INSTITUTO POLITÉCNICO DE LISBOA

Instituto Superior de Engenharia de Lisboa
Escola Superior de Tecnologia da Saúde de Lisboa

Bioburden in Aquafeeds designed for Echinoderms production in Portugal

LILIANA SOFIA FERREIRA ESTEVES
Licenciada em Biologia Marinha e Biotecnologia

Trabalho Final de Mestrado para obtenção do grau de Mestre em Análise e Controlo de Riscos Ambientais para a Saúde

Orientadores:

Doutora Carla Sofia Costa Viegas
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ABSTRACT

The aquafeeds can be a route of contamination for animals produced in aquaculture and, consequently, for humans. For this reason, it is imperative to identify the bioburden present in aquafeeds, in order to know the threat that may represent for human health. Extruded and jellified pellets and frozen microalgae were specifically designed for the potential production, in Recirculating Aquaculture Systems, of two species of echinoderms, the sea cucumber *Holothuria tubulosa* and the sea urchin *Paracentrotus lividus*. Thus, diet samples were collected to characterise their bioburden. Furthermore, water samples and sponge filter samples were also collected to identify their proliferation to water and the efficiency of filtration process used, respectively. All samples were collected 90 days after the start of the experiment. Culture-based methods were used for the characterisation of bioburden, in which mesophilic bacteria and total coliforms were enumerating for bacterial evaluation, while filamentous and xerophilic fungi were counting and identifying through micro and macroscopic characteristics for fungal assessment. *Aspergillus* sections were investigated through molecular method Real-Time Polymerase Chain Reaction. Mycotoxin analysis was carried out utilising Liquid Chromatography-Mass Spectrometry system. For mesophilic bacteria, the results reveal some spoilage activity. The results for total coliforms were actually minimal. *Fusarium verticillioides* was identified in one jellified pellet and in one frozen microalgae and *F. culmorum* was identified in the same frozen microalgae. *Aspergillus* sections were not detected in any sample. Mycotoxins were detected in the extruded pellets and in one jellified pellet, but the quantification only was possible in the extruded pellets. However, the most part of the findings of this study were below to the limits defined as harmless. So the diets used in the production systems evaluated seem to be safe to be used for feeding the studied echinoderms.

Keywords: Recirculating Aquaculture System; Aquafeed; Bacterial burden; Fungal burden; Mycotoxins.

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Resumo

As dietas utilizadas em aquacultura podem ser uma fonte de contaminação para as espécies produzidas e, sendo estas, na maioria das vezes, destinadas ao consumo humano, poderão efetivamente representar uma ameaça para a saúde humana. Portugal tem uma costa muito extensa, o que propicia que os hábitos alimentares da população Portuguesa sejam, em grande parte, sustentados pela atividade pesqueira, sendo, portanto, um dos países com maior consumo de pescado em todo o mundo. Os peixes e o marisco apresentam uma elevada qualidade nutricional, tanto ao nível do conteúdo proteico como lipídico, o que também suscita a sua procura como parte integrante de uma alimentação saudável. Assim, para que esses hábitos alimentares saudáveis sejam mantidos, de uma forma sustentável para o meio ambiente, e com o intuito de combater o declínio que se tem verificado nos *stocks* naturais decorrentes das pescas, surge a aquacultura, que visa garantir a produção das espécies aquáticas, sobretudo para o consumo humano. A produção de novas espécies, como os ouriços-do-mar e os pepinos-do-mar, também tem sido investigada, tanto em relação à manutenção da sua qualidade nutricional, através de dietas especificamente formuladas, como relativamente à sua aceitação em termos de aspeto visual e palatabilidade para consumo. Esta parece ser também uma excelente alternativa a determinadas espécies-alvo, cuja abundância se encontra ameaçada, sendo necessário continuar a investigar a sua aceitação perante a população. Uma alimentação ideal para as espécies produzidas em aquacultura seria, de facto, o mais semelhante possível ao que se encontra disponível nos ecossistemas aquáticos, garantindo as suas necessidades nutricionais e a sua qualidade. No entanto, o grande aumento do esforço pesqueiro que se tem verificado nos últimos anos sugere a procura de outros ingredientes, igualmente nutritivos, que possam substituir a farinha e o óleo de peixe, até então utilizados de forma mais abundante. Deste modo, tem-se recorrido à utilização de cereais, produzidos na agricultura, por serem uma excelente fonte de proteína com potencial para a resolução deste recente problema. Para além disso, é também uma mais-valia na produção de ração, uma vez que permite uma melhor coesão dos ingredientes e, ainda, reduz o seu teor de humidade. Isto possibilita uma menor desagregação da ração quando esta é empregue no meio aquático, minimizando a difusão de matéria orgânica no sistema de produção e, portanto, preservando a qualidade da água. Contudo, os cereais são uma fonte preocupante de

microrganismos, pois, durante o seu crescimento nas culturas, estão expostos a danos provocados por insetos, ou por ação mecânica de máquinas, e a variações climáticas que promovem temperaturas e teores de humidade propícios ao desenvolvimento de bactérias e de fungos. As bactérias desenvolvem-se muito rapidamente, quando em condições ideais e podem produzir toxinas, podendo originar várias doenças. Porém, a sua presença em dietas formuladas para alimentar espécies produzidas em aquacultura não tem sido alvo de investigação, o que incitou o interesse e a curiosidade relativamente à abordagem da contaminação bacteriana neste estudo. Os fungos, por sua vez, produzem micotoxinas, que são muito prejudiciais para a saúde animal e humana. Estas acarretam uma grande preocupação, devido ao facto de apresentarem muita estabilidade perante o calor, não sendo, portanto, eliminadas em processos de extrusão de alimento utilizados para produzir as rações. A acrescentar, ainda, a sua capacidade de persistir nas diferentes matrizes, mesmo após o desaparecimento dos fungos que as produzem, uma vez que estes apresentam uma maior sensibilidade ao processamento de alimentos. Adequadamente, as micotoxinas têm sido alvo de vários estudos em alimentação formulada especificamente para animais produzidos em cativeiro, mas ainda existem poucos estudos direcionados para a investigação da sua presença em dietas destinadas à alimentação utilizada em aquacultura. Tendo tudo isto em consideração, revela-se ser de extrema importância a identificação da contaminação biológica presente nas mais variadas dietas utilizadas em sistemas de produção de aquacultura, cuja atividade encontra-se em progressiva expansão. Assim, este estudo foi desenvolvido como forma de contribuir para um maior conhecimento científico, relativamente ao potencial risco de contaminação biológica a que as espécies produzidas em aquacultura podem, eventualmente, estar a ser expostas, através da utilização das formulações de dietas ricas em variedade de cereais. Este estudo focou-se, portanto, na caracterização da contaminação biológica presente em diferentes formulações de dietas, de modo a verificar a sua qualidade microbiológica. Para tal, foi realizada a amostragem de dietas secas, semi-húmidas e microalgas congeladas, que foram especificamente projetadas para a potencial produção de pepinos-do-mar *Holothuria tubulosa* e de ouriços-do-mar *Paracentrotus lividus*, em aquacultura com sistemas de recirculação. Além de serem recolhidas amostras de dietas, também foram recolhidas amostras de água dos tanques de produção, para uma melhor compreensão sobre a proliferação da contaminação nas diferentes matrizes, uma vez que a água é um excelente meio de ligação entre os microrganismos e as espécies de cultura. E, ainda, foram recolhidas

amostras de filtros de esponja, de modo a validar a eficiência da sua utilização nestes sistemas de produção. A amostragem de dietas, águas e filtros de esponja ocorreu 90 dias após o início dos ensaios de produção dos referidos equinodermes. A contaminação bacteriana foi investigada através da enumeração de bactérias mesófilas, que permitem estimar o nível de deterioração presente nas dietas, e de coliformes totais, cuja presença revela a falta de cuidados de higiene durante o processamento das dietas. Para o crescimento de bactérias mesófilas, utilizou-se o meio de cultura Agar Triptona de Soja com o antifúngico Nistatina, a 30 °C, e, para os coliformes totais, foi utilizado o meio de cultura Agar Bílis Vermelho Violeta, a 35 °C, que foram submetidos às condições referidas durante 7 dias. Em relação à micobiota, esta foi avaliada através da enumeração e identificação de fungos e da análise de micotoxinas. Para o crescimento de fungos filamentosos, utilizou-se o meio de cultura Agar Extrato de Malte com o antibiótico Cloranfenicol e, para os fungos xerófilos, foi utilizado o meio de cultura Agar Dicloran-Glicerol, ambos foram armazenados a 27 °C, durante 7 dias. Após o período de crescimento, as espécies fúngicas foram contadas e identificadas através das suas características micro e macroscópicas. Para a deteção das secções de *Aspergillus* foi utilizado o método molecular Reação em Cadeia da Polimerase em Tempo Real. A análise de micotoxinas foi realizada através de Cromatografia Líquida acoplada à Espetrometria de Massa. Relativamente à contaminação por bactérias mesófilas, foram detetadas na maioria das amostras analisadas, sugerindo que, de um modo geral, o seu conteúdo em grãos cereais e o teor de humidade nas dietas tenham sido fatores decisivos, assim como as possíveis flutuações de temperatura durante o transporte das dietas. Os resultados para os coliformes totais foram efetivamente mínimos, apenas uma dieta seca estava contaminada, o que se pressupõe ser proveniente dos grãos de cereais utilizados na sua formulação; e também foram detetados numa dieta congelada, presumindo-se que esta contaminação tenha ocorrido durante a amostragem ou o processamento de amostras. *Fusarium verticillioides* foi identificado numa dieta semi-húmida, que continha milho na sua composição, e numa dieta congelada e *F. culmorum* foi identificado na mesma dieta congelada. As secções de *Aspergillus* não foram detetadas em nenhuma amostra. As micotoxinas foram detetadas nas dietas secas analisadas, que continham maior teor de cereais na sua composição, e numa dieta semi-húmida, mas apenas foi possível a sua quantificação nas dietas secas. Os resultados obtidos neste estudo, na sua maioria, encontravam-se abaixo dos limites estabelecidos. Deste modo, as dietas utilizadas nos sistemas de produção avaliados parecem ser

seguras como parte integrante da alimentação dos equinodermes dos respetivos ensaios, sugerindo que não são prejudiciais para a saúde dessas espécies nem colocam em risco a Saúde Pública.

Palavras-Chave: Aquacultura com Sistema de Recirculação; Alimentação em aquacultura; Contaminação bacteriana; Contaminação fúngica; Micotoxinas.

PREAMBLE

The Instituto Politécnico de Lisboa (IPL) funded the Project "Bioburden and mycotoxigenic burden from feed applied in aquaculture – Risk assessment and control – AquaInvest" (IPL/2018/AquaInvest_ESTeSL), which allowed the development of this study. With respect to this Project, the theme "Fungal and mycotoxigenic burden from feed applied in Portuguese aquaculture" was presented with an oral communication in the 40th Mycotoxin Workshop 2018, 10th-13th June 2018, in Munich, Germany; which can be found in the Conference abstracts (page 44). Furthermore, the article about this Project is under revision.

The MARE – Marine and Environmental Sciences Centre of the Escola Superior de Turismo e Tecnologia do Mar (ESTM) of the Instituto Politécnico de Leiria (IPLeiria) is a research and development unit in the areas of Marine Biotechnology, Marine Biology and Sustainability, and Marine Food Resources. The MARE-IPLeiria is associated with innovation and empowerment of knowledge transfer to companies. Its mission is to raise awareness of the sustainability and communication of science and knowledge in the field of marine environments and resources, and to create the bridges of the sea for society. All these research activities in marine resources and their sustainability of the MARE-IPLeiria take place in the scientific and technological infrastructures built by the IPLeiria, recognised by the building of CETEMARES – Centre for research and development, training and dissemination of maritime knowledge. The building of CETEMARES is located in the port of Peniche (Portugal), allowing greater interaction with the sea and industry. It has around 2,000 m^2 of laboratories equipped with the most modern equipment in the field of biology, fisheries, aquaculture, biotechnology, chemistry, microbiology, and food technology, as well as several spaces dedicated to the formation and transfer of knowledge, making this the unique infrastructure of the West of Portugal dedicated exclusively to Science and Technology of the Sea.

The Health and Technology Research Centre (H&TRC) is a structural resource unit of the Escola Superior de Tecnologia da Saúde de Lisboa (ESTeSL) of the IPL, in Lisbon, Portugal, endowed with specific scientific and administrative autonomy under the law. Its purpose is to contribute to the development of scientific research in the field of health sciences in a multidisciplinary perspective, promoting innovation and the

dissemination of knowledge. The H&TRC is organised in an articulated way in research groups, which develop their research and development activities within their strategic lines of research. It guarantees the freedom of research of its researchers and is guided by the norms and ethical and deontological principles underlying the processes of scientific investigation, as well as by the statutory principles of the ESTeSL-IPL. Its objectives are: to promote and coordinate the execution of scientific research projects within the strategic research lines defined by its Scientific Committee; streamline innovative projects with social impact; promote cooperation, partnerships and scientific exchange with other national and international institutions, networks and researchers; to create networks of scientific and cultural extension and of intervention in the community; disseminate and promote the discussion of scientific knowledge; dynamics of student involvement in the H&TRC activities. The research team of the H&TRC is characterised by multidisciplinary expertise in genetics, biochemistry, biostatistics, chemistry, nutrition, biomedical sciences, physiotherapy, pharmacy, environmental health, endocrinology, pathology, orthoptics, radiation and medical imaging.

The Faculty of Natural Sciences of the Kazimierz Wielki University, in Bydgoszcz, Poland, cooperate with many International Scientific Centres, mostly in Europe, which has given rise to publications. The Institute of Experimental Biology collaborates, according to the formal agreements, with German and French Scientific Centres in the fields of Toxicology and Genetics. As part of the 6th Framework Programme, the European Union has created the European Network of Excellence, EVOLTREE, and the Domestic Scientific Network, DENDROGEN. The academics of this Faculty are members of many National and International Scientific Organisations and Committees, conducting several Scientific Conferences and Seminars, even the International ones; and some are regular, as the case of the International Mycotoxins Conference, since 2003.

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LIST OF ABBREVIATIONS

< – Symbol that refers to “less than”

> – Symbol that refers to “greater than”

% – Percentage

α – Alpha

α-ZOL – α-Zearalenol

β – Beta

β-ZOL – β-Zearalenol

1st – First

10^1 – A dozen (10)

10^2 – A hundred (100)

10^3 – A thousand (1,000)

10^4 – Ten thousand (10,000)

10^6 – A million (1,000,000)

10^8 – A hundred million (100,000,000)

15-AcDON – 15-Acetyldeoxynivalenol

µg – Microgram

µL – Microlitre

µm – Micrometre

ACN – Acetonitrile

AcOH – Acetic Acid

AF – Aflatoxin

AFB_1 – Aflatoxin B_1

AFB_2 – Aflatoxin B_2

AFG_1 – Aflatoxin G_1

AFG_2 – Aflatoxin G_2

API – Atmospheric Pressure Ionisation

°C – Degrees Celsius

CETEMARES – Centre for research and development of maritime knowledge

CFU – Colony-forming Units

cm – Centimetre

cm^2 – Square Centimetre

DAS – Diacetoxyscirpenol

DCPA – Dichloran Chloramphenicol Peptone Agar medium

DG-18 – Dichloran 18% Glycerol Agar medium

DNA – Deoxyribonucleic Acid

DOM-1 – Deepoxy-deoxynivalenol

DON – Deoxynivalenol or Vomitoxin

DRBCA – Dichloran Rose Bengal Chlortetracycline Agar medium

EC – European Commission

EFSA – European Food Safety Authority

ELISA – Enzyme-Linked Immunosorbent Assay

et al. – Abbreviation of “and others” in Latin

EU – European Union

FAO – Food and Agricultural Organization of the United Nations

FB – Fumonisin

FB_1 – Fumonisin B_1

FB_2 – Fumonisin B_2

FIFO – Fish-In Fish-Out Ratio

g – Gram

$\times$ g – G-force

h – Hour

H_2O – Water

ha – Hectare

HPLC – High-Performance Liquid Chromatography

HPLC-DAD – HPLC with Diode-Array Detection

HPLC-FD – HPLC with Fluorescence Detection

H&TRC – Health and Technology Research Centre

i.e. – Abbreviation of “*id est*” (equivalent to expressions like: that is, meaning, namely)

INSA – Instituto Nacional de Saúde Doutor Ricardo Jorge

IPL – Instituto Politécnico de Lisboa

IPLeiria – Instituto Politécnico de Leiria

ITRA – Itraconazole

Kg – Kilogram

Km – Kilometre

Km^2 – Square kilometre

L – Litre

LC-MS/MS – Liquid Chromatography-Tandem Mass Spectrometry system

LOD – Limits of Detection

$\log_{10}$ – Base Ten Logarithm or Common Logarithm

LOQ – Limits of Quantification

m^2 – Square Metre

MARE – Marine and Environmental Sciences Centre

MBR – Membrane Biological Reactor

ME – Matrix Effects

MEA – Malt Extract Agar medium

mg – Milligram

min – Minute

mL – Millilitre

mm – Millimetre

mM – Millimolar

MPA – Mycophenolic Acid

MS/MS – Tandem Mass Spectrometry

n – Number of times that the event occurred

NaCl – Sodium Chloride

NIV – Nivalenol

nm – Nanometre

nM – Nanomolar

No. – Number

NUTS II – Nomenclature of Territorial Units for Statistics level II

OT – Ochratoxin

OTA – Ochratoxin A

PCR – Polymerase Chain Reaction

PDA – Potato Dextrose Agar medium

POSA – Posaconazole

qPCR – Real-Time PCR

RAS – Recirculating Aquaculture System

rpm – Revolutions Per Minute

s – Second (unit of time)

SAB – Sabouraud Agar medium

S/N – Signal-to-noise Ratio

SOFIA – The State of World Fisheries and Aquaculture

sp. – Species

spp. – Subspecies

STEC – Shiga Toxin-producing *Escherichia coli* Infection

t – Tonnes

TCT – Trichothecene

TSA – Tryptic Soy Agar medium

UHPLC – Ultra-High Performance Liquid Chromatography

UV – Ultraviolet Irradiation

VORI – Voriconazole

VRBA – Violet Red Bile Agar medium

v/v – Volumetric Fraction

ZEA – Zearalenone

1. Background

1.1. Aquaculture

1.1.1. Aquaculture Activity

Aquaculture is defined as an activity that allows the cultivation or rearing of aquatic organisms (DGRM, 2016; DRAPN, 2012; Justino *et al.*, 2016; Snow *et al.*, 2012), with the application of methods that allow the progressive increase of productivity (DGRM, 2016; DRAPN, 2012; Justino *et al.*, 2016). Thus, the biogenetic cultures are characterised by the growing, reproduction, or maintenance of aquatic species in freshwater, marine or brackish waters (DR, 2007) and these activities are referred in Law No. 58/2005 of December 29th (DGRM, 2016; DR, 2005; DRAPN, 2012) and Decree-Law No. 226-A/2007 of May 31st (DGRM, 2016; DR, 2007; DRAPN, 2012). Some human intervention is necessary in the breeding process, whether through stocking, feeding, or simply protection against predators (Boison & Turnipseed, 2015; Myers, 2010).

The groups of organisms reared in freshwater, marine, and brackish environments include the finfish, molluscs, and crustaceans (Boison & Turnipseed, 2015; DGRM, 2016; Myers, 2010; Snow *et al.*, 2012). But many others aquatic animals are also reared, as the example of sea squirts and jellyfish (Boison & Turnipseed, 2015); or even aquatic plants (Boison & Turnipseed, 2015; Myers, 2010), microalgae, and seaweeds (Snow *et al.*, 2012).

The main purpose of aquaculture activity is the production of organisms with high nutritional value, in order to integrate the human diet in a sustainable way (Justino *et al.*, 2016; Liu *et al.*, 2017), given that this is one of the preferred protein sources of a great part of population (Chanpiwat *et al.*, 2016).

1.1.2. Production Systems

1.1.2.1. Extensive, Semi-intensive, and Intensive Systems

Aquaculture facilities can be distinguished in three different regimes, the extensive, semi-intensive, and intensive systems (Erzini, 2016; Ismail *et al.*, 2017; Justino *et al.*, 2016; Lekang, 2007; Parisi *et al.*, 2014), depending on the feeding,

technology, and productivity that are used in the exploitation regime selected (Ismail *et al.*, 2017; Justino *et al.*, 2016; Lekang, 2007).

