Ruvettus pretiosus*) of the Gempylidae family. Approximately 20% of oilfish's wet weight consists of indigestible lipids (wax esters), which, two hours after the ingestion, have been found to cause keriorrhea and other acute gastrointestinal symptoms, such as abdominal cramps, nausea, headache, and vomiting in susceptible subjects (Ling *et al.,* 2008; Ruiz-Gutierrez *et al*., 1997). Oilfish is usually mislabelled as codfish or grouper, either

intentionally or accidentally. Under these circumstances, outbreaks of keriorrhea associated with consumption of oilfish have been repeatedly reported in several continents (Leask *et al*., 2004; Ling *et al*., 2008; Waldman *et al*., 2006). Oilfish is of low commercial values because of their kerriorrheic properties and is considered as ‘‘not suitable for catering’’ or even banned from sale in various countries. European Union has issued special guidelines toward trading and consumption of oilfish. In conformity with EC Reg. 1021/08, fresh, prepared, frozen and processed fishery products belonging to the family Gempylidae, in particular *Ruvettus pretiosus*, may only be placed on the market in wrapped/packaged form and must be appropriately labelled to provide information to the consumer on preparation/cooking methods and on the risk related to the presence of substances with adverse gastrointestinal effects. The scientific names of the fishery products and the common names must appear on the label.

Hake (*Merluccius merluccius*) is also implicated in cases of mislabelling. It is often marketed as cod and as grouper for its similar organoleptic and morphological characteristics (Herrero *et al*., 2010; Jacquet and Pauly, 2007; Pepe *et al*., 2005; Ulrich *et al*., 2013). The marked differences in price and marketability between these species increase the opportunities for their fraudulent commercial substitution. So, numerous studies have been carried out on protein and DNA sequence analysis for a correct identification of these fish species (Dooley *et al*., 2005; Pepe *et al*., 2005). Another example of substitution of fish species may be the case of Nile perch (*Lates niloticus*) fillets which are frequently marketed as grouper (*Epinephelus* spp.) (Trotta *et al*., 2005).

Due to its lower value, Nile perch is often labelled as grouper and sold at a higher price, because of the higher popularity and quality of the latter species. In the last years, Asensio (2008) have analysed 37 grouper meals collected at the restaurant industry and 70 fillets labelled as grouper at the retailers for the identification of fish species (Asensio *et al.,* 2008a). Out of 37 purported grouper meals served at cafeterias (school and university) and restaurants, only 9 (24%) were determined to contain authentic grouper (*Epinephelus* spp.). In the case of commercial fish fillets, only 12 (17%) were determined to be grouper (*E. marginatus*). Of the remaining fillets, 34 (48,5%) were determined to be Nile perch (Asensio *et al.,* 2008a).

11. MATERIALS AND METHODS

11.1 Detection of *Staphylococcus aureus* from RTE fishery products

In the laboratories of Food Microbiology of Experimental Zooprophylactic Institute of Apulia and Basilicata located in Foggia, 99 RTE fishery products were analysed for the isolation of *Staphylococcus aureus*.

In particular, fish samples consisted of thirty-three samples of marinated anchovy fillets (*Engraulis encrasicolus*), thirty-three samples of seafood salad and thirty-three samples of smoked salmon (*Salmo salar).* Samples were collected at local retail outlets by local health officials.

For the isolation of *S. aureus,* the procedures of the following Standards were used: EN ISO 6888-2 : 1998 and EN ISO 6888- 2 Amendment 1 : 2003. Briefly: in a sterile plastic bag, 1 part test sample (10 g) was added to 9 parts of buffered peptone water (90 ml) and then was mixed. 1 ml of this initial suspension was transferred, by means of a pipette, into tubes containing 9 ml of sterile diluents to have decimal dilutions. This operation was repeated for dilutions from 10^{-1} to 10^{-3}. By means of a sterile pipette, 1 ml of each decimal dilution was transferred into a sterile Petri dish. Into each Petri dish, freshly prepared Rabbit plasma fibrinogen agar medium was immediately poured to a depth of approximately 3 mm. The *inoculum* with the culture medium was carefully mixed and left to solidify by placing the Petri dishes on a cool horizontal surface. After complete solidification, the prepared dishes were inverted and placed in the incubator set at 37°C for 24 hours. If necessary, re-incubation for 24 hours. After a sufficient incubation period, the staphylococci formed black or grey, small colonies surrounded by a halo of precipitation, indicating coagulase activity.

Five isolates of presumptive *S. aureus* were subjected to PCR analysis for the amplification of:

- *mec*A gene, encoding the PBP2a protein, able to express the methicillin-resistance (MRSA) (Murakami *et al.,*1991);
- genes of virulence encoding the staphylococcal enterotoxins (*sea, seb, sec, sed, see, seg, seh, sei, sej*) (Løvseth *et al*., 2004);
- *nuc* gene encoding the TNase (Boerema *et al*., 2006);
- 16S gene encoding the 16S rRNA (Jarroud *et al*., 2002);
- *ica*A gene encoding the PIA (Monday and Bohach, 1999);
- *pvl* gene encoding the Panton-Valentine leukocidine (Rosec and Gigaud, 2002).

For these analysis, three Multiplex-PCRs and two Single PCRs were performed (tables 3, 4, 5, 6, 7).

Table 3. Multiplex-PCR n.1: detection of *sed, see, seg, sei* genes.

Reaction Component	**Final Concentration**	**Amount for each Reaction**
Water	-----	31.2 µl
PCR Buffer	1X	5 µl
dNTP's	0.4 mM	2 µl
MgCl	2.5 mM	4 µl
Primer *sed* Forward	0.3 µM	0.3 µl
Primer *sed* Reverse	0.3 µM	0.3 µl
Primer *see* Forward	0.3 µM	0.3 µM
Primer *see* Reverse	0.3 µM	0.3 µM
Primer *seg* Forward	0.3 µM	0.3 µM
Primer *seg* Reverse	0.3 µM	0.3 µM
Primer *sei* Forward	0.3 µM	0.3 µM
Primer *sei* Reverse	0.3 µM	0.3 µM
Taq DNA Polymerase	2 U	0.4 µl
DNA	-----	5 µl
		Final Volume: 50 µl

Table 4. Multiplex-PCR n.2: detection of *sea, seb, sec, seh, sej* genes.

Reaction Component	**Final Concentration**	**Amount for each Reaction**
Water	-----	30.2 µl
PCR Buffer	1X	5 µl
dNTP's	0.4 mM	2 µl
MgCl	2.5 mM	4 µl
Primer *sea* Forward	0.3 µM	0.3 µl
Primer *sea* Reverse	0.3 µM	0.3 µl
Primer *seb* Forward	0.3 µM	0.3 µM
Primer *seb* Reverse	0.3 µM	0.3 µM
Primer *sec* Forward	0.3 µM	0.3 µM
Primer *sec* Reverse	0.3 µM	0.3 µM
Primer *seh* Forward	0.3 µM	0.3 µM
Primer *seh* Reverse	0.3 µM	0.3 µM
Primer *sej* Forward	0.3 µM	0.3 µM
Primer *sej* Reverse	0.3 µM	0.3 µM
Taq DNA Polymerase	2 U	0.4 µl
DNA	-----	5 µl
		Final Volume: 50 µl

Table 5. Multiplex-PCR n.3: detection of 16S rRNA, *nuc, mec*A genes.

Reaction Component	**Final Concentration**	**Amount for each Reaction**
Water	-----	30.6 µl
PCR Buffer	1X	5 µl
dNTP's	0.4 mM	2 µl
MgCl	2.5 mM	4 µl
Primer *mec*A Forward	0.5 µM	0.5 µl
Primer *mec*A Reverse	0.5 µM	0.5 µl
Primer *nuc* Forward	0.5 µM	0.5 µl
Primer *nuc* Reverse	0.5 µM	0.5 µl
Primer 16S Forward	0.5 µM	0.5 µl
Primer 16S Reverse	0.5 µM	0.5 µl
Taq DNA Polymerase	2 U	0.4 µl
DNA	-----	5 µl
		Final Volume: 50 µl

Table 6. Single PCR: detection of *ica*A gene.

Reaction Component	**Final Concentration**	**Amount for each Reaction**
Water	-----	15.8 µl
PCR Buffer	1X	2.5 µl
dNTP's	0.4 mM	1 µl
Primer *ica*A Forward	0.5 µM	0.25 µl
Primer *ica*A Reverse	0.5 µM	0.25 µl
Taq DNA Polymerase	1 U	0.2 µl
DNA	-----	5 µl
		Final Volume: 25 µl

Table 7. Single PCR: detection of *pvl* gene.

Reaction Component	Final Concentration	Amount for each Reaction
Water	-----	15.8 µl
PCR Buffer	1X	2.5 µl
dNTP's	0.4 mM	1 µl
Primer *pvl* Forward	0.5 µM	0.25 µl
Primer *pvl* Reverse	0.5 µM	0.25 µl
Taq DNA Polymerase	1 U	0.2 µl
DNA	-----	5 µl
		Final Volume: 25 µl

The PCR amplifications were performed in a Thermal Cycler Eppendorf using the programs reported in tables 8, 9, 10, 11.

Table 8. Program used in Multiplex-PCR n. 1 and n. 2 (detection of *sed, see, seg, sei* genes; detection of *sea, seb, sec, seh, sej* genes).

Step	Temperature (°C)	Time	Number of Cycle
Initial Denaturation	95	6 min	1
Denaturation	95	1 min	34
Annealing	62	1 min	
Extension	72	1 min	
Final Extension	72	10 min	1

Table 9. Program used in Multiplex-PCR n. 3 (detection of 16S rRNA, *nuc, mec*A genes)

Step	Temperature (°C)	Time	Number of Cycle
Initial Denaturation	95	5 min	1
Denaturation	94	30 sec	34
Annealing	55	30 sec	
Extension	72	1 min	
Final Extension	72	10 min	1

Table 10. Program used in PCR for the detection of *ica*A gene.

Step	Temperature (°C)	Time	Number of Cycle
Initial Denaturation	94	5 min	1
Denaturation	94	30 sec	29
Annealing	55	30 sec	
Extension	72	45 sec	
Final Extension	72	10 min	1

Table 11. Program used in PCR for the detection of *pvl* gene.

Step	Temperature (°C)	Time	Number of Cycle
Initial Denaturation	95	5 min	1
Denaturation	94	30 sec	29
Annealing	55	30 sec	
Extension	72	1 min	
Final Extension	72	10 min	1

PCR-amplified DNA fragments were subjected to electrophoresis. The amplicons were analysed by an horizontal 2% (wt/vol) agarose gel in 1X TAE buffer (pH 8.3; 0.09 M Tris, 0.09 M boric acid, 2.0 mM EDTA) and with 0.003% (wt/vol) ethidium bromide for DNA staining. Gel ran in 1X TBE buffer at 200 V for 30 minutes. The PCR products were visualised by a Gel Doc XR+ System transilluminator (Bio Rad, Milan, Italy).

The isolates of *S. aureus* were subjected to antimicrobial disk susceptibility test following the CLSI guidelines (CLSI, 2012). The test was performed by applying a bacterial *inoculum* of approximately $1–2\times10^8$ CFU/ml to the surface of three Mueller-Hinton with 5% sheep blood agar plates. Up to 16 commercially-prepared, fixed concentration, paper antibiotic disks were placed on the inoculated agar surfaces. Plates were incubated for 24 hours at 37°C. The antibiotic disks used from Liofilchem (Liofilchem s.r.l, Roseto d. A., Italy) are reported in table 12.

Table 12. Antimicrobials molecules used for the detection of the antimicrobial-resistance pattern in *S. aureus* isolates.

ANTIMICROBIAL	DISC CONTENT µg	ZONE DIAMETER mm		
		Susceptible	Intermediate	Resistant
		≥		≤
Amoxicillin + clavulanic acid	AUG 30	20	-	19
Ampicillin	AMP 10	29	-	28
Cephalothin	KF 30	18	15-17	14
Chloramphenicol	C 30	18	13-17	12
Clindamycin	CD 2	21	15-20	14
Enrofloxacin	ENR 5	21	17-20	16
Eritromycin	E 15	23	14-22	13
Gentamicin	CN 10	15	13-14	12
Kanamycin	K 30	18	14-17	13
Oxacillin	OX 1	13	11-12	10
Penicillin	P 10 UI	29	-	28
Streptomycin	S 10	15	12-14	11
Sulfisoxazole	ST 250	17	13-16	12
Tetracycline	TE 30	19	15-18	14
Trimethoprim-sulfamethoxazole	SXT 25	16	11-15	10
Vancomycin	VA 30	12	10-11	9

To maintain quality control of performance and reliability of the results, the standard strain of *Staphylococcus aureus* ATCC 25923 was used.

