Deliverable Report

Deliverable No:	D25.3	**Delivery Month:**	44
Deliverable Title	Impact of dietary regime on parasite resistance and mucosal defenses of greater amberjack juveniles.		
WP No:	25	**WP Lead beneficiary:**	P5. UNIABDN
WP Title:	Fish health - greater amberjack		
Task No:	25.2	**Task Lead beneficiary:**	P5. UNIABDN
Task Title:	Promoting resistance to parasitic incidence on greater amberjack		
Other beneficiaries:	P2. FCPCT		
Status:	**Delivered**	**Expected month:**	42
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Objective: Identify the effect of dietary regime on mucus immune barrier and modulate the resistance to parasite infection by adding immunostimulants to the diet.

Description: Variation of mucus immune potential has been studied in relation to different dietary regimes. Immune potential of mucus defences has been studied from the systemic point of view (including lysozyme and bactericidal activities) and from the histological point of view. The utilization of mucus stimulatory substances, such as mannan-oligosaccharides (MOS) or concentrated mannan—oligosaccharides (c-MOS), has been also evaluated. A histological study of the effects of monogenean parasitization on greater amberjack juveniles was conducted, and the potential of immunostimulants to reduce parasitic infection has been also assessed.

1.- Dietary regime for greater amberjack juveniles

A trial was conducted to determine the effects of dietary regime on mucus immunity. Dietary regime was evaluated taking into account two parameters: the feeding frequency (number of intakes per day) and the feeding rates (% of feed per day per body weight). Six hundred fish of 20.03 ± 1.5 g (mean ± SD) body weight were distributed in twenty-four 500 L tanks (25 fish/tank) and fed on the eight dietary strategies (in triplicate) for 120 days. The following feeding frequencies (number of intakes per day) and feeding rates (% of feed per day per body weight) were used: (a) apparent satiety 3 intakes/day (S3), (b) apparent satiety once a day (S1), (c) 3.5% of the biomass divided in 3 intakes/day (3.5/3), (d) 3.5% of the biomass divided in 4 intakes/day (3.5/4) (e) 3.5% of the biomass once a day (3.5/1), (f) 2.5% of the biomass divided in 3 intakes/day (2.5/3), (g) 2.5% of the biomass divided in 4 intakes/day (2.5/4), and (h) 2.5% of the biomass once a day (2.5/1).

Growth of fish of the different dietary regimes was monitored monthly. At the end of the experimental period, samples of blood were obtained by caudal sinus puncture for haematological and immunological

studies, as indicators of fish welfare. Serum lysozyme and bactericidal activity were monitored. Plasma cholesterol, lactate and triglycerides plus selected haematological enzymes were also analysed.

Fish of the S3 group presented significantly higher ($p<0.05$) growth when compared with fish of the S1 group, whereas fish fed on the 2.5% biomass feeding regime were significantly ($p<0.05$) smaller than those fed on the 3.5% biomass feeding regime, independent of the feeding frequency used. In addition, 3.5/3 and 3.5/4 fish showed similar growth to the s3 group. Two-way ANOVA analyses showed that fish final weight was affected ($P<0.05$) by the amount of feed provided but not by the number of times fish were fed. On the other hand, there was a significant ($P<0.05$) interaction between both factors. Correlation analysis of weight and nº of intakes showed significant differences ($p<0.05$; $r=0.10$); Weight=160.37 + nº of intakes x 3.055 (**Figure 1**).

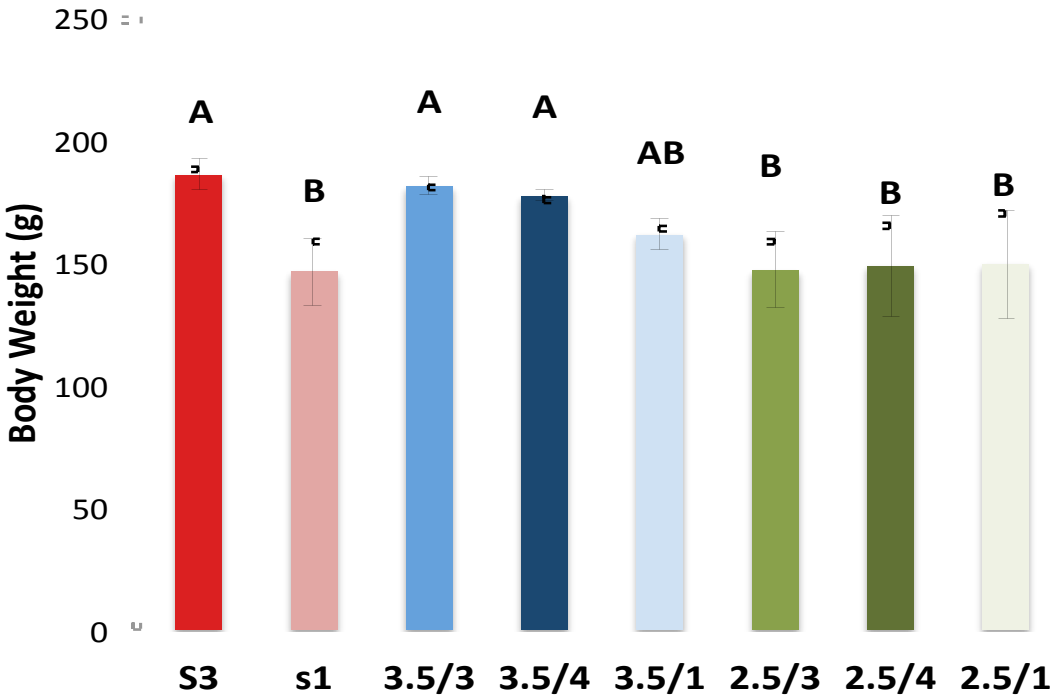

Figure 1. Final weight (g) for each treatment group at the end of the feeding trial (120 days). Different letters denote significant ($P<0.05$) differences among dietary treatments. s3: Apparent satiety 3 intakes; s1: Apparent satiety 1 intake; 3.5/3: 3.5% Biomass 3 intakes; 3.5/4: 3.5% Biomass 4 intakes; 3.5/1: 3.5% Biomass 1 intake; 2.5/3: 2.5% Biomass 3 intakes; 2.5/4: 2.5% Biomass 4 intakes; 2.5/1: 2.5% Biomass 1 intake.

Blood biochemical parameters measured showed significant differences among treatments. Fish of the 3.5/1 presented the highest cholesterol and glucose levels ($P<0.05$), whereas fish of the 2.5/4 group presented the lowest triglycerides level (**Figure 2**).
For the immunological parameters in serum, S3, S1 and 2.5/1 fish presented the highest lysozyme activity (**Figure 3**), although it was similar to that presented by 3.5/1 fish. No differences ($P>0.05$) among dietary treatments were found for peroxidase activity (**Figure 4**). Two-way ANOVA showed a significant effect of the interaction between feeding rate and feeding frequency ($P=0.021$) with no significant effect of either feeding frequency or feeding regime.