In extensive regime, the density of reared species is maintained at low levels (EC, 2012; Justino *et al.*, 2016; Lekang, 2007), as well as technology and investment (Lekang, 2007), and the input of artificial substances (Ismail *et al.*, 2017; Lekang, 2007). Thereby, the feeding used is exclusively natural (Erzini, 2016; Justino *et al.*, 2016). This system consists in maintaining natural or artificial ponds, through cleaning and fertilising the ponds or lagoons, in order to intensify the existence of microorganisms, small crustaceans and molluscs, worms and larvae that comprise the aquatic food pyramid. This allows the development of marketable organisms with higher yield comparing with natural ecosystem (EC, 2012).

In contrast, intensive regime represents a system with a really upper productivity level per unit volume (Gabriel *et al.*, 2015; Justino *et al.*, 2016; Lekang, 2007), implying the utilisation of more technology (Ismail *et al.*, 2017; Lekang, 2007) and artificial inputs, which leads to the requirement of much higher investments per unit volume (Lekang, 2007). The commercial feeds are utilised to supply the specific nutritional needs of each species (Justino *et al.*, 2016). In this way, the feeds are competently administered and their consumption and conversion are effectively monitored (Tidwell, 2012) to obtained an optimal growth conditions. The risk of diseases arising is greater, because animals are constantly under stress in order to reach their maximum performance. For that, there is a need to avoid such injury through the utilisation of disease control methods (Lekang, 2007).

However, it is possible to associate both exploitation regimes mentioned above, creating a new system called semi-intensive regime (Lekang, 2007). In this system, the production is greater than in extensive aquaculture and for that is added supplementary feed, as extruded pellets, to complement the natural feed that is available in pond (EC, 2012). In this way, the feeding consists of a mixture of natural and commercial feeds (Erzini, 2016; Ismail *et al.*, 2017; Justino *et al.*, 2016).

1.1.2.2. Open, Semi-closed, and Closed Systems

The classification of production systems may also be made according to the intervention of the producer in the control of appropriate levels of basic functions, in

terms of oxygen, temperature, and waste removal. Thereby, the systems can be categorised as open, semi-closed, or closed (Tidwell, 2012).

Open production systems use natural ecological processes to perform the basic functions mentioned above. Usually, the commercial aquaculture production utilises natural bodies of water and has a low environmental impact. The natural processes offer the sufficient oxygen supply for biomass to be supported, being provided through photosynthesis performance by natural algal communities, through diffusion, or both. The waste products are removed according to natural processes rates within the system and the water temperature is defined by the ambient temperature (Tidwell, 2012).

In semi-closed production systems, the flow of water to the production units is promoted by pumping or gravity-flowed and the water may be used once and then be discharged or be continuously cleaned and re-oxygenated through natural processes. There is a major control over water quality and management of water replenishment. It occurs the monitoring of temperature and the detection of diseases. The levels of dissolved oxygen are easily monitored and mechanical aeration can be included. The elimination of predation and control of poaching are more efficient than in extensive regime (Tidwell, 2012).

In closed production systems, human intervention acts on all basic processes and the operator is responsible for the control of all environmental variables in the production system. It is possible to keep the water temperature very near to the optimal growth temperature of rearing species, which can have a huge positive impact on growth rate and production efficiency. The water can be reused and the constant disinfection of water with ozone or ultraviolet lights reduces pathogenic organisms in a manmade culture system. The poachers and predators can be totally eliminated from the system (Tidwell, 2012).

1.1.3. Production Units

The production units used in aquaculture may be described as natural settings, like ponds and lagoons (EC, 2012; Tucker & Hargreaves, 2012); or as man-made settings, like cages and net pens (EC, 2012; Masser, 2012), longlines, rafts (EC, 2012; López *et al.*, 2017), and tanks (flow-through and recirculating systems) (Ebeling & Timmons, 2002; EC, 2012).

Ponds are considered a semi-closed system (Tidwell, 2012) and are the most common aquaculture worldwide. The production in ponds can be defined as confined bodies of standing water with small size in which are produced aquatic animals. Almost all groups of aquatic animals may be cultured in ponds, so such as crustaceans and fish, but those that adapt better to larger ranges of environmental conditions variations are best suited, mainly in relation to the water temperature. Aquatic plants can also be produced, particularly single-cell algae, as *Spirulina* sp. (Tucker & Hargreaves, 2012).

Cage culture is a production method in open sea, which consists in a “fencing-off” of a part of natural aquatic habitat. In marine environments, larger cages are used (Tidwell, 2012) and these structures may be suspended in the water column or on-bottom (Justino *et al.*, 2016). While in shallow freshwater ponds usually are used small floating cages (Tidwell, 2012). These settings are distinguished by their well-defined and rigid structures or by having a frame that supports and secures the mesh or netting. The difference between cages and net pens is the fact that net pens are made of flexible mesh or netting without whole or encasing frames (Masser, 2012).

Longline cultures are floating systems that use extensive and simple structures with ropes, culture units, and floats, and are applied in protected areas. They involve reduced investments and are easily operated (López *et al.*, 2017).

The tanks used in a flow-through system are usually made of concrete troughs and are called raceways, being considered a semi-closed system (Tidwell, 2012). A production system representative of this type of culture is compound by tanks in series, generally with rectangular form and with water flow along the extensive axis. In smaller aquacultures, habitually, the tanks are arranged in pairs for easy access and in larger farms multiple raceways are arranged in parallel series (Fornshell *et al.*, 2012). The water can be reused many times along multiple raceways in series, according to the water flow (Tidwell, 2012).

The tanks that are used in recirculating systems are constructed with moulded polyethylene, fiberglass (Ebeling & Timmons, 2002), or concrete (EC, 2012). These systems are designated by Recirculating Aquaculture Systems (RAS) and emerged as a way to solve the pollution produced by the use of conventional methods of aquaculture (Zhu *et al.*, 2016). Thereby, these are considered the production systems with major sustainability (Priyaja *et al.*, 2017), given that the water is maintaining in a closed system and is recycled (EC, 2012). RAS allows an intensive production with high population densities, which promotes the accumulation of waste products in water

(Gonçalves & Gagnon, 2011). The solid wastes produced by aquatic organisms are removed by mechanical filters (Tidwell, 2012) and the dissolved and particulate organic matter are removed through biofilter-based systems (EC, 2012; Gonçalves & Gagnon, 2011). Biofilters used in RAS carry large microorganism populations that metabolise nitrogen compounds (Summerfelt *et al.*, 2004). The use of biofilters allows the recycling of aquaculture wastewater, reducing the use of water supply. During this recombination process, nitrification occurs, in which, in a first step, ammonia is used as an energy source by ammonia-oxidizing bacteria which convert it into nitrites and, in a second step, the nitrites are used as energy source by nitrite-oxidizing bacteria and converted into nitrates. Thus, the stable and viable nitrification biofilm (population of microorganisms) guarantees the good quality of RAS (Zhu *et al.*, 2016).

Nevertheless, it is possibly to utilise the combination of advanced water treatments like Membrane Biological Reactor (MBR) (Holan *et al.*, 2014; Sharrer *et al.*, 2007; Wold *et al.*, 2014) and disinfection treatments (Aruety *et al.*, 2016; Gonçalves & Gagnon, 2011; Sharrer & Summerfelt, 2007; Summerfelt *et al.*, 2004). The MBR is a system of membrane filtration with low porosity that guarantees the retaining of high microorganism concentrations (Sharrer *et al.*, 2007). The pore size of microfiltration membranes is of 0.1 to 10 µm and the pore size of ultrafiltration membranes is of 1 to 100 nm, which distinguished by their efficiency and, therefore, the ultrafiltration membranes are preferred in aquaculture to produce the better water quality for the production system (Wold *et al.*, 2014).

Furthermore, the combination of filtration system with disinfection treatments, like Ozonation and Ultraviolet (UV) Irradiation, reduces significantly the microbiota counts (Aruety *et al.*, 2016; Martins *et al.*, 2010; Sharrer & Summerfelt, 2007), inactivating the remaining (Martins *et al.*, 2010; Summerfelt *et al.*, 2004). The Ozonation uses the ozone as an oxidizing agent that inactivates pathogens by direct contact (Summerfelt, 2003; Summerfelt *et al.*, 2004), and also degrades organic compounds to smaller compounds that are more amenable to biodegradation (Summerfelt *et al.*, 2004). The UV Irradiation destructs the residual ozone that is toxic to aquatic organisms, being converted to oxygen. This treatment produces the denaturation of microorganisms DNA, or mortality (Summerfelt, 2003; Summerfelt *et al.*, 2004), and it does not present any risk to aquatic organisms, since there is no production of toxic waste or by-products of risk (Summerfelt, 2003).

Accordingly, after undergoing these specific treatments (Martins *et al.*, 2010), it is added air or oxygen into the water (Tidwell, 2012), enabling its reuse and return to the closed system through piping systems. The organic wastes should be treated before being discarded in nature (EC, 2012).

1.1.4. Sector Development

During the year 2012, about 200 countries reported that high-value species (flatfish, prawns, shrimp, salmon, seabass, seabream, tuna) were highly traded, as well as low-value species (small pelagic), having the main purpose of exporting to developing countries, where there were low-income consumers (Ismail *et al.*, 2017).

Aquaculture is an extremely important activity, which allows to ensure the cover of the food needs of the constantly expanding world population (Gonçalves & Gagnon, 2011; Palm *et al.*, 2015), given that the fish are the most commercialised food in the World. In addition, it is important to emphasize its relevance in reducing pressure on wild fish stocks (Ismail *et al.*, 2017).

The Food and Agricultural Organization of the United Nations (FAO) made a document entitled by "The State of World Fisheries and Aquaculture (SOFIA)", mentioning that aquaculture is expanding (Crab *et al.*, 2007; FAO, 2018; Justino *et al.*, 2016; Pietsch *et al.*, 2011; Snow *et al.*, 2012) and highlighting the increase in production and quality improvements of the product produced (FAO, 2018; Justino *et al.*, 2016).

1.2. Importance in Portugal

1.2.1. Seafood/Fish Consumption

Portugal area is around 92,000 Km^2 and its coast covers 942 Km. Maybe because of this, the feeding of Portuguese population comprises many food related to the sea (Maciel *et al.*, 2016; Viegas *et al.*, 2018a submitted), having been declared that mostly of the population consumes seafood/fish more than once a week. The consumption of seafood/fish in Portugal has an average annual of approximately 54 Kg per capita (FAO, 2016), which represents a seafood/fish daily average of about 160 g (Cardoso *et al.*, 2015; Viegas *et al.*, 2018a submitted). Thereby, Portugal has the highest consumes of seafood/fish per capita worldwide, belonging to the third country in the

ranking (Almeida, 2014; Fernandes, 2017; Maciel *et al.*, 2016), afterwards Japan and Iceland (Almeida, 2014; Maciel *et al.*, 2016).

Aquaculture ensures the availability of seafood/fish protein for human consumption (Gabriel *et al.*, 2015; Ismail *et al.*, 2017; Liu *et al.*, 2017), since fishing is an activity that exploits the natural resources, which are increasingly at risk (Moreira, 2015; Viegas *et al.*, 2018a submitted). Thus, aquaculture guarantees a higher productivity and is economically more reachable for population (Boison & Turnipseed, 2015).

1.2.2. Aquaculture Establishments

In Portugal, aquaculture is represented habitually by small-scale establishments, namely the bivalve productions that are commonly found in the estuarine zones, corresponding mostly to family-based productive units (DGRM, 2016).

In 2015, the numbers of total establishments that are licensed were 1,504, but only 1,433 were active with production in this year. Whereas in 2016 was occurred an increase, there being 1,518 licensed establishments, in which 1,466 were active with production. The total area of aquaculture in 2016 (4,881 hectares), which means about 3.22 hectares per establishment, was decreased its average size at 2.0%, given that in 2015 (4,928 hectares) was of around 3.28 hectares per establishment (Annexe I) (INE, 2018).

With respect to production units, in 2016, 87.9% of the total licensed establishments (i.e. 1,335 establishments) corresponded to on-bottom production of bivalve molluscs (INE, 2018), most of which were located in Ria Formosa (DGRM, 2016; INE, 2018); 9.4% (i.e. 143 establishments) were the rearing tanks that are used for fish production; and 2.1% of the facilities (i.e. 32 establishments) corresponded to floating structures that are often used for bivalve molluscs (Annexe II) (INE, 2018).

1.2.3. Aquaculture Production

Regarding to freshwater aquaculture, in the year 2015, only 30 establishments were licensed, increasing to 34 licensed establishments and all with active production, in 2016. In both years, the production remained exclusively intensive (Annexe III) (INE, 2018).

While, in relation to marine and brackish waters, at the end of 2015, there were 1,474 licensed establishments and, in 2016, were 1,484. This reveals that the main types of water that are selected to produce aquatic organisms are marine and brackish waters. Thus, the intensive exploitation regime represented 28.4% of this production, which was ensured by 15 establishments, having decreased 5.6% comparatively to 2015; the semi-intensive exploitation regime accounted 11.3% of production, having been ensured by 76 establishments, being the volume of production similar to the previous year (11.1%); and the extensive exploitation regime counted 60.2% of production, having been ensured by 1,393 establishments, mainly by bivalve production, and the production, in 2015, was of 54.9% (Annexe III) (INE, 2018).

The total aquaculture production in Portugal, in the year 2015, was of 9,561 tonnes, with revenue of 54.1 millions of Euros, which increased to 11,259 tonnes in 2016, representing 75.2 millions of Euros (Annexe IV). These data reflect a rise of around 17.8% in volume with relation to the previous year, representing an increase of about 38.9% in monetary value, given that in 2015 were produced 9,561 tonnes that generated 54.1 millions of Euros (Annexe V) (INE, 2018).

Regarding to the major production volumes of the country, Algarve Region produced 5,967 tonnes that were exclusively from marine and brackish waters, followed by Centre Region with 3,255 tonnes, in which only 1 tonne was from freshwaters. And freshwater aquaculture was produced merely in Centre Region and in North Region with 674 tonnes (Annexe IV) (INE, 2018).

1.2.4. Production by Species

Aquaculture in marine and brackish waters remained the most relevant in 2016, signifying approximately 94.0% of total produced, in which fish production represented 37.6%, of which 84.7% were ensured by production of turbot and gilthead seabream. The largest volume of marine fish production (more 8.7% from 2015 to 2016) was mainly due to increased production of main species. In 2016, turbot production was of 2,388 tonnes, gilthead seabream was of 1,196 tonnes, and seabass was of 427 tonnes, having increased 3.8%, 8.8%, and 43.7%, respectively, relative to the preceding year (Annexe VI) (INE, 2018).

The bivalve molluscs and crustaceans represented 56.4% of production in marine and brackish waters, having increased around 32.9% from 2015 to 2016. In

2016, clams remained as the most important species, accounting 3,716 tonnes with an increase of 61.6%; followed by mussels that added 1,474 tonnes, exhibiting an increase of 12.1%. The oyster production represented 1,014 tonnes in 2016, having declined about 2.0% in relation to the previous year (Annexe VI) (INE, 2018).

The aquaculture production in freshwater decreased 24.1% in 2016, accounting 675 tonnes, representing 6.0% of total aquaculture production, in Portugal, that were exclusively ensured by trout production (Annexe VI) (INE, 2018).

1.3. Types of Feeding

1.3.1. Moisture Content in Aquafeed

The water content in aquafeeds affects their quality during storage time and their stability in water of culture. Thus, diets can be categorised depending on its humidity percentage, being designated by moist, pasty, or dry feeds (Barnabé, 1994).

Moist diets contain between 45 and 80% of water and usually are made of low market value fish and fresh plant material. These diets can be used in crude state or freezing and mincing. Habitually, fish that are used directly in diets have a great water level of about 80%. Some fish species have insufficient fat level, between 2 to 3%, requiring to mixture with fish with lipid content between 8 to 20% (Barnabé, 1994).

Semi-moist diets are composed by the same ingredients, but are added several dry products, usually meals, so such as fishmeal, yeast, and wheat millings, in order to water content is between 18 to 45%. The feed ingredients are processed through crushing and mixing, originating a paste that pass through a mincer and gets a pellet form, originating a jellified pellet that is ready to feed the fish (Barnabé, 1994).

Dry diets have a natural humidity of 7 to 13% and their lipid levels can reach 17%. Generally, are made from meals or powders that are mixed and are prepared by presses. It is further incorporated 1 to 2% of binder in the mixture that allows the cohesion of the granules (Barnabé, 1994). Thereby, is ensured a major stability of extruded pellets in water, maintaining the water quality and allowing their efficient consumption by aquatic organisms (Parker, 2012), avoiding the suspension of residual feed particles that may cause irritation of fish gills (Barnabé, 1994).

1.3.2. Feed Ingredients

All ingredients provide a particular nutrient or lend a physical property that is suitable for the diet formulation. The variety of foodstuffs utilised allows obtaining a diet nutritionally balanced, palatable, and stable in water (Ali, 2006).

Fish oil and fishmeal are the two most relevant ingredients present in the formulations of aquafeeds, because they are considered complete food and also have great digestibility, being also economical feed ingredients. However, it is not sustainable in long term to recourse to fishery activities to support the progressive growth of aquaculture production, since it would lead to the collapse of natural stocks. Thereby, the need arose to use terrestrial resources to replace part of the aquafeed formulation (Shepherd *et al.*, 2017).

Since July 1st 2013, the inclusion of non-ruminant land animal meals in aquafeeds has been allowed in Europe, as well as their by-products that gain commercial value and are more sustainable from an economic point of view (Campos *et al.*, 2017).

The hydrolysed feather meal is a rich source of protein and polyunsaturated fatty acids, being included in aquafeeds as a substitute of fishmeal. Nevertheless, rates of substitution very high can decrease the palatability and digestibility of diets, decreasing their intake (Campos *et al.*, 2017).

The feed ingredients selected for the aquaculture species diets should also take into account their sustainability, their impact on the environment, the Fish-In Fish-Out (FIFO) Ratio (Tacon *et al.*, 2011), and the acceptance of diet that may vary according to its physical appearance (Ali, 2006).

In diets for fish, may be used diatoms (genera *Phaeodactylum* and *Skeletonema*), flagellated microalgae (genera *Tetraselmis* and *Isochrysis*) (Pintado *et al.*, 2014), seaweeds (genera *Gracilaria*, *Porphyra*, *Sargassum*, and *Ulva*), peas, lupines, canola (Pereira *et al.*, 2012), cottonseed meal, soybean meal, corn meal (Marinho *et al.*, 2013; Parker, 2012), peanut meal, wheat, barley (Parker, 2012), linseed oil, rapeseed oil, soybean oil (Reis *et al.*, 2014), fish oil (Diógenes *et al.*, 2018; Enes *et al.*, 2011; Ribeiro *et al.*, 2015; Ribeiro *et al.*, 2017; Valente *et al.*, 2006), fishmeal, poultry by-products, meat meal, bone meal, and dried blood meal (Parker, 2012).

In diets for crustaceans, can be used plants, diatoms, sweet potato, peanut meal, oil cake, corn meal, wheat flour, broken rice, rice bran, cottonseed, hay (Parker, 2012), soybean meal, liver from fish (Barnabé, 1994), shrimp, and small fish (Parker, 2012).

In diets for molluscs, may be used microalgae, seaweeds (Barnabé, 1994), sunflower seeds, wheat flour, soybean meal (Parker, 2012), fish, and crustaceans (Barnabé, 1994).

1.4. Production of New Species

1.4.1. Echinoderms

1.4.1.1. Sea Cucumber

The sea cucumbers, also known as holothurians, are soft-bodied echinoderms (Santos *et al.*, 2015) and belong to marine benthic communities. They have low fat and high protein content (Santos *et al.*, 2015; Santos *et al.*, 2017), containing trace elements and amino acids essentials for human health. These marine invertebrates present several distinctive properties, as antioxidant, anti-inflammatory, antitumor (Santos *et al.*, 2015), and their suture characteristics, purported antiseptic, and wound healing properties are also valued (Brown & Eddy, 2015).

In Western cuisine, the suggestion of consume sea cucumber induces several reactions among Western consumers, alternating between curiosity and disgust (Brown & Eddy, 2015). Nevertheless, the sea cucumbers are considered a luxury food in Asian seafood markets (González-Wangüemert *et al.*, 2016; Purcell *et al.*, 2012; Rakaj *et al.*, 2018), conducing to an overfishing of natural stocks (Santos *et al.*, 2015). To ensure the Asian market demand, has been developed mainly the aquaculture of Japanese sea cucumber, *Apostichopus japonicus* (Rakaj *et al.*, 2018), as well as *Holothuria scabra*, which has also been greatly produced (Domínguez-Godino & González-Wangüemert, 2018).

