Fish & Shellfish Immunology 27 (2009) 595–602

Contents lists available at ScienceDirect

Fish & Shellfish Immunology

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

Analysis of effects induced by a pollock protein hydrolysate on early development, innate immunity and the bacterial community structure of first feeding of Atlantic halibut (*Hippoglossus hippoglossus* L.) larvae

R. Hermannsdottir [a], J. Johannsdottir [a], H. Smaradottir [c], S. Sigurgisladottir [d], B.K. Gudmundsdottir [d], R. Bjornsdottir [a,b,d,*]

[a] *Matis ohf., Icelandic Food Research and Innovation, Skulagata 4, IS-101 Reykjavik, Iceland*
[b] *University of Akureyri, Natural Resource Sciences, Borgir v/Nordurslod, IS-600 Akureyri, Iceland*
[c] *Fiskey Ltd., Hjalteyri, IS-601 Akureyri, Iceland*
[d] *Institute for Experimental Pathology, University of Iceland, Keldur v/Vesturlandsveg, IS-112 Reykjavik, Iceland*

ARTICLE INFO

Article history:
Received 22 December 2008
Received in revised form
30 April 2009
Accepted 12 May 2009
Available online 22 May 2009

Keywords:
Halibut larvae
Peptide enrichment
IgM
C3
Lysozyme
PCR–DGGE

ABSTRACT

A pollock protein hydrolysate was used for enrichment of the live feed offered to halibut larvae from the onset of exogenous feeding and the effects of treatment on selected innate immune parameters studied. The effects of treatment on the bacterial community structure of larvae were furthermore studied using the PCR–DGGE method. C3 and lysozyme were identified in larvae already at the onset of first feeding and low concentrations of IgM detected at this stage indicate maternal origin. Endogenous production of IgM was validated in the gastrointestinal tract of larvae from 29 days post onset of first feeding, with similar concentrations found in both groups. Feeding the peptide-enriched live feed stimulated production of lysozyme and affected the distribution of C3 in larval tissue but survival and normal development of halibut larvae were not affected by the treatment. *Vibrio* sp. and *Pseudoalteromonas* sp. dominated the bacterial community of larvae from both groups and peptide enrichment of the live feed was not found to affect the bacterial community structure associated with surface sterilized larvae.

1. Introduction

Constrains in morphology are a well recognized problem in the production of halibut larvae, resulting in high losses and with mean survival of only ~20% when calculated from hatching of yolk sac larvae [Fiskey Ltd., Hjalteyri, IS-601 Akureyri, Iceland]. Late maturation of the gastrointestinal tract and the intimate relationship that exists between fish larvae and the external environment containing high bacterial numbers, represent significant problems caused by opportunistic and pathogenic bacteria [1]. The specific immune system furthermore develops late in marine species, leaving the larvae to rely exclusively on innate parameters for coping with a large variety of pathogens [2]. Stimulation of innate immune parameters is therefore viewed as a promising approach for improving production methods [3]. Previous findings indicate that the ability to develop an antibody response does not necessarily correlate with the first detection of IgM positive cells but the relatively late appearance of autologous humoral IgM may, however, be compensated by maternally derived immunoglobulins that have been demonstrated in eggs and embryos of several fish species [4,5]. Experimental immunization of maternal fish has on the other hand not been found to provide the offspring protection against disease or bacterial infections [6,7]. Furthermore, the concentration of maternal immunoglobulin observed during the larval stages is generally greatly reduced from that observed during the ovarian stages [8].

The complement system is one of the first lines of immune defence and a modifier of acquired immunity [9]. C3 is the central component and is locally synthesized in various tissues [10–12].

Lysozyme isolated from fish has been found to be effective as a bacteriolytic agent against both Gram-positive and Gram-negative fish pathogens. The antibacterial properties in addition to the location of the enzyme in areas that are in frequent contact with pathogens, make lysozyme an important factor in protecting fish

* Corresponding author. Matis ohf., Icelandic Food Research and Innovation, Skulagata 4, IS-101 Reykjavik, Iceland. Tel.: +354 422 5000/+354 858 5108 (mobile); fax: +354 422 5001.
E-mail addresses: ruthermanns@hive.is (R. Hermannsdottir), jonina.johannsdottir@matis.is (J. Johannsdottir), heiddis@fiskey.is (H. Smaradottir), sjofn.sigurgisladottir@matis.is (S. Sigurgisladottir), bjarngud@hi.is (B.K. Gudmundsdottir), rannveig.bjornsdottir@matis.is, rannveig@unak.is (R. Bjornsdottir).

1050-4648/$ – see front matter © 2009 Elsevier Ltd. All rights reserved.
doi:10.1016/j.fsi.2009.05.007

against bacterial pathogens [13,14]. Leucocytes are known to synthesize and secrete lysozyme in fish and lysozyme has been found in mucus, lymphoid tissue, serum and other body fluids of halibut [15,16]. Lysozyme has also been detected in oocytes, fertilized eggs and larval stages of several fish species [5]. The presence of maternal lysozyme has futhermore been revealed in many fish species and a significant lysozyme activity has been observed at early developmental stages [17].

The gastrointestinal tract is the largest immunological organ in the vertebrate body, with cells located in the epithelial layer and lamina propria of the mucosa providing immune functions [18]. In halibut, the relatively limited numbers of IgM+ cells present in the posterior intestine are predominantly located in the epithelium rather than in the lamina propria [19].

Due to diverse bioactive properties of various products, fish protein hydrolysates in general have recently received increased attention. Protein hydrolysates derived from pollock muscle have been reported to enhance lysozyme activity of e.g. Japanese sea bass, *Lateolabrax japonicus* [20].

The objective of the present study was to investigate effects of a pollock peptide hydrolysate on early development and the bacterial community structure of halibut larvae. The hydrolysate was presented to the larvae by allowing the *Artemia* to feed on the suspension prior to being offered to the experimental fish. The parameters monitored were the bacterial community structure and the production of IgM, lysozyme and complement factor C3 in addition to larval survival and metamorphosis.