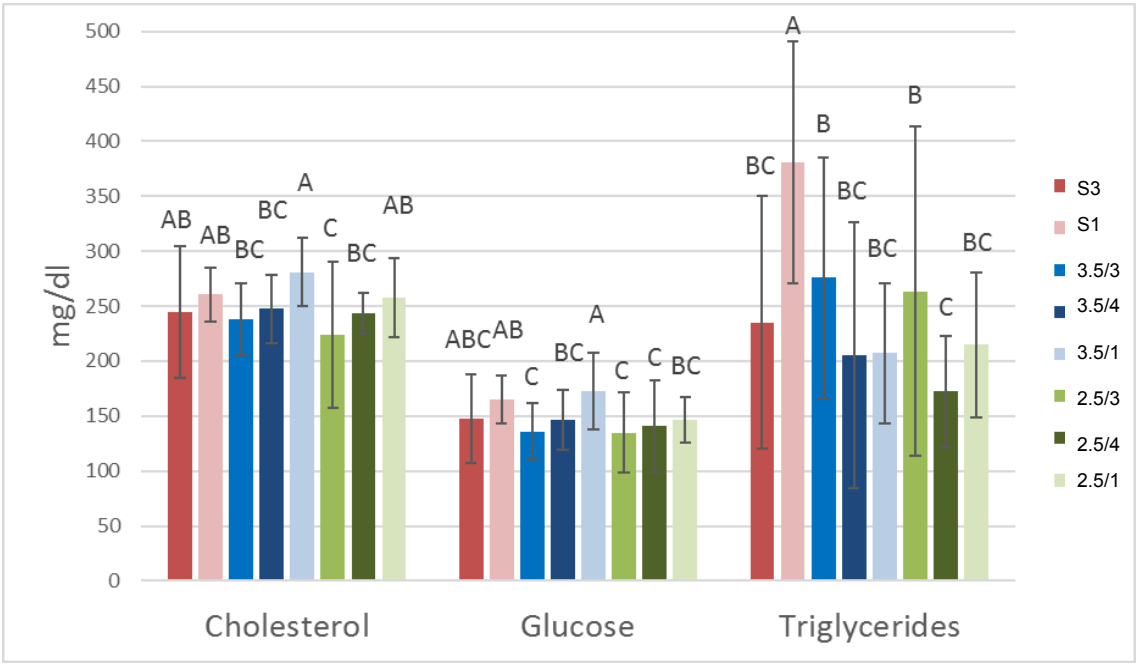

Figure 2. Blood biochemical parameters (cholesterol, glucose and triglycerides) of fish fed each dietary treatment at the end of the feeding trial (120 days). Different letters denote significant differences among dietary treatments (P<0.05).

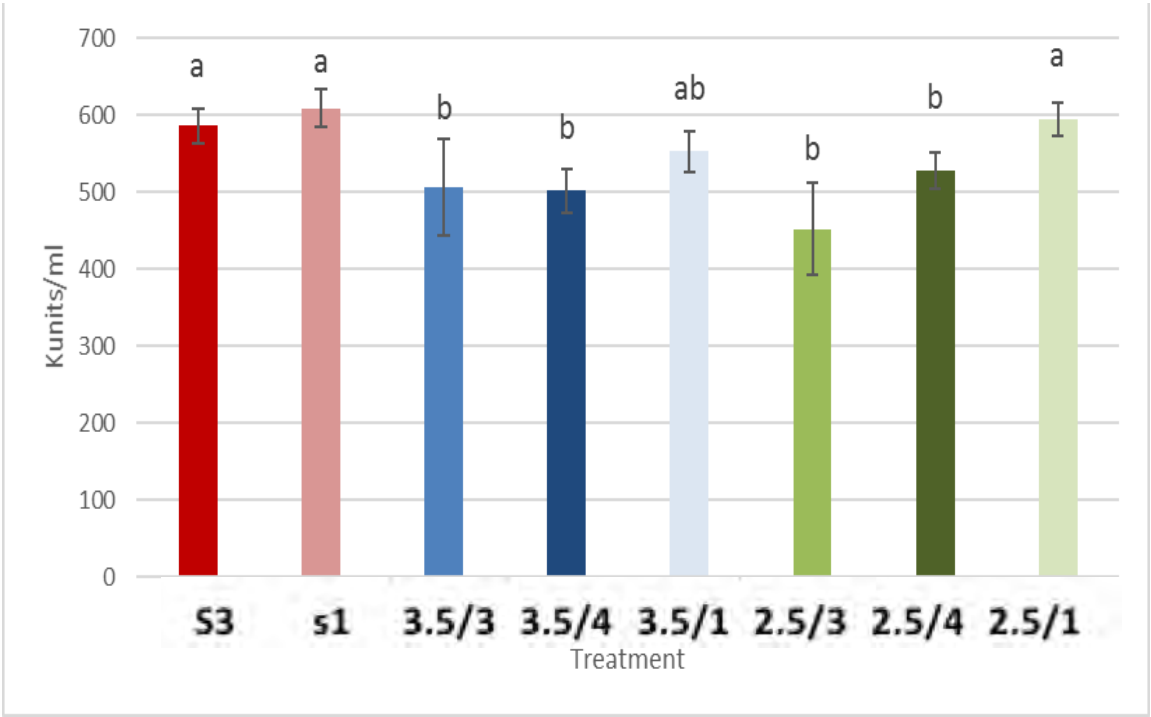

Figure 3. Serum lysozyme activity of fish fed each dietary treatment at the end of the feeding trial (120 days). Different letters indicate significant differences among dietary treatments (P<0.05).