11.2. Detection of *Listeria monocytogenes* from RTE fishery products

A total of 135 RTE fishery products were collected at local retail outlets by local health officials for the isolation of *Listeria monocytogenes*. Fish samples were analysed in the laboratories of Food Microbiology of Experimental Zooprophylactic Institute of Apulia and Basilicata located in Foggia. In particular, fish samples consisted of fourty-five samples of marinated anchovy fillets (*Engraulis encrasicolus*), fourty-five samples of seafood salad and fourty-five samples of smoked salmon (*Salmo salar).* For the isolation of *L. monocytogenes*, the procedures of the following Standards were used: UNI EN ISO 11290-2 : 1998 and ISO 11290-2 Amendment 1: 2004. Briefly: in a sterile plastic bag, 1 part test sample (10 g) was added to 9 parts of buffered peptone water (90 ml) and then was mixed. 1 ml of this initial suspension was transferred, by means of a pipette, into tubes containing 9 ml of sterile diluents to have decimal dilutions. This operation was repeated for dilutions from 10^{-1} to 10^{-3}. By means of a sterile pipette, 0,1 ml of each decimal dilution was distributed on the surface of a dish of ALOA agar medium. To estimate low numbers of *L. monocytogenes*, it was necessary to examine 1 ml of the decimal dilution (10^{-1}) distributing it on the surface of the ALOA agar medium in a large Petri dish (140mm). Carefully, the inoculums were spread over the surface of the agar plate with a spatula. Then, the dishes were inverted and placed in an incubator set at 37°C for 24 hours. If necessary, re-incubation for 24 hours. After a sufficient incubation period, *Listeria* spp. formed green-blue colonies surrounded by an opaque halo. For the confirmation of *Listeria* spp., five of the presumptive colonies on each plate were selected and streaked onto the surface of pre-dried plates of tryptone soya yeast extract agar (TSYEA). The plates were placed in the incubator set at 37°C for 24 hours or until growth was satisfactory. After the incubation, typical colonies were 1 mm to 2 mm in diameter, convex, colourless and opaque. Typical colonies were subjected to Catalase reaction. One of typical colonies was taken and suspended in a drop of hydrogen peroxide solution on a slide. The immediate formation of gas bubbles indicated a positive reaction. Then, typical colonies were subjected to Gram staining: *Listeria* spp. were revealed as Gram-positive slim, short rods (of approximately 0,4 µm diameter, and 1 µm to 2 µm length). The confirmation of *L. monocytogenes* was determined by the CAMP test and by the haemolytic reaction on sheep blood agar dishes after the incubation at 37°C for 24 hours. *L. monocytogenes* showed narrow, clear, light zone of β-haemolysis. Biochemical tests were completed with API-*Listeria* (bioMérieux) as recommended by manufacturer. The API-*Listeria* profile obtained (6510) identified the isolates as *L. monocytogenes* with 98.6% probability. The isolates of *L. monocytogenes* were subjected to serotyping with immunoprecipitation kits (Denka Seiken, Tokyo, Japan). The kits used polyvalent antisera that recognise somatic (O) and flagellar (H) antigens.

The isolates of *L. monocytogenes* were subjected to antimicrobial disk susceptibility test, following the CLSI guidelines (CLSI, 2012). The test was performed by applying a bacterial *inoculum* of approximately $1–2\times10^8$ CFU/ml to the surface of a Mueller-Hinton with 5% sheep blood agar plate. Up to 6 commercially-prepared, fixed concentration, paper antibiotic disks were placed on the inoculated agar surface. Plates were incubated for 24 hours at 37°C. The antibiotic disks used from Liofilchem (Liofilchem s.r.l, Roseto d. A., Italy) are reported in table 13.

Table 13. Antimicrobials molecules used for the detection of the antimicrobial-resistance pattern in *L. monocytogenes* isolates.

ANTIMICROBIAL	DISC CONTENT µg	ZONE DIAMETER mm		
		Susceptible	Intermediate	Resistant
		≥		≤
Ampicillin	AMP 10	20	-	19
Eritromycin	E 15	23	14-22	13
Gentamicin	CN 10	15	13-14	12
Penicillin	P 10 UI	20	-	19
Tetracycline	TE 30	19	15-18	14
Trimethoprim-sulfamethoxazole	SXT 25	16	11-15	10

To maintain quality control of performance and reliability of the results, the standard strain of *Listeria monocytogenes* ATCC 19111 was used.

11.3. Identification of fish species

11.3.1. Primer design

Merluccius merluccius, Lates niloticus, Gadus morhua, Ruvettus pretiosus, Pangasianodon hypophthalmus and *Epinephelus* spp. were the six fish species of interest subjected to the study. Polymerase Chain Reaction (PCR) was the technique used for the identification of fish species. The genomic segment to amplify and from which to create the primers was *cytochrome oxidase subunit* 1 (COI) gene. For each fish species of interest, COI sequences were obtained from GenBank

database and, then, were aligned and compared by the program BioEdit. Primers were developed by two methods. Firstly, species-specific primers to amplify fragments of 200 bp, 250 bp, 300 bp, 350 bp and 400 bp within COI gene for *M. merluccius, L. niloticus, G. morhua, R. pretiosus, P. hypophthalmus*, respectively, were designed by the program "Primer Express 3.0". The program "Primer Express 3.0" was set according to the parameters reported in table 14.

Table 14. Parameters inserted in the software "Primer Express 3.0" in order to obtain a pair of primers for the identification of *M. merluccius, L. niloticus, G. morhua, R. pretiosus, P. hypophthalmus*.

PARAMETER	VALUE
- Primer Tm	
Min Primer Tm	58
Max Primer Tm	60
Max difference in Tm of two primers	2
- Primer GC Content	
Min Primer % GC Content	30
Max Primer % GC Content	80
Max Primer 3' GC's	2
Primer 3' End Length	5
Primer 3' GC Clamp Residues	0
- Primer Length	
Min Primer Length	9
Max Primer Length	40
Optimal Primer Length	20
- Primer Composition	
Max Primer G Repeats	3
Max Num Ambig Residues in Primer	0
- Primer Secondary Structure	
Max Primer Consec Base Pair	4
Max Primer Total Base Pair	8
- Primer Site Uniqueness	

Max % Match in Primer	75
Max Consec Match in Primer	9
Max 3' Consec Match in Primer	7
- Amplicon	
Min Amplified Region Tm	0
Max Amplified Region Tm	85
Min Amplified Region Length	200 (variable)
Max Amplified Region Length	400 (variable)
- Penalty	close to zero

Secondly, COI FASTA sequences for *Epinephelus* spp. and *G. morhua* were inserted in the software "Primer – BLAST" in order to develop primers to amplify fragments of 522 bp and 562 bp, respectively. The software "Primer – BLAST" was set to create primers according to the parameters reported in table 15.

Table 15. Parameters inserted in the software "Primer – BLAST" in order to obtain a pair of primers for the identification of *Epinephelus* spp. and *Gadus morhua*.

PARAMETER	VALUE
- PCR product lenght	
Min product lenght	500
Max product lenght	600
- Primer melting temperatures (T_m)	
Min Primer Tm	57
Optimum Primer Tm	58
Max Primer Tm	59
Max Tm difference	1

Primers were commercially synthesized by Sigma Aldrich (Milan, Italy). Primers were diluted to a final concentration of 100 nM. PCR primers for each fish species of interest was created. Two pairs of primers for *Gadus morhua* were developed by both methods (table 16).

Table 16. Original species-specific primers developed for the identification of fish species.

Method	Fish speceis	Primers sequences	Length (bp)	Product size (bp)
Primer Express 3.0	*Merluccius merluccius*	FWD 5'- ATAATTGGAGGCTTCGGAAACTG -3' RVS 5'- CCAGCGTGGGCAAGATTACT -3'	23 20	200
Primer Express 3.0	*Lates niloticus*	FWD 5'- GGAGCTGGAACCGGTTGAA -3' RVS 5'- CAGCTAAGACTGGGAGGGAAAG -3'	19 22	250
Primer Express 3.0	*Gadus morhua*	FWD 5'- GGTGCACTTCTTGGTGATGATC -3' RVS 5'- ATCAACAGATGCCCCAGCAT -3'	22 20	300
Primer Express 3.0	*Ruvettus pretiosus*	FWD 5'- CGGCACATGCCTTCGTAATAA -3' RVS 5'- GGCTGCGGGTTTCATATTAATAA -3'	21 23	350
Primer Express 3.0	*Pangasius hypophthalmus*	FWD 5'- CCTTCTAGGCGACGACCAAA -3' RVS 5'- ATATTGTGAAATTGCTGGTGGTTTT -3'	20 25	400
Primer – BLAST	*Epinephelus* spp.	FWD 5'- TCTTGTATTTGGTGCCTGGG -3' RVS 5'- ACTGCTGTAATTAGGACGGC -3'	20 20	522
Primer – BLAST	*Gadus morhua*	FWD 5'- TCTCGTATTTGGTGCCTGAG -3' RVS 5'- GATACCAGCTGCTAAGACGG -3'	20 20	562

11.3.2. Fish samples for the validation of the protocol

Ten samples of each fish species of interest were obtained from wholesale fishery plants. DNA was extracted from individual sample using NucleoSpin Tissue Kit (Macherey-Nagel). All DNA samples were quantified (20 ng/µl) by Nanodrop (Thermo Scientific).

11.3.3. Polymerase chain reaction (PCR)

All samples were subjected to end-point PCR in a Thermal Cycler Eppendorf. The PCR mixture (total volume 25 µL) contained 1X PCR buffer containing 1.5 mM $MgCl_2$ (20 nm Tris-HCl pH 8.4, 50 mm KCl), 0.2 mM of dNTPs, 0.5 µM of each primers, 2 U of Phire Hot Start II DNA Polymerase (Thermo Scientific) and approximately 5 ng of DNA (table 17). PCR conditions were 98 °C for 30 s, 34 cycles of 98 °C for 5 s, 58 °C for 30 s, and 72 °C for 15 s, with a final extension at 72 °C for 1 min (table 18). The PCR amplicons were analysed by agarose gel electrophoresis by using a horizontal 2% (wt/vol) agarose gel in lX TBE buffer (pH 8.3; 0.09 M Tris, 0.09 M boric acid, 2.0 mM EDTA) and with 0.003% (wt/vol) ethidium bromide for DNA staining. PCR products were mixed with a sample buffer of 1X TBE and then applied to each well. Gel ran in 1X TBE buffer at 200 V for 30 minutes. The DNA marker used was Amplisize molecular ruler, 50% GC

content, 50-2000 bp, 10 bands (Bio Rad, Hercules, Spain). The PCR products were visualised and photographed by a Gel Doc XR+ System transilluminator (Bio Rad, Milan, Italy).

11.3.4. Sequencing

PCR products were purified using Montage PCR filter units (Millipore, Milan, Italy) and sequenced by BigDye 3.1 Ready reaction mix (Applied Biosystems, Foster City, CA) according to the manufacturer's instructions (tables 17 and 18). Sequences were imported and assembled with the BioNumerics 7.5 software (Applied Maths, Saint-Martens-Latem, Belgium) and searched for homologous sequences by BLAST search analysis (http://www.ncbi.nlm.nih.gov).

Table 17. PCR Master Mix for the identification of fish species of interest.

Reaction Component	**Final Concentration**	**Amount for each Reaction**
Water	-----	16.85 µl
PCR Buffer	1X	5 µl
dNTP's	0.2 mM	0.5 µl
Primer Forward	0.5 µM	0.5 µl
Primer Reverse	0.5 µM	0.5 µl
Taq DNA Polymerase	2 U	0.15 µl
DNA	-----	1.5 µl
		Final Volume: 25 µl

Table 18. PCR Amplification Program performed in a Thermal Cycler Eppendorf.

Step	**Temperature (°C)**	**Time**	**Number of Cycle**
Initial Denaturation	98	30 sec	1
Denaturation	98	5 sec	29
Annealing	58	30 sec	
Extension	72	15 sec	
Final Extension	72	1 min	1

11.3.5. Multiplex-PCRs

Primers were developed in order to obtain amplicons with different length (at least 50 base pairs). Duplex and Triplex PCR protocols were developed for the simultaneous analysis of more fish species using the designed primers with several combinations (table 19).

Table 19. Combinations of Duplex and Triplex PCR protocols by using the developed primers.

Multiplex-PCR	**Fish Species**	**Amplicon Lenght**
Duplex-PCR	*Lates niloticus*	250 bp
	Epinephelus spp.	522 bp
Duplex-PCR	*Lates niloticus*	250 bp
	Gadus morhua	300 bp
Duplex-PCR	*Merluccius merluccius*	200 bp
	Gadus morhua	562 bp
Duplex-PCR	*Ruvettus pretiosus*	350 bp
	Gadus morhua	562 bp
Duplex-PCR	*Ruvettus pretiosus*	350 bp
	Epinephelus spp.	522 bp
Duplex-PCR	*Pangasianodon hypophthalmus*	400 bp
	Epinephelus spp.	522 bp
Duplex-PCR	*Pangasianodon hypophthalmus*	400 bp
	Gadus morhua	562 bp
Triplex-PCR	*Merluccius Merluccius*	200 bp
	Lates niloticus	250 bp
	Pangasianodon hypophthalmus	400 bp
Triplex-PCR	*Merluccius merluccius*	200 bp
	Ruvettus pretiosus	350 bp
	Epinephelus spp.	522 bp
Triplex-PCR	*Lates niloticus*	250 bp
	Pangasianodon hypophthalmus	400 bp
	Gadus morhua	562 bp

	Lates niloticus	250 bp
Triplex-PCR	*Ruvettus pretiosus*	350 bp
	Epinephelus spp.	522 bp

11.3.6. Specificity tests

Single PCRs were performed using the designated primers for each fish species of interest with the DNA extracted from the non-target fish species (negative controls).

11.3.7. Survey on fish labelling

As regards the sampling for the evaluation of the application of labelling laws and for the detection of fraudulent actions by PCR, 43 fishery products were purchased. In particular, 18 samples (42%) at four hypermarket stores and 25 samples (58%) at five fisheries and at six local fish marketplaces. Samples purchased at hypermarket stores consisted of 6 fish skewers containing Nile perch (labeled as *Lates niloticus*), 2 breaded hake fillets (labeled as *Merluccius merluccius*), 2 fish burgers (labeled as *Gadus morhua*), 1 cod fillet (labeled as *Gadus morhua*), 1 breaded Nile perch fillet (labeled as *Lates niloticus*), 3 Nile perch fillets (labeled *Lates niloticus*), 2 salted cod fishes (labeled as *Gadus morhua*) and 1 grouper fillet (labeled as *Epinephelus marginatus*). Samples purchased at fisheries and fish marketplaces consisted of 20 fish fillets and 5 fish slices. As regards fish fillets, four were labelled as grouper, two as cod, three as Nile perch, four as catfish, five as "fillet" and two as "perch" (both without the indication of fish species). All fish slices were labelled as grouper. Samples were subjected to DNA extraction with NucleoSpin Tissue Kit (Macherey-Nagel). All DNA samples were quantified (about 20 ng/μl) by Nanodrop (Thermo Scientific) and subjected to PCR analysis with original species-specific primers developed for the identification of fish species.

