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Effect of germ-free rearing environment on gut development of larval sea bass (*Dicentrarchus labrax* L.)

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ABSTRACT

A gnotobiotic feed chain (Artemia-European sea bass) has been developed recently. To investigate the extremely complex and poorly understood modes of action of putative probiotics and pathogens *in vivo*, we quantified the effect of a germ-free controlled culture on survival and gastrointestinal tract development from day after hatching (DAH) 0 till DAH15. The larvae were kept in a germ-free static and rotating set-up, next to the conventional static group. They were sampled on DAH1, DAH6, DAH9 and DAH14. The survival of sea bass larvae on DAH14 from both germ-free treatments was not significantly higher (93 ± 3% and 82 ± 5%) compared to the conventional (51 ± 19%) group ($p = 0.058$). Morphometric data revealed a significant growth on DAH14 in germ-free static larvae (4.84 ± 0.07 mm) compared to that of conventional (4.55 ± 0.06 mm) and germ-free rotating larvae (4.54 ± 0.08 mm) ($p = 0.002$). However, histological analysis showed minor variations in regional morphology of epithelial cell types observed in the gut in individual larvae between and within treatment groups. These presented techniques combined with the germ-free sea bass model can be used as a standardised model system to study *in vivo* the mode of action of probiotics.

1. Introduction

To improve health management in larviculture, a profound insight about the host-microbial interactions has to be developed (Olafsen, 2001). Until now, antibiotics were often used to treat diseases. However, certain risks such as bacterial resistance to antibiotics became a huge concern. As antibiotic resistance represents a threat for public health (Norrby et al., 2005) and is complex to handle, alternatives such as pre- and probiotics have proven to be a successful substitute in health management of humans and terrestrial animals and are thriving in the aquaculture sector as well (Vine et al., 2006). However, the presence of the normal bacterial flora makes it impossible to define the exact role of probiotics in specific biological processes (Hooper et al., 2002). To understand the function of probiotics, an *in vivo* model system is required in order to elucidate the modes of action inside the host (Tinh et al., 2008). We have therefore developed a germ-free/gnotobiotic (with a known and controlled microbial composition) model system using the European sea bass *Dicentrarchus labrax* larvae (Dierckens et al., 2009) whose aquacultural production has doubled in the last 10 years (Darias et al., 2008).

Germ-free inbred strains of mice are commonly used to analyse host-microbial relationships (Gordon et al., 1997). Furthermore, human probiotic research has shown that bacteria have considerable effects on host gut surface. *Bacteroides thetaiotaomicron* may trigger the expression of as many as 400 genes in the gut (Heselmans et al., 2004). However, the function of most bacterial species is still unknown. Zebrafish (*Danio rerio*) genes are differently regulated in the presence of commensal microbiota or by isolated bacteria (Bates et al., 2006; Rawls et al., 2004). Rawls et al. (2004) and Bates et al. (2006) have revealed certain molecular aspects of the functional role of gut microbiota in the differentiation of the gastrointestinal tract by using the zebrafish as a vertebrate model. The germ-free zebrafish became a powerful model system to complement host-microbe research in rodents. Their digestive tract becomes already fully functional by the fifth day post fertilisation (2 to 3 days after hatching; DAH), and furthermore they do not possess a gastric region and do not require live feed. In contrast, in sea bass larvae, gastric glands appear from DAH55 onwards when the stomach reaches its definitive anatomy and histology of an adult teleost (Garcia Hernandez et al., 2001) and requires the complexity of live feed production chain. Therefore, we propose the seabass, as a more suitable model than the zebrafish for research in marine fish larviculture. As the sea bass rearing process was simplified by eliminating the initial rotifer feeding

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phase, the larvae reared in darkness can solely rely on *Artemia* nauplii as first live prey source from DAH8–9 (Chatain, 1997). Furthermore, detailed light and electron microscopic study of the gastrointestinal tract of conventional sea bass larvae from hatching until the first appearance of gastric glands has been described (Garcia Hernandez et al., 2001). However, knowledge on this marine fish larvae reared under germ-free/gnotobiotic conditions is absent. For all these reasons, we chose for the simplest food chain being still relevant to aquaculture and thus providing a proper environment to analyse the response of the host to individual microbes or/and selected microbial communities.

The objectives of this study were to evaluate the influence of germ-free environment on the development of the sea bass larvae from DAH1 until DAH15. Therefore, morphometric and volumetric assay of the whole larvae and their gastrointestinal tract was carried out in order to reveal possible differences due to microbial status. Subsequently, histological analysis was used to identify morphological alterations of the gastrointestinal tract. To our knowledge, this is the first histological study on cultured germ-free marine fish larvae fed with germ-free live feed.