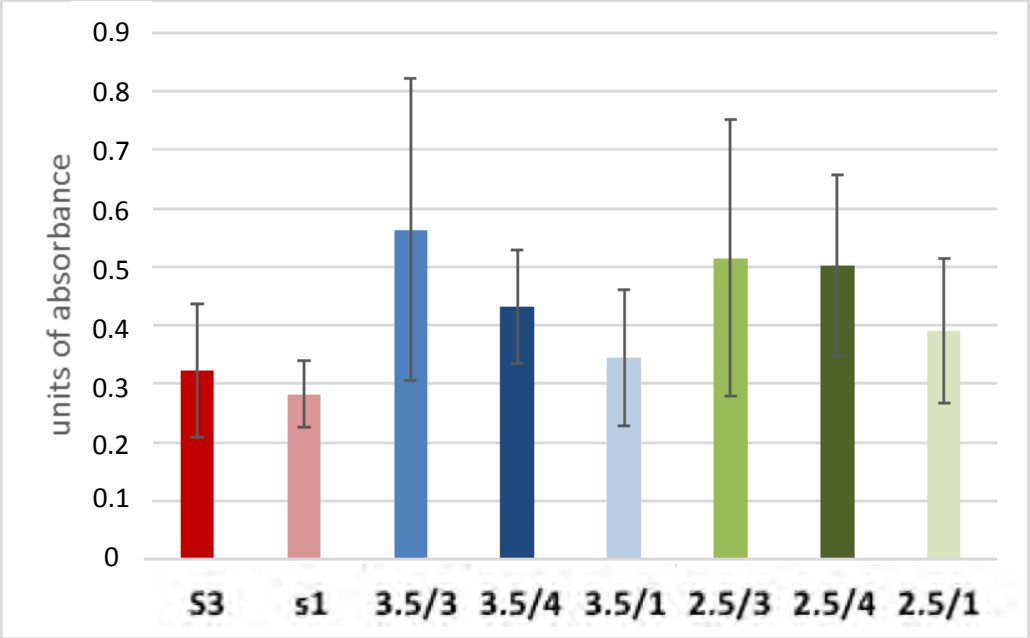

Figure 4. Serum peroxidase activity of fish fed each dietary treatment at the end of the feeding trial (120 days). No significant effect was found for this parameter.

The mucus immune (lysozyme) activity was affected by the dietary regime (**Figure 5**). Two-way ANOVA showed a direct effect of feeding rate (P=0.036) and a combined effect of feeding rate and feeding frequency (P=0.004). No effect of feeding frequency was found (P=0.16).

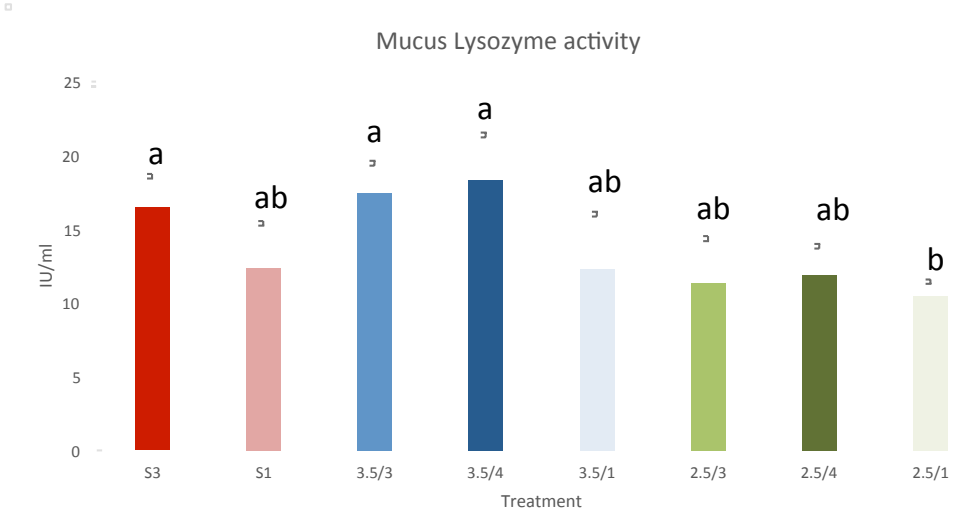

Figure 5. Mucus lysozyme activity of fish fed each dietary treatment at the end of the feeding trial (120 days). Different letters indicate significant differences among dietary treatments (P<0.05).

No effect was found on bactericidal activity (**Figure 6**), although a clear tendency to have higher bactericidal activity can be seen for animals fed to satiety or given 3.5% of body weight/day and more than one intake per day. No significant differences were found on mucus peroxidase activity (**Figure 7**).

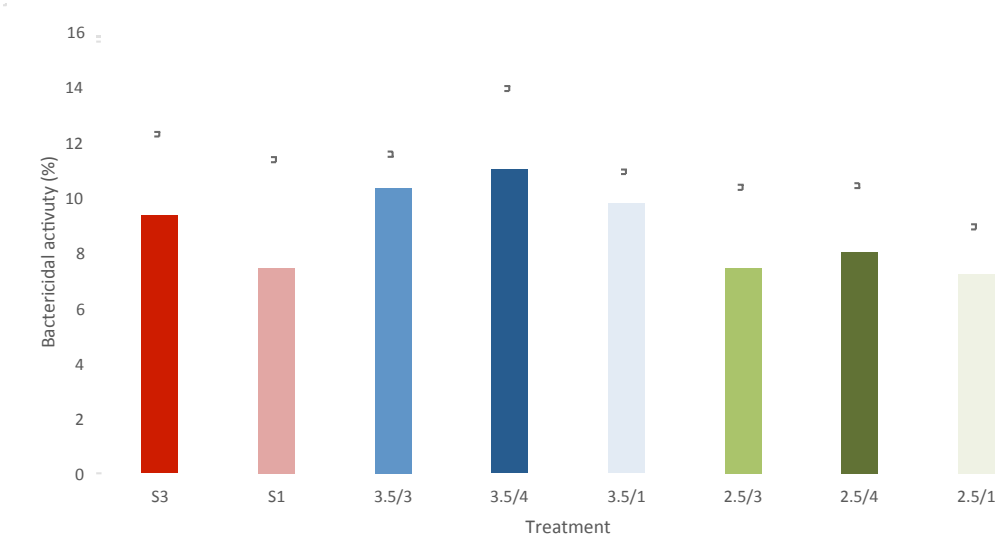

Figure 6. Mucus bactericidal activity of fish fed each dietary treatment at the end of the feeding trial (120 days). No significant effect was found on this parameter.

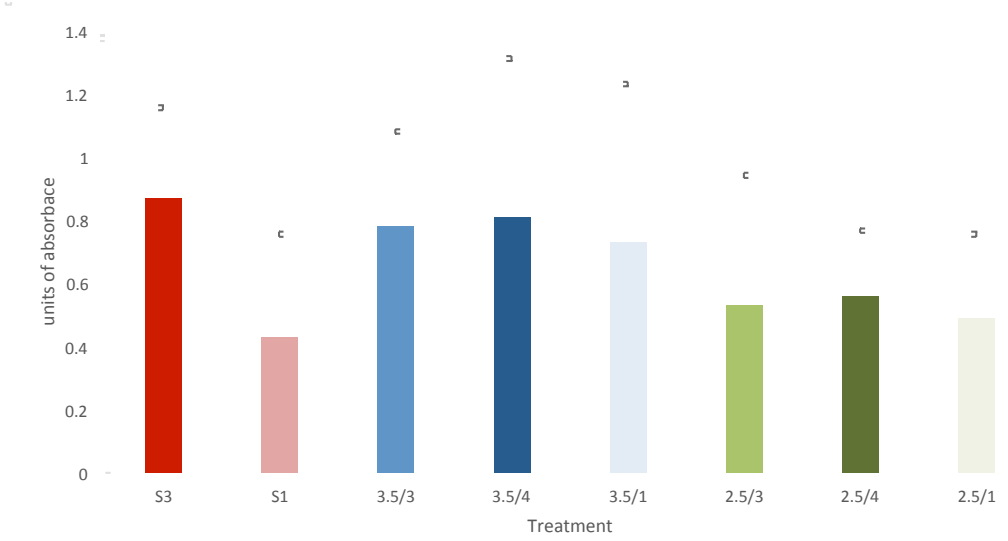

Figure 7. Mucus peroxidase activity of fish fed each dietary treatment at the end of the feeding trial (120 days). No significant effect was found on this parameter.

