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The potential of *Asparagopsis armata* to control the bacterial load associated to live feed to improve Seabream (*Sparus aurata*) larvae performance

O potencial da macroalga *Aspargopsis armata* no controlo da carga bacteriana associada ao alimento vivo melhorando a performance larvar de dourada, *Sparus aurata*

Sara Isabel Costa Castanho

Mestrado em Aquacultura e Pescas

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Sob a orientação de:

Laura Ribeiro, Investigadora auxiliar IPMA

Rodrigo Costa, Investigador auxiliar CCMAR, UAlg

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Resumo

A produção em aquacultura ainda tem margem para aumentar na medida em que as taxas de sobrevivência durante a fase larvar são baixas e podem ser melhoradas. A quantidade de bactérias oportunistas introduzidas no cultivo larvar, aquando do fornecimento de alimento vivo (*Brachionus* spp. e *Artemia* sp.) é apontada como uma das causas para a elevada mortalidade nesta fase. Várias metodologias têm sido testadas para controlar a carga bacteriana associada ao alimento vivo utilizado nas maternidades de peixes marinhos. Porém, nenhuma das técnicas utilizadas é completamente satisfatória, alguns produtos usados afectam o ambiente ou produzem compostos com efeitos secundários prejudiciais. A utilização de macroalgas marinhas como alternativa a este tipo de produtos tem crescido nos últimos anos. Algumas algas são uma fonte de diversos metabolitos, com amplo espectro de actividades biológicas e diversas aplicações. De entre várias espécies de macroalgas, a alga vermelha *Asparagopsis armata* é uma das espécies com propriedades para inibir o crescimento de bactérias patogénicas de peixes. Esta espécie de alga está amplamente distribuída e é abundante na costa Portuguesa.

O objetivo deste trabalho foi testar a aplicação indirecta desta espécie de alga na produção larvar de dourada (*Sparus aurata*), analisando o seu desenvolvimento e qualidade larvar, ao reduzir a carga bacteriana associada ao alimento vivo (*Brachionus* spp. e *Artemia* sp.).

Dois produtos de *A. armata*, um extrato preparado em laboratório e um produto comercial Ysaline®100 (YSA), normalmente utilizado na indústria cosmética, foram testados no alimento vivo a diferentes concentrações, de forma a determinar o seu efeito nos rotíferos e *Artemia* a concentracções bactericidas. Para o ensaio larvar de dourada selecionou-se a concentração de 0.5 % de YSA para tratar o alimento vivo durante 30 min após o enriquecimento. O ensaio larvar consistiu no tratamento com YSA comparando com o grupo Controlo (em água limpa), com 4 replicados cada. O ensaio decorreu desde o início com a abertura de boca (4 dias após eclosão - DAE) até aos 35 DAE. Todos os tanques foram alimentados de forma idêntica, seguindo os protocolos estabelecidos na estação piloto de piscicultura de Olhão (EPPO) do IPMA, e a quantidade de alimento foi dividida entre 4 a 5 refeições diárias.

De forma a avaliar o desenvolvimento e qualidade larvar das douradas foram recolhidas periodicamente (aos 2, 9, 15, 20, 27 e 34 DAE), amostras para biometria

(comprimento total e peso seco), insuflação da bexiga gasosa, ocorrência de anomalias físicas externas e formação da barbatana caudal. A ingestão de presas foi monitorizada e amostras para análise histológica do sistema digestivo bem como para actividade enzimática foram recolhidas em pontos-chave do desenvolvimento (aos 15 DAE apenas para análise histológica e aos 20 e 34 DAE para ambas). No final do ensaio foram recolhidas amostras para realização de testes de stress, determinação da actividade de lisozima e para testes de resistência antibacteriana. Neste ponto, foram também efectuadas análises bacteriológicas às larvas e à água de produção, utilizando placas de agar com meio de cultura geral para contagem de bactérias aeróbias (TSA) e meio de cultura selectivo para contagem de *Vibrionaceae* (TCBS).

As larvas do tratamento YSA apresentaram maior crescimento do que o Controlo ($P < 0.05$), embora uma taxa de sobrevivência menor. Apesar das diferenças de tamanho as larvas apresentaram um desenvolvimento morfológico normal sem diferenças significativas entre tratamentos. Fisiologicamente, a capacidade digestiva e resposta imunológica também não foram afectadas pelo tratamento YSA à excepção da concentração de cortisol em todo o corpo da larva. Concentrações inferiores de cortisol foram observadas nas larvas do tratamento YSA em relação às larvas do grupo Controlo. Considerando os testes antibacterianos, as larvas alimentadas com as presas tratadas com o produto comercial de *A. armata* apresentaram alguma resistência antibacteriana enquanto as larvas do controlo não apresentaram. Na análise bacteriológica as contagens de *Vibrionaceae* foram significativamente inferiores no tratamento, tanto em larvas como na água de cultivo. As contagens do número de bactérias aeróbias totais foram semelhantes entre o tratamento YSA e Controlo na análise da água de cultivo e significativamente inferiores em larvas tratadas com Ysaline®100.

O maior crescimento observado no tratamento pode estar relacionado com uma maior taxa de ingestão ou pode estar relacionado com a variação da comunidade bacteriana. Para uma maior compreensão dos resultados de sobrevivência, um maior conhecimento sobre as variações na comunidade bacteriana é necessário, bem como deve ser explorada a possibilidade de metabolitos ictiotóxicos de *A. armata* serem transportados até às larvas através do alimento vivo. O tratamento de alimento vivo com o produto comercial de *A. armata* também teve influência na concentração corporal de cortisol das larvas. A concentração de cortisol está, de uma forma geral, relacionada com respostas a situações de stress. Contudo, compreender como os mecanismos do

cortisol são afectados será importante para uma melhor compreensão do seu efeito nas larvas. Os resultados da análise bacteriológica e do teste antibacteriano mostraram a capacidade das larvas do tratamento testado (YSA) em impedir ou reduzir a colonização bacteriana do intestino das larvas. Tal, pode estar relacionado com um aumento do fitness larvar ou pode estar relacionado com a possibilidade dos metabolitos de *A. armata* serem transportados até às larvas pelo alimento vivo.

A redução da carga bacteriana conseguida com um produto comercial de *A. armata* aplicado ao alimento vivo, a uma concentração de 0.5 % durante 30 min, afectou as larvas de *S. aurata*. Apesar de este efeito não ser completamente compreendido, a possibilidade de que o tratamento pode aumentar o fitness larvar existe. Será necessário aprofundar a investigação sobre os mecanismos em que o alimento vivo tratado com *A. armata* afectam as larvas de *S. aurata* e se resultados inferiores da sobrevivência poderão ser ultrapassados.

Palavras-chave: *Asparagopsis armata*, carga bacteriana, *Sparus aurata*, alimento vivo.

Abstract

Seaweeds are a source of bioactive compounds producing a large variety of metabolites with a broad spectrum of biological activities. This work attempted to understand the use of *A. armata* metabolites in larval rearing of *Sparus aurata*, assessing its development and larval quality, by reducing the bacterial load associated with the live feed.

Two *A. armata* products (a laboratory made extract and a commercial powder, Ysaline®100 - YSA) were tested in *Brachionus* spp. and *Artemia* sp. at different concentrations. YSA at 0.5 % was selected to be used during 30 min with live feed. The gilthead seabream larvae trial comprised one treatment where live feed was bathed with YSA vs. a Control (bathed with clear water) (each n = 4). Larval quality parameters (growth, survival, swimbladder inflation, body anomalies and caudal fin development), digestive capacity (digestive system histology and enzymatic activity), immune response (lysozyme activity and cortisol concentration) and microbiological parameters were monitored. Fish larvae from YSA exhibited higher growth rate than Control ($P < 0.05$). A higher food intake or a reduction of the bacterial load might justify this result. Still, lower survival rates were observed for YSA. This result might be related with variations of the bacterial community or/and to a possible ichthyotoxic effect of *A. armata* metabolites carried by the live feed. Treatment with YSA also influenced the whole body cortisol since lower concentrations were observed for this group. Further research on how cortisol mechanisms are affected is required to fully understand its effects. Seabream larvae from YSA exhibited lower number of bacteria, either for larvae ability to prevent or reduce the bacteria colonization, which can be related to an enhancement of larval fitness, or to the possibility that *A. armata* metabolites were carried to the larvae.

The reduction of the bacterial load accomplished with live feed immersed for 30 min in YSA improved *S. aurata* larvae fitness, although the effects are not fully understood.

Keywords: *Asparagopsis armata*, bacterial load, *Sparus aurata*, live prey.

Index

Resumo II

Abstract V

Index VI

Acronyms and abbreviations VIII

1. Introduction ..... - 1 -
   1.1. General overview ..... - 1 -
   1.2. Live feeds and associated bacteria ..... - 1 -
   1.3. Methods to control bacterial load ..... - 3 -
   1.4. Assessment of larval quality ..... - 5 -
   1.5. Objectives ..... - 8 -
2. Material and methods ..... - 9 -
   2.1. Biological material ..... - 9 -
   2.2. Experimental design ..... - 10 -
   2.2.1. Effects of *Asparogopsis armata* extracts on live feed densities and pathogenic bacteria ..... - 10 -
   2.2.2. Larval rearing trial set-up ..... - 12 -
   2.2.3. Larval sampling ..... - 13 -
   2.3. Analytical methodology ..... - 14 -
   2.3.1. Fatty acid profile of live feed ..... - 14 -
   2.3.2. Survival and growth ..... - 15 -
   2.3.3. Life feed intake ..... - 15 -
   2.3.4. Morphology and development ..... - 15 -
   2.3.5. Digestive system histology ..... - 17 -
   2.3.6. Digestive enzymes activities ..... - 18 -
   2.3.7. Lysozyme activity ..... - 19 -
   2.3.8. Cortisol analysis from the stress test ..... - 20 -
   2.3.9. Bacteriological analysis ..... - 20 -
   2.3.10. Antibacterial test ..... - 21 -
   2.4. Statistical analysis ..... - 21 -

3. Results ....- 23 -
3.1. Effects of *Asparogopsis armata* extracts on live feed densities and pathogenic bacteria ....- 23 -
3.1.1. *A. armata* extracts on live feed densities ....- 23 -
3.1.2. *A.armata* extracts on pathogenic bacteria ....- 24 -
3.2. Larval rearing ....- 25 -
3.2.1. Live feed fatty acid profile ....- 25 -
3.2.2. Survival and growth ....- 27 -
3.2.3. Swimbladder ....- 27 -
3.2.4. Occurence of body anomalies ....- 28 -
3.2.5. Caudal fin formation ....- 29 -
3.2.6. Live feed intake ....- 30 -
3.2.7. Digestive system histological development ....- 31 -
3.2.8. Enzyme activity ....- 33 -
3.2.9. Lysozyme activity ....- 35 -
3.2.10. Cortisol concentration ....- 35 -
3.2.11. Bacteriological growth ....- 35 -
3.2.12. Antibacterial activity ....- 36 -
4. Discussion ....- 37 -
4.1. *A. armata* on live feed ....- 37 -
4.2. *S.aurata* larval development ....- 38 -
4.3. Larval survival and growth ....- 39 -
4.4. Larval digestive capacity and immune response ....- 41 -
5. Conclusion ....- 45 -
6. References ....- 46 -

Acronyms and abbreviations

ANOVA – Analysis of Variance

ARA – arachidonic acid

BAPNA – Nα-Benzoyl-DL-arginine-p-nitroanilide

BSA – bovine serum albumin

CF – completed flexion stage

CFU – colony forming units

Ctrl – Control group

DAE – dias após eclosão

DAH – days after hatching

DHA – docosahexanoic acid

DMSO – dimethyl sulfoxide

DNA – deoxyribonucleic acid

DO – dissolved oxygen

DW – dried weight

EF – early flexion stage

ELISA – enzyme linked immunosorbent assay

EPA – eicosapentaenoic acid

EPA – environment protection agency (United States)

EPPO – Estação Piloto de Piscicultura de Olhão

FAME – fatty acid methyl esters

FAO – Food and Agriculture Organization (United Nations)

FID – flame ionization detector

FL - flumequine

FS – flexion stage

HUFA – highly unsaturated fatty acids

Hy – hypuralia elements

IPMA – Instituto Português do Mar e da Atmosfera

LpNa – leucine-*p*-nitroanilide

Lv – Larvae

MH – Mueller Hinton agar

MUFA – monounsaturated fatty acids

OD – optical density

OT – oxitetracicline

PBS – phosphate buffered saline

pNPP – *p*-nitrophenylphosphate

psu – practical salinity units

PUFA – polyunsaturated fatty acids

SA – specific enzyme activity

SFA – saturated fatty acids

SFB – sodium phosphate buffer

TA – total enzyme activity

TCBS – thiosulfate-citrate-bile salts-sucrose agar

TSA – tryptic soy agar

TSB – tryptic soy broth

UV – Ultraviolet

VNBS – Viet Nam Brine Shrimp

WW – wet weight

YSA – Ysaline®100

1. Introduction

1.1.General overview

Aquaculture production has increased along with human population growth, and the subsequent protein demand. Fish is a nutritionally balanced source of protein and therefore its importance is increasing for human nutrition. World capture fisheries have reached a stagnant point, mainly concerning marine captures where stocks of some of the most important species are fully or over exploited (FAO, 2012). The increasing demand for marine fish implies a demand on the production of marine fish larvae. This can only be achieved with an increase in aquaculture production. In nature, only few larvae survive and become juveniles capable of integrating the stock. In aquaculture, larval survival rates are much higher but mortality rates are still significant. Thus, production efficiency has still the potential to improve in culture conditions (Planas & Cunha, 1999; Dhert *et al.*, 2001; and Uribe *et al.*, 2011).

