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UNDERSTANDING THE EFFECTS OF INTENSIVE PRODUCTION SYSTEMS ON THE SENEGALESE SOLE (*SOLEA SENEGALENSIS*) MUCOSAL HEALTH

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Understanding the effects of intensive production on Senegalese sole (*Solea senegalensis*) mucosal health

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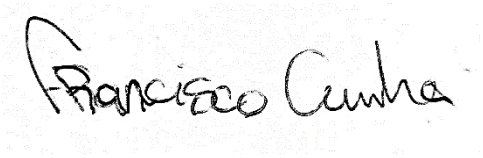

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Abstract

Aquaculture continuous growth and importance requires that an increased attention is given research that aims to promote sustainability. The production of novel species such as Senegalese sole (*Solea senegalensis*) can serve as an alternative to other oversaturated species. To further encourage sustainability, the use of substitutes for animal-based ingredients in feeds must be accomplished with the fish health in mind. This study's goal was to compare the zootechnical and humoral immune parameters of the Senegalese sole reared in production conditions for 3 months, when fed a diet with a smaller amount (25%) of fish-based ingredients. Results revealed that no changes were found on the zootechnical parameters such as specific growth rate (SGR), feed-conversion ratio (FCR) and mortality rates. The humoral immune parameters showed a decrease, with the exception of peroxidase which did not change. A possible lack of balance regarding the LC-PUFA might have contributed to the decline of these parameters over time. Overall, these results reveal feeds with lower contents of fish-based ingredients can achieve similar outcomes in regards to the fish growth. However, proper supplementation should be provided in order to counter the effects that the low n-3 content might have on the immune system.

Keywords: Senegalese sole (*Solea senegalensis*), aquaculture, immunology, mucosal health, SALT, nutrition, plant-based ingredients

Resumo

O contínuo crescimento e importância da aquacultura exige um aumento de atenção a nível da investigação que promova a sustentabilidade. A produção de novas espécies como o linguado-branco (*Solea senegalensis*) podem servir de alernativa para espécies sobressaturadas. De forma a incentivar a sustentabilidade, a utilização de substitutos de ingredientes de origem animal sem pôr em causa a saúde dos peixes. Este estudo teve como objetivo comparar os parâmetros zootécnicos e imunes humorais do linguado-branco cultivado em condições de produção durante três meses, quando alimentados com uma dieta com teor reduzido (25%) de ingredientes à base de peixe. Resultados obtidos revelaram que não foram encontradas diferenças nos parâmetros zootécnicos como a taxa de crescimento específica (SGR), taxa de conversão de alimento (FCR) e taxas de mortalidade. Os parâmetros humorais apresentaram uma diminuição, à exceção da peroxidase que não alterou. Uma falta de equilíbrio relativamente aos LC-PUFA podem ter contribuído para o decréscimo destes parâmetros ao longo do tempo. No geral, estes resultados sugerem que a utilização de rações com um teor reduzido de ingredientes à base de peixe pode atingir resultados semelhantes no que diz respeito ao crescimento do peixe. No entanto, deve ser considerada a utilização de suplementação para contrariar os efeitos que os baixos níveis de n-3 provocam no sistema imunitário.

Palavras-chave: Linguado-branco (*Solea senegalensis*), aquacultura, imunologia, saúde mucosa, SALT, nutrição, ingredientes de origem vegetal

Index

Figure/Table Index

Abbreviation List

BALT – Buccal-associated lymphoid tissue

BSA – Bovine serum albumin

FCR – Feed-conversion Rate

FM – Fish meal

FO – Fish oil

GALT – Gut-associated lymphoid tissue

GIALT – Gill-associated lymphoid tissue

IgM – Immunoglobulin M

IgT – Immunoglobulin T

LC-PUFA – Long Chain Polyunsaturated Acid

MALT – Mucosal-associated lymphoid tissue

MBW – Mean Body Weight

NALT – Nasopharynx-associated lymphoid tissue

PALT - Pharyngeal-associated lymphoid tissue

RAS – Recirculating Aquaculture System

SALT – Skin-associated lymphoid tissue

SD – Stocking Densities

SGR – Specific Growth Rate

TP – Total protein

VM – Vegetable meal

VO – Vegetable oil

1. Introduction

1.1. Aquaculture – state of the art

With the increasingly growing human population comes the subsequent need to provide an adequate food supply - particularly protein - that covers both the fields of nutrition, safety, and sustainability (Naylor *et al.*, 2021). As of now, aquaculture has become an indispensable part of the sustenance of millions of people all around the world, both financially and nutritionally (FAO, 2022). Not only that, but it is also an essential part of the way that fisheries are managed, backing the enormous demand for seafood with a more sustainable option as opposed to extracting from the environment (Thilsted *et al.*, 2016). Aquaculture has evolved not only technologically but also in terms of production numbers on the last decades. Since 1990, aquaculture has shown an enormous growth rate of 609%, while the past decade shown 57% of overall increase (FAO, 2022).

The year 2015 marked the point where aquaculture overcame fisheries in the production for human consumption, which, when paired with the increasing decline in fishing stocks, might become the norm from this point forward (FAO, 2022). Despite this, aquaculture still produces overall less than fisheries, however, it is expected that by 2025 aquaculture production overtakes fisheries captures (FAO, 2022). According to FAO (2022), fisheries and aquaculture production in 2020 decreased slightly from the maximum in 2018, which might be linked with the recent COVID-19 pandemic. It shows, however, a tendency to increase, which should be an incentive for more intensive research on how to make both industries more sustainable should be a priority.

As of 2020, Asia stands as the biggest contributor in aquaculture production worldwide, generating close to 92% of all production (including animals and algae) (FAO, 2022). Most of it originates from China, which produces close to 58% of the world's total production. In comparison, Europe contributes only near 3% of the total (FAO, 2022). Despite this, China's aquaculture production is heavily reliant on inland finfish, molluscs and algae production (FAO, 2022). On the other hand, marine species have an increasing importance in European aquaculture, especially when taking in account the weight of Norway's Atlantic salmon (*Salmo salar* Linnaeus, 1758) productions (FAO, 2022). If excluding Atlantic salmon, Europe's marine finfish production has been mostly based in few species, mainly European seabass (*Dicentrarchus labrax* Linnaeus,

1758) and gilthead seabream (*Sparus aurata* Linnaeus, 1758) *(FEAP, 2022)*. Those two species combined make close to 95% of the total marine finfish production in the Mediterranean region, with species such as turbot (*Scophthalmus maximus* Linnaeus, 1758), meagre (*Argyrosomus regius* Asso, 1801) and two sole species (*Solea solea Linnaeus, 1758;* and *Solea senegalensis Kaup, 1858*) making up for the remaining 5% (FEAP, 2022).

As for Portugal, the marine finfish production is close to half of the total marine aquaculture production, representing 44.2% (7 912 of the 17 032 total tons) of the production in 2021 (INE, 2022). Currently, the most produced species is turbot with close to 45% of the production, followed by gilthead seabream (39%) and European seabass (12%) (INE, 2022). The remaining 4% of production were disclosed as "Others", where the Senegalese sole is inserted.

1.2. Senegalese sole

*Solea senegalensis (*Kaup, 1858), commonly known as Senegalese sole, is a marine demersal flatfish (order Pleuronectiformes) belonging to the family Soleidae. It inhabits a wide array of eastern Atlantic coasts, ranging from the Biscay Bay to Senegal, including the Western Mediterranean Sea (Bjørndal *et al.*, 2016). Senegalese sole shares many similarities with its close relative, the Common or Dove sole (*Solea solea* Linnaeus, 1758), which is mostly found further north (Dinis *et al.*, 1999). Despite this, Senegalese soles tend to achieve maturation earlier and grow slightly larger than their northern counterparts, both of them aspects that are highly sought after in aquaculture (Bjørndal *et al.*, 2016; Dinis *et al.*, 1999; Howell *et al.*, 2009). The optimal water temperature for Senegalese sole is also higher than for Common sole, thus increasing Southern Europe's preference for farming Senegalese sole (Bjørndal *et al.*, 2015; Dinis *et al.*, 1999; Morais *et al.*, 2014). Temperature values between 19-21ºC are usually the optimal for the species (Dinis *et al.*, 1999; Howell *et al.*, 2011; Morais *et al.*, 2014). Temperatures above that tend to increase the propensity to pathologies (Cañavate, 2005), while temperatures rounding 15ºC might stunt growth (Howell *et al.*, 2011). Senegalese sole is an euryhaline species, meaning the salinity threshold is relatively large, being able to withstand high salinity values between 2 and 55 (Arjona *et al.*, 2007). Despite this, this species can achieve good growth rates with salinity values between 25 and 39, which decreases when salinity is lowered to 15 (Arjona *et al.*, 2009). The euryhaline capability of the Senegalese sole can be seen as an advantage not only in the wild to be able to inhabit lower

salinity areas, but also in aquaculture, being able to endure seasonal salinity changes (e.g., increased salinity during summer) and also allowing them to undergo salinity treatments for some pathologies (Arjona *et al.*, 2009; Arjona *et al.*, 2007; Dinis *et al.*, 1999).

1.2.1. Senegalese sole Aquaculture

The production of Senegalese sole began in Southern Portugal and Southern Spain during the 1970/1980s, through the use of abandoned salt ponds as tanks for extensive polycultures with gilt-head seabream and/or European seabass (Dinis *et al.*, 1999; Ferreira *et al.*, 2010; Morais *et al.*, 2014; Yúfera & Arias, 2010). On account of the advances of the aquaculture technology, stacked shallow raceways or regular tanks, either through an open or recirculation aquaculture systems (RAS), have overtaken the earth ponds (Bjørndal *et al.*, 2015; Howell *et al.*, 2009). RAS allow an easier control of the water parameters, which helps to maintain optimal environmental conditions for the fishes' growth (Bjørndal *et al.*, 2016; Bjørndal *et al.*, 2015; Howell *et al.*, 2009; Howell, 1997). Both European seabass and gilt-head seabream are immensely saturated in Southern European markets, therefore creating a space in the market for other fish species (Cañavate, 2005; Dinis *et al.*, 1999; FEAP, 2022; Howell, 1997; Imsland *et al.*, 2003; Morais *et al.*, 2014). In addition to this, due to being considered a very valuable flatfish, the Senegalese sole can be seen as an interesting alternative aquaculture product (Bjørndal *et al.*, 2015; Dinis *et al.*, 1999; Escribano *et al.*, 2020; Imsland *et al.*, 2003; Morais *et al.*, 2014). Despite this species inherent worth, Senegalese sole production is still a very small part of European aquaculture, representing only 0.2% of the total Marine Mediterranean Production in 2020 (FEAP, 2022).

Nowadays, Senegalese sole production takes place mainly in Europe, with countries such as Spain, Iceland, Portugal, and France leading in terms of tonnes produced (APROMAR, 2022). According to APROMAR (2022), a total of 1480 tonnes of Senegalese sole was produced worldwide in aquaculture in 2021, with Spain contributing around 1020 tonnes out of the total. In Portugal, Senegalese sole only made up 4.1% of the total marine finfish production in 2021, with 327 tonnes produced (INE, 2022). These numbers, however, appear to be encouraging as they have shown an increase of 116% when compared to the previous year (INE, 2021). When comparing statistics from INE (Instituto Nacional de Estatística),

the growth of Senegalese sole aquaculture in Portugal seems quite promising, growing 2415% in a 14-year span (2008-2021) (Fig. 1).

