Accepted refereed manuscript of: Leclercq E, Pontefract N, Rawling M, Valdenegro V, Aasum E, Andujar LV, Migaud H, Castex M & Merrifield D (2020) Dietary supplementation with a specific mannan-rich yeast parietal fraction enhances the gut and skin mucosal barriers of Atlantic salmon (Salmo salar) and reduces its susceptibility to sea lice (Lepeophtheirus salmonis). *Aquaculture*, 529, Art. No.: 735701. https://doi.org/10.1016/j.aquaculture.2020.735701
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Dietary supplementation with a specific mannan-rich yeast parietal fraction enhances the gut and skin mucosal barriers of Atlantic salmon (*Salmo salar*) and reduces its susceptibility to sea lice (*Lepeophtheirus salmonis*)

Running Title: Enhancement of skin defence and sea lice protection by a dietary yeast compound.

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ABSTRACT

BACKGROUND: Increasing reliance on non-medicinal interventions to control sea lice in the Atlantic salmon (*Salmo salar*) farming industry imposes a high level of skin mucosal disturbance and indirect health issues. Dietary supplementation with yeast-based MOS products is widely used to support intestinal homeostasis across farmed species. Evidence of their effect on skin mucosa is increasing in aquatic species but it remains inconsistent and someway short of a clear contribution to sea lice management. A tank-based trial was performed to test the effect of a yeast-based MOS functional compound (sMOS) on the skin mucosal layer and its protective effects against sea lice (*Lepeophtheirus salmonis*).

RESULTS: The test compound significantly increased skin mucus (+46%) and goblet cell density (+25 %) after 6 weeks of dietary supplementation when positive effects on intestinal villi-length (+10.9 %) and goblet cell density (+80.0 %) were also documented. Following dietary supplementation, a 16.6 % reduction in susceptibility to an acute standard copepodid challenge was measured alongside an earlier increase in skin lysozyme activity widely used as an index of innate immunity.

CONCLUSION: The study provides functional evidence that the benefits of dietary sMOS reach beyond the intestine to the skin mucosa. Bolstering of the Atlantic salmon skin barrier and immune functions and the resulting lower susceptibility to sea lice has the potential to reduce the need for delousing interventions and the impact of non-medicinal interventions on the animal's health and welfare.

Keywords: Atlantic salmon, functional ingredient, mucosal health, sea lice, skin mucous, yeast cell wall

1. INTRODUCTION

Naturally occurring sea lice (*Lepeophtheirus* and *Caligus* species) remains a major biological bottleneck to the expansion of the Atlantic salmon (*Salmo salar*) farming industry with *Lepeophtheirus salmonis* being the most prevalent and damaging species in the Northern hemisphere (Johnson et al., 2004; Torrissen et al., 2013). Recently, the industry has undergone a dramatic shift away from antiparasitic drugs in favour of non-medicinal interventions including hydrogen peroxide, freshwater, mechanical and thermal treatments (Overton et al., 2019a), biological control using cleaner fish (Leclercq et al., 2014; Brooker et al., 2018) and preventive cage-based technologies coercing host-parasite mismatch (Frenzl et al., 2014; Oppedal et al., 2017, Stien et al., 2018). These are deployed in combination or in rotation and integrated within comprehensive sea lice management programs. Non-medicinal based sea lice management has proved successful at controlling sea lice while generating a 78 % reduction in chemical drug use between 2014 and 2017 in Norway (Helgesen et al., 2018). However, thermal and mechanical treatments have been associated with significant health, welfare and productivity penalties in the form of external injuries, gill damage, reduced growth and elevated mortalities (Helgesen and Jansen, 2018; Overton et al., 2019a, 2019b). Beyond any direct impacts, frequent repetitive handling is likely to chronically stress and compromise the animal's physiological and immune status towards a higher risk of secondary infections (Nardocci et al., 2014). In this context and notwithstanding the continuous advancement of these novel methodologies, there is a renewed interest to bolster resilience to infectious and non-infectious challenges in an effort to reduce both the frequency and impact of delousing interventions.

Functional feeds are defined as feeds with growth, health or other physiological benefits above and beyond the levels normally achieved when basal nutritional requirements are met (Jensen et al., 2015; Martin and Król, 2017). Among these, functional ingredients derived from the

yeast cell wall (YCW) of the baker's yeast (*Saccharomyces cerevisiae*) have been extensively trialled across the aquaculture sector, validating the distinct health benefits of yeast-derived β-glucans and mannan-oligosaccharides (MOS). Yeast-derived β-1,3/1,6-glucans are conserved microbial structures recognized as non-self by the host innate immune system primarily via Dectin-1 receptors present in macrophages in mammals (Brown et al., 2003). No clear homologues to mammalian Dectin-1 have been identified in fish so far, but β-glucans have been shown to regulate a signalling pathway associated with C-type lectin receptor (CLR) and candidates β-glucan receptors with conserved Dectin-1 features have been identified (Petit et al., 2019). Upon recognition, β-glucan triggers a pro-inflammatory response stimulating phagocytosis and a number of other immune cells (Herre et al., 2004; Brown, 2006). Their potent immune-stimulatory effect is well documented in fish (Dalmo and Bøgwald, 2008; Meena et al., 2013; Kiron et al., 2016) and command a pulsed-feeding against the risk of immune desensitisation (Bricknell and Dalmo, 2005). Yeast-derived MOS-products have distinct properties and applications with three primary functionalities. Firstly, MOS function as direct blocking agents of enteropathogenic bacteria within the gut lumen preventing intestinal adhesion (Firon et al., 1983). Secondly, MOS are low-molecular-weight carbohydrates non-digestible by vertebrates but preferentially fermented by intestinal lactic acid bacteria. As such, they act as prebiotic and have indeed been shown to positively modulate the intestinal microflora in various aquaculture species (Dimitroglou et al., 2009; 2010; 2011a; Akter et al., 2016). Thirdly, yeast-derived MOS are ligands to pattern recognition receptors (PRRs) such as the endocytic mannose-receptor (MR) primarily expressed on macrophages and dendritic cells (Ringø et al., 2010). Far from being fully elucidated, the function of MR in host defence has been shown essential for both pro- and anti-inflammatory cytokines production and appears to be involved in an array of mechanisms including phagocytosis, antigen processing and cell migration as well as, importantly, homeostatic processes (Gazi and

Martinez-Pomares, 2009). These authors noted that "mannose is not a danger signal" and that "MR ligation is largely associated to the reduction of pro-inflammatory cytokines and resolution of inflammation". The benefits of MOS on intestinal health and functions are overall well established (Torrecillas et al., 2014; Guerreiro et al., 2017) as recently confirmed in the European seabass (*Dicentrachus labrax;* Torrecillas et al., 2018) and using a rainbow trout (*Oncorhynchus mykiss*) intestinal epithelial cell line (RTgutGC) model (Wang et al., 2019).