2. Materials and methods

2.1. Experimental set-up

Eggs of *D. labrax* of 2 days post fertilization were obtained from natural spawning at the hatchery of Ecloserie Marine de Gravelines (France). After arrival and acclimatisation with UV-sterilised seawater, 24,000 eggs were treated in order to obtain germ-free conditions (Dierckens et al., 2009). The eggs were rinsed with 4 L of autoclaved seawater (ASW), disinfected for 3 min with 200 mg/L glutaraldehyde (FLUKA 49629 id — Glutaraldehyde 50% solution) (2400 eggs/L), and rinsed again with 3 L ASW. Subsequently, the eggs were stored in 24 gently aerated incubation bottles for 3 days, each containing 600 eggs in 400 mL filtered ASW supplemented with 10 mg/L ampicillin and 10 mg/L rifampicin. The untreated eggs that remained under conventional conditions after acclimatisation, were put in a 10 L tank with a continuous flow-through. At the end of the incubation (60 h), larvae were collected and stocked one by one at a concentration of 12 larvae in a transparent sterile screw cap vial (total volume: 15 mL). A rifampicine solution was added together with filtered, autoclaved sea water to obtain a volume of 10 mL at a final concentration of 10 mg rifampicin/L. As preliminary results had shown that the rotating setup for untreated conventional larvae leads to high mortality from DAH4 onwards, this treatment was excluded from the experiment. In total 225 vials were used; 75 vials were placed on a rotor turning at 4 rpm (Germ-free Rotor; GfRot) with an axis tangential to the axis of the vials. The rotating set-up was used to provide aeration and avoid sedimentation. Seventy five vials were kept in a non-moving setup (Germ-free Static; GfS) and another 75 vials were kept in a non-moving setup containing the control larvae (Conventional Static; ConvS).

Throughout the experiment, eggs and larvae were kept in a temperature controlled room at 16 ± 0.5 °C in a constant dim light (10 cd steradian m^{-2}) at a salinity of 37 g/L.

Starting from DAH7 until DAH15, thirty freshly hatched germ-free *Artemia* (Marques et al., 2004) (±2.5/larva) were added to each vial, every other day. For every time point, a different set of replicates were used. Hence they were not used for subsequent observation.

2.2. Testing germ-free status

After 24 h incubation, 30 eggs were aseptically removed from the incubation bottles, homogenized for 6 min by means of a stomacher blender (400SN, Seward Medical, London, UK) in 10 mL sterile nine-salt solution (NSS; Olsson et al., 1992). Subsequently, 50 µL was plated using a spiral plater (Spiral plater™, Spiral Systems Inc, USA) on 10% marine broth (Difco Laboratories, Detroit, USA) + 15% agar (Bacteriological Grade, MP Biomedicals Inc.). In addition, 1 mL water from each incubation bottle was added to a sterile tube containing 9 mL of marine broth (10%). Agar plates and the marine broth tubes were incubated at an increasing temperature from 20 °C to 28 °C over a 72 h period and were checked for bacterial growth. Additionally, DNA extraction from the samples was carried out by using the QIAamp DNA Stool Mini Kit, according to manufacturer's instructions (Qiagen Benelux B.V., Venlo, Netherlands). 16 S rRNA gene fragments were obtained by amplifying the 16 S rRNA gene with primers P63f and P1378r (Øvreas et al., 1997). Denaturing Gradient Gel Electrophoresis (DGGE) based on the separation of PCR-amplified fragments of genes coding for 16 S rRNA was additionally carried out (Boon et al., 2002). Since bacterial primers can aspecifically bind to eukaryotic DNA, in case of amplification, PCR fragments were sequenced (ITT Biotech-Bioservice, Bielefeld, Germany). Analysis of DNA sequences and homology searches were completed with standard DNA sequencing programs and the BLAST server of the National Center for Biotechnology Information (NCBI) using the BLAST algorithm.

2.3. Sampling procedure and morphological analysis

2.3.1. Light microscopy

Histological sections were used for describing the development of the five segments of the alimentary tract, namely the oropharynx, oesophagus, gastric region, mid- and hindgut of the larvae. Acid and neutral mucin types of the pharyngeal and oesophageal goblet cells were demonstrated by histochemistry. At least twelve live fishes per treatment were sampled daily using 5 vials/day between DAH1 and DAH15. The larvae were fixed for 22 h in Bouin's fluid (saturated picric acid, formaldehyde 37%, glacial acetic acid 100%), dehydrated in graded alcohols and embedded in paraffin wax using the STP 420 Microm Tissue Processor and the embedding center EC350-1 and 2 (Microm, Prosan, Merelbeke, Belgium), respectively. For light microscopic observation longitudinal sections of 5 µm thickness and transverse sections of 8 µm thickness were cut with the HM 360 Microtome using the Section Transfer System (Microm, Prosan, Merelbeke, Belgium). The transverse sections were also used for 3D reconstruction of the larval digestive system by Amira 4.0.1. software.

For general histology the sections were stained with Haematoxylin/Eosin (HE), for both neutral and acid mucins with combined Alcian blue-periodic acid-Schiff (AB-PAS), for sialo- and sulfomucins with High Iron Diamino-Alcian blue staining, and for protein-bound amino groups with Ninhidryn-Schiff (NS). Histological analysis was performed using the Olympus BX61 light microscope and Olympus DP50 camera (Olympus Belgium, Aartselaar, Belgium).

