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Dietary β-glucan (MacroGard®) enhances survival of first feeding turbot (*Scophthalmus maximus*) larvae by altering immunity, metabolism and microbiota

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Abstract:

Reflecting the natural biology of mass spawning fish aquaculture production of fish larvae is often hampered by high and unpredictable mortality rates. The present study aimed to enhance larval performance and immunity via the oral administration of an immunomodulator, β-glucan (MacroGard®) in turbot (*Scophthalmus maximus*). Rotifers (*Brachionus plicatilis*) were incubated with or without yeast β-1,3/1,6-glucan in form of MacroGard® at a concentration of 0.5 g/L. Rotifers were fed to first feeding turbot larvae once a day. From day 13 dph onwards all tanks were additionally fed untreated *Artemia* sp. nauplii (1 nauplius ml/L). Daily mortality was monitored and larvae were sampled at 11 and 24 dph for expression of 30 genes, microbiota analysis, trypsin activity and size measurements. Along with the feeding of β-glucan daily mortality was significantly reduced by ca. 15% and an alteration of the larval microbiota was observed. At 11 dph gene expression of trypsin and chymotrypsin was elevated in the MacroGard® fed fish, which resulted in heightened tryptic enzyme activity. No effect on genes encoding antioxidative proteins was observed, whilst the immune response was clearly modulated by β-glucan. At 11 dph complement component *c3* was elevated whilst cytokines, antimicrobial peptides, toll like receptor 3 and heat shock protein 70 were not affected. At the later time point (24 dph) an anti-inflammatory effect in form of a down-regulation of *hsp 70*, *tnf-α* and *il-1β* was observed. We conclude that the administration of MacroGard® induced an immunomodulatory response and could be used as an effective measure to increase survival in rearing of turbot.

Keywords: immunostimulation, beta-glucan, fish larvae, immunity, turbot, complement component C3, trypsin, survival

1. Introduction

Turbot (*Scophthalmus maximus, Psetta maxima*) aquaculture is a steadily growing industry with a production of approximately 77,000 t in 2013 [1]. However, intensive production of marine fish larvae is still hampered due to high and unpredictable survival rates [2]. These mortalities are often pathogen-associated [3] since the immune system of the larvae is not yet fully developed [4-7]. During these immune compromised early stages, which especially in marine larvae can comprise the first 2 – 3 months post hatch, the larvae rely solely on the innate immune system, whilst the adaptive arm is not fully established [7]. This limits the number of potential treatments as well as prophylactic methods against pathogens as vaccinations are not applicable until the acquired immune system is matured and antibiotics are problematic due to environmental aspects. However, the use of immunomodulators in larval culture could present a potential method to increase immunity and survival as they enhance the non-specific, innate immune system [8, 9].

The application of immunomodulators has been widely studied in juvenile and adult fish (see [10] for review). A limited number of studies, however, have focused on early life stages [11-16]. Various routes of administration of immunomodulators to fish have been proposed, for example via feed, bath and injection. Due to the small size of fish larvae the latter method is not applicable, however, both oral and bath administration seem to be principally feasible. The disadvantage of bath treatments nevertheless lies in the large amount of substance needed due to high water volumes and water exchange rates. We therefore focused on dietary application in this study. During the early life stages most marine aquaculture fish species rely on life feed: Encapsulation of immunomodulators into rotifers has been suggested by Robles and colleagues [17] to be an effective method of administering medication to fish larvae.

Currently multiple substances are on the market that promise to have positive effects on fish health and survival. Among those, the carbohydrate β-glucan, especially β-1,3/1,6-glucan derived from yeast (*Saccharomyces cerevisiae*), is one of the most used immunomodulators.

The structure and immunostimulatory activity of β-glucan depends on its source, solubility, molecular mass, tertiary structure and the degree of branching (for review see [18]). In this study we focus on the commercially available β-glucan containing feed additive MacroGard® (Biorigin, Brazil). This product is an insoluble preparation of β-1,3/1,6-glucans from *Saccharomyces cerevisiae* and it contains a minimum of 60% β-glucans plus lipids, protein, ash and moisture and no nucleotides.

As β-glucan has been shown to increase immunity and survival in various juvenile (i.e. stage from metamorphosis to sexual maturity) and adult fish (i.e. sexually mature) both in freshwater and marine aquaculture species as well as in marine fish larvae (i.e. the stage from hatching to metamorphosis) [12] it has been suggested to be one of the most potent immune system enhancers in aquaculture [19]. In mammals but as well in fish β-glucan is detected by multiple pattern recognition receptors including toll-like receptors and complement receptor C3 [20, 21] but the main mammalian β-glucan receptor dectin-1 could not be identified in fish. In both mammals and fish β-glucan recognition results in the activation of macrophages, which induces phagocytosis, leukocyte migration and the production of cytokines (e.g. IL-1, TNFα), nitric oxide (NO) and reactive oxygen species, as well as the enhancement of complement activity [21-30]. Recent studies in juveniles and adult fishes have for example shown that yeast β-1,3/1,6-glucan in form of MacroGard® increases complement activity [31, 32] and induced an anti-inflammatory effect [27] in carp (*Cyprinus carpio*) juveniles. In addition it was also shown that β-glucan can enhance growth and leucocyte infiltration into

the epithelial layers of the gut of carp juveniles [33]. However, in juvenile turbot dietary MacroGard® did not increase protection against an infection with *Vibrio anguillarum* and complement and lysozyme activity were not influenced even though the white blood cell count was increased [34].

The immature immune system of fish larvae thus prevents inferences being made from studies focusing on juveniles to larval life stages. Nevertheless only a few studies were performed regarding earlier life stages. It was demonstrated that MacroGard® stimulates the classical complement pathway, lysozyme activity and α-2-macroglobulin in carp fry [11]. Skjermo *et al.* [12] used MacroGard® and β-glucan from *Chaetoceros mülleri* as dietary supplement in first feeding Atlantic cod (*Gadus morhua*) larvae. In contrast to MacroGard® β-glucan from *C. mülleri* increased survival whilst feeding of MacroGard® led to reduced dry weight of the larvae. Al-Gharabally and colleagues [15] investigated the effects of β-glucan and levamisole in blue-fin porgy (*Sparidentax hasta*) larvae and found reduced larval mortality and increased resistance against bacterial infection as well as enhanced lysozyme activity. In summary the data currently available demonstrates variable effects of β-glucan depending on the source of the immunomodulator, fish species tested and the age of the fish.

In the present study we hypothesized that yeast derived β-1,3/1,6-glucan (i.e. MacroGard®) has an immunomodulatory effect and can enhance survival of turbot larvae during the first stages of development and we aimed to elucidate some of the underlying mechanisms. To the best of our knowledge no data is yet available on the molecular effects of β-glucan on fish larvae. We therefore conducted an extensive analysis on the expression of genes involved in innate immunity as well as nutritional aspects. Expression of genes involved in growth, development, digestion, lipid metabolism, antioxidative activity as well as immune response

were analysed. Additionally the microbiota of the larvae was analysed using denaturing gradient gel electrophoresis. This study therefore presents the first detailed analysis of the effects of MacroGard® on first feeding turbot larvae.

2. Material& Methods

2.1 Animals

Turbot (*Scophthalmus maximus*) larvae (1 day post hatch) were obtained from Stolt Sea Farm Norway and reared in the facilities of GEOMAR Kiel, Germany. From the start of the experiment larvae were kept in six green 75 L-tanks filled with filtered 30 L North Sea water (5 µm, UV-treated, 30 ± 1 PSU). The temperature was kept constant at 18 ± 1°C and from 6 days post hatch (dph) the salinity was reduced stepwise to attain 17 ± 1 PSU at 20 dph by mixing with filtered Baltic Sea water. Larvae were kept in densities of approx. 40 larvae/L and greenwater technology (i.e. addition of *Nannochloropsis* spp.) was used in all tanks. 50% of the water was exchanged once a day with 5 µm-filtered, UV treated mix of North Sea and Baltic Sea water prior to feeding.

