Fish and Shellfish Immunology 89 (2019) 368–377

Contents lists available at ScienceDirect

Fish and Shellfish Immunology

journal homepage: www.elsevier.com/locate/fsi

Full length article

The gut mucosal barrier of zebrafish (*Danio rerio*) responds to the time-restricted delivery of *Lobosphaera incisa*-enriched diets

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ARTICLE INFO

Keywords:
Chrononutrition
Dietary lipids
Gut immunity
Microalgae
Mucosal immunity

ABSTRACT

Recent studies in mammalian models revealed compelling evidence that along with the intrinsic characteristics of diets, the time of their delivery could have a profound impact on their benefits. In this study, we explored a time-dependent modulation of the gut mucosal barrier by delivering diets enriched with the green microalga (*Lobosphaera incisa*) either in a time-restricted regime or randomly to zebrafish (*Danio rerio*). The basal diet was enriched with microalgal biomass through two inclusion levels (i.e., 10% and 15% w/w), and the feeding trial lasted for six weeks. The control group was fed with the basal diet. After collection of tissue samples at week 6, the remaining fish were challenged by intraperitoneal injection of *Streptococcus inaie*. A histological analysis of the gut structure revealed that the fish that received the microalgae randomly exhibited shorter villi length. Genes coding for immunity were modulated in the gut by dietary treatments. Notably, the transcript levels of *lysozyme, β-defensin* and *hepcidin* were significantly higher in the group subjected to the time-restricted feeding regime. Dietary microalgae affected the fatty acid content in the gut, particularly the level of arachidonic acid (ARA), and the time-restricted feeding influenced its accumulation. Groups that received diets enriched with 15% microalgae, regardless of the feeding strategy, displayed a significantly higher resistance to *S. inaie* 16 days post-infection, though differences between the delivery strategies were pronounced during the early stage of infection. In conclusion, the dietary inclusion of *L. incisa* modulated some of the features of the gut mucosal barrier of zebrafish, and the time of delivery appeared to have a considerable influence on immunomodulatory functions.

1. Introduction

Microalgae comprise a large group of photosynthetic organisms that are used as a nutritional source for many aquacultural species, particularly in larviculture. They contain essential biomolecules, such as amino acids, antioxidants, β-carotene, astaxanthin and long-chain polyunsaturated fatty acids (LC-PUFA), that support various biological functions [1]. Their value in aquaculture has increased dramatically in the last years, mainly as a consequence of the substantial change in fish diets for which alternative sources for fish meal are highly sought after.

The interplay between nutrition and the immune system is well recognised [2], and this has driven an immense interest in the strengthening of immunity through functional diets in the last decade. In addition to being an important protein source, as well as their remarkable ability to produce LC-PUFA, many microalgae species have been documented to possess immunomodulatory properties. Dietary administration of microalgae modulated the immune status of several farmed fish by targeting both the systemic and the local immunity, most markedly the cytokine cascade, bacterial defence and antioxidant capacity [3–5]. Microalgae are metabolically diverse, which plays a significant role in their ability to influence host immunity. Our knowledge about which microalgal metabolites potentially affect the immune response is still fragmentary, yet the role played by LC-PUFAs in the immunostimulation of fish is fairly well documented. This lipid group has long been established to have fundamental functions in maintaining fish health. The omega-6 (*n*-6) LC-PUFA arachidonic acid (ARA; 20:4*n*-6) is a crucial precursor of eicosanoids, lipid mediators with multiple effects on immune cells, most notably during inflammation [6]. These eicosanoids (i.e., prostaglandins, tromboxane, prostacyclins and leukotrienes) serve regulatory and homeostatic functions in the onset and resolution of inflammation, immune responses and tissue repair [7].

The oleaginous microalga *Lobosphaera incisa* is known to be the most abundant plant source of ARA with over 90% of total ARA being deposited in triacylglycerols [8]. Our laboratory has, through the years,

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https://doi.org/10.1016/j.fsi.2019.04.012
Received 26 January 2019; Received in revised form 3 April 2019; Accepted 5 April 2019
Available online 06 April 2019

been studying the immunomodulatory properties of *L. incisa*. Its dietary inclusion dramatically improved survival and stress resistance in guppy (*Poecilia reticulata*) [9,10] and disease resistance in zebrafish (*Danio rerio*) [11]. In our recent study with zebrafish [11], we have shown that the modulation of the expression of critical defence-related genes, as analysed in the kidney, had likely contributed to the immunological responses and eventual resistance to the pathogen *Streptococcus inaie*. It is yet to be evaluated whether the immunomodulatory properties of *L. incisa* can also elicit responses from mucosal surfaces and not solely from systemic immunity.

In this study, we performed a six-week-long feeding trial in which microalgae-enriched diets were administered daily to zebrafish in one of two temporal delivery strategies (i.e., time-restricted and randomly). Time-restricted delivery is based on the concept of chrononutrition in which the timing of feed intake and not only the nutritional value can impact the dietary benefits [12]. Moreover, the time-dependent delivery of immunostimulatory compounds takes into account the recent evidence that the magnitude of immune response to an exogenous stimulus is markedly influenced by the time of the day [13]. Therefore, we hypothesised that the gut barrier functions and disease resistance in zebrafish are dramatically influenced when diets enriched with microalgae possessing immunomodulatory feature is delivered at a specific time of the day rather than in a random manner.

2. Materials and methods

2.1. Ethics statement

The study was carried out in the Fish Health Laboratory at Ben-Gurion University of the Negev, Midreshet Ben-Gurion, Israel. All husbandry and experimental procedures complied with national and international legislations for the protection of animals used for scientific purposes (i.e., Directive 2010/63/EU). The experimental protocol was previously authorised by the Ben-Gurion University Committee for the Ethical Care and Use of Animals in Experiments, authorization no. IL-51-8-2008.

2.2. Zebrafish husbandry

Wild-type zebrafish (*Danio rerio* WT) were obtained from a local supplier. Upon arrival at the laboratory, fish were stocked in a 100-L container equipped with biological filters and aeration. A representative number of fish were subjected to routine bacteriological and parasitological examinations (i.e., swabs from internal organs for bacterial isolation and direct microscopic analysis of wet mounts from skin, gills and gut) to assess the health status of the stock. Fish were allowed to acclimate to laboratory conditions for 14 days under the following parameters: water temperature at 26 ± 1 °C, ammonia (0–0.2 mg L^{-1}), nitrite (0–0.025 mg L^{-1}), nitrate (0–10 mg L^{-1}), oxygen at above 80% saturation, and photoperiod at 12L:12D. The holding tank was siphoned every other day, and 10% of water exchange was carried out. During this period, fish were fed with a commercial diet (Ocean Nutrition, Essen, Belgium: 43,6% crude protein, 11,2% crude fat, 1,2% crude fibre, 15,0% moisture, 9,0% ash, 1,0% phosphorus) at about 2% of their body weight (BW) per day.

