



Improved PCR-DGGE analysis by emulsion-PCR for the determination of food geographical origin: A case study on Greek PDO “avgotaracho Mesolonghiou”

Maria-Eleni Dimitrakopoulou, Efstratia Panteleli, Apostolos Vantarakis^{*}

Department of Public Health, Medical School, University of Patras, Patras, Greece

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ABSTRACT

Greek avgotaracho Mesolonghiou (fish eggs from Flathead Mullet) is a highly valuable food product which holds Protected Destination of Origin status. The aim of this work was to use PCR-DGGE technique to examine whether there is a correlation between bacteria population in fish eggs and geographical origin. Cluster analysis of fish eggs from three geographical locations (Mesolonghi, Australia and Mauritania) discriminated samples according to their provenance. Moreover, we utilized emulsion-PCR amplification in DGGE analysis in order to investigate whether we could obtain further information about food products' bacteria communities. PCR-DGGE proved to be a suitable method for fish eggs traceability, moreover emulsion PCR-DGGE provides better results. Emulsion-PCR can face up the existing limitations of conventional PCR and thus can be demonstrated as alternative molecular technique for complex and processed matrices, regarding food traceability and authentication.

1. Introduction

Due to the growth of global trade, quality authorities, food industries, food scientists and consumers around the world are looking for reliable indicators of food products' geographical origin (El Sheikh, 2018). Moreover, consumers and food industries are increasingly interested about food quality, food traceability and authentication. Therefore, European Union has implemented schemes, such as the Protected Denomination of Origin, (PDO) Protected Geographical Identification, (PGI) and Traditional Specialties Guaranteed, (TSG), in order to guarantee typicity and quality standards and protect these food products from frauds (Di Pinto et al., 2019). Among Greek PDO brands, the traditional avgotaracho (striped grey mullet fish roe) produced in Mesolonghi lagoon, represent an important food product with a significant need for analytical approaches that determine its geographical origin and guarantee products' authentication. The eggs from fishes (commonly referred as roe) caught in the Mesolonghi lagoon are known with the trade name “avgotaracho Mesolonghiou”. The raw material of the product is derived from flathead mullet caught in Mesolonghi. Production, process and preparation of the final product is completed at the same region (Katselis et al., 2005). This product has great commercial value (over €100/kg) and constitutes one of the oldest in the

category of fresh fish, mollusks and crustaceans with protected designation of origin and geographical indication by laws of the Greek Government and the European Union (Dimitriou et al., 2016).

Among the variety of methods for food traceability, DNA-based techniques are commonly used. Moreover, DNA-based techniques are considered to be rapid, robust, sensitive, and relatively simple (Wadood et al., 2020). PCR-denaturing gradient gel electrophoresis (PCR-DGGE) has been established in the field of food traceability and authenticity in order to characterize bacteria, yeasts, and molds in food samples (El Sheikh, 2010; El Sheikh and Montet, 2016; El Sheikh and Xu, 2017; Dgge, 2010). The last decade, PCR-DGGE application has been used on different food products, such as salt (Dufossé et al., 2013), dairy (Arcuri et al., 2013; Garofalo et al., 2015; Rychlik et al., 2017), fruits (El Sheikh et al., 2012; Nganou et al., 2012), seafood (El Sheikh and Montet, 2016; Pimentel et al., 2017; Tatsadjieu et al., 2010). There are several publications, which have already provided information about the link between the analysis of microbial communities and the geographical origin of food (honey, dough, meat) (Liu et al., 2020; Sinacori et al., 2014; Van Reckem et al., 2019).

However, in conventional PCR-DGGE analysis there are some limitations concerning amplification, such as sensitivity (El Sheikh, 2019; El Sheikh and Montet, 2016; El Sheikh et al., 2018). In order to

^{*} Corresponding author.

E-mail address: avanta@upatras.gr (A. Vantarakis).