12. RESULTS

12.1. Detection of *Staphylococcus aureus* from RTE fishery products

Out of thirty-three samples of smoked salmon, *Staphylococcus aureus* was isolated from one sample (3.03%). The five isolates were confirmed to be *S. aureus* by PCR analysis on the basis of both 16S and *nuc* PCR-positive results. The strains synthesized SEB and SEC and resulted biofilm producers, giving a 188 bp band for *ica*A gene. The isolates resulted *mec*A and *pvl* negative (table 20). The isolates showed phenotypic resistance to Ampicillin and Tetracycline and were susceptible to Amoxicillin + Clavulanic acid, Cephalothin, Chloramphenicol, Clindamycin, Enrofloxacin, Eritromycin, Gentamicin, Kanamycin, Oxacillin, Penicillin, Streptomycin, Sulfisoxazole, Trimethoprim-sulfamethoxazole and Vancomycin (table 21).

Table 20. Molecular profile of the *S. aureus* isolates from smoked salmon.

Gene	**Result**
16 S	Positive (amplicon 228 bp)
*mec*A	Negative
nuc	Positive (amplicon 129bp)
sea	Negative
seb	Positive (amplicon 667 bp)
sec	Positive (amplicon 234 bp)
sed	Negative
see	Negative
seg	Negative
seh	Negative
sei	Negative
sej	Negative
*ica*A	Positive (amplicon 188 bp)
pvl	Negative

Table 21. Antimicrobial-resistance pattern of the *S. aureus* isolates from smoked salmon.

ANTIMICROBIAL	DISC CONTENT µg	ZONE DIAMETER mm			RESULT	
		Susceptible	Intermediate	Resistant		
		≥		≤		
Amoxicillin + clavulanic acid	AUG 30	20	-	19	24	S
Ampicillin	AMP 10	29	-	28	26	R
Cephalothin	KF 30	18	15-17	14	32	S
Chloramphenicol	C 30	18	13-17	12	22	S
Clindamycin	CD 2	21	15-20	14	22	S
Enrofloxacin	ENR 5	21	17-20	16	25	S
Eritromycin	E 15	23	14-22	13	26	S
Gentamicin	CN 10	15	13-14	12	22	S
Kanamycin	K 30	18	14-17	13	21	S
Oxacillin	OX 1	13	11-12	10	20	S
Penicillin	P 10 UI	29	-	28	36	S
Streptomycin	S 10	15	12-14	11	18	S
Sulfisoxazole	ST 250	17	13-16	12	24	S
Tetracycline	TE 30	19	15-18	14	14	R
Trimethoprim-sulfamethoxazole	SXT 25	16	11-15	10	24	S
Vancomycin	VA 30	12	10-11	9	14	S

12.2. Detection of *Listeria monocytogenes* from RTE fishery products

Out of fourty-five samples of smoked salmon, two samples (4.44%) were positive for the detection of *Listeria monocytogenes*. Both samples had high levels of contamination, i.e. 2000 CFU/g. The strains of *L. monocytogenes,* isolated from both samples, resulted to belong to the serovar 1/2a (table 22). The isolates of *L. monocytogenes* showed phenotypic susceptibility to all antimicrobials tested (table 23).

Table 22. Results of the serotyping of the *L. monocytogenes* strains isolated from smoked salmon.

O-antygen	**F-antigen**	**Serovar**
I, II	A, B	1/2a

Table 23. Antimicrobial-resistance profile of *L. monocytogenes* strain isolated from samples n.1 and n.2.

ANTIMICROBIAL	SYMBOL	RESISTANCE	INTERMEDIATE	SUSCEPTIBILITY	RESULT	
Ampicillin	AMP 10	19	-	20	20	S
Penicillin	P 10 UI	19	-	20	32	S
Gentamicin	CN 10	12	13-14	15	30	S
Trimethoprim / Sulfamethoxazole	SXT 25	10	11-15	16	34	S
Tetracycline	TE 30	14	15-18	19	20	S
Erythromycin	E 15	13	14-22	23	38	S

12.3 Identification of fish species and results of the survey on the application of labelling laws and for the detection of fraudulent actions

The PCR analysis allowed the detection of DNA extracted from all samples of each fish species of interest, giving fragments of the expected length. At the end of the running, the electrophoresis agarose gel showed a clear separation of amplicons due to their different size (figures 1, 2, 3, 4 and 5). After sequencing, the isolates were compared with the selected sequences of COI gene and showed a similarity ranging from 99 to 100%.

Grouper samples subjected to *Epinephelus* spp. authentication, showed 97.5% homology to *Epinephelus* costae GenBank entry (KM077928.1) and 100% homology to *Epinephelus marginatus* GenBank entry (KC500692.1).

Single PCRs performed for the specificity tests gave the expected results.

Fig. 1. Gel electrophoresis of PCR products obtained from Duplex PCR assays (lanes 1 and 2) for identification of *Ruvettus pretiosus* (350 bp) and *Epinephelus* spp. (522 bp.). Lane M: AmpliSize™ Molecular Ruler (50–2000-bp ladder; Bio-Rad). Lane (neg.): negative control.

.

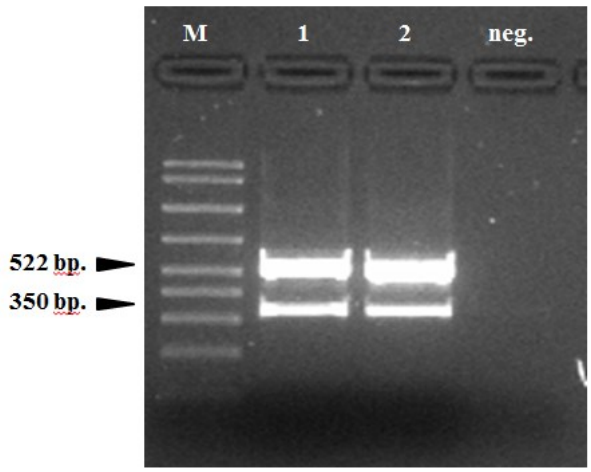


Fig. 2. Gel electrophoresis of PCR products obtained from Duplex PCR assays (lane 1) for identification of *Pangasianodon hypophthalmus* (400 bp.) and *Gadus morhua* (566 bp.). Lane M: AmpliSize™ Molecular Ruler (50–2000-bp ladder; Bio-Rad). Lane neg.: negative control.

.

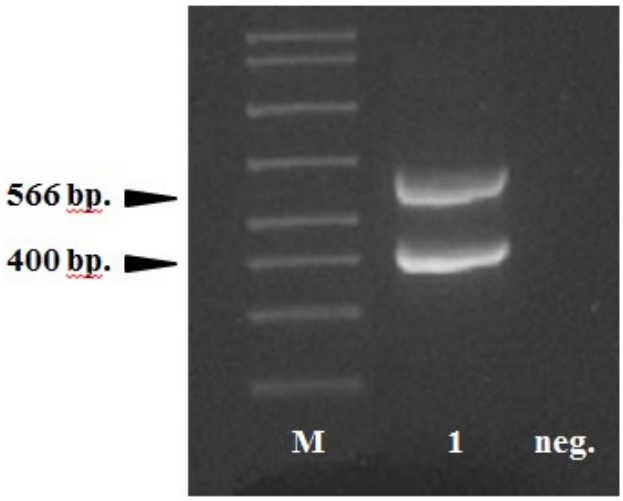


Fig. 3. Gel electrophoresis of PCR products obtained from Duplex PCR assays (lanes 1 and 2) for identification of *Lates niloticus* (250 bp.) and *Gadus morhua* (300 bp.). Lane M: AmpliSize™ Molecular Ruler (50–2000-bp ladder; Bio-Rad).

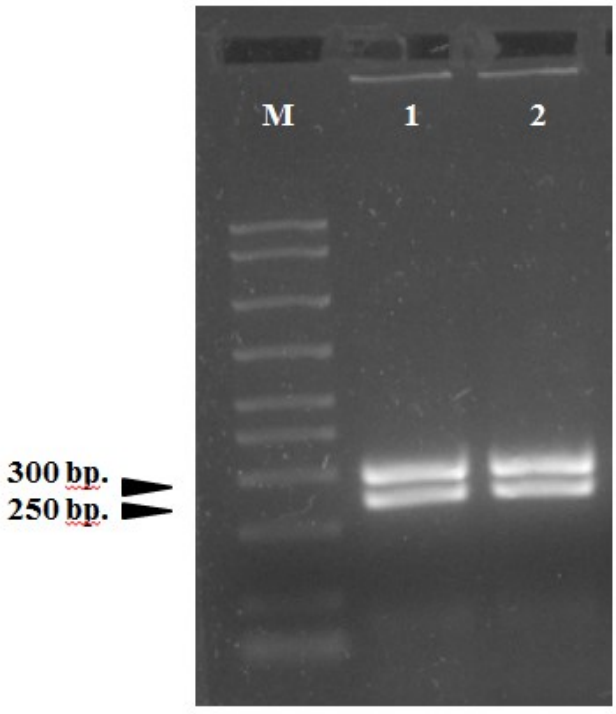


Fig. 4. Gel electrophoresis of PCR products obtained from Triplex PCR assays (lanes 1 and 2)for identification of *Merluccius merluccius* (200 bp.), *Lates niloticus* (250 bp.) and *Pangasianodon hypophthalmus* (400 bp.). Lane M: AmpliSize™ Molecular Ruler (50–2000-bp ladder; Bio-Rad). Lane neg.: negative control.

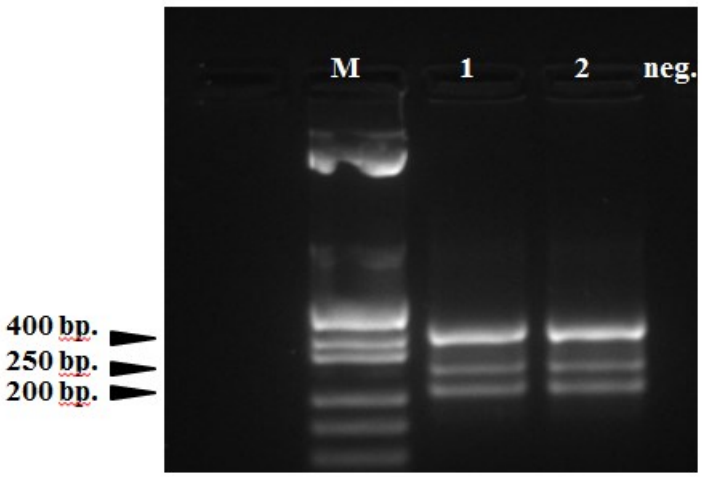
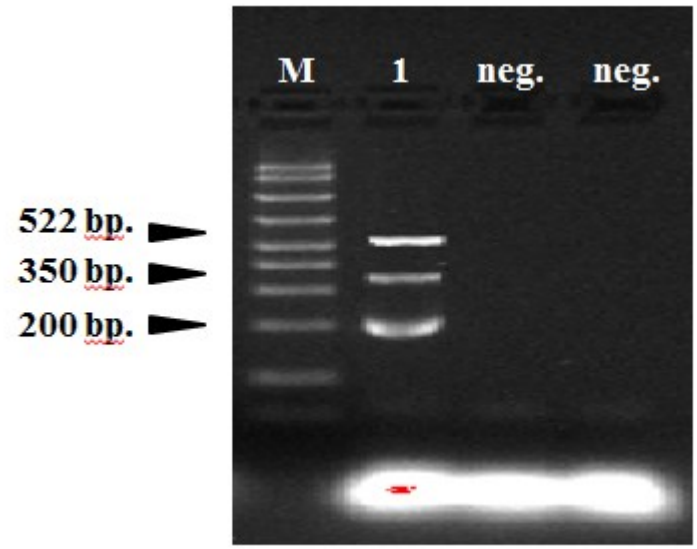


Fig. 5. Gel electrophoresis of PCR products obtained from Triplex PCR assays (lane 1) for identification of *Merluccius merluccius* (200 bp.), *Ruvettus pretiosus* (350 bp.) and *Epinephelus* spp. (522 bp.). Lane M: AmpliSize™ Molecular Ruler (50–2000-bp ladder; Bio-Rad). Lane neg.: negative controls.



Overall, out of 43 fish samples analysed, 19 (44.2%) resulted mislabelled, with 18 (41.9%) mislabelled samples from local fisheries and marketplaces and 1 (2.32%) from hypermarket stores (table 24). As regards fish samples purchased at hypermarket stores, all cod samples tested positive for *Gadus morhua* showing an amplicon of 562 bp; all Nile perch samples tested positive for *Lates niloticus* showing an amplicon of 250 bp; all hake samples tested positive for *Merluccius merluccius* showing an amplicon of 200 bp; the grouper sample tested positive for *Epinephelus* spp. showing an amplicon of 522 bp. To ascertain the existence of false positives, identifications were confirmed by sequencing. After sequencing, *Epinephelus* spp. isolates showed 100% homology to *Epinephelus diacanthus* GenBank entry (EF609520.1). Overall, out of 25 fish samples purchased at fisheries and fish marketplaces, 18 (72%) resulted mislabelled. Cases of mislabelling regarded more fish slices (100%) than fillets (65%). As regards fish fillets, three Nile perch fillets (15%) and four catfish fillets (20%) were correctly labelled. The DNA analysis on the remaining fillets showed that thirteen samples were mislabelled (65%). In particular, all samples marketed as grouper fillets (100%) showed fraudulent actions. In fact, out of four samples labelled as grouper, three (75%) tested positive for *Lates niloticus* showing an amplicon of 250 bp., and one (25%) positive for *Pangasianodon hypophthalmus* showing an amplicon of 400 bp. Both cod fillets (100%) resulted to be *Lates niloticus* showing an amplicon of 250 bp. The 5 samples labelled as "fillet" and the 2 samples labelled as "perch" were identified as *P. hypophthalmus* showing an amplicon of 400 bp. As regards grouper slices, all samples (100%) showed fraudulent species substitutions. In fact, *Ruvettus pretiosus* was found to be marketed as grouper (amplicon of 350 bp.). To ascertain the existence of false positives, identifications were confirmed by sequencing.

Table 24. Results of the survey on the application of the labelling laws and for the detection of fraudulent actions.