A week before the feeding experiment commenced, fish were transferred to 18 30-L experimental aquaria, 30 fish per aquarium. Each aquarium was equipped with biological filters, and the husbandry described for the acclimation period was similarly applied.

2.3. Production of Lobosphaera incisa WT and experimental diets

The microalga *Lobosphaera incisa* WT was obtained from the culture collection of the Microalgal Biotechnology Laboratory at Ben-Gurion University of the Negev [8]. The alga was cultivated for 14 d in 1-L glass columns in a nitrogen-depleted BG-11 medium, and thereafter, the algal biomass was broken and prepared as described by Dagar et al. [9]. The dried microalgal powder was added to the commercial fish feed (Ocean Nutrition) at two inclusion levels: 10% and 15% w/w. In brief, the microalgal biomass was added to a powdered basal diet followed by the addition of chilled double distilled water in a stepwise manner, until a paste-like consistency was achieved. The mixture was freeze-dried in a lyophiliser (Virtis, Gardiner, N.Y. Model 10-MR-TR) and then passed through a sieve with a mesh size of 500 µm. The basal diet that served as the control feed had undergone the same preparation procedure, but without microalgal inclusion. The diets were stored at −20 °C throughout the experiment to avoid lipid peroxidation.

2.4. Feeding experiment and sample collection

Each experimental diet was administered to fish entrained to a 12L:12D photoperiod (lights on at 06:00; lights off at 18:00) either in a time-restricted manner or randomly. In the time-restricted group, the daily ration of 2% body weight was given at exactly 10:00 a.m. throughout the experimental period. On the other hand, the same daily ration was given to the other group as randomly as possible during the daily cycle. Each experimental group had three replicate tanks. Feeding was synchronised 2 d before sample collection to ensure that both groups were not fed 24 h prior to sampling.

On sampling day, three fish were taken from each replicate tank and were euthanised with an overdose of clove oil (250 ppm) [11]. The intestine was collected and divided for the following downstream applications: 1) the anterior portion was fixed in 10% neutral buffered formalin for 24 h then transferred to 70% ethanol for histology; 2) the middle portion was transferred to a 1.5-ml centrifuge tube, and snap-frozen in liquid nitrogen for gene expression analysis; and 3) the remaining portion was transferred to another 1.5-ml centrifuge tube, and snap-frozen for fatty acid analysis.

2.5. Streptococcus iniae challenge

Next, 24 h after sampling, the remaining fish (n = 25) were subjected to a *Streptococcus iniae* challenge. The inoculum was prepared by streaking thawed bacterial stock onto Tryptic Soy Agar (Oxoid, Hampshire, UK), and the plate was incubated at 25 °C. A single colony was selected and inoculated into freshly prepared Tryptic Soy Broth (Oxoid) and incubated with shaking at 25 °C to the mid-logarithmic phase of growth (OD_{600nm}–0.250, corresponding to 10^8 cells mL^{-1}). A bacterial pellet was collected by centrifugation at 1500 × *g* for 10 min at 4 °C and thereafter washed and resuspended in sterile phosphate buffered saline (PBS). The final inoculum concentration of 10^3 cells mL^{-1} (previously standardised as the LC_{50} for zebrafish) was obtained by serial dilution. Bacterial cell concentration was retroactively confirmed on TSB plates. Sedated (clove oil) experimental fish were intraperitoneally injected with the inoculum in a 100-µl delivery volume. Fish mortality was recorded for two weeks.

2.6. RNA isolation, cDNA synthesis and quantification of transcript levels by real-time quantitative PCR

Total RNA from the intestine was extracted using the SV Total RNA Isolation System (Promega, Madison, WI, USA). The RNA was then quantified with a Nanodrop ND-1000 Spectrophotometer (Thermoscientific, CO, USA) and its quality assessed by electrophoresis on a 1.2% (w/v) agarose gel. First strand complementary DNA (cDNA) was synthesised from 1 µg/mL total RNA by a Verso cDNA Synthesis Kit (Thermoscientific).

All primers used in the study were earlier reported and verified (Table 1). The changes in the transcript levels of immune defence genes were quantified by real-time PCR (qPCR) using SsoAdvanced™ Universal SYBR® Green Supermix master chemistry (Bio-Rad®, CA, USA) on a Bio-Rad® CFX96 thermocycler. Diluted cDNA was run in duplicate,

Table 1
Primers used in the study.

Gene name	Abbreviation	Sequence (5′ → 3′)	Accession number	Amplification efficiency (%)	Reference
A. Target genes					
Toll-like receptor 22	*tlr22*	F: CCAGCTCTCGCCGTACCA R: TTGGGCCAGCGGATGT	AY389460	92.4	[15]
Interleukin 1β	*il1β*	F: CATTTGCAGGCCGTCACA R: GGACATGCTGAAGCGCACTT	AY340959	97.7	[15]
Nuclear factor kappa-light-chain-enhancer of activated B cells	*nfκb*	F: CGCAAGTCCTACCCACAAGT R: ACCAGACTGTGAGCGTGAAG	NM_001353873	103.3	[16]
Tumor necrosis factor alpha	*tnfa*	F: CCATGCAGTGATGCGCTTT R: TTGAGCGGATTGCACTGAAA	AY427649	98.2	[15]
Complement component 3 b	*c3b*	F: CAGTGGGAATATGTTGGCATTG R: TTAGCTGCCCTTCATAACCTGTT	AF047414	97.0	[15]
Hepcidin	*hep*	F: CCTGGCTGCTGTCGTCAT R: TGGTTCTCCTGCAGTTCTTCAC	AY130989	96.7	[17]
Beta-defensin	*defb*	F: AATGTGCATAATGCCGAAGTACA R: ACAACCATGGTGAGCAACAATATATT	NM_001081554	99.5	[18]
Alkaline phosphatase	*alp*	F: GAGCCAGCAGACCTGAACTA R: CAATGCGTCCACCTTCCA	BC052139	97.6	[19]
Lysozyme	*lys*	F: AGGCTGGCAGTGGTGTTTTT R: CACAGCGTCCCAGTGTCTTG	NM_139180	98.0	[15]
Catalase	*cat*	F: AGGGCAACTGGGATCTTACA R: TTTATGGGACCAGACCTTGG	AF170069	100.6	[20]
Glutathione peroxidase 3	*gpx3*	F: TCCAGGAAATGGATTCGTTC R: TCTCTCCTACAGGCGGACAT	NM_001137555	95.7	[21]
B. Reference genes					
Beta-actin	*actb*	F: CGAGCAGGAGATGGGAAC R: CAACGGAAACGCTCATTGC	AF057040	96.2	[15]
Elongation factor 1-alpha	*elfa*	F: CTTCTCAGGCTGACTGTGC R: CCGCTAGCATTACCCTCC	AY422992	101.4	[22]
Tubulin	*tub*	F: CCTGCTGGGAACTGTATTGT R: TCAATGAGTTCCTTGCCAAT	AF029250	100.3	[23]

including minus reverse transcriptase and no template controls. The qPCR reaction was performed according to the following thermocycling protocol: initial denaturation at 95 °C for 3 min, followed by 39 cycles of 10 s at 95 °C and 20 s at 60 °C. A five-point standard curve of a 2-fold dilution series was prepared from pooled cDNA to calculate amplification efficiencies. Cycle threshold (CT) values were calculated with the Bio-Rad® CFX96 software. Three genes were evaluated for the normalisation of the expression data. *Elongation factor alpha* (*eefa*) and *tubulin* (*tub*) were identified to be highly stable in all samples, and their geometric average generated from geNorm [14] was used for normalisation.