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obviate the limitations, emulsion PCR was proposed as a new tool to improve microbial analysis (Iacumin et al., 2020). Emulsion PCR can produce droplets of DNA molecules in thermo-stable water-in-oil (w/o) emulsion. Genetic material is divided in droplets, which contain more than one DNA fragment, and is amplified in millions of independent reactions simultaneously. Therefore, regardless the variety of lengths and concentrations of DNA fragments, limitations of PCR amplifications are reduced (El Sheikh, 2010; Ercolini et al., 2004; Nakano et al., 2003; Zhu et al., 2012). In fact, emulsion PCR is implemented in third generation PCR system, known as droplet digital PCR (ddPCR) (Vogelstein and Kinzler, 1999).

The aim of the present study is to investigate the ability of this method to associate fish eggs with geographical region, i.e. the 16S rDNA fingerprinting of bacterial communities by Polymerase Chain Reaction–Denaturing Gradient Gel Electrophoresis. The principal objective of this work is to characterize and compare by conventional and emulsion PCR–DGGE the total bacterial community of fish eggs (roe) from three locations worldwide (Mesolonghi, Australia, Mauritania) and subsequently to try linking the PCR products with the geographical origin of the fishes. In our knowledge, this is the first time, emulsion PCR–DGGE is performed regarding food traceability issues.

2. Materials and methods

2.1. Samples

Flathead mullet (*Mugil cephalus*) were collected in ponds in aquaculture farms of three different districts from Greece, Australia and Mauritania (Fig. 1). In case of Greece, Flathead mullet were caught in permanent fish devices in two years (August 2019 and 2020). The gonads from females were carefully removed from the belly of the fish and then immersed in seawater in order to be cleaned from blood and oddments of visceral organs. They remained into salt for 36–48h depending on their weight. The final step was the drying process in specially designed areas of dryers with controlled conditions (Dimitriou et al., 2016) (Fig. 2). In this stage, the roe samples were collected in bags, transferred to lab under aseptic conditions and stored at -20°C until analysis. Samples from Australia and Mauritania were purchased from local markets and stored at -20°C until analysis as well. Finally, five samples from each geographical location were included and experiments replicated at least five times.

2.2. DNA extraction

For the first experiment, bacterial DNA was isolated from roe from all areas by a commercial kit, DnEasy Powerfood Microbial Kit (Qiagen Hilden, Germany). This procedure was according to manufactures instructions and five replicates were performed for each sample from different geographical origin (Mesolonghi, Australia, Mauritania). The isolated DNA was analyzed by 0,8% (w/v) agarose gel electrophoresis. The concentration and purity of DNA extracted were determined spectrophotometrically with Nanodrop™ 1000 by measuring the absorbance at 260 nm and 280 nm.

2.3. PCR amplification

The V3 variable region of bacterial 16S rDNA from roe was amplified using primers GC338f (5'-CGCCCGCCGCGCGCGGCGGGCGGGCGGGGCGGGGACTCCTACGGGAGGCAGCAG-3', Sigma, France) and 518r (5'-ATTACCGCGGCTGCTGG-3', Sigma St. Louis, Missouri, USA) (Ampe et al., 1999; 2010). A 40-bpGC-clamp (Sigma St. Louis, Missouri, USA) was added to the forward primer. Thus, it is ensured that the fragment of DNA will remain partially double-stranded and that the region screened is in the lowest melting domain (Muyzer et al., 1993). Each mixture (final volume 50 μL) contained 100 ng of template DNA, 0, 2 μM primers, 200 μM deoxyribonucleotide triphosphate (dNTPs), 2,5 μL of 10x reaction buffer A with Mg and 5U of Taq polymerase (Sigma St. Louis, Missouri, USA). The amplification program was initial denaturation at 95°C for 3mins and 10 touchdown cycles for 1 min at 65°C (with the temperature decreasing 1°C per cycle), followed by 20 cycles of denaturing 95°C for 1min, annealing at 55°C for 1min, extension at 72°C for 10 min. Aliquots of PCR products were analyzed by conventional electrophoresis in 2% (w/v) agarose gel with TAE 1Xbuffer (Lonza, Basel, Switzerland), stained with GelRed (Biotium, Hayward, CA, USA) 0,5 $\mu\text{g}/\text{mL}$ in TAE 1X and quantified by using a standard DNA mass ladder 100bp (Lonza, Basel, Switzerland).