Retail outlet	Fishery products	N.	Species labelled	Species identified by PCR	Result
Hypermarket stores	Fish skewer	6	Nile perch (*Lates niloticus*)	*Lates niloticus*	Correctly labelled
Hypermarket stores	Fillet	2	Hake (*Merluccius merluccius*)	*Merluccius merluccius*	Correctly labelled
Hypermarket stores	Fish burger	2	Cod (*Gadus morhua*)	*Gadus morhua*	Correctly labelled
Hypermarket stores	Fillet	1	Cod *(Gadus morhua)*	*Gadus morhua*	Correctly labelled
Hypermarket stores	Fillet	1	Nile perch (*Lates niloticus*)	*Lates niloticus*	Correctly labelled
Hypermarket stores	Fillet	3	Nile perch (*Lates niloticus*)	*Lates niloticus*	Correctly labelled
Hypermarket stores	Salted fish	2	Cod (*Gadus morhua*)	*Gadus morhua*	Correctly labelled
Hypermarket stores	Fillet	1	Grouper (*Epinephelus marginatus*)	*Epinephelus diacanthus*	**Mislabelled**
Local fisheries and fish marketplaces *	Fillet	4	Grouper	*Lates niloticus (75%)* *Pangasius hypophthalmus (25%)*	**Mislabelled**
Local fisheries and fish marketplaces	Fillet	2	Cod	*Lates niloticus*	**Mislabelled**
Local fisheries and fish marketplaces	Fillet	3	Nile perch	*Lates niloticus*	Correctly labelled
Local fisheries and fish marketplaces	Fillet	4	Catfish	*Pangasius hypophthalmus*	Correctly labelled
Local fisheries and fish marketplaces	Fillet	5	Reported as “fillet”	*Pangasius hypophthalmus*	**Mislabelled**
Local fisheries and fish marketplaces	Fillet	2	Perch	*Pangasius hypophthalmus*	**Mislabelled**
Local fisheries and fish marketplaces	Fish slices	5	Grouper	*Ruvettus pretiosus*	**Mislabelled**

* = The samples sold in local fisheries and marketplaces did not show the scientific name on label.

13. DISCUSSION

From a commercial point of view, fishery products represent a wide and important category of food products since they can reach the consumers as fresh, frozen and processed products (salted, dried, smoked, canned, packaged, etc.). Fishery products can be provided in different commercial ways and this automatically can determinate the presence of numerous potential health hazards (bacterial, viral, parasitic, physical and chemical). Beyond health hazards, which can be transmitted from fishery products to consumer, due to the wide number of fish species of commercial interest and to their common morphological similarity, consumers are exposed to commercial frauds, and, sometimes, to sanitary ones. The present study shows the results of a research which had three specific aims: a) evaluation of the presence of *Staphylococcus aureus* in RTE fishery products; b) evaluation of the presence of *Listeria monocytogenes* in RTE fishery products; c) development of original protocols based on PCR analysis for the identification of some fish species widely marketed. As regards the first aim, 99 samples of RTE fishery products were analysed according to standardised protocols for the detection of *S. aureus* (EN ISO 6888- 2 : 1998 and EN ISO 6888- 2 Amendment 1 : 2003). *S. aureus* isolates were characterised by phenotypic methods (analysis of antimicrobial resistance pattern) and molecular methods (PCR-based detection of genes encoding virulence determinants). Out of 99 analysed samples, one resulted positive (3.03%). *S. aureus* isolate showed the following genetic profile: 16S+/ *nuc*+/ *seb*+/ *sec*+/ *icaA*+/ *pvl*-/ *mecA*- and resistance to Ampicillin and Tetracycline. Staphylococcal food poisoning is one of the most prevalent causes of gastroenteritis worldwide, which is caused by the ingestion of food that contains pre-formed toxins. Fish is rich in protein and its breakdown into low molecular weight peptides and amino acids supports the growth of *S. aureus* and the consequent release of staphylococcal enterotoxin(s) (SEs). Fishery products mainly involved in outbreaks are canned, smoked and salted products, boiled fish paste and fish sausages (Simon and Sanjeev, 2007). Fresh caught fish is free from *S. aureus* and contamination takes place upon handling. The contamination could be the result of a combination of improper and unsanitary handling, improper storage and cross contamination. Various authors have reported the incidence of enterotoxigenic *S. aureus* in seafood: Sanjeev *et al.* (1986) reported 68% from frozen fishery products, 48% from RTE foods including fish (Sokari, 1991), 8% from fish and shell-fish (Ayulo *et al*., 1994), 7% from shrimps, 4% from frozen cuttlefish and 4% from fish (Rodma *et al*., 1991). In Italy, Normanno reported a prevalence of 2.3% of Coagulase positive Staphylococci out of 732 fish samples analysed (Normanno *et al*., 2005). A high rate of detection of *S. aureus* in smoked fish (26%) and in other RTE products (10%) was reported by Vázquez-Sánchez in samples from Spain (Vázquez-Sánchez *et al*., 2012). In a recent work on the presence and characterisation of *S. aureus* in RTE fishery products, i.e. sushi and

sashimi, Puah *et al*. (2016) reported a positivity of 26% of the tested samples. The majority of the isolates (96%) carried virulence genes and also multi drug resistance was detected in 3.8% of the isolates (Puah *et al*., 2016). Antimicrobial resistance is a major public health problem in many countries due to the persistent circulation of resistant strains of bacteria in the environment and the possible contamination of water and food (Normanno *et al*., 2007). Antibiotic-resistant *S. aureus* involved in food contamination has been detected worldwide. According to others (Vázquez-Sánchez *et al*., 2012; Puah *et al*., 2016) our isolate was resistant to Ampicillin and Tetracycline. The presence of enterotoxigenic and antimicrobial-resistant strains in our samples highlights the high potential risk for consumers of fishery RTE especially in the absence of strict hygienic and preventive measures to avoid SEs production in foods. Regarding the presence of *L. monocytogenes* in RTE fishery products (second objective of the present thesis), we found positive 2 (4.44%) out of 135 samples. Both samples (smoked salmon) had high levels of contamination, i.e. 2000 CFU/g. The strains of *L. monocytogenes,* isolated from both samples, resulted to belong to the serovar 1/2a and were susceptible to all antimicrobial tested. Listeriosis primarily affects neonates and immune-compromised individuals, causing severe conditions such as septicaemia, encephalitis and meningitis; it also causes abortion and stillbirth in pregnancy. Since 2008 in the European Countries the number of listeriosis cases in humans has increased, with 1645 confirmed human cases, reaching the overall EU notification rate of 0.4 cases for 100,000 population. Italy recorded a statistically significant increase in human listeriosis cases between 2005 and 2009 although the overall incidence is lower than other European Member States. A high fatality rate of 16.6% was reported among the cases, with the elderly being especially affected (EFSA, 2011). The European Commission Regulation 2073/2005 set the safety criteria for *L. monocytogenes* in RTE foods intended for infants and for medical purposes and in RTE foods able to support or not to support the growth of the microorganism (included fishery products) that may pose a *L. monocytogenes* risk for public health. A survey, started in January 2010 and carried on over a two-year period in 26 EU countries, was carried out to obtain valid EU level estimates of prevalence and contamination levels of *L. monocytogenes* in RTE fishery products, such as packaged (not frozen) hot or cold smoked or gravad products. In particular, 3053 fish samples of the same batch were analysed twice, at the time of sampling (an arbitrary point in their shelf-life) and at the end of shelf-life (EFSA, 2013a). After analysis, results showed an EU prevalence of 10.4 % and 10.3 %, respectively, at both testing times (EFSA, 2013a). Cold smoked fish samples were the fishery products with the highest levels of contamination: 17.4% at the time of sampling and 16.0% at the end of shelf-life, respectively. *L. monocytogenes* was also recovered from 12.2% of gravad fish samples at both testing times (EFSA, 2013a). The proportion (and number) of fish samples with a *L. monocytogenes* count exceeding the

level of 100 CFU/g was 1% (29 samples) at time of sampling and 1.7% (52 samples) at the end of shelf-life, whose 48 (71%) batches were of processed salmons (EFSA, 2013a). In particular, out of 1793 smoked salmon samples, 294 (12.44%) resulted out of limits included in the EC Reg. 2073/2005 (EFSA, 2014a). Overall, data obtained correspond to what is reported in the literature for the high contamination levels of *L. monocytogenes* both in RTE fishery products and in smoked salmons. In fact, data reported by Latorre *et al*., after a 12-year survey (1993 through 2004) on the presence of *L. monocytogenes* in 5788 samples of several kinds of RTE foods marketed in Italy, show that 121 (2.1%) samples were contaminated with *L. monocytogenes* and the highest prevalence was found in smoked salmon (10.6%) (Latorre *et al*., 2007). However, our findings are in contrast with these results; a possible explanation could be the limited number of samples examined. Serotyping has classically been used for the subtyping of *L. monocytogenes*, based on somatic (O) and flagellar (H) antigens. *L. monocytogenes* isolates are divided into 13 serotypes (Seeliger and Hohne, 1979) but over 95% of isolates in human listeriosis and in foods belong to serotypes 1/2a, 1/2b and 4b (Parisi *et al*., 2010). Our isolates belonged to the serotype 1/2a, thus it is a potential hazard for consumers. A remarkable aspect of our findings was the very high count of *L. monocytogenes* in two positive samples (2000 CFU/g). This situation is not in conformity with EC Reg. 2073/05 that allows the presence of 100 CFU/g in RTE foods throughout the shelf-life of products. In fact, this level of contamination appears as a prudent safety limit for the food-borne risk linked to the consumption of food contaminated with *L. monocytogenes*. The detection of exceeding limit by the competent Authoritiy (official supervisor) determines the beginning of legal procedures that can have serious consequences for food business operators. The presence of *L. monocytogenes,* serovar 1/2a, in RTE fishery products can suggest to implement surveillance and control systems at all stages of the food chain, to implement instruction and training in food hygiene matters for food business operators and to limit the consumption of these products to particularly susceptible population such as young, old, pregnant, immune compromised (Y.O.P.I.) people. The importance of proper handling and storage of seafood as well as the need to control the growth of enterotoxigenic strains of *S. aureus* and *L. monocytogenes* needs to be emphasised. It is recommended to use personal protection devices, such as sanitary gloves and masks, for handling RTE foods in order to reduce the contamination by *S. aureus*. It is needed to educate workers about the importance of hygienic and sanitary conditions and implementation GMP and HACCP (Simon and Sanjeev, 2007). In fish sector, the identification of fish species throughout the production chain is of main importance, even if fishery products have been already processed. In fact, there are different ways to purchase fish and fishery products: whole, fillets, slices, skewers or mixed with other species for gastronomic dishes (seafood salad, risotto mix, fish fingers, etc.). Furthermore, the

presence of similar fish species, but very different from a nutritional and organoleptic point of view, is more frequent. In this regards, at present, commercial fishery products in Europe come from all parts of the world, meaning that accurate species identification is not always easy. In this situation, both sanitary and quality control and product traceability seem to be obstructed, because fish is not easily identifiable, with the increase of commercial (*aliud pro alio*) and sanitary frauds (commercialisation of toxic organisms). For example, oilfish (*Ruvettus pretiosus*) is seldom marketed in conformity with current EU Regulation (EC Reg. 1021/08) and it is often commercialised in place of the most popular, expensive and precious species, such as grouper (*Epinephelus* spp.). The problem of fraudulent actions in the commercialisation of foods is strongly felt at European Union level; in fact, recently a recommendation was enacted on the need to establish a "coordinated plan of supervision designed to determine the prevalence of fraudulent practices in the marketing of certain foodstuffs", including fishery products (EU Recommendation n. 1558 – 12 March 2015). Identification procedures generally include the analysis of proteins by electrophoretic techniques such as isoelectric focusing (IEF), capillary electrophoresis (CE), or immunoassay techniques such as ELISA (Trotta *et al*., 2005). Fish species identification methods based on DNA analysis have been developed as an alternative to morphological and immunological analysis. Molecular assays main advantages are: high sensitivity (detection of few DNA molecules), DNA sequence diversity (also among phylogenetically closely related species), nucleic acid material good preservation and resistance to food processes (Rasmussen and Morrissey, 2009**;** Cutarelli *et al*., 2014). In order to develop novel protocols based on PCR reaction for the genetic identification of some commercial fish species (the third aim of the present thesis), we created specific primers for the identification of: *Merluccius merluccius, Lates niloticus, Gadus morhua, Ruvettus pretiosus, Pangasianodon hypophthalmus* and *Epinephelus* spp.. The choice of genomic segment to amplify and from which to create the primers, was the mitochondrial cytochrome *c* oxidase subunit 1 (COI) gene. This genetic fragment presents very low intraspecific variability, thus permitting the unequivocal identification of fish species contained in the commercial products (Calo-Mata *et al*., 2003). Then, we applied the developed protocols to a local survey aimed to ascertain the correct labelling of fishery products marketed at local retail outlets. The development of PCR protocols has allowed to give a rapid and specific response for the identification of fish species. In fact, the time request from the arrival of the fish sample to the end of the analysis was about 6 – 8 hours. Thanks to the development of Duplex and Triplex PCR protocols, additional information may be gained from a single test run with considerable savings of time, reagents and efforts within the laboratory. Furthermore, the applicability of the assay to commercial fishery products has been demonstrated. In fact, in our survey, out of 43 investigated samples, we detected

19 (44.1%) mislabelled samples. Most of the mislabelled samples derived from local fisheries and marketplaces (41.9%) and one sample (2.32%) from hypermarket stores. Our findings are similar to the results obtained by a national seafood fraud investigations carried on in the U.S. from 2010-2012; in this survey, out of 1200 seafood samples from 674 retail outlets in 21 States, DNA testing found that one-third (33 per cent) were mislabelled (Warner *et al*., 2013). In particular, forty-four per cent of all the retail outlets visited sold mislabelled fish. Also a recent Italian investigation revealed a number of commercial frauds; for example, Cutarelli found that a sample marketed as "frozen grouper fillet" was made of halibut (*Hippoglossus hippoglossus*) instead of grouper (*E. marginatus*). Given the high demand for grouper by consumers, the prices at the subsequent wholesale and retail market levels are also high relative to other finfish species. In addition, the importation of large quantities of grouper from many foreign sources is required to meet the ever-growing demand for grouper. The strong demand for grouper, as well as its high market value, which continues to be evident on the market, is also a motivation for economic fraud. The most prevalent economic fraud associated with grouper is the selling of a cheaper finfish as grouper. In fact, the most common types of mislabelling among the grouper samples collected in US were substitutions with farmed Asian catfish (*Pangasianodon hypophthalmus*), freshwater perch (*Macquaria novemaculeata*), weakfish (*Cynoscion regalis*), bream (*Abramis brama*), and king mackerel (*Scomberomorus cavalla*), which often contains high levels of mercury (Warner *et al.*, 2013). It is important to underline that grouper is a precious fish species often item of fraud; in fact, when grouper is sold as fillet, its main features completely disappear, and its identity cannot be established on the basis of morphological features (Trotta *et al*., 2005). A survey carried out by Eurofishmarket (www.ilfattoalimentare.it) showed that around 15% of fresh/frozen grouper fillets sold on the market belonged to other species. These facts are strongly confirmed in our survey, in fact, we found that all samples marketed as grouper slices (*E. marginatus*) were slices of *R. pretiosus*. This kind of fraud is a sanitary fraud, because *R. pretiosus* is a fish known for its potential dangerousness for consumer. In fact, *R. pretiosus* also known as "oilfish", is a deep-sea fish that store large amounts of wax esters in their body for buoyancy control; the accumulation of the indigestible wax esters in the rectum through consumption of these fish produces discharges or leakage per rectum as orange or brownish green oil, but without noticeable loss of water. This physiological response is called keriorrhea (Ling *et al*., 2008). Outbreaks of keriorrhea have been repeatedly reported across continents. In EU the marketing of *R. pretiosus* is regulated by the EC Reg. 1021/08 (EC Reg. 1021/08). According to this regulation, food business operators are obliged to sell oilfish products in packaged form and to provide information on label to the consumer about their gastrointestinal adverse effects. In conclusion, our methods based on PCR analysis constitutes

an effective molecular tool for the detection of fraudulent substitution of fish species of interest applicable to raw finfish. These protocols could be applied both to quality control and to official sanitary control of fishery products and to help the anti-fraud actions controlling the traceability and labelling of fishery products (D.M. Mi.P.A.F. 27.03.2002).