2.2 Cultivation of live feed

Rotifers (*Brachionus plicatilis*) were reared in sterile filtered Baltic Sea water (salinity = 17 ‰) in tanks with conical bottoms and fed with resuspended *Nannochloropsis* spp. concentrate (BlueBiotech GmbH, Büsum, Germany). Prior to introducing individuals to the fish larval tanks, rotifers were harvested from the cultivation tank and transferred to two conical enrichment-tanks.

Artemia eggs (Premium Artemia, Sanders) were incubated for 24 hours in filtered Baltic Sea water at 30°C, harvested and the newly hatched nauplii were introduced into the fish larval tanks without any further treatment.

2.3 Experimental protocols

The larvae were fed once daily from 3 days post hatch (dph) with 3 *B. plicatilis*/ml. In the control treatment rotifers were enriched with 0.35 g/L Selco presso (INVE Aquaculture SA, Belgium) for 3 hours. Yeast β-1,3/1,6-glucan in form of MacroGard® (batch number Q511156; kindly provided by Biorigin, Brazil) was used in the second treatment and 0.5 g/L MacroGard®, as used in [12], was added to the 0.35 g/L Selco enrichment and incubated with the rotifers for 3 h. In order to ensure that MacroGard® was taken up by *B. plicatilis* the suspension of MacroGard® in sterile sea water was sonicated at 4 x 30 s at power 6 (Sonifer® cell disruptor B-30, Branson Sonic Power Co.) prior to enrichment to ensure small enough particle size (≤ 20 µm) [35]. Sonicated MacroGard® was prepared fresh every day and particle size was verified by light microscopy. The treatments were run with three tank replicates each. From day 13 dph onwards all tanks were additionally fed untreated *Artemia* sp. nauplii (1 nauplius ml/L).

In order to assess mortality rates, dead larvae were removed and counted daily. For analysis of gene expression, RNA:DNA ratio, tryptic activity and size, larvae were anaesthetised with MS 222 (Sigma, Germany) and sampled at 11 and 25 dph in the morning, before feeding, from each tank. These two life stages were chosen to evaluate short term effects of the immunomodulator (11 dph) and long-term effects during feeding of untreated Artemia (25 dph). Samples for gene expression studies and RNA:DNA analysis were stored at -80°C in RNAlater until RNA extraction or in tank water for tryptic activity.

118

119 *2.4 Growth and RNA:DNA-ratio*

120 For an analysis of growth, the standard length (SL) and width of 5 thawed larvae per tank and
121 time point was noted, followed by analyses of RNA:DNA ratio, tryptic activity and gene
122 expression. Assessing the ratio of RNA to DNA is a well-accepted index in larval research for
123 nutritional condition [36]. To do so, 11 dph turbot larvae were freeze dried for 24 h to a
124 constant weight (Alpha1-4 freeze dryer, Christ GmbH, Germany) and subsequently weighed
125 with a microbalance (SC2, Sartorius AG, Germany). Quantification of RNA and DNA was
126 performed according to Malzahn et al. [37] with modifications, where whole individual larva
127 were analysed instead of only muscle tissue. RNA:DNA-ratio was subsequently calculated of
128 individual larvae.

129

130 The individual specific growth rate (G, %/d) of the larvae was calculated based on the
131 formula:

132 (1) $G = 100 * (e^{g} - 1)$

133 where g = instantaneous growth coefficient equal to:

134 (2) $g = \frac{S_{t2} - MS_{t1}}{\Delta t}$

135 where S_{t2} equals the individual standard length of the larvae per tank at time point 2 and MS_{t1}
136 is the mean standard length of the larvae in the respective tank at time point 1. Δt = time (d)
137 between measurements [38].
138 Fulton's condition factor (K) was calculated as K = (dry weight [mg]/SL^3[mm^3])*100.

139

140

141 *2.5 Tryptic enzyme activity*

Tryptic enzyme activity of five individual fish larvae per tank and treatment was assayed following a fluorescence-method described by [39] modified for microtiter plates. In brief, 250 µL substrate (Na-benzoyl-L-arginin-4-methylcoumarinyl-7-amid, Bachem AG, Switzerland) were added to 50 µL homogenate of the individual fish larva in a 96-well-plate. After mixing and 20 min incubation at room temperature, the relative fluorescence enhancement was recorded every 2 min for 12 min using a microtiter fluorescence reader (Fluoroskan Ascent, Labsystems Thermo). The tryptic enzyme activity is given as an equivalent of hydrolysed substrate per time unit and is normalised against larval area [mm^2] (nmol hydrolysed substrate/min*larva).

2.6 Molecular genetic analysis

2.6.1 RNA extraction & cDNA synthesis

Five single larvae per tank and time point were weighed, photographed and homogenized in 1 ml Tri-Reagent (Sigma, Germany). Photographs were used to determine length and width of the larvae. After obtaining the aqueous phase by incubation with chloroform, RNA was extracted from this phase using the RNeasy Mini kit (Qiagen, Germany) following the manufacturer's instructions. The RNA concentration was determined by Nanodrop ND-1000 (Peqlab, Germany) and normalised to a common concentration with RNase free water before proceeding with cDNA synthesis. 240 ng RNA were transcribed with the Quantitec kit (Qiagen, Germany) according to the manufacturer's instructions including a genomic DNA wipe-out step. Controls for gDNA were also included and cDNA was stored at -20°C until further use.

2.6.2 Gene expression analysis using the Fluidigm Biomark system

Primers specific for immune, nutritional and house keeping genes were either taken from [40], [41] or designed with Primer 3 using sequences from Genbank (Table 1). Sequences similar to *sod*, *gpx*, and *chym* were identified via BLAST search. Expression of 30 genes in the larval samples was analysed in triplicates using the qPCR Biomark^{TM} HD system (Fluidigm) based on 96.96 dynamic arrays (GE chips). A pre-amplification step was performed with a 500 nM primer pool of all 30 primers in TaqMan-PreAmp Master Mix (Applied Biosystems) and 1.3 µl cDNA per sample. Short cDNA fragments were pre-amplified (10 min at 95°C; 14 cycles: 15 s at 95°C and 4 min at 60°C). Obtained PCR products were diluted 1:10 with low EDTA-TE buffer. 3.15 µl of the pre-amplified product was loaded onto the chip with SSofast-EvaGreen Supermix low Rox (Bio Rad) and DNA-Binding Dye Sample Loading Reagent (Fluidigm). Primers were loaded onto the chip at a concentration of 50 µM. Assay Loading Reagent (Fluidigm) and low EDTA-TE Buffer. The chip was run according to the Fluidigm 96.96 PCR protocol with a Tm of 60°C. Samples were distributed randomly across a chip and each included no template controls, controls for gDNA contamination and standards. qBase+ software was used to calculate stability of the tested housekeeping genes and gene expression was then normalised to the geometric mean of the three most stable housekeeping genes (M < 0.4). Analysis of gene expression was carried out according to the $2^{-\Delta\Delta Ct}$ method [42] whereat $\Delta\Delta Ct$ of individual samples was calculated in relation to the normalised mean ΔCt of the control group. Target genes were normalized against the reference genes *gadph* (only day 11 ph), *tubβ* (only day 24 ph) as well as *40s*, and *60s*, and x-fold change calculated in relation to the control group of each time point.

2.6.3 RT-PCR-DGGE

In order to analyse population changes of the microbiota associated with the larvae a reverse transcription polymerase chain reaction – denaturing gradient gel electrophoresis (RT-PCR-DGGE) was performed as previously described [43] with modifications.

DGGE PCR

cDNA of three to five larvae per tank were combined into two pools. A nested PCR was performed with the DGGE_f_cl [44] and DGGE677R [45] primers (Table 1) and as described in Jung-Schroers *et al.* [43] with 0.2 U hot-start KAPA 2 G robust polymerase (PeqLab), 1x KAPA A buffer, 200 nM of each primer, 200 µM of each dNTP, 18.4µl of 10x diluted cDNA in a final volume of 25 µl.