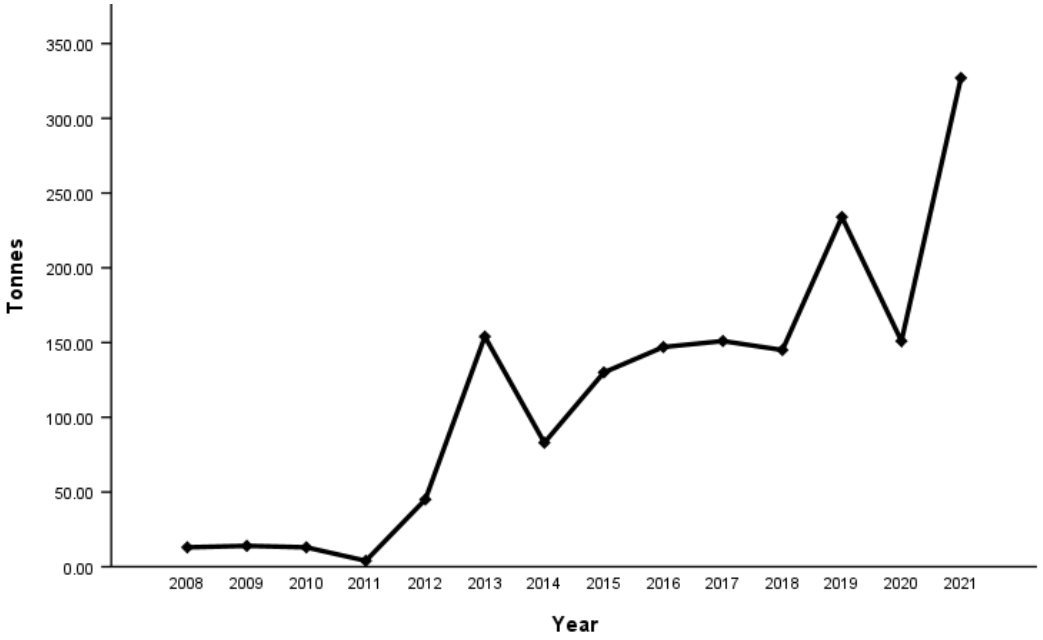

Fig. 1 – Senegalese sole production (tonnes) in Portugal, from 2008-2021. (INE)

Senegalese sole usually stands among one of the most expensive aquaculture products, ranging between 7.5 to 24€/Kg depending on the season (APROMAR, 2022; Imsland *et al.*, 2003; Mercamadrid, 2023). However, due to the similarities of both Common sole and Senegalese sole, the two are often reported as the same species in aquaculture and fisheries statistics, which may distort the real production numbers of the Senegalese sole (Bjørndal *et al.*, 2016; Bjørndal *et al.*, 2015; Morais *et al.*, 2014).

Despite sole production being practiced since the 1980s, commercial aquaculture only started growing significantly after the 2010's decade (APROMAR, 2015, 2022). Alongside the expansion of knowledge in aquaculture from a general point of view, the increased interest on promoting Senegalese sole monocultures lead to significant research and development of this species' production. This increasing investigation effort resulted in a better understanding of the species' physiology, environmental and nutritional needs (Arjona *et al.*, 2009; Dinis *et al.*, 1999; Howell, 1997; Imsland *et al.*, 2003; Navarro *et al.*, 2009). This resulted in improvements in broodstock and juveniles' production

which helps lowering the production costs of Senegalese sole, thus increasing their commercial viability to the producers (Bjørndal *et al.*, 2016; Howell *et al.*, 2009; Howell, 1997).

1.2.2. Pathologies

Another major issue with sole aquacultures has been the incidence of pathologies that may stunt the growth and incite high mortality rates (Arijo *et al.*, 2005; Mabrok *et al.*, 2022; Mabrok *et al.*, 2016; Magariños *et al.*, 1995; Morais *et al.*, 2014; Zorrilla *et al.*, 2003; Zorrilla *et al.*, 1999). The most prevalent pathologies tend to be caused by bacteria (Arijo *et al.*, 2005; Rico *et al.*, 2008; Toranzo *et al.*, 2005) such as *Tenacibaculum maritimum (Avendaño-Herrera et al., 2006; Cepeda & Santos, 2002; Vilar et al., 2012)*, *Vibrio spp. (Rico et al., 2008; Zorrilla et al., 2003)* and *Photobacterium damselae ssp. piscicida* (Magariños *et al.*, 1995; Magariños *et al.*, 2003; Zorrilla *et al.*, 1999). Aquacultures tend to lead to stressful conditions for the fish, such as improper environmental conditions, excessive stocking densities or handling-related lesions (Cañavate, 2005; Costas *et al.*, 2007; Escribano *et al.*, 2020; Mabrok *et al.*, 2016; Magariños *et al.*, 1995; Morais *et al.*, 2014; Toranzo *et al.*, 2005). These types of situations can result in a weakened immune system, thus allowing opportunistic pathogens to properly proliferate. On Senegalese sole, one of the main factors that can trigger an outbreak is water temperature (Cañavate, 2005; Mabrok *et al.*, 2016). Studies with *T. maritimum* suggest that the bacteria does not infect Senegalese sole with temperatures close to 16ºC (Mabrok *et al.*, 2016) but can lead to outbreak when above 22ºC (Cañavate, 2005; Mabrok *et al.*, 2016). Fish size/age can also influence how the effects of the pathologies. *T. maritimum* can infect both juveniles and adults, despite achieving much higher mortalities on juveniles (Avendaño-Herrera *et al.*, 2006; Bernardet *et al.*, 1994). Despite still being a relevant topic on Senegalese sole aquaculture, their impact has diminished as an increased attention is being given to subjects such as fish immunology and welfare.

1.3. Mucosal Health

Aquatic environments are typically very rich in a diverse array of pathogens, hence requiring fish to have suitable protection against them (Ellis, 2001; Esteban, 2012). Mucosal surfaces hold the microbiota, whose contribution to the animal's health holds a relevant importance, while at the same time being

able to serve as the first line of defence against pathogens (Dalmo & Bøgwald, 2022; Esteban, 2012; Gomez *et al.*, 2013; Guardiola *et al.*, 2019; Yu *et al.*, 2020). Mucus' continuous replacement does not allow pathogens to fully adhere to the fishes' skin, thus serving an important part on the physical removal of pathogens (Ellis, 2001). Besides acting as a physical barrier against the environment, it also offers innate cellular and humoral defences, making it an integral part of teleosts immunological structure (Bjorgen & Koppang, 2021; Esteban, 2012; Salinas, 2015; Salinas *et al.*, 2011; Yu *et al.*, 2020).

The mucosal-associated lymphoid tissue (MALT) is constituted of a wide range of myeloid and lymphoid cells that offer protection against pathogens and antigens (Firmino *et al.*, 2021; Gomez *et al.*, 2013). These cells are also responsible for the maintenance of the mucosa homeostasis, through symbiosis of beneficial microbiota (Gomez *et al.*, 2013; Randall & Mebius, 2014). So far, there have been identified six MALTs on teleosts, although others are currently being studied. These six include the gut (GALT), gill (GIALT), skin (SALT), nasopharynx (NALT), buccal (BALT) and pharyngeal-associated lymphoid tissue (PALT) (Firmino *et al.*, 2021; Yu *et al.*, 2020).

SALT holds both innate and specific immune structures (Fig. 2), who collaborate to eliminate potential threats (Dalmo & Bøgwald, 2022). The innate immune system is constituted by mechanisms that allow for a quick response to wounds or harmful substances (Esteban, 2012). It is constituted by components such as dendritic cells, monocytes/macrophages, granulocytes (i.e., neutrophils, basophils and eosinophils) and non-specific cytotoxic cells (Dalmo & Bøgwald, 2022; Esteban, 2012; Evans & Jaso-Friedmann, 1992; Gomez *et al.*, 2013; Yu *et al.*, 2020). The specific or adaptative immune system, on the other hand, offer a more precise immune response. The ability to create an "immunological memory" to certain antigens allow for a faster reaction upon a following infection (Stosik *et al.*, 2021; Stosik *et al.*, 2018). T cells, B cells and humoral components such as immunoglobulins (mainly IgM and to some extent IgT) are responsible for this capability, although macrophages and dendritic cells also partake on the specific immune system (Dalmo & Bøgwald, 2022; Gomez *et al.*, 2013; Salinas, 2015; Salinas *et al.*, 2011; Stosik *et al.*, 2021; Stosik *et al.*, 2018; Xu *et al.*, 2013; Yu *et al.*, 2020; Zhang *et al.*, 2010)

Teleost fish epidermis is a stratified epithelium composed by several secretory cells that produce the mucus present on their skin, such as goblet cells (Dash *et al.*, 2018; Salinas *et al.*, 2011). Mucus can be described as a complex combination of several components, such as proteins, ions, lipids, and mucins, the most prevalent molecule in mucus (Gomez *et al.*, 2013). This composition provides an environment that is ideal for the survival of commensal bacteria (Ellis, 2001; Gomez *et al.*, 2013). It contains several humoral immune defenses that aid in the inhibition of pathogens, including enzymes (such as lysozyme and peroxidase), complement proteins, proteases and immunoglobulins (Dalmo & Bøgwald, 2022; Dash *et al.*, 2018; Ellis, 2001; Esteban, 2012; Nayak, 2010). Despite this, some consider that some immunological components are only

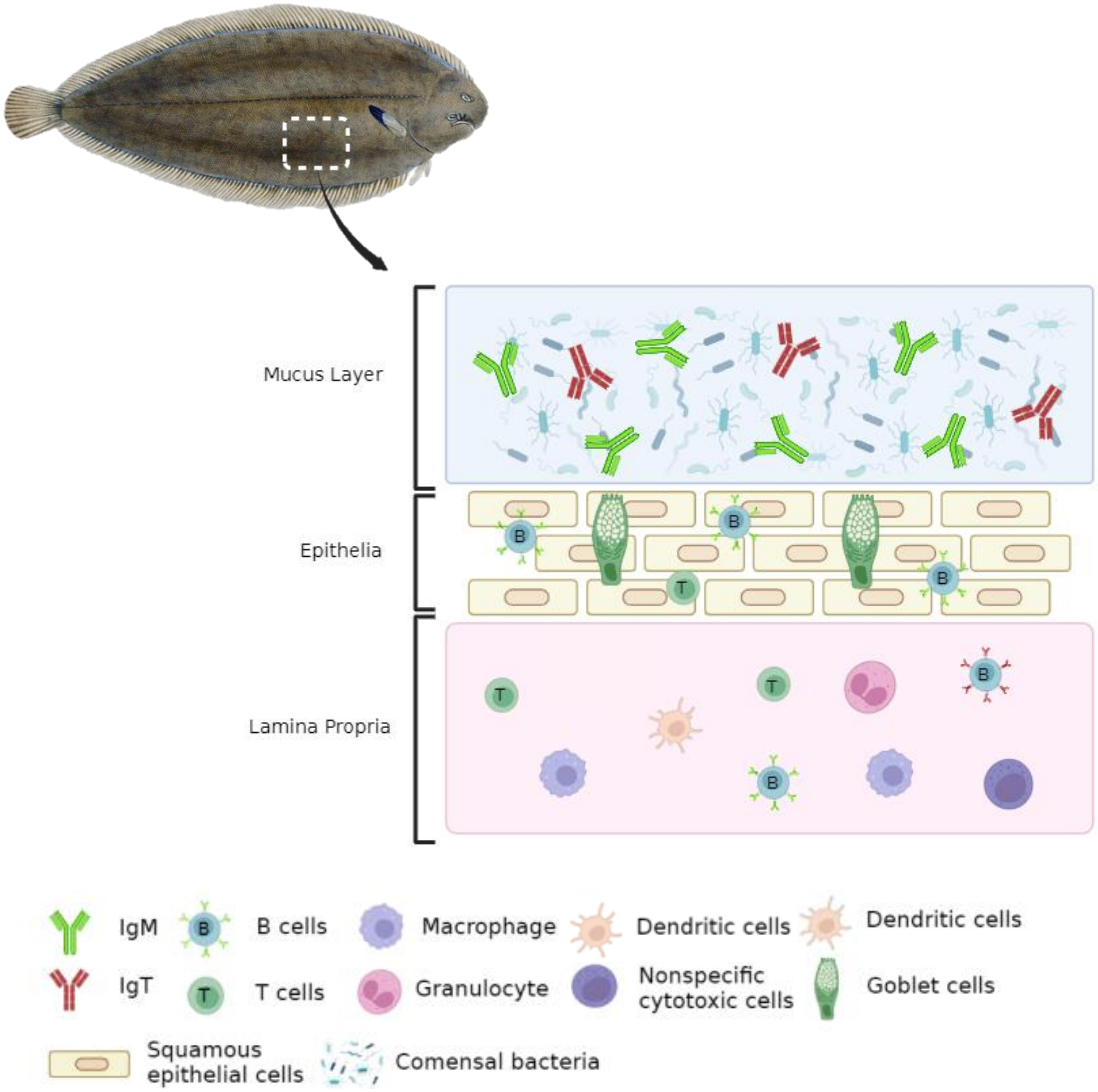

Fig. 2 - Representation of teleost's skin associated mucosal tissue (SALT). Adapted from Gomez et al. (2013) and Yu et al. (2020). Made with BioRender.com. Sole image: Whiff from The Natural History of British Fishes (1802) by Edward Donovan. Original from the New York Public Library. Digitally enhanced by rawpixel.

produced upon exposure to an infection or pathogen (Salinas *et al.*, 2011; Zhang *et al.*, 2010). Some of these components may be present on different quantities on different types of mucosal areas (Esteban, 2012). The skin mucus properties, both chemical and physical, undergo variations based on environmental factors such as water parameters and the presence of pathogens in the water (Roberts & Powell, 2005; van der Marel *et al.*, 2010).