14. REFERENCES

Adetunji, V. O., Adedeji, A. O., Kwaga, J., 2014. Assessment of the contamination potentials of some foodborne bacteria in biofilms for food products. Asian Pacific Journal of Tropical Medicine, 7, 232-237.

Akasaki, T., Yanagimoto, T., Yamakami, K., Tomonaga, H., Sato, S., 2006. Species Identification and PCR-RFLP Analysis of Cytochrome *b* Gene in Cod Fish (Order *Gadiformes*) Products. Journal of Food Science, 71(3), 190-195.

Aranishi, F., Okimoto, T., Izumi, S., 2005. Identification of gadoid species (Pisces, *Gadidae*) by PCR-RFLP analysis. Journal of Applied Genetics, 46(1), 69-73.

Argudín, M. Á., Mendoza, M. C., Rodicio, M. R., 2010. Food poisoning and *Staphylococcus aureus* enterotoxins. Toxins, 2(7), 1751-1773.

Artham, S. M., Lavie, C. J., Milani, R. V., Anand, R. G., O'Keefe, J. H., & Ventura, H. O., 2008. Fish oil in primary and secondary cardiovascular prevention. The Ochsner Journal, 8(2), 49-60.

Asao, T., Kumeda, Y., Kawai, T., Shibata, T., Oda, H., Haruki, K., Nakazawa H., Kozaki, S., 2003. An extensive outbreak of staphylococcal food poisoning due to low-fat milk in Japan: estimation of enterotoxin A in the incriminated milk and powdered skim milk. Epidemiology and Infection, 130(01), 33-40.

Asensio, L., 2007. PCR-based methods for fish and fishery products authentication. Trends in Food Science & Technology, 18(11), 558-566.

Asensio, L., González, I., García, T., Martín, R., 2008. Determination of food authenticity by enzyme-linked immunosorbent assay (ELISA). Food Control, 19(1), 1-8.

Asensio, L., González, I., Pavón, M. A., García, T., Martín, R., 2008a. An indirect ELISA and a PCR technique for the detection of grouper (*Epinephelus marginatus*) mislabeling. Food Additives and Contaminants, 25(6), 677-683.

Autio, T., Hielm, S., Miettinen, M., Sjöberg, A. M., Aarnisalo, K., Björkroth, J., Mattila-Sabdholm, T., Korkeala, H., 1999. Sources of *Listeria monocytogenes* contamination in a cold-smoked rainbow trout processing plant detected by pulsed-field gel electrophoresis typing. Applied and Environmental Microbiology, 65(1), 150-155.

Ayulo, A. M. R., Machado, R. A., Scussel, V. M., 1994. Enterotoxigenic *Escherichia coli* and *Staphylococcus aureus* in fish and seafood from the southern region of Brazil. International Journal of Food Microbiology, 24(1), 171-178.

Baird-Parker, T. C., 2000. *Staphylococcus aureus*. The microbiological safety and quality of food. Volume II, Lund, B. M., Baird-Parker, T. C., Gould, G. W. eds. Published by Aspen Publisher. p. 1317-1335.

Basti, A. A., Misaghi, A., Salehi, T. Z., Kamkar, A., 2006. Bacterial pathogens in fresh, smoked and salted Iranian fish. Food control, 17(3), 183-188.

Bean, N. H., Griffin, P. M., Goulding, J. S., Ivey, C. B., 1990. Foodborne disease outbreaks, 5-year summary, 1983-1987. Journal of Food Protection, 53(8), 711-728.

Beard, C. B., Hamm, D., Collins, F. H., 1993. The mitochondrial genome of the mosquito *Anopheles gambiae*: DNA sequence, genome organization, and comparisons with mitochondrial sequences of other insects. Insect Molecular Biology, 2(2), 103-124.

Becker, K., Friedrich, A. W., Lubritz, G., Weilert, M., Peters, G., von Eiff, C., 2003. Prevalence of genes encoding pyrogenic toxin superantigens and exfoliative toxins among strains of *Staphylococcus aureus* isolated from blood and nasal specimens. Journal of Clinical Microbiology, 41(4), 1434-1439.

Bērziņš, A., Hellström, S., Siliņš, I., Korkeala, H., 2010. Contamination patterns of *Listeria monocytogenes* in cold-smoked pork processing. Journal of Food Protection, 73(11), 2103-2109.

Bianchi, D. M., Gallina, S., Bellio, A., Chiesa, F., Civera, T., Decastelli, L., 2014. Enterotoxin gene profiles of *Staphylococcus aureus* isolated from milk and dairy products in Italy. Letters in Applied Microbiology, 58(2), 190-196.

Boerema, J. A., Clemens, R., & Brightwell, G., 2006. Evaluation of molecular methods to determine enterotoxigenic status and molecular genotype of bovine, ovine, human and food isolates of *Staphylococcus aureus*. International Journal of Food Microbiology, 107(2), 192-201.

Botzler, R. G., Cowan, A. B., Wetzler, T. F., 1974. Survival of *Listeria monocytogenes* in soil and water. Journal of Wildlife Diseases, 10(3), 204-212.

Brakstad, O. G., Aasbakk, K., & Maeland, J. A., 1992. Detection of *Staphylococcus aureus* by polymerase chain reaction amplification of the *nuc* gene. Journal of Clinical Microbiology, 30(7), 1654-1660.

Bremner, H. A., 2000. Toward practical definitions of quality for food science. Critical Reviews in Food Science and Nutrition. 40(1), 83-90.

Brooks, J. L., Jefferson, K. K., 2014. Phase variation of poly-N-acetylglucosamine expression in *Staphylococcus aureus*. PLoS Pathogens, 10(7), e1004292.

Burdge, G. C., Calder, P. C., 2005. Conversion of alpha-linolenic acid to longer-chain polyunsaturated fatty acids in human adults. Reproduction Nutrition Development, 45(5), 581-597.

Burr, M. L., Gilbert, J. F., Holliday, R. M., Elwood, P. C., Fehily, A. M., Rogers, S., Sweetnam P. R., Deadman, N. M., 1989. Effects of changes in fat, fish, and fibre intakes on death and myocardial reinfarction: diet and reinfarction trial (DART). The Lancet. 334(8666), 757-761.

Bystroń, J., Bania, J., Lis, E., Molenda, J., Bednarski, M., 2009. Characterisation of *Staphylococcus aureus* strains isolated from cows' milk. Bulletin of the Veterinary Institute in Pulawy, 53, 59-63.

Çakli, S., Kişla, D., 2003. Microbial spoilage of fishery products and prevention method. Journal of Fisheries and Aquatic Science. 20, 239–245.

Calder, P. C., 2004. n–3 Fatty acids and cardiovascular disease: evidence explained and mechanisms explored. Clinical science, 107(1), 1-11.

Calo-Mata, P., Sotelo, C. G., Pérez-Martín, R. I., Rehbein, H., Hold, G. L., Russell, V. J., Pride, S., Quinteiro, J., Rey-Méndez, J., Rosa, C., Santos, A. T., 2003. Identification of gadoid fish species using DNA-based techniques. European Food Research and Technology, 217(3), 259-264.

Céspedes, A., García, T., Carrera, E., González, I., Fernández, A., Asensio, L., Hernandez, P. E., Martín, R., 2000. Genetic differentiation between sole (*Solea solea*) and Greenland halibut (*Reinhardtius hippoglossoides*) by PCR–RFLP analysis of a 12S rRNA gene fragment. Journal of the Science of Food and Agriculture, 80(1), 29-32.

Chasseignaux, E., Toquin, M. T., Ragimbeau, C., Salvat, G., Colin, P., Ermel, G., 2001. Molecular epidemiology of *Listeria monocytogenes* isolates collected from the environment, raw meat and raw

products in two poultry-and pork-processing plants. Journal of Applied Microbiology, 91(5), 888-899.

Chmielewski, R. A. N., Frank, J. F., 2003. Biofilm formation and control in food processing facilities. Comprehensive Reviews in Food Science and Food Safety, 2(1), 22-32.

Civera, T., 2003. Species identification and safety of fish products. Veterinary Research Communications, 27, 481-489.

Clinical and Laboratory Standards Institute, 2012. Performance Standards for Antimicrobial Susceptibility Testing, 22nd Informational Supplement. M100eS22.

Comesaña, A. S., Abella, P., Sanjuan, A., 2003. Molecular identification of five commercial flatfish species by PCR–RFLP analysis of a 12S rRNA gene fragment. Journal of the Science of Food and Agriculture, 83(8), 752-759.

Cutarelli, A., Amoroso, M. G., De Roma, A., Girardi, S., Galiero, G., Guarino, A., Corrado, F., 2014. Italian market fish species identification and commercial frauds revealing by DNA sequencing. Food Control, 37, 46-50.

Da Silva, M. L., Matte, G. R., Germano, P. M. L., Matte, M.H., 2010. Occurrence of pathogenic microorganisms in fish sold in Sao Paulo, Brazil. Journal of Food Safety. 30, 94–110.

Dawnay, N., Ogden, R., McEwing, R., Carvalho, G. R., Thorpe, R. S., 2007. Validation of the barcoding gene COI for use in forensic genetic species identification. Forensic Science International 173 (1–6).

Deurenberg, R. H., Stobberingh, E. E., 2008. The evolution of *Staphylococcus aureus*. Infection, Genetics and Evolution, 8(6), 747-763.

D.M. Mi.P.A.F. 27/03/2002. Etichettatura dei prodotti ittici e sistema di controllo. Gazzetta Ufficiale n. 84 del 10 04 2002.

D.M. Mi.P.A.F. 31/01/2008. Denominazione in lingua italiana alle specie ittiche indicate nell'elenco allegato che costituisce parte integrante del presente decreto, che integra e modifica l'elenco allegato al DM del 31 gennaio 2008 come successivamente modificato e integrato dal DM del 5 marzo 2010. Gazzetta Ufficiale n. 11 del 15 gennaio 2011.

Dooley, J. J., Sage, H. D., Clarke, M. A. L., Brown, H. M., Garrett, S. D., 2005. Fish species identification using PCR-RFLP analysis and lab-on-a-chip capillary electrophoresis: application to detect white fish species in food products and an interlaboratory study. Journal of Agricultural and Food Chemistry, 53(9), 3348-3357.

Driehuis, F., Elferink, S. J. O., 2000. The impact of the quality of silage on animal health and food safety: a review. Veterinary Quarterly, 22:212-216.

EFSA BIOHAZ Panel (EFSA Panel on Biological Hazards), 2009. Assessment of the Public Health significance of methicillin resistant *Staphylococcus aureus* (MRSA) in animals and foods. doi:10.2903/j.efsa.2009.993

EFSA, 2010. Analysis of the baseline survey on the prevalence of methicillin-resistant *Staphylococcus aureus* (MRSA) in holdings with breeding pigs, in the EU, 2008 - Part B: factors associated with MRSA contamination of holdings. EFSA Journal 2010; 8(6):1597, 67 pp. doi:10.2903/j.efsa.2010.1597

EFSA NDA Panel (EFSA Panel on Dietetic Products, Nutrition and Allergies), 2010a. Scientific Opinion on Dietary Reference Values for fats, including saturated fatty acids, polyunsaturated fatty acids, monounsaturated fatty acids, trans fatty acids, and cholesterol. EFSA Journal 2010;8(3):1461, 107 pp. doi:10.2903/j.efsa.2010.1461

EFSA, 2011. Scientific opinion on the European Union summary report on trends and sources of zoonoses, zoonotic agents and food-borne outbreaks in 2009. EFSA Journal 2011;9(3):2090 [378 pp.]. doi:10.2903/j.efsa.2011.2090

EFSA Panel on Biological Hazards (BIOHAZ), 2013. Scientific Opinion on the risk posed by pathogens in food of non-animal origin. Part 1 (outbreak data analysis and risk ranking of food/pathogen combinations). EFSA Journal 2013;11(1):3025. [138 pp.] doi:10.2903/j.efsa.2013.3025

EFSA, 2013a. Scientific Opinion on the analysis of the baseline survey on the prevalence of *Listeria monocytogenes* in certain ready-to-eat (RTE) foods in the EU, 2010-2011 Part A: *Listeria monocytogenes* prevalence estimates. EFSA Journal 2013;11(6):3241, 75 pp. doi:10.2903/j.efsa.2013.3241.