The mortality observed in larvae culture is often caused by opportunistic bacteria that proliferate in the water and are mainly introduced with the live feed provided (Olsen *et al.*, 2000; Dhert *et al.*, 2001; Skjermo *et al.*, 2002; Haché & Plante, 2011). In hatcheries hygiene procedures were implemented to minimize the infection of the larvae. The water for larvae culture is treated and disinfected before it reaches the larvae, usually by UV-irradiation or ozonation. Antibiotics can also be effectively used, but their application has diminished due to the development of antibiotic-resistant bacteria (Olafsen, 2001; Shields, 2001; Magnadottir, 2010 and Attramadal *et al.*, 2012).

1.2.Live feeds and associated bacteria

Rotifers *Brachionus* spp. and brine shrimp *Artemia* sp. are the two most common organisms used to feed marine fish larvae at mouth opening. Rotifers were first identified as suitable for use in aquaculture by the Japanese since the 1960s. They are small (from 50 to 220 µm) brackish water species, capable of surviving and reproducing

in a wide range of salinity and temperature, with high fecundity. They can be produced at high densities and their non-selective filter-feeding characteristic allows bioencapsulation (the incorporation of substances in the live food organisms). Since rotifers are not the natural prey of fish larvae, bioencapsulation is a way to manipulate rotifers nutritional profile according to the nutritional requirements of different fish species. *Artemia* are a relatively primitive crustacean, also used in aquaculture due to their easiness to be produced in culture conditions and due to the possibility of bioencapsulation. *Artemia* are bigger than rotifers (400 – 800 μm) so the former generally follow the latter in the diet, when larvae are bigger and have a wider mouth opening (Pousão-Ferreira, 2009; Conceição *et al.*, 2010; Haché & Plante, 2011 and Pousão-Ferreira *et al.*, 2012).

Several bacteria have been identified in rotifer cultures, such as *Pseudomonas*, *Vibrio* or *Flavobacterium*. These are known as causing agents of some diseases and can be transferred by ingestion or through the water. There are always bacteria associated to rotifer cultures, even in recirculation systems. These systems may present a more stable microbial community then the batch systems. It has been demonstrated that a stable microbial community in rotifer cultures could have a positive effect on their own production, although it may have negative impacts on the larvae. The negative impact happens even if only non-pathogenic bacteria are present, due to the risk of providing excessive bacteria (Dhert *et al.*, 2001; Qi, *et al.*, 2009 and Haché & Plante, 2011). The high densities and feeding demands of rotifer cultures lead to a boost of the bacterial load associated with these animals, more importantly it increases the opportunistic bacteria proliferation. This is also important in the enrichment process of both rotifers and *Artemia*. The high load of organic matter in the water increases the risk of providing excessive and harmful bacteria to the fish larvae (Verdonck *et al.*, 1997; Rombaut *et al.*, 2001; Prol-García *et al.*, 2010; Haché & Plante, 2011 and Vladstein *et al.*, 2013). Dehydrated cysts of *Artemia* are also known to have associated bacteria. Usually *Artemia* cysts are decapsulated with hypochlorite before cyst incubation. This process works as disinfection but does not prevent the bacteria re-colonization and posterior boost during the enrichment procedures (Sorgeloos *et al.*, 2001; Lee, 2003; Ritar *et al.*, 2004; and Haché & Plante, 2011).

Microdiets (inert diets for fish larvae) are a possible substitution of live feeds, they are important to reduce production costs while offering the larvae high quality feed (Conceição *et al.*, 2010). Unfortunately microdiets for marine fish larvae are still a work in progress that does not fully replace the need of live feed. Along with the efforts to develop microdiets, studies have also focused on attempting to control the bacterial load associated with the live feed (Cahu & Zambonino Infante, 2001 and Conceição *et al.*, 2010).

1.3. Methods to control bacterial load

Apart from prophylactic and hygienic measures to control the bacterial load in marine fish larvae rearing systems, several techniques have been tested to handle the bacterial load in live preys used to feed fish larvae. Some of the bacteria associated with the live feeds can be simply washed off while rinsing or using fresh water baths in both rotifers and *Artemia*. But since these organisms are filter-feeders, an important part is filtered and accumulates inside them, reaching the larvae via ingestion. Different enrichment feeds or culture products can result in differences between bacteria proliferation in number and species observed. Some algae species used to feed/enrich rotifers and *Artemia* are known to inhibit some bacteria development, inducing an exchange of the existing bacteria in the gut of these preys, but when compared to commercial products algae have lower levels of essential fatty acids, important to some species of marine fish larvae (Olsen *et al.*, 2000; Ritar *et al.*, 2004 and Castanho *et al.*, 2011). Ozone treatment or some disinfectant products such as formaldehyde can also be used to control bacterial proliferation in *Artemia* cultures, but rotifers have been described to be more sensitive than *Artemia* to the disinfection methods (Dhert *et al.*, 2001). So far the rearing water is disinfected before it reaches the live feed using chemicals, UV-irradiation or ozonation. However the use of disinfectants only reduces the number of bacteria and can in some cases cause other problems (e.g. formation of by-products or toxic compounds) harmful to marine fish larval production and to the environment (Dhert *et al.*, 2001; Conceição *et al.*, 2010; and Haché & Plante, 2011).

Research on other products such pro- and prebiotics have been a major focus in recent years. Probiotics (live bacterial additives) are also being used to control the bacterial load associated with rotifers and *Artemia* by limiting the development of potential harmful opportunistic bacteria through competition for nutrients and space. The use of these products may also positively affect growth and survival of the culture populations of the live preys and colonize the fish larvae that eat them, improving their growth and survival rates. *Bacillus* spp. are commonly used as probiotics as well as yeasts. Considering that these products may affect the environment by unbalancing the microbial system, prebiotics emerged as an alternative. These products, mainly oligosaccharides, can be defined as "non-digestible (by the host) food ingredients, which beneficially affect the hosts by selectively stimulating the growth and/or the activity of specific bacteria that can improve the host health" (Dimitroglou *et al.*, 2011, p.9). Both pro- and prebiotics can work as immunostimulants as can other products that increase the immune innate responses of the marine fish larvae until the adaptive immune response is sufficiently developed. Immunostimulants are becoming important, but they also have problems as they can cause unwanted side effects such as immunosuppression if not used correctly (Touraki *et al.*, 1996; Vladstein, 1996; Gomez-Gil *et al.*, 2001; Olafsen, 2001; Shields, 2001; Skjermo *et al.*, 2002; Bricknell & Dalmo, 2005; Defoirdt *et al.*, 2007; Conceição *et al.*, 2010; Magnadottir, 2010; Defoirdt *et al.*, 2011; Dimitroglou *et al.*, 2011; Uribe et al., 2011 and Allender *et al.*, 2012).

Some antibacterial products applied in aquaculture such as hormones, antibiotics and other chemicals, are not recommended in commercial operations. They can have residual effects and affect the human consumer with undesirable side effects, and therefore there are restrictions to their use (Citarasu, 2010). Natural alternatives such as extracts from plants, marine organisms and their by-products have been shown to be effective, low cost and environment friendly alternatives (Harikrishnan *et. al.*, 2011). Seaweeds in particular are a prolific source of bioactive compounds with a broad spectrum of biological activities (Bansemir *et al.*, 2006 and Manilal *et al.*, 2009). Among several seaweed species tested in-vitro the red seaweed *Asparagopsis armata* was the most active species in inhibiting the growth of pathogenic bacteria of fish (Bansemir *et al.,* 2006). *A. armata* is abundant in the Portuguese coast in shallow subtidal areas, occasionally down to 30 m deep. However, *A. armata* can also be produced in the effluents waters of fish aquaculture as a means of bioremediation

resulting in a sustainable source of natural bioactive compounds (Mata *et al.*, 2010, 2013). *Asparagopsis* spp. antibacterial activity can be attributed to halogenated metabolites, such as halomethanes, haloacetones, haloacetates and acrylates (Kladi *et al.*, 2004). Such compounds give these seaweeds economical value since they are valuable natural ingredients for cosmetics and medicine The cosmetic industry interest has lead to the development of a patented special technique to extract compounds for several purposes (e.g. anti-acne treatment) (Mata, 2008). Metabolites such as halocetones are known enzymes inhibitors and can be used in some therapies for humans and animals (Kladi *et al.*, 2004). In this study extracts of *A. armata* will be used as a means to control the bacterial load associated to live feed to improve the Seabream (*Sparus aurata*) larvae performance and development.

1.4. Assessment of larval quality

Sparus aurata is a marine fish species reared since the 1980's, being one of the most produced marine fish species worldwide. Larval rearing was successfully improved along the last decades, but still there is opportunity for improvement since larval survival remains low (around 20 % until approximately 30 days after hatching (DAH)) (Tandler *et al.*, 1995; Cañavate and Fernández-Díaz, 2001; Colloca & Cerasi, 2005 and FAO, 2012).

Larval performance and development determine larvae quality. The larval quality can be hereditary or it can be related to culture conditions, and can be assessed by monitoring several aspects of their morphology and physiology. The swimbladder is the most important hydrostatic organ in pelagic fish allowing the animal to float and assisting the up and down movements, with low energy losses (Soares, 1995). Its inflation is a very important process in larvae development. As summarised by Planas & Cunha (1999) and Soares *et al.* (1994) abnormalities (such as non-inflation or hyperinflation) in the swimbladder can affect growth, skeletal development (hence swimming ability), or can be lethal. Non-inflation of the swimbladder in *S. aurata* was considered a severe problem in the 1970s and 1980s leading to approximately 90 % of farmed fry to be discarded. The problem was minimized with the adjustment of light, salinity, temperature or turbulence, and the installation of devices to clean the water

surface (Shields, 2001). To inflate the swimbladder larvae need to gulp atmospheric air at the surface of the water. If the water surface is not clean this may be difficult since this action is essential for a successful inflation (Planas & Cunha, 1999 and Zilberg *et al.*, 2004). Skeleton descriptions are also often used to access larval quality. Skeleton malformations are regularly analysed using specific staining methods that highlight bones and cartilages evidencing malformations externally invisible (Koumoundouros *et. al.*, 1997 and Faustino & Power, 1999). Malformations can occur due to several factors and complex, co-causative mechanisms. Swimbladder anomalies (Planas & Cunha, 1999 and Soares *et al.*, 1994), nutrition (Fernández *et al.*, 2008), temperature (Georgakopoulou *et al.*, 2010) or hereditary origin (Andrades *et al.*, 1996) are some of the important factors for skeleton malformation incidence.

An increase in larval development can also be related to an enhancement in the digestive capacity of the larvae that can also be hereditable or affected by external factors. Digestion is a physical and chemical process carried out by the oesophagus, stomach, upper and lower intestine and pyloric caeca, supported by the pancreas, gallbladder and liver. Digestive enzymes are an important part of the digestive processes along with the physical and other chemical activities that begin at ingestion and end at the excretion of feces. Enzymatic processes involve a great number of specific enzymes for the molecular breakdown of proteins, lipids and carbohydrates. Enzymes can also collaborate (directly or passively) in the transference of all types of nutrients into the enterocytes and are named according to their function or compound upon they act (e.g. lipases act on lipids) (Rust, 2002). The digestive enzymes influence what larvae are able to eat, thus they reflect their feeding habits, but what larvae eat can also influence the diversification of the digestive enzymes activity (Ribeiro *et al*, 2002, Caruso *et al.*, 2009 and Gisbert *et al.*, 2012). Algae and probiotics in the larvae diet can enhance the enzymatic activity, hence feed efficiency. Algae can trigger the digestive enzyme production or facilitate the hydrolytic functions of cell membranes (Cahu et al., 1998 and Canavate & Fernadez-Diaz, 2001), and probiotics can act through the production of supplemental digestive enzymes, metabolised by the microorganisms (Suzer *et al.*, 2008).

The immune response is also an important characteristic in fish, like the previous features it can also be of congenital origin or affected by external factors. Fish immune system consists of two components a non-specific and a specific one. The non-specific component is an innate immunity, mainly of phagocytic mechanisms associated with macrophages and granular leukocytes that can attack invading microorganisms. The specific component corresponds to the immunological memory formed by humoral and cell mediated responses. The immune response can be monitored through the lysozyme activity, a small enzyme that attacks the protective cell walls of bacteria. It is present in many places with potential for bacterial growth and travels in the blood so it can be delivered to the whole body (Goodsell, 2000). This enzyme integrates the fish's non-specific immune system and it can be found in the mucus, lymphoid tissues and serum of most fish species (Gatlin III, 2002 and Bone & Moore, 2008). Its concentration can be affected by stress or nutritional factors (Demers *et al.*, 1997, Montero *et al.*, 1999, Wu *et al.*, 2007 and Costas *et al.*, 2011).

An increase in cortisol concentration can also reflect the immune response since it activates the central nervous system to respond to stressful situations. It increases glucose and blood pressure to cope with the stress induced energy demand, and it can reduce the inflammatory/immune reaction that might lead to tissue damage (Mommsen *et al.*, 1999). Stress response can be altered by habituation to a repeated stress situation, reproductive selection or nutrition (Tort *et al.*, 2001 and Van Anholt *et al.*, 2004). Cortisol is a multifaceted hormone whose concentration is one of the stress indicators most commonly measured in fish since it can be measured easily and accurately with commercial kits such enzyme linked immunosorbent assay kits (ELISA). It is always present in vertebrates, even during non-stressful occasions but tends to increase in stressful conditions.

1.5.Objectives

This study aimed firstly to determine the concentration of *A. armata*, extract (both commercial and laboratory made), to effectively reduce bacteria without harming the live prey. Secondly, to evaluate the effect of live prey (*Brachionus* spp. and *Artemia* sp.) treated with *A. armata* derived bioactive compounds on *S. aurata* larval performance, by assessing parameters related with growth, development, digestive capacity and immune response.