2.7. Quantitative histology

Gut tissue samples, fixed in 10% neutral buffered formalin and then transferred to 70% ethanol, were processed in a microwave histoprocessor (RHS-1, Milestone, Italy). Tissues were embedded in paraffin wax blocks, sectioned (5 μm), and stained with haematoxylin and eosin (HE), and then the sections were photographed (Axioskop microscope and AxioCam MRc5; Zeiss, Oberkochen, Germany). A quantitative histomorphometry was performed in AxioVision Rel. 4.8 (Zeiss) by measuring the villi length and width, the lamina propia width, and the number of mucus cells.

2.8. Fatty acid analysis

The experimental diets and the fish gut samples collected after the six-week feeding trial were freeze-dried, weighed and directly transmethylated by adding 2 ml of a mixture of 2% H_2SO_4 in dry methanol (v/v) with 0.01% BHT (butylated hydroxytoulene) under an argon atmosphere. The fatty acid C17:0 (0.5 mg mL^{-1} in petroleum ether) was used in the reactions as an internal standard. The reaction mixture was incubated at 80 °C for 90 min, and fatty acid methyl esters (FAMEs) were then extracted by addition of *n*-hexane followed by vortexing and centrifugation at 3000 rpm for 5 min at RT. The supernatant was collected and evaporated under nitrogen to dryness. Finally, GC analyses of FAMEs were carried out using a Trace Ultra gas chromatograph (Thermoscientific) equipped with a programmable temperature vapourising injector, a flame ionisation detector, and a capillary column (Supelco-Wax, Sigma Aldrich, USA) as described earlier [10].

2.9. Statistical analyses

All statistical analyses were performed in Sigma Plot 13 (Systat Software Inc., USA). The data on gene expression, relative FA compositions, and histomorphometry were subjected to a one-way ANOVA after complying with the requirements for normal distribution and equal variance. Differences between treatment groups were further identified by Tukey's multiple comparison test. For data sets that did not follow a Gaussian distribution or did not meet the equal variance requirements, a Kruskal-Wallis one-way ANOVA on ranks followed by Dunn's multiple comparison test was used instead. The level of significance was set at $p < 0.05$. The Kaplan–Meier survival analysis was performed to compare differences in mortality rates between treatment groups following the streptococcal challenge. The level of significance was set at $p < 0.05$.

3. Results and discussion

The gut is one of the key mucosal organs in teleost fish [24]. In addition to its role in metabolism and nutrient absorption, it represents the interface between the internal and external environments and serves as an indispensable defence organ. Gut health is remarkably influenced by nutrition; hence, modern fish husbandry adopts practices that boost mucosal barrier functions through nutritional manipulations. In this study, we have shown that the gut mucosal barrier responded to microalgal inclusion in the diets. Notably, the transcription of several bacterial defence genes was significantly affected by the microalgal

diets, and these changes may have contributed to the observed improved resistance against streptococcal infection. Moreover, *L. incisa* is an excellent exogenous source of ARA, and its dietary inclusion resulted in a dramatic increase of the ARA level in the gut.

Chrononutrition is an emerging field in nutritional science. It builds on the intimate connection between endogenous circadian rhythms and metabolism [25]. The concept emphasises that the time of delivery impacts the nutritional value of the diets, in addition to the balanced nutrients/food components. Most of the studies on chrononutrition address the way that restricted feeding affects the circadian control of metabolism, thereby imparting a crucial regulatory role in the prevention of metabolic disorders—for example, obesity from a high fat diet [12,26,27]. Metabolic activity follows a daily cycle, and targeting an intake time when the physiology most efficiently harnesses and processes the dietary constituents is beneficial to the host. Fish immunity likewise follows a circadian order, and its ability to mount a response is influenced by the time of the day [13,28]. Interestingly, little is known about whether immune responses can be altered by applying a time-dependent immunomodulation through diets. In this study, we have shown that the time-restricted delivery of diets enriched with microalgae possessing immunomodulatory properties can manipulate gut barrier functionality, particularly modulating the antibacterial state of the gut. Moreover, the dynamics of *S. inaie* infection was altered in zebrafish subjected to the time-restricted feeding of microalgae-enriched diets.

3.1. *Lobosphaera incisa* WT-enriched diets modulate the expression of immune defence-related genes in the gut

Toll-like receptors (TLRs) are a class of pathogen recognition receptors (PRRs) that elicit specific responses against pathogens upon recognising pathogen-associated molecular patterns (PAMPs) [5], and Tlr22 is considered to be one of the most highly diverse “teleost-specific Tlr” families [29]. The dietary administration of *L. incisa* significantly altered the expression of *tlr22* in the group that received 15% microalgae in a time-restricted delivery strategy, for which the transcript level was higher by 60% and 37% than the control ($P = 0.039$) and the 10% ($P = 0.046$) microalgae groups, respectively (Fig. 1A). Moreover, there was a significant difference ($P = 0.033$) in the *tlr22* transcript level between feeding strategies in the group fed with 15% microalgae; the group that received the diet time-restricted showed a 2-fold higher *tlr22* expression compared with the group fed randomly. Our previous trial using the same microalgae in zebrafish showed that *tlr22* expression in the kidney was either unaffected or downregulated by dietary treatments [11]. It is plausible that the modulatory functions of *L. incisa* are more potent in the gut mucosa where interaction between tissue and dietary components is high. *Tlr22* responds to a variety of PAMPs including bacterial cell wall, dsRNA, etc. [30], and the present results revealed that it is also responsive to microalgal components, and more interestingly, the response is influenced bydelivery strategies. It is yet to be established to which microalgal component *tlr22* responds, but it is possible that it is to ARA since this is the only known variable component between the diets. Cytokines possess pleiotrophic functions, and they are well-known as crucial mediators of innate immunity. ARA is considered a pro-inflammatory LC-PUFA. Hence, one can expect that the key mediators of the pro-inflammatory cascade would be affected by its high level in the diet. Dietary microalgae did not have significant effects on the expression of three cytokine-related genes in the gut (Fig. 1B, C, D). In the time-restricted group, it is interesting to observe that the transcript level of the pro-inflammatory cytokine *il1β* had the tendency to decrease as the microalgal inclusion level increased. The 15% microalgae group that received the diet in a time-restricted manner showed significantly lower *il1β* expression than the group fed randomly ($P = 0.044$). This suggests that *L. incisa*-mediated *il1β* modulation may likely not be dependent on the inclusion level but rather the mode of delivery, and this may have significant impact on avoiding ARA-induced inflammation in the gut. Nonetheless, the study found no signs of enteric inflammation, thus corroborating the *il1β* expression data. The expression of *c3b*, a key molecule in the complement cascade, remained unchanged in all treatment groups, substantiating our previous results found in the kidney [11].