2.3.2. Morphometry

Morphometrical analysis was performed in order to show whether a bacteria-free and conventional environment have the same effect on the growth and development of the larvae. To monitor the growth of the larvae from the three treatments, morphometrical measurements on standard length were carried out on DAH1, DAH6, DAH9 and DAH14 larvae using the Olympus SZX7 stereomicroscope and Cell D software (Soft Imaging System, Olympus NV). The standard length (SL) is determined as the straight distance between the tip of the snout and the most posterior part of the notochord.

2.3.3. Stereology

Stereology was used to determine the volume of the body (V_T) of the larvae and of the digestive tract (V_D), by using two larvae per group. For this stereological application 15 to 20 transverse sections with fixed interval per fish larvae were examined and volume determination was based on the Cavalieri method (Howard and Reed, 1998; Casteleyn et al., 2007). The accuracy of the volume estimation was determined by counting the coefficient of error (CE).

If this value was lower than 10%, the volumetric estimation was considered to be accurate. Additionally, the ratio of the digestive tract volume and the total body volume was calculated.

2.4. Statistics

Statistical analysis was performed by means of SPSS software, version 15.0. Survival and standard length were given as mean values ± S.E.M. Residuals were tested for normality and subjected to non-parametric tests. Kruskal Wallis was used to compare response variables from three treatment groups. Mann–Whitney U test was used for pair-wise comparisons. Significance was accepted at $p < 0.05$.

3. Results

3.1. Quantitative analysis

3.1.1. Germ-free status

No colony forming bacteria and no turbidity were detected after plating eggs on 10% Marine Agar and incubating SW in 10% Marine Broth after 72 h. In addition, in case of PCR amplification, the retrieved sequences of PCR fragments were related to eukaryotic sequences and not to bacterial 16 S rRNA sequences (data not shown).

3.1.2. Survival

Survival of the larvae varied drastically between different replicates within one treatment, resulting in a high S.E.M (Table 1). During the course of the experiment, overall survival was lower in the control group compared to both germ-free treatments. This was significant on DAH 11 and 12 ($p<0.05$). From DAH 1 till DAH 10 the survival of conventional larvae was not significantly different compared to the two germ-free groups.

3.1.3. Morphometric measurements

On DAH1, standard body length differed little between the three groups and varied between 3.73 ± 0.06 mm and 3.86 ± 0.03 mm (Fig. 1). However, from DAH6 onwards till the end of the experiment, GfS larvae exhibited the best growth amongst the three treatments. By DAH14 the static germ-free larvae had a mean length of 4.84 ± 0.07 mm, which was significantly ($p<0.05$) longer than that of the conventional and rotating germ-free larvae, with a mean standard length of 4.55 ± 0.06 mm and 4.54 ± 0.08 mm, respectively.

3.1.4. Stereology

Volume estimations on DAH1 and DAH6 revealed a similar growth (Table 2). However, in the later phases distinction could be made between the treatment groups. From DAH9 onwards, the interindividual variation in size became very obvious. In order to estimate these differences, the ratio of V_D and V_T was calculated. On DAH9, V_D/V_T values were 8.28% in GfS larvae, whereas 7.63% and 7.64% in GfRot and ConvS larvae, respectively. On DAH14 the difference was more apparent, the GfS larva revealing the most developed digestive tract with a V_D/V_T value of 10.14%, while in GfRot and ConvS larvae the ratio amounted 7.89% and 8.29%, respectively.

Table 1
Survival (in percentage) of larvae reared in a germ-free rotating setup, germ-free static setup and conventional static setup.

	GfRot	GfS	ConvS
DAH 10	93 ± 6^a	100 ± 0^a	38 ± 21^a
DAH 11	100 ± 0^a	99 ± 1^a	20 ± 12^b
DAH 12	96 ± 3^a	98 ± 2^a	42 ± 7^b
DAH 13	79 ± 4^a	61 ± 24^a	51 ± 16^a
DAH 14	82 ± 5^a	93 ± 3^a	51 ± 19^a
DAH 15	54 ± 8^a	43 ± 8^a	19 ± 11^a

Data are shown as mean ± S.E.M. of each treatment each containing five replicates a day. Values in the same row with different superscripts are significantly different ($p < 0.05$).

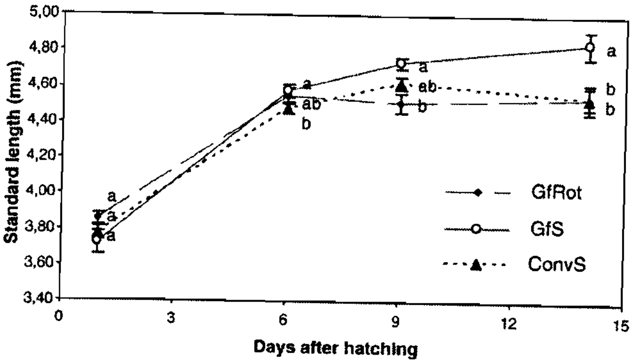


Fig. 1. Standard length (mean ± S.E.M.) of larvae on DAH1, DAH6, DAH9 and DAH14 reared in germ-free rotor (GfRot), germ-free static (GfS) and conventional static (ConvS) treatment. Different letters indicate significant differences ($p<0.05$) among sampled points for each group.