2. Material and methods

2.1. Biological material

Rotifers (*Brachionus* spp.) and *Artemia* (*Artemia* sp. nauplii and metanauplii) were produced at IPMA's aquaculture research station (EPPO; Olhão, Portugal). Rotifers were produced in a batch culture system according to EPPO's protocols. *Artemia* nauplii and metanauplii were respectively obtained from Viet Nam Brine Shrimp (VNBS from Golden Lotus Trading LLC, USA) and from Salt Lake Aquafeed (Catvis BV, Netherlands). All *Artemia* cysts were decapsulated according to the protocol described by Pousão-Ferreira (2006) and incubated at a density of 4 cysts.mL^{-1} at 27 ºC, 27 psu and strong aeration. *Artemia* nauplii were harvested at hatching to be used while *Artemia* metanauplii were harvested at hatching to be enriched. Rotifers and *Artemia* metanauplii were enriched with the commercial product RedPepper® (Bernaqua NV, Belgium) following the supplier's indications for each.

Gilthead seabream (*Sparus aurata*) eggs were obtained naturally from broodstock adapted to captivity at EPPO. Eggs were incubated at 18 ± 0.5 °C in 200 L cylindro-conical fibreglass tanks, at a density of 0.5 g.L^{-1} for 2 days. One day after hatching (1 DAH) fish larvae were distributed across eight tanks (200 L) at a density of 100 larvae.L^{-1}. Fish larvae were kept in a flow-through system with 20 to 40 % of water recirculation. Water temperature was maintained at 19.2 ± 1.23 ºC, salinity at 36 ± 1 psu, dissolved oxygen (DO) at 7.0 ± 1.05 mg/L and light intensity at approximately 800 lux. Photoperiod was of 14 hours light, starting at 9 am, and 10 hours dark. Water renewal rate ranged from 20 % to 45 % per hour, draining through an 80 μm to a 500 μm mesh, according to the type of prey used (Figure 1).

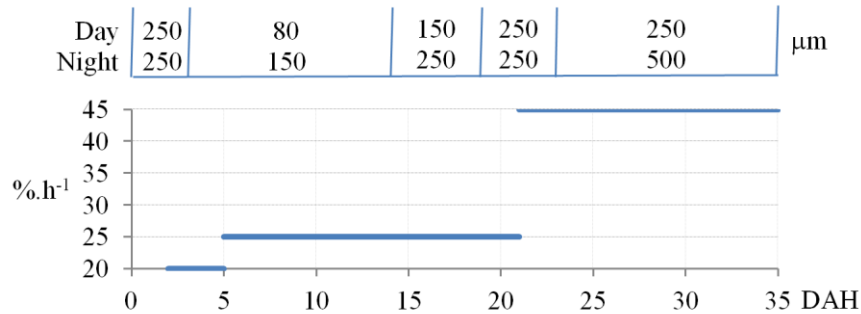


Figure 1: Water renewal and mesh size in the outlet, for all tanks throughout the larval rearing trial

Larvae opened their mouth at 4 DAH. Rotifers were given since mouth opening until 19 DAH, *Artemia* sp. nauplii were introduced at 15 DAH until 19 DAH and metanauplii were given since 20 DAH until the end of the larval rearing trial (35 DAH). Live preys were provided *ad libitum* considering that a minimum concentration of 5 rotifers and 0.5 to 1 *Artemia* nauplii per mililiter was required to balance the deficient larvae mobility in the earliest stage. The green-water technique (addition of microalgae to the rearing tanks to help maintain the water quality and the live feed nutritional profile) was applied using a mixture of *Nannochloropsis oculata* and *Isochrysis galbana* (1:1) since mouth opening until the end of the trial.

2.2. Experimental design

2.2.1. Effects of Asparogopsis armata extracts on live feed densities and pathogenic bacteria

To test the bactericide potential of the red seaweed *A. armata* against marine bacteria associated to the live prey commonly used in marine fish larvae production (*Brachionus* spp. and *Artemia* sp.), two products were used. A commercial powder from the red seaweed, Ysaline®100 (Algues & Mer, France), commonly used in the cosmetic industry, and a laboratory made extract. This extract was made from fresh seaweeds that were dried, smashed, filtered and centrifuged. Both products were used at different concentrations and for different periods of time in preliminary surveys, to select the safe

concentration to be used with the live preys and simultaneously the time needed to be effective as a bactericide.

Rotifers and *Artemia* enriched with RedPepper® were tested separately using the same protocol. After enrichment preys were harvested, washed with salt water and equally distributed between beakers of 100 mL. Preys were sampled and counted in triplicates by two operators. Sampling was made after distribution and at 30 min intervals after the addition of a product for a total of 3 h trial. During this period preys were maintained immersed in the product solution without aeration. Each concentration was tested separately with a control group (in clear water) at 5 to 10 min intervals to guarantee that the operators had time to count the live feed at the same sampling point.

As shown in Table 1, Ysaline®100 was tested using 5 different concentrations since the product has never been used on these organisms, whereas for fresh extract only two concentrations were tested since previous studies reported severe fish mortality when concentrations over 1 % of an aqueous solution of *Asparagopsis taxiformis* were used (Mata *et al.*, 2013).

Table 1: Concentrations of the Ysaline®100 (dried powder) and the laboratory made extract tested in rotifers and *Artemia* enriched with RedPepper®; "yes" indicates it was tested and "no" indicates it was not tested.

	Concentration of product (%)	Rotifers	*Artemia*
Lab made extract	1	yes	yes
	2	no	yes
Ysaline®100	5	yes	no
	1	yes	yes
	0.5	yes	no
	0.25	yes	no
	0.1	yes	no

The laboratory made extract was added directly to the beakers since it was in liquid form, whereas Ysaline®100 powder was previously diluted in salt water and then added to the beakers. To assure that the corresponding control of each treatment had the same density of rotifers or *Artemia*, salt water was added to the control beakers in the same amount as the products.

Ysaline®100 (YSA) bactericide potential is well known in its target industry but no data is available on its potential against marine bacteria commonly found in marine hatcheries. To analyse its potential in this marine environment the product was tested with a known pathological bacterium (*Photobacterium damselae* subsp. *piscicida*) isolated from an outbreak of Senegalese Sole (*Solea senegalensis*). *P. damselae* subsp. *piscicida* was cultured at room temperature in Tryptic Soy Broth medium (TSB from OXOID, USA) made with artificial seawater and used to show an exponential growth 48 h after inoculation. Concentrations of 0.01, 0.02, 0.03, 0.06, 0.125, 0.25, 0.5, 1 and 2 % were tested with the bacterium in octuplicate and compared with a control using the bacterium without YSA.

A sterile 96-well microtiter plate was filled with TSB and with overnight growing bacterial culture along with the different concentrations of YSA (except the control). Optical density (OD) readings were made at 540 nm in a Microplate reader (Syergy™ 4-Biotek, USA) at the initial point and after 24, 48 and 120 h, following the bacterial curve.

2.2.2. *Larval rearing trial set-up*

The gilthead seabream larvae trial comprised two treatments with four replicate tanks in each treatment. The trial started at mouth opening (4 DAH) and lasted until 35 DAH. All tanks were fed equally according to the protocol and the amount given per meal. Only the commercial product (YSA) was tested in this trial. YSA was selected considering two remarks. First, results from a short-term assay (until 11 DAH, Costa *et al.*, 2013) showed no significant differences in larval survival and growth between live prey treated with *A. armata* laboratory made extract and Ysaline®100

against a control group. Second, advantages in using Ysaline®100 were the maintenance of its chemical properties and availability throughout the complete larval trial.

The Ysaline®100 (YSA) treatment consisted of bathing the live preys (rotifers and *Artemia,* accordingly to feeding protocol) with YSA before their provision to fish larvae, whereas for the control treatment (Ctrl) live prey were rinsed with clear salt water. For YSA treatment live prey were bathed with 0.5 % Ysaline®100 during 30 min, after the enrichment, and then rinsed with salt water before being fed to larvae. Live prey from the control treatment (Ctrl) were rinsed with salt water following enrichment, but were maintained in clear water for 30 min to ascertain that rotifers and *Artemia* from both treatments had identical nutritional value. Live preys were distributed over 4 to 5 meals along the day. Therefore, in order to maintain the nutritional value of the enriched live preys after the 1st meal, they were stored in a cold bath of 5 ± 1 ºC for the remaining meals. These processes were performed daily during larval rearing.

Samples from rotifers, *Artemia* nauplii and metanauplii were collected before and after the 30 min bath for both treatments, for fatty acid determination. This analysis was to evaluate the impact of 30 min bath on preys' fatty acid enrichment. These samples were rinsed with distilled water, flash frozen and freeze-dried in a Lyoquest – 50 (Telstar®, Spain) freeze drier until processed for fatty acid analysis.

2.2.3. Larval sampling

To evaluate larval performance as a response to the application of a red seaweed product in the live prey, samples of 20 larvae, from each tank, for biometry (total length and dry weight) and development were collected from 2 DAH in 5 to 7 days intervals and 2 to 5 days intervals, respectively, until 34 DAH. To assess live feed intake samples (n = 15 per tank) were collected at 5, 12, 18, 23 and 30 DAH. Samples for histology and enzymatic activity (n = 15 and n = 30 respectively, per tank) were taken at 15 (only for histology), 20 and 34 DAH. Bacteriological analysis (n = 20 per tank) was performed in 34 DAH larvae and their rearing water. At the end of the trial (35 DAH) samples were also collected for stress test (n = 20 per tank), antibacterial test (n = 2 per tank), and for

lysozyme activity determination (n = 100 per tank). Fish larvae were sampled before food was added, exception made for the larvae collected to assess feed intake.

For enzymatic and lysozyme activity the starved larvae were collected, rinsed with distilled water and flash-freeze. Samples were stored at -20 ºC and processed within one month.

2.3. Analytical methodology

2.3.1. Fatty acid profile of live feed

Fatty acid methyl esters (FAME) were prepared according to Bandarra *et al.* (2009), using freeze-dried material and 5 mL of the acetyl chloride:methanol mixture (1:19, v/v). The transesterification was carried out at 80 ºC for 1 h. After cooling, 1 mL of water and 2 mL of n-heptane were added to the mixture, which was stirred and centrifuged at 2.150 g for 10 min. The organic phase was collected, filtered and dried over anhydrous sodium sulphate. The solvent was removed under nitrogen and the FAME dissolved in 0.1 mL of n-heptane. The analyses were performed in a Varian CP-3800 (Walnut Creek, CA, USA) gas chromatograph equipped with an auto sampler and fitted with a flame ionization detector at 250 ºC. The separation was carried out in an Omegawax (Supelco, USA) capillary column (25 m × 0.25 mm id). Temperature was programmed from 180 °C to 200 °C at 4 °C/min, holding for 10 min at 200 °C and heating to 210 °C at 4 °C/min, holding at 210 °C for 14.5 min with the injector and detector (FID) at 250 °C. FAME were identified by comparison with the retention times of known mixtures of standards (Supelco, FAME 37 and PUFA 3) and quantified using the area of the C21:0 internal standard with Varian software (USA).

2.3.2. Survival and growth

Larval survival was determined by counting the remaining larvae individually from all tanks at the end of the trial (35 DAH). Although dead larvae were counted every day, results were not considered to determine survival since several larvae were too degraded to be considered a reliable count.

To determine total length, larvae were measured under a Zeiss (Germany) binocular microscope provided with an ocular micrometer (precision 0.01 mm) at known magnification. Afterwards the same larvae were used to determine dry weight (DW), after being rinsed with distilled water, flash frozen in liquid nitrogen and stored in groups of 5 larvae at -20 Cº. Fifteen larvae were collected from each tank in a total of 60 larvae per treatment. At 2 DAH, since no treatments were in use, a total of 60 larvae were sampled from a common batch.

Frozen larvae were freeze-dried with a Lyoquest – 50 freeze drier and weighted in a precision balance (Sartorius Pro 11, precision 0.001/0.002 mg; Sartorius AG, Germany).

2.3.3. Life feed intake

To assess the live feed intake larvae were captured approximately 30 min after a meal and observed in a Zeiss binocular microscope to observe stomach content. This was possible because larvae were not pigmented at the surveyed sampling ages. The information was analysed according to presence or absence of food in the stomach.

2.3.4. Morphology and development

Larval morphology and development, with particular attention to swimbladder inflation, visible larval body anomalies and caudal fin formation, was assessed by collecting images of the larvae using a Canon (Japan) Power shot G5 camera linked to a Zeiss binocular microscope. These parameters are commonly monitored in hatcheries,

since they are good quality indicators that can be easily observed and are not costly or labour demanding.

Swimbladder and body anomalies were analysed by present or absent. Mortality at the surface of the tanks was also considered to assess fish larvae ability to inflate the swimbladder. Dead larvae on the water surface trapped on the oil film were counted daily.

Caudal fin formation was classified according to 3 stages: an early flexion stage (EF) was considered when hypuralia elements were evident in a straight notochord (Figure 2A and A'), the flexion stage (FS) was considered when the posterior part of the notochord presented an upward curvature (Figure 2B and B') and the last stage (CF) was considered when the flexion was concluded.

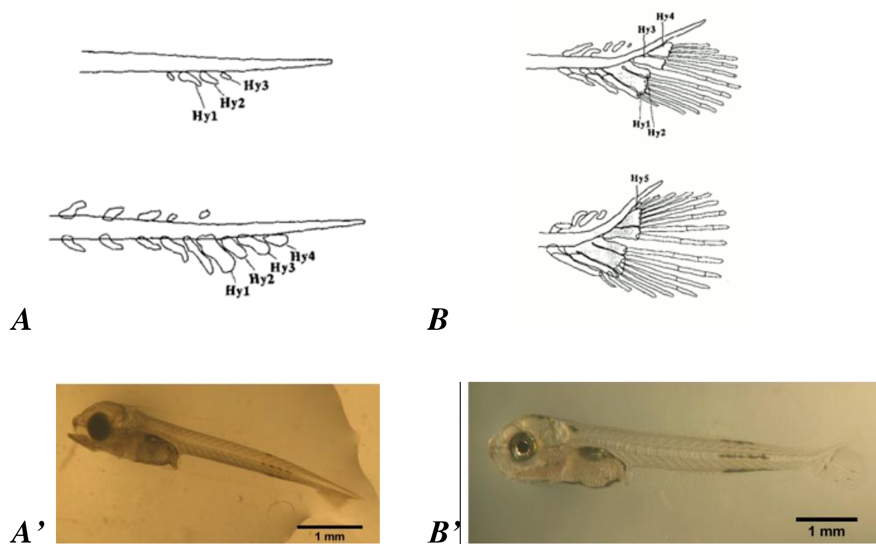


Figure 2: Development stages of the caudal complex of *S. aurata* larvae. ***A***. **-** early flexion stage (EF) with the presence of hypuralia (Hy) elements; ***B.*** **-** flexion stage (FS) with an upward curvature of the posterior part of the notochord (in Koumoundouros *et. al.*, 1997). A' and B' **-** examples of observed larvae at EF and FS respectively.