Bacterial defence factors are crucial components of the immunological repertoire in the gut. *Hepcidin* and *β defensin* are members of a large class of antimicrobial peptides (AMPs) that protect against potential pathogens. They are ubiquitously expressed in teleost, and their presence in mucosal tissues emphasises their role in defence [31]. They are involved in other biological functions, such as iron regulation for *hepcidin* [32] and the mediation of host-microbiota interaction and chemotaxis of immune cells for *β defensin* [33,34]. The transcription of both *hep* and *defb* in the gut was significantly modulated by dietary administration of *L. incisa*, especially in the groups that received the diet enriched with 15% microalgae (Fig. 2A and B). Expression of *hep* significantly increased by at least 60% in fish that received the diet with 15% microalgae compared with the 10% microalgae ($P = 0.022$) and control ($P = 0.030$) diet-fed fish in the time-restricted group (Fig. 2A). Moreover, the groups that received diets enriched with 15% microalgae but delivered differently displayed striking significant differences ($P = 0.015$)—*hep* expression was 40% higher in the group that received the diet enriched with 15% microalgae in a time-restricted strategy than in the group that received the same diet randomly. On the other hand, *defb* transcript levels significantly changed in randomly ($P = 0.020$) and time-restricted ($P = 0.018$) fed fish groups. In the groups that received 15% microalgae in the diet, *defb* expression significantly increased by 36% in randomly fed group while an increase of 25% was identified in the time-restricted groups, when compared with their respective controls. Furthermore, the expression of *defb* in the group fed with 15% microalgae was significantly higher ($P = 0.022$) than the group that received 10% microalgae in the time-restricted group. However, we did not observe significant differences in the expression between delivery strategies. Several studies in fish have already shown that *defb* expression responded to microalgal inclusion in the diets [4,35], and the current study corroborates this association; moreover, it offered compelling insights into the influence of intake timing on the observed immunomodulatory functions. Lysozyme is a ubiquitous antibacterial molecule in teleost and is one of the most studied components of innate immunity [36]. It possesses strong hydrolytic activity that targets the β1→4 glycosidic bond between the *N*-acetylglucosamine and *N*-acetylmuramic acid residues of many bacterial cell walls. The expression of *lys* was modulated by microalgae-enriched diets in the time-restricted group ($P = 0.048$) but not in the randomly fed group (Fig. 2D). Specifically, the transcript level of *lys* increased by almost 50% in fish fed with 15% microalgae compared with the control group. When comparing delivery strategies, the groups that received the diets in a time-restricted manner showed significantly higher *lys* expression ($P = 0.038$ for 10%; $P = 0.034$ for 15%) than the randomly fed group, regardless of the inclusion level. Lysozyme is both a direct effector and a synergist of AMP activity. Its lytic activity allows AMPs to enter and permeabilise the cell [37]. Dietary microalgae affected the expression of both AMPs (i.e., *hep* and *defb*) and *lys*, and the synergy previously described may likely occur and influence the overall defence mechanism in the gut. Additionally, the influence of time-restricted delivery on the expression of these immune genes indicates that they may exhibit temporal sensitivity. Though this is not a well-explored aspect of fish immunity [13,28], our current understanding suggests that the ability of the immune system to mount a response is time-dependent, thus perhaps accounting for why some genes were modulated more when the immunomodulatory compound was delivered in a specific time frame. Even though the expression of *alp* appeared to be affected by microalgal feeding in the randomly fed fish, the changes were not statistically significant (Fig. 2C).

Dietary additives can sometimes cause tissue oxidative stress. During this condition, cells produce antioxidant molecules, such as

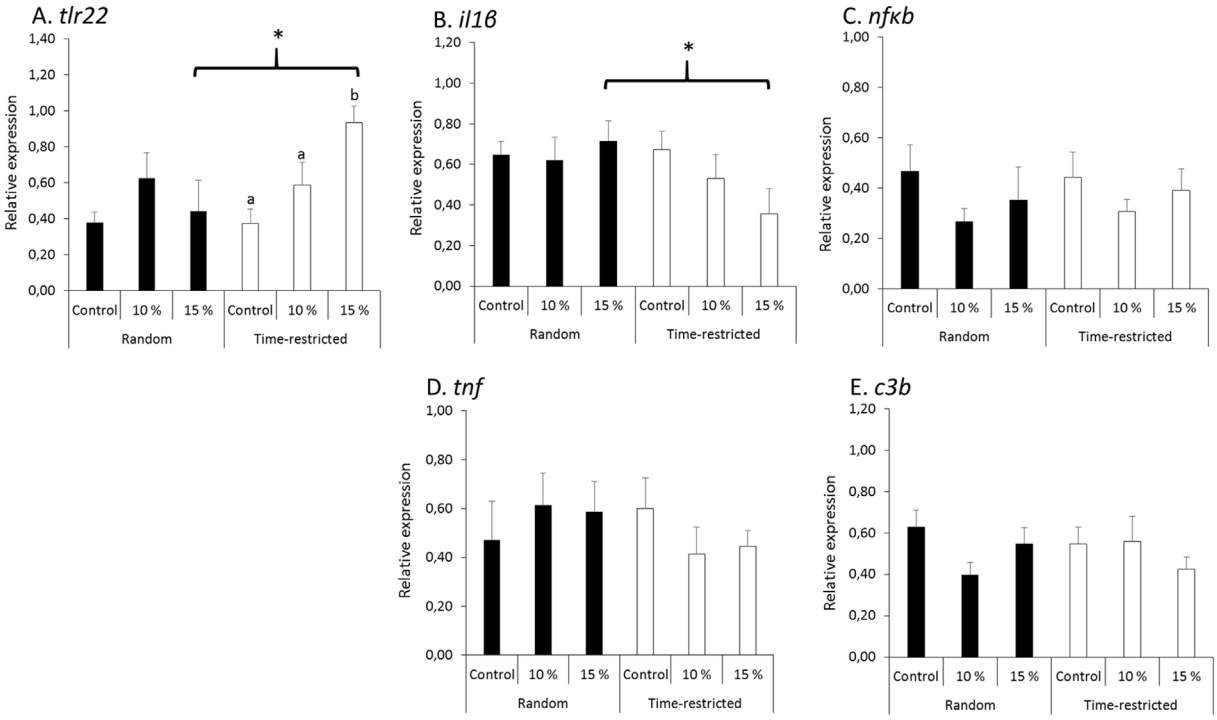

Fig. 1. Expression of *tlr22*, cytokines and complement genes in the gut of zebrafish fed with diets enriched with *Lobosphaera incisa* WT and delivered either randomly or in a time-restricted manner. Relative transcript levels are presented as mean ± SD from six individual fish. Asterisks (*) represent significant differences between groups that received the same inclusion level of the microalgae but delivered in a different manner. *tlr22*: toll-like receptor 22; *il1β*: interleukin 1β; *nfκb*: nuclear factor kappa-light-chain-enhancer of activated B cells; *tnf*: tumor necrosis factor; *c3b*: complement component 3.

catalase, superoxide dismutase (SOD), and glutathione peroxidase, to counteract the presence of reactive oxygen species in the immediate environment. High levels of ARA may be toxic to cells and may cause oxidative stress [38]. The transcript levels of *cat* and *gpx3* were not affected by the dietary treatments (Fig. 3). Both inclusion levels and the delivery strategy resulted in insignificant changes to the expression of these marker genes. This indicates that the normal redox state of the gut was not disturbed by the dietary treatments and, further, did not cause oxidative stress. This observation is corroborated by the histology as no serious tissue damage was observed (Section 3.2).