Intensive aquaculture conditions can be detrimental for the fish's immune system when considering the stress caused by the high densities and handling, which could increase the risk of pathologies (Costas *et al.*, 2007; Ellis, 2001; Liu *et al.*, 2019; Long *et al.*, 2019; Montero *et al.*, 1999; Salas-Leiton *et al.*, 2010). Given this, an increased attention must be given to procedures who can promote the farmed fishes' welfare and subsequent immune system performance (Esteban, 2012; Gomez *et al.*, 2013). Currently, one of the most interesting procedures is the use of immunomodulating functional feeds, through the use of probiotics on aquaculture feeds (Fernandez *et al.*, 2015; García de La Banda *et al.*, 2010; Nayak, 2010; Nimalan *et al.*, 2022; Ramos-Pinto *et al.*, 2019). The use of probiotics can also provide an important contribution to the current shift of the use of fishmeal and fish-oil to a more sustainable source of nutrients, such as plant protein/oil (García de La Banda *et al.*, 2010). Plant-based protein may lacks of certain amino-acids and other important nutritional components such as Long-Chain Polyunsaturated Fatty Acids (LC-PUFA) that provoke negative effects both on the growth and immunity of fish (Kiron, 2012; Sitjà-Bobadilla *et al.*, 2005; Torrecillas *et al.*, 2017a; Valente *et al.*, 2016). However, the use of probiotics could serve as a supplement to cover those deficiencies (Batista *et al.*, 2016; García de La Banda *et al.*, 2010; Nimalan *et al.*, 2022).

Regarding the mucosal health and immunity on flatfish, more specifically Senegalese sole, the studies and information are still scarce (Escribano *et al.*, 2020), and in particular there is a huge lack of knowledge regarding different leucocyte populations at the mucosal sites (e.g., different B cell subsets) and immunoglobulin types. On the last decade more studies started emerging regarding these topics, mainly on the mucosal immune response (Escribano *et al.*, 2020; Guardiola *et al.*, 2017; Guardiola *et al.*, 2019). When taking in account that the flatfish's skin is in contact with substrate most of the time, a deeper focus should be given on their skin mucosa, which remains poorly studied (Escribano *et al.*, 2020). Senegalese sole's increasing importance in aquaculture should also be

an aspect to take in account to further improve the knowledge of their skin mucosa immune responses.

1.4. Objective

The main focus of this study was to evaluate the evolution of the fish's skin mucosa's humoral parameters during a small period on a production cycle, as there is still a lack of studies on the Senegalese sole's mucosal health on a production scale. This experiment took place alongside of a dry feed trial, with the purpose of formulating a feed that had a smaller amount of fishmeal (FM) (replaced by vegetable meal) and fish oil (FO), as a way to promote a more sustainable practice (Ansari *et al.*, 2021). As such, there was also an intent to test the effects of the two different diets on the growth rates and humoral parameters of the sole.

2. Material and Methods

2.1. Experimental Design

Senegalese soles were obtained with a mean weight of 20 g, from Safistela S.A. (Póvoa de Varzim, Portugal). They were reared on Aquacria Arousa S.A. (Cambados, Spain) until reaching around 85 g, to be able to consume the intended dry feed. The rearing conditions were the same for all the fish, and similar to the conditions they were going to remain during the following experimental period, given that the same RAS was used for both. Once the size was achieved, they were randomly transferred to four PVC square tanks, and reared in regular production conditions during four months (December 2022 – March 2023, 94 days in total), on a 5 m^3 s^{-1} flow and an initial mean stocking density of 7.95 kg m^{-2}.

All the tanks were connected to one of the production's RAS, thus maintaining the same water conditions for all the tanks. During the experimental period, the environmental parameters were kept, in average, with a temperature of 19.7ºC, salinity of 26, 7.42 pH and above 95% oxygen saturation. Despite seasonal variations, the mean water parameters values were kept in optimal conditions for the species (Arjona *et al.*, 2009; Morais *et al.*, 2014). Other parameters such as nitrates and ammonia levels were tested every day to prevent it reaching undesired levels. A natural photoperiod (12L/12D) was present throughout the whole experiment. The fish were subjected to daily production

routines, which included cleaning the tanks, feeding and collecting/documenting the mortality.

2.2. Feed

Two different dry feeds were used during the experiment, both of them produced by the same company. A standard commercially available feed was given to the two control tanks, while a novel feed was tested on two test tanks. The test feed contained 25% less fish meal, which had been swapped for a plant-based meal. The test feed was both isoenergetic and isoproteic. Both types of feed had the same size pellets, with 3mm diameter. Feeding took place through automatic 12h belt feeders, dispensing dry feed from 12:00H to 00:00H. Feed quantities were automatically calculated via software.

2.3. Sampling

The mucus samples were collected once a month for three months, following Palaksha *et al.* (2008) and Guardiola *et al.* (2014) methods. Twelve fish from each tank (n=12) were randomly selected one by one for mucus collection, being later released onto a box inside the tank to prevent sampling the same individual. The mucus was gently scraped out of the ocular and blind sides using a rubber spatula while avoiding the collection of particles such as feces or any other unwanted particles that could be present in the water. All the samplings took place at the same hour of the day, to reduce the influence of the daily cortisol fluctuations (Oliveira *et al.*, 2013; Sanchez-Vazquez *et al.*, 2019). Once collected, the samples were frozen at -80ºC until the three sets of samples were complete and ready to analyze.

After each mucus sampling, a random set of 50 fish (n=50) from every tank was weighted one by one to assess the growth rates. Weighting before the mucus sampling was avoided in order to minimize stress or any alteration of the humoral parameters.

2.4. Zootechnical Parameters

Stocking densities (SD), feed conversion rate (FCR) and specific growth rates (SGR) (Ramos-Pinto *et al.*, 2019) were obtained through the following calculations:

- $SD\ (kg\ m^{-2}) = \frac{[Total\ Fish\ Number * Mean\ Body\ Weight\ (MBW)(kg)]}{Tank\ Area\ (m^2)}$

- $FCR = \frac{Total\ Feed\ Weight\ (kg)}{(Mean\ Final\ Weight\ (kg) - Mean\ Initial\ Weight\ (kg))}$
- *SGR* (% day^{-1}) = $(e^{g} - 1) * 100$
    - $g = \frac{[Ln(Mean\ Final\ Weight\ (g)) - Ln(Mean\ Initial\ Weight\ (g)]}{Time\ (days)}$

The mortality rates and mean body weights (MBW) were also taken in consideration for the zootechnical parameters.

2.5. Mucus Humoral Parameters Analysis

2.5.1. Mucus Samples Preparation

After all the mucus samples were collected, they were prepared for the analysis. The preparation was based on Azeredo *et al.* (2017) methods, also relative to skin mucus. The samples were homogenized using a Tris-buffered saline (TBS, 50 mM Tris–HCl, 150 mM NaCl, pH 8.0) on a 1:1 ratio. Afterwards, the homogenized samples were shaken using a vortex on high intensity and later centrifuged (1500 rpm, 10 min, 4 ºC). The supernatant obtained was carefully transferred to new tubes, which were frozen at -80ºC to prepare for the lyophilization (0.940 mBar, -77ºC, 24h). Once complete, the lyophilized skin mucus was resuspended using ultra-pure water with a 1:1 ratio according to the amount of mucus and vigorously mixed with a vortex to ensure it was homogenized. In certain cases, the use of the centrifuge was necessary to break bigger mucus masses. After this process, the samples were frozen at -80ºC until the analysis began.

2.5.2. Total Protein

The first parameter measured was total protein (TP) using a Thermo Scientific™ Pierce™ BCA Protein Assay Kit. It uses a colorimetric method to detect TP on a sample when mixed with the working reagent, comparing with a standard of bovine serum albumin (BSA) with known protein content. It was used a dilution of the prepared samples, on a 1:5 ratio with ultrapure water. Using a 96-wells plates, 25 µL of both standard or sample were pipetted into each well, followed by adding 200 µL of working reagent. Once mixed, the samples and standard samples are incubated for one hour at a 25ºC temperature. Afterwards they were placed on the spectrophotometer to be read at a 562 nm. The BSA standard line's equation is used to calculate the sample's TP concentration using the obtained absorbance values, taking in account the dilution factor. The samples were then

all adjusted to contain 500 μg mL^{-1} of TP (Azeredo *et al.*, 2017). Samples whose contents were under that value were lyophilized once again and diluted on a smaller dilution factor in order to obtain a more concentrated sample.

2.5.3. Peroxidase Activity

An absorbance-based technique was used to quantify the peroxidase activity (U mL^{-1} of mucus), based on Quade and Roth (1997) and Azeredo *et al.* (2017) protocols. A volume of 50 μL of sample was diluted on 100 μL of HBSS Ca^{2+} or/and Mg^{2+} free in a 96 well plate, with 3 wells being kept with 150 μL of HBSS Ca^{2+} or/and Mg^{2+} free to serve as blank. Afterward, 50 μL of TMB 10mM and 50 μL H_2O_2 were added to each well and 2 minutes had to be waited before moving to the next step. As soon as the time passed, the reaction was stopped using 50 μL of H_2SO_4 2M. Once complete, the absorbance was measured at 450 nm, with the presumption that 1 unit of peroxidase would produce a shift of 1 unit in optical density.

2.5.4. Lysozyme Activity

To determine the lysozyme concentration (μg ml^{-1}), a turbidimetric method adapted from Esteban *et al.* (2001) was used to determine the lysozyme lysis activity against a known bacteria *Micrococcus lysodeikticus* (Sigma M3770-5g) on a known amount 0.25 mg mL^{-1}. A standard line using Hen egg white lysozyme (HEWL) with known amounts of lysozyme was prepared in order to compare to the sample. On a 96 well plate, both the HEWL solution (25 μL of each different concentration HEWL + 135 bacterial suspension) and the controls (two controls with 160 μL buffer and bacterial suspension 160 μL). Once the bacterial suspension was added, the solution was immediately read on the spectrophotometer on 450nm. In order to assess the lysozyme activity, two readings were done, the first at 0.5 minutes and the second at 10 minutes. It should be expected that the concentration of bacteria would drop on the second reading due to the lysis effect. Once the standard line was complete, the same procedure was taken with the samples instead of the HEWL solution. The concentration of sample lysozyme was determined using the previously obtained standard line equation.

2.5.5. Bactericidal Activity

The bactericidal activity was determined using an adapted protocol from Sunyer and Tort (1995). *Tenacibaculum maritimum* was cultured for 48 h at 25ºC in a concentration of $2x10^8$. Once grown, it was diluted with Marine Broth (MB) (Condalab, Madrid, Spain) to a concentration of $1x10^7$ (≈ 0.200 abs). In a 96-well round-bottom plate, 40 μL of mucus and 20 μL of *T. maritimum* were incubated in duplicates for 2.5h at 25ºC. Two positive controls were used, replacing the mucus sample for 40 μL of HBSS (Hank's Balanced Salt Solution) and 20 μL of *T. maritimum*, and another with 40 μL of MB and 20 μL of *T. maritimum*, both in triplicates. Once the incubation was complete, 25 μL of MTT (3-(4,5 dimethyl-2-yl)-2,5-diphenyl tetrazolium bromide, 1 mg mL^{-1}) was added to each well and incubated again for 10 min at 25ºC. The plates were then centrifuged at 2000 x *g* for 10 min. The precipitate obtained was dissolved in 200 μL of DMSO (dimethyl sulfoxide), from which 100 μL were transferred to a 96-well flat-bottom plate followed by an absorbance reading at 560 nm. The absorbance obtained from the samples was compared to the positive control sample (only bacteria) in order to calculate the percentage of bactericidal capacity (or percentage of non-viable bacteria).

2.6. Statistical Analysis

The statistical data was analysed using the IBM SPSS Statistics software [version 29.0.0.0 (241)]. Descriptive data was presented as mean value ± standard error.

All the data was tested for normality and homogeneity of variance through Shapiro-Wilk and Levene tests, respectively. In cases where they did not achieve those criteria, outliers were removed and, if necessary, data was logarithmically transformed before proceeding with two-way analysis of variance (ANOVA), where the sampling time and dietary treatment were defined as fixed variables and the tank was used as a random factor. A Tukey HSD test was performed when significant differences were found, with the purpose of identifying significantly different groups.