Beyond the local intestinal effects of dietary MOS, several studies showed elevated systemic (humoral) immunity including in European seabass (Torrecillas et al., 2007; 2011), red drum (*Sciaenops ocellatus*; Zhou et al., 2010), rainbow trout (Staykov et al., 2007) and freshwater species (Welker et al., 2011; Akrami et al., 2012; Razeghi et al., 2012; Liu et al., 2013). Evidence is also emerging of an effect of certain MOS products on the skin and gill mucosa and of enhanced protection against associated pathogens. Dietary MOS were reported to decrease the susceptibility of greater amberjack (*Seriola dumerili*) to the skin fluke *Neobenedenia girellae* (Fernández-Montero et al., 2019), increased survival of juvenile red drum when challenged with the marine ectoparasite *Amyloodinium ocellatum* (Buentello et al., 2010) and channel catfish (*Ictalurus punctatus*) when challenged with *Flavobacterium columnare* with indications of mannose-associated signalling pathways recruitment, inflammatory resolution and enhanced epithelial repair documented in the gill (Zhao et al., 2015). In rainbow trout, MOS increased skin mucus excretion, circulating immunity and survival to *Aeromonas salmonicida* (Rodriguez-Estrada et al., 2013). In Atlantic salmon, MOS significantly reduced sea lice susceptibility under a heavy natural challenge (Dimitroglou et al., 2011b) but had no apparent effect under a moderate natural challenge using a distinct yeast-based MOS product at lower incorporation rate (Refstie et al., 2010) as was also reported under controlled laboratory conditions (Jensen et al., 2014). Dietary MOS was found to affect the skin mucus proteome of seawater Atlantic salmon with calreticulin-like protein described as a

multi-functional protein directly involved in mucin synthesis (Micallef et al., 2017) with possible participation in immunity and T-cell adaptive response in particular (Porcellini et al., 2006).

The response of Atlantic salmon to sea lice infection involves a combination of chronic stress, impaired healing, innate and adaptive immune components (Mustafa et al., 2000; Skugor et al., 2008). Interestingly, the expression of a MR (Macrophage mannose receptor 1, MRC1) and of several mucins were recently found highly up-regulated at sea lice attachment site suggesting increased mucus secretion and a possible route to enhancing protection (Robledo et al., 2018). Similarly, mechanical wound-healing in Atlantic salmon involves mucous cell recruitment at the border of the healing wound and secretion of an adherent mucous layer in concomitance with a characteristic early innate immune response (Sveen et al., 2019).

Accumulating evidence of an effect of MOS on skin mucosal surface and of enhanced protection against external pathogens support the concept of cross-communication towards a degree of cross-protection between mucosal barriers (Iijima and Kiyono, 2001; Salinas et al., 2011; Rombout et al., 2014). The prospect that the established effects of MOS on intestinal homeostasis and immunity may, in part, cross-over to the skin mucosa raises strong interest particularly towards enhanced sea lice protection and wound-healing in Atlantic salmon. However, published studies on the effect of MOS on Atlantic salmon skin mucosa remains surprisingly seldom and with contrasting findings therefore warranting further attention given the current challenges faced by the industry.

The aim of the study was to document the effect of a specific MOS product on the skin barrier function and susceptibility of Atlantic salmon to sea lice while documenting the relationship between intestinal, skin health and sea lice protection as a prerequisite to any further mechanistic studies.

2. MATERIAL AND METHODS

Animals were investigated and handled in accordance with the Animals (Scientific Procedures) Act 1986 (ASPA) revised to transpose European Directive 2010/63/EU as currently in force since 1 January 2013 in Scotland.

2.1 System and fish

The experiment was carried out at the Machrihanish Marine Environmental Research Laboratory (MERL; Institute of Aquaculture, University of Stirling, Scotland, UK) within a flow-through indoor tank system (600 L circular, self-cleaning central drain) supplied with pumped-ashore, pre-treated natural seawater under a simulated natural photoperiod (16:8 h light:darkness). Water flow was set at 2 L/min and individual tanks equipped with oxygen-sensor. Dissolved oxygen saturation was maintained above 80 %, water temperature and salinity were measured daily and averaged 14.1 ± 0.4 °C and 33.9 ± 0.3 ppt respectively over the trial's duration. Following on-site acclimation, locally sourced Atlantic salmon post-smolts (Buckieburn hatchery, Stirling, Scotland, UK) originating from a single size-graded population were randomly distributed into the experimental units (40 fish / tank; mean initial body-weight, BW_i = 252 ± 4 g; mean intra-tank and inter-tank coefficient of variation; CV_{intra} = 16.0 ± 1.8 %; CV_{inter} = 1. 69 % at trial's start).

2.2 Experimental design and sea lice challenge

The trial lasted 65 days testing two diets in quadruplicate: a basal diet (control diet) and the same basal diet supplemented pre-extrusion with a specific commercial MOS product incorporated at 4 kg/T feed pre-extrusion (sMOS diet; Lallemand SAS, Blagnac, France). This product is obtained from the primary fermentation of *S. cerevisiae* and typically contains 26 % Mannans, 24 % β-glucans (18 % β-1,3-glucans and 7 % β-1,6-glucans), 1 % chitin and 25 %

of proteins. The structure of this YCW product shows 26 % of interaction with an Atomic Force Microscopy (AFM) tip functionalised with Concanavalin A (a lectin binding to α-mannose units), mannan-chains of unfolded median length of 32 nm and a mean elasticity's modulus of 637 kPa.

The basal diet was formulated to the Atlantic salmon post-smolt requirements, the diets were prepared by BioMar (Ø 3 mm; Tech-Center, Brande, Denmark), randomly allocated to one of four experimental units and hand-fed to visual satiation 5 to 6 times daily over the trial's duration. Mortalities were removed daily and did not exceed 5 % / tank (2 fish / tank) over the trial's duration. A standard sea lice (SL) infection challenge was performed at day 46 using laboratory bred free-swimming *L. salmonis* copepodids. Within each tank, fish were crowded to half the initial rearing volume, exposed to an acute standard copepodid challenge (3,000 copepodids / tank) and maintained for 2 h under low water volume, low water exchange to favour parasite settlement.

