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Field Observations on the Effect of a Mannan Oligosaccharide on Mortality and Intestinal Integrity of Sole (*Solea senegalensis*, Kaup) Infected by *Photobacterium damselae* subsp. *piscicida*

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Abstract

This study was conducted in order to investigate the effect of a mannan oligosaccharide (MOS) on the intestinal morphology of sole (*Solea senegalensis*, Kaup) reared under commercial conditions. The dietary inclusion rate for MOS was 0.4% and it was used either alone or in combination with a vaccination regime against bacterial diseases (*Pasteurella* spp. and *Vibrio* spp.). One week after the start of the experimental period, a natural outbreak of pasteurellosis, caused by *Photobacterium damselae* subsp.*piscicida*,occurred in all the groups of fish.A two-way ANOVA showed that only MOS supplementation reduced fish mortality by ca. 8% (P = 0.050). Additionally, light microscopy examination of the intestine revealed that MOS supplementation significantly increased the mucosal folding by 29% (P = 0.016) in the anterior intestinal region and by 33% (P = 0.002) in the posterior intestinal region. Scanning electron microscopy demonstrated that both MOS supplementation and vaccination significantly increased microvilli density on the enterocyte surfaces in the anterior intestinal regionby 13% (P = 0.028) and 30% (P = 0.001) respectively. In the posterior intestinal region neither MOS supplementation nor vaccination significantly affected the microvilli density (P = 0.005).The present study suggests that dietary MOS supplementation protects the intestinal morphology of infected sole and hinders the development of pathogenic infection, possibly by binding with *Photobacterium damselae* subsp. *piscicida*, resulting in reduced mortality of infected fish.

Keywords: Dietary mannan oligosaccharide; MOS; Flatfish; Intestinalhistology; *Photobacterium damselae* subsp. *piscicida*

Introduction

The commercial production of sole currently takes place mainly in Spain and Portugal with *Solea senegalensis* being the preferred species. Production levels of this species increased from 110 tons in 2008 to 500 tons per year in 2010 [1]. However disease control and feed related problems such as poor growth rates and feed conversion ratios (FCR's) are the main factors affecting production performance and these are critical factors to the commercial success of any aquaculture business.

The gastrointestinal (GI) tract has been recognized as one of the major routes of infection in fish [2,3].Understanding of the GI structure and its function and how it interacts with infectious diseases, anti-nutritional dietary components or stress in fish is a major factor in improving feed utilisation and protecting fish health in applied aquaculture practices.

There have been several approaches to disease prevention in aquaculture. The development and use of vaccines has expanded over the years both with regard to fish species and microbial diseases and vaccination is considered an effective tool in a farms strategy for disease control. However in the culture of new species the availability of a functional vaccine for the disease encountered may not be a realistic option and vaccines developed for other species specific pathogens may not be applicable until further development work is undertaken.

In addition to vaccination there has been a major initiative towards the appraisal of functional dietary supplements in commercial feeds for fish and crustacean species not only for their role in improving disease resistance but for improving nutrient utilisation. Many of these types of products are extracted from yeast [4,5]. Mannan oligosaccharide (MOS) has been shown to be an effective tool and works in several different ways within the digestive tract of poultry and swine [6-8] and more recently in aquaculture [9-12].

In order for an infection to occur pathogenic bacteria have to adhere to the enterocyte cell wall in the intestine [13] and this is mediated by their interaction with carbohydrates present on the cell surface [14]. Dietary MOS has been used to bind pathogens with a mannose –specific Type 1 fimbrae therefore preventing the bound bacteria from colonizing the intestine and allowing them to pass through the intestine and be excreted[15,16]. This has resulted in the use of MOS to reduce the incidence and severity of infections of salmonella [17] and *Pasteurella multocida* in broilers [18].

MOS has also been shown to bind to a number of bacterial strains, including Aeromonas spp., *Vibrio* spp. and *Photobacterium damselae* subsp. *piscicida* (#17911), known to cause disease in fish and shrimp [Dr Newman, Venture Laboratories, personal communication].Torrecillas et al. [11,19] demonstrated that dietary MOS supplementation reduced infectionby reducing pathogen translocation across the intestinal tract of *Vibrio* alginolyticus and *L. anguilarum* in European sea bass (*Dicentrarchus labrax*) when challenged via exposure by anal canalization or cohabitation.