3.2. Qualitative analysis

3.2.1. Histology — ontogenesis of the digestive tract

3.2.1.1. DAH1. On DAH1, the digestive tract appeared as an undifferentiated tube with a large yolk sac. Apart from the gastric area, which was slightly curved, the oropharynx, oesophageal region and the intestine consisted of a straight duct when observed dorsally (Fig. 2). At this phase, the mouth and anus were closed. Except for the pharyngeal area which was widened and lined with simple cuboidal cells, the rest of the alimentary tract showed a round lumen lined by a simple epithelium with an inner layer which varied in height surrounded by a layer of mesodermal cells. The region of the oesophagus was lined with simple columnar cells with basal nuclei and several dorsally located lighter cells. More caudally, in the mid- and hindgut area, epithelial cells were columnar to cuboidal. Along the whole alimentary tract scattered mitotic figures were observed. The most caudal region of the gut was curved ventrally and the anus was lined with a pseudostratified epithelium. In general, no differences were observed in the morphological developmental pattern between larvae reared in conventional and germ-free static/rotor conditions.

Table 2
Volume estimation of the digestive tract, total body volume and the ratio between the volume of the digestive tract and total body volume based on stereology using the Cavalieri method.

		GfRot ($n=2$)		GfS ($n=2$)		ConvS ($n=2$)	
		V	CE	V	CE	V	CE
DAH1	V_D	0.002	0.051	0.002	0.074	0.002	0.078
	V_T	0.106	0.053	0.095	0.118	0.102	0.072
	V_D/V_T	1.89%		1.89%		1.76%	
DAH6	V_D	0.012	0.041	0.011	0.045	0.011	0.041
	V_T	0.149	0.085	0.145	0.089	0.138	0.066
	V_D/V_T	8.05%		7.59%		7.68%	
DAH9	V_D	0.010	0.046	0.014	0.029	0.011	0.049
	V_T	0.131	0.048	0.169	0.063	0.144	0.065
	V_D/V_T	7.63%		8.28%		7.64%	
DAH14	V_D	0.012	0.038	0.015	0.041	0.010	0.054
	V_T	0.152	0.059	0.148	0.068	0.121	0.084
	V_D/V_T	7.89%		10.14%		8.29%	

V: volume in mm^3, CE: coefficient of error, V_D: volume of the digestive tract mucosa; V_T: volume of the total body; V_D/V_T: ratio between the volume of the digestive tract and total body volume.

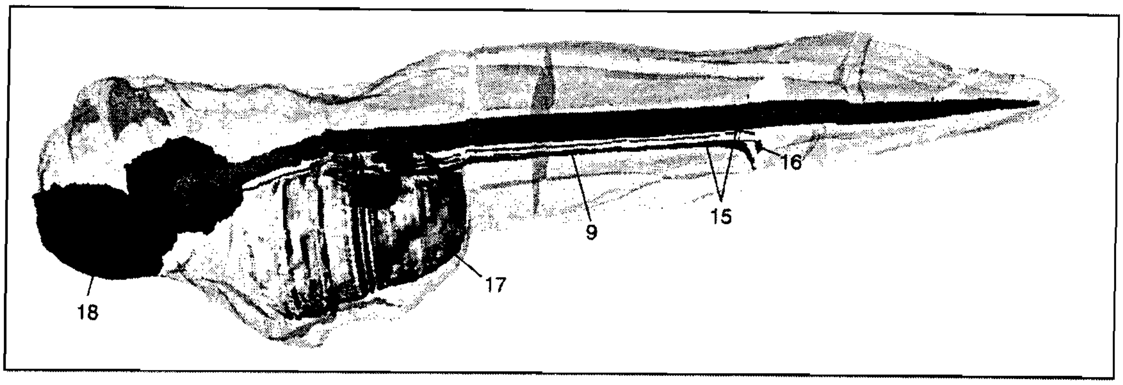


Fig. 2. General view of DAH1 larva (GfS) showing the notochord, yolk sac, an undifferentiated digestive tube and urinary tubules (reconstruction with Amira software).

3.2.1.2. DAH6. Parallel with the resorption of the yolk sac, the sea bass larvae underwent rapid developmental changes leading to a differentiation of the alimentary tract into five distinct segments, namely the oropharynx, oesophagus, gastric region, midgut and hindgut (Fig. 3). The oropharynx contained the oral cavity with a large mouth opening and the pharyngeal region. The oral cavity was lined by a simple squamous epithelium with few taste buds, whereas the pharynx was covered with a stratified epithelium containing several goblet cells. The oesophageal mucosa presented longitudinal folds and was lined by a stratified cuboidal epithelium with goblet cells, surrounded by a prominent lamina propria and a layer of circular smooth muscle cells. The AB-PAS staining demonstrated that the scattered goblet cells present in the mucosa of the pharynx and oesophagus, contained neutral (magenta) or acid (purple-blue) mucins. Other goblet cells, containing both acid and neutral mucins (blue-magenta) were detected as well. Other regions of the digestive tract, such as midgut and hindgut contained no goblet cells in this early phase of development. Further histochemical investigation evidenced in both control and germ-free animals the exclusive presence of a single acid mucin subtype, namely sulfomucins. Sialomucins were absent in both conventional and germ-free treatments throughout the experiment (data not shown).