In the Mediterranean and North-eastern Atlantic, the aquaculture production of sea cucumber has high potential, due to the great diversity of species that exists there with high commercial value (Domínguez-Godino & González-Wangüemert, 2018). The new target species, such as *Holothuria mammata*, *H. tubulosa*, *H. polii* (Rakaj *et al.*, 2018), *H. arguinensis*, *H. sanctori*, and *Parastichopus regalis* (or *Stichopus regalis*) (Santos *et al.*, 2017), are considered a good nutrient sources and also suitable for human

consumption (Rakaj *et al.*, 2018). The exploitation of *H. forskali*, an European species, also presents an increase interest (Brown & Eddy, 2015).

Holothuria mammata can be found in Northern Spain, Canary Islands, Azores, and Madeira (González-Wangüemert *et al.*, 2016). *H. tubulosa* is a species actively harvested in Spain, Italy, Greece, Turkey, and other countries (Rakaj *et al.*, 2018).

Holothuria arguinensis was well-successful in reproduction (Domínguez-Godino & González-Wangüemert, 2018; Rakaj *et al.*, 2018) and in juveniles rearing (Domínguez-Godino & González-Wangüemert, 2018); and can be found in Azores, centre of Portugal, and Canary Islands (González-Wangüemert *et al.*, 2016).

1.4.1.2. Sea Urchin

Sea urchins represent a prised delicacy in the Mediterranean, Asia, Chile, Polynesia (Castilla-Gavilán *et al.*, 2018), and Barbados. Their gonads, an edible portion typically restricted to testis and ovary, are often termed as roe in culinary (Brown & Eddy, 2015). They are rich in β-carotene and polyunsaturated fatty acids (Rahman *et al.*, 2013) and have high commercial value (Cirino *et al.*, 2017), being used raw in sushi and sashimi (Brown & Eddy, 2015; Castilla-Gavilán *et al.*, 2018). Thereby, the Japanese market absorbs 80% of their production and France is the second country with major consumes in the World (Castilla-Gavilán *et al.*, 2018).

Aquaculture methods are utilised to produce the juveniles, enhancement of the gonads, or to improve natural stocks. The challenging is essentially the roe appearance and taste, but the product value may be raise improving the roe quality with feeding kelp in controlled setting (Brown & Eddy, 2015).

Some sea urchins as *Holopneustes purpurascens* and *Heliocidaris tuberculata* are poor candidates for commercialisation, given that their aquaculture production is unpredictable and they have frequently poor gonad quality. *Psammechinus miliaris* is easy to culture and its gonads have acceptable taste and colour, but its relatively small size did not allow its commercial development due to the lack of market acceptance (Brown & Eddy, 2015).

Despite the good gonadal and somatic growth of the large and globular *Echinus esculentus*, when reared with artificial diets, has a poor taste (Brown & Eddy, 2015).

Strongylocentrotus nudus, *S. intermedius*, and *Pseudocentrotus depressus* are appropriate species for complete aquaculture, with rapid growth and high commercial value, being possible to breed from eggs to harvest size (Brown & Eddy, 2015).

Paracentrotus lividus, as known as purple sea urchin (Brown & Eddy, 2015), is quite large and is traditionally consumed on the Mediterranean coast. Its harvesting occurs essentially in Portugal, Croatia, and Ireland for export (Lawrence, 2007). This species is becoming a relevant choice in Europe for emerging aquaculture industry (Ghisaura *et al.*, 2016), being its cultivation well-known, mainly in Scotland (Brown & Eddy, 2015).

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2. STATE OF ART

2.1. Bacteriota

The aquafeeds are an input in aquaculture production and can lead to exposure of aquatic organisms to biological agents that are a human health issue (Sapkota *et al.*, 2008). The intensive aquaculture is more susceptible to be affect by microbial diseases (Priyaja *et al.*, 2017). Wherefore, studies about bacterial contamination present in aquafeeds and their feed ingredients are of major importance, but there are no studies from Portugal neither from European countries with this specific objective.

The dry feeds used for aquaculture can be compared with poultry feeding, given that the raw materials used can be the same, mainly with regard to cereals.

The animal feed has been frequently associated to contamination by foodborne bacterial pathogens (Crump *et al.*, 2002). Bacterial infections in birds have been related to feeds with contaminated feed ingredients, being a concern to affect the productivity and conduct to economic losses (Udhayavel *et al.*, 2017).

The poultry feed and other animal feeds were reported with *Salmonella enterica*, namely animal protein-based feeds and vegetable protein-based feeds, but the distinction is not always made between isolated serotypes (Crump *et al.*, 2002). This bacterium is often isolated from cereals and their whole grains, being wheat the raw material most affected. Davies & Wales (2013) investigated *Salmonella* Typhimurium contamination in cereal ingredients, having obtained only 2 positive results for 581 samples of wheat and no positive results for 96 samples of barley. The heat treatments in feed processing can kill a large part of bacteria, but the recontamination of feeds by *Salmonella* genus may also occur during storage or cooling. This is a concern to Public Health and has been described the link between the contamination of animal feeds and livestock species (Davies & Wales, 2013).

The soil may be considered an initial contamination source of spore-forming bacteria in silages and can be distinguished as strictly anaerobic (*Clostridium* genus) or aerobic (*Bacillus* and other related genera). They reduce the shelf-life and the food spoilage that originate changes in texture or even the development of off-flavours. In a study in which samples of soil were collected in dairy farms from Canada revealed that contained 5.2 log $CFU.g^{-1}$ of *Clostridium* genus. Another study from Pittsburgh reveal

that the presence of *C. perfringens* in soil samples ranged from 0 to 10^3 CFU.g^{-1} (Heyndrickx, 2011). Udhayavel *et al.* (2017) studied the contamination of *C. perfringens* in poultry feed ingredients. Samples of meat and bone meal had 39 positive results out of 91 samples; bone meal had 13 positive results out of 29 samples; fishmeal had 21 positive results out of 38 samples; dry fish had 5 positive results out of 13 samples; and all results were negative for samples of soybean meal, rapeseed meal, and maize (Udhayavel *et al.*, 2017).

Clostridium botulinum of proteolytic group I and non-proteolytic group II cause human botulism and both groups can origin outbreaks from foods. *C. botulinum* of non-proteolytic group II grows at low temperatures and produces spores, being a concern in chilled food storage. The contaminations with its spores are associated to seafood/fish products, meat, and potato peels, being this last one a reflection of their presence in soil (Heyndrickx, 2011).

Bacillus cereus is found in cereals, rice, fish, meat products, vegetables, but also in sediments, soil, and dust. The raw plant materials may be utilised for animal feed. *B. cereus* spores, as well as all bacterial spores, are resistant to desiccation and heat and can survive to food-processing steps, but its vegetative cells are reduced or eliminated. The soil has been greatly contaminated with its spores, revealing ranges from 4 to 6 $\log_{10}.g^{-1}$ (Heyndrickx, 2011).

The harvesting methods, human settlements, nutritional quality, amount of food supply, and temperature variation influence the development of opportunistic bacteria in crops (Huicab-Pech *et al.*, 2017). Their inoculation in feed materials can occur during growing, dispersal, harvesting, processing, and storage of feeds. Their development is influenced mainly by moisture content of feed formulations (Maciorowski *et al.*, 2007).

Overall, opportunistic pathogens can cause many effects in fish species, namely haemorrhagic septicaemia, circling or erratic swimming, decreased appetite or anorexia, uncoordinated movements, corneal opacity, abdominal inflammation, softening of liver and brain, epithelial hyperplasia in gills, and necrosis in organs (liver, kidney, heart, brain, spleen, and musculature) (Huicab-Pech *et al.*, 2017).

Bacteria use the water as precursor vehicle in aquaculture systems, which allow fish-to-fish outbreaks through direct contact. Fish have bacteria in their normal digestive flora. However, the stress induced by additional organic matter and consequent poor water quality and also the interaction between fish, aquatic

environment, and pathogens increase the incidence of diseases (Huicab-Pech *et al.*, 2017). The opportunistic bacteria can become pathogenic originating diseases and they can cause mortality, mainly in RAS (Wold *et al.*, 2014). The pathogenicity degree depends on resistance of farmed species and also environmental conditions (Huicab-Pech *et al.*, 2017). Feeds are great contributors to the organic load in tank water and the appearance of bacteria in marine hatchery tanks can be promoted by aquafeed and intake of water (Wold *et al.*, 2014).

Streptococcus iniae, *S. parauberis*, and *Aeromonas salmonicida* may contaminate fish by-products and low-value fish (Kim *et al.*, 2013). *S. iniae* is associated to morbidity and mortality of wild and farmed fish, infecting very cultured fish species (Aruety *et al.*, 2016).

The genera *Aeromonas*, *Pseudomonas*, and *Staphylococcus* are potential bacterial pathogens that can induce epidemic outbreaks and *A. hydrophila* is considered the most important pathogen in aquaculture, being able to originate weakness and anorexia, haemorrhages in skin and gills, vision loss (Huicab-Pech *et al.*, 2017). *Vibrio* genus is also a frequent cause of bacterial disease in finfish and shellfish of aquaculture (Priyaja *et al.*, 2017). *Vibrio anguillarum*, *V. harveyi*, *Flavobacterium columnare*, and *Edwardsiella tarda* are some pathogenic bacteria that normally cause mortality in fish. *Edwardsiella* genus provokes septicaemia, opaque eyes, and abdominal inflammation. The Gram-negative bacteria are considered the main source of bacterial disease (Huicab-Pech *et al.*, 2017).

The high choice for fish as a protein primary source of large population conduct to a concern about bacterial pathogens present in fish (Chanpiwat *et al.*, 2016). Bacteria can infect human by their invasion in edible tissues of target-animals that are destined to human consumption (Crump *et al.*, 2002). Fish products have been associated to outbreaks of foodborne illness, with infections caused mainly by pathogenic bacteria *Escherichia coli* and *Listeria monocytogenes*. *E. coli* is a classic indicator of faecal contamination and has been associated to contaminated meat and seafood/fish products, but not all *E. coli* strains are pathogens for human. *Listeria* spp. has been also detected in food for human consumption, including fish products (Chanpiwat *et al.*, 2016).

In a study developed by Chanpiwat *et al.* (2016), the fish samples were contaminated with a mean of 1.57 log CFU.g^{-1} *Escherichia coli* and 1.36 log CFU.g^{-1} *Listeria*. Regarding to the fish samples that they obtained from market (open air and

supermarket), they detected a mean of 2.06 log CFU.g^{-1} *E. coli* and 1.93 log CFU.g^{-1} *Listeria*, whereas the fish samples that they collected at aquaculture operations presented a mean of 1.12 log CFU.g^{-1} *E. coli* and 0.83 log CFU.g^{-1} *Listeria*. Comparing fish samples from open air markets and supermarket, they verified that the differences between them were not significative. From open air markets, they found a mean of 2.26 log CFU.g^{-1} *E. coli* and 1.77 log CFU.g^{-1} *Listeria* and from supermarkets the mean were of 1.96 log CFU.g^{-1} *E. coli* and 2.02 log CFU.g^{-1} *Listeria*. The undercooked or improperly prepared fish revealed to be a risk for Public Health (Chanpiwat *et al.*, 2016).

The genera *Micrococcus* and *Staphylococcus* can also be found in fish, given that they are related to the lack of hygiene during the fish management, due to they are commonly detected in human skin. Besides this, they can cause alimentary intoxications in humans (Huicab-Pech *et al.*, 2017).

Other bacterial species that may be found in fish and can also be a concern for Public Health are *Aeromonas hydrophila*, *Vibrio cholerae*, *V. parahaemolyticus*, *V. vulnificus*, *Pseudomonas* spp., *Streptococcus iniae*, *Flexibacter* sp., *Edwardsiella* sp., and *Flavobacterium* sp. (Huicab-Pech *et al.*, 2017).

2.2. Mycobiota

There are few studies around the World about the fungi, moulds, and mycotoxins present in aquafeeds and only one of these studies was performed in Portugal.

Almeida *et al.* (2011) analysed the natural mycobiota and the Aflatoxin (AF) contamination in plant-based aquafeed distributed in Portugal that is used for production of seabass. For mycological examination, they enumerated the fungi growth on media Dichloran Rose Bengal Chlortetracycline Agar (DRBCA) and Sabouraud Agar (SAB) with Chloramphenicol and their identification was realised by macro and microscopic morphology characterisation and with taxonomic keys. For AF detection and quantification, they use the standard method and the determination of Aflatoxins B_1 (AFB_1), B_2 (AFB_2), G_1 (AFG_1), and G_2 (AFG_2) was executed by isocratic reversed-phase High-Performance Liquid Chromatography (HPLC). They found moulds in less than half of samples, in which *Aspergillus flavus* was in 35 out of 87 (40.2%) fish feed samples with an average of 2.7 log_{10} CFU.g^{-1}, followed by *A. niger* (39.1%) with an

average of 2.2 log_{10} $CFU.g^{-1}$, *A. glaucus* (29.9%) with an average of 1.9 log_{10} $CFU.g^{-1}$, *Penicillium* spp. (28.7%) with an average of 2.0 log_{10} $CFU.g^{-1}$, *Cladosporium* spp. (28.7%) with an average of 1.9 log_{10} $CFU.g^{-1}$, and *Fusarium* spp. (25.3%) with an average of 1.8 log_{10} $CFU.g^{-1}$. No sample contained AFs (Almeida *et al.*, 2011).

Pietsch *et al.* (2013) examined the concentrations of Deoxynivalenol (DON) and Zearalenone (ZEA) in commercial feed and the possible impact in health of aquatic organisms. The fish feed was from Central Europe and was designed for carp feeding. For mycotoxins analysis, DON and its metabolite Deepoxy-deoxynivalenol (DOM-1) were analysed using the HPLC with Diode-Array Detection (HPLC-DAD) and ZEA was determined utilising the HPLC with Fluorescence Detection (HPLC-FD). DON levels in fish feed ranging from 0 to 825 $\mu g.Kg^{-1}$ and, wherefore, were below to the values of guidance for complete feedings. DOM-1 was not detected in samples. ZEA was detected in all samples; its concentrations ranging from 3 to 511 $\mu g.Kg^{-1}$ ZEA and, therefore, did not exceed the recommended values by the European Commission (EC) (Pietsch *et al.*, 2013).

Greco *et al.* (2015) investigated the toxicogenic mycobiota, namely mould genera and species, and quantified the six most relevant mycotoxins in commercial aquafeed for rainbow trout in Argentina. For mycobiota analysis, they used the DRBCA medium to count total cultivable fungi, the Dichloran 18% Glycerol Agar (DG-18) medium to count xerophilic fungi, and the Dichloran Chloramphenicol Peptone Agar (DCPA) medium for selective isolation of species of *Fusarium* and *Alternaria*. For mycotoxins analysis, they utilised the Enzyme-Linked Immunosorbent Assay (ELISA) analytical test kits for the quantitative analysis of AF, Ochratoxin A (OTA), T-2 toxin, Fumonisin (FB), DON, and ZEA. They found fungi at moderate levels in 26 out of 28 feed samples and the mycotoxigenic that predominated were *Eurotium repens*, *E. rubrum*, and *Penicillium* genus. Mycotoxins were discovered in all samples and 93% of feed samples had the co-occurrence of 2 out of 6 mycotoxins; 7% of samples had the co-occurrence of six mycotoxins (Greco *et al.*, 2015).

Nácher-Mestre *et al.* (2015) quantified 18 mycotoxins in aquafeeds for two fish species based on processed animal proteins from non-ruminants (produced in Central Europe) and plant-proteins (produced in United Kingdom). For mycotoxins analysis, they used Ultra-High Performance Liquid Chromatography (UHPLC) linked to a Tandem Mass Spectrometry (MS/MS). FB levels in corn ranged from 0.011 to 4.9 $mg.Kg^{-1}$. DON levels in corn and wheat ranged from 139 to 814 $\mu g.Kg^{-1}$ and from 17 to

504 μg.Kg^{-1}, respectively. OTA levels in corn, pea protein, and wheat products ranged from 0.4 to 5.2 μg.Kg^{-1}. For seabream diets, one of the main sources of DON was contaminated wheat with 371 μg.Kg^{-1}; the main source of FBs and 15-Acetyldeoxynivalenol (15-AcDON) was corn with 139 μg.Kg^{-1} and 53 μg.Kg^{-1}, respectively; the substitution of fishmeal by plant oils had no effects on mycotoxins level. For the Atlantic salmon diets, corn was contaminated with 403 μg.Kg^{-1} FBs and pea proteins were contaminated with 1.8 μg.Kg^{-1} OTA (Nácher-Mestre *et al.*, 2015).

Marijani *et al.* (2017) studied the co-occurrence of mycotoxins in fish feed and feed ingredients from East Africa. They isolated the fungi on DG-18 medium and obtained a pure culture of each type of colony that sub-cultured on Potato Dextrose Agar (PDA) medium. They identified macro and microscopic characteristics of pure fungal isolates that were sub-cultured on Malt Extract Agar (MEA) medium to isolate pure cultures for purification and extraction of Deoxyribonucleic Acid (DNA). The identification of pure fungal cultures was based on their morphology that was confirmed through DNA sequencing. Fungal genera were known through Polymerase Chain Reaction (PCR) assay. The multi-mycotoxins analysis was realised using Liquid Chromatography-Tandem Mass Spectrometry (LC-MS/MS) system. Ten fungal species were detected in feed samples. *Aspergillus flavus* was the most prevalent species and was identified in 54.5% of samples. Fourteen mycotoxins were detected in feed samples, being DON, AFs, and FBs the most prevalent. AFB_1 levels in feeds ranged from <2 to 806 μg.Kg^{-1}, whereas AFB_2 ranged from <2 to 74.4 μg.Kg^{-1}. DON-contaminated feeds ranged from 69.1 to 984.3 μg.Kg^{-1}. FB concentrations ranged from 0.033 to 3.97 mg.Kg^{-1}. Sunflower seed cake contained the highest levels of AFB_1 with 806.9 μg.Kg^{-1}. Cottonseed cake was contaminated with OTA at levels ranged from 6.5 to 24.42 μg.Kg^{-1}. T-2 toxin level was of 36.5 μg.Kg^{-1} in feed ingredients (Marijani *et al.*, 2017).

The feed materials can be inoculated with moulds and fungi during growing, dispersal, harvesting, processing, and storage of feeds. The moisture content influences the fungal development in feeds, but fungi can also grow in stored grains without free water (Maciorowski *et al.*, 2007).

An additional challenge is the fungicide resistance that can build up through the survival and spread of rare fungal mutants in crops used in feed production. Not only is the prevalence of azole fungicide use worrisome for the potential negative impacts on human health, as fungicide resistance may be of concern to those treating

medical mycoses. Furthermore, the resistant fungal species can also lead to mycotoxin-contaminated feed (Doyle *et al.*, 2006; Viegas *et al.*, 2018a submitted).

The presence of mycotoxins in edible tissues of fish and crustaceans and their possible persistence may be considered potential risks for Public Health (Almeida *et al.*, 2011; Santacroce *et al.*, 2008). Thus, the most important mycotoxins in animal production, according to their occurrence and toxicity, are the AFs, ZEA, FBs, OTA, DON, and T-2 toxin (Greco *et al.*, 2015).

AFs are produced mainly by *Aspergillus flavus* that is a common contaminant found in agriculture (Almeida *et al.*, 2011; Gonçalves *et al.*, 2018a), but other *Aspergillus* species are also found only less often (Gonçalves *et al.*, 2018a; Gonçalves *et al.*, 2016). AFs usually appear in corn, wheat, cottonseed, peanut, and soybean (Gonçalves *et al.*, 2016; Santacroce *et al.*, 2008). The most common in aquafeeds are AFB_1, AFB_2, AFG_1, and AFG_2 (Gonçalves *et al.*, 2018a; Gonçalves *et al.*, 2016). Regarding to AFB_1, have already been detected 67 $\mu g.Kg^{-1}$ in fishmeal, 31 $\mu g.Kg^{-1}$ in soybean, 12 $\mu g.Kg^{-1}$ in wheat, and 9 $\mu g.Kg^{-1}$ in pellet feed (Matejova *et al.*, 2017). This is the most hazardous AFs (Matejova *et al.*, 2017; Santacroce *et al.*, 2008) that origins mutagenic, hepatotoxic, carcinogenic, and immunosuppressive effects in aquatic vertebrates and other animals. Sea bass exposed to 18 $\mu g.Kg^{-1}$ AFB_1, during 42 days, induced reduction of plasma proteins and liver damage. Common carp exposed to 200 $\mu g.Kg^{-1}$ AFB_1, during 120 days, provoked kidney damage and circulation disturbances. But more fish species were affected (Matejova *et al.*, 2017). The aflatoxicosis was already occurred in fish that consumed contaminated feed with 20 $\mu g.Kg^{-1}$ AFB_1 (Marijani *et al.*, 2017).