198 Table 1: Primers utilized for gene expression analysis by real-time PCR

Group	Name		Sequence	Accesion	Name & function
House keeping	40S	FW	GAAACAGCCCACCATCTTCC	DQ848873.1	ribosomal subunit 40S
		RV	GTAAGTGCCATCAATAGCCTCTC		
	60S	FW	GATGGTCCGCTACTCTCTCG	DQ848879.1	ribosomal subunit 60S
		RV	CACGGGTGTTCTTGAAGTGA		
	Tubß	FW	GAACACGGAATTGACCCAAC	DQ848894.1	Tubulin β
		RV	GGGCACGTATTTACCACCTG		
	GAPDH	FW	CAGTGTATGAAGCCAGCAGAG	DQ848904.1	Glyceraldehyde-3-Phosphate Dehydrogenase
		RV	ACCCTGGATGTGAGAGGAG		
Antioxidant	SOD	FW	AAACAATCTGCCAAACCTCTG	HS029499.1	Superoxide dismutase
		RV	CAGGAGAACAGTAAAGCATGG		
	Prx6	FW	TCAGAGAGCGAGGGAATGAC	GU561990.1	Peroxiredoxin
		RV	CCGATGAGATAGACAAGGATGG		
	GPX	FW	CCCTGATGACTGACCCAAAG	HS032063.1	Glutathione peroxidase
		RV	GCACAAGGCTGAGGAGTTTC		
	nkef	FW	AGCACACTGACAAACACGGCGA	EU747733.1	natural killer cell enhancing factor
		RV	TGCGGCAGAACATCAAGGAGACC		
Growth	GHr	FW	CCCTCATCCAAACCCAAAC	AF352396.1	Growth hormone receptor
		RV	GGGCATAACATTGCTGACCT		
	GHP	FW	ATTCCATCCAAACTGCCTGAG	EF467362.1	Growth hormone precursor

		RV	GTAGGTTCCATAAGGAGCGAG		
	GH	FW	GAGCAAAGTTCAGAACCTTCA	EF467362.1	Growth hormone
		RV	TCGGATGGAATCTGGAACCT		
	IGF2	FW	GAATGTTGTTTCCGTAGCTG	JN032705.1	Insulin-like growth factor 2
		RV	TCGGGACTTCCTGTTTTAGT		
Bone development	Ost	FW	GGTTTGACTGTGGAGAAGGA	AY663810.1	Osteocalcin
		RV	AGTCGTGTCCATCATTTCCTC		
	ALPL	FW	CAAAATGGTCAACGGTGCGAGGGA	DQ848861.1	Alkaline phosphatase
		RV	GGGGGCCTTTAGCAAACACAGCA		
Vision	RH1	FW	AAAGGGCTGAGAGGGAAGTC	KF312147.1	Rhodopsin
		RV	CAATGGGTTGTAGATGGAGGA		
Lipid metabolism	LPL	FW	TCCCTTTGTTATGCCTGTCC	JQ690822.1	lipoprotein lipase
		RV	GCTGATGATTGAGTCCTTCTCC		
	ApoE	FW	AGGCCACCGCTAAGGAGCTTTTCA	AJ236883.1	Apolipoprotein E
		RV	TTCCCAACCTGCTCTTGGATCTGGG		
cell differentiation, development, metabolism	PPARa	FW	CTGCAAGGGTTTCTTCAG	JQ901838.1	Peroxisome proliferator-activated receptor
		RV	GTTGCGGTTCTTCTTCTG		
Digestion	Chym	FW	TACAACGCTCCCAGCACTC	HS030320.1	Chymotrypsin, proteolysis
		RV	TTCTCGCACACCAGAGGAC		
	Tryp	FW	ATCTCTGCTGCTCACTGCTG	DQ848906.1	Trypsinogen, protein digestion
		RV	GTCCTGTAGTCGTAACTCTGATGC		

Fatty acid synthesis	d6FAD	FW	TGGAGAGTCACTGGTTTGTGTGGGT	AY546094.1	Delta-6 fatty acyl desaturase
		RV	AGGTGGCCTGTAGCTGCATGGTTA		
Immune response	C3	FW	TGACAATGGTGTCGCTGTACT	DQ400678.1	Complement component C3, alternative pathway
		RV	CAATAGGTCAAGGTCATTTGTGTTA		
	IL1β	FW	ACCAGACCTTCAGCATCCAGCGT	AJ295836.2	Interleukin β, pro-inflammatory cytokine
		RV	TTCAGTGCCCCATTCCACCTTCCA		
	TNFα	FW	AAAAGAAGTCGGCTACGGGGTGGA	FJ654645.1	Tumor necrosis factor alpha, pro-inflammatory cytokine
		RV	TTCCAGTGCCAAGCAAAGAGCAGG		
	Hep1	FW	CGAGTCACATCAGGCAGAAG	JQ219840.1	Hepcidin 1, antimicrobial peptide
		RV	TCCTCAGAACTTGCAGCAGA		
	gLys	FW	TCTCATTGCTGCCATCATCTC	HQ148717.1	g-type Lysozyme, bactericidal
		RV	CCACTCGGATTAACATCAACCT		
	LysC	FW	GAACGCTGTGAATTGGCCCGACT	AB355630.1	c-type Lysozyme, bactericidal
		RV	GTTGGTGGCTCTGGTGTTGTAGCTC		
	HSP70	FW	CCGCTGCTGCTATTGCCTATGGT	EF191027.1	Heat shock protein 70, stress protection
		RV	TGCCGCCACCGAGATCAAAGATG		
	IRF7	FW	TCACAGTCAAGGTGGTCCCGCT	HQ424129.1	Interferon regulatory factor 7, induction of interferon
		RV	TGAGATCGTAGAGGCTGTTGTGCGA		
	TLR3	FW	GACGTGCTGATCCTGGTCTTTCTGG	FJ009111.1	Toll like receptor 3, pattern recognition receptor
		RV	AGCTCAGGTAGGTCCGCTTGTTCA		
	unBact_16s	FW	AGGATTAGATACCCTGGAGTCCA	Multiple sequences	Universal bacteria
		RV	CATGCTCCACCGCTTGTGC		

Microbiota analysis	Aerom_16s	FW	GCGAAGGCGGCCCCCTGGACAAAGA	Multiple sequences	*Aeromonas* spp.
		RV	CCACGTCTCAAGGACACAGCCTCCAAATC		
	Flav_16s	FW	GGGATAGCCCAGAGAAATTTGGAT	Multiple sequences	Flavobacterium spp.
		RV	AGTCTTGGTAAGCCGTTACCTT		
	Vib _16s	FW	GTTTGCCAGCGAGTAATGTC	Multiple sequences	*Vibrio* spp.
		RV	TAGCTTGCTGCCCTCTGTATGCG		
	DGGE_f_cl	FW	CGCCCGCCGCGCGCGGCGGGCGGGGCGG GGGCACGGGGGGCCTACGGGAGGCAGCAG	Multiple sequences	Universal bacteria for DGGE
	DGGE677R	RV	ATMTCTACGCATTTCACCGCTAC		

199

200 *2.6.4 Denaturing gradient gel electrophoresis (DGGE)*

201 The larval microbiota was analysed using a Biostep TV400 DGGE vertical electrophoresis
202 system with 16.5 x 17.5 cm gels. PCR products were run on an 8% (w/v in 1x TAE)
203 polyacrylamide gel containing a 40-60% gradient of denaturing agent (100% denaturant
204 contains 7 M urea an 40% w/v formamide) at 60°C for 15 h at 100V. Gels were stained with
205 0.11 M silver nitrate for band visualisation [46].

206

207 *2.6.5 Analysis of DGGE profiles*

208 RT-PCR-DGGE banding profiles were analysed on the basis of presence and absence of
209 bands at certain positions in each lane. Using the program DendroUPGMA
210 (http://genomes.urv.cat/UPGMA/) [47] a similarity matrix and a resulting dendrogram were
211 constructed using the Pearson coefficient of correlation. The gel was digitalized and the band
212 intensities evaluated using the program Bionumerics 7.5 (Applied Maths). In order to analyse
213 the bacterial community the Simpson index of diversity were calculated as well as the
214 richness of the community. For each lane the Simpson index was calculated with $D = 1 - \sum pi^2$, where *pi* represents the relative intensity of bacterial amount in *i*. This index of diversity
216 is weighted towards most abundant species. Values can range from 0 to 1 and increasing
217 values indicate an increasing diversity. The number of bands in a lane was defined as the
218 species richness in this community.