Towards the presumptive stomach, the simple cuboidal epithelium of the most caudal part of oesophagus was continuous with the dilated gastric region lined by simple cuboidal to columnar epithelium devoid of goblet cells. At this stage a primordial pyloric sphincter already developed separating the incipient stomach from the cranial portion of the midgut. Furthermore, the gut was divided by anlage of the ileo-rectal valve into a prevalvular intestine (midgut) and the postvalvular intestine (hindgut). The two intestinal segments showed no histological differences at this age, both being lined by a high columnar epithelium with a strongly eosinophilic and PAS positive brush border and containing neutral mucins. At this stage no histological differences were observed in the alimentary tract of the three treatment groups.

3.2.1.3. DAH9. Compared to DAH6, no considerable variations were observed in the first segment of the alimentary tract, except for the more numerous taste buds and goblet cells in the pharynx and goblet cells in the oesophagus. The larvae began exogenous feeding on DAH7, while their yolk sac was still present at that time. Some histological alterations were detected in the mid- and hindgut (Fig. 4a–c). The intestinal epithelium was thickened at several points showing variations in epithelial cell types not only among different groups, but also between larvae reared under the same conditions. More specifically, the midgut of conventional larvae consisted of a simple columnar to cuboidal epithelium in the cranial portion, in contrast to a cuboidal to squamous epithelium in the caudal portion. The hindgut was lined by a simple cuboidal–columnar epithelium with a tall brush border on the apical surface. The hindgut enterocytes of one larva exhibited supranuclear vacuoles.

Germ-free larvae reared in a static setup exhibited high columnar cells in the cranial part of the midgut, and cuboidal cells with very tall brush border in the caudal segment of the midgut. Germ-free rotor larvae were characterized by cuboidal to columnar epithelium with a very tall brush border. The midgut enterocytes contained lipid inclusions. In both germ-free groups the hindgut demonstrated cuboidal to columnar cells with a better developed brush border than in the conventional larvae.

3.2.1.4. DAH14. At this age, all the compartments of the alimentary canal were clearly distinguishable (Fig. 5a,b). Furthermore, midgut enterocytes contained more numerous lipid vacuoles indicating absorption and storage of lipids (Fig. 5c,d,g). In the hindgut Ninhydrin-Schiff positive large supranuclear vacuoles as a result of pinocytotic absorption of nutrients were more visible from this age onwards (Fig. 5h). At this phase, no goblet cells were present in the mid- and hindgut.

In general, inconsistencies were present in the types of epithelium of the gut between individuals within a treatment group (Fig. 5c–f).

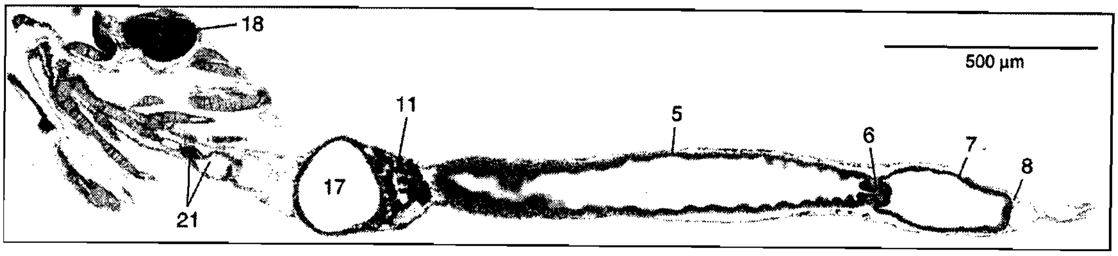


Fig. 3. General view of the mid- and hindgut of a DAH6 larva (median section; GfS).