Peroxidase activity and zootechnical parameters distribution did not follow normality and homogeneity requirements despite the removal of outliers and data transformation; therefore, a Kruskal-Wallis test was performed. For the

peroxidase activity, the Kruskal-Wallis test was applied to compare the mean values of the control and test groups. To evaluate the peroxidase activity and zootechnical parameters, the same test was applied to all four samplings (including the initial for the weight) between control and test groups.

Due to an outbreak of *Tenacibaculum maritimum* in one of the two test tanks before the first sampling that could affect the bactericidal activity results, a second ANOVA test was performed excluding this one in order to prevent any bias on that specific parameter.

A level of significance of $p \leq 0,05$ was used on all the statistical analysis.

3. Results

3.1. Zootechnical Parameters

The mean weights of both groups of fish remained consistent throughout the whole experiment (Fig. 3). Initial weighting landed on 83.29 ± 2.13 g (n=50) for the control group and 84.29 ± 1.93 g (n=50) for the test group. The first sampling revealed a slight decrease on the test group mean weight (1st sampling: 104.57 ± 2.45 g; n=50) in comparison the control group (1st sampling: 107.72 ± 2.67 g). The final weighting showed an increase on the mean weight of the test group (163.48 ± 4.31 g, n=50 against the control group (157.69 ± 3.98 g, n=50). No significant differences were found between diets on the four samplings (Initial sampling: $p = 0.51$; 1st sampling: $p = 0.45$; 2nd sampling: $p = 0.88$; 3rd sampling: $p = 0.37$, $\alpha = 0.05$).

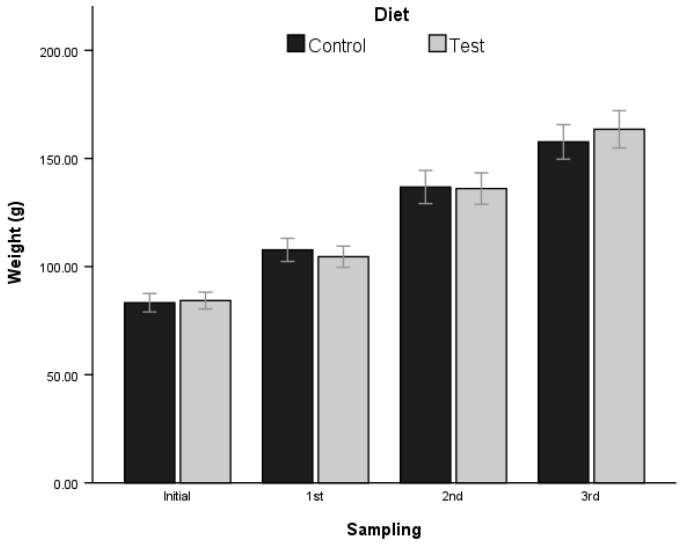

Fig. 3 – Mean body weight (g) of Senegalese sole fed on two different diets. Initial sampling represents the day they were transferred to the tanks. Bars represent the mean ± standard error of mean.

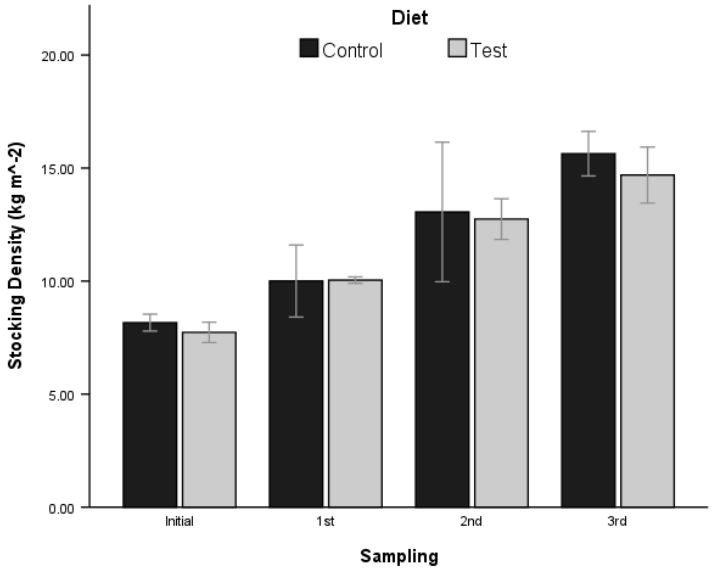

Fig. 4 - Mean stocking density (kg m^{-2}) of Senegalese sole fed on two different diets. Initial sampling represents the day they were transferred to the tanks. Bars represent the mean ± standard error of mean.

Stocking densities increased a mean of 1.9-fold over the experimental period (Fig. 4). No differences were found on SD between diets (p = 0.43, α=0.05). All SD increases between samplings proved to be significant, with the exception to the first sampling on the control group (p = 0.10, α=0.05) and the third sampling on the test group (p = 0.09, α=0.05).

Test tanks achieved a better FCR (1.26 ± 0.08) when compared to the control tanks (1.49 ± 0.05) (Table 1). The test tanks also obtained a slightly higher SGR (0.71 ± 0.002 % day^{-1}) than the control tanks (0.69 ± 0.01% day^{-1}) (Table 1). The mortalities, however, presented a higher percentage on the test tanks (1.26 ± 1.00%) than on the control tanks (0.57 ± 0.03%) (Table 1). No significant differences were revealed on any of the parameters.

Table 1 – Average means of Feed Conversion Rate (FCR), Specific Growth Rate (SGR) and Mortality on Senegalese sole fed two different diets (Control and Test).

	Diet	
	Control	Test
	Mean ± Std. Error	Mean ± Std. Error
FCR (%)	1.49 ± 0.05	1.26 ± 0.08
SGR (% day^{-1})	0.69 ± 0.01	0.71 ± 0.00
Mortality (%)	0.57 ± 0.03	1.26 ± 1.01

3.2. Humoral Immune Parameters

3.2.1. Peroxidase Activity

Overall peroxidase activity values showed higher values on the control group (5.41 ± 0.18 U mg^{-1}, n=56) than the test group (4.74 ± 0.15 U mg^{-1}, n=54) (p=0.01, α=0.05). When comparing the diets between sampling times, there were only significant differences on the first sampling between control (5.70 ± 0.18 U mg^{-1}, n=13) and test diet (4.33 ± 0.33 U mg^{-1}, n=13) (p=0.02, α=0.05) (Fig. 5).

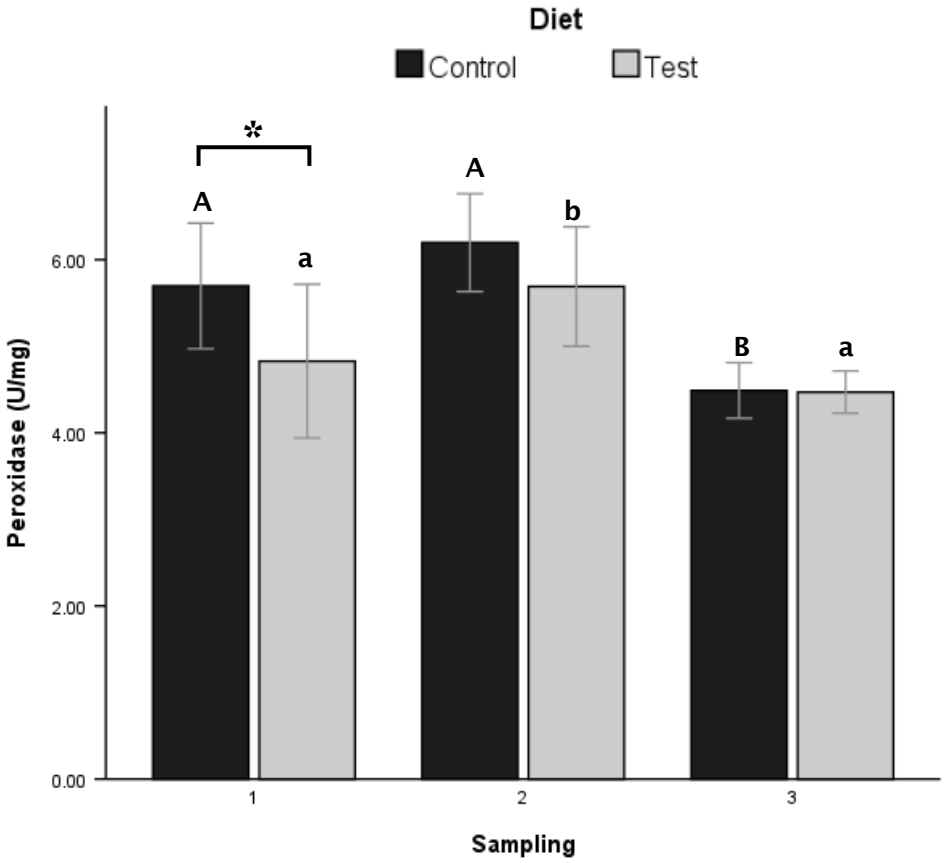

Fig. 5 - Peroxidase activity (U mg^{-1}) on Senegalese sole skin mucus fed on two different diets. Bars represent the mean ± standard error of mean. Asterisks represent significant differences between control and test diet in each sampling on two-way ANOVA test ($p<0.05$, α=0.05). Capital and small letters represent significant differences between sampling times for control and test group, respectively.

For the test diet, differences on the overall values (p=0.03, α=0.05). Statistical tests revealed a significant increase between the 1st and 2nd sampling (p=0.02, α=0.05), followed by a decrease between 2nd and 3rd sampling (p=0.02, α=0.05).

The same was noticed for the control diet, where significant differences were found on the overall peroxidase activity between samplings ($p<0.001$,

α=0.05). The control diet peroxidase activity decreased on the third sampling, showing significant differences between 1st and 3rd (p=0.005, α=0.05)., and 2nd and 3rd (p<0.001, α=0.05). No differences were found between 1st and 2nd sampling (p=0.26, α=0.05).

3.2.2.Lysozyme Activity

Overall lysozyme activity displayed a higher mean value on the control group (4.81 ± 0.16 μg ml $^{-1}$, n=51) than the test group (4.27 ± 0.20 μg ml $^{-1}$, n=41) (Fig. 6), with the differences proving to be significant (p=0.04, α=0.05). The two-way ANOVA test showed no significant effect of the sampling times on the lysozyme activity (tanks: p= 0.051, α=0.05; samplings: p=0.46, α=0.05; n=92). It did, however, show that the diet had a significant effect on lysozyme activity (p=0.04, α=0.05).

On the first sampling, the mean lysozyme activity value showed a higher value on the test group (5.33 ± 0.26 μg ml $^{-1}$, n=22) than on the control group (3.74 ± 0.39 μg ml $^{-1}$, n=13). The second sampling proved the opposite, with the values being higher on the control group (5.75 ± 0.35 μg ml $^{-1}$, n=17) than the test group (2.93 ± 0.23 μg ml $^{-1}$, n=6), In both samplings, there were significant differences (p<0.001, α=0.05). During the third sampling, an increase of lysozyme activity on the test group (4.54 ± 0.25 μg ml $^{-1}$) was noticeable, although maintaining a smaller value than the control group (4.94 ± 0.14 μg ml $^{-1}$), which led to a loss of significant differences between both groups.