Once the dietary regime for greater amberjack juveniles was determined, the next step was to determine the effect of the addition of mucus-stimulating products to the diet on parasitic infection. The aim of this study was to determine the effect of different commercial prebiotics on the greater amberjack immune system and the impact on resistance to an experimental infection against the ectoparasite *Neobenedenia girellae*, as this monogenean parasite is one of the most important parasite species affecting greater amberjack in sea cages. For this purpose, two commercial prebiotics were studied including one mannan-oligosacharide (MOS) and one concentrate of mannan-oligosaccharides (c-MOS). In addition, a morphological study on how greater amberjack skin is changed by the action of the monogenean, and the site of action of this parasite species was also conducted to understand how this parasite damages fish skin and alters the mucus immune barrier.

2. Morphological study on the incidence of monogenean parasite in greater amberjack skin. Optical and electron microscopy studies.

Parasitized animals were sacrificed and skin collected from two different regions; cranial and dorsal. These sites were selected because of their difference in the prevalence of the parasite, and since they are also the most common places where the parasite is attached. Tissue samples were fixed in buffered formaldehyde (4%) for subsequent histological studies (**Figure 8**).

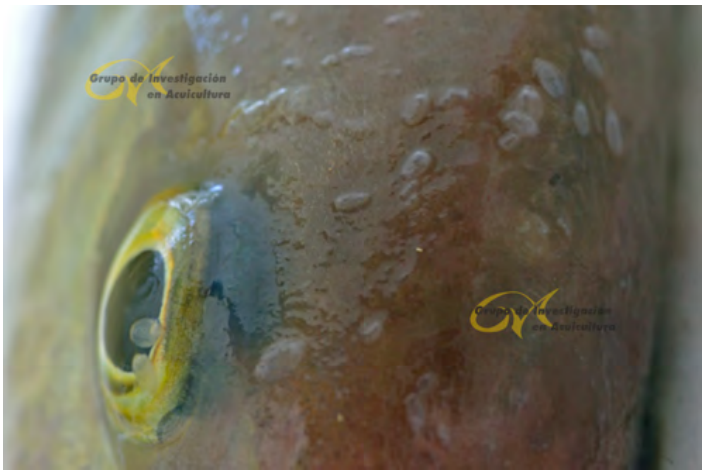

Figure 8. *Neobenedenia girellae* adults attached to the head of *Seriola dumerili*.

For the microscopic analyses, glycol methacrylate resin embedding (Technovit 7100, Kulzer) was conducted, since the use of plastic blocks produces an increase of the resolution power of the images. For observation of the goblet cells, the sections were stained with Alcian-PAS, using GIEMSA for staining contrast instead of hematoxylin. The microscope used for the observations was an OLYMPUS CX41RF with an adapted camera OLYMPUS U-MDOB3.

For the description of the parasite species, morphological methods were used. The main characteristic for distinguishing between the genus *Neobenedenia* and *Benedenia* is size, with *Benedenia* being bigger than other parasites infecting amberjacks. Secondly, the anterior structure is slightly concave or flat, starting with the horizontal top of their anterior suckers hooded. *Neobenedenia* on greater amberjack skin is shown in **Figure 9**.

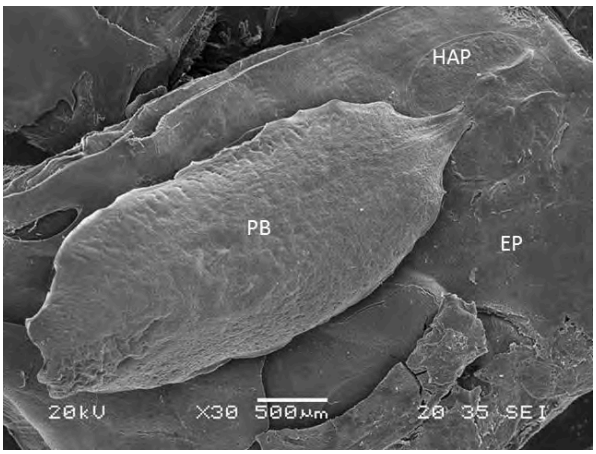

Figure 9. Electron scanning microscopy (SEM) picture of *Neobenedenia girellae*. HAP: Haptor; PB: Parasite body; EP: Epidermis.

The comparison between the dorsal and the cranial region epithelium of juvenile greater amberjack showed that dorsal region presented a thinner epidermis than the cranial region. More goblet cells were observed in the epidermal layer in the dorsal region and the dermis layer in the dorsal region was thinner than in the cranial region (**Figure 10**).

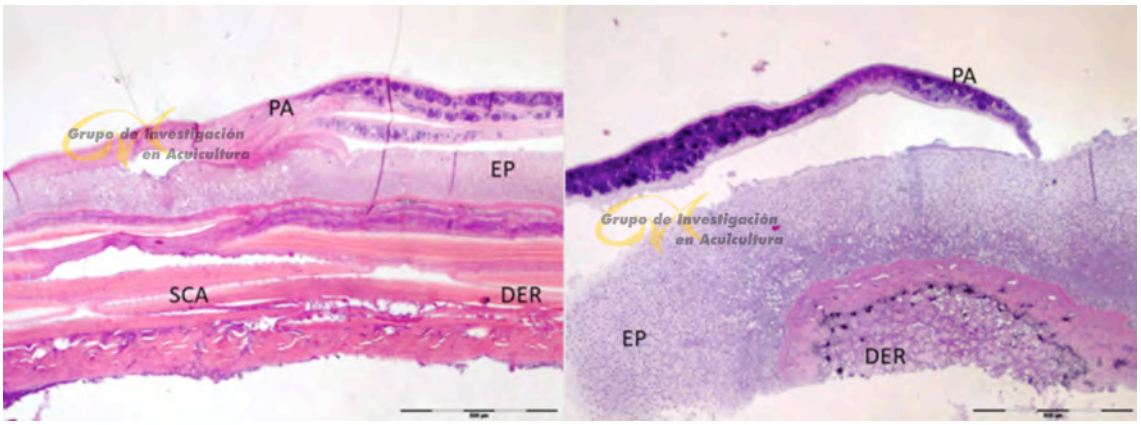

Figure 10. Dorsal (left) and cranial (right) region. PA: parasite; EP: epidermis; SCA: scales; DER: Dermis

Parasite damage on greater amberjack skin: At the cellular level after parasite infection, a hydropic degeneration was observed around the parasite site of adhesion. Vacuoles presented a water imbalance and occupied almost all the cytoplasm, moving the nucleus and all the cellular organelles to the cell extremes. This hydropic vacuole degeneration could cover big areas and produce spongiosis with intercellular edema (**Figure 11**).