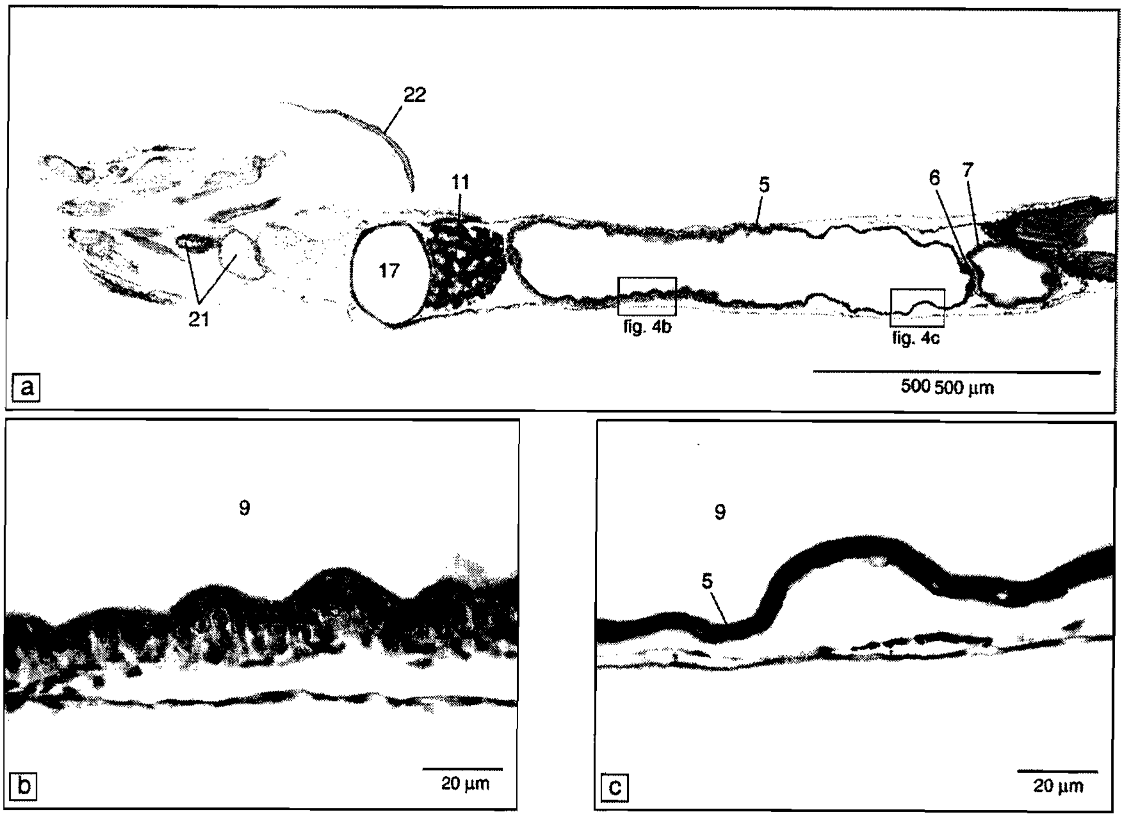


Fig. 4. a General view of the mid- and hindgut of a DAH9 larva (median section; GfS). b–c. High magnification of the cranial and caudal parts of the midgut epithelium. Note the high columnar epithelium in the cranial region, and the squamous epithelium in caudal region.

However, it can be stated that conventional larvae exhibited a slightly higher intestinal epithelium in the midgut compared to germ-free larvae. The midgut of control larvae consisted of a cuboidal to columnar epithelium, whereas the midgut of both groups of germ-free larvae was lined with a cuboidal to squamous epithelium. Moreover, the hindgut of the control larvae was characterised by a cuboidal to columnar epithelium with a visible brush border, whereas in germ-free static larvae the hindgut was lined by simple columnar epithelium with a very tall brush border. The hindgut of rotating germ-free larvae was lined by a simple squamous to cuboidal epithelium.