3.2. Morphometry of gut barrier features in microalgae-fed zebrafish

The gut was in a good health condition as no major histopathological changes were documented in any treatment group. As a very plastic organ, mucosal surfaces such as the gut undergo phenotypic alterations as a response to dietary changes [24], including microalgal supplementation [39]. These alterations are usually associated with the adaptive response of the epithelial barrier functionality and for efficient nutrient uptake. We performed a quantitative histomorphometry of key gut features including villus length and width, lamina propia width and number of mucus cells (Table 2). The overall results revealed that the mucosal barrier features were not dramatically affected by dietary feeding. Nonetheless, it is interesting to observe that increasing the dietary inclusion level of microalgae resulted in significantly shorter villi length ($P = 0.021$), particularly when the diet was delivered randomly. Average villus length decreased by at least 35% in the microalgae-enriched group compared with the control ($P = 0.004$ for 10%; $P = 0.003$ for 15%). Earlier reports indicate that the shortening of villi affected the absorptive capacity of the gut [40]. We do not have sufficient data to expound upon to what extent villi shortening affects the absorptive capacity of the gut, but this may have some implications in tissue ARA accumulation, which is discussed further in Section 3.3. We can further speculate that shortening of the villi may be a negative adaptive response in the randomly-fed group to erratic feeding schedules.

3.3. Lipid profile in the gut is affected by diets and delivery strategy

Fatty acid analysis revealed that the inclusion of microalgae altered some of the fatty acid components of the basal diet (Table 3). The most abundant fatty acids in the basal diet included palmitic acid (C16:0), oleic acid (C18:1*n*-9) and linoleic acid (C18: 2*n*-6). Microalgae inclusion resulted in a decrease in the levels of palmitic acid and docosahexaenoic acid (C22:6*n*-3) in the diets. Nonetheless, the levels were still within the range that supports the normal physiological functions of many fish species [41,42]. The oleaginous microalga *L. incisa* is known to contain high levels of ARA, and its inclusion increased the ARA level about 15-fold and 23-fold in the 10% and 15% microalgae inclusion groups, respectively, compared with the basal diet.

We likewise subjected the gut of the experimental fish to a fatty acid (FA) analysis to determine whether the changes in FA in the diets would also be reflected in the tissue, particularly in the ARA levels. Palmitic acid and oleic acid (C18:1*n*-9) are two of the most abundant FAs in the gut of zebrafish, and their levels did not change following dietary manipulation (Table 4). These fatty acids constituted at least 50% of the total fatty acid composition in the gut. The levels of ARA in the gut were significantly affected by the dietary inclusion of *L. incisa* (Fig. 4). In the randomly fed group, fish that received the diet with 15% microalgae registered a significant increase ($P = 0.047$) of about 62% in their gut ARA content compared with the control group. Although there was also an obvious increase in fish fed with 10% microalgae, the changes were not significant compared with the control and the 15% microalgae-enriched groups. In the time-restricted group, there was a clear tendency that the increase in the level of ARA in the diet also resulted in a significant increase ($P = < 0.001$) of tissue ARA content. The ARA level in the gut of fish fed with 10% microalgae was 41.6% significantly higher ($P = 0.017$) than in the control. The increment was more

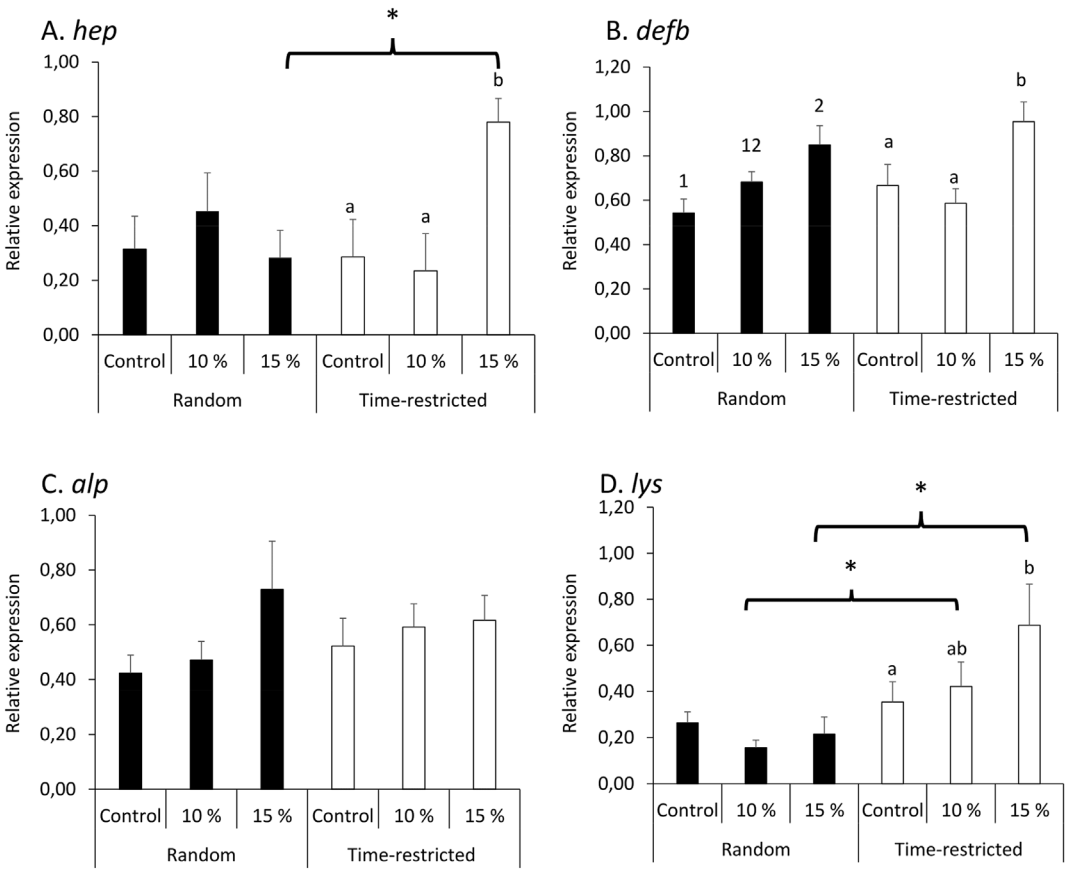

Fig. 2. Expression of bacterial defence genes in the gut of zebrafish fed with diets enriched with *Lobosphaera incisa* WT and delivered either randomly or in a time-restricted manner. Relative transcript levels are presented as mean ± SD from six individual samples. Different numbers indicate significant differences between treatment groups within the randomly fed group, whereas different letters refer to significant differences between treatment groups within the time-restricted group. Asterisks (*) represent significant differences between groups that received the same inclusion level of the microalgae but delivered differently. *hep*: hepcidin; *defb*: defensin; *alp*: alkaline phosphatase; *lys*: lysozyme.