2.3 Sampling schedule

At stocking (T_0; trial start), all fish were individually measured for BW (± 0.1 g) and fork-length (FL; ± 1 mm) under light sedation (MS-222, 30 ppm, ~ 1 min). Two days prior SL-challenge (T_1; T_0 + 44 days), 10 fish / tank were randomly netted and sedated for BW and FL measurements, of which 4 fish were returned to their original tank following intermediary recovery holding and 6 fish were sampled for skin mucus prior being sacrificed by cranial concussion for skin and intestinal tissue sampling. One week after SL-challenge (T_2; T_0 + 53 days); 15 fish / tank were randomly netted and sedated for measurement of BW, FL and SL assessment, of which 9 fish were returned to their original tank and 6 fish were sacrificed for skin mucus and tissue sampling. At the end of trial (T_3; T_0 + 65 days), all remaining fish (17 to 19 fish/tank) were individually measured for BW and FL, of which 15 fish / tank were

randomly selected for SL assessment and skin mucus sampling, and of those 6 fish / tank were randomly selected for skin and intestinal tissue sampling.

2.4 Sampling procedures

Sea lice assessment was performed blindly by the same two trained scientists at all time-points with fish carefully examined using a macroscope. For each fish examined (15 fish / tank / time-point T_2 and T_3), the number and life-stage of sea lice was determined and skin mucous was sampled after body-size (T_1) or sea lice (T_2 and T_3) assessment from the left-side flank preserved from any unnecessary handling disturbance. After removing any sea lice using a tweezer, a spatula was consistently wiped over a standard body-area, i.e. from the edge of the operculum to the anal pore, and the accumulating mucus transferred into a 1 ml pre-weighed syringe, weighted (± 0.001 g) and snap-frozen at -80 °C until further analysis. The collected, crude skin mucus weight was expressed relative to individual fish standard length (mg of mucus / cm of fish) for comparison of relative skin mucus level between experimental groups. Skin and distal intestine were sampled as follow. A skin sample of ~1 cm^2 was excised from the dorsal region between the head and dorsal fin. A transversal section of distal intestine (~1 cm length) was then excised, stripped of digesta and washed in PBS. Skin and intestinal sample were fixed in 10 % formalin, kept at 4 °C for 48 hours prior storage in 70 % ethanol at 4 °C until processing.

2.5 Analytical protocols

Skin mucous protein concentration was determined using a Protein Assay kit (Pierce™ BCA, ThermoFisher Scientific) in accordance with the manufacturer's recommendations. Lysozyme activity of the epidermal mucus was determined using a turbidimetric assay based upon the lysing activity of *Micrococcus Lysodeikticus* according to Ellis (1990). Formalin-fixed skin

and intestinal samples were processed following standard histological procedures. In brief, samples were dehydrated, embedded in paraffin wax for transversal sectioning at 5 µm thickness and stained with combined haematoxylin eosin, alcian blue and van Gieson to ensure visible contrast between mucin cells and the surrounding tissue. Images were captured on a Leica DMD 108 digital microscope at x40 magnification for measurement of the following parameters by image analysis (Image J 1.47v, National Institutes of Health, Bethesda, Maryland, USA). From the distal intestine sections (2 sections/fish), villus height was determined as the average height of four complete villus; lamina propria width was calculated as the average of three measurements per villi (bottom, middle and top of the villi) from four complete villus; and goblet cell abundance determined across a 200 µm length of five distinct villus starting from the apex (Fig. 1a). Goblet cell coverage in the intestinal tissue section was performed by computer-assisted image analysis (Image J 1.47v) for automated measurement of tissue surface area on a black and white image and of goblet cell coverage on the same fluorescent image (Fig. 1b, 1c). From the skin sections (2 sections/fish), goblet cell abundance was measured across a 400 µm section from the tip of a scale pocket (Fig. 2a). To determine goblet cell coverage (%), an in-house script was used (Image J 1.47v) for automated goblet cell separation onto a white background (Fig. 2b) and determination of the total area covered by goblet cells. The area of the dermis was then measured on the original image using the freehand-draw tool (Fig. 2c) to calculate goblet cell coverage as follows: Goblet cell coverage (%) = (total area of goblet cells ÷ dermal tissue area) x 100.

2.6 Calculations and statistics

Fulton's condition factor (K) was calculated as $K = (100\ BW) / FL^3$ with BW (g) and FL (cm); specific growth rate (SGR) as SGR (% / day) = $100\ (e^g - 1)$; where $g = (LnBWf - LnBWi)/t$; with BWf and BWi as the mean final and initial body-weight (g) respectively and t the trial's

duration (day); thermal growth coefficient (TGC) as TGC = 1000 (($BWf^{1/3}$ - $BWi^{1/3}$) /dd) where dd is the total degree-day over the trial's duration.

A 1-way analysis of variance (ANOVA) manipulated by a general linear model was applied to test the effect of diet on body-size parameters at trial start and end as well as growth indices over the trial's duration. A mixed linear ANOVA model was applied on skin mucus, histology parameters and lice count with diet and time as fixed factor and tank as random factor. Prior analyses, proportions were arcsin-transformed; datasets were checked for normality using the Kolmogorov–Smirnov test and for homogeneity of variance using Levene's test. Where differences occurred, post-hoc analyses were carried-out using Bonferroni-corrected t-test. These statistical analyses were applied using IBM® SPSS® Statistics v24. Linear regression between relative skin mucus level pre-challenge (T_1) and sea-lice count at T_2 (7-day post challenge) were conducted using SigmaPlot v11.0 to test the significance of the linearity and determine the adjusted R-squared (R^2) value of the regression model using relative skin mucus level as an independent variable and sea-lice count as a dependent variable. A significance level of 5 % ($p < 0.05$) was applied, data are presented as mean ± SEM of replicates tanks.

3. RESULTS

3.1 Performance

There was no statistical difference in body-size parameters between groups at the start of the trial (Table 1a). The test diets had no significant effect on body-sizes and growth but a trend for a positive effect of sMOS diet on SGR and TGC (+ 11.3 %) was observed and associated with a better maintenance of Fulton's condition factor at the end of the trial (Table 1b).

3.2 Distal intestine cyto-architecture

Distal intestine villi length (Fig. 3a) was significantly higher in the sMOS compared to the control group across time (+ 10.0 ± 4.6 %; $p = 0.028$), prior, as well as 3 weeks after the SL-challenge. Goblet cell density and coverage were also significantly higher in the sMOS compared to control group across time (Fig. 3b; + 65 ± 26 %; $p < 0.001$; + 31 ± 21 %, $p < 0.01$ respectively across time) and statistically decreased following the sea lice challenge (T_2 compared to T_1) in both treatments. Subsequently at T_3, goblet cell density returned to pre-challenge levels and their coverage remained stable in the control while both parameters further decreased in the sMOS group although remaining significantly higher than in the control at that time point (+ 35.1 % and + 25.2 % respectively; $p < 0.001$).