2. Materials and methods

2.1. Experimental design and evaluation of larval success

Due to the highly variable survival rates and overall success of larvae commonly observed when larvae of different parenteal origin are compared, the experiment was carried out in commercial size 3500 L production units of first feeding larvae of common silo origin (sibling tank units). Larvae were fed enriched *Artemia franciscana* nauplii (Great Salt Lake, Utah, USA) two times a day for a period of ~60 days at 11 °C when weaning onto formulated feed was started. The peptide hydrolysate was added to the fatty acid enrichment medium of *Artemia* (0.02 g L^{-1} with pray densities of 300 000 pray L^{-1}) prior to enrichment for 24 h. Approximately ~1/3 of the cultures was then offered to larvae in the first of two daily feedings (24 h *Artemia*), with environmental shading in larval tanks provided by inorganic clay. The remaining 2/3 of the cultures were grown for additional 8 h and then offered to larvae in the second of two daily feedings (32 h *Artemia*). The same medium, however, without the addition of the peptide hydrolysate, was used for enrichment of the 24 h and 32 h *Artemia* offered to the control group of larvae (untreated). The peptide hydrolysate was manufactured from pollock fillets by Iceprotein Ltd. in Iceland, using the Hultin-Process method based on changes in the pH of the solution in a chilled environment [21]. Slight modification was made with the addition of the Alcalase 4.1 enzyme (Novozymes) and Protomex (Novo Nordisk a/s) during the production process. The dry weight of larvae throughout the first feeding period was estimated by measuring the weight of between ~150 (50 days post hatch, dph) to ~15 (50 days post onset of first feeding, dpff) larvae from individual tanks at approximately weekly intervals throughout the first feeding period. The larvae were enumerated and then dried for 4–5 days at 70–75 °C before weighing (dry weight).

Survival through first feeding was estimated at the onset of weaning ~60 dpff, and was calculated as the percentage of normal larvae originally transferred to the first feeding tanks. The success of metamorphosis was estimated at the onset of weaning and was calculated as the percentage of juveniles developing to successfully metamorphosed fry, using normal eye migration and pigmentation as success criteria.

2.2. Sampling and preparation of samples

Larvae were collected at weekly intervals throughout the first feeding period. Samples were transferred to sterile jars filled with water from the respective tank and transported on ice to the laboratory for processing within 4 h post collection. The larvae were killed by an overdose of Hypnodil (51 μg mL^{-1}), followed by surface sterilization and sample preparation as previously described [22]. Two 1 mL aliquots of homogenized samples (0.1 g larvae mL^{-1}) were then transferred to sterile eppendorf tubes and stored at −80 °C for later analysis of the bacterial community structure and IgM concentration. For measuring the concentration of IgM in larvae, thawed homogenates were further homogenized by sonication on ice, using tapered microtip (3 mm), ultra high intensity and 20% amplitude, with pulse ON set for 1 s and pulse OFF for 1 s for a total of 59 s (Sonics Vibra cell 750 W model, Sonics & Materials Inc.). The homogenates were then centrifuged at 10,000*g* for 10 min and the aqueous phase collected.

For the immunohistochemistry analysis, 5 larvae from each sample were embedded each in a plastic tube (diameter 15 mm, length 30 mm) and then covered in a TissueTek medium (Sakura Fintek Europe). The blocks were frozen in liquid nitrogen for approximately 15 s and then stored at −80 °C for later analysis. The protein content of the purified halibut IgM standard and the anti-halibut IgM antibody solutions (mouse and rabbit anti-halibut IgM) was measured using a Bradford reagent (Sigma B6916) and calculated based on a standard curve made of bovine serum albumin (BSA, Sigma A7906) according to the Bradford method [23].

2.3. IgM detection by sandwich ELISA

The concentration of IgM in larval extracts was measured using a sandwich ELISA. In each step the wells of a flat-bottom microtitre plates (Nunc) were supplemented with 100 μL of the respective solutions. Phosphate buffered saline (PBS) containing 0.2% w/v BSA and 0.05% v/v Tween®20 (Sigma P9416) (PBSA–Tween) was used for further dilution of antigen and antibody solutions. All incubations were carried out in a humid atmosphere and the wells were washed three times between each step using PBS containing 0.05% v/v Tween®20 (PBS–Tween). The wells were coated with rabbit anti-halibut IgM antibodies diluted to 20 μg protein mL^{-1} in a sodium carbonate buffer (0.015 M Na_2CO_3, 0.035 M $NaHCO_3$, pH adjusted to 9.6 using 2 M HCl) and incubated over night at 15 °C. Unbound sites were saturated using 2% w/v BSA in PBS–Tween and larval homogenates (0.1 and 0.01 g larvae mL^{-1}) then incubated on the plate at room temperature for 2 h. All samples were analysed in quadruple with PBSA–Tween used as a negative control and purified halibut IgM as a positive control (0.024–1.56 ng μL^{-1}) on each plate. The capture antibody (mouse anti-halibut IgM) and the secondary antibody (alkaline phosphatase conjugated rabbit anti-mouse IgG, Sigma A1902) were used 1:100 and 1:1000 diluted, respectively, and incubations carried out at room temperature for 2 h. Incubation with the substrate solution (1 mg mL^{-1} *p*-nitrophenyl phosphate dissolved in substrate buffer consisting of 0.1 M Tris, 0.1 M NaCl and 50 mM $MgCl_2$ with pH adjusted to 9.5) was carried out for 1 h at room temperature followed by reading the optical density at 405 nm using Multiscan EX ELISA plate reader (Thermo Electron Corporation, Finland). The value of blanks was subtracted from the reading and the final values calculated as the mean of four repeats for each sample.

2.4. Immunohistochemistry (IHC)

Embedded larvae were cryosectioned using a Leica CM 1800 (Leica Instruments GmbH), producing ~9 μm thick sections that were collected onto poly L-lysine coated microscope slides (Polysine Menzel gläser, Thermo Scientific, Menzel GmbH & Co). The first section was collected after sectioning through the eyes, three sections in a row then arranged on one slide each and the next 10 sections discarded, with 1–2 sections in between collected for negative controls. This was repeated until cutting through the distal end of the digestive system. The slides were dried at room temperature for 1 h and then stored at −80 °C until staining. Frozen sections were warmed up to room temperature and then fixed in acetone (chilled on ice prior to use) for 10 min prior to air drying for 30 min and subsequent staining.