In addition MOS has been shown to improve gut function and health by increasing the villi height, uniformity and integrity [9,10,20-24]. As a result,

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the feed within the digestive tract can be more efficiently digested, leading to superior nutrient absorption and improved growth [25-27]. Furthermore it has been stated that MOS can modulate the intestinal and systemic immune systems, by acting as a non-pathogenic microbial type antigen with an adjuvant like effect [28].

This study took place at a commercial farm where sole have been susceptible to infection with pasteurellosis in the past and this has led to considerable mortality and reduced performance.The aim of the study therefore was to investigate the prophylactic effect of MOS on the intestinal morphologyand fish health status. The impact of the use of an experimental vaccine for pasteurellosis, a common Gram negative bacterial disease which is caused by *Photobacterium damselae* subsp. *piscicida* (former Pasteurella *piscicida*), on the control and MOS treated fish was also investigated.

Materials and Methods

Animals and experimental design

The experiment was conducted in Spain under commercial conditions. Twelve round plastic tanks of 3m diameter and $2m^3$ water volume, were supplied with ambient sea water, filtered to approximately 10 μm, and renewed two times per hour.

Fish for this trial were acclimatised in the tanks (850 fish per tank of average weight 45.0g) and the fish in half the tanks vaccinated using an experimental vaccine manufactured by HIPRA (AMER, Spain), which targets Pasteurella spp. and *Vibrio* spp. The dose was injected (endoperitoneal) according to manufacturer instructions. The fish in all tanks were fed the control diet prior to the start of the trial 14 days later.

Fish were subjected to one of four different treatment regimes, in triplicate, for a period of 10 weeks. During the trial, mortality was recorded daily and fish weighed at the start and end of the trial and weekly in-between. Mean water temperature was 18^0C at the start of the trial and this gradually increased to 26 0C by the end of the trial. A natural photoperiod was used throughout the trial period (14 hr light: 10 dark hr at the start and 15:9 by the end of the trial).

Dietary treatments

All fish were fed using the same standard commercial diet (Skretting LE Europa 18%, 2mm) (Table1) with or without the addition of 0.4% MOS (Bio-Mos® Alltech Inc.).

The first treatment group consisted of feeding non-vaccinated fish the standard commercial control diet (CON). The second treatment group consisted of vaccinated fish fed with the control diet(CON-V). The third treatment groupconsisted of vaccinated fish fed the control diet supplemented with 0.4% MOS (MOS-V). The fourth treatment group consisted of unvaccinated fish which were fed the control diet supplemented with 0.4% MOS (MOS).

A week after the start of the trial, as the water temperature increased to 19 – 21°C, an outbreak of pasteurellosis occurred. This was caused by *Photobacterium damselae* subsp. *piscicida* and was confirmed and verified in a representative number of moribund fish from all treatment groups by the veterinary laboratory of Skretting Spain (TrouwEspaña, Cojobar, 09620 Burgos). Further inlet water samples also confirmed the presence of this opportunistic pathogen in the water supply to the farm.

The feeding rate was reduced from 1.5% of body weight at the start of the trial to 0.8% during the disease outbreak due to the loss of fish appetite during this period.

Diet proximate analysis	
Protein	57.0 %
Fat	18.0 %
Ash	11.5 %
Energy	19.9 MJ kg^{-1}
Vitamin A	5,000 U.I. Kg^{-1}
Vitamin D^{3t}	750 U.I. Kg^{-1}
Vitamin E*	250 mg Kg^{-1}

*Vitamin E was supplied as alpha-tocoferol acetate

Table 1: The basal (control) diet composition and vitamin profile.

Data collection

At the end of the trial period, intestinal samples (4 fish per experimental tank, 12 fish per treatment) were retained for histological examination by light and scanning electron microscopy (LM and SEM). Anterior and posterior intestinal sections were examined. Anterior intestinal samples were excised approximately 1 cm after the stomach and the posterior samples were excised approximately 1 cm before the anus. Material for LM was fixed in 4% saline formalin, embedded in paraffin wax and cut into 8 μm thickness sections. The sections were stained using Mallory's Trichrome staining technique as described by Handy et al. [29] and screened under low power magnification microscope (Olympus SZH-ILLD) with an attached digital camera (Nikon CoolPix 990).