3.3 Skin mucus and histology

There were significant overall diet effect in the form of higher relative skin mucus level (Fig. 4a; + 22.8 ± 12.2 %; $p = 0.002$), goblet cell density (Fig. 4b; + 10.7 ± 7.1 %; $p < 0.001$) and goblet cell coverage (Fig. 4c; + 41.0 ± 25.4 %; $p = 0.029$) in the sMOS compared to control group across time-points. Skin mucus level was significantly higher in the sMOS compared to the control prior as well as 3 weeks after the SL-challenge (T_1: + 46.2 %; $p = 0.019$; T_3: +15.1 %; $p = 0.018$) and remained steady over time in both treatments. Similarly, goblet cell coverage was significantly higher in the sMOS group prior and 3 weeks after the challenge (T_1: + 81.1 %; $p < 0.001$; T_3: + 48.1 %; $p = 0.007$) with a transient increase and a transient decrease were observed at 7-days post challenge (T_2) in the control and sMOS group respectively. In comparison, goblet cell density was significantly higher in the sMOS group at pre-challenge only (+ 24.5 %; $p < 0.001$) and increased following the sea lice challenge in the control.

The skin mucus protein concentration was not affected by diet ($p = 0.266$) but significantly varied over time (Fig. 5a; $p < 0.001$) showing in both groups a transient increase 7-days post-challenge (T_2; + 26 % across groups) followed by a reduction towards pre-challenge levels at

T_3. Skin lysozyme activity (Fig. 5b) significantly varied over time ($p < 0.001$) being, in particular, 2.2-fold higher at T_3 compared to T_1 across experimental groups. Further, there was a significant overall diet effect ($p = 0.012$) being significantly higher in the sMOS compared to the control group at T_2 (+ 203 %; $p < 0.001$). At that time, lysozyme activity remained at pre-challenge level in the control but had increased to levels observed at T_3 in the sMOS group, albeit with a high variability across rearing suggesting the onset of lysozyme up-regulation.

3.4 Sea lice count

Sea lice development was homogenous within and between tanks at each time-point with all stages being chalimus at 7-day post-challenge (T_2) and pre-adult at 3-week post-challenge (T_3; data not shown). There was an overall significant effect of diet on sea lice count ($p = 0.002$) being significantly lower in the sMOS compared to the control group at T_2 (-16.6 %; $p = 0.004$) but not T_3 (-9.8 %; $p = 0.152$). Sea lice count significantly decreased between T_2 and T_3 in the control only (Fig. 6; Control: - 16.2 %, $p = 0.005$; sMOS: - 9.4 %, $p = 0.175$). There was a weak negative relationship between relative skin mucus level pre-challenge (T_1) and sea lice count at T_2 (correlation coefficient r = - 0.587; r^2 = 0,345; adjusted-r^2 = 0.214; $p = 0.166$).

4. DISCUSSION

Using a limited number of practical parameters, the study provided applied scientific evidence indicating that sMOS supplementation reinforced the skin mucosa prior and in response to sea lice resulting in enhanced protection against the larval chalimus stage. No negative impact of the diet on growth was observed but a positive impact on the intestinal cyto-architecture was confirmed. This supports emerging evidence that the protective effects of dietary yeast-based MOS reach beyond the intestinal to the skin mucosa and warrants further research on the mechanisms and factors involved.

315

316 *4.1 Intestinal cytoarchitecture and growth*

317 The effects of yeast-based MOS products on the intestinal cytoarchitecture, i.e. increased villi-
318 height and goblet cell density, were previously reported in various aquaculture species
319 including salmonids (Refstie et al., 2010; Dimitroglou et al., 2011b; Rawling et al., 2017) and
320 are widely associated with enhanced intestinal health and functions. In particular, a higher
321 goblet cell density and surface coverage suggest a higher level of mucus secretion which has
322 an essential role in lubricating food passage and providing physical protection to the underlying
323 intestinal wall against external damage from e.g. toxins and infectious agents (Pérez-Sánchez
324 et al., 2013). More than a simple static physical barrier, goblet cell-secreted mucus actively
325 sustain mucosal epithelial homeostasis by promoting the growth and maintenance of epithelial
326 cells and therefore act as an integral player in innate and adaptive immunity in particular
327 delivering foreign luminal antigens to lamina propia dendritic cells (Shan et al., 2013;
328 Pelaseyed et al., 2014; McCauley and Guasch, 2015). Being immuno-driven, increased
329 intestinal surface area using functional yeast fractions is expected to convey superior animal
330 performance in particular when exposed to challenging conditions. In this study, the apparent
331 improvement in growth (+11.3% in SGR) and maintenance in condition (K) measured with the
332 sMOS diet was particularly encouraging considering the short-duration of the pre-challenge
333 phase and the acute sea lice challenge applied. However, the growth achieved over the trial's
334 duration was insufficient (below 2-fold increase in body-weight) to appropriately assess a diet
335 effect on performance; and this was due to the repetitive interventions inherent to the
336 experimental aims.

337

338 *4.2 Skin mucosal protection: Pre-challenge*

Following 6 weeks of dietary supplementation and prior to sea lice challenge, sMOS was associated with higher levels of skin mucus secretion, goblet cell density and relative surface area with no alterations in the mucus protein concentration and lysozyme activity. At that time, the apparent proliferation of epidermal goblet cells by dietary sMOS was concomitant with observations in the intestinal mucosa. Such coinciding responses across distinct mucosal tissues corroborate the concepts of an integrated mucosal immune response whereby the different mucosal-associated lymphoid-tissue (MALT) are inter-linked and cross-communicate with stimulation of one MALT resulting in similar responses in other distant MALT (Iijima and Kiyono, 2001). This arena primarily refers to mucosal anti-body response in the context of oral or mucosal immunization against targeted pathogens with evidence of cross-mucosal response in various studies; albeit with a clear compartmentalization within and between MALTs (Salinas et al., 2011). Recently, different studies in aquaculture species have reported enhanced anti-microbial defense of the skin using in-feed functional ingredients (e.g. Cerezuela et al., 2016; Micallef et al., 2017; Saeidi et al., 2017). However, this is the first report of a diet-induced proliferation of goblet cells co-occurring in the local gut and distal skin epithelium. This reinforces the notion of inter-connectivity between intestinal and external mucosa and strengthens current evidence of a contribution of yeast-based functional ingredients beyond their intestinal effect.