Ochratoxins (OTs) are produced by the genera *Aspergillus* and *Penicillium*. OTA is the most toxic and abundant in this group and is found in barley, wheat, maize, soybean, and peanuts. The main target-organs in fish are the kidney and liver. Juvenile channel catfish exposed to 2 and 4 $mg.Kg^{-1}$ OTA, during 6 weeks, induced immunosuppressive effects. Adverse effects are expected to occur in fish fed with 1 $mg.Kg^{-1}$ OTA (Matejova *et al.*, 2017).

The *Fusarium* mycotoxins are toxic and hazardous metabolites of fungi from *Fusarium* genus, in which are included ZEA, FBs, and Trichothecenes (TCTs) (Greco *et al.*, 2015; Matejova *et al.*, 2017).

ZEA is produced by several *Fusarium* species (Gonçalves *et al.*, 2016) and is detected in maize. ZEA and its metabolites are estrogenic compounds that mimic

natural oestrogens (Matejova *et al.*, 2017) and *F. graminearum* is the main responsible to oestrogenic effects identified in farmed animals (Gonçalves *et al.*, 2016). The toxicokinetic effects in fish are influenced by the water temperature (Woźny *et al.*, 2017). Juvenile rainbow trout exposed to 10 $mg.Kg^{-1}$ during 24, 72, and 168 hours prolonged clotting time and promoted low iron concentrations in ovary and liver (Matejova *et al.*, 2017).

FBs are produced by *Fusarium verticillioides*, *F. nygamai*, and *F. proliferatum* (Gonçalves *et al.*, 2016). This last one is frequently found in maize (Matejova *et al.*, 2017). The most abundant FB is the Fumonisin B_1 (FB_1) that reduces growth rate, feed efficiency and feed consumption ratio (Gonçalves *et al.*, 2016), and causes mortality by direct tissue damage or immunosuppression. Common carp exposed to 0.5 and 5 $mg.Kg^{-1}$ FB_1 during 42 days promoted a decrease of body weight gain and changes in platelet count and red blood cell parameters (Matejova *et al.*, 2017).

TCTs encompass the DON (or Vomitoxin), T-2 toxin, HT-2 toxin (that is a deacetylated metabolite of T-2 toxin), Diacetoxyscirpenol (DAS), Neosolaniol, and Nivalenol (NIV) and its 3- and 15-acetyl derivate. The most common and relevant TCTs in fish are the DON and T-2 toxin (Matejova *et al.*, 2017).

DON is produced by the genera *Fusarium*, *Cephalosporium*, *Myrothecium*, *Verticimonosporium*, and *Stachybotrys* (Pietsch *et al.*, 2014). It is frequently found in cereal grains (Gonçalves *et al.*, 2016), such as barley, wheat, and maize (Sanden *et al.*, 2012). Aquafeeds with contaminated wheat are a pathway to affect farmed fish (Matejova *et al.*, 2017), inducing decrease in feed intake, protein and energy utilisation, growth, and feed efficiency (Gonçalves *et al.*, 2016). Rainbow trout exposed to 2 $mg.Kg^{-1}$ during 23 days revealed severe hyaline degeneration in renal tubules (Matejova *et al.*, 2017).

T-2 toxin is produced by several *Fusarium* species. It infects wheat, maize, oats, and barley. Channel catfish exposed to 630 $\mu g.Kg^{-1}$ during 2 weeks induced reduction in body weight gains and feeds with 1.3, 2.5, and 5 $mg.Kg^{-1}$ promoted low haematocrit values. Rainbow trout exposed to 15 $mg.Kg^{-1}$ during 16 weeks revealed enlarged gall bladders and spleens and focal intestinal haemorrhaging (Matejova *et al.*, 2017).

3. OBJECTIVE

The main objective of this study was to evaluate bacterial, fungal, and mycotoxigenic burden in diets with different composition and moisture content (extruded pellets, jellified pellets, and frozen microalgae) designed to the production in RAS of sea cucumbers *Holothuria tubulosa* and sea urchins *Paracentrotus lividus*, in Portugal.

Samples of sponge filters (biofilters) and rearing waters from these RAS were also performed and investigated to complement the diet results.

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4. Materials and Methods

4.1. Description of Production Systems

In the Aquaculture Laboratories of the MARE – Marine and Environmental Sciences Centre of the Instituto Politécnico de Leiria (IPLeiria), located in the building of CETEMARES (Peniche, Portugal), two nutritional trials were conducted focusing on the production of echinoderms in RAS. The trials were performed during four months.

One of the trials was developed in production systems with the sea cucumbers *Holothuria tubulosa*, in which extruded pellets (samples D1 and D2) and frozen microalgae (samples D6 and D7) were provided, as shown in Table 1. They were fed three times a week and the objective of this trial was to evaluate which diet allows the maintenance of the animals in captivity for reproductive trials purposes. The trial with the sea cucumbers was composed by one RAS with three rearing tanks of 400 L each and a 400 L sump tank.

In relation to the other trial, it was developed in production systems with the sea urchins *Paracentrotus lividus*, in which jellified pellets (samples D3, D4, and D5) were provided, as described in Table 1. They were fed daily *ad libitum* and the objective of this trial was to evaluate allows the best gonadal quality, in terms of growth, nutritional profile, appearance, and palate, for human consumers. The trial with sea urchins was composed by three RAS, in which each RAS had associated three rearing tanks of 60 L each and a 70 L sump tank.

All these diets were especially designed for each species of echinoderms, according to their specific nutritional needs.

Each RAS contained mechanical filtration and biofilters (sponge filters and bio balls), aeration, skimmer pump, and the water flow was kept at 50 L/h in each rearing tank. The water temperature was of 20.5 °C and the sponge filters had a time of use of 90 days, in both production systems.

4.2. Sampling

The above-mentioned samples of diets were collected, during these nutritional trials of these echinoderm species in the Aquaculture Laboratories of MARE-IPLeiria, and it was also performed the complementary sampling of sponge filters (biofilters) and rearing waters from these RAS, which were analysed as referred in Table 1.

For all samples, asepsis conditions were maintained. Sterile plastic vials (for diets and waters) and sterile bags (for sponge filters) were used to reserve and transport the samples that were collected with the use of gloves.

Two extruded pellets, three jellified pellets, and two frozen microalgae were sampling, each sample with 105 g.

One sponge filter was collected from the sea cucumber tanks and three sponge filters were sampled from the sea urchin tanks. Each sample with 49 cm^2 (7×7 cm) was collected from the middle layer of each sponge.

One water sample was sampled before entering the rearing tanks and it was the negative control, four water samples were collected from the sea cucumber tanks, and three water samples were sampled from the sea urchin tanks. Each sample had 60 mL and it was collected from the centre of each rearing tank.

The samples of diets, sponge filters, and waters were collected 90 days after the beginning of the experiments and before feeding the animals. The last feeding event had occurred 20 hours before the sampling.

The transport of samples to the Health and Technology Research Centre (H&TRC) of the Instituto Politécnico de Lisboa (IPL), where the sample processing took place, was carried out in refrigerated thermal boxes.

Table 1 – Sample reference with respective characterisation; and storage location and storage time of diet samples.

Sample Reference	Sample Characterisation	Storage Location	Storage Time*
D1	Extruded pellet: 60% fishmeal, 10% fish oil, 10% corn meal, 5% wheat gluten, 5% soy protein concentrate, 5% pea meal, and 5% sunflower meal	Ambient temperature	3 months
D2	Extruded pellet: 40% fishmeal, 20% fish oil, 10% corn meal, 10% wheat gluten, 10% soy protein concentrate, 5% pea meal, and 5% sunflower meal	Ambient temperature	8 months
D3	Jellified pellet: 47% canned corn, 47% fresh spinach, and 6% agar	Fridge	1 week
D4	Jellified pellet: 22% canned corn, 22% fresh spinach, 50% acorn, and 6% agar	Fridge	2 weeks
D5	Jellified pellet: 22% canned corn, 22% fresh spinach, 50% pumpkin, and 6% agar	Fridge	3 weeks
D6	Frozen microalgae: microalga *Tetraselmis* sp.	Freezer -20 °C	10 months
D7	Frozen microalgae: diatom *Phaeodactylum tricornutum*	Freezer -20 °C	9 months
F1	Sponge filter of sea cucumber system	-	-
F2	Sponge filter of sea urchin tanks fed with the diet D3	-	-
F3	Sponge filter of sea urchin tanks fed with the diet D5	-	-
F4	Sponge filter of sea urchin tanks fed with the diet D4	-	-
W1	Water collected before entering the rearing tanks	-	-
W2	Water collected in rearing tank of sea cucumbers fed with the diets D1 and D2	-	-
W3	Water collected in rearing tank of sea cucumbers fed with the diet D7	-	-
W4	Water collected in rearing tank of sea cucumbers fed with the diet D6	-	-
W5	Water collected after passing the sponge filter common to three rearing tanks of sea cucumbers fed with the diets D1, D2, D6, and D7	-	-
W6	Water collected after passing the sponge filter of three rearing tanks of sea urchins fed with the diet D3	-	-
W7	Water collected after passing the sponge filter of three rearing tanks of sea urchins fed with the diet D5	-	-
W8	Water collected after passing the sponge filter of three rearing tanks of sea urchins fed with the diet D4	-	-

*Storage time corresponds to the production batch of diets.

4.3. Characterisation of Bioburden

The seven diet samples were maintained under the conditions set forth in Table 1, during eight weeks, after which, 4.4 g of each diet sample (not oven-dried prior to processing, thus retaining natural water content) were washed and diluted in 40 mL of Sterilised Distilled Water for their extraction, for 20 min at 200 rpm. Then, 150 µL were seeded on 2.0% MEA medium supplemented with 50 $mg.L^{-1}$ Chloramphenicol and on DG-18 medium at 27 °C, for 7 days, for the growth of filamentous fungi (Viegas *et al.*,

2012) and xerophilic fungi (Greco *et al.*, 2015; Greco *et al.*, 2014), respectively; and 150 μL were also inoculated on Tryptic Soy Agar (TSA) medium supplemented with 0.2% Nystatin at 30 °C and on Violet Red Bile Agar (VRBA) medium at 35 °C, for 7 days, for the growth of mesophilic bacteria and total coliforms (Gram-negative bacteria), respectively (Ribeiro *et al.*, 2018; Viegas *et al.*, 2018c).

One piece of each sponge filter with around 2 cm^2 (1.4×1.4 cm) was cut and introduced into a sterile 15 mL falcon to be washed with 10 mL of 0.1% Tween™ 80 Saline Solution (NaCl 0.9%), in order to remove the content present within the sponge filters, since it works as a detergent. After this, it was performed their extraction, for 30 min at 250 rpm, on an Orbital Laboratory Shaker (Edmund Bühler SM-30, Hechingen, Germany) (Viegas *et al.*, 2017b; Viegas *et al.*, 2018c). For the eight weeks of storage, 25% Glycerol (2.5 mL) were added to each suspension of sample to preserve the cells during freezing, since without Glycerol the cells would be destroyed in this step. The suspensions with the Glycerol were stored at -80 ºC in the freezer. Before inoculation and after being frozen, the centrifugation step was ensured for 30 min at 3,500 rpm.

For water samples, 1.1 mL of Glycerol were added to 4.4 mL of each water sample and then frozen at -80 °C, for eight weeks post-extraction; 3 min at approximately 1,500 x g (Kinsey *et al.*, 1999).

For inoculation of the suspensions of sponge filter and water samples, 150 μL were seeded on 2.0% MEA medium supplemented with 50 $mg.L^{-1}$ Chloramphenicol and on DG-18 medium at 27 °C, for 7 days, for the growth of filamentous fungi (Viegas *et al.*, 2012) and xerophilic fungi (Greco *et al.*, 2015; Greco *et al.*, 2014), respectively; and 150 μL were inoculated on TSA medium supplemented with 0.2% Nystatin at 30 °C and on VRBA medium at 35 °C, for 7 days, for the growth of mesophilic bacteria and total coliforms (Gram-negative bacteria), respectively (Ribeiro *et al.*, 2018; Viegas *et al.*, 2018c).

All samples (diets, sponge filters, and waters) were inoculated on media for Azole-resistance Screening, in which 150 μL of their suspensions were seeded on one SAB medium and on three SAB medium supplemented, respectively, with 4 $mg.L^{-1}$ Itraconazole (ITRA), 1 $mg.L^{-1}$ Voriconazole (VORI), and 500 $\mu g.L^{-1}$ Posaconazole (POSA). These SAB media supplemented with Azole Antifungals were incubated at 27 °C, for 7 days, to evaluate the susceptibility or non-susceptibility of many fungal species (Viegas *et al.*, 2018c).

After incubation, the bacterial and fungal densities (Colony-forming Units, CFU/m^2/g/mL) were determined through their growth on their specific media. The fungal species were identified morphologically through the analysis of their macro and microscopic characteristics (Hoog *et al.*, 2000; Viegas *et al.*, 2015; Viegas *et al.*, 2012).

This technique was performed through a partnership with the H&TRC of the IPL, who provided the results obtained, so that this work could be developed.

4.4. Molecular Detection of Specific *Aspergillus* Sections

Molecular detection of *Aspergillus* sections (*Flavi*, *Fumigati*, *Circumdati*, and *Versicolores*) was performed since previous studies reported an increased prevalence in feed for animal production from Portuguese swine (Viegas *et al.*, 2018b) and in one feed industry that supply several Portuguese poultry industries (Viegas *et al.*, 2016).

Molecular identification of the different fungal species/strains was achieved by Real-Time PCR (qPCR) using the Via 7 Real-time PCR System (Applied Biosystems) on all samples collected. Reactions included 1× iQ™ Supermix (Bio-Rad), 500 nM of each primer (Table 2), and 375 nM of TaqMan™ probe in a total volume of 10 µL. Amplification followed a three-step PCR: 50 cycles with denaturation at 95 °C for 30 s, annealing at 52 °C for 30 s, and extension at 72 °C for 30 s. A non-template control was used in every PCR reaction. As positive controls of amplification, DNA samples were obtained from reference strains from the Mycology Laboratory of the Instituto Nacional de Saúde Doutor Ricardo Jorge (INSA) (Viegas *et al.*, 2014; Viegas *et al.*, 2015).

This technique was performed through a partnership with the H&TRC of the IPL, who provided the results obtained, so that this work could be developed.

Table 2 – Sequence of primers and TaqMan^{TM} probes used for Real-Time PCR.

***Aspergillus* Sections Targeted**	**Sequence**	**Reference**
Flavi (Toxigenic Strains)		
Forward Primer	5' – GTCCAAGCAACAGGCCAAGT – 3'	(Mayer *et al.*, 2003)
Reverse Primer	5' – TCGTGCATGTTGGTGATGGT – 3'	
Probe	5' – TGTCTTGATCGGCGCCCG – 3'	
Fumigati		
Forward Primer	5' – CGCGTCCGGTCCTCG – 3'	(Cruz-Perez *et al.*, 2001)
Reverse Primer	5' – TTAGAAAAATAAAGTTGGGTGTCGG – 3'	
Probe	5' – TGTCACCTGCTCTGTAGGCCCG – 3'	
Circumdati		
Forward Primer	5' – CGGGTCTAATGCAGCTCCAA – 3'	(Viegas *et al.*, 2017a)
Reverse Primer	5' – CGGGCACCAATCCTTTCA – 3'	
Probe	5' – CGTCAATAAGCGCTTTT – 3'	
Versicolores		
Forward Primer	5' – CGGCGGGGAGCCCT – 3'	(EPA, 2014)
Reverse Primer	5' – CCATTGTTGAAAGTTTTGACTGATCTTA – 3'	
Probe	5' – AGACTGCATCACTCTCAGGCATGAAGTTCAG – 3'	

4.5. Mycotoxin Analysis

Nineteen samples were screened for mycotoxins presence. Water samples (100 µL) were directly diluted 1:7 (v/v) with a mixture of Acetonitrile (ACN): Water (H_2O): Acetic Acid (AcOH) (39.5:60:0.5). Samples of diets and sponge filters (500 mg) were shaken with 2.0 mL of ACN: H_2O: AcOH (79:20:1) for 60 minutes (Viegas *et al.*, 2017b).

Raw extracts were diluted with the same amount of water, mixed, centrifuged and injected into the LC-MS/MS system. Detection of mycotoxins was carried out using HPLC Nexera (Shimadzu, Tokyo, Japan) with a mass detector of Atmospheric Pressure Ionisation (API) 4,000 (Sciex, Foster City, CA, USA) (Viegas *et al.*, 2017b).

Mycotoxins were separated on a Chromatographic Column Gemini NXC18 (150×4.6 mm, 3 µm) (Phenomenex, Torrance, CA, USA); mobile phase (A: H_2O + 5 mM Ammonium Acetate + 1% AcOH; B: Methanol + 5 mM Ammonium Acetate + 1% AcOH) mobile phase flow rate: 750 $\mu L.min^{-1}$, injection volume: 7 µL (Viegas *et al.*, 2017b).

The mycotoxin concentration was calculated using external calibration and standard solutions of available compounds (Viegas *et al.*, 2017b). Thirty-six mycotoxins were assessed, namely: Patulin, Nivalenol, Deoxynivalenol-3-glucoside, Deoxynivalenol, Fusarenon-X, α-Zearalanol, β-Zearalanol, β-Zearalenol, α-Zearalenol, Zearalanone, Zearalenone, T-2 tetraol, Deepoxy-deoxynivalenol, Neosolaniol, 15-Acetyldeoxynivalenol, 3-Acetyldeoxynivalenol, Monoacetoxyscirpenol,

Diacetoxyscirpenol, Aflatoxin M_1, Aflatoxin B_1, Aflatoxin B_2, Aflatoxin G_1, Aflatoxin G_2, Fumonisin B_1, Fumonisin B_2, Fumonisin B_3, T-2 triol, Roquefortine C, Griseofulvin, T-2 toxin, HT-2 toxin, Ochratoxin A, Ochratoxin B, Mycophenolic acid, Mevinolin, and Sterigmatocystin.

The Limits of Detection (LOD) and Quantification (LOQ) obtained for each mycotoxin, with the analytical method used, are presented in Table 3 and were estimated from mycotoxins standards spiked into a blank feed extract. The values of LOD and LOQ were calculated based on Signal-to-noise (S/N) Ratios of 3:1 and 10:1, respectively, using the Analyst 1.6.2 Software. The Matrix Effects (ME) reveals the effect of matrix on the way the analysis was conducted and on the quality of the results obtained, and is presented in Table 3. ME were calculated by comparison of the slopes obtained from analytical curves prepared in pure solvent and in blank feed extract using the following equation (Rodríguez-Carrasco *et al.*, 2016):

$$ME\ (\%) = \frac{slope\ curve\ in\ feed\ matrix}{slope\ curve\ in\ pure\ solvent} \times 100$$

This technique was performed through a partnership with the Department of Physiology and Toxicology of the Kazimierz Wielki University, in Bydgoszcz, Poland, who provided the results obtained, so that this work could be developed.

Table 3 – Limits of Detection (LOD), Limits of Quantification (LOQ), and Matrix Effects (ME) for mycotoxins and their metabolites in blank feed matrix.

Mycotoxins	Limit of Detection, LOD (μg.Kg^{-1})	Limit of Quantification, LOQ (μg.Kg^{-1})	ME (%)
Patulin	1.1	3.6	88.9
Nivalenol	4.5	14.9	84.7
Deoxynivalenol-3-glucoside	5.4	17.8	87.3
Deoxynivalenol	2.7	8.9	85.4
Fusarenon-X	4.8	15.8	80.7
Deepoxy-deoxynivalenol	4.2	14.0	81.5
α-Zearalanol	2.0	6.6	77.1
β-Zearalanol	0.9	3.0	78.1
β-Zearalenol	1.4	4.6	77.5
α-Zearalenol	1.0	3.3	78.9
Zearalanone	0.5	1.7	76.4
Zearalenone	0.2	0.7	79.0
T-2 tetraol	5.4	17.8	100.6
Neosolaniol	0.1	0.3	97.6
15-Acetyldeoxynivalenol	0.8	2.6	87.4
3-Acetyldeoxynivalenol	0.8	2.6	89.0
Monoacetoxyscirpenol	0.1	0.3	92.4
Diacetoxyscirpenol	0.3	1.0	97.3
Aflatoxin M_1	0.1	0.3	83.0
Aflatoxin B_1	0.1	0.3	80.9
Aflatoxin B_2	0.1	0.3	89.1
Aflatoxin G_1	0.1	0.3	82.4
Aflatoxin G_2	0.1	0.3	82.9
Fumonisin B_1	0.5	1.7	105.9
Fumonisin B_2	0.4	1.3	106.4
Fumonisin B_3	0.5	1.7	103.5
T-2 triol	0.3	1.0	95.2
Roquefortine C	0.2	0.7	82.2
Griseofulvin	0.1	0.3	91.8
T-2 toxin	0.1	0.3	93.8
HT-2 toxin	0.3	1.0	96.9
Ochratoxin A	0.1	0.3	99.6
Ochratoxin B	0.1	0.3	114.2
Mycophenolic Acid	0.2	0.7	110.8
Mevinolin	0.1	0.3	107.8
Sterigmatocystin	0.1	0.3	113.2

4.6. Data Analysis

The provided data were managed using the Software Microsoft Office Excel. Frequency analysis (n; %) were executed for qualitative and quantitative data, respectively.