Primary antibodies were visualized using horseraddish peroxidase (HRP) or fluorescent labelled secondary antibodies. Preparing sections for staining included washing three times in PBS–Tween followed by blocking of active sites for protein binding by incubation for 30 min with PBS–Tween containing 10% w/v normal goat serum (Sigma G9023). All incubations were carried out at room temperature and the sections were washed with PBS–Tween between steps. The antibody solutions were diluted in PBS–Tween containing 0.2% w/v normal goat serum and incubated for 1 h. The primary antibodies (diluted 1:100) were rabbit anti-halibut IgM and mouse anti-halibut C3 and rabbit anti-cod lysozyme. Sections incubated with the dilution solution were used as negative controls.

Analysis of primary antibody binding included blocking of endogenous peroxidaxe activity by incubation for 15 min with 3% v/v H_2O_2 diluted in PBS prior to blocking of active sites for protein binding. The sections were then incubated in a solution of the respective primary antibody followed by a single washing step and incubation with HRP labelled secondary goat anti-rabbit Ig (Dako P0448) or rabbit anti-mouse Ig (Dako P0161) antibodies, diluted according the manufacturer's instructions (1:50 multiplied by the dilution factor of 1.0). Following washing, the sections were incubated for 10 min with Na-acetate buffer (6.8 g L^{-1} Na-acetate and pH brought to 5 using 2 M acetic acid). Excess liquid was poured off and the sections then incubated with a substrate solution containing 3-amino-9-ethyl-cabazole (AEC: 17.33 mg mL^{-1} dissolved in N<N-dimethylformamide, 750 μL of the solution then transferred to 32.5 mL aliquotes of Na-acetate buffer and 16.25 μL of 30% v/v H_2O_2 finally added). After incubation for 10 min, the sections were rinsed with distilled water, counterstained with hematoxylin for 10 s and then rinsed under running tap water for 10 min. The slides were then mounted using Clarion Mounting Medium (Sigma, USA) and analysed using Leica DMRA2 microscope with pictures taken using a Leica DC300F digital camera. Red color represents a positive reaction and the cell nuclei will stain blue.

For analysis of primary antibody binding using fluorescent labelled secondary antibodies, the sections were incubated with a solution of the respective primary antibody followed by washing and incubation for 30 min with a solution of the fluorescent labelled secondary antibodies (goat anti-rabbit Ig, Sigma F0382 or rabbit anti-mouse Ig, Sigma F0257) diluted according to the manufacturer's instructions. From this step onwards the slides were carefully protected from light. Following washing, the sections were mounted and analysed as described above.

2.5. Analysis of the bacterial community of surface sterilized larvae

For analysis of the bacterial community structure of surface sterilized larvae, thawed homogenates were analysed using PCR and the denaturing gradient gel electrophoresis method (PCR–DGGE) as previously described [22]. A relative mobility standard was run on each gel, consisting of PCR products from laboratory subcultures and type strains with various GC content and hence resolving in different regions of the gel. The relative mobility standard was represented by A: *Pseudoalteromonas elyakovii* (99% similar to GenBank accession no. AB000389), B: *Vibrio* sp. (100% similar to GenBank accession no. DQ146994, C: *Marinovum algicola* (DSM 10251) and D: *Shewanella baltica* (99% similar to GenBank accession no. CP000891). For characterization of amplified products, a small core from bands of interest was excised and DNA fragments sequenced [22].

2.6. Statistical analysis

The data were analysed using SigmaStat® release 3.5 (Systat Software, Inc. CA 94804-2028, USA). The normality of the data distribution was analysed using the Kolmogorov–Smirnov test. A *t*-test was used to analyse the success of larvae in individual incubators compared to mean values for success in all production units during the period ($n = 15$). The concentration of IgM in larvae from the two groups is expressed as mean ± S.D. and a *t*-test was used to compare the two groups at individual sample points. Differences were considered statistically significant when $p < 0.05$.

3. Results

3.1. Larval survival and development

Feeding peptide-enriched live feed was not found to affect the survival of first feeding larvae, with 66% and 63% survival observed in the treated and untreated group, respectively ($p > 0.05$). The selected treatment schedule may, however, have negatively affected the normal development of larvae with only 72% of treated larvae developing to successfully metamorphosed fry as compared to 93% in the untreated group. The difference between the groups was found to be significant ($p = 0.014$).

3.2. IgM concentrations of treated and untreated larvae

Low concentrations of IgM were detected in larvae already at the onset of feeding (Fig. 1). IgM was not detected between 8 and 36 dpff but increasing concentrations were observed in larvae from

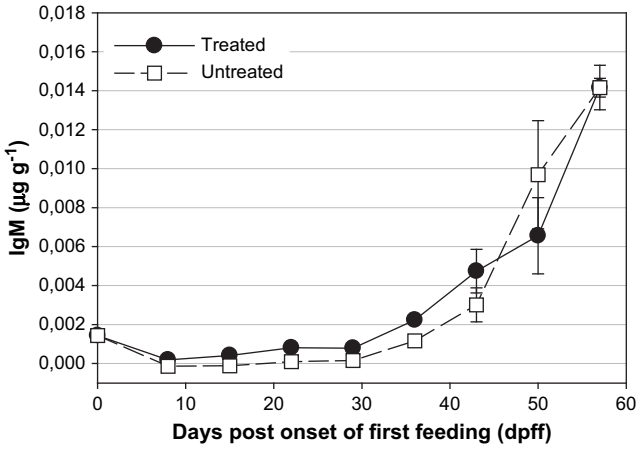

Fig. 1. Concentration of IgM in larvae (μg IgM in each g wet weight of larvae) sampled throughout the first feeding period. Shown are mean values ± S.D. in two samples from a pool of larvae collected at various samplings through the first weeks of exogenous feeding. The larvae were either fed peptide-enriched (treated) or traditional (untreated) live feed. IgM concentrations were measured by the ELISA method, using affinity purified IgM as a standard.

both groups from 36 dpff and onwards. An analysis of concentration of IgM in larvae sampled at various sampling points throughout the first feeding period revealed no significant difference between the two groups ($p = 0.33–1.00$).

Immunohistochemistry analysis using specific antibodies against halibut IgM revealed a positive response in the kidney and spleen as well as in the muscularis externa, the connective tissue and other cellular layers of the digestive system of larvae already at the onset of feeding. In the spleen, the observed response was restricted to clearly defined centers within the organ (Fig. 2).

Immunohistochemistry studies using specific antibodies revealed higher individual variability in the presence of IgM observed in larvae from the untreated group as compared to larvae from the treated group.