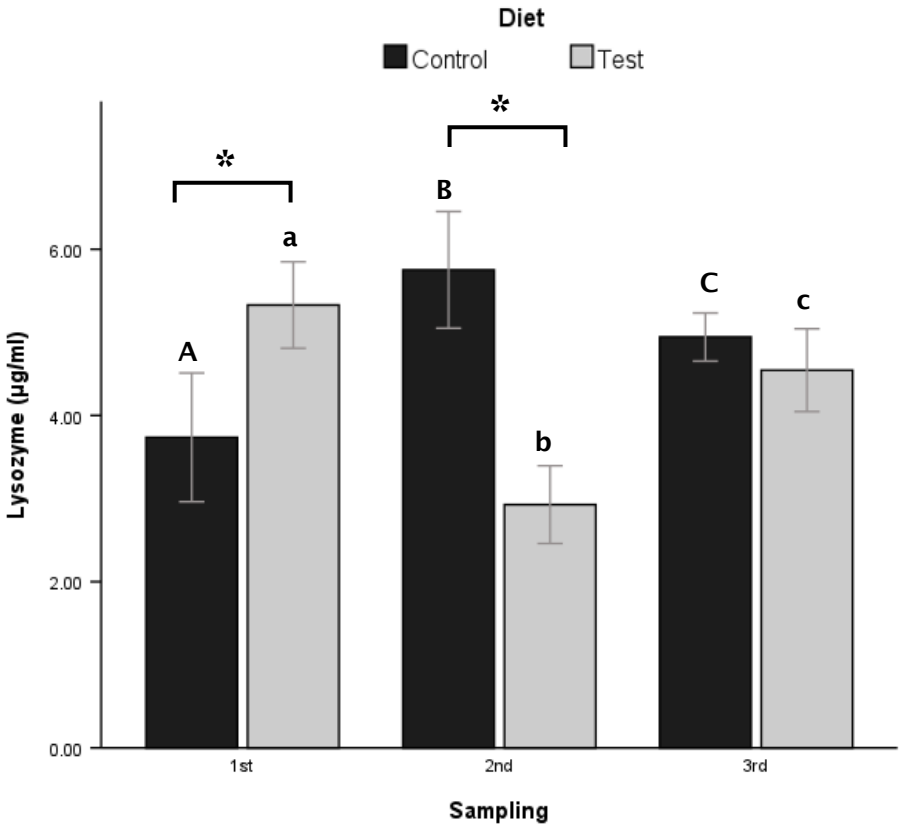

Fig. 6 – Lysozyme activity (µg ml $^{-1}$) on Senegalese sole skin mucus fed on two different diets. Bars represent the mean ± standard error of mean. Asterisks represent significant differences between control and test diet in each sampling on two-way ANOVA test ($p<0.05$, $\alpha=0.05$). Capital and small letters represent significant differences between sampling times for control and test group, respectively.

3.2.3. Bactericidal Activity

Based on the descriptive analysis, the overall bactericidal activity against *T. maritimum* was lower in fish fed the test diet (15.78 ± 1.16 %, n=58) than the ones that received the control diet (18.78 ± 1.11 %, n=57) regardless sampling time, with the differences proving to be significant between both test groups ($p=0.04$, $\alpha=0.05$). The test group maintained lower percentages of bactericidal activity through all the samplings except the first sampling (Fig. 7), where it was registered a mean of 24.74 ± 1.97% on the test group (n=14) against on the control group 22.73 ± 2.30% (n=14), although they were not significant ($p=0.49$, $\alpha=0.05$). The remaining sampling times all showed significant differences between test groups, with the control group achieving higher bactericidal activities (Second sampling: $p=0.003$; Third sampling: $p=0.05$, $\alpha=0.05$).

When comparing the bactericidal activity of each group between sampling times, the test group revealed a significant decrease from the first to the second

sampling (p<0.001, α=0.05), followed by a slight drop from the second to the third sampling, despite no significance (p=0.056, α=0.05). The control group showed no differences between first and second sampling, although it decreased between second and third sampling (p=0.003, α=0.05).

The second ANOVA showed that the lack of difference between test and control diets was maintained (p=0.43, α=0.05), although this time with an increased value for the test group during the first sampling (25.44 ± 2.58 %, n=7).

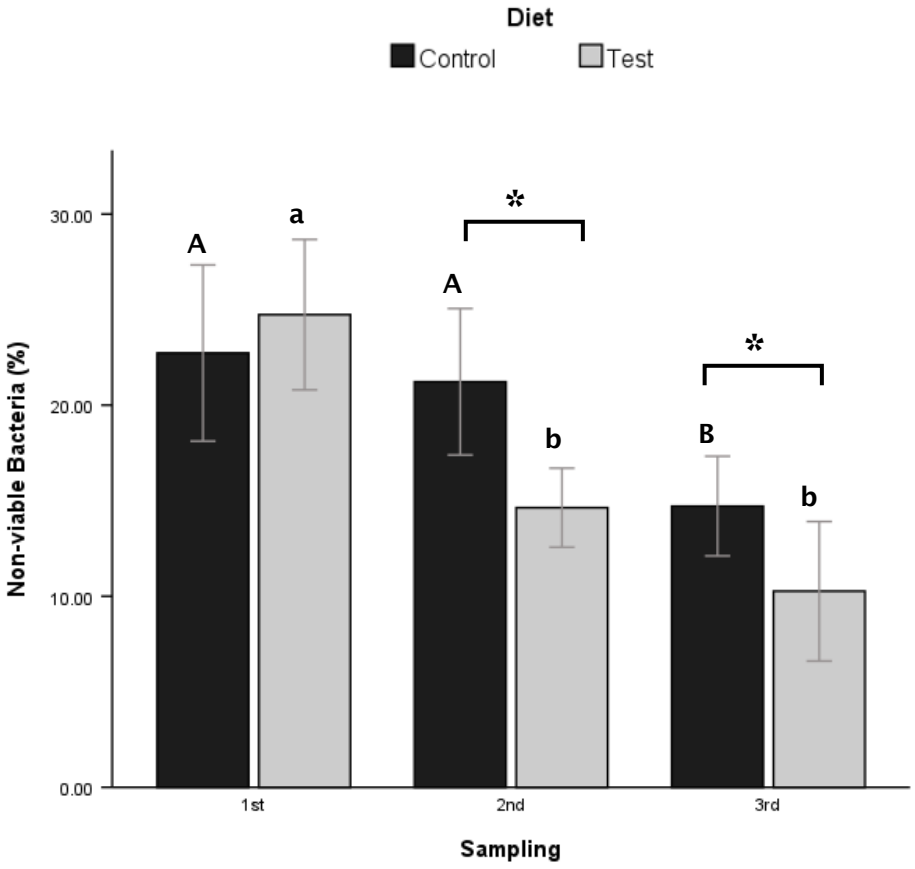

Fig. 7 - Bactericidal activity (% of non-viable bacteria) on Senegalese sole skin mucus fed on two different diets. Bars represent the mean ± standard error of mean. Asterisks represent significant differences between control and test diet in each sampling on two-way ANOVA test (p<0.05, α=0.05). Capital and small letters represent significant differences between sampling times for control and test group, respectively.

4. Discussion

4.1. Zootechnical Parameters

Results obtained showed that both groups had slight average weight differences throughout the whole experiment, with none of them being statistically significant. This goes in agreement with several studies where plant protein feed was used, not only with Senegalese sole (Batista *et al.*, 2016; Valente *et al.*, 2016), but also with other important aquaculture species (Parma *et al.*, 2016; Torrecillas *et al.*, 2017a). Batista *et al.* (2016) tested the use of two feeds with different percentage of plant protein which evidenced that Senegalese sole can achieve regular growth rates using up to 72% of plant protein inclusion. On the same note, Valente *et al.* (2016) demonstrated that Senegalese sole could be effectively fed with feeds using up to 75% plant protein inclusion. Furthermore, on the same study, it was shown that using a 100% plant protein feed led to not only a decrease in growth rates, but also an inferior flesh quality. Montero *et al.* (2015) investigated the effects of the total replacement of FO by two different vegetable oils (VO) - linseed oil and soybean oil - on a feed for Senegalese soles. It was reported that despite not reaching significant levels, the soles fed with VO feed shown slightly reduced weight, especially the ones fed with soybean oil.

Stocking densities increases were expected, with no differences being found between diets. The SD values achieved on the final sampling grew around 1.9-fold since the start of the experiment, reaching a SD that has been reported to not have effects on the growth of the Senegalese sole, both being considered as a low SD level (Andrade *et al.*, 2015; Salas-Leiton *et al.*, 2008). The lack of differences between control and test group also goes in agreement with the SGR results obtained on the present study.

The FCR showed no significant differences between diets, however, the test group had a lower value than the control group. Plant-based diets tend to have a lower digestibility on carnivorous fishes, which can lead to increased FCR values due to the lack of effectiveness on the absorption of nutrients (Colombo, 2020). The lack of differences in FCR values on these trials could suggest that Senegalese sole can effectively take advantage of this specific diet. This goes in agreement with studies such as the one performed by Parma *et al.* (2016), where increasing amounts of soybean meal in gilthead seabream feed did not increase the FCR. The same lack of changes in FCR was pointed by Batista *et al.* (2016)

when comparing two Senegalese sole diets with different contents of plant ingredients. The authors pointed that only the supplementation of the feed led to significant changes. Some authors suggested that the effects of plant-based ingredients in diets depend on the specific ingredients (such as supplements) and concentration (Batista *et al.*, 2016; Collins *et al.*, 2013; Colombo, 2020). On the other hand, Pratoomyot *et al.* (2010) found that smaller amounts of FM led to a reduction of the FCR during the first 8 weeks of trial, which increased after that point. On the present study, the fact that the transition period between the previously consumed diet and the novel one was not noticeable could mean that the implementation of this diet during a production cycle would not hinder the fish's growth on a short-term.

Pratoomyot *et al.* (2010) pointed out that a decrease in FM inclusion on different diets led to a decrease in SGR on the first 8 weeks of trial (from the 8th week forward no differences were noted). On the present study, however, both groups maintained roughly the same SGR, which goes in agreement with several other studies (Costas *et al.*, 2007; Parma *et al.*, 2016; Salas-Leiton *et al.*, 2010; Salas-Leiton *et al.*, 2011; Torrecillas *et al.*, 2017a). When also rearing Senegalese sole, some authors achieved similar SGR values than the ones obtained on these trials, meaning the test diet used on this study could be a potential alternative for commercial feeds (Costas *et al.*, 2007; Salas-Leiton *et al.*, 2010; Salas-Leiton *et al.*, 2011). Torrecillas *et al.* (2017a) managed to reduce the FM and FO usage on European seabass feed up to 10% and 3% respectively without reducing the growth rates. Yamamoto *et al.* (2023) showed interesting results after being able to increase SGR on rainbow trout (*Oncorhynchus mykiss* Walbaum, 1792) fed plant-based diets by selectively breeding fishes previously fed those types of feed, with the SGR increasing on latter generations. For this study, in addition to the mean weight, this could mean that this specific reduction in fish-based protein could still be used as an alternative diet formula that does not interfere with the growth rates of the fish, one of the most important factors in aquaculture.

The higher mortality rate on the treatment group seems to have been linked with the *T. maritimum* outbreak in one of the treatment tanks during the first month, having the mortality rates kept quite low throughout the rest of the experiment. The outbreak was likely caused by an increase in water temperature

as it has been reported before (Cañavate, 2005; Mabrok *et al.*, 2022), although it is still unclear why the rest of the tanks were not affected.

4.2. Humoral Immune Parameters

Mean peroxidase activity was, overall, significantly lower on the test group, with the biggest gap between diets being found on the first sampling. The values, however, tended to slightly decrease over time on both groups when comparing to the first sampling. This went with agreement with other feed trial tests where peroxidase values also decreased over time on skin mucus (Ramos-Pinto *et al.*, 2019). Senegalese sole has also been reported as a species with low quantities of skin mucus peroxidase (Guardiola *et al.*, 2019). Additionally, Cordero *et al.* (2016) showed that lyophilizing skin mucus samples may lead to significant reductions of peroxidase values. When looking further into the results of the present study, in both diets there were decreases on the peroxidase activity between the second and third sampling, which could be an effect of the increasing SD.

Regarding lysozyme, it was observed an overall lower activity on test groups. This goes in agreement with other experiments with Senegalese soles fed plant based diets, such as Batista *et al.* (2016). However, during the first sampling the lysozyme activity was significantly higher on the treatment group. As mentioned before, one of the treatment tanks had been affected by a *T. maritimum* outbreak before first sampling, which led to some mortality. Given the bactericidal role of the lysozyme (Dalmo & Bøgwald, 2022), the previous bacterial infection might have led to increases in the immune parameters, which would go in agreement with studies such as Escribano *et al.* (2020). The inconsistencies between samplings on both groups could also be linked with the *T. maritimum* outbreak, as it has been shown by Escribano *et al.* (2020) that 3 weeks following an infection there are is a decrease in lysozyme. Some studies also point to the possibility that teleost fish can only produce an efficient antibody response when exposed to a pathogen or infection (Salinas *et al.*, 2011; Zhang *et al.*, 2010), which could also help explain the increase on the test group during the first month of sampling.