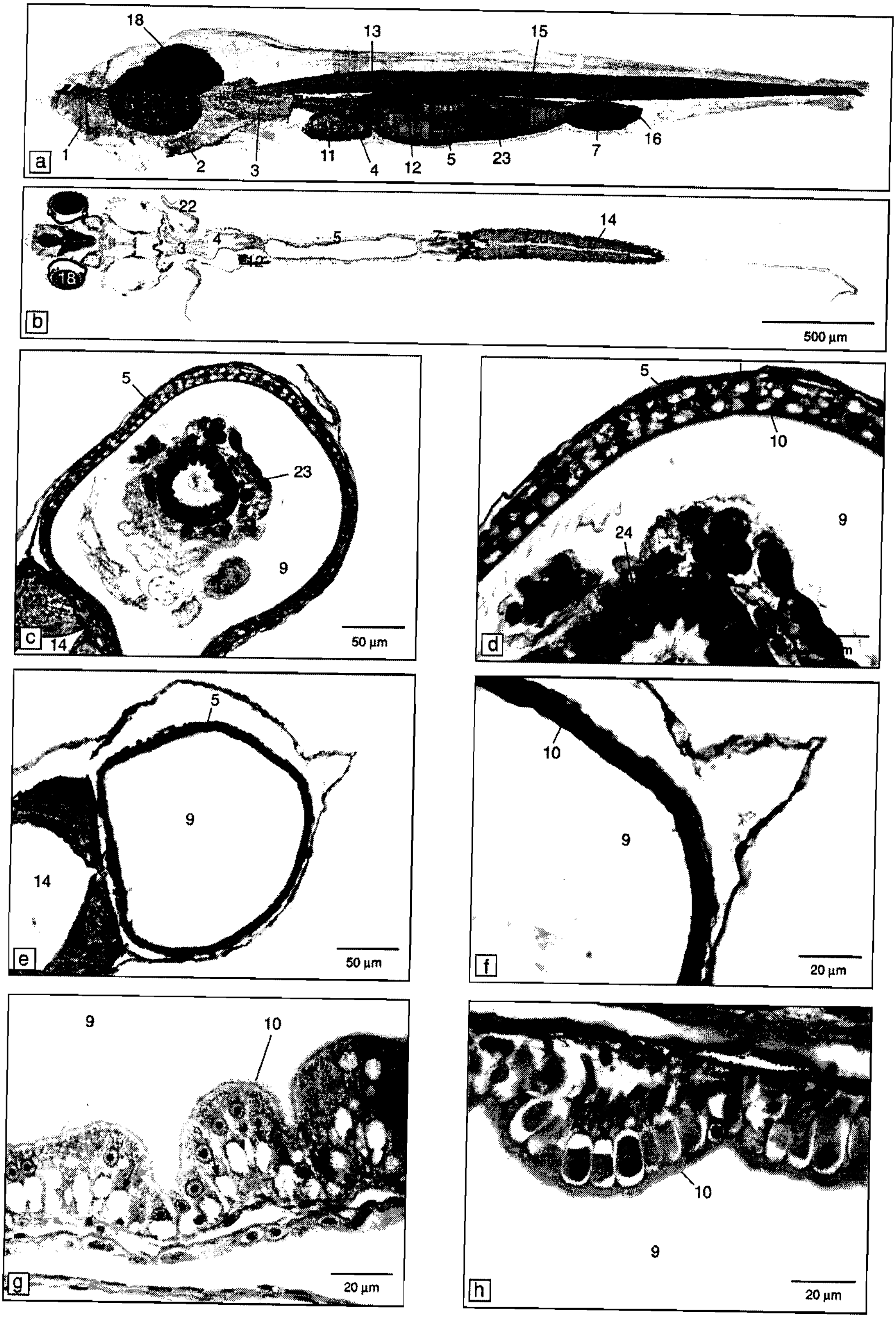
4. Discussion

In the current study, we investigated whether a germ-free environment has an influence on the survival and development of sea bass larvae in the first 15 days of development. The survival of the larvae from both germ-free treatments was higher, which is in accordance with the results on low mortality of turbot larvae reared in absence of culturable bacteria (Munro et al., 1995).

To reveal the influence on the development, a histological and stereological approach was used in order to emphasize the slight differences which may occur in the structure and size of the gut wall of germ-free and conventional animals. Morphometrical analysis clearly revealed that germ-free static larvae were significantly larger than the other two groups. Stereological data demonstrated that these larvae were not only bigger, but had the highest ratio between the digestive tract volume and the total body volume on DAH14.

Several mechanisms can contribute to these effects. One of the possible explanations for the better development of germ-free larvae might be cell kinetics. In germ-free birds mitotic activity of enterocytes seemed to be enhanced (Koutsos and Arias, 2006). In contrast to birds, gut bacteria contribute to an increased mucosal cell replacement rate in mammals (Banasaz et al., 2001, 2002). The intestines of germ-free rats and zebrafish are characterized by a reduced cell proliferation rate (Uribe et al., 1997; Rawls et al., 2004), slower renewal of epithelial cells, and more slender villi with a thinner lamina propria in germ-free rodents (Berg, 1996). No differences in apoptotic rate of gut enterocytes were observed in germ-free and conventional zebrafish larvae (Rawls et al., 2004). As the reactions of the host to the presence of bacteria is so divergent, an immunohistochemical assay is needed to clarify the effect of bacteria on cell proliferation rate and programmed cell death in germ-free and conventional sea bass larvae.

Fig. 5. a. General view of a DAH14 germ-free static larva showing the compartmentalized digestive tract (reconstruction with Amira software). Note the presence of *Artemia* nauplii in the midgut (GfS). b. General view of the digestive tract of a DAH14 larva (median section, GfS). c–d. Germ-free static DAH14 larva with the characteristic lipid vacuoles (arrows) in the columnar epithelium of the midgut. Note the presence of *Artemia* nauplii in the lumen with its respective midgut. e–f. DAH14 larva without the presence of *Artemia* with flattened epithelium originating from the same germ-free static group as the larva from Fig. 5c–d. g–h. Epithelium of the mid- and hindgut of DAH15 larva lined by a columnar epithelium with a distinct brush border and without a mucus layer. Note the lipid vacuoles (arrows) in the midgut and supranuclear inclusions (arrowheads) in the hindgut (GfS). Legend: 1. oral cavity; 2. pharynx; 3. oesophagus; 4. gastric region; 5. midgut; 6. ileo-rectal valve; 7. hindgut; 8. anus; 9. lumen of the digestive tract; 10. brush border; 11. liver; 12. pancreas; 13. swim bladder; 14. notochord; 15. urinary duct; 16. urinary bladder; 17. yolk sac; 18. eye; 19. brain; 20. muscle tissue; 21. heart; 22. pectoral fin; 23. *Artemia* nauplii; 24. epithelial cells of the midgut of *Artemia* nauplii.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20 µm