3.3. Detection of C3 in treated and untreated larvae

Immunohistochemistry studies revealed the presence of C3 in larvae already at the onset of feeding when C3 was observed in the digestive system as well as in the kidneys. After eight days in feeding and onwards, C3 was clearly more prominent in the digestive system of treated compared with untreated larvae. In untreated larvae, C3 appeared to be present only in the connective tissue beneath the epithelium cells of the digestive tract, while a positive response was also detected between the epithelium cells of the digestive tract in treated larvae. At 29 dpff, a positive reaction in the intestines of larvae was more prominent and widespread in the five treated compared to the five untreated larvae examined. A positive reaction was furthermore detected in the muscularis externa as well as throughout the connective tissue beneath the epithelium cells of the digestive tract in treated larvae. In untreated larvae the response was mainly restricted to the muscularis externa. At 43 dpff, the pyloric caecae are appearing and the foldings are turning conical (Fig. 3). The intestines contain small folds in the uppermost part where no obvious differences were detected in treated and untreated larvae with respect to the presence of C3. A weaker reaction with respect to the presence of C3 was observed in the oesophagus of larvae compared to the previous sampling a week before. Highly variable response was observed in the untreated larvae at this sampling, but a positive response was

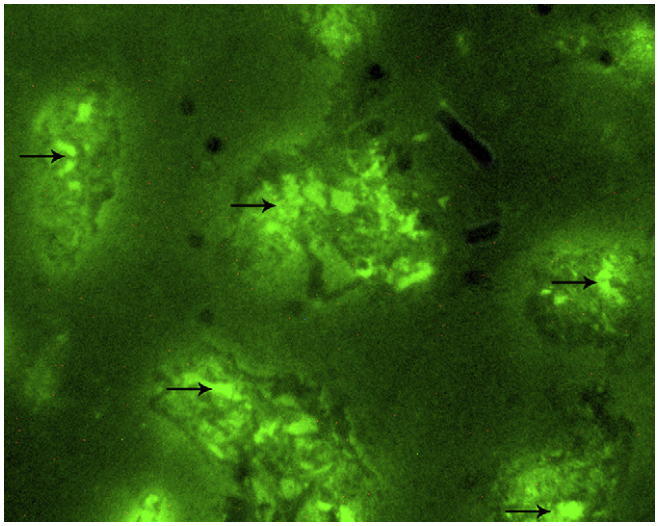

Fig. 2. Immunostaining using specific antibodies against IgM and fluorescent labelled secondary antibodies. Shown is a section of the spleen of a larvae prior to the onset of exogenous feeding (1000× magnification). Arrows indicate positive response observed in clearly defined centers within the spleen tissue.

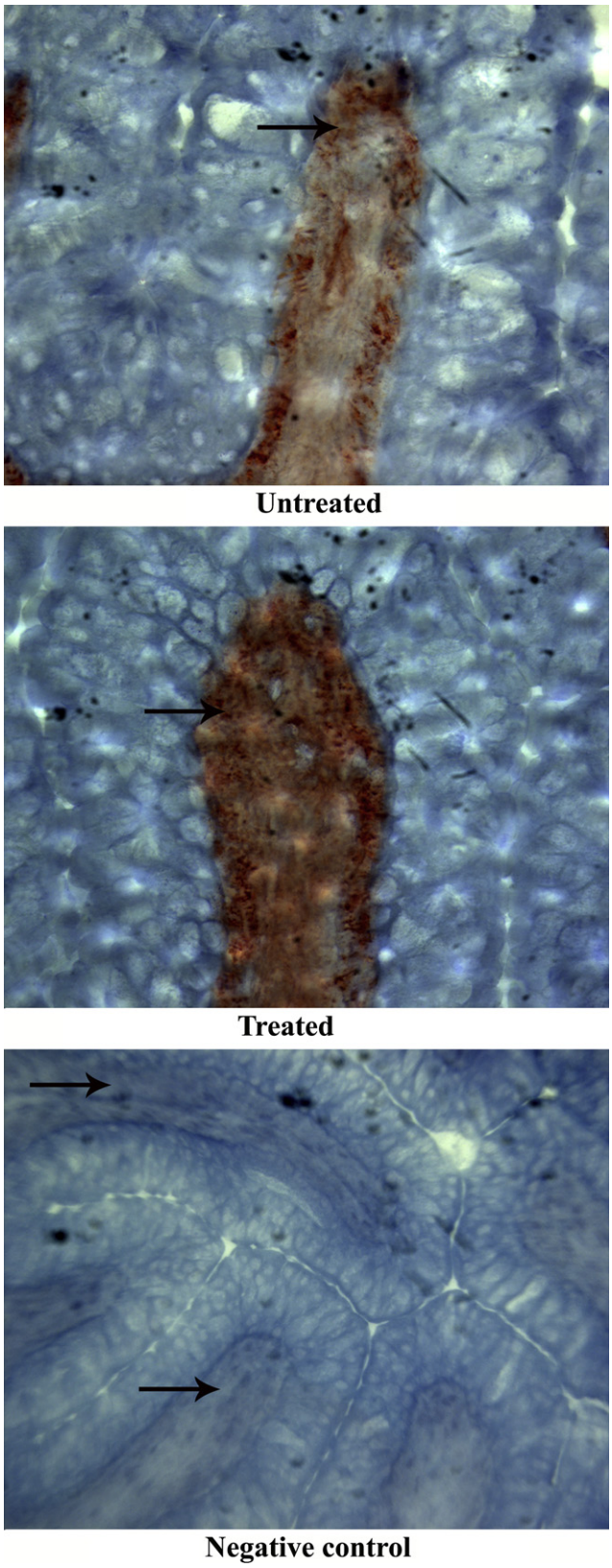

Fig. 3. Immunostaining of sections of larvae sampled 43 days post onset of first feeding. The binding of anti-C3 antibodies was visualized by horseradish peroxidase labelled secondary antibodies. Shown is the oesophagus of treated and untreated larvae (1000× magnification). Also shown is a negative control of treated larvae (400× magnification). Arrows indicate the connective tissue of the intestinal wall.

observed in all five treated larvae examined (Fig. 3). At 50 dpff, a similar reaction was observed in the intestines of larvae compared to the previous sampling a week before. No obvious differences could be detected when treated and untreated larvae were compared at this timepoint.