The same tendency was observed on the bactericidal activity. Significant differences between diets were observed, with the test group having the lowest values on the last two samplings. On the first sampling however, a slightly higher

value (although not significant) of bactericidal activity was achieved on the test group. The same point made for the lysozyme activity values could be applied here, having the bactericidal activity increased due to the recent *T. maritimum* outbreak before the first sampling. Considering the fact that fish were reared in a RAS system, the pathogen was spread throughout the whole system, however, it was more prominent in one of the test tanks. Escribano *et al.* (2020) reported that Senegalese sole showed an increase in skin mucus bactericidal activity 14 days after being challenged with a *T. maritimum* bath. van der Marel *et al.* (2010) suggested that an increase of pathogen load in the water can also be a factor that promotes the increase of humoral immune properties of skin mucus, which could potentially explain the overall higher bactericidal activity during the first sampling. However, Mabrok *et al.* (2016) claimed that the Senegalese sole may not possess the suitable bactericidal compounds to defend themselves against *T. maritimum*. The skin mucus has been proven to modify its properties according to the water parameters, such as low salinity decreasing skin mucus viscosity on salmonids (Roberts & Powell, 2005). The current study took place in a RAS system, however, there were seasonal fluctuations on environmental parameters such as temperature and salinity that could potentially have an effect on humoral immune parameters and physico-chemical properties of the mucus. Salinity, in particular, suffered some fluctuations during January, prior to the first sampling. It was, however, always kept with a mean value above of what is considered optimal conditions (Arjona *et al.*, 2009). Guardiola *et al.* (2019) reported that sole's bactericidal activity might be altered by the viscosity of the skin mucus, as a higher viscosity may lead to decreases in the bactericidal activity. However, there still needs to be a higher understanding of how these properties change on Senegalese sole based on the water parameters.

Through the three samplings, there was an overall decrease on the peroxidase and bactericidal activity values between the first and last sampling. This decrease could be linked to a possible imbalanced ratio of LC-PUFA, mainly n-3 and n-6. VM and VO tend to have low amounts of n-3 and be rich in n-6, thus creating nutritional imbalances with consequences to the immune system, has it has been reported by several authors (Bell *et al.*, 2002; Lin & Shiau, 2007; Machado *et al.*, 2019; Montero *et al.*, 2015; Montero & Izquierdo, 2010; Torrecillas *et al.*, 2017b; Yıldız *et al.*, 2018). In the present study, the inclusion of these type of ingredients in the test diet could have led to a decrease of the

humoral immune parameters. Montero *et al.* (2015) suggested that the total substitution of FO by VO in a Senegalese sole feed might have led to an inflammatory process in the gut, resulting in an over-expression of immune-related genes. Lin and Shiau (2007) reported similar results with grouper (*Epinephelus malabaricus* Bloch & Schneider, 1801), with the use of only VO in the feed leading to decrease in humoral immune parameters. On the same study, however, the authors reported that a blend of FO and VO (50% FO and 50% VO) resulted in an increase of the immune parameters. These immunity boosts tend to be linked with the use of probiotics or other types of supplementations to reduce the lack of n-3 on plant-based diets. This was the case on a study conducted by Ramos-Pinto *et al.* (2019), where dietary supplementation on a fishmeal-free feed proved to have short-termed immunity boosts on *S. aurata* juveniles. On the same note, Nya and Austin (2011) reported that the immunity boost of dietary garlic on rainbow trout lasted only 28 days, starting to decline after that period. A study by Sharifuzzaman and Austin (2009) revealed similar results, with the probiotic effect on immune parameters in rainbow trout fed the probiotic for two weeks.

The increasing SD might have had some impact on the humoral immune parameters. Several studies have proven that high stocking densities lead to significant decreases on the immune parameters of several species (Jia *et al.*, 2016; Liu *et al.*, 2019; Long *et al.*, 2019). On Senegalese sole, it has also been reported that high SD tend to increased stress and a reduction of humoral immune parameters, which can lead to a higher susceptibility to diseases (Costas *et al.*, 2013; Costas *et al.*, 2007). Costas *et al.* (2013; 2007) have also pointed out that high SD showed lower humoral immune parameters such as lysozyme and peroxidase than the ones reared with a low SD. Salas-Leiton *et al.* (2010) stated that despite the crowding stress that high SD provokes on soles, it can be counteracted by increasing the ration size. Regarding this study, the SD during the experimental period maintained what some authors would consider low SD (Andrade *et al.*, 2015; Salas-Leiton *et al.*, 2008), while others have determined that the SD after the 2nd sampling would be a high value (Costas *et al.*, 2013; Costas *et al.*, 2007). When taking in consideration the immune parameters values obtained, only peroxidase and bactericidal activity showed a decrease on the last sampling, with lysozyme actually increasing.

5. Conclusion

The aim of the present study was to evaluate the evolution of Senegalese sole mucosal health during a production cycle with two different diets differing in the FM inclusion level. Regarding a zootechnical point of view, the plant-based diet showed promising results, has it managed to maintain growth ratings similar to those with a commercially available diet. In relation to the humoral immune parameters, the decreases might be connected to the nutritional imbalances, mainly on LC-PUFA, which tend to have a negative impact on the immune system. However, this could be prevented by providing a different type of supplementation or probiotic. Being an aquaculture species with an increasing importance on European aquacultures, further studies regarding on the improvement of their immune system could potentially improve production levels. Considering that it is a minimally invasive process, skin mucus analysis could prove to be an important tool for immunity and health assessment especially on an aquaculture level, reducing possible mortalities from more invasive processes.

6. References

Andrade, T., Afonso, A., Pérez-Jiménez, A., Oliva-Teles, A., de las Heras, V., Mancera, J. M., Serradeiro, R., & Costas, B. (2015). Evaluation of different stocking densities in a Senegalese sole (*Solea senegalensis*) farm: Implications for growth, humoral immune parameters and oxidative status. *Aquaculture*, *438*, 6-11. https://doi.org/10.1016/j.aquaculture.2014.12.034

Ansari, F. A., Guldhe, A., Gupta, S. K., Rawat, I., & Bux, F. (2021). Improving the feasibility of aquaculture feed by using microalgae. *Environ Sci Pollut Res Int*, *28*(32), 43234-43257. https://doi.org/10.1007/s11356-021-14989-x

APROMAR. (2015). Aquaculture in Spain 2015. *Asociasión Empresarial de Acuicultura de España*.

APROMAR. (2022). Aquaculture in Spain 2022. *Asociasión Empresarial de Acuicultura de España*. www.apromar.es

Arijo, S., Chabrillón, M., Díaz-Rosales, P., Rico, R. M., Martínez-Manzanares, E., Balebona, M. C., Toranzo, A. E., & Moriñigo, M. A. (2005). Bacteria isolated from outbreaks affecting cultured sole, *Solea senegalensis* (Kaup). *Bulletin of the European Association of Fish Pathologists*, *25*, 148-154.

Arjona, F. J., Vargas-Chacoff, L., Ruiz-Jarabo, I., Gonçalves, O., Páscoa, I., Martín del Río, M. P., & Mancera, J. M. (2009). Tertiary stress responses in Senegalese sole (Solea senegalensis Kaup, 1858) to osmotic challenge: Implications for osmoregulation, energy metabolism and growth. *Aquaculture*, *287*(3-4), 419-426. https://doi.org/10.1016/j.aquaculture.2008.10.047

Arjona, F. J., Vargas-Chacoff, L., Ruiz-Jarabo, I., Martin del Rio, M. P., & Mancera, J. M. (2007). Osmoregulatory response of Senegalese sole (Solea senegalensis) to changes in environmental salinity. *Comp Biochem Physiol A Mol Integr Physiol*, *148*(2), 413-421. https://doi.org/10.1016/j.cbpa.2007.05.026

Avendaño-Herrera, R., Toranzo, A. E., & Magariños, B. (2006). Tenacibaculosis infection in marine fish caused by *Tenacibaculum maritimum*: a review. *DISEASES OF AQUATIC ORGANISMS*, *71*, 255-266.

Azeredo, R., Machado, M., Guardiola, F. A., Cerezuela, R., Afonso, A., Peres, H., Oliva-Teles, A., Esteban, M. A., & Costas, B. (2017). Local immune response of two mucosal surfaces of the European seabass, *Dicentrarchus labrax*, fed tryptophan- or methionine-supplemented diets. *Fish Shellfish Immunol*, *70*, 76-86. https://doi.org/10.1016/j.fsi.2017.09.016

Batista, S., Ozório, R. O. A., Kollias, S., Dhanasiri, A. K., Lokesh, J., Kiron, V., Valente, L. M. P., & Fernandes, J. M. O. (2016). Changes in intestinal microbiota, immune- and stress-related transcript levels in Senegalese sole (*Solea senegalensis*) fed plant ingredient diets intercropped with probiotics or immunostimulants. *Aquaculture*, *458*, 149-157. https://doi.org/10.1016/j.aquaculture.2016.03.002

Bell, J. G., Henderson, R. J., Tocher, D. R., McGhee, F., Dick, J. R., Porter, A., Smullen, R. P., & Sargent, J. R. (2002). Substituting fish oil with crude palm oil in the diet of Atlantic salmon (*Salmo salar*) affects muscle fatty acid composition and hepatic fatty acid metabolism. *J Nutr*, *132*(2), 222-230. https://doi.org/10.1093/jn/132.2.222

Bernardet, J.-F., Kerouault, B., & Michel, C. (1994). Comparative Study on *Flexibacter maritimus* Strains Isolated from Farmed Sea Bass (*Dicentrarchus labrax*) in France. *Fish Pathology*, *29*, 105-111.

Bjorgen, H., & Koppang, E. O. (2021). Anatomy of teleost fish immune structures and organs. *Immunogenetics*, *73*(1), 53-63. https://doi.org/10.1007/s00251-020-01196-0

Bjørndal, T., Guillen, J., & Imsland, A. (2016). The potential of aquaculture sole production in Europe: Production costs and markets. *Aquaculture Economics & Management*, *20*(1), 109-129. https://doi.org/10.1080/13657305.2016.1124939

Bjørndal, T., Guillen, J., Imsland, A., & Øiestad, V. (2015). Sole: Production and Markets. In FAO (Ed.), *GLOBEFISH Research Programme* (Vol. 118, pp. 72).

Cañavate, J. P. (2005). Opciones del lenguado senegalés *Solea senegalensis* Kaup, 1858 para diversificar la acuicultura marina. *Boletín. Instituto Español de Oceanografía*, *21*, 147-154.

Cepeda, C., & Santos, Y. (2002). First isolation of *Flexibacter maritimus* from farmed Senegalese sole (*Solea senegalensis*, Kaup) in Spain. *Bulletin of the European Association of Fish Pathologists*, *22*, 388-392.

Collins, S. A., Øverland, M., Skrede, A., & Drew, M. D. (2013). Effect of plant protein sources on growth rate in salmonids: Meta-analysis of dietary inclusion of soybean, pea and canola/rapeseed meals and protein concentrates. *Aquaculture*, *400-401*, 85-100. https://doi.org/10.1016/j.aquaculture.2013.03.006

Colombo, S. M. (2020). Physiological considerations in shifting carnivorous fishes to plant-based diets. In *Aquaculture* (pp. 53-82). https://doi.org/10.1016/bs.fp.2020.09.002

Cordero, H., Cuesta, A., Meseguer, J., & Esteban, M. A. (2016). Changes in the levels of humoral immune activities after storage of gilthead seabream (*Sparus aurata*) skin mucus. *Fish Shellfish Immunol*, *58*, 500-507. https://doi.org/10.1016/j.fsi.2016.09.059

Costas, B., Aragão, C., Dias, J., Afonso, A., & Conceição, L. E. (2013). Interactive effects of a high-quality protein diet and high stocking density on the stress response and some innate immune parameters of Senegalese sole *Solea senegalensis*. *Fish Physiol Biochem*, *39*(5), 1141-1151. https://doi.org/10.1007/s10695-013-9770-1

Costas, B., Aragão, C., Mancera, J. M., Dinis, M. T., & Conceição, L. E. C. (2007). High stocking density induces crowding stress and affects amino acid metabolism in Senegalese sole *Solea senegalensis* (Kaup 1858) juveniles. *Aquaculture Research*, *39*(1), 1-9. https://doi.org/10.1111/j.1365-2109.2007.01845.x

Dalmo, R. A., & Bøgwald, J. (2022). Innate Immunity. In K. Buchmann & C. J. Secombes (Eds.), *Principles of Fish Immunology* (pp. 31-103). Springer, Cham. https://doi.org/10.1007/978-3-030-85420-1_2

Dash, S., Das, S. K., Samal, J., & Thatoi, H. N. (2018). Epidermal mucus, a major determinant in fish health: a review. *Iranian Journal of Veterinary Research*, *19*, 72-81.