2.3.5. *Digestive system histology*

Larvae were fixed in 4 % buffered formaldehyde with 10 times more volume than the sample volume (10:1). Samples were kept in a fridge overnight. Afterwards they were washed, first with phosphate buffered saline (PBS) pH 7.4 (3 x 15 min) and after with tap water (15 min) and stored in 70 % ethanol at 8 ºC.

The 20 and 34 DAH larvae were decalcified before embedding in paraffin. To be decalcified the samples were rinsed in tap water (30 min for 1 h) then they were sunken in the decalcification solution (1:1 of 8 % hydrochloric acid and 8 % formic acid) during 2 h and 4 – 5 h for 20 and 34 DAH larvae respectively. After decalcification larvae were again stored in 70 % ethanol in the fridge. Afterwards all samples were dehydrated in graded ethanol before equilibration in xylene and then embedded in paraffin wax. The paraffin was cut in 5 µm thick sections and stained with haematoxylin-eosin before observed under a light microscope.

Digital images of fish larvae sections were captured with a digital camera Nikon DS-F12 connected to a Nikon (Japan) Eclipse Ci binocular light microscope. The abdominal cavity images were examined (40 x and 100 x) and analysed using the image analysis software ImageJ 1.47t (NIH, USA) with particular attention to the liver and to the intestine. Liver and intestinal images were taken at a 400 x magnification from different sections (n = 4) of each larva (n = 3) from each tank (n = 4) of the two treatment groups sampled at different ages.

Liver was evaluated according to the hepatocyte density. Their nuclei were counted automatically with the software, in a known area (0.01 mm^2) of the photo to assess the nutrient reserves (lipid and/or glycogen) content in the liver, assuming that a higher nuclei density always means smaller hepatocyte with lower nutrient reserves content. Intestinal morphology was evaluated according to its epithelium height as a way to assess differences in the absorption capability of the larvae.

2.3.6. *Digestive enzymes activities*

Before analysis, 34 DAH larvae were sectioned on a cold surface, to obtain the abdominal cavity as illustrated in Figure 3. Samples of the sectioned body cavity and whole body larvae (20 DAH) were then weighted and homogenised with 15 x higher volume of deionised water than their corresponding weight, using an vortexer (Ultra Turrax T10 from IKA®, Germany). The solutions were centrifuged (5000 rpm, 4 ºC) during 5 min in a table top refrigerated centrifuge (Z383K from HERMLE Labortechnik, Germany) and afterwards the homogenates were separated from the pellets and stored at -20 ºC between analyses. Trypsin, amylase, aminopeptidase, acid phosphatase and alkaline phosphatase activity were determined.

Figure 3: 34 DAH larvae, in whole body (above) and sectioned (below) with the abdominal cavity in the middle section.

Trypsin activity was measured at 407 nm at 25 °C using BAPNA (Nα-Benzoyl-DL-arginine-p-nitroanilide) as substrate in Trizma-$CaCl_2$ buffer (20 mM), pH 8.2 as described by Holm *et al.* (1988). To determine amylase activity the homogenates were combined with a starch solution (3 $g.L^{-1}$ starch) as substrate (reaction tubes) (Méthais & Bieth, 1968). The same solution was prepared without the homogenates (blank tubes). After 30 min at 37 ºC the reaction was stopped in all tubes by adding hydrochloric acid (HCl) at 1 M. The homogenate was then added to the black tubes and an iodine working solution at N/3000 was added to all tubes. The absorbance of all solutions was read at 580 nm and the results from the reaction tubes were compared to the results from the blank tubes to calculate amylase activity.

Aminopeptidase activity was determined using LpNa (leucine-*p*-nitroanilide) at 2 mM with DMSO (dimethyl sulfoxide) as substrate and Tris-HCl at 100 mM as the buffer solution combined with the homogenates (Maroux *et al.*, 1973). Acid and alkaline phosphatase activities were determined using pNPP (*p*-nitrophenylphosphate) at 5.5 mM with $MgCl_2.6H_2O$ as substrate and citric acid at 0.1 M with sodium citrate at 0.1 M (acid phosphatise activity, Terra *et al.*, 1979) or Na_2CO_3 at 30 mM (alkaline phosphatise activity, Bessey *et al.*, 1946) as the buffer solutions combined with the homogenates. Absorbance was read at 405 λ for aminopeptidase and acid phosphatase and at 407 λ for alkaline phosphatase, during approximately 10 min.

Absorbance readings were performed in a in a Multiskan® Go apparatus (Thermo Scientific™, USA). Enzyme activity was calculated in micromoles of substrate hydrolysed (mU) and expressed as specific (mU/mg protein) and total activity (mU/larva). Protein was determined according to the Bradford method (Bradford, 1976) using bovine serum albumin (BSA) as a standard.

2.3.7. Lysozyme activity

Whole body larvae were weighted and homogenised with 4 x higher volume of phosphate buffer than the corresponding weight, using a vortexer (Ultra Turrax T25 from IKA®, Germany). The solutions were centrifuged (17000 rpm, 4 ºC) during 30 min in a table top refrigerated centrifuge Z383K and afterwards the homogenates were separated from the pellets. Lysozyme activity was measured using a method based on the ability of lysozyme to lyse the bacterium *Micrococcus lysodeikticus* (Ellis, 1990). In a 96-well microplate, 70 µL of the samples homogenate and 200 µL of the substrate (0.5 $mg.mL^{-1}$ of *M. lysodeikticus* suspension (Sigma, USA) in 0.05 M sodium phosphate buffer at pH 6.2 (SFB)) were added in triplicates. The reduction in absorbance at 450 nm was measured in approximately 1 min intervals for a total of 4 min at 25 ºC using a Multiskan® Go. Lyophilized hen egg white lyzozyme (Sigma) was serially diluted in SFB and used to develop a standard curve whose formula was used to calculate the lysozyme amount in the samples.

2.3.8. *Cortisol analysis from the stress test*

At the end of the trial, collected larvae were kept in two groups of 15 larvae (30 larvae per tank) until calm behaviour was observed. One of the groups was subjected to a stress situation of 30 s without water. Both groups were rinsed with distilled water, flash frozen and stored at -20 ºC.

The samples were unfrozen to be homogenized in 500 µL of deionised water using a vortexer (Ultra Turrax 8G from IKA®, Germany). In 10 mL capacity extraction tubes 4 mL of diethyl ether was added to the sample homogenate and stirred (10 min). Afterwards samples were centrifuged at 500 rpm at 4 ºC (5 min). To separate the aqueous phase from the formed pellet the tubes were submerged in liquid nitrogen to freeze the pellet enabling the separation of the ether to another test-tube. The pellet was submitted to a second extraction after which the test-tubes were kept in a dry bath of 37 – 40 ºC overnight. After all the ether evaporated 250 µL of 0.9 % NaCl solution were added and the extracted samples were stored at -20 ºC. Cortisol concentration was determined *in vitro* through immunoassay using the Cortisol Saliva ELISA kit (IBL, Germany).

2.3.9. *Bacteriological analysis*

Larvae and water from the larval rearing tanks were sampled at 34 DAH to assess bacterial growth. Twenty larvae were disinfected with benzalkonium chloride (0.1 %) for 30 s and then washed with sterilized saline solution (1.5 %). Afterwards larvae were homogenized with a pestle (Potter-Elehjem Tissue Homogenizer – PTFE from VWR, USA) in a final volume of 1 mL with sterile artificial sea water. This solution and the water samples were sequentially diluted three-fold and plated. Triplicates of 100 µL of each dilution were spread on agar plates, tryptic soy agar (TSA from Merck, USA) was used to obtain the total number of aerobic bacteria, and thiosulfate-citrate-bile salts-sucrose agar (TCBS from OXOID, USA) was used to isolate and count *Vibrionaceae*. Concentrations of 10^{-1} to 10^{-3} and 10^{-2} to 10^{-4} were used for TCBS and TSA respectively for the homogenized larvae. Water samples were applied at a concentration of 10 to 10^{-2} for TCBS and 10^{-1} to 10^{-3} for TSA. Plates were

incubated at 22ºC for 7 days and CFUs (colony forming units) were counted after this period.

2.3.10. Antibacterial test

Four larvae from each tank were collected in starvation at the end of the trial to access antibacterial activity. The larvae were weighted, smashed and placed onto a disc in groups of two. The discs were placed onto Mueller Hinton agar (MH, OXOID) plates for each replicate, previously prepared with a pathogenic bacterium (*Photobacterium damselae* subsp. *piscicida*) isolated from an outbreak of Senegalese Sole (*Solea senegalensis*). Discs with antibiotics (Oxitetracicline and Flumequine, OXOID) were also placed in the plates to be used as a control. Plates were incubated at 22 ºC for two days and the data were treated as presence or absence of a resistance halo.

2.4. Statistical analysis

The data obtained from total length, DW, mortality at the surface of the water tank, cortisol, fatty acids, histology analysis, enzyme and lysozyme activity and bacteriological analysis were subjected to log transformation before statistical analysis. The data obtained in percentages such as preys population and survival after subject to YSA treatment, larval survival, live feed intake, swimbladder inflation, body anomalies occurrences and caudal fin formation were statistically analysed after an arcsine square root transformation.

Student's *t*-test was applied to results from survival at the end of the trial and at the surface of the water tank and to the bacteriology results. A Mann-Whitney Rank Sum test was applied to Lysozyme activity results since one of the replicates was lost (no equally of variance). One Way Analysis of Variance (One Way ANOVA) was applied to fatty acids and cortisol results and Two Way Analysis of Variance (Two Way ANOVA) was applied to results from the first trial to define the adequate concentration

of an *A. armata* product to be used in live feed. This last method was also used to compare fatty acids, length and DW, live feed intake, swimbladder inflation, body anomalies occurrences, caudal fin formation, cortisol, histological analysis and enzyme activity. When appropriate both ANOVA's were followed by an all pairwise Multiple Comparison Procedures (Holm-Sidak method).

3. Results

3.1. Effects of *Asparogopsis armata* extracts on live feed densities and pathogenic bacteria

3.1.1. A. armata extracts on live feed densities

Rotifers population was not affected by the laboratory-made *A. armata* extract. The percentage of active/live rotifers was of 100 % after 2 h, decreasing to 91.8 ± 6.09 % of the initial number after 3 h. *Artemia* metanauplii presented 100 % survival for the two tested concentrations of extract after 3 hours.

Artemia metanauplii also presented 100 % survival with the commercial product (Ysaline®100). Results obtained for rotifers subjected to the different concentrations of YSA tested are shown in Figure 4. The 5 % concentration was not included since no rotifer survival was observed after 30 min at this concentration. At 1 % concentration, the number of rotifers decreased 50 % after 30 min and no living rotifers were observed after 60 min. No significant differences were observed between the remaining concentrations during the experimental period.

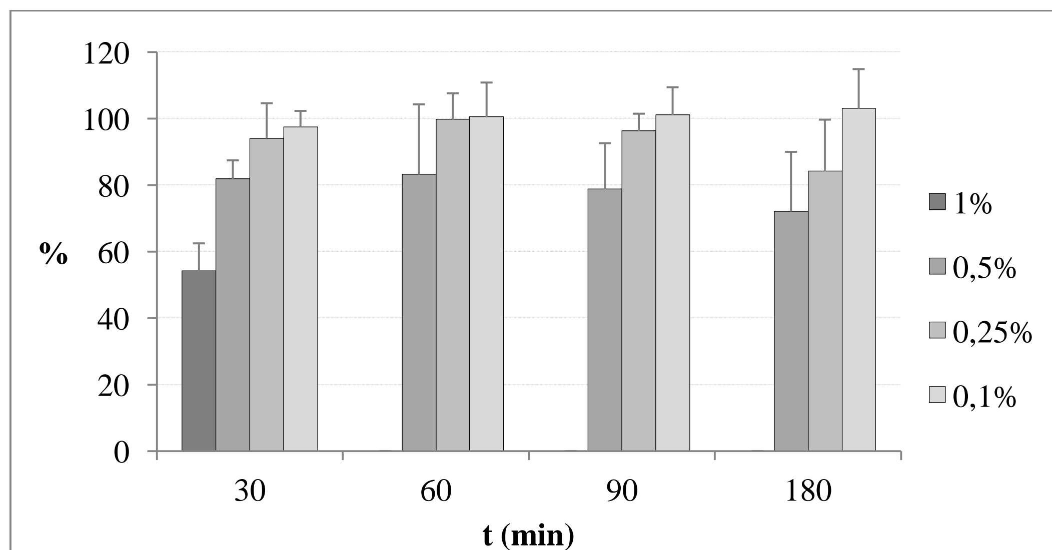


Figure 4: Percentage of rotifers from the initial population number at 30, 60, 90 and 180 min submerged in a bath with the commercial product Ysaline®100 at different concentrations. Values are presented as mean ± standard deviation (n = 3).