EFSA NDA Panel (EFSA Panel on Dietetic Products, Nutrition and Allergies), 2014. Scientific Opinion on health benefits of seafood (fish and shellfish) consumption in relation to health risks associated with exposure to methylmercury. EFSA Journal 2014;12(7):3761, 80 pp. doi:10.2903/j.efsa.2014.3761

EFSA, 2014a. Scientific Opinion on the analysis of the baseline survey on the prevalence of *Listeria monocytogenes* in certain ready-to-eat foods in the EU, 2010-2011 Part B: analysis of factors related to prevalence and exploring compliance. EFSA Journal 2014;12(8):3810 [73 pp.]. doi:10.2903/j.efsa.2014.3810

EFSA, NDA Panel (EFSA Panel on Dietetic Products, Nutrition and Allergies), 2015. Statement on the benefits of fish/seafood consumption compared to the risks of methylmercury in fish/seafood. EFSA Journal 2015;13(1):3982

Eklund, M.W., Peterson, M.E., Poysky, F.T., Paranjpye, R.N., Pelroy, G.A., 2004. Control of bacterial pathogens during processing of cold-smoked and dried salmon strips. Journal of Food Protection 67, 347–351.

El-Shenawy, M. A., 1998. Sources of *Listeria* spp. in domestic food processing environment. International Journal of Environmental Health Research. 8:241-251.

Elvevoll, E. O., Osterud, B., 2003. Impact of processing on nutritional quality of marine food items. Forum of Nutrition 56:337-40.

European Commission, 2003. Opinion on Staphylococcal Enterotoxins in milk products, particularly cheeses (adopted on 26-27 March 2003).http://ec.europa.eu/food/fs/sc/scv/out61_en.pdf

European Commission, 2014. A pocket guide to the EU's new fish and aquaculture consumer labels. Luxembourg: Publications Office of the European Union. 2014 - 16 pp. - 20 x 20 cm. ISBN 978-92-79-43893-6. doi: 10.2771/86800

European Commission Recommendation No. 1558 of the European Commission of 12 March 2015 on a coordinated control plan with a view to establishing the prevalence of fraudulent practices in the marketing of certain foods. P 0001 – 0017.

European Commission Regulation (EC) No. 178/2002 of the European Parliament and of the Council of 28 January 2002 laying down the general principles and requirements of food law,

establishing the European Food Safety Authority and laying down procedures in matters of food safety**.** Official Journal of the European Union L 031 , 01 February 2002 P. 0001 – 0024.

European Commission Regulation (EC) No. 852/2004 of the European Parliament and of the Council of 29 April 2004 on the hygiene of foodstuffs. Official Journal of the European Union L 139, 30 April 2004**,** pp. 1-54.

European Commission Regulation (EC) No. 853/2004 of the European Parliament and of the Council of 29 April 2004 laying down specific hygiene rules for food of animal origin. Official Journal of the European Union L 139, 30 April 2004**,** pp. 55-205.

European Commission Regulation (EC) No. 854/2004 of the European Parliament and of the Council of 29 April 2004 laying down specific rules for the organisation of official controls on products of animal origin intended for human consumption. Official Journal of the European Union L 139, 30 April 2004**,** pp. 206-320.

European Commission Regulation (EC) No. 2073/2005 of the European Parliament and of the Council of 15 November 2005 on microbiological criteria for foodstuffs. Official Journal of the European Union L 338, 22 December 2005, pp. 1-26.

European Commission Regulation (EC) No. 2074/2005 of 5 December 2005 laying down implementing measures for certain products under Regulation (EC) No. 853/2004 of the European Parliament and of the Council and for the organisation of official controls under Regulation (EC) No. 854/2004 of the European Parliament and of the Council and Regulation (EC) No. 882/2004 of the European Parliament and of the Council, derogating from Regulation (EC) No. 852/2004 of the European Parliament and of the Council and amending Regulations (EC) No. 853/2004 and (EC) No. 854/2004. Official Journal of the European Union L 338, 22 December 2005**,** pp. 27-59.

European Commission Regulation (EC) No. 1021/2008 of the European Parliament and of the Council of 17 October 2008 amending Annexes I, II and III to Regulation (EC) No. 854/2004 laying down specific rules for the organisation of official controls on products of animal origin intended for human consumption and Regulation (EC) No. 2076/2005 as regards live bivalve molluscs, certain fishery products and staff assisting with official controls in slaughterhouses. Official Journal of the European Union L 277, 18 October 2008, pp. 15-17.

European Council Regulation (EC) No. 1224/2009 of 20 November 2009 establishing a Community control system for ensuring compliance with the rules of the common fisheries policy, amending

Regulations (EC) No. 847/96, (EC) No. 2371/2002, (EC) No. 811/2004, (EC) No. 768/2005, (EC) No. 2115/2005, (EC) No. 2166/2005, (EC) No. 388/2006, (EC) No. 509/2007, (EC) No. 676/2007, (EC) No. 1098/2007, (EC) No. 1300/2008, (EC) No. 1342/2008 and repealing Regulations (EEC) No. 2847/93, (EC) No. 1627/94 and (EC) No. 1966/2006. Official Journal of the European Union L 343, 22 December 2009**,** pp. 0001-0113.

European Commission Implementing Regulation (EU) No 404/2011 of 8 April 2011 laying down detailed rules for the implementation of Council Regulation (EC) No 1224/2009 establishing a Community control system for ensuring compliance with the rules of the Common Fisheries Policy. Official Journal of the European Union L 112, 30 April 2011 P. 0001 – 0153.

European Commission Regulation (EU) No. 1129/2011 of 11 November 2011 amending Annex II to Regulation (EC) No. 1333/2008 of the European Parliament and of the Council by establishing a Union list of food additives. Official Journal of the European Union L 295, 12 November 2011 P. 0001 – 0177.

European Regulation (EU) No. 1169/2011 of the European Parliament and of the Council of 25 October 2011 on the provision of food information to consumers, amending Regulations (EC) No. 1924/2006 and (EC) No. 1925/2006 of the European Parliament and of the Council, and repealing Commission Directive 87/250/EEC, Council Directive 90/496/EEC, Commission Directive 1999/10/EC, Directive 2000/13/EC of the European Parliament and of the Council, Commission Directives 2002/67/EC and 2008/5/EC and Commission Regulation (EC) No. 608/2004. Official Journal of the European Union L 304, 22 November 2011**,** pp. 0001-0088.

European Regulation (EU) No. 1379/2013 of the European Parliament and of the Council of 11 December 2013 on the common organisation of the markets in fishery and aquaculture products, amending Council Regulations (EC) No. 1184/2006 and (EC) No. 1224/2009 and repealing Council Regulation (EC) No. 104/2000. Official Journal of the European Union L 354, 28 December 2013 P. 0001 – 0325.

European Commission Implementing Regulation (EU) No. 1420/2013 of 17 December 2013 repealing Regulations (EC) No. 347/96, (EC) No. 1924/2000, (EC) No. 1925/2000, (EC) No. 2508/2000, (EC) No. 2509/2000, (EC) No. 2813/2000, (EC) No. 2814/2000, (EC) No. 150/2001, (EC) No. 939/2001, (EC) No. 1813/2001, (EC) No. 2065/2001, (EC) No. 2183/2001, (EC) No. 2318/2001, (EC) No. 2493/2001, (EC) No. 2306/2002, (EC) No. 802/2006, (EC) No. 2003/2006, (EC) No. 696/2008 and (EC) No. 248/2009 following the adoption of Regulation

(EU) No. 1379/2013 of the European Parliament and of the Council on the common organisation of the markets in fishery and aquaculture products. Official Journal of the European Union L 353, 28 December 2013**,** pp. 0001-0082.

Evenson, M. L., Hinds, M. W., Bernstein, R. S., Bergdoll, M. S., 1988. Estimation of human dose of staphylococcal enterotoxin A from a large outbreak of staphylococcal food poisoning involving chocolate milk. International Journal of Food Microbiology, 7(4), 311-316.

FAO, 2014. FAO yearbook. The State of World Fisheries and Aquaculture. FAO, Rome.

Farber, J. M., Peterkin, P. I., 1991. *Listeria monocytogenes*, a food-borne pathogen. Microbiological Reviews, 55(3), 476-511.

Feldhusen, F., 2000. The role of seafood in bacterial foodborne diseases. Microbes and Infection. 2(13), 1651-1660.

Fetsch, A., Contzen, M., Hartelt, K., Kleiser, A., Maassen, S., Rau, J., Kraushaar, B., Layer, F., Strommenger, B., 2014. *Staphylococcus aureus* food-poisoning outbreak associated with the consumption of ice-cream. International Journal of food Microbiology, 187, 1-6.

Fu, K., Su, B., He, D., Lu, X., Song, J., Huang, J., 2012. Pollution assessment of heavy metals along the Mekong River and dam effects. Journal of Geographical Sciences, 22(5), 874-884.

Genigeorgis, C.A., 1989. Present state of knowledge on staphylococcal intoxication. International Journal of Food Microbiology, 9: 327-360.

Giovannacci, I., Ragimbeau, C., Queguiner, S., Salvat, G., Vendeuvre, J. L., Carlier, V., Ermel, G., 1999. *Listeria monocytogenes* in pork slaughtering and cutting plants: use of RAPD, PFGE and PCR–REA for tracing and molecular epidemiology. International Journal of Food Microbiology, 53(2), 127-140.

GISSI-Prevenzione Investigators., 1999. Dietary supplementation with n-3 polyunsaturated fatty acids and vitamin E after myocardial infarction: results of the GISSI-Prevenzione trial. The Lancet. 354(9177), 447-455.

Goulet, V., Hebert, M., Hedberg, C., Laurent, E., Vaillant, V., De Valk, H., Desenclos, J. C., 2012. Incidence of listeriosis and related mortality among groups at risk of acquiring listeriosis. Clinical Infectious Diseases, 54: 652-660.

Graham, A., But, P. P. H., 2008. Rapid detection of oilfish and escolar in fish steaks: a tool to prevent keriorrhea episodes. Food chemistry, 110(2), 538-546.

Grigoryan, K., Badalyan, G., Andriasyan, D., 2010. Prevalence of *Staphylococcus aureus* in fish processing factory. Potravinarstvo 4, 25–28.

Gründling, A., Burrack, L. S., Bouwer, H. A., Higgins, D. E., 2004. *Listeria monocytogenes* regulates flagellar motility gene expression through MogR, a transcriptional repressor required for virulence. Proceedings of the National Academy of Sciences of the United States of America, 101(33), 12318-12323.

Guallar, E., Sanz-Gallardo, M. I., Veer, P. V. T., Bode, P., Aro, A., Gómez-Aracena, J., Kark J. D., Riemersma, R. A., Martin-Moreno, J. M., Kok, F. J., 2002. Mercury, fish oils, and the risk of myocardial infarction. New England Journal of Medicine, 347(22), 1747-1754.

Guimarães, C. F. M., Máralém, E. T., Monteiro, M. L. G., Lemos, M., Mano, S. B., Conte Junior, C. A., 2015. The chemical quality of frozen Vietnamese *Pangasius hypophthalmus* fillets. Food Science & Nutrition.

Gutiérrez, D., Delgado, S., Vázquez-Sánchez, D., Martínez, B., Cabo, M. L., Rodríguez, A., Herrera J. J., García, P., 2012. Incidence of *Staphylococcus aureus* and analysis of associated bacterial communities on food industry surfaces. Applied and Environmental Microbiology, 78(24), 8547-8554.

Haldimann, M., Alt, A., Blanc, A., Blondeau, K., 2005. Iodine content of food groups. Journal of Food Composition and Analysis, 18(6), 461-471.

Hammad, A. M., Watanabe, W., Fujii, T., Shimamoto, T., 2012. Occurrence and characteristics of methicillin-resistant and-susceptible *Staphylococcus aureus* and methicillin-resistant coagulase-negative staphylococci from Japanese retail ready-to-eat raw fish. International Journal of Food Microbiology, 156(3), 286-289.

Hassanien, F. S., Ahmed, A. A., Helmy, N. A. E. H., 2014. Food poisoning microorganisms in fried seafood. Benha Veterinary Medical Journal. vol. 27, n. 2:116-125.

Henderson, R. J., Tocher, D. R.,1987. The lipid composition and biochemistry of freshwater fish. Progress in Lipid Research, 26(4), 281-347.

Hennekinne, J. A., De Buyser, M. L., Dragacci, S., 2012. *Staphylococcus aureus* and its food poisoning toxins: characterization and outbreak investigation. FEMS Microbiology Reviews, 36(4), 815-836.

Herrero, B., Madriñán, M., Vieites, J. M., Espiñeira, M., 2010. Authentication of Atlantic cod (*Gadus morhua*) using real time PCR. Journal of Agricultural and Food Chemistry, 58(8), 4794-4799.

Hsieh, H. S., Chai, T. J., Hwang, D. F., 2007. Using the PCR–RFLP method to identify the species of different processed products of billfish meats. Food Control, 18(4), 369-374.

Hubalkova, Z., Kralik, P., Tremlova, B., Rencova, E., 2007. Methods of gadoid fish species identification in food and their economic impact in the Czech Republic: a review. Veterinarni Medicina Praha, *52*(7), 273.

Huss, H.H., Ababouch, L., Gram, L., 2003. Assessment and management of seafood safety and quality. In: Huss, H.H., Ababouch, L., Gram, L. (Eds.), FAO Fisheries Technical Paper, 444. Food Agriculture Organization of the United Nations, Rome, Italy.

Iida, T., Kanzaki, M., Maruyama, T., Inoue, S., Kaneuchi., C., 1991. Prevalence of *Listeria monocytogenes* in intestinal contents of healthy animals in Japan. The Journal of Veterinary Medical Science. 53:873-875.

ISO 11290-2:1998 : Microbiology of food and animal feeding stuffs -- Horizontal method for the detection and enumeration of *Listeria monocytogenes* -- Part 2: Enumeration method.

ISO 11290-2:2004 / Amendment 1:1004: Modification of the enumeration medium. Microbiology of food and animal feeding stuffs -- Horizontal method for the detection and enumeration of *Listeria monocytogenes* -- Part 2: Enumeration method.