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5. RESULTS

5.1. Bacterial Contamination

The mesophilic bacteria (growth on TSA medium supplemented with antifungal Nystatin) were found in all types of samples. They were detected in 6 out of 7 (85.7%) diet samples (Figure 1), being jellified pellet D5 and frozen microalga D6 the most contaminated, with more than 10^3 CFU.g^{-1}; followed by frozen microalga D7 with 8.1x10^2 CFU.g^{-1}, jellified pellet D4 with 2.6x10^2 CFU.g^{-1}, jellified pellet D3 with 1.6x10^1 CFU.g^{-1}, extruded pellet D2 with 1.1x10^1 CFU.g^{-1}, and extruded pellet D1 with no detection (Table 4).

With respect to sponge filter samples, 3 out of 4 (75.0%) were contaminated (Figure 1) with more than 10^3 CFU.m^{-2} and there was no detection in sample F2 (Table 4).

The mesophilic bacteria were found in 5 out of 8 (62.5%) water samples (Figure 1), being samples W2 and W3 the most contaminated, with more than 10^3 CFU.mL^{-1}; followed by water sample W5 with 5.7x10^2 CFU.mL^{-1}, water sample W8 with 1.5x10^2 CFU.mL^{-1}, water sample W7 with 1.3x10^2 CFU.mL^{-1}, and water samples W1, W4, and W6 with no detection (Table 4).

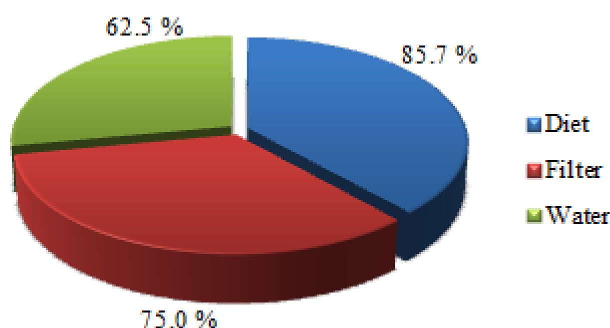

Figure 1 – Percentage of mesophilic bacteria present in samples of diets, sponge filters, and waters inoculated on TSA medium.

The total coliforms (growth of Gram-negative bacteria on VRBA medium) were uniquely found in diet samples (Figure 2), in which 2 out of 7 (28.6%) were contaminated, more precisely the extruded pellet D1, with 1 CFU.g^{-1}, and the frozen microalga D7, with 4 CFU.g^{-1} (Table 4).

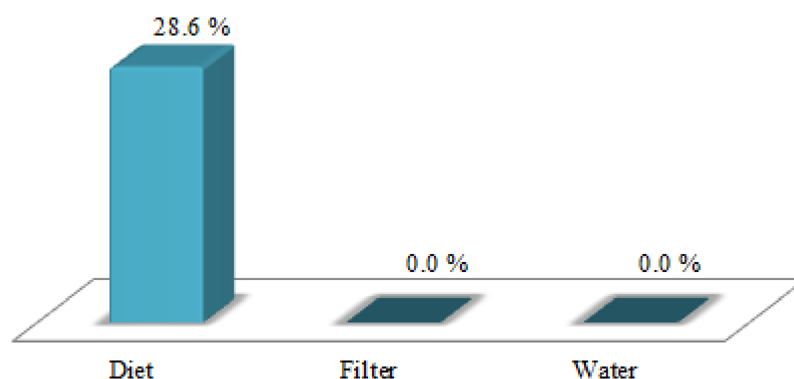

Figure 2 – Percentage of total coliforms present in samples of diets, sponge filters, and waters inoculated on VRBA medium.

Table 4 – Bacterial densities present in samples of diets (CFU.g^{-1}), sponge filters (CFU.m^{-2}), and waters (CFU.mL^{-1}).

Sample Reference	Sample Characterisation	Mesophilic Bacteria	Total Coliforms
D1	Extruded pellet: 60% fishmeal, 10% fish oil, 10% corn meal, 5% wheat gluten, 5% soy protein concentrate, 5% pea meal, 5% sunflower meal	<1	1
D2	Extruded pellet: 40% fishmeal, 20% fish oil, 10% corn meal, 10% wheat gluten, 10% soy protein concentrate, 5% pea meal, 5% sunflower meal	$1.1x10^1$	<1
D3	Jellified pellet: 47% canned corn, 47% fresh spinach, 6% agar	$1.6x10^1$	<1
D4	Jellified pellet: 22% canned corn, 22% fresh spinach, 50% acorn, 6% agar	$2.6x10^2$	<1
D5	Jellified pellet: 22% canned corn, 22% fresh spinach, 50% pumpkin, 6% agar	$>10^3$	<1
D6	Frozen microalgae: microalga *Tetraselmis* sp.	$>10^3$	<1
D7	Frozen microalgae: diatom *Phaeodactylum tricornutum*	$8.1x10^2$	4
F1	Sponge filter of sea cucumber system	$>10^3$	<1
F2	Sponge filter of sea urchin tanks fed with the diet D3	<1	<1
F3	Sponge filter of sea urchin tanks fed with the diet D5	$>10^3$	<1
F4	Sponge filter of sea urchin tanks fed with the diet D4	$>10^3$	<1
W1	Water collected before entering the rearing tanks	<1	<1
W2	Water collected in rearing tank of sea cucumbers fed with the diets D1 and D2	$>10^3$	<1
W3	Water collected in rearing tank of sea cucumbers fed with the diet D7	$>10^3$	<1
W4	Water collected in rearing tank of sea cucumbers fed with the diet D6	<1	<1
W5	Water collected after passing the sponge filter common to three rearing tanks of sea cucumbers fed with the diets D1, D2, D6, and D7	$5.7x10^2$	<1
W6	Water collected after passing the sponge filter of three rearing tanks of sea urchins fed with the diet D3	<1	<1
W7	Water collected after passing the sponge filter of three rearing tanks of sea urchins fed with the diet D5	$1.3x10^2$	<1
W8	Water collected after passing the sponge filter of three rearing tanks of sea urchins fed with the diet D4	$1.5x10^2$	<1

5.2. Fungal Contamination

The filamentous fungi (growth on MEA medium supplemented with antibiotic Chloramphenicol) were found only in diet samples (2 out of 7, i.e. 28.6%), jellified pellet D4 and frozen microalga D6, in which *Fusarium verticillioides* was the fungal species identified, with 1 $CFU.g^{-1}$ in each one (Table 5).

The xerophilic fungi (growth on DG-18 medium) were found in one diet sample, one sponge filter sample, and one water sample (Table 5). *Fusarium culmorum* was detected in frozen microalga D6, with 1 $CFU.g^{-1}$. The genera *Penicillium* and *Aureobasidium* were both present in the water sample W1, with 1 $CFU.mL^{-1}$; and the *Cladosporium* genus was found in the sponge filter sample F2, with 1 $CFU.m^{-2}$.

Regarding to the Azole-resistance Screening, on SAB medium, *Fusarium verticillioides* was identified in the frozen microalga D7, with 1 $CFU.g^{-1}$, and *Penicillium* sp. was identified in the water sample W4, with 1 $CFU.mL^{-1}$. On SAB medium supplemented with 4 $mg.L^{-1}$ ITRA, *F. verticillioides* was identified in the frozen microalga D6, counting with 1 $CFU.g^{-1}$.

The *Aspergillus* sections (*Flavi*, *Fumigati*, *Circumdati*, and *Versicolores*) were not identified by molecular tools used.

Table 5 – Fungal burden present in samples of diets, sponge filters, and waters.

Sample Reference	Sample Characterisation	Filamentous Fungi	Xerophilic Fungi
D1	Extruded pellet: 60% fishmeal, 10% fish oil, 10% corn meal, 5% wheat gluten, 5% soy protein concentrate, 5% pea meal, 5% sunflower meal	-	-
D2	Extruded pellet: 40% fishmeal, 20% fish oil, 10% corn meal, 10% wheat gluten, 10% soy protein concentrate, 5% pea meal, 5% sunflower meal	-	-
D3	Jellified pellet: 47% canned corn, 47% fresh spinach, 6% agar	-	-
D4	Jellified pellet: 22% canned corn, 22% fresh spinach, 50% acorn, 6% agar	*Fusarium verticillioides*	-
D5	Jellified pellet: 22% canned corn, 22% fresh spinach, 50% pumpkin, 6% agar	-	-
D6	Frozen microalgae: microalga *Tetraselmis* sp.	*Fusarium verticillioides*	*Fusarium culmorum*
D7	Frozen microalgae: diatom *Phaeodactylum tricornutum*	-	-
F1	Sponge filter of sea cucumber system	-	-
F2	Sponge filter of sea urchin tanks fed with the diet D3	-	*Cladosporium* sp.
F3	Sponge filter of sea urchin tanks fed with the diet D5	-	-
F4	Sponge filter of sea urchin tanks fed with the diet D4	-	-
W1	Water collected before entering the rearing tanks	-	*Aureobasidium* sp. *Penicillium* sp.
W2	Water collected in rearing tank of sea cucumbers fed with the diets D1 and D2	-	-
W3	Water collected in rearing tank of sea cucumbers fed with the diet D7	-	-
W4	Water collected in rearing tank of sea cucumbers fed with the diet D6	-	-
W5	Water collected after passing the sponge filter common to three rearing tanks of sea cucumbers fed with the diets D1, D2, D6, and D7	-	-
W6	Water collected after passing the sponge filter of three rearing tanks of sea urchins fed with the diet D3	-	-
W7	Water collected after passing the sponge filter of three rearing tanks of sea urchins fed with the diet D5	-	-
W8	Water collected after passing the sponge filter of three rearing tanks of sea urchins fed with the diet D4	-	-

5.3. Mycotoxin Contamination

The mycotoxin contamination only occurred in diet samples. The two extruded pellets D1 and D2 had present five to six mycotoxins, respectively; and the jellified pellet D4 contained merely one mycotoxin, the Mycophenolic Acid (MPA) (Table 6).

Therefore, in detail, the extruded pellet D1 had five mycotoxins (DON, ZEA, FB_1, Fumonisin B_2 or FB_2, and MPA), in which the concentration of ZEA was of 2.10 $\mu g.Kg^{-1}$ and the rest of mycotoxins were below of the LOQ, being only within the LOD. The extruded pellet D2 had six mycotoxins (DON, ZEA, FB_1, FB_2, T-2 toxin, and

OTA), being the diet sample with greater mycotoxin contamination. And the jellified pellet D4 had MPA within the LOD, but below of the LOQ.

Regarding to the extruded pellet D2, DON was the most prevalent mycotoxin (Figure 3), meaning that had the greater values, with 62.99 $\mu g.Kg^{-1}$. Furthermore, also ZEA (14.38 $\mu g.Kg^{-1}$), FB_1 (7.51 $\mu g.Kg^{-1}$), FB_2 (14.66 $\mu g.Kg^{-1}$), T-2 toxin (2.51 $\mu g.Kg^{-1}$), and OTA (0.94 $\mu g.Kg^{-1}$) presented values within the LOQ.

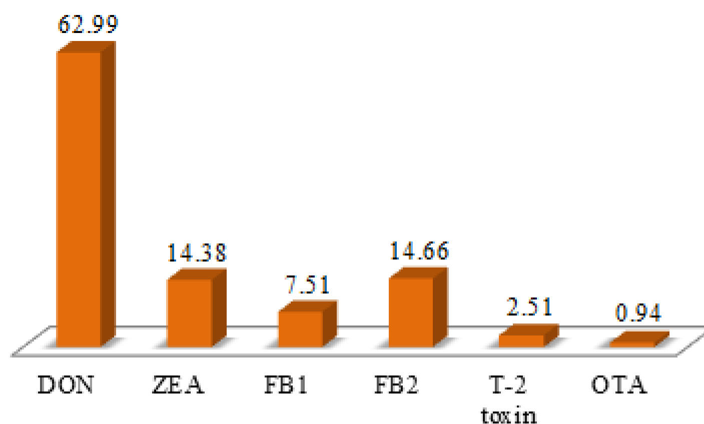

Figure 3 – Concentrations of mycotoxins present in the extruded pellet D2, with the following units: $\mu g.Kg^{-1}$.

Table 6 – Distribution of fungal species and mycotoxin contamination in samples of diets, sponge filters, and waters.

Sample Reference	Sample Characterisation	Fungal Species	Mycotoxins
D1	Extruded pellet: 60% fishmeal, 10% fish oil, 10% corn meal, 5% wheat gluten, 5% soy protein concentrate, 5% pea meal, 5% sunflower meal	-	DON, ZEA, FB_1, FB_2, MPA
D2	Extruded pellet: 40% fishmeal, 20% fish oil, 10% corn meal, 10% wheat gluten, 10% soy protein concentrate, 5% pea meal, 5% sunflower meal	-	DON, ZEA, FB_1, FB_2, T-2 toxin, OTA
D3	Jellified pellet: 47% canned corn, 47% fresh spinach, 6% agar	-	-
D4	Jellified pellet: 22% canned corn, 22% fresh spinach, 50% acorn, 6% agar	*Fusarium verticillioides*	MPA
D5	Jellified pellet: 22% canned corn, 22% fresh spinach, 50% pumpkin, 6% agar	-	-
D6	Frozen microalgae: microalga *Tetraselmis* sp.	*Fusarium culmorum* *Fusarium verticillioides*	-
D7	Frozen microalgae: diatom *Phaeodactylum tricornutum*	-	-
F1	Sponge filter of sea cucumber system	-	-
F2	Sponge filter of sea urchin tanks fed with the diet D3	*Cladosporium* sp.	-
F3	Sponge filter of sea urchin tanks fed with the diet D5	-	-
F4	Sponge filter of sea urchin tanks fed with the diet D4	-	-
W1	Water collected before entering the rearing tanks	*Aureobasidium* sp. *Penicillium* sp.	-
W2	Water collected in rearing tank of sea cucumbers fed with the diets D1 and D2	-	-
W3	Water collected in rearing tank of sea cucumbers fed with the diet D7	-	-
W4	Water collected in rearing tank of sea cucumbers fed with the diet D6	-	-
W5	Water collected after passing the sponge filter common to three rearing tanks of sea cucumbers fed with the diets D1, D2, D6, and D7	-	-
W6	Water collected after passing the sponge filter of three rearing tanks of sea urchins fed with the diet D3	-	-
W7	Water collected after passing the sponge filter of three rearing tanks of sea urchins fed with the diet D5	-	-
W8	Water collected after passing the sponge filter of three rearing tanks of sea urchins fed with the diet D4	-	-

6. DISCUSSION

The food industry has the seafood/fish products as the most commercialised food in worldwide. Nevertheless, the reduction of fishery efforts on wild fish stocks is tremendously important for the recovery of natural resources (Ismail *et al.*, 2017; Viegas *et al.*, 2018a submitted). Thereby, the aquaculture activity comes to allow to support the demand of world population that is expanding, being its growth essential for sustainability of sea stocks (Gonçalves & Gagnon, 2011; Palm *et al.*, 2015).

The feed safety is truly important to economic activity of aquaculture, given that it has a great influence on animal productivity (Almeida *et al.*, 2011; Kumar *et al.*, 2013). The feed demand for aquaculture has been increasing. The legal restrictions on utilisation of meat and bone meals for aquafeed formulations, specifically in the European Union (EU), conduct to an effort of use of more vegetable ingredients in their formulations (Almeida *et al.*, 2011). Plant feed materials increase the risk of developing bacterial burden in aquafeeds (Correia, 2015; Ebeneezar *et al.*, 2018), as well as the risk of fungal burden (Almeida *et al.*, 2011; Hooft & Bureau, 2017; Kumar *et al.*, 2013).

The contamination of feeds is a concern for animal and humans, because is a potential path of transmission of diseases (Maciorowski *et al.*, 2007). Any failure in manufacturing practices of feeds, such as the effectiveness of the heat treatments used or storage conditions, inevitably lead to an increase the bioburden, which affects the durability of food preservation (Correia, 2015).

The seafood/fish products for human consumption have been associated to foodborne illness outbreaks and infections caused by pathogenic bacteria (Chanpiwat *et al.*, 2016; Sapkota *et al.*, 2008). The most investigated bacteria in food are the mesophilic bacteria, which allow knowing the spoilage present in foods, and total coliforms that indicate the lack of hygiene care (Correia, 2015).

With respect to bacteriota, two media were used for bacterial burden, in which the TSA medium supplemented with the antifungal Nystatin allowed the development of mesophilic bacteria that are characterised by having an optimal growth temperature ranging 30 and 37 °C, encompassing the most of the bacteria that are pathogenic to humans, being a threat to Public Health. However, the mesophilic bacteria can growth in the temperature range of 20 to 45 °C (Correia, 2015). The antifungal Nystatin in this medium is used to inhibit several fungal species, making it more selective for the

bacteria concerned (Modrzewska *et al.*, 2017). Whereas the VRBA medium allows the development of total coliforms that are abundant in also warm-blooded animal faeces and rarely origin sickness, being often used as indicators of microbial co-contaminating occurrence of enteropathogenic bacteria (Bej *et al.*, 1990; Divya & Solomon, 2016; Ebeneezar *et al.*, 2018; González *et al.*, 2003; Maciorowski *et al.*, 2007; Molina *et al.*, 2015).

For mesophilic bacteria found in this study, a large number of samples (14 out of 19) were contaminated, indicating some decomposition activity (Correia, 2015; Santos *et al.*, 2005). Thereby, only one diet sample, one sponge filter sample, and three water samples were not contaminated.

Regarding to extruded pellets that were preserved at ambient temperature, the sample D2 ($1.1x10^{1}$ $CFU.g^{-1}$) had mesophilic bacteria, while the sample D1 (<1 $CFU.g^{-1}$) did not have any contamination. The fishmeal present in formulation of extruded pellet D2 may have contributed to the spoilage activity (Udhayavel *et al.*, 2017) and also the presence of wheat gluten and soy protein concentrate. Grains have been often detected with diverse microflora with concentrations between $5x10^{3}$ and $1.6x10^{8}$ $CFU.g^{-1}$ and the bacterial populations mostly isolated are the genera *Pseudomonas*, *Bacillus*, *Flavobacterium*, *Streptococcus*, *Enterobacter*, *Escherichia*, *Salmonella*, and *Clostridium*. The plant material is normally inoculated during crops pre-harvest (Maciorowski *et al.*, 2007).

The extruded pellets have lower moisture content than the other diets, which induces a lesser spoilage activity and, consequently, allows a major storage time. However, drying process may not completely remove the pathogenic populations (Maciorowski *et al.*, 2007) and, in fact, the extruded pellet D2 was contaminated with mesophilic bacteria, but with lowers concentrations than the others diet samples.

Furthermore, the fact that sample D2 is stored at ambient temperature and had a major storage time than sample D1 may have contributed to the bacterial growth (Carvalho *et al.*, 2000; Maciorowski *et al.*, 2007). One of the strategies that bacteria use to survive in animal feeds under storage conditions are the sporulation cycles (*Clostridium perfringens* and *C. botulinum*), resistance to desiccation (*Escherichia coli*), or even mechanism to resist acid (*Listeria monocytogenes*), which allows the colonisation of animal intestines for their posterior development (Maciorowski *et al.*, 2007).

The extruded pellets D1 and D2 were both used in sea cucumber tanks and the water that was collected from these rearing tanks (water sample W2) had upper than 10^3 $CFU.mL^{-1}$; thereby, the contamination of mesophilic bacteria in this water sample was probably originated by the extruded pellet D2 that was the only one contaminated.

About frozen microalgae that were preserved at -20 °C in the freezer, the sample D6 had upper than 10^3 $CFU.g^{-1}$ and the sample D7 had $8.1x10^2$ $CFU.g^{-1}$. In both were detected mesophilic bacteria, which can be related to the possible temperature fluctuations during transport or storage that may have allowed the occurrence of an alteration on bacterial flora, having been possible their development when the temperatures were favourable for their growth (Correia, 2015; Heyndrickx, 2011).