Thicker skin mucus coverage is expected to provide a stronger physical barrier against sea lice settlement. Indeed, infective *L. salmonis* copepodids initially settles to the host using hooked second antennae driven into the epidermis, followed by attachment to the epithelial basement via a new frontal filament produced at each chalimus molt (Bron et al., 1991; Gonzáles-Alanis et al., 2001). Accordingly in this study, a higher relative skin mucus level and goblet cell coverage at time of copepodid challenge (T_1) was observed alongside a significantly lower chalimus count 1-week after challenge (T_2) in the supplemented group. However, the negative

relationships between skin mucus level and chalimus count were not statistically significant suggesting the contribution of other protective factors in the skin mucus. With sessile chalimus predominantly feeding on skin mucus (Heggland et al., 2020), differences in susceptibility may also pertain to the presence, in the skin mucus, of immune relevant molecules (Brinchmann, 2016) or of other factors such as of agents blocking the secretion of protease from *L. salmonis* (Fast et al., 2003). Further studies should address the dietary modulation of skin mucus composition by the MOS product tested in this study in both naïve and infected Atlantic salmon.

4.3 Skin mucosal response to sea lice and diet effect

The host mucosal response to sea lice, as observed in the control group, did not involve an apparent alteration in the level of skin mucus excretion but was characterized, within 7 days of copepodid exposure, by a rapid proliferation of skin goblet cells accompanied by a transient increase in goblet cell coverage and mucus protein concentration together indicating a reinforcement of the skin physical barrier. This apparent primary response partly dissipated at a later stage and upon the recruitment of antimicrobial-defence, i.e. increased lysozyme activity, which could constitute a more steady state response to an established, mobile stages infection as observed at T_3 in this study.

In comparison in the sMOS group, estimated skin mucus level and goblet cell coverage initially decreased to the values measured in the control group following copepodid exposure. This temporary loss of beneficial dietary effect may have been linked to handling and short-term starvations associated with the challenge protocol or to the immune-modulation of the host by the parasites secretory/excretory system. Indeed, *L. salmonis* secrete different immunomodulatory compounds to evade the host immune response (Firth et al., 2000; Fast et al., 2007; Fast 2014; Hamilton et al., 2018) and these may have more active and discernible effects within an immunologically active mucosa as was the case in the sMOS group pre-

challenge. In any case, these suppressions were only transient and not below the basal levels observed in the control group. Interestingly, goblet cell density remained consistently high with no further proliferation upon sea lice exposure while an earlier increase in lysozyme activity was observed compared to the control together indicating the preparation and reinforcement of the skin mucosal response to sea lice by the sMOS product tested.

4.4 Continuous lice protection

A significant 16.6 % reduction in copepodid settlement, as measured at the chalimus stage, was achieved by the test compounds under the controlled conditions of the study. Surprisingly few studies have tested the effect of yeast-based functional ingredients against sea lice in general and *L. salmonis* in particular. Previous studies using yeast-based MOS products showed contrasting results varying from significant reductions to no apparent effects (Refstie et al., 2010; Dimitroglou et al., 2011b; Covello et al., 2012; Jensen et al., 2014) albeit under a variety of trials' set-up. The phytochemical glucosinolate reduced *L. salmonis* by 17 % to 25 % (Jodaa Holm et al., 2016), a commercial product containing plant-derived compounds reduced *Caligus rogercresseyi* count by ~22 % (Nùñes-Acuña et al., 2015) and an oil-top coated commercial mixture of natural identical compounds reduced *L. salmonis* infection by up to 20 % (Jensen et al., 2014). Non-specific immune-modulators that potentiate the host innate immunity system and allow continuous preventive applications such as MOS (this study, Torrecillas et al., 2014) evidently have a distinct role and expected level of efficacy compared to short-term intervention therapies against sea lice. Besides their potential benefits against other infectious agents, the efficacy of preventive solutions over a single sea lice infection challenge does not express their actual benefit over their intended continuous application. Salmon-lice propagation is essentially host-density dependant such that the infection pressure within a farm is essentially internal and to a lower extent from neighbouring farms (Jansen, et al., 2012;

414 Aldrin et al., 2018). Accordingly, commercial sea-sites typically experience limited events of
415 salmon lice recruitment from wild hosts but often suffer from successive infection waves and
416 on-site amplification of their internal or local lice population. In that context, the impact of
417 continuous mitigation measures on the standing parasite population will also amplify over its
418 successive generations. This could be expressed as a cumulative efficacy coefficient $C_n = 1 - (1-c)^n$; where c is the efficacy of the control method against parasite-host colonization and *n*
419
420 the number of generation or infection wave for which the method is applied. In the present
421 study, sMOS had a 16.6 % efficacy against sea lice settlement translating, at the 3rd and 5th
422 internal wave of infection into a reduction of the standing lice population of $C_3 = 42$ % and $C_5 =$
423 60 % respectively. This corresponds to the approximate number of successive generations of
424 salmon-lice over the 6 to 9 month warmer-water period in Southern Norway and Scotland
425 based on a generation time of 4 weeks at 18 °C to 8-9 weeks at 6 °C (Hayward et al., 2011)
426 and 7 week at 12 °C (Tully; 1989). Such cumulative efficacy would remain valid regardless of
427 the frequency or efficacy of any successive intervention therapies over the period and applies
428 to each salmon-lice cohort from their initial recruitment from wild-stock. It illustrates that the
429 residual salmon lice population will be increasingly lower as the grow-out cycle progress under
430 a scenario of self-reinfection and co-infection with neighbouring sea-sites, ultimately reducing
431 the frequency of interventions where such continuous strategies are applied.
432 Beyond sea lice susceptibility, increased mucosal robustness in the form of a thicker skin
433 mucus layer and of bolstered mucosal immunity, as documented here with dietary sMOS, is
434 expected beneficial against the risk of mucosal damages and of secondary infections associated
435 with direct and stress-related impact of non-medicinal interventions and handling. The practical
436 health and welfare contribution of such prophylactic functional ingredients could be quantified
437 by long-term studies under commercial conditions.
438

5. CONCLUSION

In conclusion, dietary sMOS induced goblet cell proliferation in the distal intestine and skin mucosa, promoted skin mucus excretion and an earlier up-regulation of its lysozyme activity which were associated with a lower susceptibility to the larval chalimus stage of the sea lice *L. salmonis*. Such practical evidence of a dietary enhancement of the skin mucosal defence by sMOS supports its contribution against sea lice propagation and suggests its broader contribution as prophylactic functional ingredients in support of mucosal integrity, animal health and welfare under repetitive handling conditions.

6. ACKNOWLEDGEMENTS

The authors would like to thank William Roy, Chessor Matthew and Sally Boyd from the Institute of Aquaculture (Stirling University; Machrihanish Marine Environmental Research Laboratory) for excellent field and administrative support.