2.4. Emulsion-PCR

For this experiment, droplet digital PCR was used (Bio-Rad QX100™ ddPCR™ System). To generate the droplets, DNA template, master mix and droplet generator oil were placed into the droplet generator plate, followed by PCR amplification. The PCR amplification protocol was as described above, except the concentration of Taq polymerase, which was increased 20X. Publications exist, which propose that excess

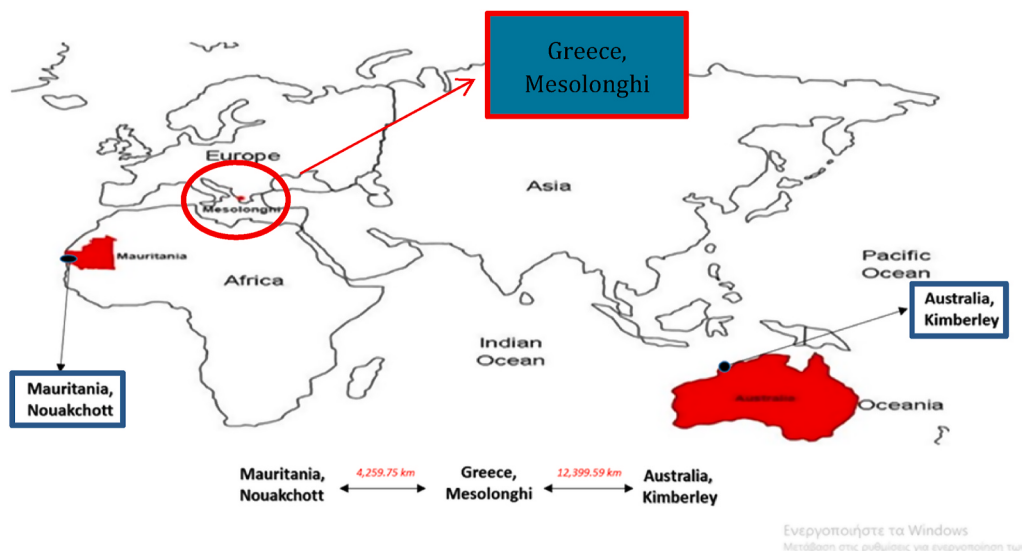


Fig. 1. Geographical distribution of roe samples.



Fig. 2. Flow diagram of avgotaracho Mesolonghiou production.

polymerase enzyme can increase amplification efficiency (Beverung et al., 1999; Siu et al., 2021). Following PCR, 20 μ l elution buffer and 70 μ l chloroform were added to the PCR products and vortexed for 1 min vigorously. Subsequently, PCR products were centrifuged at 25 °C 14000g for 10 min in order to break the water-oil emulsion. Two μ l from the upper phase which contains the DNA, were amplified again at the same conditions and analyzed by electrophoresis in 2% agarose gel (Iacumin et al., 2020).

2.5. Denaturing gradient gel electrophoresis conditions

PCR amplicons from conventional PCR were separated by DGGE on 8% polyacrylamide gel (acrylamide: bisacrylamide, 37.5:1) with 30%–60% denaturing gradients (100% denaturant contained 7 mol/L urea and 40% (v/v) formamide) (Bekaert et al., 2015; Le Nguyen et al., 2008; Nguyen et al., 2008). As far as the appropriate ratio time: voltage concerns, time has been proven of providing a noteworthy impact on the final band pattern (Green et al., 2010; Sigler et al., 2004). Therefore, two different electrophoresis combinations of time and voltage were carried out in the VS20-DGGE system (Clever Scientific) in 0.5 x TAE buffer at 60 °C, 65 V for 16 h and 190V for 5, 3h. After electrophoresis, gel was stained with GelStar nucleic acid (Lonza, Basel, Switzerland) for 30 min. After validation of running time and voltage, the best protocol was chosen, based on the band pattern resulted, for DGGE analysis of emulsion-PCR amplicons.