Samples for the SEM were rinsed in 1% S-carboxymethyl-L-cysteine for 30 s in order to remove epithelial mucus and then fixed in 2.5% glutaraldehyde with 0.1 M cacodylate buffer and 1% NaCl, and adjusted to pH 7.2. The samples were treated using an Emitech K850 critical point drier, with ethanol as the intermediate fluid and CO_2 as the transition fluid. All samples were covered with gold in Emitech K550 sputter coater and were observed with a Jeol JSM 5600 LV electron microscope at 15 kV.

Analysis

Digital images were taken and analysed using appropriate software (ImageJ 1.34, National Institutes of Health, USA). Each tissue sample was analysed using 3 – 5 images. Images from the light microscopy were analysed to determine the perimeter ratio (PR) between the internal perimeter (IP) of the intestinal lumen and the external perimeter (EP) of the intestine (PR = IP/EP, arbitrary units). A high PR value indicates high villi length, increased mucosal folding or both. SEM images were used to calculate the density of the microvilli structures (MD) of the enterocytes on top of the villi by calculating the ratio between the microvilli (foreground, FG) and the background (BG) (MD = FG/BG). Two-way ANOVA (SPSS 15.0; SPSS Inc., USA) was used for the statistical analysis of the data.Significance was accepted at the $P<0.05$ level.

Results and Discussion

The initial stocking number, final number and cumulative mortality figures are shown in table 2. The cumulative mortality figures were high due to the natural disease outbreak of pasteurellosis, one week after the start of the trial. MOS treated fish without vaccination (MOS) had the lowest mortality at 47.78%, followed by 53.22% in the MOS+ vaccination (MOS-V) group, 55.952% in the control fed (CON) group and 61.58% in the control feed + vaccination (CON-V) group. Statistical analysis showed that there was a significant effect ($P = 0.050$) of MOS supplementation on the total mortality which tended to be lower (by ca. 8%) in MOS treated fish compared to the control. The effect of vaccination

Treatment	CON	CON-V	MOS-V	MOS
Initial stocking	2581	2488	2625	2482
Final stocking	1137	956	1228	1296
Total mortalities	55.95 ± 0.82%^{a}	61.58 ± 4.75%^{a}	53.22 ± 3.31%^{b}	47.78 ± 7.29%^{b}

Table 2: Stocking numbers and cumulative mortalities for each treatment group of fish (mean ± SD). Different superscript letters indicate a statistical difference ($P<0.05$, n=3).

and the interaction effect between MOS supplementation and vaccination had an insignificant effect on fish mortalities (P = 0.120 and P = 0.494 respectively).

The increased mortalities observed in the vaccinated fish both from the control (CON –V) and the MOS group (MOS-V) is possibly due to the stress of vaccination combined with exposure to a disease challenge from the natural environment within three weeks of vaccination before optimal immunity could be developed. Endoperitoneal vaccination has been reported to be a stressful procedure and should be avoided in cases of diseased fish [30]. This is especially true with juvenile flatfish vaccination, such as sole, as it is an exceptionally difficult husbandry procedure due to fish morphology. Previous studies have reported that MOS enhances the survival of shrimp and trout respectively under non-challenged conditions [27,31]. In the present study it was observed that MOS reduced the negative effect that vaccination had on the mortality.

In the anterior and the posterior intestinal region MOS supplementation displayed a significant increase of PR (P = 0.016 and P = 0.002, respectively; Table 3 and Figures 1 and 2) indicating longer mucosal foldings. The effect of vaccination and the interaction effect between MOS supplementation and

	PR*	MD*		
Treatment	Anterior	Posterior	Anterior	Posterior
CON	3.44 ± 1.01^{a}	3.16 ± 0.42^{a}	2.11 ± 0.60^{a}	3.21 ± 0.49^{a}
CON-V	4.16 ± 0.68^{a}	4.14 ± 0.62^{a}	3.03 ± 0.69^{b}	3.19 ± 0.73^{a}
MOS-V	4.80 ± 0.25^{a}	4.87 ± 0.30^{a}	3.66 ± 1.11^{a}	3.12 ± 0.56^{a}
MOS	5.00 ± 0.17^{b}	4.86 ± 0.42^{b}	2.71 ± 0.57^{b}	2.72 ± 0.50^{a}

*Arbitrary units, PR = IP/EP and MD = FG/BG.

Table 3: Intestinal lumen perimeter ratio (PR) and microvilli density (MD) (mean ± SD) of sole. Different superscript letters indicate statistical differences between values within the same column (P<0.05, n=12).