Dinis, M. T., Ribeiro, L., Soares, F., & Sarasquete, C. (1999). A review on the cultivation potential of *Solea senegalensis* in Spain and in Portugal. *Aquaculture*, *176*, 27-38.

Ellis, A. E. (2001). Innate host defense mechanisms of fish against viruses and bacteria. *Dev Comp Immunol*, *25*, 827-839.

Escribano, M. P., Ramos-Pinto, L., Fernandez-Boo, S., Afonso, A., Costas, B., & Guardiola, F. A. (2020). Mucosal immune responses in Senegalese sole (*Solea senegalensis*) juveniles after *Tenacibaculum maritimum* challenge: A comparative study between ocular and blind sides. *Fish Shellfish Immunol*, *104*, 92-100. https://doi.org/10.1016/j.fsi.2020.05.080

Esteban, M. Á. (2012). An Overview of the Immunological Defenses in Fish Skin. *ISRN Immunology*, *2012*, 1-29. https://doi.org/10.5402/2012/853470

Esteban, M. A., Cuesta, A., Ortuno, J., & Meseguer, J. (2001). Immunomodulatory effects of dietary intake of chitin on gilthead seabream (*Sparus aurata* L.) innate immune system. *Fish Shellfish Immunol*, *11*(4), 303-315. https://doi.org/10.1006/fsim.2000.0315

Evans, D. L., & Jaso-Friedmann, L. (1992). Nonspecific cytotoxic cells as effectors of immunity in fish. *Annual Rev. of Fish Diseases*, *2*, 109-121. https://doi.org/10.1016/0959-8030(92)90059-7

FAO. (2022). *The State of World Fisheries and Aquaculture 2022*. https://doi.org/10.4060/cc0461en

FEAP. (2022). *FEAP Annual Report 2022*.

Fernandez, I., Lopez-Joven, C., Andree, K. B., Roque, A., & Gisbert, E. (2015). Vitamin A supplementation enhances Senegalese sole (*Solea senegalensis*) early juvenile's immunocompetence: New insights on potential underlying pathways. *Fish Shellfish Immunol*, *46*(2), 703-709. https://doi.org/10.1016/j.fsi.2015.08.007

Ferreira, H., Ribeiro, A. R., Dias, J., Yúfera, M., Arias, A. M., Falcão, M., Serpa, D., Aires, T., Pousão-Ferreira, P., Cunha, M. E., Valente, L. M. P., Dinis, M. T., & Conceição, L. E. C. (2010). Sustainable semi-intensive polyculture of seabream and sole in earthen ponds. *Aquaculture Europe*, *35*, 17-21.

Firmino, J. P., Galindo-Villegas, J., Reyes-Lopez, F. E., & Gisbert, E. (2021). Phytogenic Bioactive Compounds Shape Fish Mucosal Immunity. *Front Immunol*, *12*, 695973. https://doi.org/10.3389/fimmu.2021.695973

García de La Banda, I., Lobo, C., León-Rubio, J. M., Tapia-Paniagua, S., Balebona, M. C., Moriñigo, M. A., Moreno-Ventas, X., Lucas, L. M., Linares, F., Arce, F., & Arijo, S. (2010). Influence of two closely related probiotics on juvenile Senegalese sole (*Solea senegalensis*, Kaup 1858) performance and protection against Photobacterium damselae subsp. piscicida. *Aquaculture*, *306*(1-4), 281-288. https://doi.org/10.1016/j.aquaculture.2010.05.008

Gomez, D., Sunyer, J. O., & Salinas, I. (2013). The mucosal immune system of fish: the evolution of tolerating commensals while fighting pathogens. *Fish Shellfish Immunol*, *35*(6), 1729-1739. https://doi.org/10.1016/j.fsi.2013.09.032

Guardiola, F. A., Cuartero, M., Del Mar Collado-Gonzalez, M., Diaz Banos, F. G., Cuesta, A., Moriñigo, M. A., & Esteban, M. A. (2017). Terminal carbohydrates abundance, immune related enzymes, bactericidal activity and physico-chemical parameters of the Senegalese sole (*Solea*

senegalensis, Kaup) skin mucus. *Fish Shellfish Immunol*, *60*, 483-491. https://doi.org/10.1016/j.fsi.2016.11.025

Guardiola, F. A., Cuesta, A., Abellan, E., Meseguer, J., & Esteban, M. A. (2014). Comparative analysis of the humoral immunity of skin mucus from several marine teleost fish. *Fish Shellfish Immunol*, *40*(1), 24-31. https://doi.org/10.1016/j.fsi.2014.06.018

Guardiola, F. A., Mabrok, M., Machado, M., Azeredo, R., Afonso, A., Esteban, M. A., & Costas, B. (2019). Mucosal and systemic immune responses in Senegalese sole (*Solea senegalensis* Kaup) bath challenged with *Tenacibaculum maritimum*: A time-course study. *Fish Shellfish Immunol*, *87*, 744-754. https://doi.org/10.1016/j.fsi.2019.02.015

Howell, B., Conceição, L. E. C., Prickett, R., Cañavate, P., & Mañanós, E. (2009). Sole farming: nearly there but not quite?! A report of the 4th Workshop on the Cultivation of Soles. *Aquaculture Europe*, *34*, 24-27.

Howell, B., Prickett, R., Cañavate, P., Mañanós, E., Dinis, M. T., Conceição, L. E. C., & Valente, L. M. P. (2011). Sole farming: There or thereabouts! A report of the 5th workshop on the cultivation of soles. *Aquaculture Europe*, *34*, 24-27.

Howell, B. R. (1997). A re-appraisal of the potential of the sole, *Solea solea* (L.), for commercial cultivation. *Aquaculture*, *155*, 355-365.

Imsland, A. K., Foss, A., Conceição, L. E. C., Dinis, M. T., Delbare, D., & White, P. (2003). A review of the culture potential of *Solea solea* and *S. senegalensis*. *Reviews in Fish Biology and Fisheries*, *13*, 379-407. https://doi.org/10.1007/s11160-004-1632-6

INE. (2021). *Estatísticas de Pesca 2021*.

INE. (2022). *Estatísticas da Pesca 2022*.

Jia, R., Liu, B. L., Feng, W. R., Han, C., Huang, B., & Lei, J. L. (2016). Stress and immune responses in skin of turbot (*Scophthalmus maximus*) under different stocking densities. *Fish Shellfish Immunol*, *55*, 131-139. https://doi.org/10.1016/j.fsi.2016.05.032

Kiron, V. (2012). Fish immune system and its nutritional modulation for preventive health care. *Animal Feed Science and Technology*, *173*(1-2), 111-133. https://doi.org/10.1016/j.anifeedsci.2011.12.015

Lin, Y.-H., & Shiau, S.-Y. (2007). Effects of dietary blend of fish oil with corn oil on growth and non-specific immune responses of grouper, *Epinephelus malabaricus*. *Aquaculture Nutrition*, *13*, 137-144.

Liu, B., Fei, F., Li, X., Wang, X., & Huang, B. (2019). Effects of stocking density on stress response, innate immune parameters, and welfare of turbot (*Scophthalmus maximus*). *Aquaculture International*, *27*(6), 1599-1612. https://doi.org/10.1007/s10499-019-00413-2

Long, L., Zhang, H., Ni, Q., Liu, H., Wu, F., & Wang, X. (2019). Effects of stocking density on growth, stress, and immune responses of juvenile Chinese sturgeon (*Acipenser sinensis*) in a recirculating aquaculture system. *Comp Biochem Physiol C Toxicol Pharmacol*, *219*, 25-34. https://doi.org/10.1016/j.cbpc.2019.02.002

Mabrok, M., Algammal, A. M., Sivaramasamy, E., Hetta, H. F., Atwah, B., Alghamdi, S., Fawzy, A., Avendano-Herrera, R., & Rodkhum, C. (2022).

Tenacibaculosis caused by *Tenacibaculum maritimum*: Updated knowledge of this marine bacterial fish pathogen. *Front Cell Infect Microbiol*, *12*, 1068000. https://doi.org/10.3389/fcimb.2022.1068000

Mabrok, M., Machado, M., Serra, C. R., Afonso, A., Valente, L. M., & Costas, B. (2016). Tenacibaculosis induction in the Senegalese sole (*Solea senegalensis*) and studies of *Tenacibaculum maritimum* survival against host mucus and plasma. *J Fish Dis*, *39*(12), 1445-1455. https://doi.org/10.1111/jfd.12483

Machado, M., Castro, C., Oliva-Teles, A., & Costas, B. (2019). Interactive effects of dietary vegetable oil and carbohydrate incorporation on the innate immune response of European seabass (*Dicentrarchus labrax*) juveniles subjected to acute stress. *Aquaculture*, *498*, 171-180. https://doi.org/10.1016/j.aquaculture.2018.08.050

Magariños, B., Pazos, F., Santos, Y., Romalde, J. L., & Toranzo, A. E. (1995). Response of *Pasteurella piscida* and *Flexibacter maritimus* to skin mucus of marine fish. *DISEASES OF AQUATIC ORGANISMS*, *21*, 103-108. https://doi.org/doi:10.3354/dao021103

Magariños, B., Romalde, J. L., Romalde, S. L., Moriñigo, M. A., & Toranzo, A. E. (2003). Pathobiological characterisation of *Photobacterium damselae* subsp. *piscicida* isolated from cultured sole (*Solea senegalensis*). *Bulletin of the European Association of Fish Pathologists*, *23*, 183-190.

Mercamadrid. (2023). *Product Statistics*. https://www.mercamadrid.es/estadisticas/

Montero, D., Benitez-Dorta, V., Caballero, M. J., Ponce, M., Torrecillas, S., Izquierdo, M., Zamorano, M. J., & Manchado, M. (2015). Dietary vegetable oils: effects on the expression of immune-related genes in Senegalese sole (Solea senegalensis) intestine. *Fish Shellfish Immunol*, *44*(1), 100-108. https://doi.org/10.1016/j.fsi.2015.01.020

Montero, D., & Izquierdo, M. (2010). Welfare and health of fish fed vegetable oils as alternative lipid source to fish oil. In G. Turchini, W. Ng, & D. Tocher (Eds.), *Fish Oil Replacement and Alternative Lipid Sources in Aquaculture Feeds*.

Montero, D., Izquierdo, M. S., Tort, L., Robaina, L., & Vergara, J. M. (1999). High stocking density produces crowding stress altering some physiological and biochemical parameters in gilthead seabream, *Sparus aurata*, juveniles. *Fish Physiol Biochem*, *20*, 53-60.

Morais, S., Aragão, C., Cabrita, E., Conceição, L. E. C., Constenla, M., Costas, B., Dias, J., Duncan, N., Engrola, S., Estevez, A., Gisbert, E., Mañanós, E., Valente, L. M. P., Yúfera, M., & Dinis, M. T. (2014). New developments and biological insights into the farming of *Solea senegalensis* reinforcing its aquaculture potential. *Reviews in Aquaculture*, *8*(3), 227-263. https://doi.org/10.1111/raq.12091

Navarro, D. B., Rubio, V. C., Luz, R. K., Madrid, J. A., & Sánchez-Vázquez, F. J. (2009). Daily feeding rhythms of Senegalese sole under laboratory and farming conditions using self-feeding systems. *Aquaculture*, *291*(1-2), 130-135. https://doi.org/10.1016/j.aquaculture.2009.02.039

Nayak, S. K. (2010). Probiotics and immunity: a fish perspective. *Fish Shellfish Immunol*, *29*(1), 2-14. https://doi.org/10.1016/j.fsi.2010.02.017

Naylor, R. L., Hardy, R. W., Buschmann, A. H., Bush, S. R., Cao, L., Klinger, D. H., Little, D. C., Lubchenco, J., Shumway, S. E., & Troell, M. (2021). A 20-year retrospective review of global aquaculture. *Nature*, *591*(7851), 551-563. https://doi.org/10.1038/s41586-021-03308-6

Nimalan, N., Sørensen, S. L., Fečkaninová, A., Koščová, J., Mudroňová, D., Gancarčíková, S., Vatsos, I. N., Bisa, S., Kiron, V., & Sørensen, M. (2022). Mucosal barrier status in Atlantic salmon fed marine or plant-based diets supplemented with probiotics. *Aquaculture*, *547*. https://doi.org/10.1016/j.aquaculture.2021.737516

Nya, E. J., & Austin, B. (2011). Development of immunity in rainbow trout (*Oncorhynchus mykiss*, Walbaum) to *Aeromonas hydrophila* after the dietary application of garlic. *Fish Shellfish Immunol*, *30*(3), 845-850. https://doi.org/10.1016/j.fsi.2011.01.008