3.4. Detection of lysozyme in treated and untreated larvae

Lysozyme was detected in the digestive system of larvae already at the onset of feeding, however, with considerable differences in the distribution and magnitude of the reaction observed when individual larvae were compared. A positive reaction was observed in the digestive system of some of the five larvae examined in each group, while a response was hardly detectable in other larvae. After eight days in feeding, the response was generally stronger in the digestive tract of treated compared with untreated larvae, mainly in the muscularis externa and surrounding the epithelium cells of the digestive system. No obvious differences could be detected between the groups at 15 dpff and 29 dpff, but a considerable individual variability was observed in larvae from the untreated as compared with the treated group. A distinct positive response was observed in treated larvae compared to the untreated group at 50 dpff (Fig. 4). A positive response was mainly detected in the connective tissue as well as beneath and in between the goblet cells of the intestinal wall in treated larvae, while very little response was observed in the five untreated larvae examined. The positive response in the connective tissue may reflect lysozyme producing cells or glands within the connective tissue (Fig. 4).

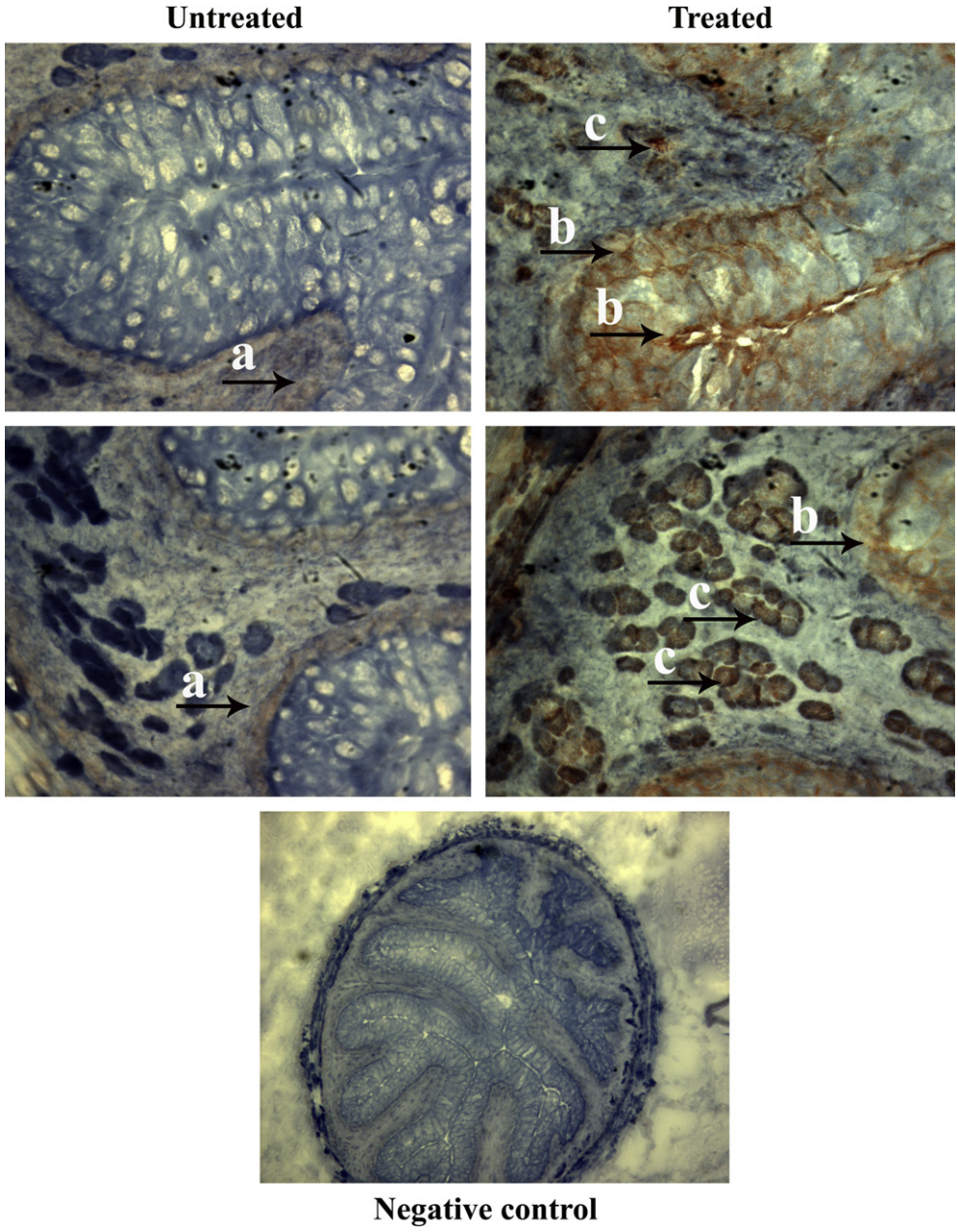

Fig. 4. Immunostaining of sections of treated and untreated larvae sampled 50 days post onset of first feeding (1000× magnification). Shown are sections from the oesophagus, at the junctions to the stomach, with binding of anti-lysozyme antibodies visualized by horseradish peroxidase labelled secondary antibodies. Also shown is a negative control of treated larvae (200× magnification). Arrows indicate positive response in the epidermal layer (a), in between the goblet cells (b) as well as in mucus producing glands found within the connective tissue of the intestinal wall (c).

3.5. The bacterial community of surface sterilized larvae

The PCR–DGGE profiles of the bacterial community of surface sterilized larvae revealed a considerable diversity in numbers of amplified products visible in the gels (Fig. 5). *Vibrio* sp. and *Pseudoalteromonas* sp. dominated the bacterial community in both groups, together with *Tenacibaculum/Flexibacter/Sphingobacteriales* that were observed in samples of larvae collected from 28 dpff and onwards (Table 1). The overall results do not indicate a difference in the bacterial community of treated and untreated larvae sampled at various days throughout the first feeding period (Fig. 5).

16S rDNA sequencing of the 254 bp products excised from the DGGE gels commonly proved to be too short for a decisive identification of bacterial species (Table 1). The products are therefore generally identified to the family or genus level and only occasionally to the species level. In some cases identification was not possible due to the presence of more than one group in some of the excised products. These products are not identified in the gel (Fig. 5).