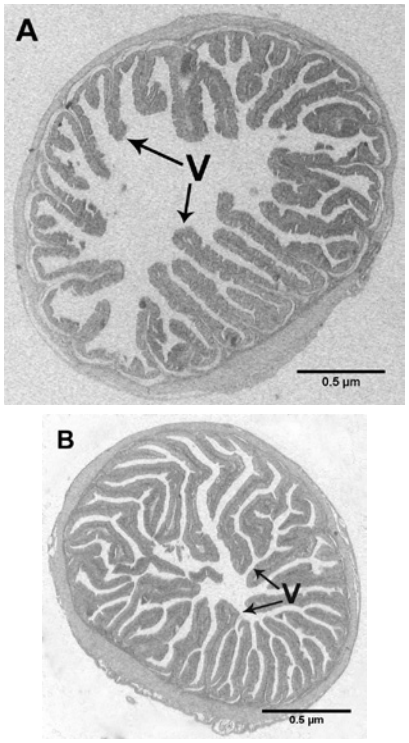

Figure 1: Comparative LM sections from the anterior intestinal region presenting the positive effect of MOS supplementation. Section A Fish fed the control diet (CON). Arrows indicate uneven intestinal folding. Section B Vaccinated fish fed the control diet with MOS supplementation (MOS-V). Arrows indicate improved intestinal folding.

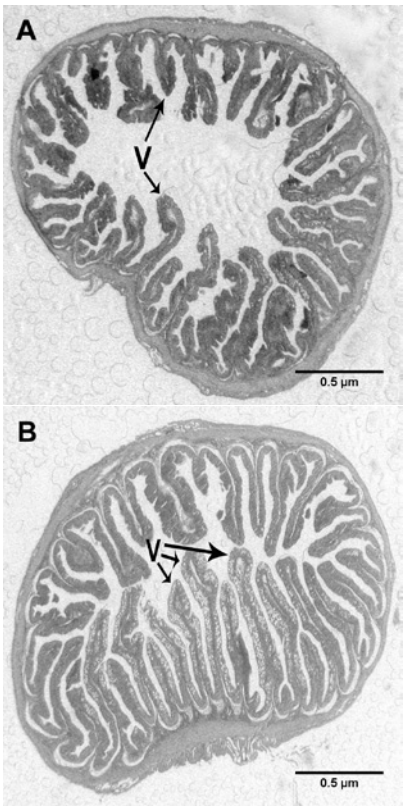

Figure 2: Comparative LM sections from the posterior intestinal region presenting the positive effect of MOS supplementation. Section A Vaccinated fish fed the control diet(CON-V). Arrows indicate uneven intestinal folding. Section B. Fish fed the control diet with MOS supplementation (MOS). Arrows indicate improved intestinal folding.

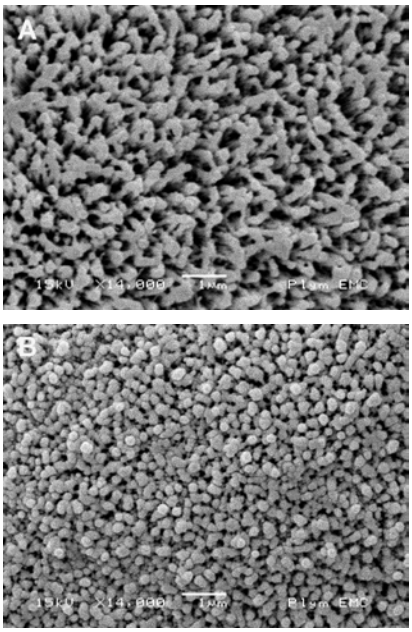

Figure 3: Comparative SEM micrographs from the anterior intestinal region presenting the positive effect of MOS supplementation. Micrograph A is from fish fed the control diet (CON) whereas micrograph B is from fish fed with the control diet with MOS supplementation (MOS).

vaccination on PR was insignificant for the anterior intestinal region (P = 0.493 and P = 0.242, respectively) and the posterior intestinal region (P = 0.095 and 0.101, respectively). In the anterior intestinal region PR increased from 3.44 ± 1.01 AU in the CON group to 4.16± 0.68 AU in the CON-V group, 4.80 ± 0.25 AU in the MOS-V group and 5.00 ± 0.17 AU in the MOS group. Similarly, in the posterior intestinal region the PR increased from 3.16± 0.42 AU in the CON group to 4.14 ± 0.62 AU in the CON-V group, 4.87± 0.30 AU in the MOS-V group and 4.86 ± 0.42 AU in the MOS group. A higher PR indicates a better intestinal condition profile and with longer mucosal folding morphology. More extensive and longer villi structures of the intestine would support the hypothesis of better nutrient uptake and utilization [32,33].