3.1.2. A. armata extracts on pathogenic bacteria

Photobacterium damselae subsp. *piscicida* growth was affected by YSA at concentrations higher than 0.25 % (Figure 5). Bacterial growth was inhibited during 120 h at concentrations of 2 and 1 %. YSA at 0.5 % concentration inhibited bacterial growth efficiently during 48 h. At 0.25 % YSA concentration, bacterial growth could be observed during the first 24 h of incubation, but at an inferior rate than lower concentrations. Concentrations of 0.125 % YSA and lower (not shown) presented a similar bacterial growth in comparison with the control.

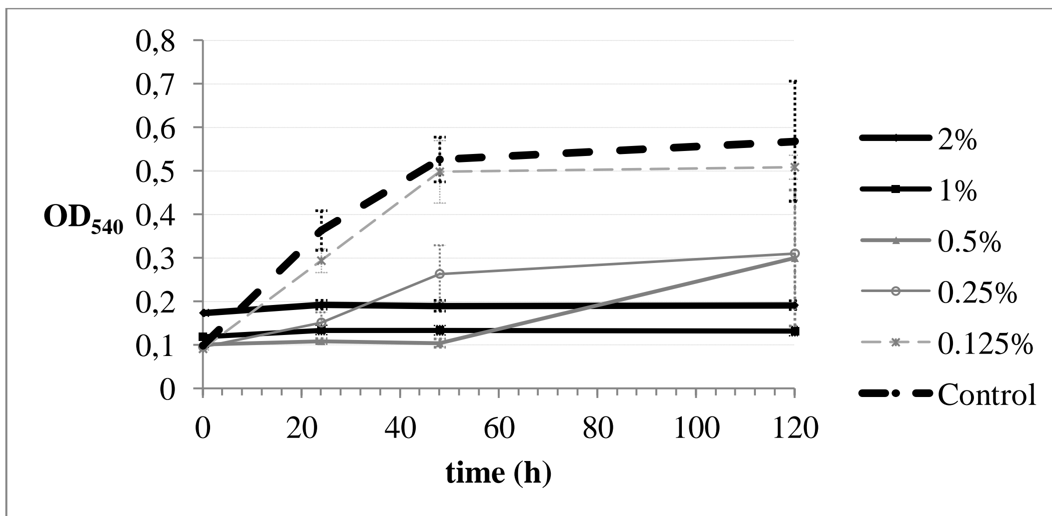


Figure 5: Optical density at 540 nm (OD_{540}) growth curves of *Photobacterium damselae* subsp. *piscicida* growth during 120 h, using different concentrations of Ysaline®100 (YSA). Control = no YSA. Values are presented as mean ± standard deviation (n = 8).

3.2. Larval rearing

3.2.1. Live feed fatty acid profile

Live prey fatty acids are presented in Table 2. No significant differences were observed for any fatty acid in rotifers, except for total ω3, with a lower value in rotifers after 30 min in clear water. In *Artemia* nauplii, most of the fatty acids were significantly ($P < 0.05$) lower after 30 min in an YSA bath. However, the main PUFA's (polyunsaturated fatty acids) for fish larvae namely DHA (docosahexanoic acid), EPA (eicosapentaenoic acid) and ARA (arachidonic acid), presented no significant differences before and after the 30 min, with and without YSA for this prey. *Artemia* metanauplii main PUFA also presented no significant differences, but for most fatty acids significant differences were observed after 30 min in clear water and YSA bath.

Table 2: Fatty acids profile in mg.g^{-1} of dry weight of the preys (Rotifers, *Artemia* nauplii and *Artemia* metanauplii) used in the larval rearing trial before (0) and after a 30 min period for control (30 Ctrl) and YSA (30 YSA) treatments. Values are presented as mean ± standard deviation. Different superscripts indicate significant differences ($P < 0.05$) in the All pairwise Multiple Comparison Procedures (Holm-Sidak method) within the same fatty acid for each live feed.

Fatty acid	Rotifers			***Artemia* nauplii**			***Artemia* metanauplii**		
	t = 0	t = 30 Ctrl	t = 30 YSA	t = 0	t = 30 Ctrl	t = 30 YSA	t = 0	t = 30 Ctrl	t = 30 YSA
14:0	4.7 ± 2.13	2.9 ± 0.97	5.3 ± 1.07	5.2 ± 1.16[a]	5.4 ± 0.78[a]	2.5 ± 0.33[b]	6.8 ± 0.43[a]	3.4 ± 1.06[b]	2.8 ± 0.57[b]
16:0	28.9 ± 11.97	19.5 ± 5.43	31.1 ± 4.36	30.6 ± 5.29[a]	34.3 ± 2.91[a]	18.7 ± 1.73[b]	53.1 ± 3.38[a]	29.9 ± 7.26[b]	26.3 ± 4.96[b]
18:0	4.1 ± 1.16	3.6 ± 0.78	4.4 ± 0.35	6.9 ± 1.22[a]	8.1 ± 0.61[a]	5.9 ± 0.16[b]	12.0 ± 0.59[a]	8.6 ± 1.38[b]	8.6 ± 1.19[b]
Other SFA	3.1 ± 1.35	2.5 ± 0.55	3.6 ± 0.50	19.6 ± 2.96[a]	21.7 ± 1.99[a]	12.2 ± 1.13[b]	7.9 ± 0.36[a]	5.1 ± 0.93[b]	5.0 ± 0.79[b]
Σ SFA	40.8 ± 16.57	28.6 ± .7.01	44.4 ± 6.15	62.2 ± 10.39[a]	69.5 ± 5.62[a]	39.4 ± 3.33[b]	79.8 ± 4.74[a]	47.0 ± 10.56[b]	42.7 ± 7.46[b]
16:1 (n9+n7)	4.0 ± 1.78	2.5 ± 0.75	4.6 ± 0.69	31.6 ± 4.56[a]	35.8 ± 4.08[a]	19.4 ± 2.03[b]	5.7 ± 0.44[a]	3.2 ± 0.83[b]	3.0 ± 0.58[b]
18:1 (n9+n7)	11.3 ± 3.32	9.1 ± 1.68	12.9 ± 1.02	46.9 ± 4.36[a]	54.1 ± 4.52[a]	39.0 ± 1.43[b]	39.9 ± 2.12[a]	27.3 ± 4.57[b]	28.2 ± 4.22[b]
Other MUFA	2.3 ± 0.14	2.2 ± 0.39	2.2 ± 0.04	2.2 ± 0.40	2.3 ± 0.31	1.9 ± 0.23	2.0 ± 0.15	1.3 ± 0.49	1.2 ± 0.35
Σ MUFA	17.6 ± 5.17	13.8 ± 2.43	19.7 ± 1.59	80.6 ± 8.73[a]	92.2 ± 8.17[a]	60.3 ± 3.46[b]	47.6 ± 2.65[a]	31.8 ± 5.85[b]	32.4 ± 4.44[b]
18:2 n 6	13.9 ± 4.26	9.8 ± 2.63	15.2 ± 1.29	5.0 ± 0.88[ab]	5.8 ± 0.33[a]	4.1 ± 0.18[b]	13.6 ± 0.78[a]	9.2 ± 1.49[b]	9.4 ± 1.46[b]
18:3 n 3	0	0	0	2.0 ± 0.18[ab]	2.4 ± 0.23[a]	1.9 ± 0.07[b]	43.7 ± 2.65[a]	30.1 ± 4.70[b]	31.0 ± 4.92[b]
20:4 n 6_ARA	3.1 ± 0.46	3.0 ± 0.58	3.2 ± 0.18	5.2 ± 0.60	5.7 ± 0.45	4.9 ± 0.11	5.6 ± 0.24	4.8 ± 0.39	5.0 ± 0.51
20:5 n 3_EPA	3.5 ± 056	3.1 ± 0.38	3.6 ± 0.23	16.3 ± 1.03	18.1 ± 1.39	18.6 ± 0.45	11.0 ± 0.37	9.7 ± 0.77	10.0 ± 1.00
22:5 n 6	7.5 ± 0.29	6.8 ± 0.97	6.8 ± 0.25	0	0	0	7.9 ± 0.14	8.1 ± 0.09	8.2 ± 0.54
22:6 n 3_DHA	19.8 ± 0.86	18.6 ± 1.46	18.1 ± 0.51	0	0	0.3 ± 0.45	19.9 ± 0.23	20.9 ± 1.01	20.9 ± 1.47
Other PUFA	6.0 ± 1.72	5.2 ± 0.65	6.2 ± 0.63	32.3 ± 3.80[ab]	36.1 ± 3.60[a]	24.4 ± 3.70[b]	13.4 ± 0.84[a]	10.0 ± 1.13[b]	10.4 ± 1.30[b]
Σ PUFA	53.9 ± 5.82	46.3 ± 4.45	53.1 ± 1.56	60.9 ± 5.81[a]	68.2 ± 5.36[a]	50.5 ± 1.72[b]	115.2 ± 5.15[a]	92.9 ± 7.39[b]	94.8 ± 9.70[b]
Total ómega 3 (ω3 or n3)	28.0 ± 0.94[a]	25.7 ± 1.06[b]	26.3 ± 0.24[ab]	23.4 ± 1.81	25.9 ± 1.95	22.4 ± 0.46	86.2 ± 3.86[a]	69.4 ± 5.38[b]	69.5 ± 7.40[b]
Total ómega 6 (ω6 or n6)	25.4 ± 4.55	20.1 ± 3.37	26.1 ± 1.30	11.3 ± 1.56	12.7 ± 0.80	10.0 ± 0.37	27.5 ± 1.16[a]	22.5 ± 1.80[b]	22.9 ± 2.03[b]
Ratio ω3/ω6	1.1 ± 0.16	1.3 ± 0.16	1.0 ± 0.04	2.1 ± 0.15	2.0 ± 0.12	2.2 ± 0.04	3.1 ± 0.01[a]	3.1 ± 0.02[ab]	3.0 ± 0.06[b]
Ratio DHA/EPA	5.8 ± 1.11	6.1 ± 0.84	5.1 ± 0.39	-	-	-	1.8 ± 0.05	2.2 ± 0.29	2.1 ± 0.16

3.2.2. Survival and growth

Larval survival at the end of the trial was significantly higher ($P < 0.05$) in the control treatment (Ctrl) when compared to YSA. The results obtained were 16.5 ± 2.72 % and 8.2 ± 1.80 % for Ctrl and YSA treatments, respectively.

Larval growth in total length and dry weight was similar for both treatments since the beginning of the trial until 15 DAH. From this age onward until the end of the trial fish larvae from YSA exhibited a higher weight and length than larvae from Ctrl ($P < 0.05$) (Figure 6).

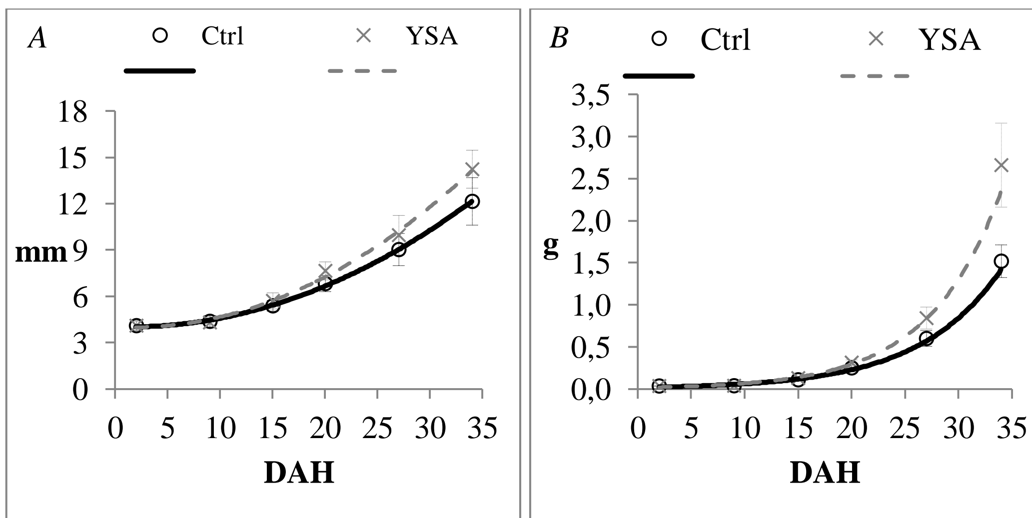


Figure 6: Larval growth in total length (*A*) and in dry weight (*B*) for the different treatments (Ctrl and YSA) along the trial period. Values are presented as mean ± standard deviation (n = 45 for total length and n = 12 for dry weight).

3.2.3. Swimbladder

The swimblader inflation was detected since 9 DAH in more than 80 % of seabream larvae, as shown in Figure 7A. Dead larvae on the water surface of the tanks (Figure 7B) were observed between 10 and 17 DAH. These patterns and the number of dead larvae were similar for both treatments ($P > 0.05$).

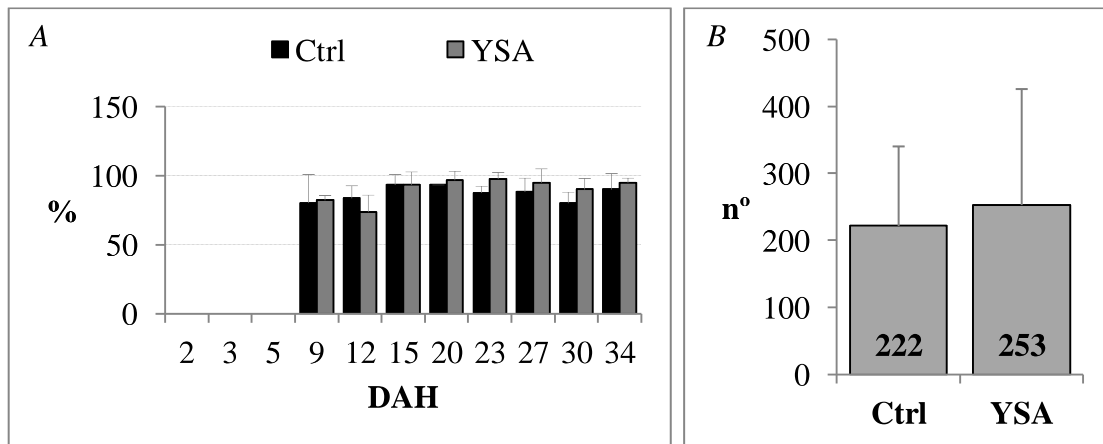


Figure 7: *A* – Percentage of larvae with the swimbladder inflated in both treatments tested, along the trial. *B* – Total number of dead larvae at the surface of the water for each treatment throughout the trial. Values are presented as mean ± standard deviation (n = 45 and n = 4 for *A* and *B* respectively).