Table 1
Groups identified as a part of the bacterial community of first feeding halibut larvae. The products identified are represented by 16S rDNA sequences covering the variable region 4 of the gene (bp 533–787).

Product	BLAST identification	Division (% similarity)	genbank
1	*Vibrio* sp.	γ-Proteobacteria (100%)	AB457058
2	*Vibrio* sp.	γ-Proteobacteria (94%)	FJ178085
3	*Vibrio* sp.	γ-Proteobacteria (92%)	DQ658900
4	*Formosa* sp.	Flavobacteria (95%)	EU419942
	Flavobacteriaceae	Flavobacteria (95%)	DQ993346
	Mesoflavibacter	Flavobacteria (95%)	AB265181
	Uncultured Flavobacteriaceae	Flavobacteria (95%)	DQ189641
5	Flavobacteriaceae	Flavobacteria (92%)	FM162927
	Tenacibaculum sp.	Flavobacteria (92%)	AF493678
6	*Tenacibaculum* sp.	Flavobacteria (95%)	AB274770
	Flexibacter aurantiacus	γ-Proteobacteria (95%)	AB078044
	Sphingobacteriales	Sphingobacteria (94%)	EU361312
7	*Vibrio* sp.	γ-Proteobacteria (88%)	DQ357823
8	Uncultured *Pseudoalteromonas*	γ-Proteobacteria (90%)	AM941173
	Pseudoalteromonas sp.	γ-Proteobacteria (90%)	AB055797
9	*Vibrio* sp.	γ-Proteobacteria (91%)	EF492024
10	Uncultured bacterium clone	(97%)	EU540612

4. Discussion

Increasing levels of IgM were detected in larvae from ~29 dpff onwards and feeding peptide-enriched *Artemia* was not found to affect the measured concentration. Low levels of IgM were detected already at the onset of feeding but IgM was not detected in larvae sampled between 8 and 26 dpff, indicating the maternal origin of IgM detected at the onset of feeding. Interestingly, maternal IgM was found in clearly defined centers within the spleen, indicating an organized accrual of IgM molecules within this organ at early developmental stages. Previous studies of salmon have shown that the low levels of maternal IgM detected prior to hatching reached non-detectable levels just prior to the onset of feeding when autologous IgM production started [24]. Maternal IgM has furthermore been seen dispersed throughout the yolk of the embryo as well as within the external membrane of the egg in channel catfish and salmon, which may indicate separate functional roles [24,25]. Furthermore, the lack of protection observed in maternal antibodies of larvae and fry indicates a different or additional role attributed to the molecules [5]. An increase in the levels of IgM has been reported during early developmental stages of fish larvae, where Ig+ cells have been verified [19]. However, although surface Ig positive B cells were detectable at 14 dph, they were not able to develop into plasma cells of carp until 30 dph [26]. Maternal IgM has to our knowledge not previously been detected in halibut larvae.

C3 was detected throughout the larvae at the onset of exogenous feeding, but a faint response indicated only low levels present at this stage. Previous studies report the presence of C3 in columnar

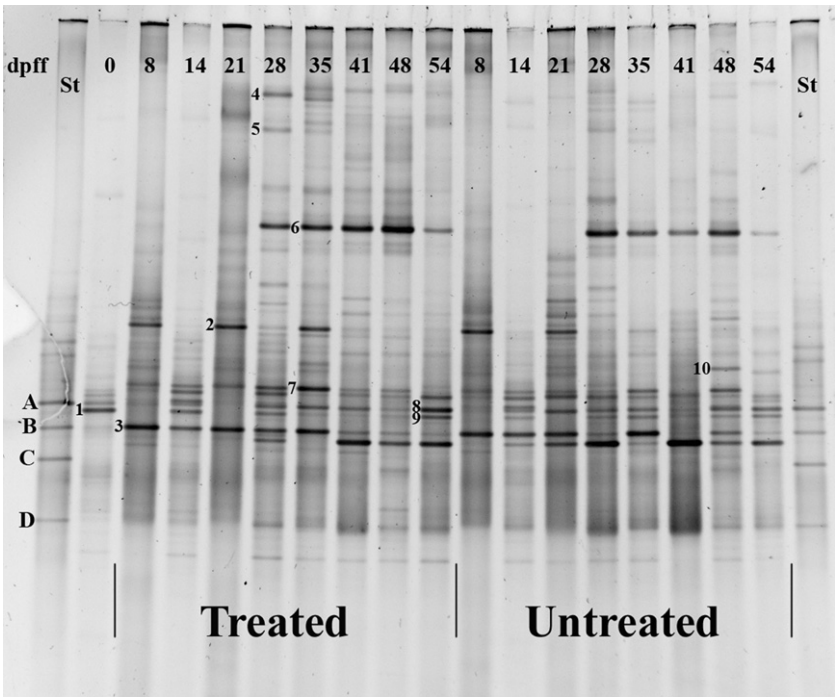

Fig. 5. DGGE profiles in a pool of ~100 (0 days post onset of first feeding, dpff) to ~15 (54 dpff) surface sterilized larvae sampled throughout the first feeding period. Shown are the profiles of larvae fed peptide-enriched (treated) and normal (untreated) live feed. Also shown are relative mobility standards A-D (St). The bacteria are represented by 16S rDNA sequences covering the variable region 4 of the gene (bp 533–787).

epithelial cells of the gut (oesophagus, stomach and intestine) as well as in epithelial and mucosal cells of the skin of halibut larvae at the onset of feeding [5]. In the present study the main difference observed in treated and untreated larvae was the presence and distribution of C3 within the space between the epithelial cells lining the digestive tract. In the uppermost part, i.e. in the oesophagus and stomach, the presence of C3 was more prominent in treated larvae, but no differences were observed further down the digestive tract when treated and untreated larvae were compared. The response in the muscularis externa of the digestive system was similar in treated and untreated larvae. The response within the connective tissue beneath the epithelial cells was, however, substantially weaker in larvae from the untreated group and no response was detected in some of the larvae from this group while all larvae sampled from the treated group showed marked response. The present results therefore strongly indicate that feeding peptide-enriched live feed resulted in stimulation of C3 production in the larvae.

The complement factors have been found to play a role in the formation and generation of different organs in fish and complement components are synthesized locally in various tissues throughout the development [5,27–29]. As the organs of the immune system are not fully developed all at the same time, local synthesization of C3 may explain the variation in time when the complement factor is detected in various parts of the larvae in the present study.