2.6. Data analysis

Images were acquired in the UVP gel documentation system. All the gel images' individual lanes were straightened and aligned via UVP Visionworks Software. This software provides identification of the relative positions of bands. Banding patterns were standardized with reference pattern of *Escherichia coli* extracted DNA. The similarity between each PCR products from different geographical origins was obtained from the analysis based on a dendrogram computed with Jaccards' similarity and the unweighted pair group method with mathematic average (UPGMA) as the agglomerative clustering (Fromin et al., 2002). The reproducibility value was determined as the average similarity for all five templates from each provenance.

3. Results

Bacterial diversity of fish eggs from three different geographical

origins (Mesolonghi, Australia, Mauritania) was compared. Bacterial DNA populations have been isolated from each sample, amplified by PCR and emulsion PCR in parallel and analyzed by DGGE.

At first, in order to estimate quality of bacterial DNA isolated, electrophoresis in 0,8% agarose gel was performed, while purity and concentration of each sample was measured by Nanodrop™ 1000. Average DNA yield of samples from Mesolonghi was 317,5 ng/ μ l, from Mauritania was 302,8 ng/ μ l and from Australia was 248,2 ng/ μ l. Ratio of A260/280 of all samples was 0,78-0,91. DNA isolated from samples were subjected to PCR amplification and analyzed by electrophoresis in 2% agarose gel. Fig. 3A shows 0,8 agarose gel electrophoresis, while Fig. 3B shows 2% agarose gel electrophoresis.

The fingerprints obtained from DGGE analysis of all different origins from overnight electrophoresis and from 5,3h electrophoresis are shown (Fig. 4A and B). A qualitative analysis of the abundant bacterial communities of all samples is obtained and indicated that Greek PDO avgotaracho Mesolonghiou is easily discriminated. Also, it is validated that long run electrophoresis time, provides better band profile patterns.

The dendrogram resulting from the Cluster Analysis performed among samples taking into account the presence and absence obtained by DGGE analysis, from electrophoresis run 16 h, 65V is shown in Fig. 5. Cluster analysis of fish eggs samples from three different geographical regions, showed the bacterial population similarity among the different sample provenance. At 51%, two main clusters were observed. The first cluster includes samples from Australia and the second cluster includes samples from Mauritania and Mesolonghi. In addition, at 62%, fish eggs from Mesolonghi and Mauritania are discriminated by a second cluster. However, the five replicates from each geographical origin had similar DGGE pattern.

In the second part of the experiment, the goal was to examine whether emulsion PCR could overcome the limitations of conventional PCR and provide higher efficiency. More in detail, after analysis of conventional PCR-DGGE, emulsion PCR was performed and PCR products were analyzed by DGGE as well. DGGE patterns obtained from emulsion PCR, revealed better amplification efficiency and the presence of additional three bands in Greek PDO sample Fig. 6. The results obtained demonstrate that, amplicons amplified with emulsion PCR, provide a more informative fingerprint of samples' bacteria community. Moreover, emulsion PCR provided more effective and accurate results in DGGE patterns, regardless complexity of species in samples or food matrices.