Differences in larval body size may also be attributed to the toxic metabolic by-products of the resident gut bacteria, such as ammonia or phenolic and aromatic compounds which are considered to have a growth-depressing effect on the host (Anderson, 2002; Collier et al., 2003). Moreover, competition for energy and amino acids between the microbial communities and the host may have a considerable impact as well (Anderson, 2002; Collier et al., 2003). Consequently, it is likely that commensal microbiota cause poorer growth (Muramatsu et al., 1987). Germ-free animals might grow better because they do not contain any microbiota which can produce toxic products or can compete for nutritional components. This hypothesis is not supported by our findings on germ-free rotating larvae, which exhibited a similar growth as conventional larvae. This might be explained by the rotating set-up which may cause stress to the larvae and therefore may diminish growth. However, further investigation is needed to strengthen this hypothesis.

The high variation in intestinal epithelium height may be due to bacterial status of the larvae and nutritional conditions. Unequal success in prey capture may result in nutritional differences within a group. Quantitative and qualitative studies have been carried out to elucidate the effect of starving and delayed first feeding in larvae of several fish species; *Paralichthys dentatus* (Bisbal and Bengston, 1995), *Paralichthys californicus* (Gisbert et al., 2004), *Seriola lalandi* (Chen et al., 2007), *Amphiprion melanopus* (Green and McCormick, 1999), *Theragra chalcogramma* (Theilacker and Porter, 1995) and *Tinca tinca* (Ostaszewska et al., 2006). It is suggested that the condition and height of intestinal epithelial cells are good indicators for malnutrition or starvation, as enterocyte height actually decreases with food deprivation, and proteolysis of the enterocytes eventually leads to lower absorption of nutrients due to reduced surface area (Green and McCormick, 1999). Less compact intestinal mucosa with shrinkage of enterocytes and irregular brush border results in reduced mucosal thickness (Bisbal and Bengston, 1995). These studies also stated that lipid vacuoles in the midgut and supranuclear protein vacuoles in the hindgut are absent in food-deprived larvae. In our experiment, from DAH14 onwards, only some larvae from all three groups contained such vacuoles. Hence, we suppose that not all larvae had the same amount of food intake. This might be the explanation for the variation in epithelium height, but further research is needed. Fasting resistance is age-dependent and larvae seem to be more vulnerable to food deprivation in the earliest stages, up to two to three weeks, and are more tolerant after metamorphosis (Theilacker and Porter, 1995; Chen et al., 2007).

The presence of mucins in the digestive tract is of particular importance as an innate defence barrier (Cebra, 1999). It is suggested that acid mucins, particularly sulfated mucins, are least degradable by bacterial glycosidases and host proteases, and therefore they are most protective against bacterial translocation in the pharynx and oesophagus (Roberton and Wright, 1997). However, these sulfated mucins were found in both conventional and germ-free larvae unaffected by microbial status. Neutral and acid AB-PAS positive goblet cells were visible in the pharyngeal and oesophageal epithelium before the start of exogenous feeding (DAH6). This is in contrast with the findings of Micale (2006) who observed PAS-positive goblet cells only on DAH11 in common pandora, few days after the start of first feeding. In the present study no mucins were visible in the midgut and hindgut of sea bass larvae in the first 15 days after hatching. This is in accordance with Garcia Hernandez et al. (2001), who found goblet cells, containing neutral mucins, only from DAH25 onwards in this area. Therefore, we can state that in early stages of development, surely till DAH15, European sea bass cannot rely on the mucous defence in this region. As microbial genes may contribute to the regulation of glycan secretion in the host which is an important component of mucins (Hooper and Gordon, 2001; Hooper et al., 2002), the production of carbohydrate structures may be a suitable method to investigate host-microbe interactions (Hooper and Gordon, 2001).

In our study, in germ-free and conventional sea bass larvae, the number of goblet cells increased in time and showed similar numbers in both groups. According to Bates et al. (2006) and Rawls et al. (2004), however, germ-free zebrafish elicited lower numbers of goblet cells compared to conventional animals. Future tests should focus on changes in mucin histochemistry, because mucins may influence bacterial translocation (Ottesen and Olafsen, 2000).

In conclusion, in order to evaluate growth and gastrointestinal tract development affected by germ-free/conventional environment we succeeded to reveal some differences between control and germ-free sea bass larvae. Morphometrical and stereological methods proved to be good diagnostic tools demonstrating that germ-free static larvae were longer, larger and had a more developed gastrointestinal tract compared to the other two treatments. However, no real qualitative differences in morphology of the gastrointestinal tract have been found between germ-free and control larvae by light microscopy. Differences of mucin content observed in the pharyngeal and oesophageal mucosa, suggest a microbial regulation of glycoprotein production in the gastrointestinal tract, but further investigation is needed to validate this finding.

The model system described in the present paper forms the basis to study in detail the putative influence of probiotics on morphology, physiology, innate immunity, resistance to microbial pathogens etc. in marine fish larvae.

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