Oliveira, C. C., Aparicio, R., Blanco-Vives, B., Chereguini, O., Martin, I., & Javier Sanchez-Vazquez, F. (2013). Endocrine (plasma cortisol and glucose) and behavioral (locomotor and self-feeding activity) circadian rhythms in Senegalese sole (*Solea senegalensis* Kaup 1858) exposed to light/dark cycles or constant light. *Fish Physiol Biochem*, *39*(3), 479-487. https://doi.org/10.1007/s10695-012-9713-2

Palaksha, K. J., Shin, G. W., Kim, Y. R., & Jung, T. S. (2008). Evaluation of non-specific immune components from the skin mucus of olive flounder (*Paralichthys olivaceus*). *Fish Shellfish Immunol*, *24*(4), 479-488. https://doi.org/10.1016/j.fsi.2008.01.005

Parma, L., Candela, M., Soverini, M., Turroni, S., Consolandi, C., Brigidi, P., Mandrioli, L., Sirri, R., Fontanillas, R., Gatta, P. P., & Bonaldo, A. (2016). Next-generation sequencing characterization of the gut bacterial community of gilthead sea bream (*Sparus aurata*, L.) fed low fishmeal based diets with increasing soybean meal levels. *Animal Feed Science and Technology*, *222*, 204-216. https://doi.org/10.1016/j.anifeedsci.2016.10.022

Pratoomyot, J., Bendiksen, E. Å., Bell, J. G., & Tocher, D. R. (2010). Effects of increasing replacement of dietary fishmeal with plant protein sources on growth performance and body lipid composition of Atlantic salmon (*Salmo salar* L.). *Aquaculture*, *305*(1-4), 124-132. https://doi.org/10.1016/j.aquaculture.2010.04.019

Quade, M. J., & Roth, J. A. (1997). A rapid, direct assay to measure degranulation of bovine neutrophil primary granules. *Veterinary Immunology And Immunopathology*, *58*(1997), 239-248.

Ramos-Pinto, L., Martos-Sitcha, J. A., Reis, B., Azeredo, R., Fernandez-Boo, S., Perez-Sanchez, J., Calduch-Giner, J. A., Engrola, S., Conceição, L. E. C., Dias, J., Silva, T. S., & Costas, B. (2019). Dietary tryptophan supplementation induces a transient immune enhancement of gilthead seabream (*Sparus aurata*) juveniles fed fishmeal-free diets. *Fish Shellfish Immunol*, *93*, 240-250. https://doi.org/10.1016/j.fsi.2019.07.033

Randall, T. D., & Mebius, R. E. (2014). The development and function of mucosal lymphoid tissues: a balancing act with micro-organisms. *Mucosal Immunol*, *7*(3), 455-466. https://doi.org/10.1038/mi.2014.11

Rico, R. M., Tapia-Paniagua, S., Martinéz-Manzanares, E., Balebona, M. C., & Moriñigo, M. A. (2008). Characterization of *Vibrio harveyi* strains

recovered from diseased farmed Senegalese sole (*Solea senegalensis*). *J Appl Microbiol*, *105*(3), 752-760. https://doi.org/10.1111/j.1365-2672.2008.03786.x

Roberts, S. D., & Powell, M. D. (2005). The viscosity and glycoprotein biochemistry of salmonid mucus varies with species, salinity and the presence of amoebic gill disease. *J Comp Physiol B*, *175*(1), 1-11. https://doi.org/10.1007/s00360-004-0453-1

Salas-Leiton, E., Anguis, V., Manchado, M., & Cañavate, J. P. (2008). Growth, feeding and oxygen consumption of Senegalese sole (*Solea senegalensis*) juveniles stocked at different densities. *Aquaculture*, *285*(1-4), 84-89. https://doi.org/10.1016/j.aquaculture.2008.08.001

Salas-Leiton, E., Anguis, V., Martin-Antonio, B., Crespo, D., Planas, J. V., Infante, C., Cañavate, J. P., & Manchado, M. (2010). Effects of stocking density and feed ration on growth and gene expression in the Senegalese sole (*Solea senegalensis*): potential effects on the immune response. *Fish Shellfish Immunol*, *28*(2), 296-302. https://doi.org/10.1016/j.fsi.2009.11.006

Salas-Leiton, E., Anguís, V., Rodríguez-Rua, A., & Cañavate, J. P. (2011). High stocking density and food restriction have minimum impact on size dispersal of cultured Senegalese sole (Solea senegalensis, Kaup 1858) juveniles. Evidence for individual growth being regulated by population structure. *Aquacultural Engineering*, *45*(2), 43-50. https://doi.org/10.1016/j.aquaeng.2011.06.002

Salinas, I. (2015). The Mucosal Immune System of Teleost Fish. *Biology (Basel)*, *4*(3), 525-539. https://doi.org/10.3390/biology4030525

Salinas, I., Zhang, Y. A., & Sunyer, J. O. (2011). Mucosal immunoglobulins and B cells of teleost fish. *Dev Comp Immunol*, *35*(12), 1346-1365. https://doi.org/10.1016/j.dci.2011.11.009

Sanchez-Vazquez, F. J., Lopez-Olmeda, J. F., Vera, L. M., Migaud, H., Lopez-Patino, M. A., & Miguez, J. M. (2019). Environmental Cycles, Melatonin, and Circadian Control of Stress Response in Fish. *Front Endocrinol (Lausanne)*, *10*, 279. https://doi.org/10.3389/fendo.2019.00279

Sharifuzzaman, S. M., & Austin, B. (2009). Influence of probiotic feeding duration on disease resistance and immune parameters in rainbow trout. *Fish Shellfish Immunol*, *27*(3), 440-445. https://doi.org/10.1016/j.fsi.2009.06.010

Sitjà-Bobadilla, A., Peña-Llopis, S., Gómez-Requeni, P., Médale, F., Kaushik, S., & Pérez-Sánchez, J. (2005). Effect of fish meal replacement by plant protein sources on non-specific defence mechanisms and oxidative stress in gilthead sea bream (Sparus aurata). *Aquaculture*, *249*(1-4), 387-400. https://doi.org/10.1016/j.aquaculture.2005.03.031

Stosik, M., Tokarz-Deptula, B., & Deptula, W. (2021). Immunological memory in teleost fish. *Fish Shellfish Immunol*, *115*, 95-103. https://doi.org/10.1016/j.fsi.2021.05.022

Stosik, M. P., Tokarz-Deptula, B., & Deptula, W. (2018). Specific humoral immunity in Osteichthyes. *Cent Eur J Immunol*, *43*(3), 335-340. https://doi.org/10.5114/ceji.2018.80054

Sunyer, J. O., & Tort, L. (1995). Natural hemolytic and bactericidal activities of sea bream *Sparus aurata* serum are effected by the alternative complement pathway. *Veterinary Immunology And Immunopathology*, *45*, 333-345.

Thilsted, S. H., Thorne-Lyman, A., Webb, P., Bogard, J. R., Subasinghe, R., Phillips, M. J., & Allison, E. H. (2016). Sustaining healthy diets: The role of capture fisheries and aquaculture for improving nutrition in the post-2015 era. *Food Policy*, *61*, 126-131. https://doi.org/10.1016/j.foodpol.2016.02.005

Toranzo, A. E., Magariños, B., & Romalde, J. L. (2005). A review of the main bacterial fish diseases in mariculture systems. *Aquaculture*, *246*(1-4), 37-61. https://doi.org/10.1016/j.aquaculture.2005.01.002

Torrecillas, S., Mompel, D., Caballero, M. J., Montero, D., Merrifield, D., Rodiles, A., Robaina, L., Zamorano, M. J., Karalazos, V., Kaushik, S., & Izquierdo, M. (2017a). Effect of fishmeal and fish oil replacement by vegetable meals and oils on gut health of European sea bass (*Dicentrarchus labrax*). *Aquaculture*, *468*, 386-398. https://doi.org/10.1016/j.aquaculture.2016.11.005

Torrecillas, S., Roman, L., Rivero-Ramirez, F., Caballero, M. J., Pascual, C., Robaina, L., Izquierdo, M. S., Acosta, F., & Montero, D. (2017b). Supplementation of arachidonic acid rich oil in European sea bass juveniles (*Dicentrarchus labrax*) diets: Effects on leucocytes and plasma fatty acid profiles, selected immune parameters and circulating prostaglandins levels. *Fish Shellfish Immunol*, *64*, 437-445. https://doi.org/10.1016/j.fsi.2017.03.041

Valente, L. M. P., Cabral, E. M., Sousa, V., Cunha, L. M., & Fernandes, J. M. O. (2016). Plant protein blends in diets for Senegalese sole affect skeletal muscle growth, flesh texture and the expression of related genes. *Aquaculture*, *453*, 77-85. https://doi.org/10.1016/j.aquaculture.2015.11.034

van der Marel, M., Caspari, N., Neuhaus, H., Meyer, W., Enss, M. L., & Steinhagen, D. (2010). Changes in skin mucus of common carp, *Cyprinus carpio L.*, after exposure to water with a high bacterial load. *J Fish Dis*, *33*(5), 431-439. https://doi.org/10.1111/j.1365-2761.2010.01140.x

Vilar, P., Failde, L. D., Bermudez, R., Vigliano, F., Riaza, A., Silva, R., Santos, Y., & Quiroga, M. I. (2012). Morphopathological features of a severe ulcerative disease outbreak associated with *Tenacibaculum maritimum* in cultivated sole, *Solea senegalensis* (L.). *J Fish Dis*, *35*(6), 437-445. https://doi.org/10.1111/j.1365-2761.2012.01360.x

Xu, Z., Parra, D., Gomez, D., Salinas, I., Zhang, Y. A., von Gersdorff Jorgensen, L., Heinecke, R. D., Buchmann, K., LaPatra, S., & Sunyer, J. O. (2013). Teleost skin, an ancient mucosal surface that elicits gut-like immune responses. *Proc Natl Acad Sci U S A*, *110*(32), 13097-13102. https://doi.org/10.1073/pnas.1304319110

Yamamoto, T., Matsunari, H., Murashita, K., Yoshinaga, H., Oku, H., Furuita, H., Kaji, N., Aoyagi, T., & Miura, M. (2023). Effect of feeding plant-based diet to broodstock on the utilization of plant-based diet in the offspring of rainbow trout *Oncorhynchus mykiss*. *Aquaculture*, *568*. https://doi.org/10.1016/j.aquaculture.2023.739330

Yıldız, M., Eroldoğan, T. O., Ofori-Mensah, S., Engin, K., & Baltacı, M. A. (2018). The effects of fish oil replacement by vegetable oils on growth

performance and fatty acid profile of rainbow trout: Re-feeding with fish oil finishing diet improved the fatty acid composition. *Aquaculture*, *488*, 123-133. https://doi.org/10.1016/j.aquaculture.2017.12.030

Yu, Y., Wang, Q., Huang, Z., Ding, L., & Xu, Z. (2020). Immunoglobulins, Mucosal Immunity and Vaccination in Teleost Fish. *Front Immunol*, *11*, 567941. https://doi.org/10.3389/fimmu.2020.567941

Yúfera, M., & Arias, A. M. (2010). Traditional polyculture in "esteros" in the Bay of cádiz (Spain). Hopes and expectancies for the prevalence of a unique activity in Europe. *Aquaculture Europe*, *35*, 22-25.

Zhang, Y. A., Salinas, I., Li, J., Parra, D., Bjork, S., Xu, Z., LaPatra, S. E., Bartholomew, J., & Sunyer, J. O. (2010). IgT, a primitive immunoglobulin class specialized in mucosal immunity. *Nat Immunol*, *11*(9), 827-835. https://doi.org/10.1038/ni.1913

Zorrilla, I., Arijo, S., Chabrillón, M., Diaz, P., Martinéz-Manzanares, E., Balebona, M. C., & Moriñigo, M. A. (2003). *Vibrio* species isolated from diseased farmed sole, *Solea senegalensis* (Kaup), and evaluation of the potential virulence role of their extracellular products. *Journal of Fish Diseases*, *26*, 103-108.

Zorrilla, I., Balebona, M. C., Moriñigo, M. A., Sarasquete, C., & Borrego, J. J. (1999). Isolation and characterization of the causative agent of pasteurellosis, *Photobacterium damsela* ssp. *piscida* from sole, *Solea senegalensis* (Kaup). *Journal of Fish Diseases*, *22*, 167-172.