219

220 *2.6.6 Occurrence of some important bacteria in larval microflora*

221 *RT-qPCR*

In order to evaluate the occurrence of some important pathogenic or opportunistic bacteria in the larval microflora a targeted RT-qPCR was performed using primers amplifying a region of the 16S rDNA specific for *Vibrio* spp., *Aeromonas* spp and *Flavobacteria* spp. (Table 1). The proportional occurrence of these bacteria was calculated in relation to the overall bacterial load evaluated using a universal bacteria primer (Table 1) as described earlier [48].

2.7 Statistical analyses

All data are presented as mean ± SEM (standard error of the mean). Statistical data analysis was carried out using Statistica 8 (StatSoft, Inc. 2008) and R 3.1.1 [49]. Data were tested for normality and homogeneity of variances. Daily mortality was calculated and was analysed in R using Kaplan-Meier estimates of survival [50]. Multivariate analysis (MANOVA) were performed to test for differences in the entire gene expression pattern of all 30 genes but also divided into functional gene classes (Groups see Table 1). 2-way nested ANOVAs (tank nested in treatment) were performed to test for significant effects of tank, treatment, time and time*treatment interactions. For data sets that displayed significant time*treatment interactions nested ANOVAs were performed for the individual time points. Significance was defined as $p \leq 0.05$ and gene expression results were Bonferroni corrected for multiple testing. For gene expression studies statistical analysis was performed on Δct values, whilst graphs represent x-fold gene expression relative to the control group. Graphs were constructed with GraphPad Prism 5 and data are plotted as independent data points (n = 18), whilst statistical analysis controlled for potential tank effects through nested ANOVA.

3. Results

3.1 Survival

246 The survival at the end of the experiment in the MacroGard® (22.8 ± 5.4%) fed group was
247 approximately three times higher than in the control group (7.4 ± 4.7%). The two treatments
248 were significantly different (χ^2 = 98.7, df = 1, p <0.001).

249

250 *3.2 Growth*

251 The size parameters (standard length, width, weight) of the larvae at the different sampling
252 points are shown in table 2. No significant differences were found between treatments for the
253 size parameters. Similarly no differences were detected in the condition factor as well as the
254 RNA:DNA ratio on day 11 ph. SGR from 11 to 24 dph tended to be higher in the
255 MacroGard® treated group than in the control fed larvae (df = 1, F = 3.78, p = 0.06).

256

257 Table 2: Size and growth parameters of turbot larvae (n = 18) fed rotifers enriched with or
258 without 0.5 g/L MacroGard®. Values represent mean ± SEM of independent data points.

	11 dph		24 dph	
Parameters	Control	MacroGard®	Control	MacroGard®
Weight [mg]	0.30 ± 0.03	0.33 ± 0.04	12.20 ± 1.70	13.65 ± 1.11
Length [mm]	3.68 ± 0.09	3.51 ± 0.12	7.97 ± 0.24	8.48 ± 0.17
Width [mm]	0.71 ± 0.09	0.60 ± 0.05	3.60 ± 0.37	4.28 ± 0.14
Area [mm^2]	2.46 ± 0.23	2.52 ± 0.27	29.62 ± 2.78	36.86 ± 3.13
SGR	14.87 ± 0.82	13.30 ± 1.08	33.12 ± 1.89	38.25 ± 1.34
Condition [mg/cm^3]	0.61 ± 0.05	0.74 ± 0.04	2.21 ± 0.16	2.19 ± 0.07
RNA:DNA	3.97 ± 0.19	3.99 ± 0.18		

259

260 *3.3 Gene expression*

261 All studied genes were expressed at 11 and 24 dph and multivariate analysis revealed that
262 gene expression was effected by the treatments (df = 1, F = 3, $p \leq 0.05$), the experimental
263 duration (df = 1, F = 5335.4, $p \leq 0.0001$) and their interaction (df = 1, F = 4.9, $p \leq 0.05$) but

not by tank dependent effects. Expression of genes involved in growth, development, digestion, lipid metabolism, antioxidative activity as well as immune response were analysed.

The MANOVA also revealed that genes involved in growth (*gh*, *ghp*, *ghr*, *igf2*, *rh1*, *ppar*), antioxidative activity (*sod*, *gpx*, *nkef*, *prx6*) and lipid metabolism (*apo e*, *d6fad*, *lpl*) were not influenced by the immunomodulator (see supplementary data in Pangea database).

Genes related to development however were significantly regulated due to the time ($F = 2821.2$, $p \leq 0.0001$), treatment and their interaction (both $F < 5$, $p \leq 0.01$; all $df = 1$). This was expressed in the enhancement of osteocalcin (*ost*) expression, a gene involved in mineralisation of bones, 24 days post hatch (10.16 ± 2.32 –fold, $\Delta ct = 12.27 \pm 0.40$; $df = 1$, $F = 13.94$, $p = 0.003$) compared to the control (1.62 ± 0.42 –fold, $\Delta ct = 15.09 \pm 0.44$) but not at 11 dph (Figure 2).

Interestingly administration of β-glucan influenced genetic pathways involved in digestion ($F = 3.3$, $p \leq 0.01$). These genes were also influenced by experimental period ($F = 1641.4$, $p \leq 0.0001$) and the interaction of time and treatment ($F = 6.0$, $p \leq 0.001$; all $df = 1$). Chymotrypsin (2.20 ± 0.22-fold, $\Delta ct = -0.48 \pm 0.24$; $df = 1$, $F = 25.99$, $p < 0.0001$) and trypsin (3.74 ± 0.7-fold, $\Delta ct = 12.47 \pm 0.26$; $df = 1$, $F = 16.56$, $p < 0.0001$) were enhanced on day 11 ph compared to the control (1.13 ± 0.13, 1.22 ± 0.19 -fold respectively and $\Delta ct = 0.42 \pm 0.17$ and 14.0 ± 0.22, respectively) (Figure 3A). Enzymatic trypsin activity was also measured on day 11 and 24 dph and normalised against the area of the larvae. Activity of the enzyme was significantly heightened in the MacroGard® fed fish (0.52 ± 0.08 /mm^2) on day 11 ph compared to the control (0.25 ± 0.07 /mm^2). At 24 dph trypsin activity was not different between the two groups (Figure 3B).

MacroGard® feeding interacting with administration time led to modulation of the immune system dependent on the diet (df = 1, F = 4.4, $p < 0.0001$). Among the immune relevant genes, β-glucan feeding did not modulate the expression of genes encoding for bactericidal enzymes (*glys, lys c*), antimicrobial peptide (*hep1*) and pattern recognition receptor (*tlr3*, Fig. 4). However, at 11 days post hatch MacroGard® feeding led to an approximately doubling of the gene expression of complement component *c3* (2.22 ± 0.38-fold, Δct = 4.73 ± 0.37; df = 1, F = 6.81, p = 0.015) compared to the control (1.32 ± 0.24-fold, Δct = 5.31 ± 0.28). Contrarily on day 24 dph dietary β-glucan reduced gene expression of interleukin 1 (*il-1β*, Δct = 9.59 ± 0.24) and tumor necrosis factor α (*tnf-α*, Δct = 15.15 ± 0.23) by about 50 - 60% compared to the control (*il1β:* Δct = 8.20 ± 0.34 and *tnf-α*: 13.95 ± 0.21; df = 1, F = 9.13 and 8.17, $p \leq 0.05$). In parallel gene expression of heat shock protein 70 (*hsp70*) was down-regulated at 24 dph (0.82 ± 0.04-fold, Δct = 1.46 ± 0.06) compared to the control (1.01 ± 0.04-fold, Δct = 1.16 ± 0.06 ;df = 1, F = 11.95, p = 0.005).