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TABLES

Table 1: Body-size parameters and growth performance (Mean ± SEM, n = 4)

		Control	sMOS
a. Body and population size parameters			
Initial (T_0; day 0)			
Body-weight	(g)	255 ± 2	249 ± 1
Fork-length	(cm)	28.5 ± 0.1	28.5 ± 0.1
Fulton's K		1.09 ± 0.02	1.07 ± 0.01
Population	(n/tank)	40	40
Pre-challenge (T_1; day 44)			
Body-weight	(g)	38.9 ± 2	38.5 ± 2
Fork-length	(cm)	32.7 ± 0.4	32.6 ± 0.4
Fulton's K		1.10 ± 0.03	1.10 ± 0.03
Sampled population	(n/tank)	10	10
End-point (T_4; day 65)			
Body-weight	(g)	399 ± 19	408 ± 12
Fork-length	(cm)	33.7 ± 0.2	33.6 ± 0.3
Fulton's K		1.04 ± 0.04	1.07 ± 0.01
Population	(n/tank)	17 ± 1	18 ± 0
b. Growth performance			
Pre-challenge period (T_0 to T_1)			
SGR	(%/day)	0.98 ± 0.11	1.00 ± 0.07
TGC		1.54 ± 0.19	1.57 ± 0.11
Challenge period (T_1 to T_4)			
SGR	(%/day)	0.11 ± 0.39	0.29 ± 0.09
TGC		0.18 ± 0.62	0.46 ± 0.15
Whole trial (T_0 to T_4)			
SGR	(%/day)	0.68 ± 0.07	0.76 ± 0.04
TGC		1.09 ± 0.05	1.21 ± 0.05

K: Condition factor, SGR: Specific growth rate; TGC: Thermal growth coefficient; T_0, T_1 and T_4: Sampling points 0 (trial start), 1 (day 44, 2 days prior sea lice challenge) and 4 (trial end) respectively.

FIGURES LEGENDS

Figure 1: Transversal cut of Atlantic salmon distal intestine illustrating **a)** the measurements performed for cyto-architecture assessment: Lamina propria (LP) width, mucosal fold height, and goblet cell density (n / 200 µm from villi apex) and image transformation to determine **b)** tissue surface area (white) and **c)** goblet cell surface area (fluorescent) for calculation of goblet cell coverage (%) in the intestinal tissue section. Scale bar represents 100 µm. sm: sub-mucosa; mp: muscularis propia.

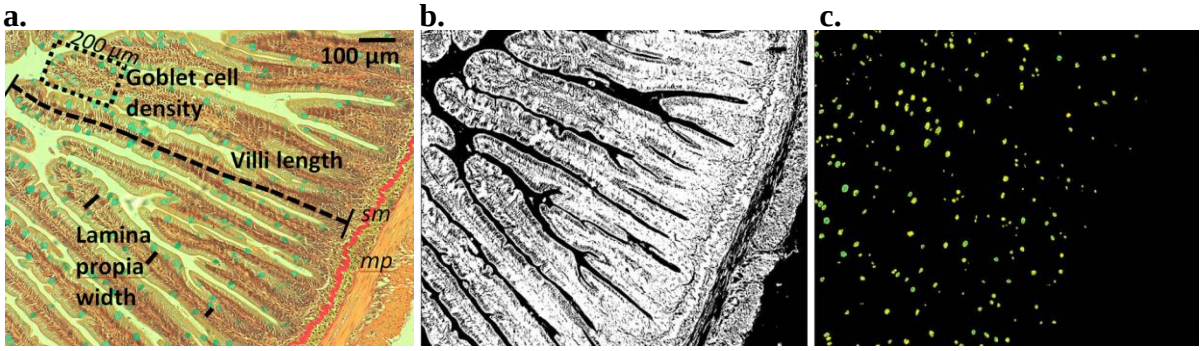
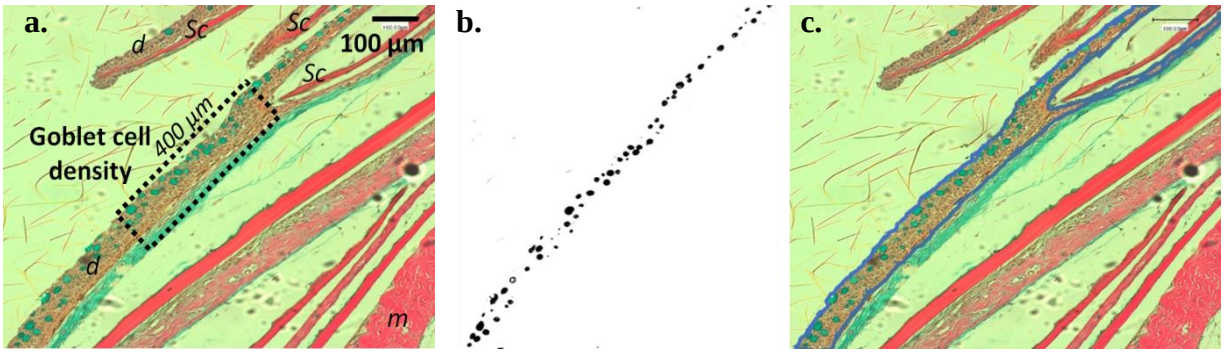


Figure 2: Transversal cut of Atlantic salmon skin illustrating **a)** goblet cell density measurement (n / 400 µm) and image transformation to determine **b)** goblet cell surface area (black surface area) and **c)** dermis surface area (purple outline) for calculation of goblet cell coverage (%) in the skin section. Scale bars represent 100µm. d: dermis, Sc: scale; m: muscle.



738 **Figure 3**: Distal intestine **a)** fold-length and **b)** goblet cells density at T_1 (day 44); T_2 (day 53)
739 and T_3 (day 65) with sea lice challenge applied at day 46. Dot-plot of individual data (grey bar);
740 box-plot of individual data and mean ± SEM of replicate tanks mean (n =4; black-dot).
741 Different letter indicate significant differences between groups and time-points.