Frozen microalgae D6 and D7 were utilised in other two sea cucumber tanks. The water collected from rearing tank fed with diet D6 (water sample W4) had no detection (<1 $CFU.mL^{-1}$), which may be due to an efficient feeding conversion, so did not occur dissemination of feed contamination to tank water (Tidwell, 2012). With regard to the water collected from rearing tank fed with diet D7 (water sample W3), it had amounts of mesophilic bacteria higher than 10^3 $CFU.mL^{-1}$, which suggests the transfer of bacteria from aquafeeds to water column (Summerfelt *et al.*, 2004).

The sponge filter (biofilter) of sea cucumber system (sample F1) that receives the water from three sea cucumber tanks, mentioned above, had a concentration upper than 10^3 $CFU.m^{-2}$, being also in accordance with the remaining results. It should be taken into account that biofilters naturally have bacterial populations that grow at system temperature (20.5 °C), which allow the nitrification processes in an aerobic environment, removing nitrogen compounds. However, the pathogenic bacteria may also be within RAS (Summerfelt *et al.*, 2004). In this study, it was evaluated the presence of mesophilic bacteria that are a concern for human health, because of their optimal temperature growth. These results obtained only allow to estimate the bacterial retention that is allowed by the sponge filter, since water was collected before and after passing the sponge filter. Thus, the water collected after passing the sponge filter common to the three sea cucumber tanks that were fed with extruded pellets and frozen microalgae (water sample W5) had concentrations of mesophilic bacteria of $5.7x10^2$ $CFU.mL^{-1}$, which reveals that the efficiency of sponge filter (sample F1) on removal of bacteria was not effective. Possibly, it was overloaded due to the accumulation of particles from uneaten food and faeces, decreasing its efficacy and providing the

proliferation of bacteria in RAS, due to the presence of organic matter that is an energy source (Wold *et al.*, 2014), but was able to eliminate a large portion of them.

For greater efficiency in their removal, minimising the bacterial densities present, probably the addition of more sponge filters, one to each rearing tank, or even the implementation of an advanced water treatment in RAS, like MBR (Holan *et al.*, 2014; Sharrer *et al.*, 2007; Wold *et al.*, 2014), followed by Ozonation (Aruety *et al.*, 2016; Gonçalves & Gagnon, 2011) and UV Irradiation (Sharrer & Summerfelt, 2007; Summerfelt *et al.*, 2004). These water treatments have been used in RAS for more effectiveness in removal of bacteria from the rearing water, recycling it, improving the water quality, and ensuring the health of farmed animals (Aruety *et al.*, 2016; Gonçalves & Gagnon, 2011; Holan *et al.*, 2014; Martins *et al.*, 2010; Sharrer *et al.*, 2007; Summerfelt *et al.*, 2004; Wold *et al.*, 2014).

In relation to jellified pellets that were maintained in the fridge, samples D4 and D5 had less 25% of canned corn and less 25% of fresh spinach than sample D3 ($1.6x10^1$ $CFU.g^{-1}$); in addition, sample D4 was composed by 50% of acorn ($2.6x10^2$ $CFU.g^{-1}$) and sample D5 by 50% of pumpkin ($>10^3$ $CFU.g^{-1}$). The moisture content of these diets may have to led to the development of mesophilic bacteria (Hyun *et al.*, 2018; Maciorowski *et al.*, 2007), given that these are semi-moist diets and, therefore, have higher water activity (Maciorowski *et al.*, 2007). It is also possible that was occurred temperature fluctuations during transport that may have permitted the growth of mesophilic bacteria on favourable temperature (Correia, 2015; Heyndrickx, 2011). Besides this, the maize has been reported with bacterial contamination by *Escherichia coli*, *Listeria monocytogenes*, and the *Salmonella* genus. And *L. monocytogenes* has also been found in improperly canned corn (Maciorowski *et al.*, 2007). Furthermore, the greater amounts of mesophilic bacteria found in jellified pellet D5 suggest that possibly the pumpkin offered higher bacterial load (Hyun *et al.*, 2018).

The rearing waters from sea urchin tanks were always collected after passing the sponge filter corresponding to a system of three rearing tanks. The jellified pellet D3 was used in the tank water referenced as sample W6 (<1 $CFU.mL^{-1}$), which did not present any contamination; and the sponge filter corresponding to this system was the sample F2 (<1 $CFU.m^{-2}$) that was also not contaminated, being all these results in agreement. Nevertheless, if the nitrifying aerobic bacteria were not present in sufficient quantity or are not feasible, the production system can be compromised and the nitrification process is not complete, which can compromise the water quality,

becoming a harmful and toxic to the health of reared species. In this case, other filtration process has to be used (Zhu *et al.*, 2016).

The jellified pellet D4 was used in tank water referenced as sample W8, having a contamination of mesophilic bacteria of $1.5x10^2$ $CFU.mL^{-1}$, and the sponge filter corresponding to this system was the sample F4 with more than 10^3 $CFU.m^{-2}$; which seems to reveal a satisfactory efficiency of the sponge filter used. The jellified pellet D5 was used in tank water referenced as sample W7, presenting $1.3x10^2$ $CFU.mL^{-1}$, and the sponge filter corresponding to this system was sample F3 that had upper than 10^3 $CFU.m^{-2}$. Therefore, in general, the sponge filters used seem to have an acceptable performance in the recirculating systems, given that the results obtained had higher bacterial load than the achieved for samples of diets and rearing waters.

The higher concentrations of mesophilic bacteria found in diet samples of this study belonged to the jellified pellet D5 and the frozen microalga D6 that had concentrations greater than 10^3 $CFU.g^{-1}$. According to Santos *et al.* (2005), the levels of mesophilic bacteria in some feed ingredients below to 10^4 $CFU.g^{-1}$ are considered satisfactory (Santos *et al.*, 2005). Therefore, it is not known if these diet samples would be inferior to 10^4 $CFU.g^{-1}$ to be below these established values, but it seems that the moisture content and the transport conditions were the major contributors for their contaminations, respectively (Correia, 2015; Maciorowski *et al.*, 2007). However, the remaining diet samples analysed can be considered satisfactory results, suggesting that have a good microbiological quality.

Besides that, the results obtained for water samples, except for samples W2 and W3 ($>10^3$ $CFU.mL^{-1}$), were below to the results obtained in other studies that are reported by Gorlach-Lira *et al.* (2013), in which the culture water had values in the order of 10^4 to 10^6 $CFU.mL^{-1}$ (Gorlach-Lira *et al.*, 2013). The bacterial contamination observed in tank waters of this study may also be caused by failure to clean and disinfect sump tanks (Carvalho *et al.*, 2000).

There is a gap with regard to legislation on mesophilic bacteria. There are no defined concentrations for animal feed, indicating the need of more research to establish these limits. However, the limits admitted in some foodstuff are according to the bacterial species, which was not identified in this study. For feed ingredients that can be used in aquafeeds, limits only exist for *Listeria monocytogenes* present in foodstuffs that would allow them to grow and the results of microbiological test are considered satisfactory if all observed values were below than 100 $CFU.g^{-1}$, in the case of the

manufacturer demonstrates that the product will not exceed that limit until the end of useful life; or 0 CFU.25 g^{-1}, if the manufacturer cannot demonstrate that the product will not exceed the limit of 100 CFU.g^{-1} until the end of useful life. For *Salmonella* present in vegetable products, the results are considered satisfactory if all observed values were 0 CFU.25 g^{-1} (CE, 2007). In relation to legislation of water, the concentrations of water supply for food industry should be 0 CFU.100 mL^{-1} for *Clostridium perfringens* and its spores (DR, 2017).

Beyond that in only 2 out of 19 samples were detected total coliforms, in which both two are diets. More specifically, the extruded pellet D1 that had only 1 CFU.g^{-1} and the frozen microalga D7 that had 4 CFU.g^{-1}. For sample D1, the bacterial load may have arisen from cereal grains (Maciorowski *et al.*, 2007) and, with regard to sample D7, probably occurred a contamination during the sampling or sample processing. However, these values are considered very low, because commonly total coliform bacterial counts less than 10^2 CFU.g^{-1} are considered acceptable in some foodstuffs (Arthur *et al.*, 2011; Santos *et al.*, 2005), having been results very acceptable and within the established limits.

The enumeration of total coliforms on VRBA medium not allow to distinguish the presence of *Escherichia coli* from other coliforms (Arthur *et al.*, 2011; González *et al.*, 2003), needing of additional tests to determine it (González *et al.*, 2003). This was not considered necessary given the good results obtained that were all values very below of the established limits, being acceptable and insurance. Therefore, the requirements of production and storage of diets must have been controlled and adequate. The hygiene care must have been maintained throughout the processing and probably there was no post-process contamination on diets (Arthur *et al.*, 2011; Correia, 2015; González *et al.*, 2003).

There is also a gap with regard to legislation on total coliforms. In relation to animal feed, there are no defined limits, indicating the need of more research to establish these limits. For feed ingredients used in aquafeeds, limits only exist for *Escherichia coli* in vegetable products, in which a minimum of five sample units must be collected and the results of microbiological test considered satisfactory shall be less than 100 CFU.g^{-1}. The results may also be considered acceptable, if, in the case of five units, two to be between 100 and 1.000 CFU.g^{-1} and the other three shall be less than 100 CFU.g^{-1} (CE, 2007). *E. coli* is an indicator of faecal contamination. Regarding to

water legislation, the concentrations of water supply for food industry should be of 0 CFU.100 mL^{-1} for total coliforms, *Enterococcus*, and *Escherichia coli* (DR, 2017).

Animal feeds have been contaminated by *Salmonella* that is regularly found in cereal ingredients, being *Salmonella* Typhimurium (STM) the most common in wheat and barley (Davies & Wales, 2013). Other bacteria that are involved in spoilage of vegetables belong to the genera *Erwinia* and *Pseudomonas* (Correia, 2015). A high bacterial proportion can be killed through heat treatments used in feed processing, but is common the recontamination of feeds during their cooling and storage (Davies & Wales, 2013).

Udhayavel *et al.* (2017) analysed poultry feed ingredients and fishmeal had the highest contamination for *Clostridium perfringens*. Its incidence seems to be related to high protein contents of animal origin. The vegetable protein sources, such as maize, soybean meal, and rapeseed meal had the lowest level of *C. perfringens*. This bacterium provokes necrotic enteritis in poultry (Udhayavel *et al.*, 2017).

The bacteria are normally present in digestive flora of fish, but the accumulation of organic matter in aquatic environment decreases the water quality, increasing the susceptibility of aquatic animal to pathogenic bacteria (Huicab-Pech *et al.*, 2017). The opportunistic pathogens can origin disease in aquatic organisms under environmental stress (Blancheton *et al.*, 2013). The resistance to bacterial pathogenicity varies according to species and environmental conditions. Generally, *Aeromonas hydrophila* causes haemorrhagic septicaemia syndrome, weakness, anorexia, and vision loss, being known as an opportunistic pathogen of aquatic environments (Huicab-Pech *et al.*, 2017). *A. salmonicida* provokes furunculosis infection (Kim *et al.*, 2013). *Streptococcus agalactiae* origins meningoencephalitis and septicaemia in fish (Huicab-Pech *et al.*, 2017). *S. iniae* has been associated to morbidity and mortality of fish, infecting several farmed fish species (Aruety *et al.*, 2016). *Edwardsiella* sp. origins septicaemia in external and internal organs (Huicab-Pech *et al.*, 2017). The bacterial pathogen most prominent in aquaculture of finfish and shellfish is *Vibrio* sp. (Priyaja *et al.*, 2017). Several bacterial genera have impact in fish health and are also considered a threat to human health (Huicab-Pech *et al.*, 2017). Some bacterial pathogens that can bioaccumulate in muscle tissues of aquatic animals and that are human pathogens are the genera *Escherichia*, *Vibrio*, *Shigella*, *Enterococcus* (Sapkota *et al.*, 2008), *Campylobacter*, *Salmonella* (Sapkota *et al.*, 2008; Udhayavel *et al.*, 2017), *Clostridium* (Correia, 2015; Udhayavel *et al.*, 2017), and *Bacillus* (Correia, 2015).

According to the European Food Safety Authority (EFSA), in the year 2016, there were 246,307 confirmed cases of *Campylobacter* in humans, *Salmonella* had reported 94,530 cases, for *Yersinia* were 6,861 cases, Shiga toxin-producing *Escherichia coli* (STEC) infections were reported in 6,378 cases, and there were 2,536 confirmed cases with *Listeria* (EFSA, 2017).

The *Campylobacter* genus is one of the most often sources of human foodborne illness (Maciorowski *et al.*, 2007), originating abdominal pain, and is the main bacterium responsible for bacterial diarrhoea in the EU (Correia, 2015). Non-typhoid *Salmonella* spp. is one of the most regularly sources of foodborne illness in humans (Maciorowski *et al.*, 2007). *Salmonella* genus can originate gastroenteritis, including nausea, vomiting, diarrhoea, headache, fever, and abdominal pain (Maciorowski *et al.*, 2006). *Yersinia enterocolitica* can originate severe abdominal pain and diarrhoea, fever, throat inflammation, bloody stools, rash, nausea, headache, generalized weakness, joint pain, and vomiting (Correia, 2015). *Escherichia coli* can cause septicaemia, cellulitis, swollen head syndrome (Maciorowski *et al.*, 2007), gastroenteritis, and haemorrhagic colitis. *Listeria monocytogenes* causes meningeal infections, persistent fevers, nausea, vomiting, and diarrhoea (Correia, 2015). *Clostridium perfringens* and *C. botulinum* are linked to necrotic enteritis in poultry and bloat in primates. *C. botulinum* strains produce toxins that are associated to botulism in animals (Maciorowski *et al.*, 2007).

The pathogen transmission through contaminated feed ingredients used in animal feeds causes infections on farmed animals (Udhayavel *et al.*, 2017). Feed is a preferred pathway for bacteria, when they have favourable conditions for their development (Correia, 2015). Thus, moist and semi-moist feeds are more palatable to animals, but are more susceptible to spoilage (Parker, 2012). The chemical composition of feeds is altered according to bacterial growth, which causes visual, odoriferous, and palatability changes, due to the spoilage process (Correia, 2015). To avoid their deterioration and keep their best quality, they must be continuously maintained at low temperature (Parker, 2012). The national and international transport of contaminated feed mixing or animal feed ingredients increase the opportunity of more animals being exposed to contaminated feed (Maciorowski *et al.*, 2006), which results in economic losses and low production performance (Udhayavel *et al.*, 2017). The possibility of animals destined to human consumption be contaminated represents a threat for Public Health (Maciorowski *et al.*, 2006; Udhayavel *et al.*, 2017).

For fungal burden, it has been known that the risk of exposing the farmed species to fungi and mycotoxins have been amplified, due to the presence of cereal grains in formulated aquafeeds (Almeida *et al.*, 2011; Hooft & Bureau, 2017; Kumar *et al.*, 2013). The effects of multi-mycotoxins co-contamination on aquatic species still not very well-known (Gonçalves *et al.*, 2016; Viegas *et al.*, 2018a submitted). Aquafeeds have high cost due to the utilisation of fishmeal and fish oil in their formulations and, as a way of fighting the reducing availability of fish resources and the production costs, these two important feed materials have been substituted by plant protein ingredients and vegetable oils. Mainly, the replacement of fishmeal by plant-based ingredients increases the risk of mycotoxin contamination (Marijani *et al.*, 2017; Matejova *et al.*, 2017; Pietsch *et al.*, 2011; Pietsch *et al.*, 2013). Toxigenic fungi grow on diverse crops of cereals grains, essentially maize, wheat, and soybean and they naturally produced mycotoxins. Thereby, contaminated aquafeed affects health of aquatic organisms, through contamination of their tissue, representing a risk to food safety (Matejova *et al.*, 2017).

Regarding to mycobiota, two media were used for fungal growth, in which the MEA medium supplemented with the antibiotic Chloramphenicol allows the development of filamentous fungi and the antibiotic Chloramphenicol restricts a large spectrum of Gram-positive and Gram-negative bacteria (Viegas *et al.*, 2012); and DG-18 medium allows the development of xerophilic fungi (Greco *et al.*, 2015; Greco *et al.*, 2014) that are adapted to extreme conditions (Díaz-Valderrama *et al.*, 2017) and the antifungal Dichloran restricts the colony size of the fast-growing fungal genera, enabling the growth of different species and a broader characterisation of fungal load in highly contaminated matrices (Caetano *et al.*, 2018; Viegas *et al.*, 2018c).

In this study, only 4 out of 19 samples were contaminated with fungal burden, more specifically two diet samples (one jellified pellet and one frozen microalga), one sponge filter sample, and one water sample, demonstrating that, in general, the aquaculture production system is going well.

With respect to filamentous fungi, *Fusarium verticillioides* was found in the jellified pellet D4, with 1 $CFU.g^{-1}$, and in the frozen microalga D6, with 1 $CFU.g^{-1}$. The moisture content of these diets may have been a great influence on growth of moulds (Maciorowski *et al.*, 2007; Matejova *et al.*, 2017). The filamentous fungi have an excellent ability to secrete numerous enzymes, which gives them the ability to grow rapidly on simple substrates (Balabanova *et al.*, 2018). *F. verticillioides* is regularly

found in maize and normally develops during growth period in field (Matejova *et al.*, 2017). This species is multi-phytopathogenic extensively distributed in worldwide in association with cereals and also cereal-based food products (Deepa & Sreenivasa, 2017; Viegas *et al.*, 2018a submitted).

The contamination of *Fusarium verticillioides* in the jellified pellet D4 may has been provided by the presence of corn in its formulation and should be also taken into account that this is the only diet with acorn. It can be explained by several factors that seems to contribute to the development of moulds in crops, such as the mechanical damage, utilisation of pesticides, the damage by insects, spore load, substrate composition, and plant variety (Matejova *et al.*, 2017). Thus, must be considered the exposure of cereal crops to contamination and ensuing colonisation by fungi along different development phases of plant, during harvesting, transport, storage (Batista *et al.*, 2003), and, in the final phase, as a food product or as feed (Pinotti *et al.*, 2016).

While the detection of *Fusarium verticillioides* in the frozen microalga D6 may be occurred by a contamination during the sampling or sample processing.

The storage conditions may have influence on the growth of this moulds in these diets (Almeida *et al.*, 2011; Maciorowski *et al.*, 2007), given that the fungal growth frequently occurs when the storage conditions are poor (Gonçalves *et al.*, 2018b).

For xerophilic fungi, *Fusarium culmorum* was found in the frozen microalga D6, with 1 CFU.g^{-1}. This diet may have been contaminated during the sampling or sample processing.

Few fungi are adapted to flourish in conditions of low water activity (a_w), where the free water is scarce. The fungi that are able to grow on a substrate with the a_w lower than 0.85 are denominated by xerophilic (Díaz-Valderrama *et al.*, 2017). Generally, the xerophilic fungi have rapid growth when the a_w is above 0.77 and have slow growth when the a_w is between 0.68 and 0.75. The free water may contribute to the spoilage activity and, if the storage conditions are not adequate, the moisture content increases and, consequently, also increases the fungal growth, as well as the proliferation of xerophilic fungi (Greco *et al.*, 2014).

The genera *Penicillium* and *Aureobasidium* were detected with 1 CFU.mL^{-1} each, both in the water sample W1, corresponding to the water before entering the rearing tanks, that was a negative control. But no fungus was detected in other water

samples, which can indicate that some type of contamination occurred in water sample W1 during its collection or during the sample processing.

The temperature of the water supply may interfere with the microbiological processes. In the summer months, the water temperature is higher than the winter months and, therefore, it is assumed that there is also an increase in biological activity during this period. Long residence times can also result in the raise of microbial growth in distribution networks (Zlatanović *et al.*, 2017).

The detection of the *Cladosporium* genus occurred only in one sponge filter sample (sample F2), with 1 CFU.m^{-2}, which may indicate the previous occurrence of contamination in the water sample W6, probably originated by the jellified pellet D3. Almeida *et al.* (2011) detected the *Cladosporium* genus in 28.7% of fish feed samples (Almeida *et al.*, 2011). Therefore, there may have been accumulation in the sponge filter, since the experiment was already running 90 days ago.

In the study developed in Portugal by Almeida *et al.* (2011), 40.2% of their samples of fish feed had fungi. They obtained 25.3% of positive results for *Fusarium* genus, but the genera *Aspergillus*, *Penicillium*, and *Cladosporium* were detected in major percentages, being *A. flavus* with 40.2% of positive results. Regarding to material feed used in fish feed formulations, corn samples had the highest mould contamination (Almeida *et al.*, 2011).