Lysozyme was detected in the digestive tract as well as in skin mucus and other parts of the larvae already at the onset of feeding. Previous studies reveal that lysozyme may be detected in mucus, serum and ova as well as in lymphoid tissue of a number of fish species [14–17]. Previous studies furthermore show that lysozyme activity may be enhanced in fish after immunostimulation [30]. Increased lysozyme activity has also been detected in the offspring of immunized compared to non-immunized parents [31]. The present result indicate stimulated production of lysozyme in the gastrointestinal tract of larvae after feeding peptide-enriced live feed for only a few days but the stimulation did not proceed further into the first feeding.

The bacterial community structure of surfaced sterilized larvae was dominated by *Vibrio* sp. and *Pseudoalteromonas* sp. as previously reported by our group [22]. The intestinal bacterial community of marine larvae is established by the ingestion of bacteria by drinking long before the larvae actually start feeding and the microbial community of the gastrointestinal tract of larvae has been found to affect growth and survival as well as the normal development of fish larvae [32,33]. The results of the immunohistochemistry studies indicate stimulated production of C3 and lysozyme in the digestive system of treated compared with untreated larvae. Peptide enrichment of the live feed represents an increase in organic nutrient availability that has been found to support the multiplication of opportunistic bacteria [34]. Treating larvae through the live feed may furthermore be expected to evoke responses primarily in the digestive system. However, offering peptide-enriched *Artemia* nauplii to larvae from the onset of exogenous feeding was neither found to affect the bacterial community structure nor the normal development and survival of first feeding larvae. The present results therefore indicate a direct immunostimulatory effect of feeding peptide-enriched *Artemia* nauplii, rather than indirect effects through modulation of the bacterial community of the gut. Hydrolysates from cod muscle as well as emptied stomachs of cod have previously been found to stimulate the activity of Atlantic salmon head kidney leucocytes in addition to benefits obtained in growth performance [35,36]. Protein hydrolysates derived from pollock muscle have furthermore been reported to enhance lysozyme activity of e.g. sea bass [20]. However, a certain concentration of the pollock hydrolysates in the diet provoked immunostimulating effects, while higher and lower levels did not significantly affect the immune response. Other studies have reported the lack of effects of a dietary protein hydrolysate on the innate immune functions of juvenile coho salmon [37]. Numerous findings, however, report biologically active peptides with immunostimulating and antibacterial properties being produced during the hydrolyzing procedure [35–39] and the present results indicate immunostimulating effects in first feeding halibut larvae.

5. Conclusion

It can be concluded from this study that maternal IgM of halibut larvae is located in clearly defined centers within the spleen tissue already at the onset of first feeding. The overall results indicate that feeding peptide-enriched live feed to larvae stimulated the production of lysozyme and C3 during the first weeks in feeding that represent the main bottleneck in the production. However, there were indications of overstimulation due to the continuous offering of peptide-enriched *Artemia* to larvae throughout the first feeding period, with reduced ratio of surviving larvae successfully developing into normal fry. The appropriate concentrations for treatment will have to be examined further and feeding prevalence adjusted.

Acknowledgements

This research was partially supported by a grant obtained from the Biotechnology network of the University of Akureyri (Grant No. R 063-07) and partially by the University of Akureyri Research Fund. The authors would like to thank Bergljót Magnadóttir and her co-workers at the Institute for Experimental Pathology, University of Iceland at Keldur for kindly donating purified halibut IgM and specific mouse anti-halibut IgM and mouse anti-halibut C3 antibodies used in the study. Dr. Merete Bjørgan Schrøder and her co-workers at the Norwegian College of Fisheries, University of Tromsø in Norway also receive thanks for kindly donating the specific rabbit anti-halibut IgM and rabbit anti-cod lysozyme antibodies used in the study. Furthermore, we thank Eydis Elva Thorarinsdottir for her contribution in the laboratory.

References

[1] Vadstein O, Mo T, Bergh O. Microbial interactions, prophylaxis and diseases. In: Moksness E, Kjorsvik E, Olsen Y, editors. Culture of cold-water marine fish. Blackwell Publishing; 2004. p. 28–73.
[2] Magnadottir B. Innate immunity of fish (overview). Fish Shellfish Immunol 2006;20:137–51.
[3] Gullian M, Thompson F, Rodriguez J. Selection of probiotic bacteria and study of their immunostimulatory effect in *Penaeus vannamei*. Aquaculture 2004;233:1–14.
[4] Chantanachookhin C, Seikai T, Tanaka M. Comparative study of the ontogeny of the lymphoid organs in 3 species of marine fish. Aquaculture 1991;99:143–55.
[5] Magnadottir B, Lange S, Gudmundsdottir S, Bogwald J, Dalmo R. Ontogeny of humoral immune parameters in fish. Fish Shellfish Immunol 2005;19:429–39.
[6] Lillehaug A, Sevatdal S, Endal T. Passive transfer of specific maternal immunity does not protect Atlantic salmon (*Salmo salar* L.) fry against yersiniosis. Fish Shellfish Immunol 1996;6:521–35.
[7] Tanaka T, Furukawa K, Suzuki Y, Aida K. Transfer of maternal antibody from mother to egg may have no protective meaning for larvae of red sea bream *Pagrus major*, a marine teleost. Fish Sci 1999;65:240–3.
[8] Picchietti S, Taddei A, Scapigliati G, Buonocore F, Fausto A, Romano N. Immunoglobulin protein and gene transcripts in ovarian follicles throughout oogenesis in the teleost *Dicentrarchus labrax*. Cell Tissue Res 2004;315:259–70.
[9] Nakao M, Yano T. Structural and functional identification of complement components of the bony fish, carp (*Cyprinus carpio*). Immunol Rev 1998;166:27–38.