742

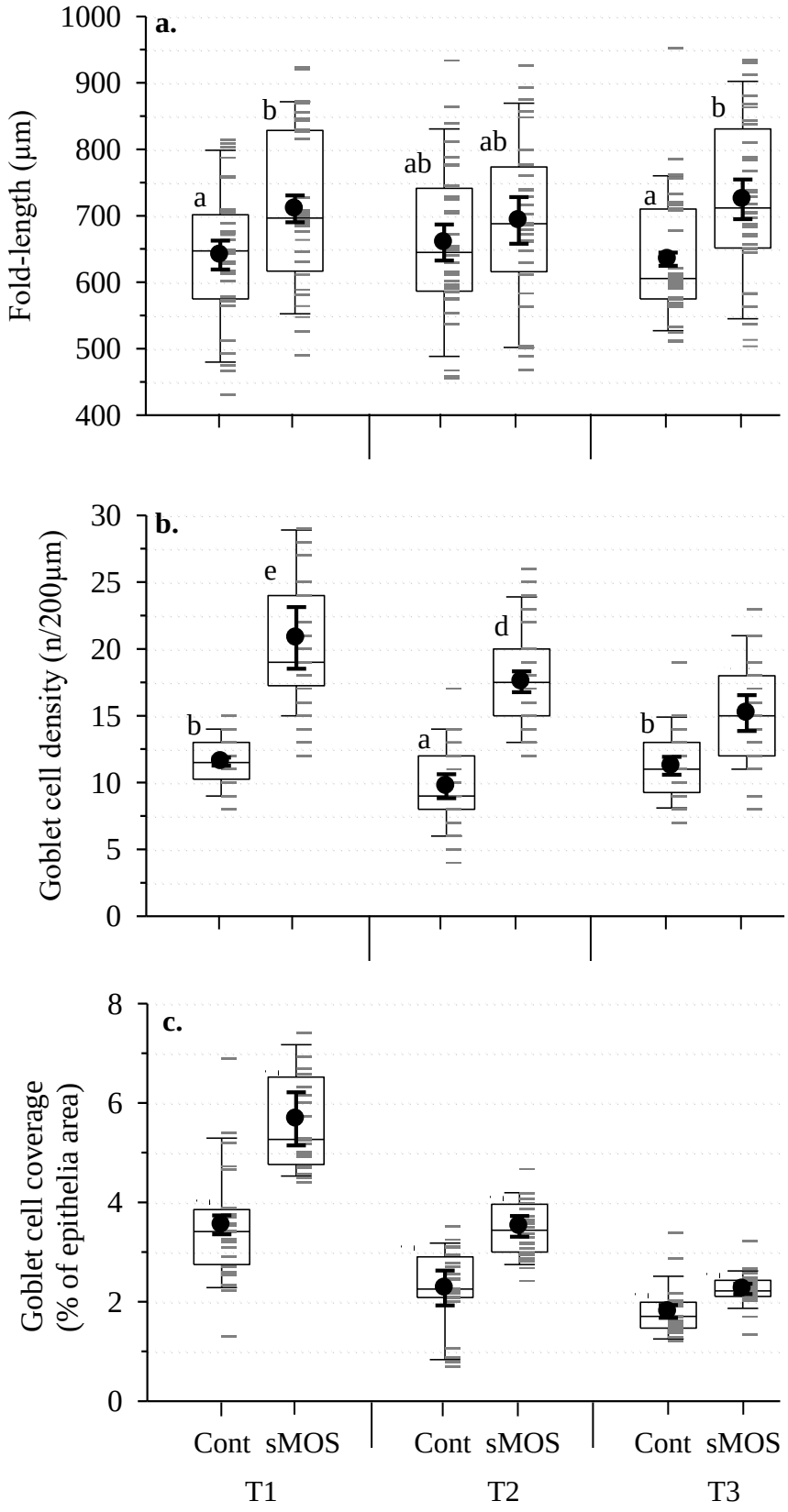


Figure 4: a) Relative skin mucus level; **b)** goblet cell density and **c)** goblet cell coverage in the epidermis at T_1 (day 44); T_2 (day 53) and T_3 (day 65) with sea lice challenge applied at day 46. Dot-plot of individual data (grey bar); box-plot of individual data and mean ± SEM of replicate tanks mean (n =4; black-dot). Different letter indicate significant differences between groups and time-points.