ISO 6888-2:1999 : Microbiology of food and animal feeding stuffs -- Horizontal method for the enumeration of coagulase-positive staphylococci (*Staphylococcus aureus* and other species) -- Part 2: Technique using rabbit plasma fibrinogen agar medium.

ISO 6888-2:1999 / Amendment 1:2003: Inclusion of precision data. Microbiology of food and animal feeding stuffs -- Horizontal method for the enumeration of coagulase-positive staphylococci (*Staphylococcus aureus* and other species) -- Part 2: Technique using rabbit plasma fibrinogen agar medium.

Jacquet, J. L., Pauly, D., 2007. The rise of seafood awareness campaigns in an era of collapsing fisheries. Marine Policy, 31(3), 308-313.

Jarraud, S., Mougel, C., Thioulouse, J., Lina, G., Meugnier, H., Forey, F., Nesme, X., Etienne, J., Vandenesch, F., 2002. Relationships between *Staphylococcus aureus* genetic background, virulence factors, *agr* groups (alleles), and human disease. Infection and Immunity, 70(2), 631-641.

Jeffrey, B. G., Weisinger, H. S., Neuringer, M., Mitchell, D. C., 2001. The role of docosahexaenoic acid in retinal function. Lipids 36, 859–871.

Jérôme, M., Lemaire, C., Verrez-Bagnis, V., Etienne, M., 2003. Direct sequencing method for species identification of canned sardine and sardine-type products. Journal of Agricultural and Food Chemistry, 51(25), 7326-7332.

Junttila, J. R., Niemelä, S. I., Hirn, J., 1988. Minimum growth temperatures of *Listeria monocytogenes* and non-haemolytic *Listeria*. Journal of Applied Bacteriology, 65(4), 321-327.

Kadariya, J., Smith, T. C., Thapaliya, D., 2014. *Staphylococcus aureus* and staphylococcal food-borne disease: an ongoing challenge in public health. BioMed research international, vol. 2014, Article ID 827965, 9 pages. http://dx.doi.org/10.1155/2014/827965

Karl, H., Lehmann, I., Rehbein, H., Schubring, R., 2010. Composition and quality attributes of conventionally and organically farmed *Pangasius* fillets (*Pangasius hypophthalmus*) on the German market. International journal of Food Science & Technology, 45(1), 56-66.

Kerouanton, A., Hennekinne, J. A., Letertre, C., Petit, L., Chesneau, O., Brisabois, A., De Buyser M. L., 2007. Characterization of *Staphylococcus aureus* strains associated with food poisoning outbreaks in France. International Journal of Food Microbiology, 115: 369–375.

Keto-Timonen, R., Tolvanen, R., Lunden, J., Korkeala, H., 2007. An 8-year surveillance of the diversity and persistence of *Listeria monocytogenes* in a chilled food processing plant analyzed by amplified fragment length polymorphism. Journal of Food Protection, 70(8), 1866-1873.

Klinbunga, S., Khamnamtong, N., Tassanakajon, A., Puanglarp, N., Jarayabhand, P., Yoosukh, W., 2003. Molecular genetic identification tools for three commercially cultured oysters (*Crassostrea belcheri, Crassostrea iredalei,* and *Saccostrea cucullata*) in Thailand. Marine Biotechnology, 5(1), 27-36.

Kluytmans, J., Van Belkum, A., Verbrugh, H., 1997. Nasal carriage of *Staphylococcus aureus*: epidemiology, underlying mechanisms, and associated risks. Clinical Microbiology Reviews, 10(3), 505-520.

Kluytmans, J. A. J. W., Wertheim, H. F. L., 2005. Nasal carriage of *Staphylococcus aureus* and prevention of nosocomial infections. Infection, 33(1), 3-8.

Kvasnička, F., 2005. Capillary electrophoresis in food authenticity. Journal of Separation Science, 28:813–825.

Lado, B. H., Yousef, A. E., 2007. Characteristics of *Listeria monocytogenes* important to food processors. Food Science and Technology- New York- Marcel Dekker-, 161, 157.

Lakra, W. S., Goswami, M., Gopalakrishnan, A., 2009. Molecular identification and phylogenetic relationships of seven Indian Sciaenids (Pisces: *Perciformes, Sciaenidae*) based on 16S rRNA and cytochrome *c* oxidase subunit I mitochondrial genes. Molecular Biology Reports, 36(5), 831-839.

Latorre, L., Parisi, A., Fraccalvieri, R., Normanno, G., La Porta, M. C., Goffredo, E., Palazzo, L., Ciccarese, G., Addante, N., Santagada, G., 2007. Low prevalence of *Listeria monocytogenes* in foods from Italy. Journal of Food Protection, 70(6), 1507-1512.

Lawrence, D. T., Dobmeier, S. G., Bechtel, L. K., Holstege, C. P., 2007. Food poisoning. Emergency Medicine Clinics of North America, 25(2), 357-373.

Le Loir, Y., Baron, F., Gautier, M., 2003. *Staphylococcus aureus* and food poisoning. Genetics and Molecular Research, 2(1), 63-76.

Leask, A., Yankos, P., Ferson, M. J., 2004. Fish, so foul! Foodborne illness caused by combined fish histamine and wax ester poisoning. Quarterly Report, 83, vol. 28 (1) 82 – 85.

Lenstra, J. A., Lees, M., 2003. DNA methods for identifying plant and animal species in food. Food Authenticity and Traceability, 34-53.

Lianou, A., Sofos, J. N., 2007. A review of the incidence and transmission of *Listeria monocytogenes* in ready-to-eat products in retail and food service environments. Journal of Food Protection, 70(9), 2172-2198.

Lin, W. F., Hwang, D. F., 2008. Application of species-specific PCR for the identification of dried bonito product (*Katsuobushi*). Food Chemistry, 106(1), 390-396.

Lin, Y. S., Poh, Y. P., Lin, S. M., Tzeng, C. S., 2002. Molecular techniques to identify freshwater eels: RFLP analyses of PCR-amplified DNA fragments and allele-specific PCR from mitochondrial DNA. Zoological Studies-Taipei-, 41(4), 421-430.

Ling, K. H., Cheung, C. W., Cheng, S. W., Cheng, L., Li, S. L., Nichols, P. D., Ward, R. D., Graham, A., But, P. P. H., 2008. Rapid detection of oilfish and escolar in fish steaks: a tool to prevent keriorrhea episodes. Food Chemistry, 110(2), 538-546.

Lockley, A. K., Bardsley, R. G., 2000. DNA-based methods for food authentication. Trends in Food Science & Technology, 11(2), 67-77.

Loncarevic, S., Tham, W., Danielsson-Tham, M. L., 1995. Prevalence of *Listeria monocytogenes* and other *Listeria* spp. in smoked and'gravad'fish. Acta Veterinaria Scandinavica, 37(1), 13-18.

Løvseth, A., Loncarevic, S., & Berdal, K. G., 2004. Modified multiplex PCR method for detection of pyrogenic exotoxin genes in staphylococcal isolates. Journal of Clinical Microbiology, 42(8), 3869-3872.

Lundén, J., Autio, T., Markkula, A., Hellström, S., Korkeala, H., 2003. Adaptive and cross-adaptive responses of persistent and non-persistent *Listeria monocytogenes* strains to disinfectants. International Journal of Food Microbiology, 82(3), 265-272.

McClure, P. J., Roberts, T. A., Oguru, P. O., 1989. Comparison of the effects of sodium chloride, pH and temperature on the growth of *Listeria monocytogenes* on gradient plates and in liquid medium. Letters in Applied Microbiology, 9(3), 95-99.

McLauchlin, J., Rees, C. E. D., 2009. Genus I. *Listeria*, p. 244-257. In Bergey's Manual of Systematic Bacteriology, second ed., vol. 3. Springer, New York, NY.

Mead, P. S., Slutsker, L., Dietz, V., McCaig, L. F., Bresee, J. S., Shapiro, C., Griffin, P. M., Tauxe, R. V., 1999. Food-related illness and death in the United States. Emerging Infectious Diseases, 5: 607-625.

Michelini, E., Cevenini, L., Mezzanotte, L., Simoni, P., Baraldini, M., De Laude, L., Roda, A., 2007. One-step triplex-polymerase chain reaction assay for the authentication of yellowfin (*Thunnus albacares*), bigeye (*Thunnus obesus*), and skipjack (*Katsuwonus pelamis*) tuna DNA from fresh, frozen, and canned tuna samples. Journal of Agricultural and Food Chemistry, 55(19), 7638-7647.

Miettinen, M. K., Siitonen, A., Heiskanen, P., Haajanen, H., Björkroth, K. J., Korkeala, H. J., 1999. Molecular epidemiology of an outbreak of febrile gastroenteritis caused by *Listeria monocytogenes* in cold-smoked rainbow trout. Journal of Clinical Microbiology, 37(7), 2358-2360.

Mitchell, D..C., Niu, S. L., Litman, B. J., 2003. Enhancement of G protein-coupled signaling by DHA phospholipids. Lipids 38, 437–443.

Miya, S., Takahashi, H., Ishikawa, T., Fujii, T., Kimura, B., 2010. Risk of *Listeria monocytogenes* contamination of raw ready-to-eat seafood products available at retail outlets in Japan. Applied and Environmental Microbiology, 76(10), 3383-3386.

Monday, S. R., & Bohach, G. A., 1999. Use of multiplex PCR to detect classical and newly described pyrogenic toxin genes in staphylococcal isolates. Journal of Clinical Microbiology, 37(10), 3411-3414.

Monecke, S., Coombs, G., Shore, A. C., Coleman, D. C., Akpaka, P., Borg, M., Chow, H., Ip, M., Jatzwauk, L., Jonas, D., Kadlec, K., Kearns, A., Laurent, F., O'Brein, F. G., Pearson, J., Ruppelt, A., Schwarz, S., Scicluna, E., Slickers, P., Tan, H. L., Weber, S., Ehricht, R., 2011. A field guide to pandemic, epidemic and sporadic clones of methicillin-resistant *Staphylococcus aureus*. PloS one, 6(4), e17936.

Moore, J. C., Spink, J., Lipp, M., 2012. Development and application of a database of food ingredient fraud and economically motivated adulteration from 1980 to 2010. Journal of Food Science, 77(4), R118-R126.

Moran, P., Garcia-Vazquez, E., 2006. Identification of highly prized commercial fish using a PCR-based methodology. Biochemistry and Molecular Biology Education, 34(2), 121-124.

Murakami, K., Minamide, W., Wada, K., Nakamura, E., Teraoka, H., Watanabe, S., 1991. Identification of methicillin-resistant strains of staphylococci by polymerase chain reaction. Journal of Clinical Microbiology, 29(10), 2240-2244.

Nakamura, H., Hatanaka, M., Ochi, K., Nagao, M., Ogasawara, J., Hase, A., Kitase, T., Haruki, K., Nishikawa, Y., 2004. *Listeria monocytogenes* isolated from cold-smoked fish products in Osaka City, Japan. International Journal of Food Microbiology, 94(3), 323-328.

Noguch, T., & Arakawa, O., 2008. Tetrodotoxin–distribution and accumulation in aquatic organisms, and cases of human intoxication. Marine Drugs. 6(2), 220-242.

Nolan, D. A., Chamblin, D. C., Troller, J. A., 1992. Minimal water activity levels for growth and survival of *Listeria monocytogenes* and *Listeria innocua*. International Journal of Food Microbiology, 16(4), 323-335.

Normanno G., Firinu, A., Virgilio S., Mula G., Dambrosio A., Poggiu A., Decastelli L., Mioni R., Scuota S., Bolzoni G., Di Giannatale E., Salinetti A. P., La Salandra G., Bartoli M., Zuccon F., Pirino T., Sias S., Parisi A., Quaglia N.C., Celano G.V., 2005. Coagulase-positive Staphylococci and *Staphylococcus aureus* in food products marketed in Italy. International Journal of Food Microbiology, 98(1), 73-79.

Normanno, G., Dambrosio, A., Quaglia, N. C., Lorusso, V., Merico, A., Germinario, G. L., Abenante, R. P., Narcisi, D., Firinu, A., Celano, G. V., 2006. Detection of staphylococcal enterotoxin a from ricotta cheese by cell culture. Journal of Food Quality 29, 374–382.

Normanno, G., Corrente, M., La Salandra, G., Dambrosio, A., Quaglia, N. C., Parisi, A., Greco, G., Bellacicco, A.L., Virgilio, S., Celano, G. V., 2007. Methicillin-resistant *Staphylococcus aureus* (MRSA) in foods of animal origin product in Italy. International Journal of Food Microbiology, 117(2), 219-222.

Normanno, G., Dambrosio, A., Lorusso, V., Samoilis, G., Di Taranto, P., Parisi, A., 2015. Methicillin-resistant *Staphylococcus aureus* (MRSA) in slaughtered pigs and abattoir workers in Italy. Food Microbiology, 51, 51-56.

Olsen, S. F., 2002. Low consumption of seafood in early pregnancy as a risk factor for preterm delivery: prospective cohort study. BMJ. 324(7335), 447.

Onmaz, N. E., Abay, S., Karadal, F., Hizlisoy, H., Telli, N., Al, S., 2015. Occurrence and antimicrobial resistance of *Staphylococcus aureus* and *Salmonella* spp. in retail fish samples in Turkey. Marine Pollution Bulletin, 90(1), 242-246.

Orsi, R. H., Borowsky, M. L., Lauer, P., Young, S. K., Nusbaum, C., Galagan, J. E., Birren, B. W., Ivy, R. A., Sun, Q., Graves, L. M., Swaminathan, B., Wiedmann, M., 2008. Short-term genome evolution of *Listeria monocytogenes* in a non-controlled environment. BMC Genomics. 9:539.

Ortega, E., Abriouel, H., Lucas, R., Gálvez, A., 2010. Multiple roles of *Staphylococcus aureus* enterotoxins: pathogenicity, superantigenic activity, and correlation to antibiotic resistance. Toxins, 2(8), 2117-2131.