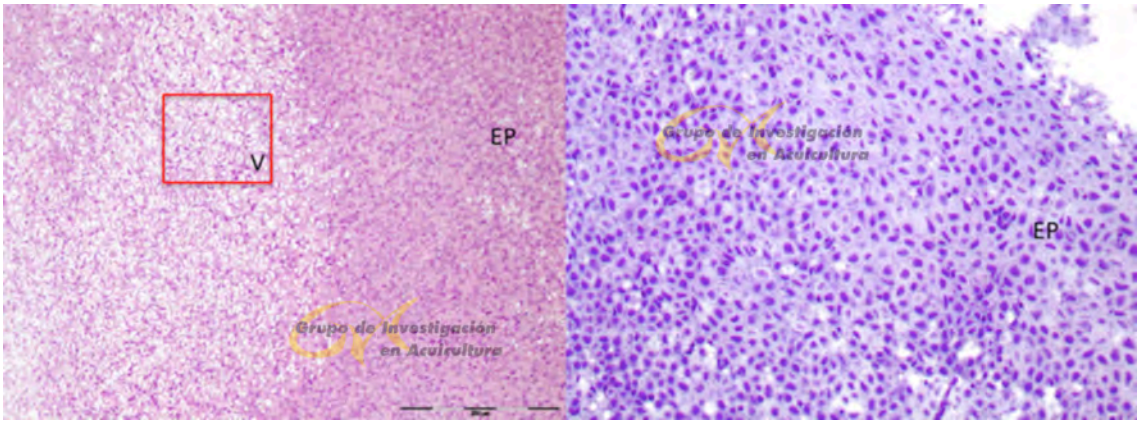

Figure 11. Vacuolization in the epidermis of *greater amberjack*. High level of hydropic degeneration that develops into spongiosis (left) *vs* epidermis of a non parasitized animal (right). V: Vacuolization; MCLT: Mononuclear cells - lymphocytic type.

At a tissue level, a clear disruption of the epidermal layer was observed. The haptor with the attachment structures produce an overpressure in the region where the parasite is fixed, which results in disruption of the typical structure of the tissue and the cells (**Figure 12**).

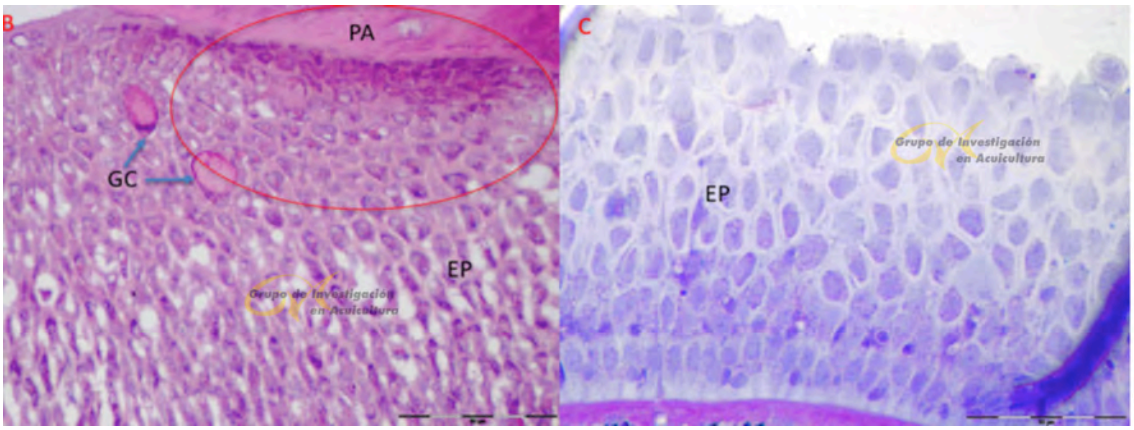

Figure 12. Disruption of the tissue and the cells (left) compared with a non parasitized animal (right). PA: parasite; EP: epidermis; GC: Goblet cells.

Mechanical damage produced by the attachment structures, the hooks (hamulus) and the anchors, was also observed (**Figure 13**). A disorganization of tissue can be seen.

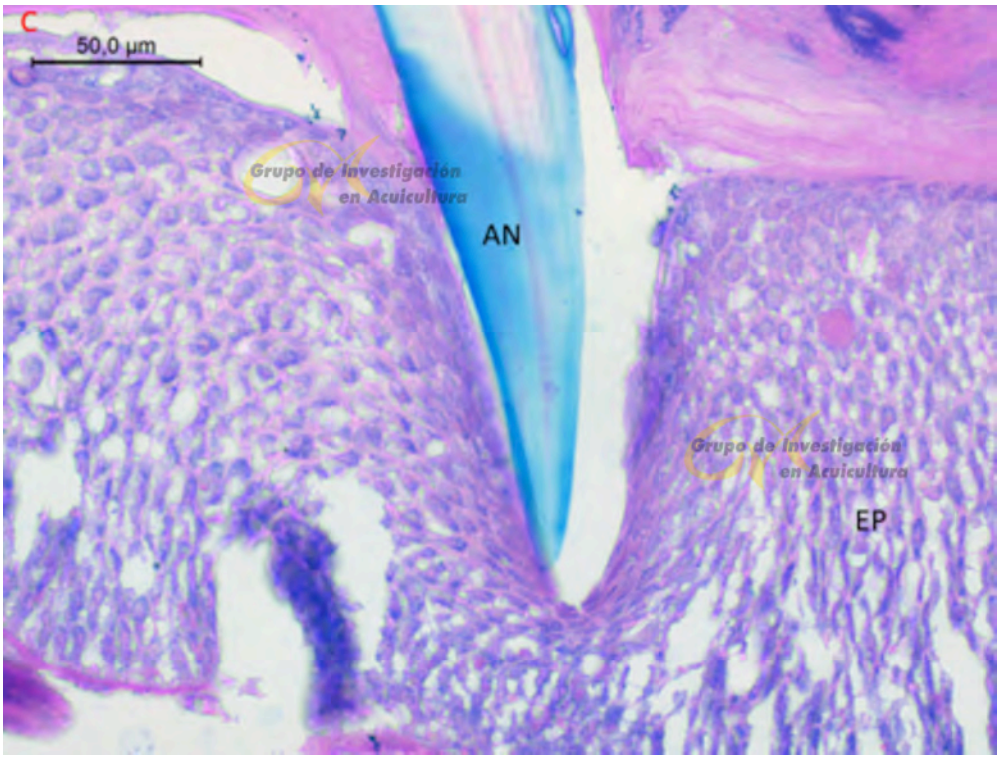

Figure 13. Mechanical damage produced at the epidermis by the attachment structures. AN: Anchor; EP: Epidermis.