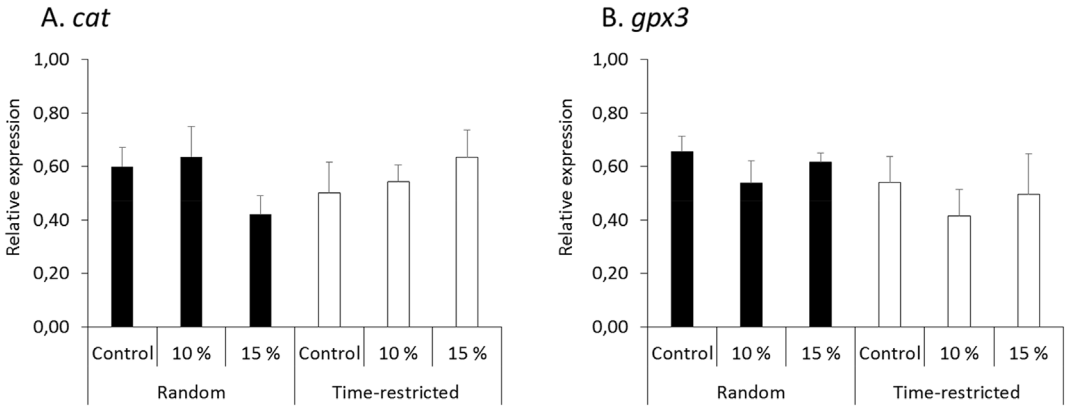

Fig. 3. Expression of antioxidant defence genes in the gut of zebrafish fed with diets enriched with *Lobosphaera incisa* WT and delivered either randomly or in a time-restricted manner. Relative transcript levels are presented as mean ± SD from six individual samples. *cat*: catalase; *gpx3*: glutathione peroxidase.

striking in the group fed with 15% microalgae, for which the difference from the control group was about 63.8% higher (P = < 0.001). There was also a significant difference between the 10% and 15% microalgae-enriched groups, for which the ARA level in the latter was significantly higher (P = 0.005) by 38% than in the former. The tissue enrichment of ARA as a consequence of its increased level in the diet suggests that the fish were able to efficiently accrue the FA. Higher levels of ARA may influence the physiological processes in the gut for which ARA plays a key mediator role—for example, inflammation and membrane integrity, among many others [43]. Moreover, the increased ARA level in the gut may partly explain the heightened antibacterial state in the mucosa, as this LC-PUFA is known to be a potent immunological stimulus [7,38]. The shortening of the intestinal villi (Table 2) in the randomly fed fish did not show any significant influence on the ARA absorptive potential of the gut, as the ARA content of the fish from this group was similar to that of the time-restricted group. We cannot exclude the possibility, however, that villi shortening may negatively impact the absorption of other dietary constituents that were not quantified in the current study. Nonetheless, if there was malabsorption of nutrients following villi shortening in the microalgae-fed fish, the

Table 2
Morphometry of gut barrier features.

	Random			Time-restricted		
	Control	10%	15%	Control	10%	15%
Villus length (μm)	273 ± 58[a]	184 ± 50[b]	178 ± 47[bc]	204 ± 40	175 ± 31	200 ± 48
Villus width (μm)	82 ± 22	64 ± 18	66 ± 9	75 ± 11	70 ± 15	76 ± 23
Lamina propia width (μm)	46 ± 13	35 ± 7	39 ± 5	45 ± 9	36 ± 7	70 ± 9
Mucus cells[a]	8 ± 2	8 ± 3	5 ± 4	7 ± 2	8 ± 2	6 ± 2

[a] mucus cell number is expressed as the number of mucus cells per mucosal fold. NOTE: different letters indicate significant difference.

Table 3
Fatty acid composition and content (%) of the basal and the microalgae-enriched diets used in the study. *L. incisa* WT biomass was incorporated in the basal diet at two levels (10%; 15% w/w). Values are shown as % of total fatty acid (TFA).

Fatty acid	Control	10%	15%
14:0	3.5	3.0	2.8
16:0	17.7	16.4	15.8
16:1	4.1	3.4	3.1
16:2	0.3	0.3	0.3
16:3	0.3	0.5	0.6
16:4	0.3	0.3	0.2
18:0	3.1	2.9	2.8
18:1*n*-9	16.1	15.7	15.6
18:1*n*-7	2.6	2.8	2.9
18:2x	0.3	0.2	0.2
18: 2*n*-6	18.0	16.8	16.8
18:3*n*-6	0.1	0.2	0.3
18:3*n*-3	2.6	2.4	2.3
18: 4*n*-3	1.5	1.3	1.2
20:0	0.3	0.2	0.2
20:1	4.4	3.8	3.3
20: 2	0.3	0.4	0.4
20:3*n*-6	0.1	0.2	0.3
20:4*n*-6 (ARA)	0.6	8.5	12.6
20:3*n*-3	0.2	0.2	0.2
20:4*n*-3	0.5	0.4	0.4
20:5*n*-3	6.3	5.5	5.0
22:0	0.4	0.4	0.4
22:1	5.1	4.2	3.8
22:4*n*-6	0.2	0.3	0.3
22:5*n*-3	0.9	0.7	0.6
22:6*n*-3	9.5	8.1	7.1
%TFA/dw	15.6	17.3	18.6
ARA (mg/g)	1.0	14.7	23.3

changes were likely not that crucial as the fish remained healthy and robust, as supported by the survival data after the pathogen challenge. Though it appeared that increased levels of ARA in the gut affected the ratio of DHA and EPA, the changes observed were not statistically significant.