3.4 Microbiota analysis

The band pattern shown on the RT-PCR-DGGE gel (Figure 5) differed between samples. In total 86 bands were detected with the highest number (i.e. 32 bands) occurring in the pooled sample b of control tank 2 at 11 dph and the lowest number (15 bands) being found in the pool b of control tank 1 at 24 dph. The dendrogram shows a separate cluster for the control samples from 11 dph. The MacroGard® fed larvae from tank 1 seemed to have a similar microbiota to these control samples whilst the other two MacroGard® fed tanks were at 11 dph more similar to the microbiota found in the samples from 24 dph. Correspondingly the microbiota of the MacroGard® fed larvae sampled at 24 dph represented an own cluster with similarity to the 24 dph control samples. Two outliers (11 dph MacroGard® tank 3 pool b and 24 dph control tank 2 pool a) were found.

Various ecological indexes were calculated from the microbial fingerprinting of whole larvae of the two feeding regimes (Table 3). The multivariate analysis did not show any tank effects and no time dependent effects except for richness ($df = 1$, $F = 4.55$, $p \leq 0.05$). However, treatment and time*treatment interactions were observed for Richness and Simpson. The species richness (represented by the number of bands observed) indicates differences between feeding regimes, which became significant at 24 dph. The diversity of the microbiota (represented by Simpson index) was more similar between the two feeding regimes at 24 dph compared to the microbiota from control and MacroGard® fed fish sampled at 11 dph.

Table 3: Ecological indexes of larval microbiota from the RT-PCR-DGGE analysis

Index	**Day**	**Treatment**		**Statistics for treatment effect**
		Control	**MacroGard®**	
Richness	11	25.8 ± 3.9	21.0 ± 4.1	df = 1, F = 4.86, p = 0.069
	24	18.5 ± 2.4	23.0 ± 1.1	df = 1, F = 13.26, p = 0.011
Simpson	11	0.95 ± 0.01	0.93 ± 0.02	df = 1, F = 5.07, p = 0.065
	24	0.93 ± 0.01	0.94 ± 0.00	Not significant

The proportional contribution of some important pathogenic or opportunistic bacteria (Fig. 6) differed between sampling days but not between treatments as *Vibrio* spp. constituted 3.93 ± 1.77% of the overall microbiota at 11 dph but 32.13 ± 4.96% at 24 dph ($df = 1$, $F = 65.4$, $p < 0.0001$).

4. Discussion

During the first weeks of turbot larval development, aquaculture farms experience high losses of 70-100% [51], which largely reflect the losses observed in nature as well and therefore

might be regarded as a natural phenomenon. Additionally, in aquaculture, these losses might be related to unsuitable feed or infections due to opportunistic pathogens, facilitated by high stocking densities. Hence, the present study aimed to enhance survival of turbot larvae during the first stages of development and investigate underlying mechanisms.

Larval survival was enhanced by 15% due to the administration of β-glucan. As the deviation in mortality rates between the two treatment groups started to occur at around 5 dph, thus after the first phase of high mortality, we can conclude that dietary MacroGard® did not affect survival of the larvae during their first phase of development. Presumably survival in this early phase could be enhanced by combining larval and maternal immunostimulation as suggested by Vadstein [8] and demonstrated in rainbow trout (*Oncorhynchus mykiss*) by Ghaedi et al [16]. Below we discuss possible physiological and immunological mechanisms underlying the enhanced survival rate observed after 5 dph.

Survival and growth of fish larvae are closely linked in nature [52]. In the present study the MacroGard® treated larvae had a slightly higher SGR from 11 dph to 24 dph, however none of the other size parameters such as RNA:DNA ratio, length and weight differed between the treatments. The effect of β-glucan on larval and juvenile growth seems to vary between species. For example dietary β-glucan increased growth of larval *S. hasta* [15], juvenile carp [33] and juvenile olive flounder (*Paralichthys olivaceus*) [53], but decreased growth in cod larvae [12] and no effect on size parameters was observed in juvenile turbot [54].

Correspondingly to our results on growth, genes involved in growth and development were not influenced by the β-glucan treatment. This is the first time β-glucan effects on growth hormone gene expression were studied in fish but it is in accordance to studies in pigs, where β-glucan also had no effect on growth hormone production [55, 56] Nevertheless, our data

suggest that bone mineralisation (i.e. osteocalcin gene expression) might be increased in turbot larvae fed with MacroGard® during the later time period (24 dph). To the authors knowledge no data is yet available on the effects of β-glucan on skeletal development in fish larvae. As β-glucan does not have any nutritional value, it is possible that the enhanced osteocalcin expression is a secondary effect due to the altered gut microflora in the MacroGard® fed fish. In sea bass (*Dicentrarchus labrax*) for example it was shown that bone development was enhanced in response to probiotic lactic acid bacteria [57]. In future research it should therefore be investigated if β-glucan can enhance bone structure and prevent malformations.

Protein and lipid uptake are important factors influencing survival and performance of fish larvae. Whilst genes related to lipid metabolism were not affected by the feeding of the carbohydrate β-glucan, protease activity and associated gene expression was enhanced in MacroGard® fed larvae. This is concordant with previous observations that dietary β-glucan enhances trypsin activity in red snapper (*Lutjanus peru*) [58]. In the aforementioned study the authors used a different β-glucan formulation (i.e. Fibrosel®) compared to the present study, we therefore assume that the yeast carbohydrate stimulated the digestive system and not any other components of the feed additive. This increased peptidase activity results in increased supply of amino acids to the organism [59], which in turn enhances survival.

This effect of increased trypsin activity however disappeared at 24 dph when tryptic activity as well as gene expression of digestive enzymes was not influenced. This might be due to the alternative feed (untreated *Artemia*) offered, which at this stage should have been the main food source for the larvae.

In contrast to earlier studies [58, 60], we found no effect of β-glucan on the gene expression of antioxidants even though functionality of antioxidant enzymes in turbot larvae was demonstrated previously [61].

The enhancement of survival post the 5th day in β-glucan fed fish could be facilitated by the immunomodulatory activity of this compound. However, beside complement component C3, most of the studied immune genes (*il-1β*, *tnfα*, *hep 1*, *glys*, *lysc*, *irf7*, *tlr3*) were not affected in larval turbot during the first 11 days of the experiment, which is in contrast to most studies conducted on fish juveniles. Our data therefore highlight, once again, the discrepancy between immunological studies on fish larvae and juvenile or adult fish. To the best of our knowledge no data is yet available on the maturation of immunocompetence in turbot larvae. Padrós and Crespo [62] demonstrated that whilst the pronephros was present from 0 dph, spleen and thymus were not detected until 10 dph and 10-30 dph respectively at ca. 18°C. However, it is the functioning not the appearance of organs that determines immunocompetence.

For example TLR3, which has been suggested to be involved in β-glucan recognition in carp [63] was not affected by the treatment. It can therefore be speculated that in turbot larvae recognition of β-glucan is rather associated with complement component C3, as it has been demonstrated in mice [20]. It is unknown yet if the mechanisms of β-glucan recognition depend on life stage resp. maturity of the immune system.

Activation of the alternative complement pathway also seems to be a general effect of β-glucan administration in fish. For example, Chettri *et al.* [64] observed *c3* up-regulation in rainbow trout fry after bath exposure to β-glucan. This is also in accordance to the studies conducted by Pionnier and colleagues [31, 32], in which the authors demonstrate an up-regulation of *c3* in response to MacroGard® feeding in juvenile carp. In their study *c3* up-

404 regulation was accompanied by enhanced activation of the alternative complement pathway.
405 In the present study it can therefore be assumed that MacroGard® feeding activated the
406 complement pathway and hence increased opsonisation of pathogens. This increase in
407 immunity most probably contributes to the elevated survival rates observed in the present
408 study. In future studies endpoint analyses of cellular immunity such as phagocytic activity as
409 well as pathogen resistance should also be included to elucidate the overall effects of β-glucan
410 on immuncompetence.