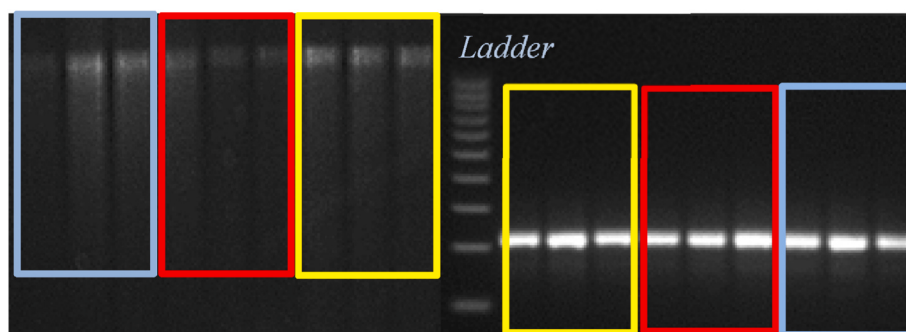


Fig. 3. A. 0,8% agarose gel electrophoresis of isolated DNA from samples B.2% Agarose gel electrophoresis of PCR products(237bp). Five samples from each geographical origin. Highlighted with Red: Greek PDO, Yellow: Mauritania, Blue: Australia.. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

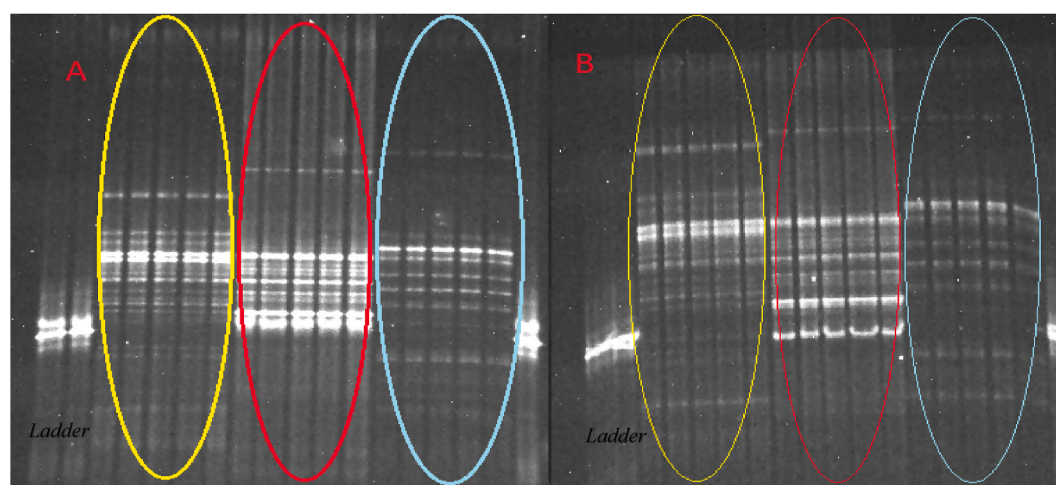


Fig. 4. A. PCR-DGGE 16S rRNA band profiles of fish eggs from 3 different geographical origins (16h at 65V) and B. PCR-DGGE 16S rRNA band profiles of fish eggs from 3 different geographical origins (5,3h-190V). **Highlighted with Red: Greek PDO, Yellow: Mauritania, Blue: Australia.** . (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