3.4. Dietary microalgae improve disease resistance and alter the infection dynamics

S. inaie is an opportunistic pathogen in the ornamental fish industry. Earlier reports from our laboratory demonstrated that administration of *L. incisa* had a dramatic influence on the resistance of ornamental fish, such as guppy and zebrafish, to challenging conditions (i.e., stress, bacterial infection) [9–11]. We followed the mortality for two weeks after experimental infection with *S. inaie* and found that the fish fed the microalgae-supplemented diets had survival rates higher than 50% (Fig. 5). Groups that received diets with 15% microalgae displayed the highest survival rates amongst the groups, regardless of the delivery strategy. The result supported the previous trial that showed improved protection against streptococcal infection following the dietary administration of *L. incisa* [11]. The group that received the control diet in a time-restricted manner demonstrated the lowest survival rate (40.5%), though it was not statistically different from the randomly fed control group. It appeared that the groups with 10% microalgae displayed higher survival rates than the control groups, nonetheless, the difference was not statistically significant. The delivery strategy had no noticeable impact on fish survival 16 d post-infection. However, striking differences were observed in the first week post-challenge. Survival rates in all microalgae-fed groups (above 75%) were significantly higher ($P = 0.041$ for 19%; $P = 0.038$) than in the control groups (approximately 68%) at 7 d post-infection, regardless of the inclusion level and delivery strategy. At 8 d post-infection, a prominent difference was observed within the groups fed with 10% microalgae for which fish fed in a time-restricted manner demonstrated a significantly higher ($P = 0.043$) survival rate than their randomly fed counterparts. This tendency was pronounced until day 10. Collectively, the survival profiles showed that microalgal supplementation, particularly at the 15% inclusion level, significantly improved the resistance against streptococcal infection. These observations lend support to previous claims that dietary microalgae can improve the disease resistance of several farmed fish, hence their estimation as beneficial dietary supplements in addition to being a promising protein source [1,9,11,44]. Microalgae have both antibacterial and immunostimulatory properties [44], and these beneficial features likely contribute to the improved disease resistance. We have observed an enhanced antibacterial state in the gut as shown by the elevated transcription of bacterial defence genes following dietary treatment, and this may have played a significant role in priming the immunity against bacterial infection. Though the delivery strategy did not show remarkable impacts on these observations at the later stage of infection, the influence was striking in the early stage, especially in the group fed with 10% microalgae for which the time-restricted delivery exerted a significantly higher resistance to the pathogen. Moreover, the survival profiles demonstrated that infection-related mortality can be delayed by the inclusion of microalgae in the diet. To our knowledge, this is the first report in fish to have shown that the time of delivery of immunostimulatory diets exhibited an influence on the infection dynamics, especially in the early stage.

3.5. Conclusion

In modern fish husbandry, nutritional immunomodulation is practised as a means of promoting health and improving disease resistance. Current strategies target the mucosal barriers as mounting evidence points to their importance in the overall health of farmed fish. The present study has added new evidence for the immunomodulatory properties of the microalga *L. incisa*. The results revealed that its potential to modulate the functions of the gut mucosal barrier can be manipulated not only by its level in the diet but also by the time of delivery. Time-restricted feeding remarkably affected the magnitude of immunostimulatory functions in the gut, especially in altering the antibacterial state. To our knowledge, this is the first report in fish to have employed the concept of chrononutrition, particularly time-restricted feeding, in dietary immunostimulation. The data presented offer relevant insights into promising methods of how to promote health

Table 4
Fatty acid composition (% of TFA ± SD) and content in the gut of zebrafish fed with the basal and microalgae-enriched diets for six weeks either randomly or in a time-restricted manner.

Fatty acid	Random			Time-restricted		
	Control	10%	15%	Control	10%	15%
14:0	2.8 ± 0.4	3.6 ± 1.0	4.3 ± 2.4	4.6 ± 3.0	2.0 ± 0.5	3.1 ± 0.3
14:1	1.7 ± 0.2	1.8 ± 0.2	1.5 ± 0.2	1.6 ± 0.2	1.9 ± 0.2	1.3 ± 0.1
15:0	0.4 ± 0.1	0.6 ± 0	0.5 ± 0.1	0.6 ± 0.2	0.5 ± 0.1	0.5 ± 0
16:0	20.9 ± 0.2	18 ± 0.1	17.6 ± 0.9	20.1 ± 0.7	20.3 ± 1.7	19.5 ± 0.9
Σ16:1[a]	3.8 ± 0.4	3.5 ± 0.8	2.6 ± 1.5	2.8 ± 0.3	3.9 ± 0.4	2.6 ± 0.1
16:2	0.1 ± 0	0.2 ± 0	0.2 ± 0	0.2 ± 0.1	0.2 ± 0.1	0.1 ± 0
16:3	0.3 ± 0	0.4 ± 0	0.4 ± 0.1	0.4 ± 0	0.4 ± 0.1	0.5 ± 0
18:0	6.5 ± 1.3	5.1 ± 1	5.9 ± 0.7	7 ± 0.4	5.1 ± 0.7	7.1 ± 0.4
18:1*n*-9	29.4 ± 6.1	31.7 ± 2.1	33.8 ± 3.7	26.3 ± 5.5	33.8 ± 4.2	28.4 ± 2.4
18:1*n*-7	1.7 ± 0.4	2.2 ± 0.2	1.7 ± 0.2	2.1 ± 0	2 ± 0.3	1.8 ± 0.2
18:2x	0.2 ± 0	0 ± 0	0.2 ± 0.1	0.3 ± 0.1	0.2 ± 0.1	0.2 ± 0
18:2n-6	10.4 ± 0.3	12 ± 0.8	10 ± 0.8	10.6 ± 0.4	11.8 ± 0.6	9.7 ± 0.5
18:3*n*6	0.2 ± 0	0.4 ± 0.1	0.3 ± 0.1	0.1 ± 0.1	0.3 ± 0.1	0.3 ± 0
18:3*n*3	1.2 ± 0.2	1.3 ± 0.2	1.1 ± 0.1	1.1 ± 0.2	1.1 ± 0.5	0.8 ± 0.3
18:4*n*-3	0.6 ± 0.3	1.9 ± 0	1.5 ± 1	0.4 ± 0.3	1.1 ± 0.7	0.7 ± 0.1
20:0	0.2 ± 0	0.2 ± 0	0.2 ± 0	0.2 ± 0.1	0.2 ± 0	0.2 ± 0
20:1	0.9 ± 0.5	1.8 ± 0.1	1.1 ± 0.4	1.8 ± 0.1	1.8 ± 0.1	1.5 ± 0
20:2	0.3 ± 0.1	0.3 ± 0	0.1 ± 0.1	0.3 ± 0.1	0.3 ± 0.1	0.4 ± 0
20:3*n*-6	0.7 ± 0.3	0.4 ± 0	0.4 ± 0.1	0.7 ± 0.1	0.3 ± 0.1	0.6 ± 0.1
20:4*n*-6	1.5 ± 0.5 B	2.7 ± 0.6 AB	3.3 ± 0.4 A	1.5 ± 0.5 b	2.1 ± 0.8 b	5.2 ± 0.7 a
20:4*n*-3	0.3 ± 0	0.3 ± 0	0.3 ± 0	0.4 ± 0	0.3 ± 0	0.3 ± 0
20:5*n*-3	2.5 ± 0.9	2 ± 0.1	1.6 ± 0.2	2.5 ± 0.6	1.9 ± 0.4	1.8 ± 0.1
22:0	1.1 ± 0.1	1.3 ± 0.1	1.1 ± 0.3	1.3 ± 0.1	1.1 ± 0.5	1 ± 0.2
22:4*n*-6	0.3 ± 0.1	0.5 ± 0.1	0.4 ± 0	0.4 ± 0.2	0.3 ± 0.1	0.6 ± 0
22:5*n*-6	0.2 ± 0	0.3 ± 0	0.2 ± 0	0.5 ± 0.3	0.2 ± 0	0.3 ± 0.1
22:5*n*-3	0.9 ± 0.2	0.8 ± 0.1	0.8 ± 0	1.3 ± 0.5	0.8 ± 0.2	0.9 ± 0.1
22:6*n*-3	10.6 ± 3.9	6.7 ± 1.3	7.7 ± 1.2	10.8 ± 2.5	6.1 ± 1.8	10.3 ± 0.8

Note: Different letters in upper and lower case indicate significant ($p < 0.05$) difference between treatments.
[a] Total of isomers.