411

412 Complement component C3 is part of the complement system and its binding to pathogen
413 surfaces activates the alternative pathway, which leads to opsonisation and destruction of
414 pathogens. Transcription of complement *c3* can be activated by immunostimulants directly or
415 indirectly via cytokines. In the present study β-glucan administration led to enhanced mRNA
416 levels of *c3* at 11 dph whilst expression of cytokines was not affected suggesting direct
417 detection of β-glucan by C3 as discussed above. It is however possible, that alternative or
418 additional pathways were not detected owing to the limited number of immune genes studied
419 or that the modulated microbiota led to these changes [65].

420

421 We did not find any indications that the immunostimulating effect of dietary MacroGard® led
422 to increased metabolic costs (e.g. decreased growth and survival) of the fish larvae.
423 Nevertheless, as mounting an immune response could be costly (see [66] for discussion) the
424 dietary composition should be monitored carefully and adapted if necessary.

425

Interestingly at day 24 ph, i.e. after *Artemia* as live feed were introduced, transcription levels of important pro-inflammatory cytokines (*tnf-α*, *il-1β*) were lower in the MacroGard® treated group than in the control group. In previous studies such lowered expression patterns of pro-inflammatory cytokines (*tnf-α*, *il-1β)* were also observed after feeding juvenile carp for 14 and 25 days with MacroGard® [27, 63] and *in vitro* in murine microglia cells after treatment with β-glucan [67]. The majority of studies regarding this immunomodulator however describe an induction of an inflammatory response [68, 69]. The here observed anti-inflammatory effect is also associated with reduced levels of heat shock protein 70 gene expression. This is as well in contrast to previous observations in fish and mammals where HSP 70 protein levels were increased after β-glucan administration [70, 71]. This apparent reduction in inflammatory response (i.e. down-regulated cytokines) and stress response (i.e. reduced *hsp70* expression) in MacroGard® fed larvae compared to the control treatment could indicate that fish of the former group are healthier and/or possess a health promoting intestinal microbiota. Previous histopathological studies on turbot larvae demonstrated that intestinal lesions, which can occur during gut development, are often associated with mortalities due to pathogenic gut bacteria [3]. On the other hand Kühlwein et al. [33, 72] demonstrated that in carp β-glucan feeding was associated with an altered gut microbiota and enhanced microvilli length and density.

In the present study histological effects were not investigated but fingerprinting of larval microbiota revealed that feeding of MacroGard® was associated with an altered bacterial composition as well as a change in richness and diversity. The presented data, esp. the similarity analysis, propose that administration of MacroGard® led to a faster maturation of the larval microbiota compared to the control fed fish. It is current knowledge that dietary β-glucan can lead to changes in a matured microbiota [43, 72] and this is the first study indicating an influence of this immunomodulator on the developing larval microbiota.

Nevertheless, a more detailed study of the larval microbiota by next generation sequencing is needed to elucidate this possible maturation effect further.

Although MacroGard® feeding had no effect on occurrence of opportunistic bacteria studied, it is possible that the administration of β-glucan enhanced intestinal health in the larvae, which could explain the anti-inflammatory effect on immune gene expression and increased larval survival.

On the other hand larval survival could also be increased due to the direct effect of β-glucan on rotifers. Little knowledge is yet available on the influence of immunomodulators on live feed but β-glucan administration seems to protect *Artemia* nauplii against pathogens [73, 74]. It can be speculated that similar effect could be observed in rotifers and hence heightened performance of turbot larvae might be due to the feeding of healthier life feed. Furthermore the influence of β-glucans leaked into the tank water due to gut passage of the rotifers should not be underestimated as this might change the microbial community of the larval tanks.

In conclusion this study shows a clear enhancement of survival in turbot larvae in response to MacroGard® feeding. This demonstrates the viability of administering immunomodulators via encapsulation in life feed as suggested by Robles *et al.* [17]. In addition, the effects observed demonstrate that MacroGard® at a concentration of 0.5 g/L enhances systemic performance in turbot larvae. This concentration has also been shown to positively increase growth rate, immune factors and stress resistance in *S. hasta* larvae [15] but had no effects in first feeding cod larvae [12]. It thus needs to be elucidated if this is a species or feeding protocol dependent effect.

474 This enhanced survival due to dietary MacroGard® was associated with a modulation of the
475 physiology, immunity and microbiota of the larvae. We therefore propose that administration
476 of immunomodulators as prophylactic measure should be considered in turbot larvae
477 hatcheries. However, more knowledge of the development of the gut microbiota and the
478 innate immune system is needed for this life stage in turbot to elucidate if the decrease in
479 mortality can be associated with a more potent immune defence directly induced by
480 MacroGard®.

481 Additionally our study highlights the similar and dissimilar immunomodulatory effects of β-
482 glucan dependent on life stage and thus the need for more studies focussed on immune
483 responses of early life stages.

484

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Figure Captions

Figure 1: Feeding treatment and survival rates. Turbot larvae were fed with rotifers enriched with β-glucan (■) or without β-glucan (●). The feeding scheme shows the timing of the different feed treatments. Symbols indicate mean of three tanks and lines indicate SEM.

Figure 2: Gene expression of osteocalcin in turbot larvae after feeding β-glucan. Turbot larvae were fed 0.5 g/L MacroGard® encapsulated in rotifers (black bars) or untreated rotifers (white bars). Larval samples were taken at 11 and 24 dph. The figure displays the x-fold gene

720 expression to the control and the bars represent mean ± SEM of independent data points.
721 Asterisks represent levels of significance: **: $p \leq 0.01$.

722

723 Figure 3: A) Gene expression of chymotrypsin and trypsin and B) tryptic activity in turbot
724 larvae after feeding β-glucan. Turbot larvae were fed 0.5 g/L MacroGard® encapsulated in
725 rotifers (black bars) or untreated rotifers (white bars). Larval samples were taken at 11 and 24
726 dph. The figures display the x-fold gene expression to the control. The bars represent
727 mean ± SEM. Asterisks represent levels of significance: **: $p \leq 0.01$, ***: $p \leq 0.001$.

728

729 Figure 4: Gene expression of immune-related genes in turbot larvae after feeding β-glucan.
730 Turbot larvae were fed 0.5 g/L MacroGard® encapsulated in rotifers (black bars) or untreated
731 rotifers (white bars). Larval samples were taken at 11 and 24 dph. The figures display the x-
732 fold gene expression to the control. The bars represent mean ± SEM. Asterisks represent
733 levels of significance: *: $p \leq 0.05$, **: $p \leq 0.01$.