It should be highlighted the detection of the *Fusarium* genus amongst the found fungal burden in this study, in which *F. verticillioides* was predominant amongst *Fusarium* isolates. The identification of *Fusarium* species is in agreement with the fact of the *Fusarium* genus to be one of the most often moulds detected in animal feeds (Almeida *et al.*, 2011).

Regarding to the Screening of Azole-resistance, the samples were inoculated on the azole-supplemented media for a susceptibility testing to triazole fungicides (Balajee *et al.*, 2007). In this study, these media were incubated at 27 °C, and not at between 34 to 37 °C (as defined in clinical guidelines), which allowed to reveal a wider mycobiota diversity (Viegas *et al.*, 2018c).

In this study, *Fusarium verticillioides* was identified on SAB medium supplemented with ITRA seeded with the frozen microalga D6, counting with 1 CFU.g^{-1}. The susceptibility testing revealed that this species seems to have decreased its susceptibility to the ITRA, but it seems to be susceptible to the VORI and POSA. Thus, *F. verticillioides* is not susceptible to the concentration of ITRA used in this culture

medium. However, this contamination may have occurred during the sample processing or inoculation, since it is a frozen microalga.

Fusarium verticillioides causes various diseases in corn crops. The mycotoxins that it produces, as FB_1, can be recognised in plant tissues (particularly in cereal grains), with and without symptoms, being harmful to animal and human health. The azole fungicide inhibits fungal growth through its interaction with the heme-iron of cytochrome P450 sterol 14α-demethylase (CYP51), which is an important enzyme in the biosynthesis of ergosterol that is the main sterol in fungal membranes. Thus, the resistance exhibited by fungal species determine the efficacy of the azole fungicide, triazole or imidazole. *F. verticillioides* is resistant or tolerant to triazole fungicides (as ITRA, VORI, and POSA), as well as imidazole, which are fungicides that are habitually used to control the diseases of maize (Fan *et al.*, 2014).

The systemic application of fungicides is extensive in crops production. Nonetheless, a major disadvantage is that several fungal pathogens developed resistance to generality of the fungicides used (Kano *et al.*, 2015; O'Neill, 2015). The fungicide-resistance is considered a challenge in production of plant, representing a major threat to animal and human health, because of the dissemination of antifungal resistance found in fungi that infect human and animal populations, and, therefore, the consequent increase of fungal resistance risk to azole therapy and also treatment failure (Verweij *et al.*, 2009; Viegas *et al.*, 2018a submitted).

Currently, there is a great concern regarding antifungal resistance that appears to be an emerging risk and, therefore, epidemiological surveillance is mandatory (Sabino *et al.*, 2016). The *Fusarium* species are emerging, opportunistic, and life-threatening pathogens and are the most often cause of fungemia joined with metastatic skin lesions, being the most common presentation the disseminated fusariosis that manifests mainly in immunocompetent individuals. The *Fusarium* pathogens usually express wide *in vitro* resistance to antifungal agents with high variability, being amongst the most resistant fungi (Taj-Aldeen *et al.*, 2016).

Thereby, the vigilance of strains of fungi with azole-resistant must be ensued in microbiota evaluations in general, namely in aquaculture, with the purpose of acknowledge and also to prevent animal, the exposure of workers and consumers to fungicide-resistant fungi in critical levels (Alborch *et al.*, 2010; Halstensen, 2008).

The quantification of fungi through the utilisation of conventional methods (culture-based methods) may lead to underestimation of results and the fungal

identification through morphological characteristics requires considerable expertise. Thereby, the utilisation of molecular methods for the detection and quantification of DNA of fungi has increased. The molecular methods are more specific, sensitive, and fast, but, even so, conventional methods are necessary to characterise fungal distribution in the poorly studied environments (Rodrigues *et al.*, 2011; Viegas *et al.*, 2012).

In this study, it was investigated the identification of *Aspergillus* sections, due to the biological and medical importance of the *Aspergillus* genus and its spread and diversity (Ashtiani *et al.*, 2017). This genus has extreme importance in terms of pathogenicity in humans, animals, and plants; as producers of bioactive and mycotoxins (toxic secondary metabolites); as agents of spoilage of food commodities; and in industrial bioprocesses (biotechnological exploration) and food fermentations (Rodrigues *et al.*, 2011; Samson *et al.*, 2006). The *Aspergillus* species cause life-threatening infections of immunocompromised patients, such as the aspergillosis that is frequently caused by *A. fumigatus* (Balajee *et al.*, 2007; Sabino *et al.*, 2017). This species is the most important pathogenic and is grouped in the *Aspergillus* section *Fumigati*. The *A. ochraceus* group are from *Aspergillus* section *Circumdati* and are important, given that they produce several mycotoxins, such as OTA, Penicillic Acid, Vioxanthin, Viomellein, and Xanthomegnin (Samson *et al.*, 2006). The *Aspergillus* section *Flavi* holds the species that produce the AFs, which are a group of highly toxigenic compounds (Rodrigues *et al.*, 2011). Thus, the species *A. flavus, A. parasiticus*, and *A. nomius* are the most common and important producers of AFs in food and feed (Rodrigues *et al.*, 2011; Samson *et al.*, 2006).

The molecular method qPCR was used for the identification of *Aspergillus* sections, because it presented greater precision, sensitivity, and speed and lower risk of contamination (Dizon *et al.*, 2018). However, no *Aspergillus* sections were amplified by the qPCR assay, in which were used specific primers and probes for target *Aspergillus* sections (*Flavi*, *Fumigati*, *Circumdati*, and *Versicolores*), which is good for the production systems. This molecular method has the advantage of specifically identifying fungal DNA in all fungal structures that contain DNA (Viegas *et al.*, 2012), but it can only be detected one species at a time, able to become more susceptible to potential PCR inhibitors, which may have been the cause of non-detection. The possible existence of complex environmental substances in the analysed matrices may have inhibited the PCR amplification, which has already occurred in highly contaminated environments (Viegas *et al.*, 2018c).

It would be interesting to carry out research in order to identify other fungal species by molecular methods, as is the case of the *Fusarium* genus that has also great relevance (Taj-Aldeen *et al.*, 2016).

Regarding to mycotoxin contamination, five to six mycotoxins were detected in the two extruded pellets D1 and D2, both with high cereal content. Although there are no fungi in these extruded pellets, the presence of mycotoxins suggests that were previously present (Alborch *et al.*, 2010; Halstensen, 2008; Pinotti *et al.*, 2016). The feed procedures with physical treatments, such as extrusion (i.e. high temperature and pressure), that reduce levels of fungal burden in animal feeds (Almeida *et al.*, 2011; Greco *et al.*, 2015; Hooft & Bureau, 2017), or even inactivate the fungi (Greco *et al.*, 2015). The high cereal content of these extruded pellets may have been the determining factor for the previous occurrence of fungi that produced the mycotoxins that persisted in the matrix (Matejova *et al.*, 2017). The mycotoxins are heat stable and for that reason can persist in these diet samples (Greco *et al.*, 2015). The elimination or inactivation of mycotoxins through their exposition to extreme temperatures, so such as roasting or boiling processes, is also very difficult (Peraica *et al.*, 1999; Viegas *et al.*, 2018a submitted). For that reason, the mycotoxins are able to remain in feeds for a long time after the disappear of fungal species through which they were produced (Alborch *et al.*, 2010; Halstensen, 2008; Pinotti *et al.*, 2016), which could explain their detection in these diet samples.

The extruded pellet D2 was the diet sample with greater mycotoxin contamination in this study. The concentration of DON was of 62.99 $\mu g.Kg^{-1}$, which was a much lower value than the levels found in other studies. Pietsch *et al.* (2013) obtained an average value of 289 $\mu g.Kg^{-1}$ in fish feed with more than 80% of samples contaminated with DON (Pietsch *et al.*, 2013). In Greco *et al.* (2015) study, they found 230 $\mu g.Kg^{-1}$ in fish feed samples (Greco *et al.*, 2015). Gonçalves *et al.* (2016) identified an average of 109 $\mu g.Kg^{-1}$ in aquafeed samples (Gonçalves *et al.*, 2016). Nácher-Mestre *et al.* (2015) identified a ranging of 17 to 504 $\mu g.Kg^{-1}$ for wheat samples and a ranging from 139 to 814 $\mu g.Kg^{-1}$ for corn samples (Nácher-Mestre *et al.*, 2015). Marijani *et al.* (2017) detected a range of 69.1 to 984.3 $\mu g.Kg^{-1}$ in fish feed samples (Marijani *et al.*, 2017). The EC recommends that DON levels in complete feeding stuff for animal should be below 5 $mg.Kg^{-1}$ (EC, 2006; Pietsch *et al.*, 2013; Sanden *et al.*, 2012; Smith *et al.*, 2016) and, therefore, the results obtained in this study are good and in agreement with the recommended values.

The detection of DON in extruded pellets D1 and D2 may reveal the previous presence of *Fusarium* species, like *F. culmorum* (Ferrigo *et al.*, 2016; Matejova *et al.*, 2017; Tančić *et al.*, 2015) and *F. graminearum* (Matejova *et al.*, 2017; Pietsch *et al.*, 2014; Sanden *et al.*, 2012; Smith *et al.*, 2016), or even other fungal genera, such as *Cephalosporium*, *Myrothecium*, *Verticimonosporium*, and *Stachybotrys* (Hooft & Bureau, 2017; Matejova *et al.*, 2017; Pietsch *et al.*, 2011; Sanden *et al.*, 2012). DON is a concern for being the most prevalent TCT in Europe, making it one of the most important mycotoxins (Smith *et al.*, 2016). This mycotoxin is the most predominant TCT contaminant in maize, wheat (Hooft & Bureau, 2017; Matejova *et al.*, 2017; Pietsch *et al.*, 2011; Sanden *et al.*, 2012), and soybean meal (Hooft & Bureau, 2017), and these are feed ingredients present in the formulations of these two extruded pellets, revealing that they may have been the source of this contamination.

The impact of DON in farmed fish has been studied and different species revealed different sensitivities (Sanden *et al.*, 2012). Rainbow trout is extremely sensitive to DON-contaminated feeds (Matejova *et al.*, 2017; Sanden *et al.*, 2012) and, after being exposed to a concentration of 2.0 $mg.Kg^{-1}$ during 23 days, they presented severe hyaline droplet degeneration in the tubular epithelial cells of renal tubules in caudal kidney and significant changes in haematological and biochemical parameters (Matejova *et al.*, 2017). Rainbow trout exposed to a 2.6 $mg.Kg^{-1}$ during 56 days showed changes on nutritional status with reduction on crude protein (Pietsch *et al.*, 2014), reduced feed intake, and deleterious metabolic effects (Hooft & Bureau, 2017). Salmonids showed lesions and histopathological changes in liver when exposed to 1.4 $mg.Kg^{-1}$ and also higher concentrations. Carps exhibited effects on immune system when concentrations ranging 352 and 953 $\mu g.Kg^{-1}$ (Pietsch *et al.*, 2014).

In Pietsch *et al.* (2014) study, carps had a metabolic crisis in their livers that was caused by damage of tissues and influenced their nutritional status (Pietsch *et al.*, 2014). DON harms fish health, growth performance (Gonçalves *et al.*, 2018b; Pietsch *et al.*, 2014; Sanden *et al.*, 2012), immune suppression, and liver alterations (Gonçalves *et al.*, 2018b); and it was already reported mycotoxicosis in fish (Matejova *et al.*, 2017). The wide occurrence of this TCT, even at low levels, can become a concern, due to the immunotoxic effects and growth retardation that provokes in fish. Regular monitoring of its presence in feed ingredients and fish feed is recommended (Marijani *et al.*, 2017).

The toxic effects of DON in mammals are generally malabsorption of nutrients, diarrhoea, and emesis (Pietsch *et al.*, 2014). In humans, during pregnancy, DON can be

transported to foetus through the placental barrier and, at cellular level, the DNA damage is related to the oxidative stress (Sanden *et al.*, 2012).

The concentration of T-2 toxin present in extruded pellet D2 was of 2.51 $\mu g.Kg^{-1}$ that is much lower than Greco *et al.* (2015) detected in their study, with an average of 70.08 $\mu g.Kg^{-1}$ (Greco *et al.*, 2015), and also than Marijani *et al.* (2017) study, in which they found 36.5 $\mu g.Kg^{-1}$ in fish feed ingredients (Marijani *et al.*, 2017).

The detection of T-2 toxin in extruded pellet D2 may reveal the previous presence of *Fusarium* species, like *F. acuminatum*, *F. poae*, *F. equiseti*, *F. langsethiae*, and *F. sporotrichioides*, that infect maize and wheat (Matejova *et al.*, 2017), and these are two feed ingredients present in the formulation of this diet, which may have been the cause of this contamination.

Some effects have been reported to fish fed with contaminated diets with T-2 toxin. Rainbow trout exposed to 2.5 $mg.Kg^{-1}$ revealed low haematocrit and blood haemoglobin and decrease in feed consumption and growth, while the exposure to 10 $mg.Kg^{-1}$ caused regurgitation and gastrointestinal bleeding (Greco *et al.*, 2015) and its exposure to 15 $mg.Kg^{-1}$ during 16 weeks revealed enlarged gall bladders and spleens, and focal intestinal haemorrhaging. Channel catfish exposed to 630 $\mu g.Kg^{-1}$ for 2 weeks showed significant decreases in body weight gain and in concentrations upper than 1.25 $mg.Kg^{-1}$ increased the gastric lesions in number and severity. Common carp exposed to 2.45 $mg.Kg^{-1}$ exhibited changes in the oxidative status and significantly reduction of body weight (Matejova *et al.*, 2017).

DON and T-2 toxin are the most common and relevant TCTs (Matejova *et al.*, 2017), but T-2 toxin is one of the most toxic (Gonçalves *et al.*, 2016).

The concentrations of FB_1 and FB_2 present in extruded pellet D2 were of 7.51 and 14.66 $\mu g.Kg^{-1}$, respectively. Regarding to the FBs, Gonçalves *et al.* (2016) identified an average of 326 $\mu g.Kg^{-1}$ in aquafeeds (Gonçalves *et al.*, 2016). Nácher *et al.* (2015) found FBs in corn with a ranging from 11.1 to 4,901 $\mu g.Kg^{-1}$ and one wheat gluten sample had 13.2 $\mu g.Kg^{-1}$ (Nácher-Mestre *et al.*, 2015). Marijani *et al.* (2017) detected a range of 33.2 $\mu g.Kg^{-1}$ to 3.97 $mg.Kg^{-1}$ in fish feed samples (Marijani *et al.*, 2017). According to the EC guidelines, the sum of FB_1 and FB_2 in feed materials (maize) should not exceed 60 $mg.Kg^{-1}$ (EC, 2006; Smith *et al.*, 2016). Thus, with respect to FBs, the extruded pellet D2 is in agreement with the limit values.

The detection of FBs in the two extruded pellets D1 and D2 may reveal the previous presence of *Fusarium verticillioides* (Ferrigo *et al.*, 2016; Matejova *et al.*,

2017; Tančić *et al.*, 2015) and *F. proliferatum* that are found in maize and normally grow in crops pre-harvest (Gonçalves *et al.*, 2018b; Matejova *et al.*, 2017; Smith *et al.*, 2016) and these two extruded pellets had corn in their formulations. The FBs are the most predominant mycotoxin in grains that is the most often feed ingredient used in aquafeed formulations. The FB_1 is the most toxic and abundant FBs and it was present in both extruded pellets (Matejova *et al.*, 2017).

Generally, the FBs are the most predominant mycotoxin present in cereal grains that are the most common feed ingredient in aquafeed formulations (Matejova *et al.*, 2017).

The effects that FB_1 cause in fish have been reported. Channel catfish exposed to 20 $mg.Kg^{-1}$ for 10 weeks demonstrated reduced body weight gain, while the exposure to 40 $mg.Kg^{-1}$ or more induced histological changes and an increase of liver glycogen, and to 80 $mg.Kg^{-1}$ presented significantly lower disease resistance. Common carp exposed to 500 $\mu g.Kg^{-1}$ exhibited changes in platelet count and red blood cell parameters and dose-dependent changes in biochemical profile. Nile tilapia exposed to 40 $mg.Kg^{-1}$ or more revealed significantly lower mean weight gain (Matejova *et al.*, 2017).

In mammals, FBs provoke disruptive effects on liver and neural tissues (Matejova *et al.*, 2017).

The concentration of ZEA found in this study was of 14.38 $\mu g.Kg^{-1}$ in the extruded pellet D2 and of 2.10 $\mu g.Kg^{-1}$ in the extruded pellet D1. In the study conducted by Pietsch *et al.* (2013), they found contamination of ZEA in 100% of their fish feed samples with an average value of 67.9 $\mu g.Kg^{-1}$ (Pietsch *et al.*, 2013). Greco *et al.* (2015) found 87.97 $\mu g.Kg^{-1}$ in samples of fish feed (Greco *et al.*, 2015). Gonçalves *et al.* (2016) identified an average of 42 $\mu g.Kg^{-1}$ in aquafeed samples (Gonçalves *et al.*, 2016). The guidance values recommended by the EC for ZEA levels in feed materials (cereals) destined to animal feeds should not exceed 2 $mg.Kg^{-1}$, excepting maize by-products that have a maximum of 3 $mg.Kg^{-1}$ (EC, 2006; Pietsch *et al.*, 2013; Smith *et al.*, 2016), and both results of this study were below of these values.

The detection of ZEA in the two extruded pellets D1 and D2 may reveal the previous presence of *Fusarium* species, so such as *F. verticillioides*, *F. culmorum*, *F. graminearum* (Gonçalves *et al.*, 2016; Matejova *et al.*, 2017; Smith *et al.*, 2016), *F. sporotrichioides* (Gonçalves *et al.*, 2016), *F. cerealis*, *F. incarnatum*, and *F. equiseti*.

This mycotoxin is regularly found in maize (Matejova *et al.*, 2017) and this feed ingredient was used in the formulation of both extruded pellets.

The ZEA has been identified in fish feed and feed materials (Woźny *et al.*, 2017). In a study evaluating commercial fish feed from Europe Central, the concentrations of ZEA did not exceed an average level of 68 μg.Kg^{-1}, which did not appear to be harmful to fish produced in aquaculture (Matejova *et al.*, 2017).

The impact of ZEA in fish health has been reported. Juvenile rainbow trout exposed to 10 mg.Kg^{-1} described lowered iron concentrations in liver and ovary and a slight tendency to prolonged clotting time. Atlantic salmon exposed to ZEA and α-Zearalenol (α-ZOL) revealed greater expression of zona radiata protein in plasma and a dose-dependent increment in vitellogenin (Matejova *et al.*, 2017) and these are major structural elements of oocyte (Gonçalves *et al.*, 2016).

ZEA and its metabolites, α-ZOL and β-Zearalenol (β-ZOL), have the ability of mimics the natural oestrogens, being α-ZOL that has the greater binding affinity to the oestrogen receptors. In mammals, ZEA causes reproductive malfunctions and, in humans, the oestrogen toxicity is related to reproductive problems (Matejova *et al.*, 2017).

The *Fusarium* mycotoxins (as TCTs, FBs, and ZEA) are stable under food processing conditions (Gonçalves *et al.*, 2018b; Pietsch *et al.*, 2011), being resistant to heat (Matejova *et al.*, 2017; Sanden *et al.*, 2012), and, therefore, they may persist in finished feeds. The improper storage conditions are also a contributor for the mycotoxin problem, mainly because of cereal grains in finished feeds (Gonçalves *et al.*, 2018b; Pietsch *et al.*, 2011).

The concentration of OTA present in the extruded pellet D2 was of 0.94 μg.Kg^{-1} that is lower than the average value obtained in Greco *et al.* (2015) study, in which they found 5.26 μg.Kg^{-1} in fish feed samples (Greco *et al.*, 2015). Gonçalves *et al.* (2016) identified an average of 1 μg.Kg^{-1} in aquafeed samples (Gonçalves *et al.*, 2016). Nácher-Mestre *et al.* (2015) detected a range of 0.4 to 5.2 μg.Kg^{-1} for corn, wheat, and pea protein samples (Nácher-Mestre *et al.*, 2015). Marijani *et al.* (2017) found OTA contamination in cottonseed cake samples with a ranging from 6.50 to 24.42 μg.Kg^{-1} (Marijani *et al.*, 2017). The recommended values of the EC for OTA levels present in feed materials (cereals) for animal feeds are a maximum of 0.25 mg.Kg^{-1} and the extruded pellet D2 is in agreement with the established (EC, 2006; Smith *et al.*, 2016).