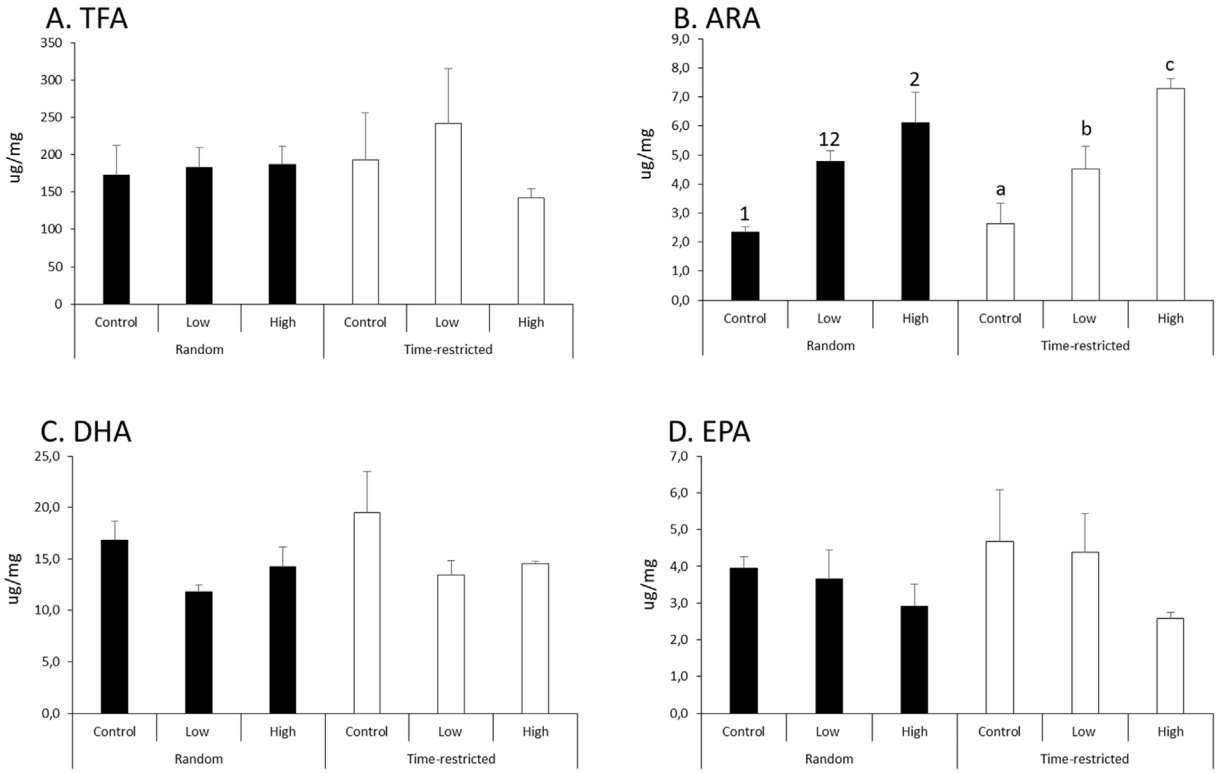

Fig. 4. Long-chain polyunsaturated fatty acid contents in the gut of zebrafish fed with the basal and microalgae-enriched diets. (A) Total fatty acid (TFA), (B) arachidonic acid (ARA), (C) docosahexaenoic acid (DHA) and (D) eicosapentaenoic acid (EPA). The values are micrograms of fatty acid per mg of whole gut tissue, and are shown as means ± SD (n = 6). For statistical notations, refer to Fig. 1 for details.

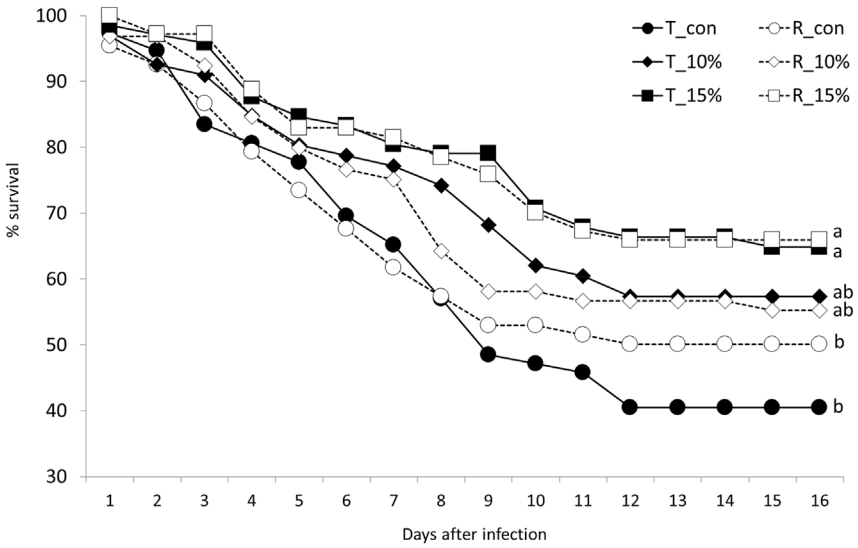

Fig. 5. Percentage survival of zebrafish fed with diets enriched with *Lobosphaera incisa* WT following *Streptococcus iniae* challenge. Fish were injected intraperitoneally with 10^3 cfu/ml of *S. iniae* in a 100-µl delivery volume. Mortality was recorded over a two-week period. T and R in the legends represent time-restricted and random feeding regimes, respectively. Significant differences between treatments 16 d post-infection were denoted with different letter notations. Note: For clarity, notations for significant differences between groups in days 7–9 were not provided in the figure, but descriptions can be found in the main text.

through nutritional immunostimulants in farmed fish. It is worth exploring in the future other time-points for time-restricted delivery as the one tested in the present study may not be the optimal time period.

Acknowledgments

C.C.L. would like to thank the Jacob Blaustein Center for Scientific Cooperation for his postdoctoral fellowship. We would like to thank Shoshana Didi-Cohen for assistance in the lipid analysis, Tamar Sinai for technical assistance and in fish husbandry, and Dr. Marta Bou for her insights on the FA data.

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