734

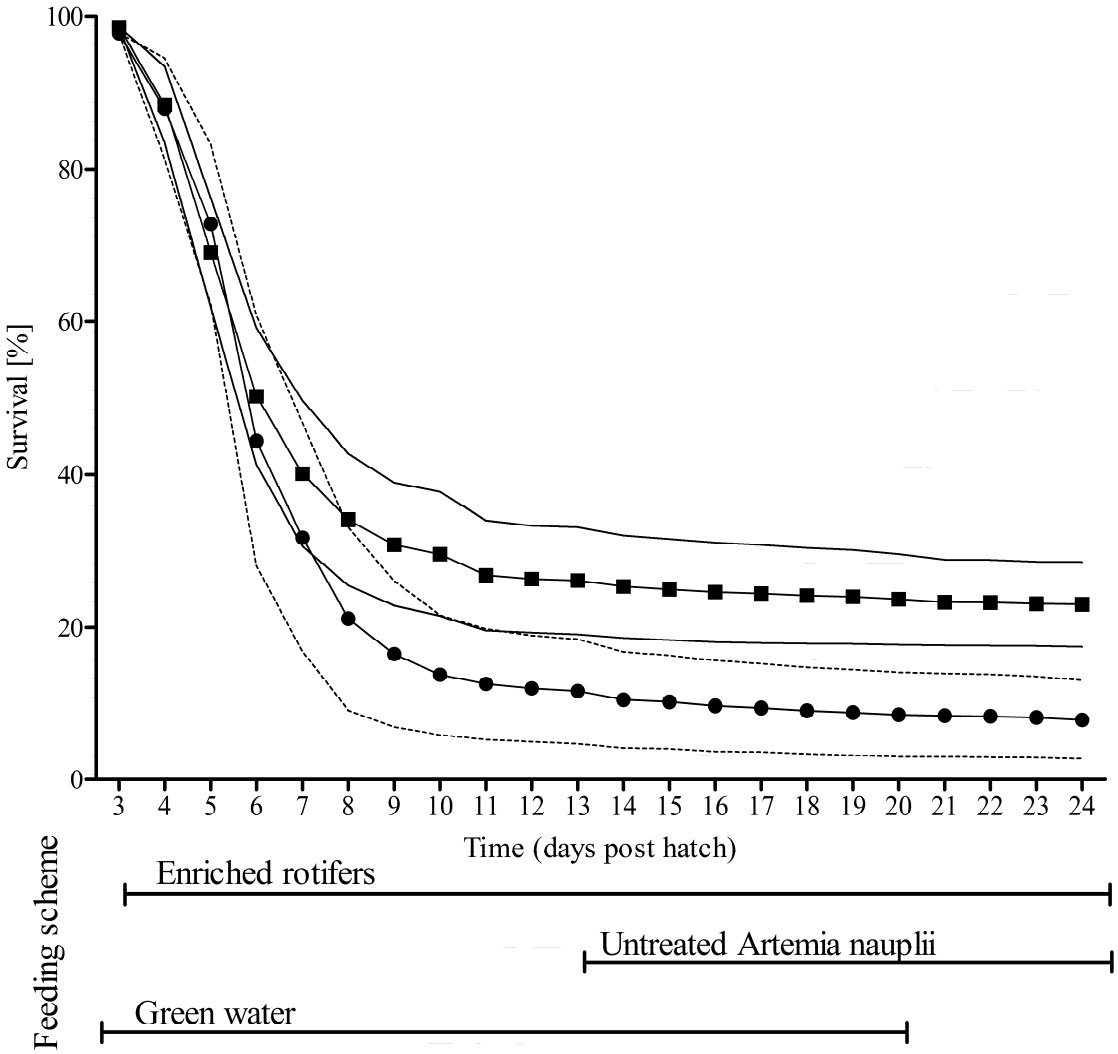
735 Figure 5: RT-PCR-DGGE analysis of larval microbiota from turbot, which were fed with
736 rotifers that were enriched with or without 0.5 g/L MacroGard®. Three to five samples from
737 each experimental tank were combined into two pools (a & b). The dendrogram shows the
738 similarity between samples according to the Pearson coefficient.

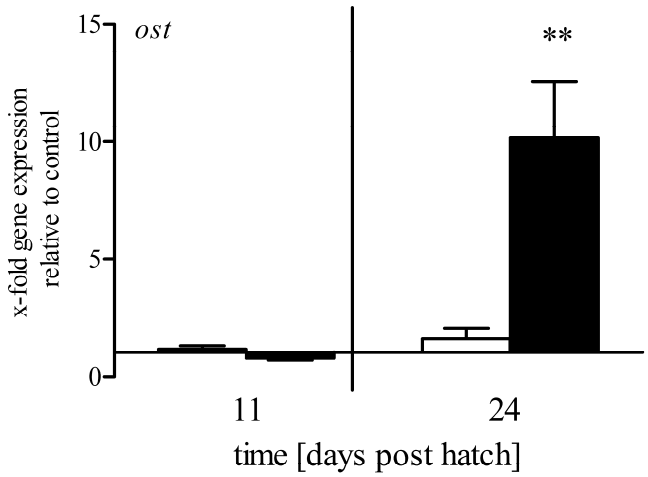
739

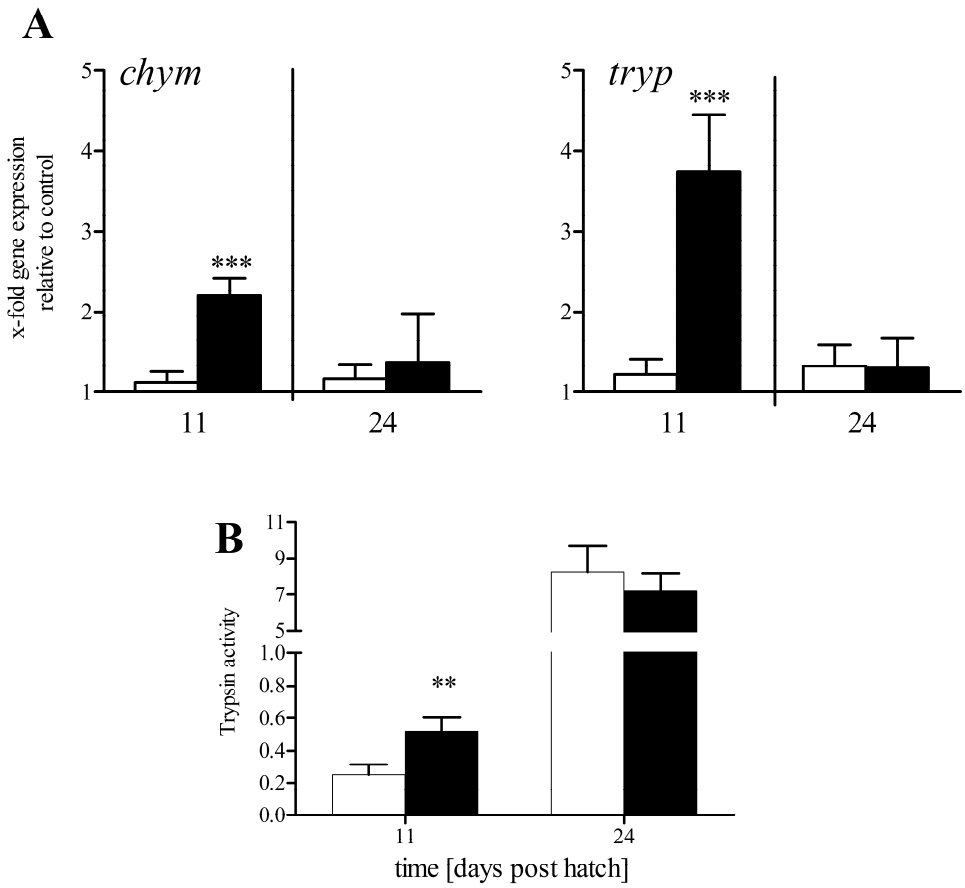
740 Figure 6: Proportional contribution of important pathogenic bacteria species to the larval
741 microbiota. ■: *Vibrio* spp, ■: *Aeromonas* spp., ■: Flavobacteria spp., □: others. ***:

