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Microbial spoilage investigation of thawed common cuttlefish (*Sepia officinalis*) stored at 2 °C using next generation sequencing and volatilome analysis

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ABSTRACT

Cephalopods are highly appreciated with increasing demand seafood, but are also very perishable and deteriorate fast mainly due to microbiological spoilage. For this reason exploration of bacterial communities through 16S Next Generation Sequencing (NGS) and Volatile Organic Compounds (VOCs) analysis was performed. Furthermore, sensory evaluation, classical microbiological analysis, Total Volatile Base-Nitrogen/TVB-N and Trimethylamine-Nitrogen/TMA-N determination were also carried out. Shelf-life of thawed cuttlefish (*Sepia officinalis*) stored at 2°C determined by sensory evaluation was 4 days. Aerobic Plate Counts (APC) reached the levels of 6.6 log cfu/g. The initial and final population of all spoilage microorganisms enumerated with selective media was under detectable levels with the exception of *Pseudomonas*. Based on 16S NGS analysis, *Psychrobacter* were the dominants among others, e.g. *Pseudomonas*, *Shewanella, Comamonas, Carnobacterium, Vagococcus,* of the initial microbiota. *Psychrobacter* was also the dominant microorganisms of the spoiled cuttlefish. TVB-N and TMA-N increased considerably only at the late stages of storage. A plethora of VOCs were produced and some exhibited an increasing profile throughout storage, making them promising molecules as freshness indicators in contrast to TVB-N and TMA-N. The application of next generation sequencing revealed the microbiota that escapes the classic microbiological methodologies, showing that other microorganisms different from those determined on selective culture media might be the main cause of microbiological spoilage.

1. Introduction

Cephalopods are a great source of valuable proteins and lipids (Sykes et al., 2009). They are also used as sources of high bioactive compounds with nutraceutical value and food application potential (known as functional ingredients) (Freitas et al., 2012). These reasons seem to have increased the interest for cephalopods landings and consumption worldwide. Especially the last two years there has been a great increase in cephalopods demand and consumption in Asia, Europe and USA, while the major suppliers increased their production from 15 up to 53% (Anonymous, 2018).

However, seafood is very perishable product and the deterioration of sensory attributes occurs quickly after catch. Thus, cephalopods, which often captured by long-distance fishing, are usually distributed as frozen, and allowed to defrost until their final processing or consumption. Hydrogen sulfide (H_2S)-producing bacteria have been reported as the main spoilage bacteria of ice-stored cuttlefish (*Sepia officinalis*) and broadtail shortfin squid (*Illex coindetii*) based on enumeration of microorganisms on culture media (Vaz-Pires et al., 2008). *Pseudoalteromonas*, *Photobacterium phosphoreum*, *Shewanella putrefaciens* and *Pseudomonas*, identified by 16S rDNA sequencing after growing on plates, were found to constitute the spoilage microbiota of ice-stored squid (*Todaropsis eblanae*) (Paarup et al., 2002). However, culture-dependent methods are time-consuming and many fish spoilage microorganisms are unable to grow on general culture media (Broekaert et al., 2011; Emerson et al., 2017), while stressed microorganisms due to various processing factors such as freezing are not able to grow on selective media (Boziaris and Adams, 2001; Wesceie et al., 2009). The

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introduction of next generation sequencing technology platforms that permits the sequencing of millions of DNA fragments in parallel with affordable costs, resolve the problem allowing the determination of the microbial diversity based on their differences in the 16S rDNA extracted directly from the samples without the need to culture them in laboratory media.

Various volatile organic compounds such as alcohols, aldehydes, ketones, organic acids, esters, ammoniac and sulfur compounds have been reported as products of bacterial action and/or chemical activity during fish/seafood spoilage (Duflos et al., 2006; Fratini et al., 2012; Kuuliala et al., 2018; Miks-Krajnik et al., 2016; Parlapani et al., 2014, 2015a; 2015c, 2017; Tuckey et al., 2013). Various individual or groups of VOCs have the potential to be used as spoilage/freshness indicators. Solid Phase Micro-Extraction coupled with Gas Chromatography/Mass Spectrometry (SPME-GC/MS) is one of the methodologies used to study their profile during storage (Duflos et al., 2006; Edirisinghe et al., 2007; Fratini et al., 2012; Miks-Krajnik et al., 2016; Parlapani et al., 2014, 2015a; 2015c; Tuckey et al., 2013). TVB-N and TMA-N have been also reported as spoilage indicators for teleost fish, crustaceans and molluscs (Boziaris et al., 2011; Lapa-Guimarães et al., 2005; Olafsdottir et al., 1997). However, TVB-N and TMA-N increase only at the late stages of cephalopods (Lapa-Guimarães et al., 2005; Sykes et al., 2009) or teleost fish storage (Castro et al., 2006; Parlapani and Boziaris, 2016) making them unsuitable for freshness assessment and remaining shelf-life determination (Oehlenschläger, 2014, 2014). Hence, it is essential to study the VOCs profile throughout storage in order to indicate the compounds that are present at the beginning of storage and increase until the rejection of the product.

Although there is an increasing demand for cephalopods consumption, only few studies regarding their spoilage assessment have been carried out (Bouletis et al., 2015; Lapa-Guimarães et al., 2005; Paarup et al., 2002; Ruíz-Capillas et al., 2002; Sykes et al., 2009; Vaz-Pires et al., 2008). However, to our knowledge, there is no study concerning the microbial diversity using NGS and the investigation of VOCs production of cuttlefish (*Sepia officinalis*). The aim of this study was to investigate (i) the microbial diversity through 16S rRNA amplicon sequencing method using the Illumina's MiSeq platform, (ii) the VOCs profile using SPME-GC/MS, to reveal any potential freshness indicator and (iii) the sensory, microbiological and physico-chemical (pH, TVB-N, TMA-N) changes, to assess thawed common cuttlefish spoilage status and quality changes during chilled storage.