3.2.4. Occurence of body anomalies

The incidence of visible body anomalies (Figure 8) represented 2.5 % of the total larvae analysed, with higher incidence until 12 DAH. No statistical differences were observed between treatments ($P > 0.05$).

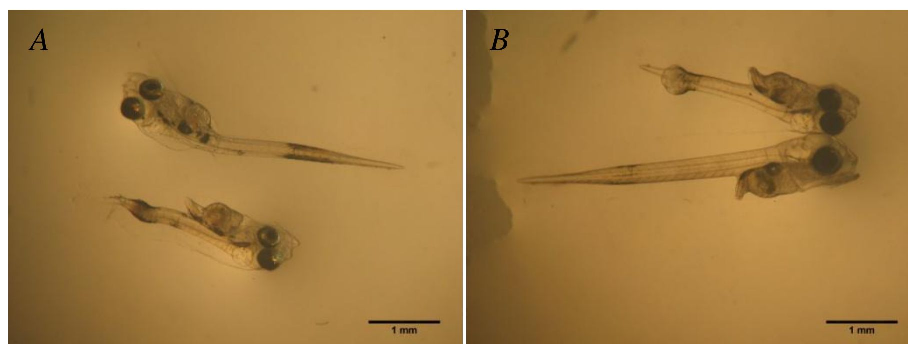


Figure 8: Examples of deformed and normal larvae from both treatments at two different ages: *A* – larvae from the control treatment at 9 DAH with an abnormal larva below and *B* – larvae from YSA treatment at 12 DAH with an abnormal larva above.

3.2.5. Caudal fin formation

Caudal fin formation started between 9 and 15 DAH, (Figure 9), significant differences were observed ($P < 0.05$) between different ages, by the end of the trial (34 DAH) all YSA larvae presented a caudal fin flexion completed while 91.7 ± 8.39 % of the control larvae completed flexion. But when comparing at different size classes (Figure 10) no significant differences were observed.

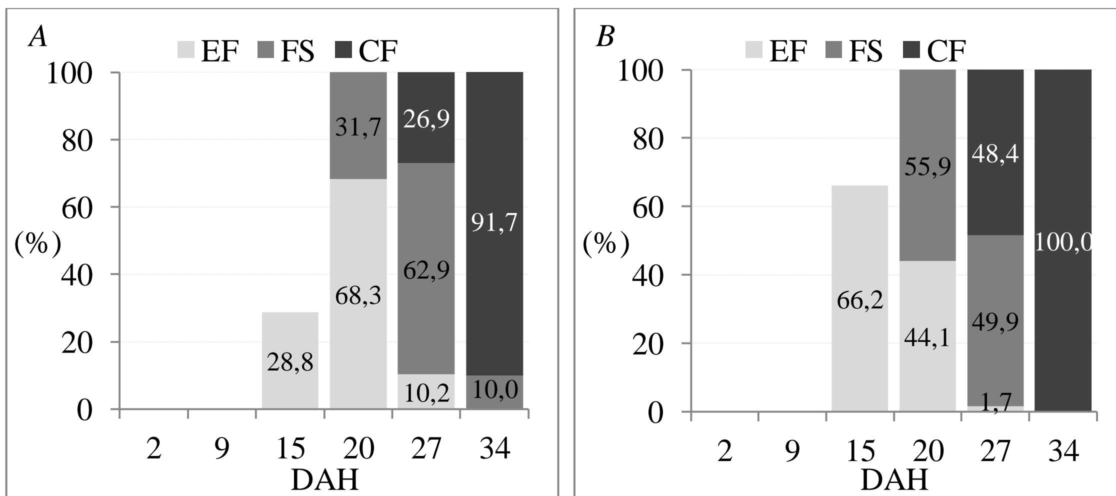


Figure 9: Percentage of larvae in early flexion stage (EF), flexion stage (FS) and with completed flexion (CF) at different ages through the trial for control (Ctrl) (*A*) and YSA (*B*) treatments.

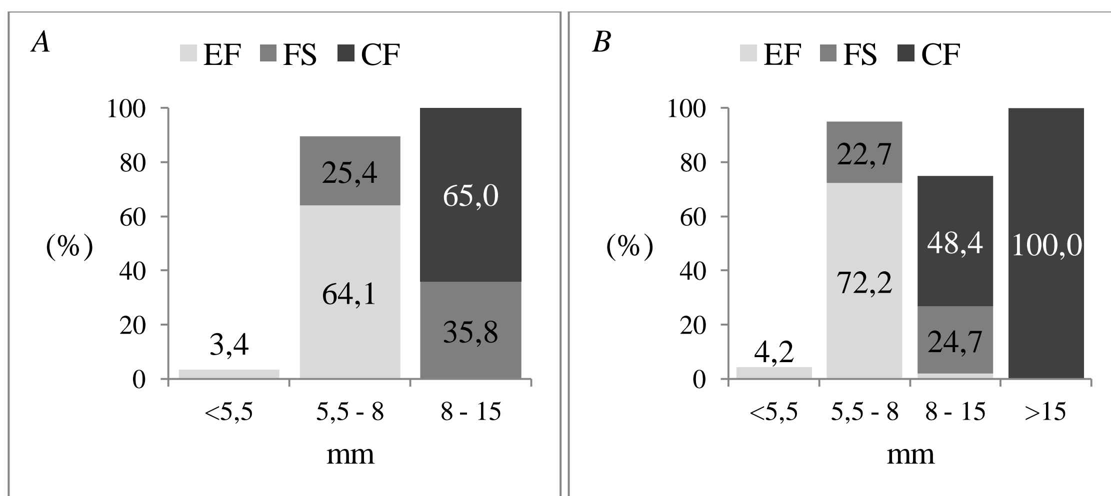


Figure 10: Percentage of larvae in early flexion stage (EF), flexion stage (FS) and with completed flexion (CF) at different size classes for control (Ctrl) (*A*) and YSA (*B*) treatments.

3.2.6. *Live feed intake*

At 5 DAH approximately 50 % of the larvae presented food on their digestive tract. From 12 DAH onward over 90 % of the observed larvae presented a full digestive tract. No significant differences were observed between treatments at the same age ($P > 0.05$). Figure 11 shows examples of larvae of different ages of the two treatments.

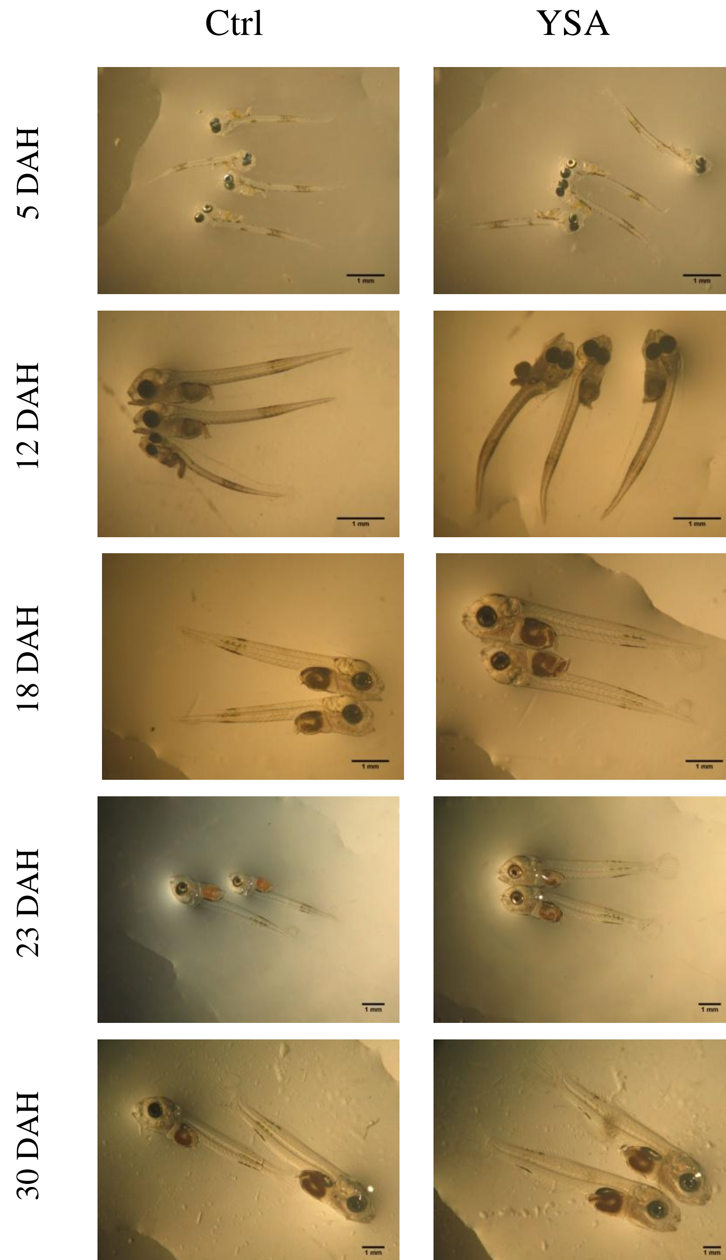
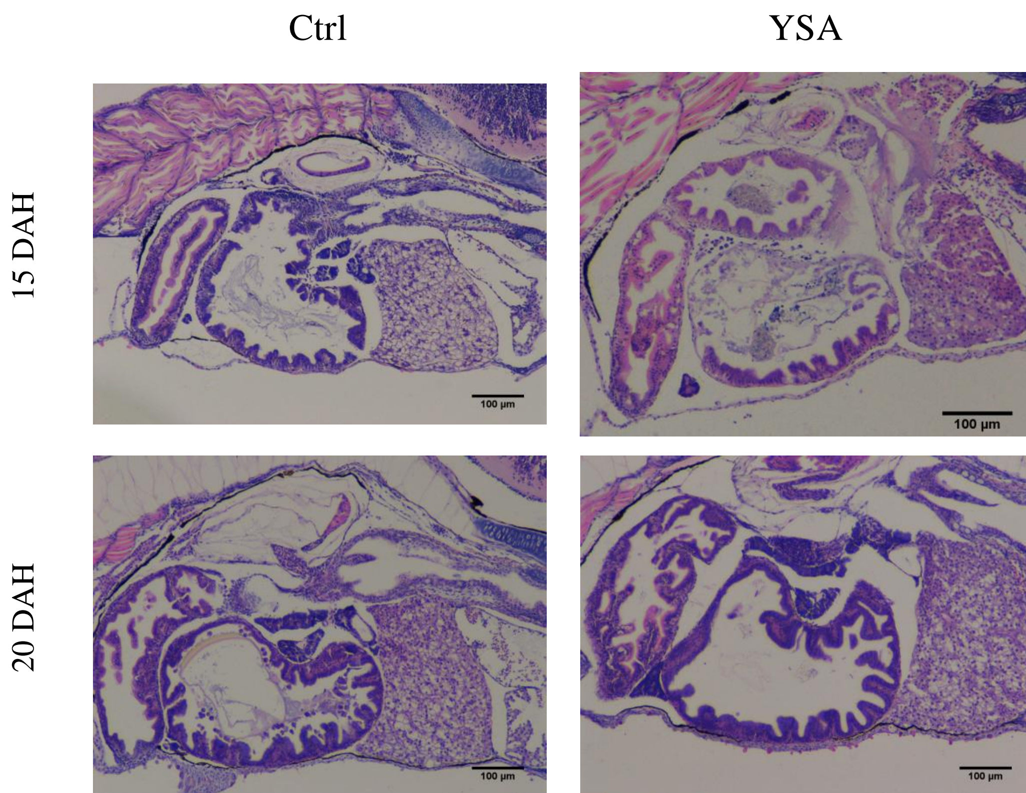


Figure 11: Larvae of the two tested treatments (Ctrl on the left and YSA on the right) at different ages (5, 12, 18, 23 and 30 DAH, respectively from the top to the bottom) showing food in their digestive tract.

3.2.7. Digestive system histological development

From the examination of sections (Figure 12) it was possible to observe that from 15 DAH onwards larvae presented an oesophagus, an anlage stomach, an anterior and posterior intestine, liver, pancreas and spleen with similar degree of development regardless the type of treatment. Until the end of the trial the striking events consisted of an increase of the intestine mucosal folds and the appearance of gastric glands in the stomach, regardless the type of treatment. It was also observed that some of the analysed larvae presented remains of food in the digestive tract.



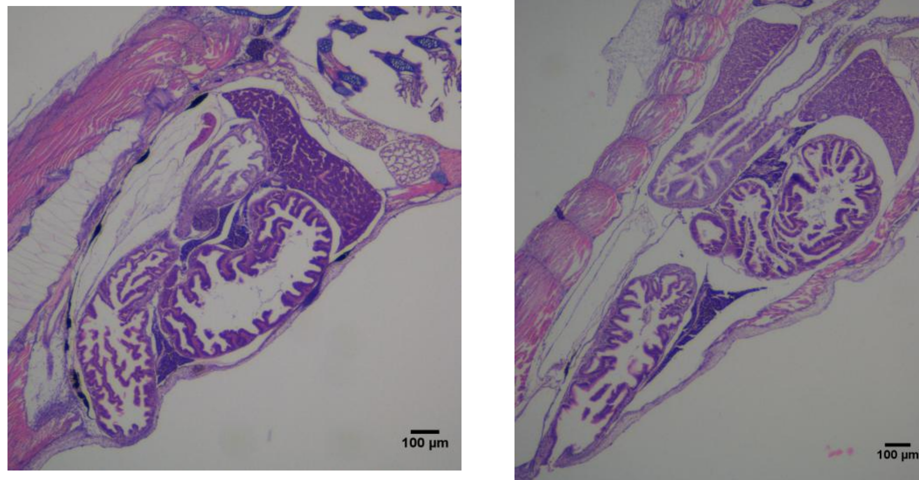


Figure 12: Example of histological sections of the abdominal cavity in whole body larvae from Ctrl (on the left) and YSA (on the right) treatments of 15, 20 (100 x) and 34 DAH larvae (40 x), in that order from the top to the bottom.