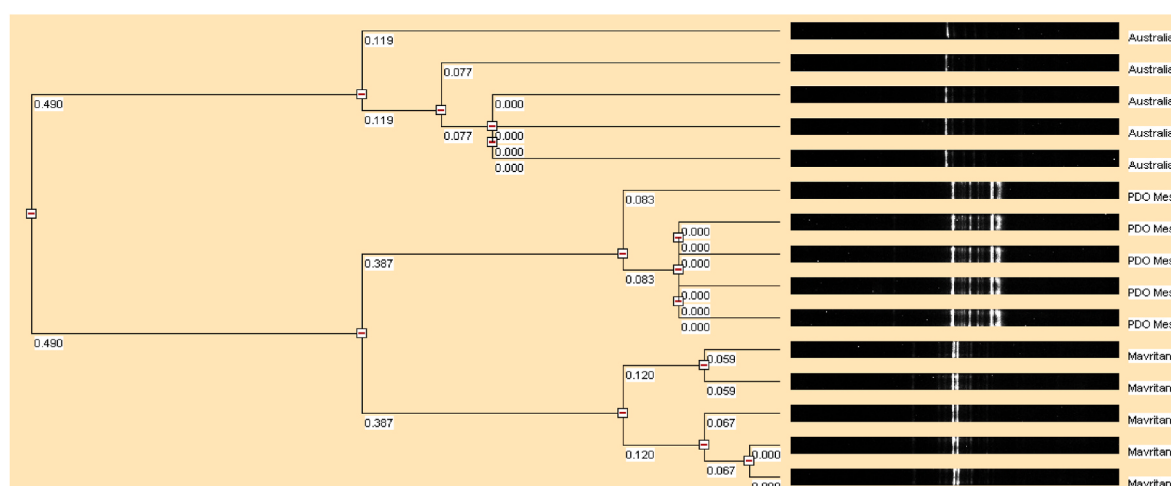


Fig. 5. Cluster analysis of 16S rDNA banding profiles for fish eggs bacterial communities from 3 geographical origins. Five replicates from each location.

4. Discussion

DGGE fingerprinting was used to determine the geographical origin of fish roe from three regions (Mesolonghi, Mauritania, Australia). The use of this technique has been already widely demonstrated to determine geographical origin of food products. Moreover, emulsion PCR has been described as improving amplification gene pattern in related studies (Boers et al., 2015; Chai, 2019; Chai and Oh, 2015).

When comparing the band patterns observed by PCR-DGGE from samples from different locations, a high heterogeneity regarding samples' provenance, is revealed (El Sheikh and Montet, 2016; Le Nguyen et al., 2008). Nevertheless, electrophoretic conditions can affect the band patterns, so two different combinations of time and voltage were performed (Green et al., 2010). Our results indicated that overnight electrophoresis run provide better quality of band profiles of these food samples. Longer electrophoresis run time (over 10h), proved to be optimal for DGGE analysis of bacterial communities isolated from other food matrixes as well, such as bananas, seabass, peach, milk kefir, Gotija cheese (Bigot et al., 2020; Chombo-Morales et al., 2016; El Shobaky et al., 2015; Garofalo et al., 2015; Pimentel et al., 2017).

A dendrogram analysis of all samples, grouped them into three main clusters according to their geographical origin. All five replications of

each geographical origin had statistically similar DGGE patterns. Interestingly, Greek PDO and Mauritania samples clustered more closely than samples from Australia. This can be explained, by the fact that the kilometric distance from Greece to Australia is way longer than that to Mauritania. From the results obtained, PCR-DGGE analysis can be a promising tool for a potential biological barcode of fish eggs regarding food traceability.

Moreover, to PCR-DGGE analysis, we investigated whether emulsion-PCR-DGGE could offer more information about bacteria diversity of food samples. Emulsion-PCR-DGGE considered to be a two-step PCR application. The technique is more time consuming, laborious and technically more demanding, however it is more sensitive, and it can be more efficient in complex food matrixes. Furthermore, emulsion-PCR can provide amplification of low abundance genes and so, specifically regarding food traceability and authentication a food biological barcode could be discovered (Iacumin et al., 2020; Nakano et al., 2003; Williams et al., 2006). However, there are no publications that utilized emulsion PCR-DGGE approach for identify food geographical origin. In our case emulsion-PCR did provide an improved amplification pattern, however, we believe that further sample analysis could verify the observed results. In relation to molecular food traceability and biological barcode approaches, especially when complex matrixes are