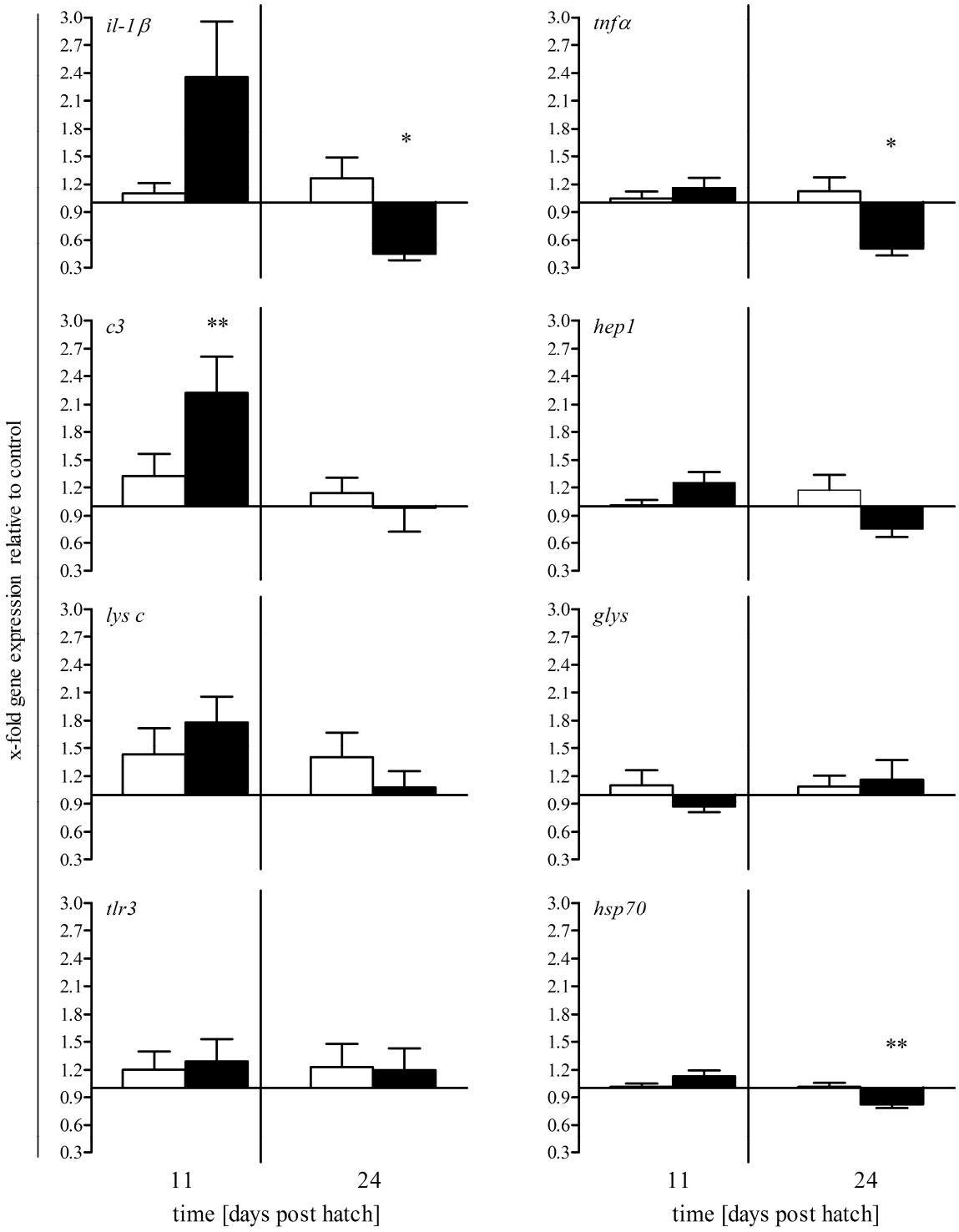
742 significantly different to the same treatment group at 11 dph with $p \leq 0.0001$ (i.e. significant
743 difference between sampling days).

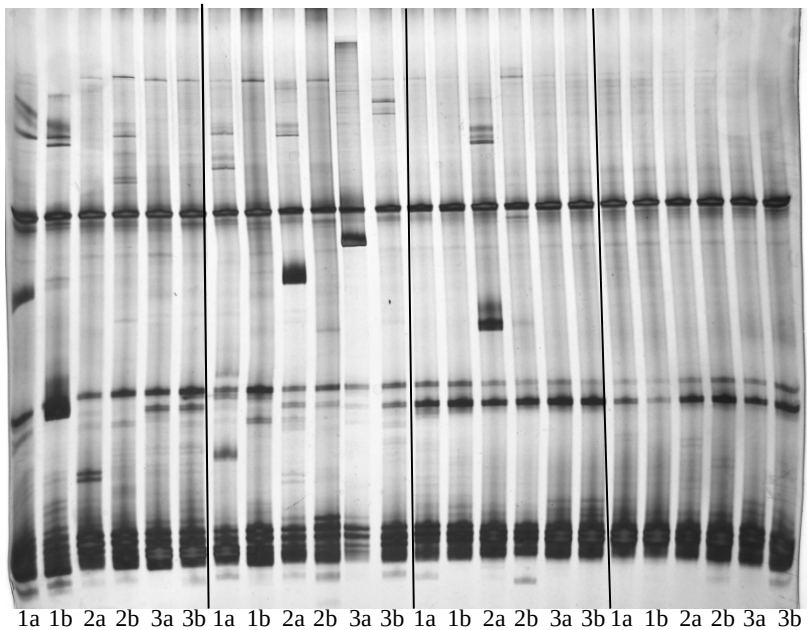
744

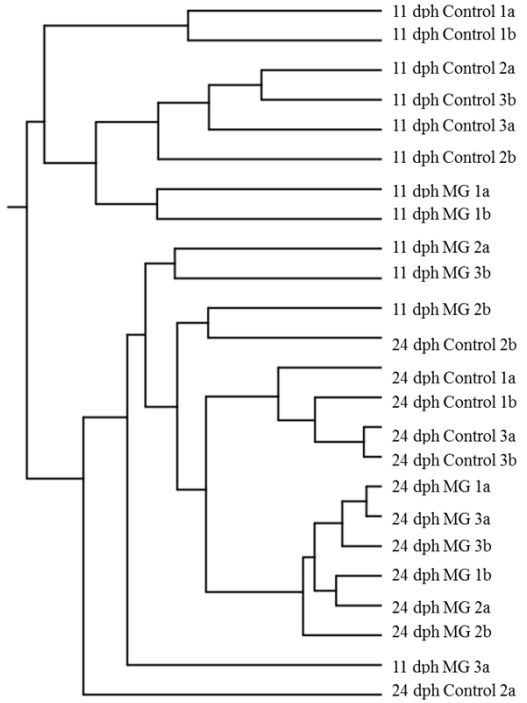

100
80
60
40
20
0
Survival [%]
3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24
Time (days post hatch)
Feeding scheme
Enriched rotifers
Untreated Artemia nauplii
Green water




A
chym
tryp
x-fold gene expression
relative to control
***
***
5
4
3
2
1
11
24
B
Trypsin activity
**
11
9
7
5
1.0
0.8
0.6
0.4
0.2
0.0
11
24
time [days post hatch]


il-1β
tnfα
c3
hep1
lys c
glys
tlr3
hsp70
*
*
**
**
3.0
2.7
2.4
2.1
1.8
1.5
1.2
0.9
0.6
0.3
x-fold gene expression relative to control
11
24
time [days post hatch]
11
24
time [days post hatch]


1a 1b 2a 2b 3a 3b 1a 1b 2a 2b 3a 3b 1a 1b 2a 2b 3a 3b 1a 1b 2a 2b 3a 3b
Control
MacroGard®
Control
MacroGard®
11 dph
24 dph


11 dph Control 1a
11 dph Control 1b
11 dph Control 2a
11 dph Control 3b
11 dph Control 3a
11 dph Control 2b
11 dph MG 1a
11 dph MG 1b
11 dph MG 2a
11 dph MG 3b
11 dph MG 2b
24 dph Control 2b
24 dph Control 1a
24 dph Control 1b
24 dph Control 3a
24 dph Control 3b
24 dph MG 1a
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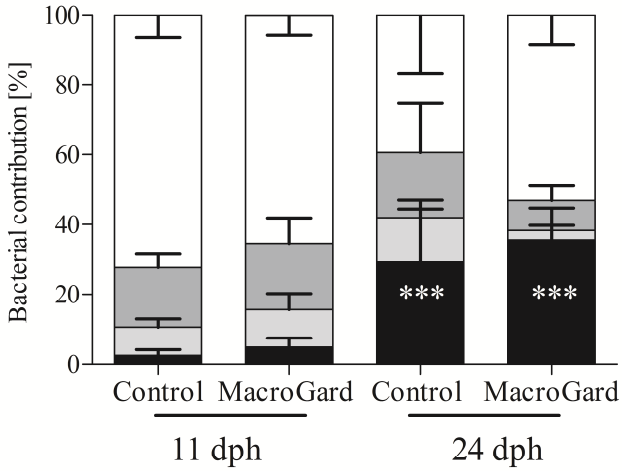
24 dph MG 2b
11 dph MG 3a
24 dph Control 2a



Highlights

- Dietary MacroGard® increases survival rate in turbot larvae
- MacroGard® feeding alters larval microbiota
- MacroGard® modulates genes involved in immunity, digestion and development
- MacroGard® enhances activity of digestive enzymes