The hepatic tissue of 15 and 20 DAH larvae was well vascularised, compact and continuous. Hepatocytes presented a large unstained or slightly stained area and their nucleus was displaced from the centre of the cells. 34 DAH larvae presented some intercellular spaces, their hepatocytes area was predominantly stained and a more central nucleus was observed. These structural alterations occurred regardless the type of treatment. As presented in Table 3 no significant differences of the nuclei count were observed between treatments at the analysed ages. Significant differences were age related, 20 DAH larvae presented a lower number of nuclei for a similar area of liver.

Table 3: Number of nuclei per 0.01mm^2 of analysed liver of 15, 20 and 34 DAH larvae of control (CTRL) and YSA treatments. Values are presented as mean ± standard deviation (n = 12).

DAH	Ctrl	YSA
15	141 ± 38.1	205 ± 117.0
20	123 ± 32.7	91 ± 14.9
34	227 ± 13.9	160 ± 26.6

The intestine of the larvae of the analysed ages appeared as columnar epithelium bordered by a distinct brush border composed of microvilli, observed despite treatment. Intestinal epithelium height (Table 4) was similar between treatments for the studied ages (15, 20 or 34 DAH). Significant differences were age related, 15 DAH larvae presented a thinner epithelium when compared to 20 and 34 DAH larvae.

Table 4: Intestinal epithelium height (µm) of seabream larvae aged 15, 20 and 34 DAH, from control (CTRL) and YSA treatments. Values are presented as mean ± standard deviation (n = 12).

DAH	Ctrl	YSA
15	10.86 ± 0.534	10.77 ± 1.369
20	11.95 ± 0.560	12.05 ± 0.559
34	12.15 ± 0.291	13.02 ± 0.846

3.2.8. Enzyme activity

Scattered values of trypsin activity were obtained along the kinetic reaction, instead of a linear reaction. Since trypsin has an important role in protein digestion during the first 4 weeks of larvae development, it seems that the absence of trypsin activity is a result of the methodology, maybe due to the instability of the substrate.

Aminopeptidase, acid and alkaline phosphatase specific activities presented no significant differences between treatments. For these enzymes specific activity differences were age induced. A similar pattern was observed for the total activity of these enzymes with an exception. Differences were observed between treatments at 34 DAH. Amylase pattern was different from the other enzymes, no differences were observed in the specific activity of Ctrl between ages and no differences were observed at 34 DAH between treatments for total enzyme activity (Figure 13).

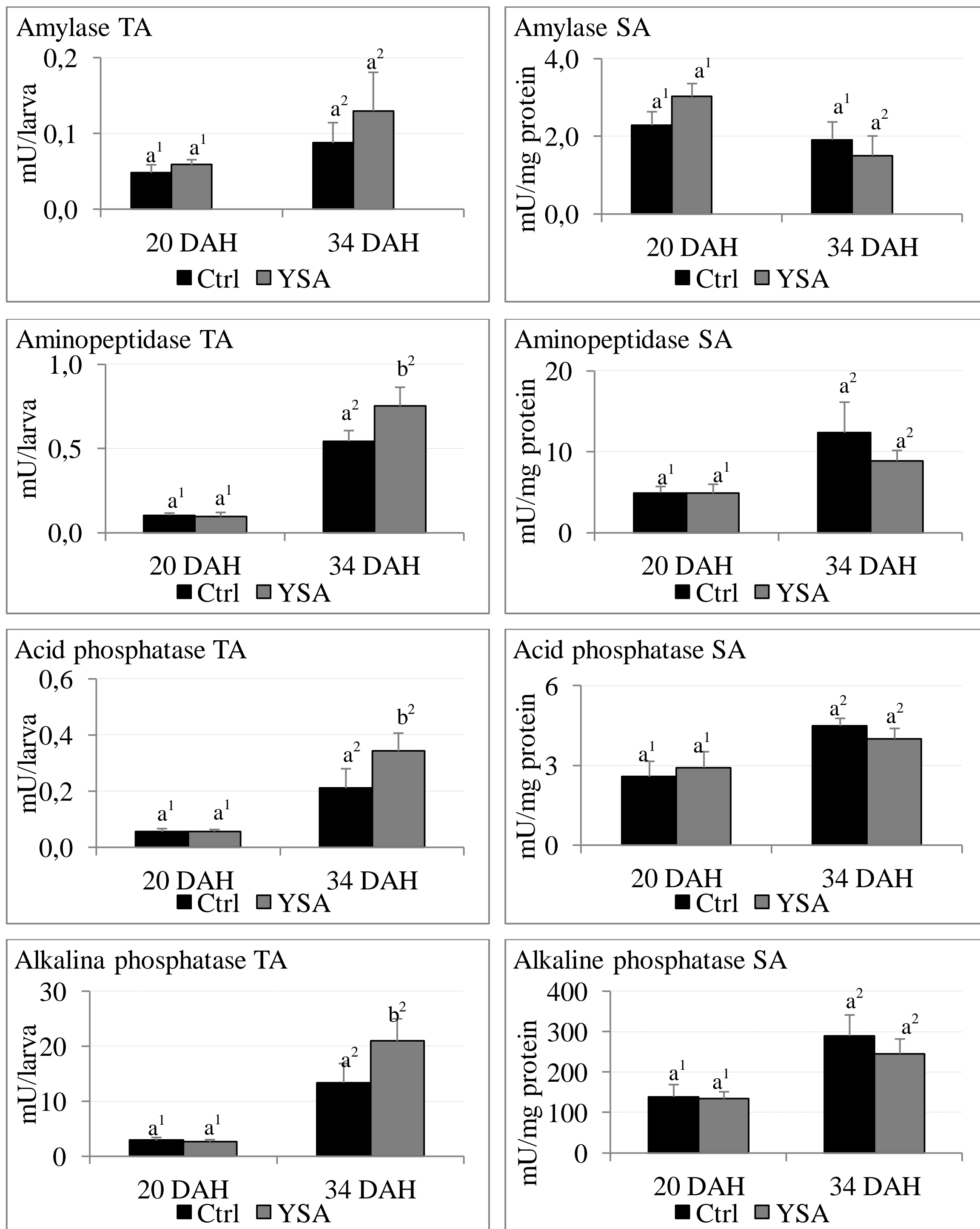


Figure 13: Total (TA) and specific (SA) enzyme activity of amylase, aminopeptidase, acid phosphatase and alkaline phosphatase at 20 and 34 DAH for control (Ctrl) and YSA treatments. Values are presented as mean ± standard deviation (n = 4). The letters (a, b) and the numbers (^{1},^{2}) were used to indicate significant differences ($P < 0.05$) for the factors 'treatment' and 'age', respectively.

3.2.9. *Lysozyme activity*

Lysozyme activity (Units.mg^{-1}) was determined in whole body 35 DAH larvae for both treatments. No significant differences (P > 0.05) were observed. The results obtained were 1790 ± 515.6 and 1814 ± 628.5 Units.mg^{-1} for Ctrl and YSA treatments, respectively.

3.2.10. *Cortisol concentration*

No significant differences in cortisol concentrations were observed before and after the stress test for any of the treatments. Significant differences were observed between treatments before and after the challenge, as presented in Table 5.

Table 5: Cortisol concentration (mean ± standard deviation (n = 4)) expressed in ng.g^{-1} DW, pre- and post-stressor for both treatments, control (Ctrl) and YSA.

	Pre-stressor	Post-stressor
Ctrl	5.28 ± 0.121	5.25 ± 0.110
YSA	2.88 ± 0.136	2.86 ± 0.135

3.2.11. *Bacteriological growth*

For a reliable count, plates with CFU's between 30 and 300 were counted. The 10^{-2} dilution was the optimum dilution for counting, for both media and both larvae and water samples. CFU's counts for *Vibrionaceae* were significantly lower in YSA for both water and sampled larvae and in total number of aerobic bacteria for larvae only. No significant differences were found in total number of aerobic bacteria in the water, as shown in Table 6.

Table 6: CFUs counts of water (CFU/mL) and larvae (CFU/g) samples of both treatments control (Ctrl) and YSA. Values are presented as mean ± standard deviation (n = 4).

	Water		Larvae	
	TSA	TCBS	TSA	TCBS
Ctrl	5833.3 ± 1997.96	1089.2 ± 122.70	3685.4 ± 1407.54	547.9 ± 180.04
YSA	5291.7 ± 1647.08	711.7 ± 75.30	1584.5 ± 732.11	163.9 ± 42.37

3.2.12. Antibacterial activity

Some antibacterial activity was observed in 50 % of the plates with whole body larvae from the YSA treatment, at the end of the trial as exemplified in Figure 14, where the halos around the discs mean reduced or no bacterial growth. No activity was observed for Ctrl, against a *P. damselae* subsp. *piscicida* strain sensitive to flumequine and with some resistance to oxitetracicline.

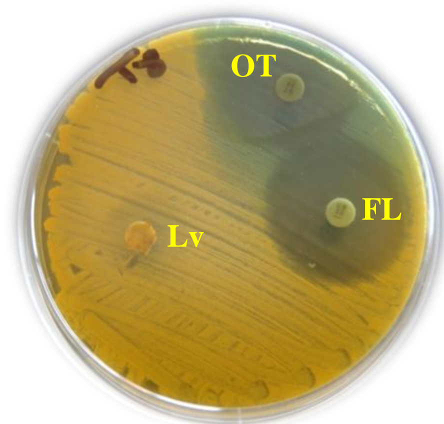


Figure 14: Plate from an YSA replicate with oxitetracicline (OT) and flumequine (FL) discs and a disc with whole body larvae (Lv).

4. Discussion

4.1. *A. armata* on live feed

A. armata bactericide potential is known but it was not tested in live feed for larval rearing up till now. The two *A. armata* products tested in live prey (the laboratory made extract and a commercial powder, Ysaline®100) were found to be applicable to treat live feed without compromising their viability. Further use of Ysaline®100 in extended trials would render more reliability given its known composition, the possibility of working with accurate metabolite concentrations and ease of handling. Thus the commercial product was selected for further testing.

The concentration of 0.5 % was selected to treat both rotifers and *Artemia* in the larvae rearing trial Ysaline®100 since bacterial growth (*P. damselae* subsp. *piscicida*) was inhibited and concentrations of 1 % or higher showed to be harmful to rotifers. The selected concentration was efficient in inhibiting *P. damselae* subsp. *piscicida* growth for at least 48 h. However, the period selected for live feed treatment was 30 min, to assure that live prey maintain their nutritional value in HUFA's (highly unsaturated fatty acids, a subset of Poly Unsaturated Fatty Acids or PUFA's) after that period and to minimize the delay in the first meal given to the larvae, (without affecting the hatchery daily activities). Docosahexaenoic acid (DHA, 22:6n3), eicosapentaenoic acid (EPA, 20:5n3) and arachidonic acid (ARA, 20:4n6) are the three most important HUFA's for marine fish larvae that have to be delivered through live feed (Sargent *et al.*, 1997, 1999). Although some fatty acids were lost after 30 min in starvation, mainly in *Artemia* metanauplii, DHA, EPA and ARA profiles were maintained at similar levels. For *Artemia* nauplii significant differences were observed between treatments for most of analysed fatty acids. This energy loss for YSA treatment may not be of high importance since *Artemia* nauplii were provided simultaneously with rotifers and for a short period of time (5 days), without significant effect on the most important HUFA. Considering the fatty acid profile of eggs or of the yolk sac of a newly hatched *S.aurata* larvae as the required fatty acid profile to a nutritional balanced feed (Sargent *et al.*, 1999, Cahu & Zambonino Infante, 2001), close or higher values of total PUFA were obtained in the live prey after enrichment and after 30 min in starvation, for both treatments, when compared to Pousão-Ferreira (2009) *S. aurata*

eggs (56.0 μg.mg^{-1}). Therefore, the required nutritional profile of the prey was maintained after the 30 min treatment.

4.2. *S. aurata* larval development

Seabream larvae from both treatments presented a full digestive tract confirming that treating live prey with YSA did not affect seabream feeding behaviour. The lower percentage of larvae with full stomach at 5 DAH can be attributed to the fact that larvae between 4 and 7 DAH still have reduced swimming ability and feed both endogenously (using the yolk sac) and exogenously (Elbal *et al.*, 2004). Thus, in this adaptation period they were not completely depended on the live feed provided, using their reserves and exogenous feeding, while their swimming ability and capture success evolves. Yet their acceptance to the external feed (rotifers and *Artemia*) was similar.

Seabream larvae do not present a functional swimbladder at hatching, its inflation is a decisive process in the early life history of *S. aurata* to obtain normal and healthy larvae (Soares, 1995). The swimbladder's development is made in two stages, first there's a primary inflation and then an expansion of this organ. During the two steps, larvae need to gulp atmospheric air thus they need to surpass the water/air barrier. This barrier is sometimes foiled by a greasy film that makes the process difficult or traps larvae, leading to their death. Larvae mortality observed at the surface of the tank was within the normal period of swimbladder inflation, which according to Soares (1995) takes place between 5 and 15 DAH for *S. aurata* larvae, but can vary with the culture conditions. This indicates that most of these larvae died trying to inflate. *S. aurata* larvae ability to inflate the swimbladder is no longer considered a major problem for its production, but inflation problems can be severe (Soares, 1995) making its monitoring of most importance, especially when testing new products for rearing these species. Since Ysaline®100 has never been used in larval rearing, its effect on the larvae ability to inflate was unknown and consequently it was monitored. The inflation process occurred in the normal period reported for this fish species with a similar process described by Soares (1995). Since no differences were observed in the swimbladder inflation, treatments had no influence in this process nor did external factors had an

important impact (e.g. poor water quality with a foiled surface), once a high percentage (95 ± 3.3 %) of larvae inflated their swimbladder.