[10] Del Rio-Tsonis K, Tsonis P, Zarkadis I, Tsagas A, Lambris J. Expression of the third component of complement, C3, in regenerating limb blastema cells of urodeles. J Immunol 1998;161:6819–24.
[11] Colten H. Immunology: drawing a double-edged sword. Nature 1994;371:474–5.
[12] Fishelson Z, Attali G, Mevorach D. Complement and apoptosis. Molec Immunol 2001;38:207–19.
[13] Yousif A, Albright L, Evelyn T. *In vitro* evidence for the antibacterial role of lysozyme in salmonid eggs. Dis Aquat Org 1994;19:15–9.
[14] Grinde, Lie O, Poppe T, Salte R. Species and individual variation in lysozyme activity in fish of interest in aquaculture. Aquaculture 1988;68:299–304.
[15] Murray C, Fletcher T. The immunohistochemical localization of lysozyme in plaice (*Pleuronectes platessa* L.) tissues. J Fish Biol 1976;9:329–34.
[16] Lange S, Gudmundsdottir B, Magnadottir B. Humoral immune parameters of cultured Atlantic halibut (*Hippoglossus hippoglossus* L.). Fish Shellfish Immunol 2001;11:523–35.
[17] Balfry SK, Iwama GK. Observations on the inherent variability of measuring lysozyme activity in coho salmon (*Oncorhynchus kisutch*). Comp Biochem Physiol Part B 2004;138:207–11.
[18] Takahashi I, Kiyono H. Gut as the largest immunologic tissue. J Parenteral Enteral Nutr 1999;23:S7–12.
[19] Grove S, Johansen R, Reitan L, Press CM. Immune- and enzyme histochemical characterisation of leukocyte populations within lymphoid and mucosal tissues of Atlantic halibut (*Hippoglossus hippoglossus*). Fish Shellfish Immunol 2006;20:693–708.
[20] Liang M, Wang J, Chang Q, Mai K. Effects of different levels of fish protein hydrolysate in the diet on the nonspecific immunity of Japanese sea bass, *Lateolabrax japonicas* (Cuvieret Valenciennes, 1828). Aquac Res 2006;37:102–6.
[21] Kristinsson H, Rasco B. Fish protein hydrolysates: production, biochemical, and functional properties. Crit Rev Food Sci Nutr 2000;40:43–81.
[22] Bjornsdottir R, Johannsdottir J, Coe J, Smaradottir H, Agustsson T, Sigurgisladottir S, et al. Survival and quality of halibut larvae (*Hippoglossus hippoglossus* L.) in intensive farming: possible impact of the intestinal bacterial community. Aquaculture 2009;286:53–63.
[23] Bradford MM. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem 1976;72:248–54.
[24] Olsen Y, Press C. Degradation kinetics of immunoglobulin in the egg, alevin and fry of Atlantic salmon, *Salmo salar* L., and the localisation of immunoglobulin in the egg. Fish Shellfish Immunol 1997;7:81–91.
[25] Hayman J, Lobb C. Immunoglobulin in the eggs of the channel catfish (*Ictalurus punctatus*). Dev Comp Immunol 1993;17:241–8.
[26] Koumans-van Diepen J, Taverne-Thiele J, van Rens B, Rombout J. Immunocytochemical and flow cytometric analysis of B cells and plasma cells in carp (*Cyprinus carpio* L.); an ontogenetic study. Fish Shellfish Immunol 1994;41:19–28.
[27] Lange S, Bambir S, Dodds A, Magnadottir B. An immunohistochemical study on complement component C3 in juvenile Atlantic halibut (*Hippoglossus hippoglossus* L.). Dev Comp Immunol 2004;28:593–601.
[28] Lange S, Bambir S, Dodds A, Magnadottir B. The ontogeny of complement component C3 in Atlantic cod (*Gadus morhua* L.): an immunohistochemical study. Fish Shellfish Immunol 2004;16:359–67.
[29] Lange S, Magnadottir B. Spontaneous haemolytic activity of Atlantic halibut (*Hippoglossus hippoglossus* L.) and sea bass (*Dicentrarchus labrax*) serum. Comp Biochem Physiol Part B 2003;136:99–106.
[30] Misra CK, Das BK, Pradhan J, Pattnaik P, Sethi S, Mukherjee SC. Changes in lysosomal enzyme activity and protection against *Vibrio* infection in *Macrobrachium rosenbergii* (De Man) post larvae after bath immunostimulation with [beta]-glucan. Fish Shellfish Immunol 2004;17:389–95.
[31] Hanif A, Bakopoulos V, Dimitriadis G. Maternal transfer of humoral specific and non-specific immune parameters to sea bream (*Sparus aurata*) larvae. Fish Shellfish Immunol 2004;17:411–35.
[32] Huys L, Dhert P, Robles R, Ollevier F, Sorgeloos P, Swings J. Search for beneficial bacterial strains for turbot (*Scophthalmus maximus* L.) larviculture. Aquaculture 2001;193:25–37.
[33] Picchietti S, Mazzini M, Taddei AR, Renna R, Fausto AM, Mulero V, et al. Effects of administration of probiotic strains on GALT of larval gilthead seabream: immunohistochemical and ultrastructural studies. Fish Shellfish Immunol 2007;22:57–67.
[34] Olafsen JA. Interactions between fish larvae and bacteria in marine aquaculture. Aquaculture 2001;200:223–47.
[35] Bøgwald J, Dalmo R, McQueen Leifson R, Stenberg E, Gildberg A. The stimulatory effect of a muscle protein hydrolysate from Atlantic cod, *Gadus morhua* L., on Atlantic salmon, *Salmo salar* L., head kidney leucocytes. Fish Shellfish Immunol 1996;6:3–16.
[36] Gildberg A, Bøgwald J, Johansen A, Stenberg E. Isolation of acid peptide fractions from a fish protein hydrolysate with strong stimulatory effect on Atlantic salmon (*Salmo salar*) head kidney leucocytes. Comp Biochem Physiol Part B 1996;114:97–101.
[37] Murray AL, Pascho RJ, Alcorn SW, Fairgrieve WT, Shearer KD, Roley D. Effects of various feed supplements containing fish protein hydrolysate or fish processing by-products on the innate immune functions of juvenile coho salmon (*Oncorhynchus kisutch*). Aquaculture 2003;220:643–53.
[38] Daoud R, Dubois V, Bors-Dodita L, Nedjar-Arroume N, Krier F, Chihib N-E, et al. New antibacterial peptide derived from bovine hemoglobin. Peptides 2005;26:713–9.
[39] Coste M, Huneau J, Mahe S, Tomé D. Interactions between milk protein peptides and intestinal mucosa. In: Paubert-Braquet M, Dupont C, Paoleti R, editors. Foods, Nutrition and immunity, 1. Basel: Krager; 1992. p. 96–103. Dyn Nutr Res.