2. Materials and methods

2.1. Cephalopods provision and storage

Two different batches of whole frozen cuttlefish (≈500–700 g) were provided from a Greek company and transferred to the Laboratory of Marketing and Technology of Aquatic Products and Foods (Dept. of Ichthyology and Aquatic Environment, School of Agricultural Sciences, University of Thessaly) in June and August 2016. The cephalopods were block frozen for not more than a month and left to defrost for 72 h, in a refrigerator operating at 4°C. Then, the thawed cephalopods were placed on plastic trays covered with film suitable for food use and stored aerobically in incubators operating at 2°C for 8 days.

At every sampling point, four (4) individuals were taken for analyses (2 individuals from each different batch). For identification of bacterial diversity using 16S rRNA amplicon sequencing, pooled flesh of 25 g from two individuals from each batch were taken at the beginning of the experiment and rejection time point, and subsequently stored at −20 °C.

2.2. Sensory evaluation and determination of shelf-life

Sensory evaluation was carried out every day in order to determine the rejection time point. Sensory evaluation was performed by five trained panelists. The sensory attributes which evaluated were skin appearance and colour, odour and muscle texture based on European Community (EC) freshness grading system for cephalopods (EU, 1996). Rating of each sensory attribute was scored using a 1 to 5 scale with 1 being the highest quality and 5 the lowest. Scale 1, 2 and 3 corresponds to freshness categories E, A and B, while 4 was considered as the score for rejection, and 5 the totally spoiled sample. The samples were rejected when at least one of the panelist scored with 4.

2.3. Microbiological media and analysis

All microbiological media were obtained from LAB M (Lancashire, UK), apart from STAA (Biolife Italiana srl, Milano, Italy). Iron Agar (IA) was prepared according to Gram et al. (1987).

Twenty-five (25) g samples (flesh tissue) were mixed with 25 ml of MRD (Maximum Recovery Diluent, 0.1% w/v peptone, 0.85% w/v NaCl) and homogenized in a laboratory blender. Then the homogenates were transferred to a stomacher bags with additional 200 ml of MRD and homogenized for 1 min using a Stomacher (Bug Mixer, Interscience, London, UK) to produce the 1st dilution. Spread plating method (0.1 ml of serial dilutions in MRD) was used for enumeration of the following microorganisms a) aerobic plate count (APC) on TSA (Tryptone Soy Agar), incubated for 48–72 h at 25°C, (b) *Pseudomonas,* on cetrimide-fucidin-cephaloridine agar (CFC), incubated for 48 h at 25 °C and (c) *Brochothrix thermosphacta*, on STAA, incubated for 48–72 h at 25 °C. Pour plating method (1 ml of serial dilutions in MRD) was used for enumeration of (a) H_2S producing bacteria on IA by counting only black colonies, after incubation at 25 °C for 72 h, (b) Lactic Acid Bacteria (LAB) on De Man, Rogosa, Sharpe agar (MRS) after incubation at 25 °C for 72 h and (c) Enterobacteriaceae on Violet Red Bile Glucose agar (VRBGA), incubated at 37 °C for 24 h. TSA was used for APC enumeration due to its ability to give almost ten-fold higher numbers of colonies compared to other non-selective agar media such as PCA (Kakasis et al., 2011). The results were expressed as mean log cfu/g ± standard deviation of 4 replicates (2 × 2 = 4).

2.4. NGS analysis

2.4.1. Samples preparation for DNA extraction

Twenty five (25) grams of each pooled sample (n = 4 individuals, 2 from each batch) were mixed with 50 ml sterile deionized H_2O (1:2 dilution) and homogenized in a laboratory blender. Forty (40) ml of homogenized seafood suspension were transferred aseptically to sterile centrifuge tubes and centrifuged twice 30 × g for 5 min at 20 °C to remove any flesh particles and the supernatant was transferred to sterile centrifuge tubes and centrifuged at 2200 × g for 15 min at 20 °C, according to Parlapani et al. (2018). Finally, the pellet was resuspended in 3 ml of sterile deionized H_2O.

The colonies of TSA plates at the beginning of the experiment and time of sensory rejection were also taken for DNA extraction and subsequent molecular analysis of microbial cultures. All the colonies from each plate were aseptically collected, suspended in 35 ml sterile deionized H_2O and centrifuged at 2200 × g for 15 min at 20 °C. The pellet was resuspended in 3 ml of sterile deionized H_2O and treated according to Parlapani et al. (2018). The samples were designated as C1 (fresh flesh), C2 (spoiled flesh), CC1 (cultures of fresh flesh) and CC2 (cultures of spoiled flesh).

2.4.2. DNA extraction and library preparation

For each sample, 200 μl of diluted pellet were used for bacterial DNA extraction with ZR Soil Microbe DNA MicroPrep (ZYMO RESEARCH; Irvine, CA, USA) according to the manufacturer's instructions. DNA concentration was measured on a Qubit 2.0 Fluorimeter using the Qubit® dsDNA BR assay kit (Invitrogen, Carlsbad, CA, USA) and its integrity was evaluated by electrophoresis on a 0.8% agarose gel.

Bacterial diversity was assessed by sequencing the V3–V4 region of