Considering the importance that malformations have on larval quality and knowing that visible anomalies are associated with malformations (Koumoundouros *et al.*, 1997), fish larvae were monitored throughout development. Only a low percentage of observed larvae presented evident body anomalies and the higher percentage was found in younger larvae, without significant differences between treatments. This indicates that the treatments did not affect the normal larvae physical development. It also suggests that the main anomalies observed were of congenital origin and most of these larvae did not survived.

The observation of the development of the caudal fin complex was also used as an indicator of a normal larval development. This structure was selected because it is easy to see without the use of staining methods and its development could be monitored during the trial period. Koumoundouros *et al.* (1997) describes the caudal fin complex development with larval size. Larval growth is usually accompanied by larval development, when larvae don't grow they remain in a stagnant development stage. Conversely, a faster growth means faster development. This explains why significant differences were observed according to age and not according to size classes. Considering the caudal fin complex, the treatments did not affect its development, which followed the normal progress described by Koumoundouros *et al.* (1997).

4.3.Larval survival and growth

To avoid the effect reported by Mata *et al.* (2013), which observed fish mortality when using *Asparagopsis taxiformis*, the extract of *A. armata* was used in the live feed and washed off before provided to *S. aurata* larvae. Rotifers and *Artemia* suffered no major harm, using a concentration of the product that was effective in inhibiting a known pathogenic bacterium. However, even without a direct contact with the larvae, the use of *A. armata* metabolites in the treatment of the live feed negatively affected larval survival during the trial. The presented results, along with the chemical similarities between *A. armata* and *A. taxiformis*, seem to indicate that *A. armata* may

have the same ichthyotoxic effect in small concentrations, causing a lower survival in larvae fed with YSA-treated rotifers and *Artemia*. Further research is necessary to understand how the larvae are affected by the treatment and at which age/stage they are more susceptible. The lack of a reliable count of the dead larvae seems to indicate that the younger larvae may be more susceptible to the YSA treatment. It is more likely that small, rather than big, dead larvae have been missed while counting, but this presumed methodological caveat needs to be verified in future studies. The possibility that rotifers and *Artemia* can somehow transport the metabolites to the larvae, increasing larval mortality, e.g. by ingesting the dissolved particles, needs to be explored. These organisms are non-selective filter feeders and thirty minutes may not alter the nutritional content of the preys but even a small portion of the product could have been filtered and reached the larvae via ingestion. According to YSA technical sheet the particle size of the product is < 300 µm, bigger than the rotifers, but no information is provided on the size of the particles after dissolution in salt water. Also, the extract is primarily constituted by bromoform ($CHBr_3$), as described in the product's patent, produced by the genus *Asparagopsis* at a concentration of 1 % (EPA, 1989) or over 2 % of the total seaweed's dry weight, varying with environment conditions (e.g. CO_2 concentrations) (Mata, 2008). Bromoform is a halogenated compound known to be toxic to fish since the 1980's. It has been reported to accumulate in some fish and shrimp in concentrations of 3 to 50 fold the above water concentration and can depurate within 2 days of cessation of exposure (EPA, 1989). According to the EPA (1989) report, bromoform is scarcely soluble in water (about 3 $g.L^{-1}$ at 25 ºC) and is expected to be lost by volatilization. It can also be significantly removed by biodegradation under aerobic and anaerobic conditions especially in the presence of an electron acceptor, such as nitrate. In general, this substance can be absorbed through the respiratory tract, skin and gastrointestinal tract reaching the blood and distributed throughout the body, it can be found in adipose tissues and several organs, including the brain (EPA, 1989).

At the end of the trial YSA larvae were significantly bigger than the control larvae. This work intends to test an *A. armata* product to regulate bacterial load associated to the live feed (and to monitor larval wellbeing under the tested circumstances), as probiotics do, even if in a different way. Suzer *et al.* (2008) suggested that the use of probiotics potentiated larval growth due to an enhanced digestion and increased food absorption. Alternatively, survival can also influence larval

growth by changing the larval density and the larva/prey ratio. With a low larva/prey ratio, thus more prey available per individual, larvae tend to maximise ingestion. Higher food ingestion could lead to a higher weight gain but it can also lead to a lower food residence time in the gut, where living preys can still be found in the faeces (Parra & Yúfera, 2000). Higher densities and lower prey densities (within safe limits), however, tend to potentiate weight gain due to an increase in feed efficiency (Parra & Yúfera, 2000 and Yilmaz *et al.*, 2010). Live food intake analysis showed that undigested preys were sporadically observed in the larvae of both treatments, suggesting that feed efficiency was not altered with the larva/prey ratio. The higher weight gain observed in YSA larvae appears to be related to a higher food intake or to an alteration of the bacterial load that could have enhanced digestion and food absorption.

4.4.Larval digestive capacity and immune response

Histology was a tool to examine the digestive tract structures. Through this method, it is possible to evaluate the intestine morphology and absorption area and the liver condition. A visual evaluation of the tissues, mainly liver and intestine, was made. Liver is one of the target organs for bromoform toxicity, resulting in morphology or functional abnormalities, whereas intestinal lesions have also been observed in mice (EPA, 1989). Here all observed tissues appeared healthy without lesions in any organ. No perceptible differences in the liver structure were observed between treatments at any age. Differences were age related with 20 DAH larvae presenting lower nuclei density. Considering that a lower nuclei density indicates bigger hepatocytes with more nutrient reserves content (lipid and/or glycogen), the lower nuclei density in the 20 DAH larvae in comparison to the 15 DAH, seem to indicate an increase on the liver reserves, thus well-nourished larvae. Regarding the 34 DAH larvae, they presented a higher nuclei density (similar to the 15 DAH), which may be a result of the normal liver growth after cellular division, resulting in smaller hepatocytes. On the other hand, smaller hepatocytes could be a result of the reserves being reabsorbed, indicating that the larvae used them e.g. for growth. The liver is a good indicator of the nutritional composition of the food provided as their reserves are the first energy sources to be used, after the yolk sac and oil globule depletion, as a response to deficient diets

(Gisbert *et al.*, 2008). The nutritional requirements of larvae are different from the nutritional requirements of juvenile fish (Cahu & Zambonino Infante, 2001), at 34 DAH larvae with 12 and 14 mm (Ctrl and YSA, respectively) are considered post-larvae, a stage between larval and juvenile stages. Moreover *S. aurata* larvae at 34 DAH are usually eating inert diets (Yúfera *et al.*, 2000 and Pantazis *et al.*, 2014) which can cope with the changes in the nutritional requirements. In this trial inert diets were deliberately not used so they would not interfere with the results outcome, but at this stage the provided feed could have started to be insufficient for the fish's development. In the intestine structure, differences were visible between different ages, which may correspond to the larval development with an increasing absorptive capacity, but no differences were evident between treatments. YSA did not affect negatively nor positively the physical digestion of the larvae.

The analysis of the digestive enzyme activity was used as an indicator of food acceptance and digestive capacity. In this work some of the most common digestive enzymes activities were analysed. Differences observed in the total activity at 34 DAH were due to a higher weigh of the YSA treatment. When analysed in specific activity, the weigh effect was removed and no significant differences were observed. *A. armata* metabolites did not inhibit nor did potentiate the activity of the analysed enzymes. These findings together with the live feed intake observations support that feed efficiency was not altered and the higher weight gain in the YSA treatment was due to a higher food intake.

Immune response analysis of *S. aurata* larvae has shown that lysozyme activity was not significantly different between treatments. This indicates that the use of *A. armata* derived metabolites to treat live food did not inhibit or potentiated the lysozyme activity of *S. aurata* larvae. For cortisol measurements, the lack of significant differences before and after a stressful situation, suggests that the induced challenge was not enough to trigger a stress response, or that the interval between the challenge and the larvae's death was not sufficient (no more than 10 min) for the cortisol to be released. The observed concentrations (5.28 ± 0.121 and 2.88 ± 0.136 $ng.g^{-1}$ DW for control and YSA respectively) were lower than the results obtained by Szisch *et al.* (2005) (7.53 ± 0.92 $ng.g^{-1}$ WW) for larvae at a similar development stage,

without a stress challenge. It is important to note that, since the authors presented their results in WW, the difference between the results are higher, an adjustment to the presented cortisol concentrations to WW would result in lower values. The stress response could have possibly been observed using a longer challenge time and/or longer time before collection, e.g. Van Anholt *et al.* (2004) exposed their 28 and 50 DAH seabream larvae to a 90 s period, 3 times higher than the period used in this work, and obtained significant differences between pre- and post-stress after a 20 and 30 min period before collection. Nevertheless, significant differences were observed between treatments with lower cortisol level in the YSA treatment. When a stressor is applied repeatedly a decrease in cortisol levels is usually observed (Tort *et al.*, 2001), the presence of ichthyotoxic metabolites such as bromoform (considering that the metabolites reach the larvae) could be considered a chronicle stressful situation. However, this could have not been the cause of the lower cortisol concentration in YSA, since larvae need energy to cope with the stress (Mommsen *et al.*, 1999), which could have prevented the higher growth that was observed in the YSA larvae. Cortisol is always present in vertebrates, it is an essential multifaceted hormone that plays important physiological and metabolic roles also interacting with many other hormones at several levels. Its synthesis involves microsomal enzymatic pathways that may be disrupted by some environmental contaminants (Mommsen *et al.*, 1999) also it should be present that some *A. armata* metabolites are enzymes inhibitors (Kladi *et al.*, 2004). To understand how cortisol concentration is affected by the YSA treatment further study on these mechanisms and their actual effect on larvae is necessary.

Bacteriological analyses indicate that the use of YSA in the live feed was efficient in reducing the bacterial load in the intestine of the *S. aurata* larvae, even without affecting the total bacteria present in the water. As mentioned earlier, it is important to reduce the bacterial load associated to the live feed, as they are a source of potential pathogenic bacteria that can negatively affect larvae rearing (Haché & Plante, 2011 and Vladstein *et al.*, 2013). But, as explained by Vladstein *et al.* (2013) knowing which bacterium are present and in which proportion they exist, can be more important than knowing the total bacterial number. Reduction of the total bacterial load can be followed by an increase of opportunistic species that can multiply more rapidly (r-strategy) than other species, thus affecting marine larval rearing. Samples were collected to investigate which bacteria are present and which species are dominant in

the larvae and water of the performed trial, using a pyrosequencing technique (DNA sequencing by synthesis using pyrophosphate release detection). This information, still in process, will help to elucidate if the remaining bacteria in the YSA treatment were a possible cause of the poor larval survival, rather than the possibility that *A. armata* toxic metabolites were carried to the larvae through the live prey. On the other hand, the present bacteria could have contributed to fitter larvae (bigger and more resistant to pathogenic bacteria) aside the mortality effect.

The disc diffusion method is a common method most used to access the antibacterial activity in the mucus of fish, including *S. aurata* specimens (Magariños *et al.*, 1995), used in this work to access antibacterial activity by *S. aurata* larvae. The differences observed between treatments indicate that the larvae from YSA treatment have some antibacterial capacity. This could be a result of the YSA contribution to fitter larvae, which would be able to inhibit bacterial growth using as-yet unknown or untested mechanisms. But, considering that in the histological observation some larvae still presented food in the digestive tract, the antibacterial activity observed in this test could have been influenced by the direct action of the bactericide properties of *A. armata* carried to the larvae through the preys, corroborating the possibility that the live prey carry the product's metabolites. Whether the metabolites in the prey present in the digestive tract contribute to the antibacterial properties observed, or the metabolites are transferred and accumulate in the larvae is unknown. If the metabolites are transferred to the larvae, can their antibacterial properties contribute to an enhancement of the larvae fitness e.g. higher growth?

5. Conclusion

A. armata metabolites act as an efficient bactericide that can be applied in safe concentrations to effectively control the pathogenic bacteria load associated to live prey. However, *S. aurata* larval survival was negatively affected by live prey treated with YSA. Metabolites present in *A. armata* are known for its toxicity to marine fish larvae, a reason why YSA was not directly used in the rearing of the larvae. Seabream's development was not affected in most of the analysed parameters except growth, cortisol concentration and antibacterial activity, apart from survival. The mechanisms through which treated live prey affected these parameters are yet to be fully understood. Further research should be conducted to understand if the seaweed metabolites are carried to the larvae by the larvae prey and if so if they accumulate in them. Another question that emerges from this work is whether larval survival was directly affected by the toxicity of the metabolites or indirectly by changes in the bacterial community. Thus, the factors that likely underpin effects on larval survival should also be subject of more investigation. If the changes in the bacterial load were not the cause of poor survival, could they potentiate growth, improving larval fitness? If so, can the negative impact on the larval survival be overcome by any favourable effect on the bactericidal potential of *A. armata*, and can that negative impact be suppressed? The lower cortisol concentration in YSA treatment should also be investigated in order to understand how it is affected and if the observed effect is positive, contributing to the larvae fitness, or if the lower cortisol concentration is an effect of a negative impact on the larvae metabolism, inhibiting some important enzymatic pathways.

Analyses in progress of the bacterial diversity present in tank water, live feed and larvae using 454-pyrosequencing will help unveil some of these questions. Identifying which bacteria are present in the larvae's gut and rearing water will answer if those bacterial are potentially harmful to the larvae and if they were the cause of their poor survival, or if they are potentially beneficial bacteria that could have contributed to a higher larval growth. Thus if *A.armata* metabolites can be used to regulate the bacterial load and diversity in the live feed provided to fish larvae and what needs to be overcome to increase eventual positive effects in their rearing.

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