Paine, M. A., McDowell, J. R., Graves, J. E., 2007. Specific identification of western Atlantic Ocean scombrids using mitochondrial DNA cytochrome *c* oxidase subunit I (COI) gene region sequences. Bulletin of Marine Science, 80(2), 353-367.

Paine, M. A., McDowell, J. R., Graves, J. E., 2008. Specific identification using COI sequence analysis of scombrid larvae collected off the Kona coast of Hawaii Island. Ichthyological Research, 55(1), 7-16.

Parfentjev, I. A., Catelli, A. R., 1964. Tolerance of *Staphylococcus aureus* to sodium chloride. Journal of Bacteriology, 88(1), 1-3.

Parisi, A., Latorre, L., Normanno, G., Miccolupo, A., Fraccalvieri, R., Lorusso, V., Santagada, G., 2010. Amplified fragment length polymorphism and multi-locus sequence typing for high-resolution genotyping of *Listeria monocytogenes* from foods and the environment. Food Microbiology, 27(1), 101-108.

Parisi, A., Latorre, L., Fraccalvieri, R., Miccolupo, A., Normanno, G., Caruso, M., Santagada, G., 2013. Occurrence of *Listeria* spp. in dairy plants in Southern Italy and molecular subtyping of isolates using AFLP. Food Control, 29(1), 91-97.

Pava-Ripoll, M., Pearson, R. E. G., Miller, A. K., Ziobro, G. C., 2012. Prevalence and relative risk of *Cronobacter* spp., *Salmonella* spp., and *Listeria monocytogenes* associated with the body surfaces and guts of individual filth flies. Applied and Environmental Microbiology, 78(22), 7891-7902.

Pepe, T., Trotta, M., Di Marco, I., Cennamo, P., Anastasio, A., Cortesi, M. L., 2005. Mitochondrial cytochrome *b* DNA sequence variations: an approach to fish species identification in processed fish products. Journal of Food Protection, 68(2), 421-425.

Phan, L. T., Bui, T. M., Nguyen, T. T., Gooley, G. J., Ingram, B. A., Nguyen, H. V., Nguyen, P. T., De Silva, S. S., 2009. Current status of farming practices of striped catfish, *Pangasianodon hypophthalmus* in the Mekong Delta, Vietnam. Aquaculture, 296(3), 227-236.

Puah, S. M., Chua, K. H., Tan, J. A. M. A., 2016. Virulence Factors and Antibiotic Susceptibility of *Staphylococcus aureus* Isolates in Ready-to-Eat Foods: Detection of *S. aureus* Contamination and a High Prevalence of Virulence Genes. International Journal of Environmental Research and Public health, 13(2), 199.

Rasmussen, R. S., Morrissey, M. T., 2008. DNA-based methods for the identification of commercial fish and seafood species. Comprehensive Reviews in Food Science and Food Safety, 7(3), 280-295.

Rego, I., Martínez, A., González-Tizón, A., Vieites, J., Leira, F., Méndez, J., 2002. PCR technique for identification of mussel species. Journal of Agricultural and Food Chemistry, 50(7), 1780-1784.

Rehbein, H., 1990. Electrophoretic techniques for species identification of fishery products. Zeitschrift für Lebensmittel-Untersuchung und Forschung, 191(1), 1-10.

Robbins, J. B., Fisher, C. W., Moltz, A. G., Martin, S. E., 2005. Elimination of *Listeria monocytogenes* biofilms by ozone, chlorine, and hydrogen peroxide. Journal of Food Protection, 68(3), 494-498.

Rocourt, J., BenEmbarek, P., Toyofuku, H., Schlundt, J., 2003. Quantitative risk assessment of *Listeria monocytogenes* in ready-to-eat foods: the FAO/WHO approach. FEMS Immunology & Medical Microbiology, 35(3), 263-267.

Rodma, P., Satjapala, T., Suwanvitaya, P., 1991. Study of *Staphylococcus aureus* in frozen food product. Food (Thailand). 21 (3), 197-204.

Rola, J. G., Sosnowski, M., Ostrowska, M., Osek, J., 2015. Prevalence and antimicrobial resistance of coagulase-positive staphylococci isolated from raw goat milk. Small Ruminant Research, 123(1), 124-128.

Rørvik, L. M., Caugant, D. A., Yndestad, M., 1995. Contamination pattern of *Listeria monocytogenes* and other *Listeria* spp. in a salmon slaughterhouse and smoked salmon processing plant. International Journal of Food Microbiology, 25(1), 19-27.

Rosec, J. P., Gigaud, O., 2002. Staphylococcal enterotoxin genes of classical and new types detected by PCR in France. International Journal of Food Microbiology, 77(1), 61-70.

Ruiz-Gutierrez, V., Perez-Zarza, M. C., Muriana, F. J. G., Bravo, L., 1997. Lipid and fatty acid composition of muscle and internal organs from *Ruvettus pretiosus*. Journal of Fish Biology, 50(6), 1353-1357.

Saito, E., Yoshida, N., Kawano, J., Shimizu, A., Igimi, S., 2011. Isolation of *Staphylococcus aureus* from raw fish in relation to culture methods. Journal of Veterinary Medical Science. 73, 287–292.

Sanjeev, S., Mahadeva Iyer, K., Rao, C. C. P., James, M. A., 1986. Occurrence of enterotoxigenic Staphylococci in frozen fishery products. Fishery Technology, *23*(2), 164-166.

Sanjuan, A., Comesaña, A. S., 2002. Molecular identification of nine commercial flatfish species by polymerase chain reaction-restriction fragment length polymorphism analysis of a segment of the cytochrome b region. Journal of Food Protection, 65(6), 1016-1023.

Santaclara, F. J., Cabado, A. G., Vieites, J. M., 2006. Development of a method for genetic identification of four species of anchovies: *E. encrasicolus, E. anchoita, E. ringens* and *E. japonicus*. European Food Research and Technology, 223(5), 609-614.

Sauders, B. D., Pettit, D., Currie, B., Suits, P., Evans, A., Stellrecht, K., Dryja, D. M., Slate, D., Wiedmann M., 2005. Low prevalence of *Listeria monocytogenes* in human stool. Journal of Food Protection, 68:178-181.

Schelin, J., Wallin-Carlquist, N., Thorup Cohn, M., Lindqvist, R., Barker, G. C., 2011. The formation of *Staphylococcus aureus* enterotoxin in food environments and advances in risk assessment. Virulence, 2(6), 580-592.

Schoder, D., Melzner, D., Schmalwieser, A., Zangana, A., Winter, P., Wagner, M., 2011. Important vectors for *Listeria monocytogenes* transmission at farm dairies manufacturing fresh sheep and goat cheese from raw milk. Journal of Food Protection, 74(6), 919-924.

Scott, W. J., 1953. Water relations of *Staphylococcus aureus* at 30 C. Australian Journal of Biological Sciences, 6(4), 549-564.

Seeliger, H. P. R., Höhne, K, 1979. Serotyping of *Listeria monocytogenes* and related species. Methods in Microbiology. 13, 31–49.

Semeraro A.M., 2011. Frodi alimentari: aspetti tecnici e giuridici. Rassegna di Diritto, Legislazione e Medicina Legale Veterinaria, 10(2).

Shaikevich, E. V., Zakharov, I. A., 1993. Polymorphism of mitochondrial COI and nuclear ribosomal ITS2 in the *Culex pipiens* complex and in *Culex torrentium* (Diptera: *Culicidae*). ISSN 1993-0771, ISSN 1993-778X.

Simon, S. S., Sanjeev, S., 2007. Prevalence of enterotoxigenic *Staphylococcus aureus* in fishery products and fish processing factory workers. Food Control, 18(12), 1565-1568.

Singh, A., Ward, O. P., 1997. Microbial production of docosahexaenoic acid. Advances in Applied Microbiology, 45, 271-312.

Sokari, T., 1991. Distribution of enterotoxigenic *Staphylococcus aureus* in ready-to-eat foods in eastern Nigeria. International Journal of Food Microbiology, 12(2), 275-279.

Spies, I. B., Gaichas, S., Stevenson, D. E., Orr, J. W., Canino, M. F., 2006. DNA-based identification of Alaska skates (*Amblyraja, Bathyraja* and *Raja*: *Rajidae*) using cytochrome *c* oxidase subunit I (COI) variation. Journal of Fish Biology, 69(sb), 283-292.

Storelli, M. M., Normanno, G., Barone, G., Dambrosio, A., Errico, L., Garofalo, R., Giacominelli-Stuffler, R., 2012. Toxic metals (Hg, Cd, and Pb) in fishery products imported into Italy: suitability for human consumption. Journal of Food Protection, 75(1), 189-194.

Strauss, R. E., Bond, C. E., 1990. Taxonomic methods: morphology. Methods for fish biology. American Fisheries Society, Bethesda, Maryland, 109-140.

Teletchea, F., 2009. Molecular identification methods of fish species: reassessment and possible applications. Reviews in Fish Biology and Fisheries, 19(3), 265-293.

Tobe, S. S., Kitchener, A. C., Linacre, A. M., 2010. Reconstructing mammalian phylogenies: a detailed comparison of the cytochrome *b* and cytochrome oxidase subunit I mitochondrial genes. PloS one, 5(11), e14156.

Tranter, H. S., 1990. Foodborne staphylococcal illness. The Lancet, 336(8722), 1044-1046.

Trotta, M., Schönhuth, S., Pepe, T., Cortesi, M. L., Puyet, A., Bautista, J. M., 2005. Multiplex PCR method for use in real-time PCR for identification of fish fillets from grouper (*Epinephelus* and *Mycteroperca* species) and common substitute species. Journal of Agricultural and Food Chemistry, 53(6), 2039-2045.

Ulrich, R. M., John, D. E., Barton, G. W., Hendrick, G. S., Fries, D. P., Paul, J. H., 2013. Ensuring seafood identity: Grouper identification by real-time nucleic acid sequence-based amplification (RT-NASBA). Food Control, 31(2), 337-344.

Uyttendaele, M., Busschaert, P., Valero, A., Geeraerd, A. H., Vermeulen, A., Jacxsens, L., Goh, K. K., De Loy, A., Van Impe, J. F., Devlieghere, F., 2009. Prevalence and challenge tests of *Listeria monocytogenes* in Belgian produced and retailed mayonnaise-based deli-salads, cooked meat

products and smoked fish between 2005 and 2007. International Journal of Food Microbiology, 133(1), 94-104.

Valnot, I., von Kleist-Retzow, J. C., Barrientos, A., Gorbatyuk, M., Taanman, J. W., Mehaye, B., Rustin, P., Tzagoloffl, A., Munnich, A., Rötig, A., 2000. A mutation in the human heme A: farnesyltransferase gene (COX10) causes cytochrome *c* oxidase deficiency. Human Molecular Genetics, 9(8), 1245-1249.

Van Cleef, B. A. G. L., van Benthem, B. H. B., Verkade, E. J. M., Van Rijen, M., Kluytmans-van den Bergh, M. F. Q., Schouls, L. M., Duim, B., Wagenaar, J.A., Graveland, H., Bos, M. E. H., Heederik, D., Kluytmans, J. A. J. W., Heederik, D., 2014. Dynamics of methicillin-resistant *Staphylococcus aureus* and methicillin-susceptible *Staphylococcus aureus* carriage in pig farmers: a prospective cohort study. Clinical Microbiology and Infection, 20(10), O764-O771.

Van Leeuwen, S. P. J., Van Velzen, M. J. M., Swart, C. P., Van der Veen, I., Traag, W. A., De Boer, J., 2009. Halogenated contaminants in farmed salmon, trout, tilapia, pangasius, and shrimp. Environmental Science & Technology, 43(11), 4009-4015.

Vázquez-Sánchez, D., López-Cabo, M., Saá-Ibusquiza, P., Rodríguez-Herrera, J. J., 2012. Incidence and characterization of *Staphylococcus aureus* in fishery products marketed in Galicia (Northwest Spain). International Journal of Food Microbiology, 157(2), 286-296.

Viñas, J., Tudela, S., 2009. A validated methodology for genetic identification of tuna species (genus *Thunnus*). PLOS one, 4(10), e7606.

Waldman, W., Chodorowski, Z., Kujawska, H., Anand, J. S., 2006. Acute intoxication with oilfish (*Ruvettus pretiosus*). Przeglad Lekarski, 63(6), 462.

Walker, S. J., Archer P., Banks J. G., 1990. Growth of *Listeria monocytogenes* at refrigeration temperatures. Journal of Applied Bacteriology. 68:157-162.

Warner, K., Timme, W., Lowell, B., Hirshfield, M., 2013. Oceana study reveals seafood fraud nationwide. Oceana. Retrieved on August, 11, 2014.

Williams, C. M., Burdge, G., 2006. Long-chain n− 3 PUFA: plant v. marine sources. Proceedings of the Nutrition Society, 65(01), 42-50.

Winn W., A. S., Janda W., Koneman E., Procop G., Schreckenberger P., Woods G., 2006. Koneman's Color Atlas and Textbook of Diagnostic Microbiology Lippincott Williams & Wilkins, Philadelphia.

Yoshida, T., Sugimoto, T., Sato, M., Hirai, K. 2000. Incidence of *Listeria monocytogenes* in wild animals in Japan. Journal of Veterinary Medical Science, 62(6), 673-675.

Zarei, M., Maktabi, S., Ghorbanpour, M., 2012. Prevalence of *Listeria monocytogenes*, *Vibrio parahaemolyticus, Staphylococcus aureus*, and *Salmonella* spp. in seafood products using multiplex polymerase chain reaction. Foodborne Pathogens and Disease, 9(2), 108-112.

Zhang, J., Cai, Z., 2006. Differentiation of the rainbow trout (*Oncorhynchus mykiss*) from Atlantic salmon (*Salmo salar*) by the AFLP-derived SCAR. European Food Research and Technology, 223(3), 413-417.

Zimmermann, M. B., 2012. The effects of iodine deficiency in pregnancy and infancy. Paediatric and Perinatal Epidemiology, 26(s1), 108-117.

14.1. Web references

http://www.ilfattoalimentare.it/filetti-cernia-falsi-denunciaeurofishmarket

http://www.ismea.it/flex/cm/pages/ServeBLOB.php/L/IT/IDPagina/9509