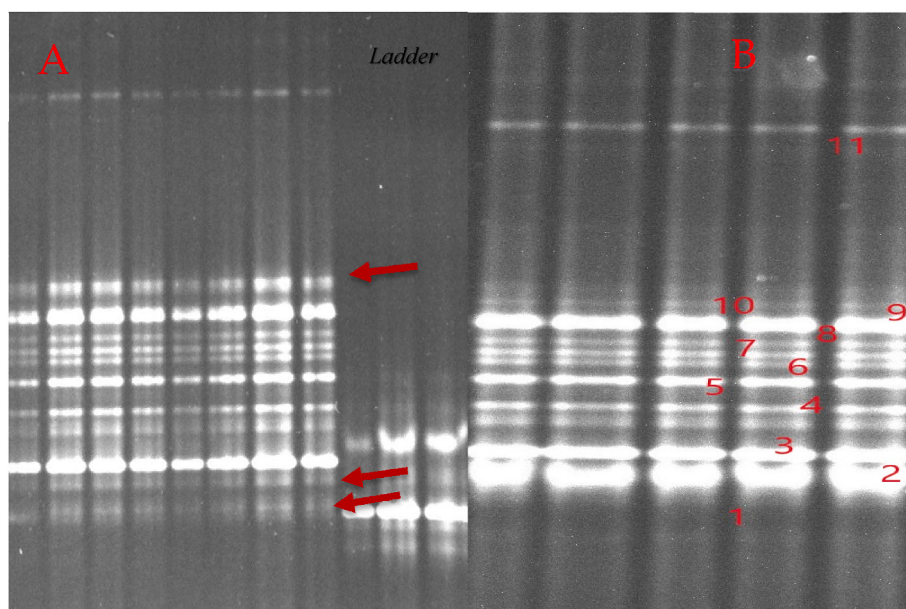


Fig. 6. A. Emulsion PCR-DGGE 16s rDNA band profiles of Greek PDO (16h at 65V) B. Conventional PCR-DGGE 16s rDNA band profiles of Greek PDO (16h at 65V).

concerned, emulsion PCR-DGGE could result an improved analysis. Apart complex matrices, there are certain food products with high bacterial communities, which could benefit from an analysis that promotes comprehensive gene amplification. Although, PCR-DGGE has already been established for food traceability and authentication issues (El Sheikh, 2010; El Sheikh et al., 2011a,b), emulsion PCR-DGGE could overcome the existing limitation of conventional PCR, and thus could deliver biological barcodes for every food type regardless the level of food processing.

5. Conclusion

The bacterial population of “avgotaracho” PDO Mesolonghiou was analyzed and compared with bacteria of fish eggs from Australia and Mauritania. This approach offers the ability to establish markers of PDO from DNA microbes, which can differentiate among other products. Bacterial DNA, extracted from Greek PDO product and two other regions, and analyzed by PCR-DGGE. In addition, emulsion-PCR was performed in order to obtain a more informative biological barcode of a special and highly valued food product such as Greek PDO “avgotaracho Mesolonghiou”.

Our results indicated the presence of unique microorganisms in fish eggs from Mesolonghi, which could be used as a marker for its’ traceability and authentication. PCR-DGGE analysis of bacterial communities allowed Greek PDO to be distinguished from Australia and Mauritania. As far as emulsion PCR-DGGE concerns, it can be suggested to it can reduce PCR bias as well as allowing to obtain a more accurate information of food bacteria diversity. Since emulsion-PCR can be automated by droplet digital PCR system, it can be easily performed avoiding technical demanding procedures. Thus, this methodology can be applied in a variety of food products not only for food traceability and authenticity purposes, but also for food safety and quality issues.

Author contributions

Conceptualization, M.D and A.V; methodology, M.D and E.P; software, M.D; formal analysis, M.D; writing—original draft preparation, M. D; writing—review and editing, E.P and A.V; supervision, A.V.; funding acquisition, A.V. All authors have read and agreed to the published version of the manuscript.

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CRedit authorship contribution statement

Maria-Eleni Dimitrakopoulou: Conceptualization, Methodology, Software, Formal analysis, Writing – original draft, preparation, Writing – review & editing. **Efstratia Panteleli:** Software, Writing – review & editing, Supervision. **Apostolos Vantarakis:** Methodology, Supervision, Funding acquisition.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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