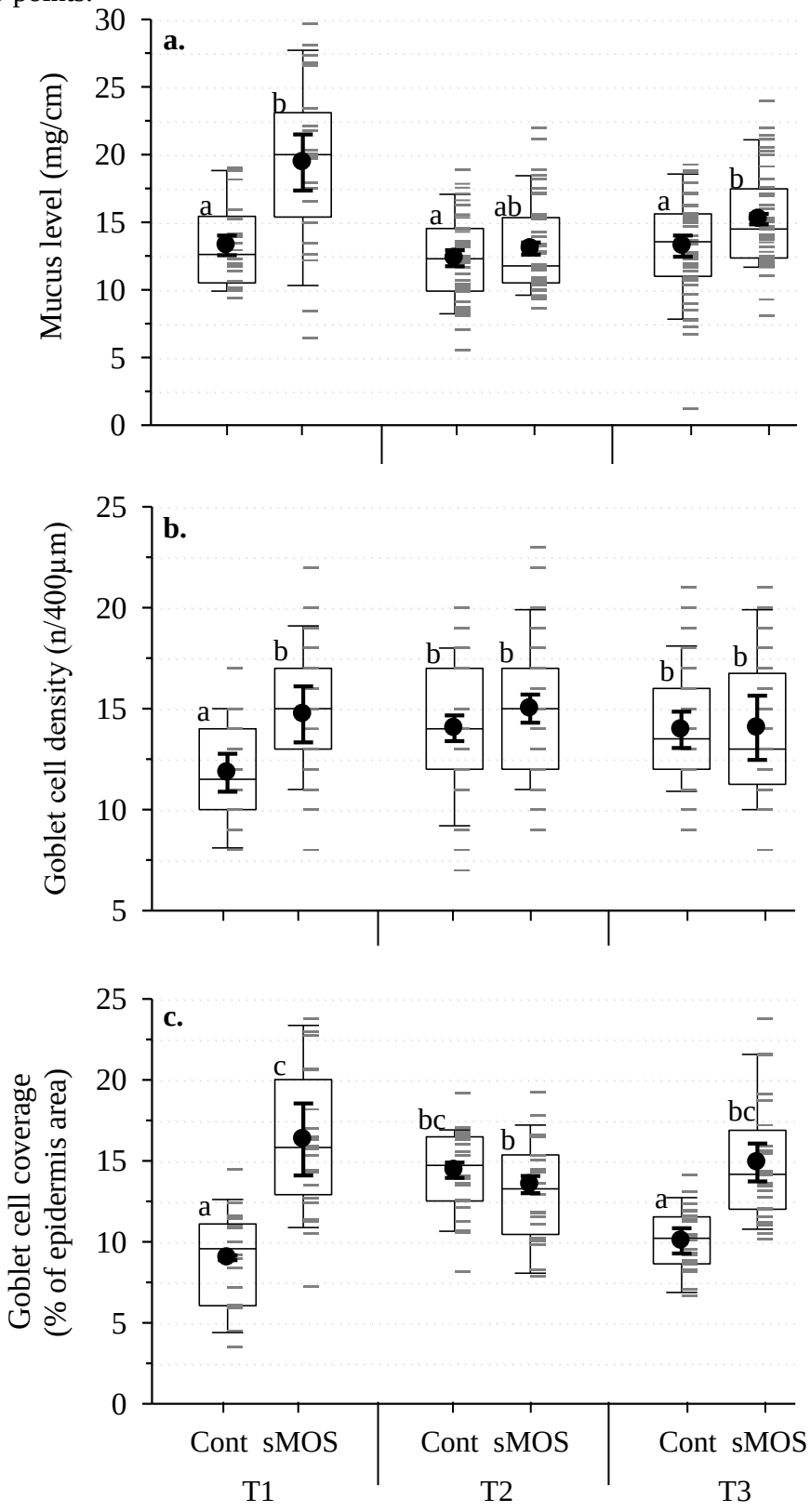


Figure 5: Skin mucus **a)** protein concentration and **b)** lysozyme activity at T_1 (day 44); T_2 (day 53) and T_3 (day 65) with sea lice challenge applied at day 46. Dot-plot of individual data (grey bar); box-plot of individual data and mean ± SEM of replicate tanks mean (n =4; black-dot). Different letter indicate significant differences between groups and time-points.

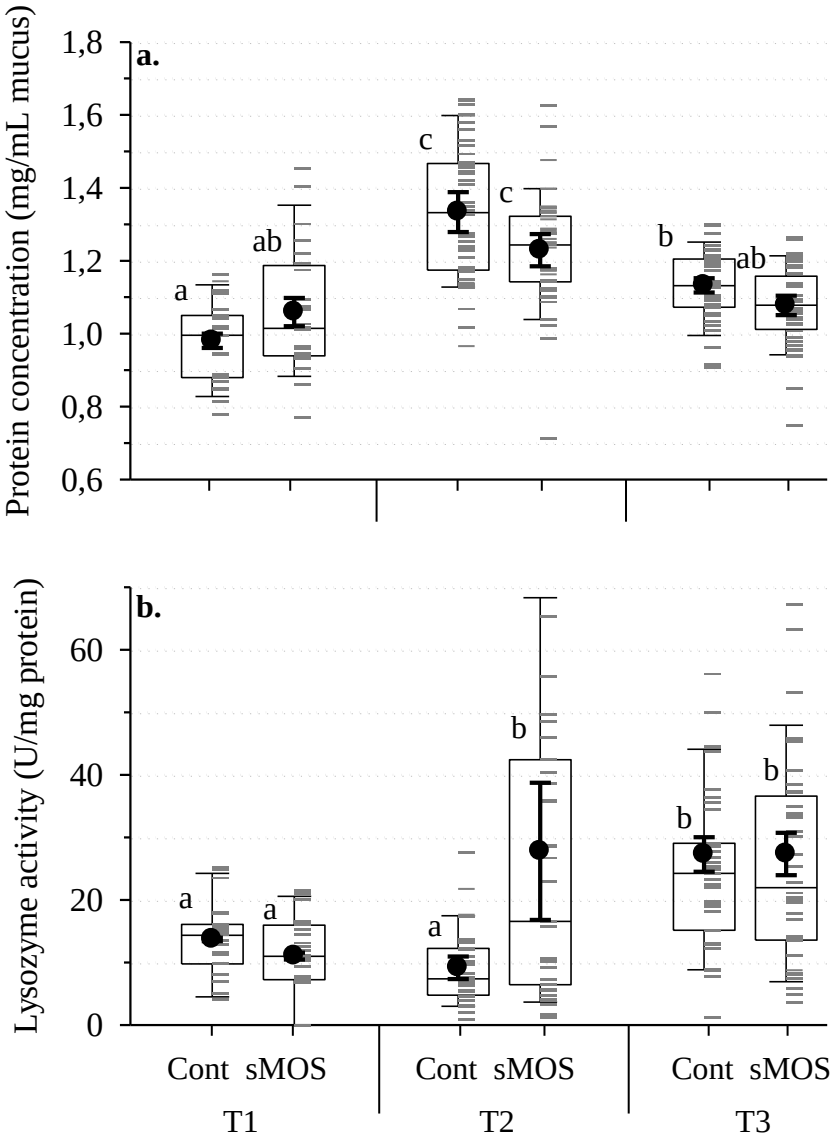
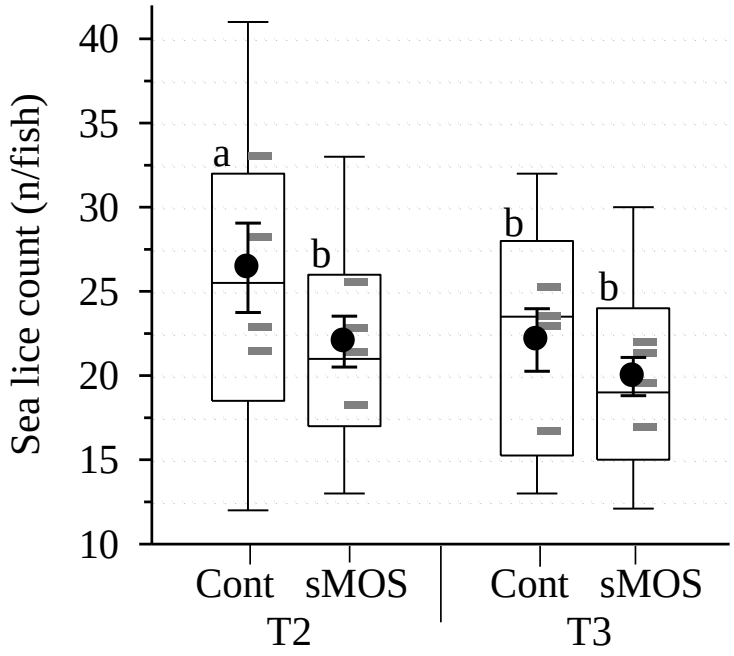


Figure 6: Sea lice count showing box-plot of individual fish count, dot-plot (grey bar) of mean sea lice count per tank and mean ± SEM of replicate tank per treatment and time-point (n =4 with 15 fish/tank/time-point assessed). Different letter indicate significant differences between groups and time-points.



760 **Figure 7:** Relationship between mean relative skin mucus level pre-challenge (T_1) and mean
761 sea-lice count 7-day post challenge (T_2) within individual tanks. The linear regression model
762 had an adjusted-r^2 value of 0.214 and was not significant (p = 0.166).

763