The detection of OTs in the extruded pellet D2 may reveal the previous presence of the *Aspergillus* species, such as *A. ochraceus*, *A. carbonarius*, and *A. niger*, or the *Penicillium* species, so such as *P. viridicatum*, *P. verrucosum*, and *P. nordicum* (Zhang *et al.*, 2016). The most toxic and abundant OT is OTA that was the only one found in this study (Gonçalves *et al.*, 2016; Matejova *et al.*, 2017; Smith *et al.*, 2016). The OTA habitually occurs in maize, wheat, and soybean (Matejova *et al.*, 2017) that are feed ingredients present in the formulation of the extruded pellet D2.

Frequently, Eastern European feed has been reported with high prevalence of OTA. The fish feeds have been contaminated with OTA and used in aquaculture (Matejova *et al.*, 2017).

The impact of OTA in fish health has been reported and, in general, the target-organs are the kidney and liver (Gonçalves *et al.*, 2016; Matejova *et al.*, 2017). Rainbow trout exposed to 8 $mg.Kg^{-1}$ showed necrosis in haematopoietic tissue, glomeruli, and tubules of the kidney. Juvenile channel catfish exposed to 2 $mg.Kg^{-1}$ for 2 weeks revealed significant decreases in body weight gain and the exposure to 4 $mg.Kg^{-1}$ during 21 days displayed significantly greater mortality (Matejova *et al.*, 2017). Common carp exposed to 15 $\mu g.Kg^{-1}$ demonstrated reduction of feed utilisation parameters and decreasing growth performance (Gonçalves *et al.*, 2016).

In mammals, the target-organ of OTA is often the kidney and display enterohepatic circulation. In humans, OTA is associated to potentially fatal kidney disease (Matejova *et al.*, 2017).

In the jellified pellet D4 was detected MPA, which may reveal the previous presence of the *Penicillium* species, like *P. roquefortin* that is an important source of this mycotoxin. The presence of corn in its formulation seems to have been the cause of this contamination. MPA is a suitable marker for the occurrence of *Penicillium* in food and feed (Smet *et al.*, 2011).

The *Penicillium* mycotoxins (as OTA and MPA) are a concern in storage of food and feed with poor conditions (Smet *et al.*, 2011).

The feed ingredients that may have promoted the mycotoxin contamination in the extruded pellets D1 and D2 were corn meal, wheat gluten, and soy protein concentrate and in the jellified pellet D4 was canned corn. Approaching other studies, it can be understood that these are feed ingredients commonly used in animal feeds and that really add significant levels of mycotoxins (Gonçalves *et al.*, 2018b; Marijani *et al.*, 2017; Nácher-Mestre *et al.*, 2015). The exposure of aquatic animals to mycotoxins has

been raised through the increases of utilisation of plant sources in aquafeeds (Gonçalves *et al.*, 2018b; Hooft & Bureau, 2017).

The mycotoxin contamination noted in these diets agrees with the trend of other settings associated to animal production. With regard to the Europe, a great percentage of feed samples were reported as being contaminated with numerous mycotoxins. Nevertheless, regulations of food and feed are formulated based only on toxicity data of isolated mycotoxins. Mostly, the concentrations obtained were low sufficient to guarantee compliance with maximum admitted levels or guidance values of the EU (Pinotti *et al.*, 2016; Viegas *et al.*, 2018a submitted).

The cereals and cereal-based products are considered important feed ingredients to be used in animal feeds and are a possible source of mycotoxin contamination (Pinotti *et al.*, 2016). The cereal grains used in diet samples analysed in this study were probably the cause of contamination (Giorgi, 2006; Viegas *et al.*, 2018a submitted).

Based on 107 articles, the presence of mycotoxins was more studied in raw and processed cereals, representing this group of commodity types 80% of the data reported, with about 50% coming from Europe (Smith *et al.*, 2016). The results obtained in this study show that they are in agreement with the trend verified in the studies developed in Europe.

The utilisation of different methodologies for the detection and quantification of mycotoxins in different studies should be emphasized, which makes it difficult to compare the results between studies, since they confer differences in sensitivity and accuracy. In some studies there is a focus on only certain mycotoxins and other studies are developed in non-targeted approaches, revealing comparative complications in qualitative and quantitative terms (Smith *et al.*, 2016).

In this study, the mycotoxins have not been detected in samples of waters and sponge filters, which may be related to an efficient feeding conversion, or that the method used is not sensitive enough to detect the presence of mycotoxins in these matrices. The fact that it was not possible to quantify all mycotoxins detected in diet samples is also related with the sensitivity of the methods used, namely the ranging of LOD and LOQ. To increase the sensitivity of the LC-MS/MS system, instead of using the HPLC, the UHPLC could be used. The UHPLC combines fast detection, high sample throughput, and quantification with increased resolution, sensitivity, and selectivity, given that it has a smaller particle size of column packing material, which

would make it possible a better physical separation of substances and the quantification of lower concentrations. The number of reports of UHPLC-MS/MS as multi-mycotoxin method is expanding (Pamel *et al.*, 2011; Seifrtová *et al.*, 2010).

Regulations still continue with a gap and the mycotoxins co-contamination may promote harmful effects on animals, because of possible interactions that result in synergistic or additive effects (Matejova *et al.*, 2017). Studies about the effects of mycotoxin mixtures were realised in cell lines and observed that the combination of various mycotoxins origin synergistic or additive effects (Korkalainen *et al.*, 2017; Yang *et al.*, 2017) on cell inhibition, at high and/or moderate and/or low level (Yang *et al.*, 2017), and the mycotoxin regulation should consider this (Korkalainen *et al.*, 2017; Yang *et al.*, 2017).

Previously developed studies have shown that 116 combinations of mycotoxins have already been found in samples of cereals and derived cereal products. The most present combinations were AF+FB (21 times, or 23%), DON+ZEA (14 times, or 15%), AF+OTA (12 times, or 13%), and FB+ZEA (11 times, or 12%) that were cited in 91 articles. In addition, a recent survey by the BIOMIN Company revealed that the most prevalent mycotoxins among the 6,844 analysed agricultural commodity samples in the world are DON (66%), FB (56%), and ZEA (53%). The mycotoxin toxicological interactions should be taken into account (Smith *et al.*, 2016).

For European samples, 105 mycotoxin combinations were highlighted in 67 publications and 24% of the publications reported the presence of the combination AF+OTA, 15% described DON+ZEA, 13% reported DON+NIV, 12% described DON+T-2 toxin, and the other combinations were reported in less than 10% of these articles (Smith *et al.*, 2016).

The legislation is based on individual mycotoxins. However, usually mycotoxin contamination occurs by their combination and usually not individually. This shows how extremely important it is to assess the toxicological impact of mycotoxin combinations, in order to better understand the risks that food and feed carries to animal and human health (Smith *et al.*, 2016).

The contaminated aquafeed may affect the health of aquatic animals, through contamination of their tissue (Gonçalves *et al.*, 2018b; Matejova *et al.*, 2017). Edible tissues and organs of fish have been reported with the presence of residues of mycotoxins (Anater *et al.*, 2016). AFs, ZEA, and *Fusarium* mycotoxins have been identified in ovaries, muscles, liver, and head of fish (Nomura *et al.*, 2011; Tolosa *et al.*,

2014; Woźny *et al.*, 2013). This can contribute to the mycotoxin intake in humans, representing a risk to human health (Almeida *et al.*, 2011; Greco *et al.*, 2015; Hooft & Bureau, 2017; Matejova *et al.*, 2017; Pulina *et al.*, 2014), because of the long-term cumulative or chronic effects that cause on human health (Prencipe *et al.*, 2018).

There is a growing interest in mycotoxin detoxification strategies and the need to implement mycotoxin remediation strategies, being necessary further research (Zutter *et al.*, 2016). The utilisation of insects in formulations of animal feeds may be the solution to the mycotoxin problem, since insects have shown the ability to detoxify various toxic metabolites. Insects are an alternative source of protein and can supplement other sources, such as fishmeal, grain, soy, and maize. The insects with the greatest immediate potential for the production of large-scale feed are house crickets (*Acheta domesticus*), common housefly (*Musca domestica*) larvae, black soldier fly (*Hermetia illuscens*) larvae, and yellow mealworm (*Tenebrio molitor*). There are producers of flies in China, the United States, Spain, and South Africa, and are already producing large quantities for aquaculture and poultry feeds, because they have the ability to bioconvert organic waste. However, insects are not yet specifically mentioned in animal feed legislation (Raamsdonk *et al.*, 2017). Usually, insects live in close proximity of the TCTs-producing fungi and toxicity, since they remain in contact with the toxic secondary metabolites of *Fusarium* species. However, knowledge about their detoxification strategies is still lacking and more studies should be conducted (Zutter *et al.*, 2016).

In a general way, with regard to bacterial and fungal burden found in diet samples of this study, it would be expected that open systems are subject to a higher bioburden than closed systems, as the RAS, since the removal of the organic matter is dependent of natural processes and does not depend on human intervention, as in closed systems (Tidwell, 2012). Moreover, it would be expected that the bioburden found in the intensive regime be greater than in the extensive regime, because of the higher productivity per unit volume (Gabriel *et al.*, 2015; Justino *et al.*, 2016; Lekang, 2007) that induces stress in farmed aquatic organisms, becoming more susceptible to the appearance of diseases that can be originate through an input in the production system, such as the contaminated commercial feeds (Lekang, 2007).

Probably, passing these experimental cultures of echinoderms in RAS for a business sector in open sea, it would be expected that the results were better and, consequently, that there were lower risks to the health of farmed species, given that

these diets, even contaminated, would be much more diluted in the huge amounts of water, suggesting that there would be detected lower concentrations of bioburden in the production systems (Tidwell, 2012). It can also be emphasised the relevance of investigating the different production phases, in which formulations of diets and frequency of feeding used are different and, therefore, will have another impact on the system (Lekang, 2007).

Accordingly, it highlights the importance of aquaculture activity for Portugal, revealing that enlarged studies must be performed on aquafeeds, in order to understand the potential impact on health of animals and, consequently, Public Health.

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7. CONCLUSION

The characterisation of bioburden in aquafeeds is extremely important to know and to preserve the health of species produced in aquaculture systems. In this way, it becomes possible to control the risks that inputs, such as diets formulated with ingredients from agriculture, produce in aquaculture systems. Thereby, it is possible to make improvements in order to minimise such exposure for aquatic organisms and also for Public Health.

Mesophilic bacteria were detected in the majority of samples analysed, which reveal that diet samples had some spoilage activity. It seems that the higher amounts of mesophilic bacteria found were due to the cereal and moisture content, and possibly due to some fluctuations of temperature during their transport, suggesting that there should be better conditioned. However, the results obtained were, for the most part, within the suggested limits, proposing that are safe for the echinoderms produced in the aquaculture systems analysed in this study.

Total coliforms were found in only two samples, being both diet samples. With regard to the extruded pellet, it appears that the contamination came from cereals and, for frozen microalga, it seems that occurred a contamination during the sampling or sample processing. Both had minimal levels, clearly very below to the limits suggested as harmless, signifying that good hygiene care existed throughout their processing and storage.

The *Aspergillus* sections were not detected in any sample, which is good for the production systems analysed.

Two *Fusarium* species, *Fusarium verticillioides* and *F. culmorum*, were identified in a frozen microalga and the species *F. verticillioides* was also found in a jellified pellet. It seems that the cause of contamination was the moisture content and the presence of corn in jellified pellet. While the frozen microalga may has been contaminated during the sampling or sample processing.

Mycotoxins were detected in two extruded pellets and in one jellified pellet. The quantification of mycotoxin contamination it only was possible in the two extruded pellets, due to the sensibility of the method used. These contaminations suggest, once again, that cereal content is a potential source of contamination by fungi and, consequently, mycotoxins. The non-detection of fungi in the extruded pellets reveals

that the feed processing can eliminate them, through high temperatures, while the persistence of mycotoxins in the matrix confirms their resistance and heat stability. Nevertheless, all mycotoxin concentrations were below to the guidance values and, therefore, do not appear to be harmful to echinoderms produced in these RAS.

These preliminary results come from a short study with few samples, which highlights the importance of conducting a study with more samples and with sampling in different periods of the year, in order to allow the statistical analysis and the crossing of data.

In a general way, the results obtained for the bacterial, fungal, and mycotoxigenic burden reveal the importance of continuing to evaluate the different types of aquafeeds, as well as the filtration processes and the rearing waters, because they allow to know the proliferation of bioburden in the different matrices and to improve all methodologies.

More studies should be performed with focus on feed ingredients and aquafeeds where they will be used, in order to allow the real source of contamination. The identification of bacterial species and their pathogenicity should be investigated to better understand the risks of exposure to these aquafeeds, as well as the investigation of other fungal species through molecular methods. There is also a need of an enlarged study on detection of mycotoxins in extruded pellets, with a more sensitive analytical method.

Future studies could be carried out to evaluate the persistence of bioburden in edible tissues of aquatic organisms produced in aquaculture, as a way to estimate the biomagnification, more specifically, the hazard for the health of animals that are produced for human consumption and, therefore, consequently, to better know the impact on human health. It should also be evaluated the effect that each mycotoxin may possibly have on exposure of consumers.

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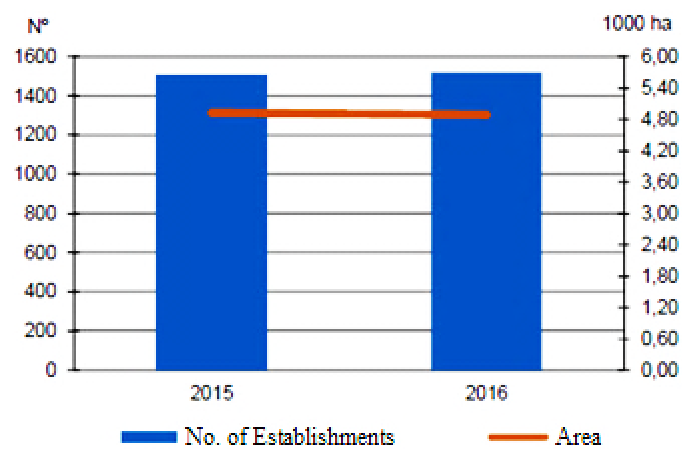
ATTACHMENTS

Annexe I

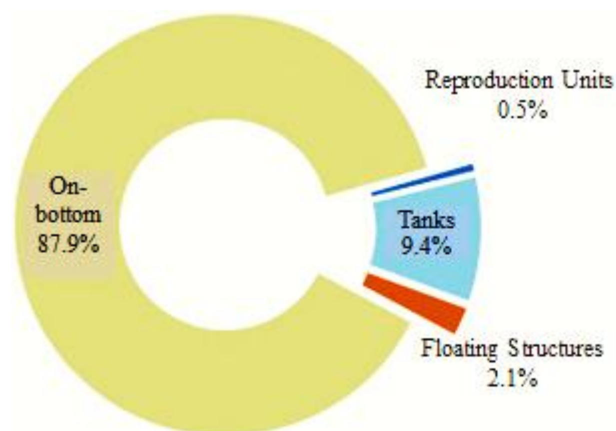
Graphical representation of the number of establishments and total areas (in thousands of hectares) referred to the Portuguese aquaculture establishments, in the years 2015 and 2016.



Adapted from INE, 2018.

Annexe II

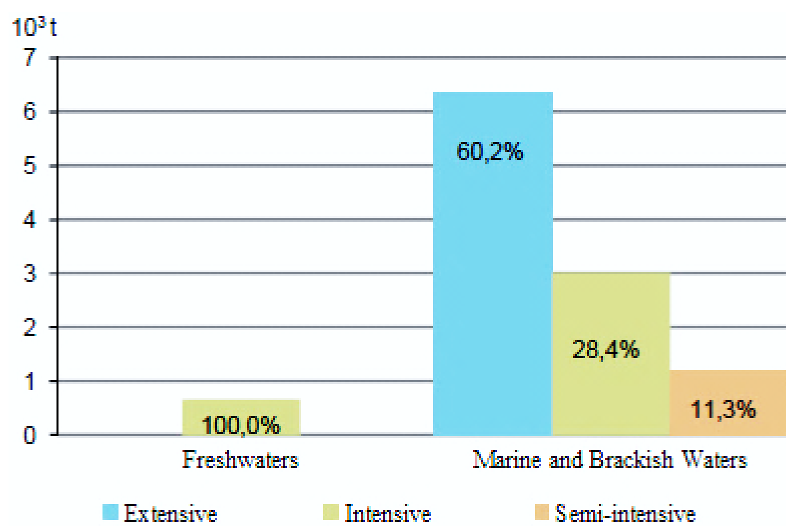
Graphical representation of aquaculture structures used in Portugal (in percentage), in the year 2016.



Adapted from INE, 2018.

Annexe III

Graphical representation of type of water and exploitation regime (in percentage and thousands of tonnes) referred to the Portuguese aquaculture production, in the year 2016.



Adapted from INE, 2018.

Annexe IV

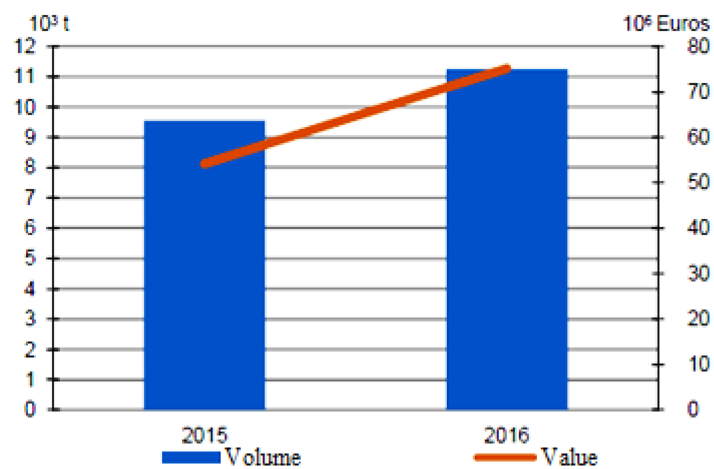
Data relating to the volume (in tonnes) and monetary value (in thousands of Euros) produced through aquaculture in inland and offshore waters from Portugal (2015-2016), by Nomenclature of Territorial Units for Statistics level II (NUTS II).

NUTS II		Total		Freshwaters		Marine and brackish waters	
Production		t	1,000 Euros	t	1,000 Euros	t	1,000 Euros
Portugal	**2015**	**9,561**	**54,135**	**890**	**2,138**	**8,671**	**51,997**
	2016	**11,259**	**75,198**	**675**	**1,817**	**10,583**	**73,380**
Continent		**10,873**	**73,567**	**675**	**1,817**	**10,197**	**71,749**
North Region		716	2,234	674	1,809	41	424
Centre Region		3,255	24,227	1	8	3,254	24,219
Lisbon Metropolitan Area		766	3,425	0	0	766	3,425
Alentejo		169	1,348	0	0	169	1,348
Algarve Region		5,967	42,333	0	0	5,967	42,333
Autonomous Region of Madeira		**386**	**1,631**	**0**	**0**	**386**	**1,631**

Adapted from INE, 2018.

Annexe V

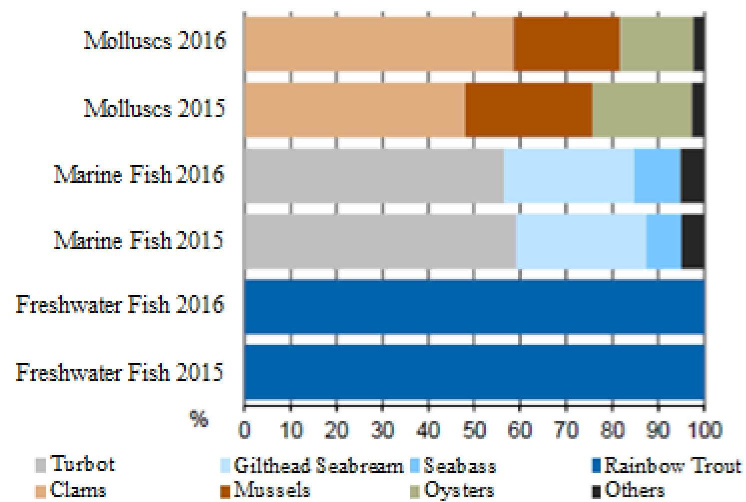
Graphical representation of volume (in thousands of tonnes) and monetary value (in millions of Euros) referred to the Portuguese aquaculture production, in the years 2015 and 2016.



Adapted from INE, 2018.

Annexe VI

Graphical representation of the production by species (in percentage), namely molluscs and marine and freshwater fish, referred to the Portuguese aquaculture, in the years 2015 and 2016.



Adapted from INE, 2018.