The parasite infection also induced an increase in the number of goblet cells (**Figure 14**).

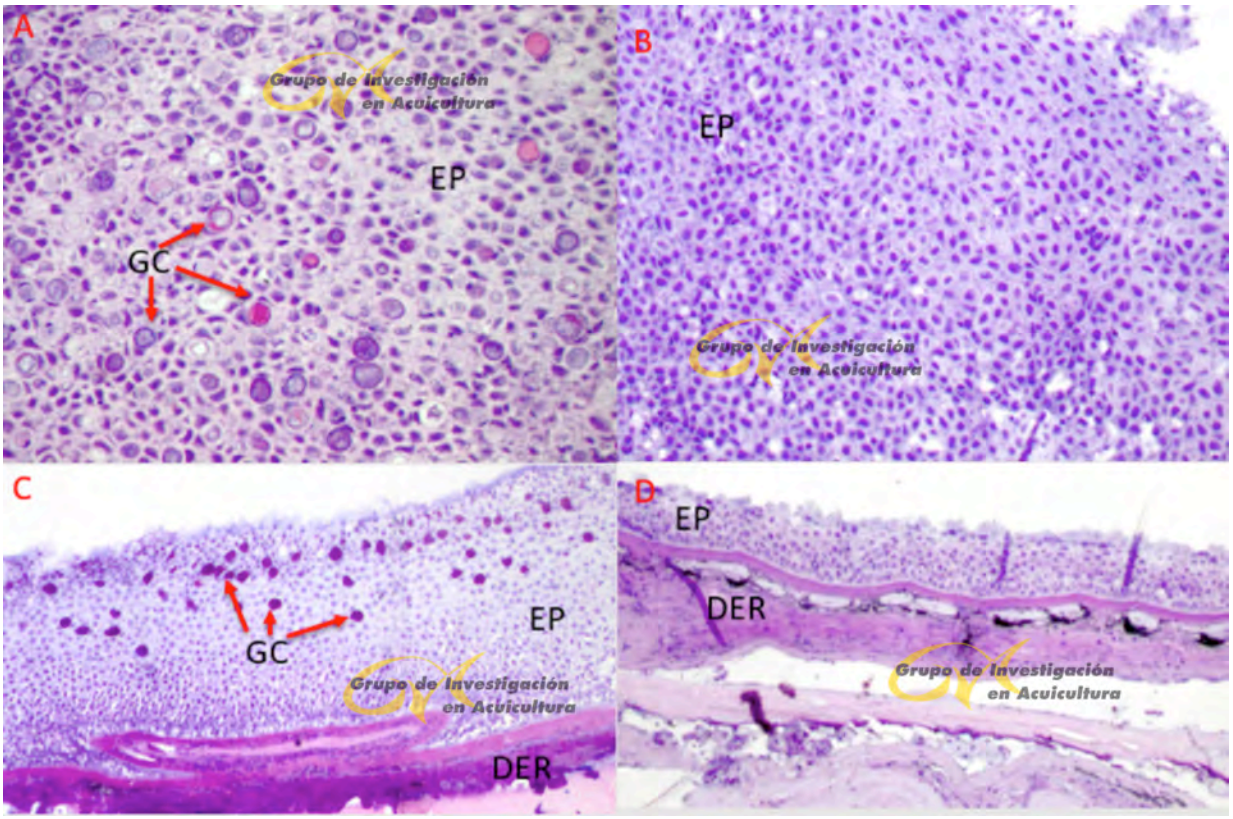

Figure 14. Longitudinal section of the epidermis layer of a parasitized (A) and non-parasitized (B) greater amberjack. Transverse section of a parasitized (C) and non-parasitized animal (D) with different amounts of goblet cells. GC: Goblet cell; EP: Epidermis; DER: dermis.

At the immunological level or immune defence barrier some modifications were clearly observed when the parasite got attached to the host. Mononuclear cell lymphocytic type mobilization was observed near the regions where the anchors and hooks were introduced into the host. This immune cell mobilisation is a focal extravasation of lymphocytes related with blood vessels, producing a perivascular dermatitis (**Figure 15**).