SEM was used to determine the density of the microvilli. The results, in Table 3 and Figure 3, showed that in the anterior region of the intestineof fish fed MOS supplementation alone and vaccination had a significant effect on the microvilli density (P = 0.028 and P = 0.001, respectively). There was an insignificant interaction effect between the MOS supplementation and the vaccination on the anterior intestinal region (P = 0.960). The microvilli density increased from 2.11 ± 0.60 AU in the CON group to 2.71 ± 0.57 AU in the MOS group, 3.03 ± 0.69 AU in the CON-V group and 3.66 ± 1.11 AU in the MOS-V group. In the posterior intestinal region neither MOS supplementation nor vaccination significantly (P = 0.188 and P = 0.371 respectively) affected the condition of the microvilli under these conditions.

Fish internal organs such as liver, kidney and spleen in sole (*Solea senegallensis*), sea bream (*Sparus aurata*) and turbot (*Scophthalmus maximus*) have been examined histologically to investigate the effect of pasteurellosis [34,35] however there is limited information on the effect of pasteurellosis on the GI tract.

Photobacterium damselae subsp. *piscicida* has been shown to infect yellowtail (*Seriola quinqueradiata*) and snake-head fish (*Channa maculata* Lacepede), through the oral route, via the GI tract [36-38]. There is evidence that *Photobacterium damselae* subsp. *piscicida* strongly binds to the intestinal mucosa [39]. Naka et al. [40] demonstrated that *Photobacterium damselae* subsp. *piscicida* has strong adherence to the fish intestine possibly due to the mannose sensitive hemaglutinin (*msh*A) on the bacterial cell. Infection with *Photobacterium damselae* subsp. *piscicida* has been shown to cause damage in the GI tract of European sea bass [41] and GI inflammation as evidenced by elevated expression of pro-inflammatory (TNF-α and IL-1β) genes [42].

The virulence of Gram negative pathogenic bacteria is linked to its serotype and this is related to the surface components of the bacteria such as lipopolysaccharides [43] and may reflect the ability of the bacterial surface antigens to interact with the host's tissues.The main molecule involved in antigen recognition and the binding process in antigen presenting cells is the mannose receptor [44]. Indeed, Torrecillas et al. [19] demonstrated that MOS can reduce pathogen translocation across the GI tract. The improved intestinal condition demonstrated in this study, supported by evidence from other work [9,10,12],indicates that MOS hinders the development of pathogenic infection. Therefore the reduced mortality observed in the MOS fed groups may have been induced by a reduction of infection (via the GI tract) rather than a reduction in mortality of infected fish.

Bacterial diseases often affect the morphology of the posterior intestine by altering its physiological condition, i.e. necrosis and sloughing of the intestinal mucosa [45,46]. However, during the healing process, the intestine will return to its normal condition and structure. In the posterior intestine, all treatments produced significantly higher PR compared to the control group, indicating an improved overall intestine condition. Qualitative analysis of the LM sections of the posterior intestine indicated that the MOS and MOS-V treated fish exhibited an elevated number of supranuclear vacuoles in the enterocytes in comparison to the CON and CON-V treated fish. The presence of such vacuoles has been related with the absorption of lipids, proteins and polypeptides in the intestine, leading to a superior nutrient utilization [47]. The observed histological changes suggest that MOS may work positively to affect the recovery process in the posterior intestine however further quantitative analysis would be required.

Overall, the results from this study indicate that MOS affects both the anterior and posterior intestinal regions of sole grown under commercial production conditions. It appears that the anterior part of the intestine is more responsive than the posterior region with respect to morphological features. MOS appears to confer a positive modulatory effect on stressed and diseased sole and possesses recovery properties against Gram negative bacterial infections encountered under culture conditions. The use of prebiotics, such as MOS, offer the commercial farmer nutritional strategies, in addition to vaccination, that can help recovery from disease and improve and promote better fish health. However the effect of MOS on disease pathogens needs to be further assessed under controlled challenge conditions to confirm the findings here at a commercial farm.

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