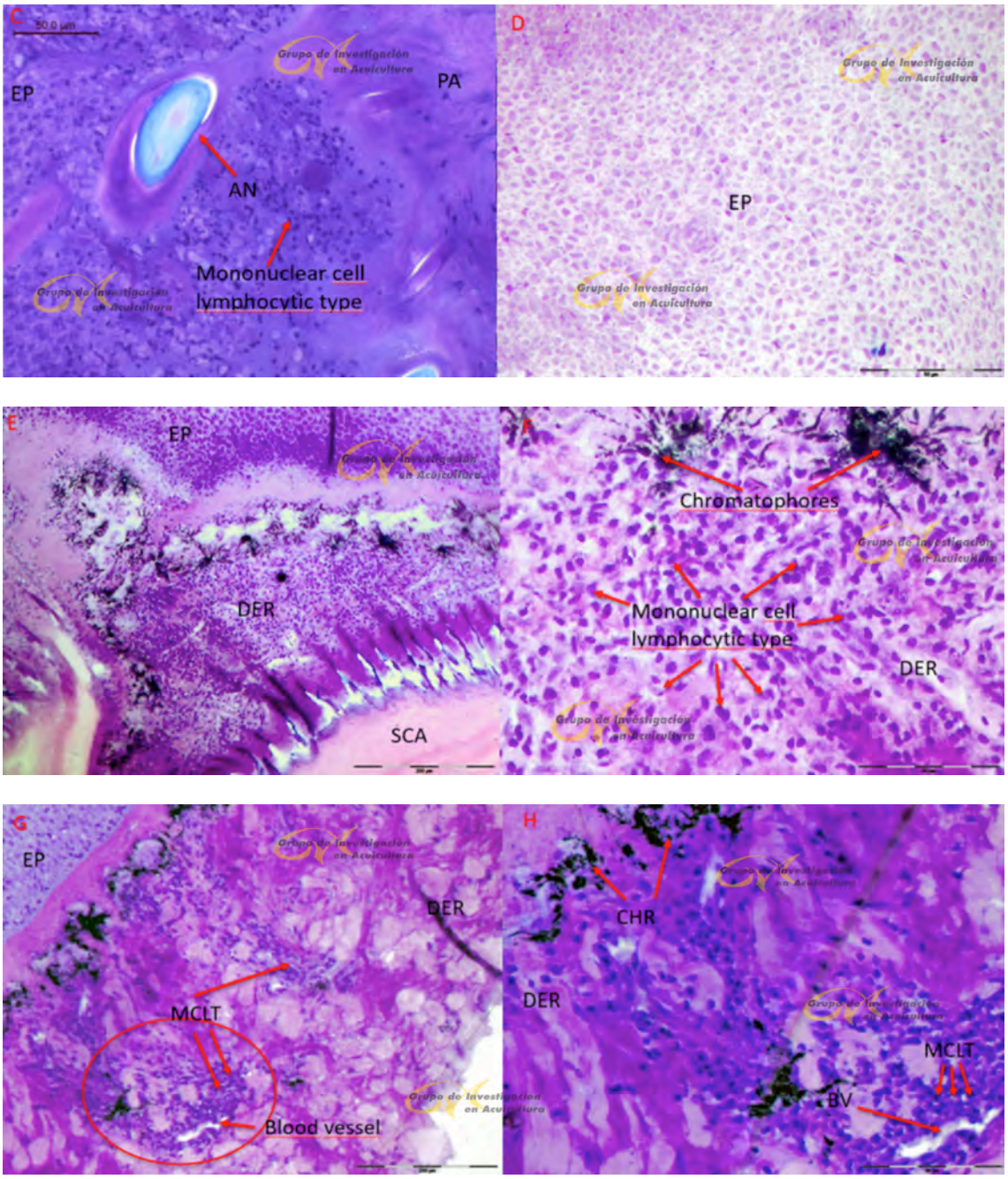

Figure 15. Mononuclear cell lymphocytic type infiltrations (MCLTI) around the anchors and hooks (C), compared with a non-parasitized animal (D). Massive MCLTI in the dermis layer (E,F), and focused extravasation related to blood vessels (G,H). MCLT: mononuclear cell lymphocytic type; CHR: Chromatophores; BV: Blood vessels.

3. Dietary utilization of mucus stimulating products in diets for greater amberjack juveniles.

Three hundred and twenty four fish of 331 ± 30 g (mean ± SD) were distributed in eighteen 1,000 l tanks (18 fish/tank) and fed to apparent satiety 3 times per day for 90 days. The experimental diets used were: greater amberjack base control diet (A), supplemented with MOS (D) and supplemented with C-MOS (E). Feed intake was monitored daily while growth performance and feed efficacy were recorded monthly.

At the end of the experimental period, samples of skin mucus and serum were collected. For this challenge, fish from the diet trial were infected by cohabitation with heavily parasitized greater amberjack infected with

N. girellae for 10 days, and prevalence and parasite level recorded. Growth parameters showed no significant differences among groups (P>0.05) after 90 days of the trial. However, there was a trend for diet E fish to have higher values (**Figure 16**).

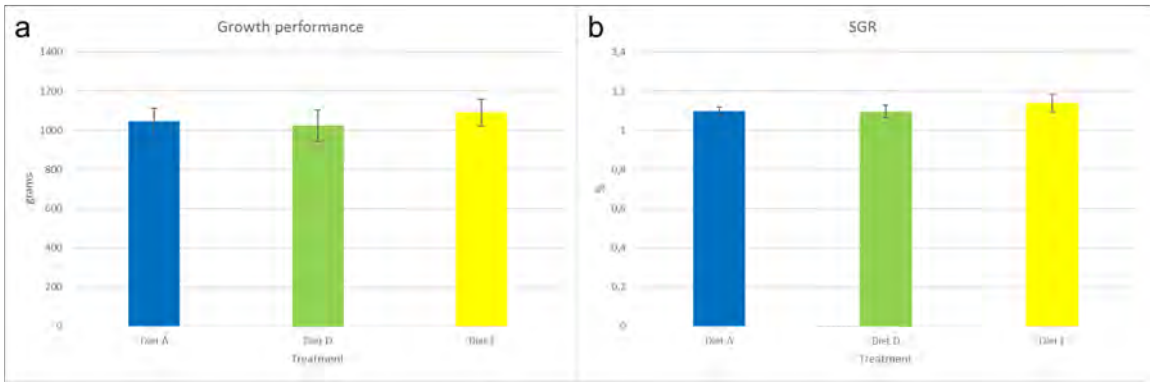

Figure 16. Growth performance (a) and Specific growth rate (SGR) (b) of fish fed with different prebiotics during a 90 days trial. Diet A: Control with no prebiotic; Diet D: MOS; Diet E: C-MOS.

The utilization of prebiotics (either MOS or c-MOS) induced a significant (P<0.05) increase of serum bactericidal activity whereas no effect (P>0.05) was found on lysozyme or peroxidase activities (**Figure 17**).

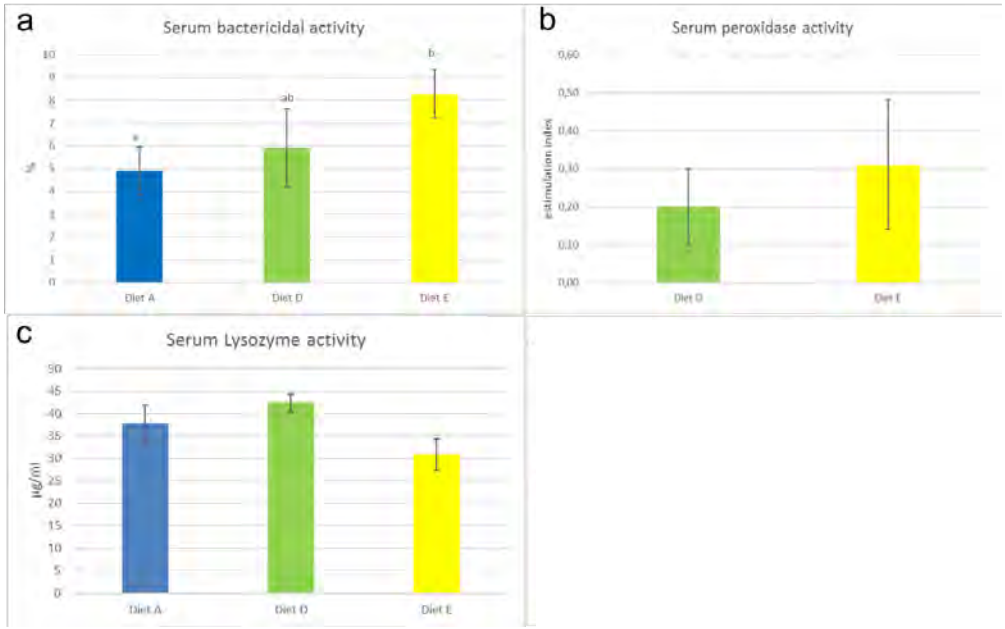

Figure 17. Serum bactericidal activity (a), serum peroxidase activity (b), and serum lysozyme activity (c) of fish fed each prebiotic treatment at the end of the feeding trial (90 days). Different letters denote significant differences among treatments (P<0.05). Diet A: Control with no prebiotic; Diet D: MOS; Diet E: C-MOS.

Regarding mucus immunity, a significant (P<0.05) effect of the addition of prebiotics was found on mucus bactericidal activity, increasing the immune potential of the skin mucus against bacteria (**Figure 18a**), whereas no effect was found on mucus lysozyme activity (**Figure 18b**).

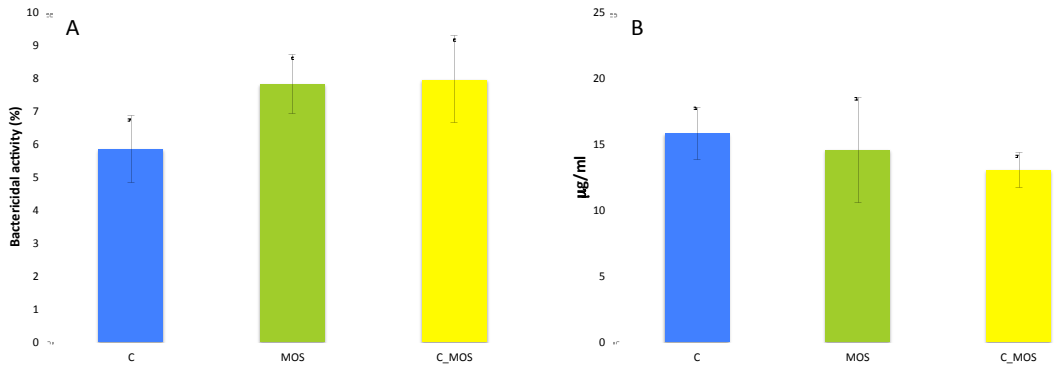

Figure 18. Mucus bactericidal activity (A), and mucus lysozyme activity (B) of fish fed each prebiotic treatment at the end of the feeding trial (90 days). Different letters denote significant differences among treatments (P<0.05). Diet A: Control with no prebiotic; Diet D: MOS; Diet E: C-MOS.

Ectoparasite challenge was conducted at the end of the trial. Prior to the challenge, standardization of the monogenean culture was done as follows: Eggs of *Neobenedenia* were collected from the adults using two methods: A) a 53-µm net was placed in tanks with parasitized *greater amberjack* juveniles, where the adult parasites produced eggs that became entangled in the nets and were easy to collect. B) Removal of adult parasites with tweezers was also conducted, placing them in petri dishes with filtered seawater and waiting for a few hours until the adults started producing eggs. Eggs obtained were incubated at 20ºC for 7 days in filtered seawater (**Figure 19**). With those controlled monogenea, an experimental infection was undertaken using fish cohabited with experimental fish, ensuring all the experimentally infected fish were exposed to the same parasites at the same stage of development.

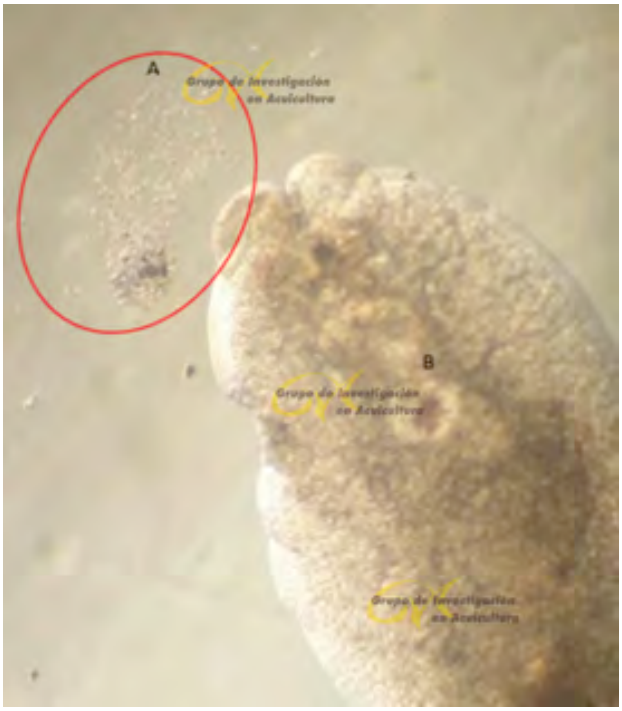

Figure 19. A: eggs produced by an adult parasite of *Neobenedenia girellae.* B: Adult parasite.

Ectoparasite challenge was conducted at the end of the trial and differences in the parasitization level among groups were found. Diet E (c-MOS) presented the lowest (P<0.05) parasitization level compared with the control and diet D (MOS) groups (**Figure 20**).

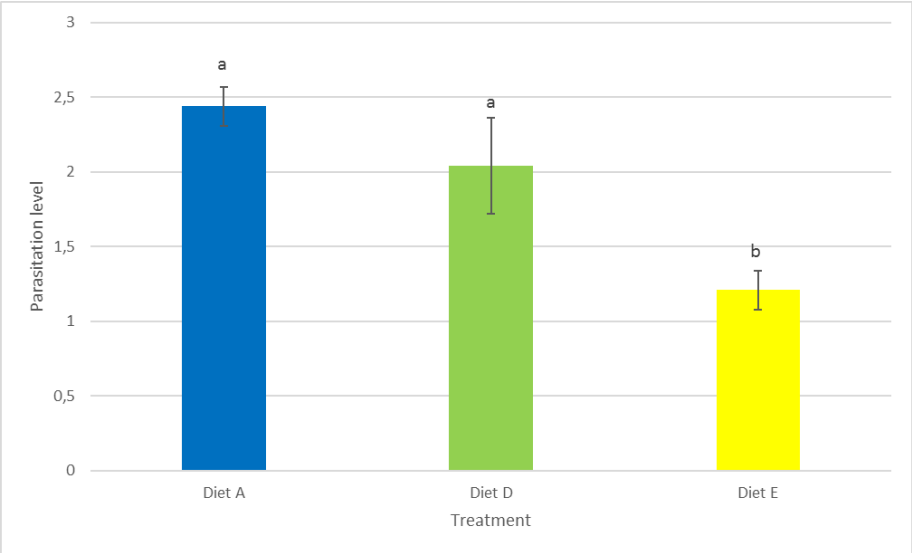

Figure 20. Parasite challenge conducted after 90 days of feeding prebiotic diets. Parasitation level; 1: 1-5 parasites; 2: 6-10; 3: >10. Different letters indicate significant differences among treatments ($P<0.05$). Diet A: Control with no prebiotic; Diet D: prebiotic 1; Diet E: prebiotic 2.

Conclusions

Dietary regime can alter mucus immunological properties. The addition of mucus stimulating products, and especially those based on concentrated mannan-oligosaccharides, enhance mucus immune potential and resistance to the ectoparasite *Neobenedenia girelliae*.

Co-funded by the Seventh Framework Programme of the European Union