Morphological characteristics of the digestive tract of gnotobiotic Artemia franciscana nauplii

R.A.Y.S. Asanka Gunasekara, Anamaria Rekecki, Pieter Cornillie, Maria Cornelissen, Patrick Sorgeloos, Paul Simoens, Peter Bossier, Wim Van den Broeck

Department of Morphology, Faculty of Veterinary Medicine, Ghent University, Salisburylaan 133, B-9820, Merelbeke, Belgium
Corresponding author. Tel.: +32 92647716; fax: +32 92647790.
E-mail address: wim.vandenbroeck@UGent.be (W. Van den Broeck).

1. Introduction

Successful aquaculture is still hampered by diseases of the larval phases, leading to massive mortalities and considerable economic losses (Marques et al., 2005). Antibiotic therapy and disinfectants have only had limited success in the prevention or cure of aquatic diseases (Defoirdt et al., 2004). Moreover, the frequent use of these chemicals results in rapid development of resistance (Dias et al., 1995; Molina-Aja et al., 2002; Vattanaviboon et al., 2003; Vivekanandhan et al., 2002) of their digestive tracts. The alimentary tract of gnotobiotic Artemia nauplii, fed with dead Aeromonas hydrophila and wild type strain of Saccharomyces cerevisiae, is a hooked, tubular structure which is composed of three clearly distinguishable parts, i.e. the foregut, midgut and hindgut that are freely suspended in haemolymph. The epithelium lining of the entire gut consists of a single cell layer. Enteroctyes of the foregut and hindgut are cuboidal and lined by a thin cuticle, whereas midgut enterocytes are cuboidal to columnar and possess an apical brush border. The fore- and hindgut mainly display characteristics suggestive for mechanical functions, whereas the midgut shows characteristics of absorption, storage and secretion. The gnotobiotic Artemia rearing system is most useful to investigate the effects of micro-organisms on the development of nauplii. The knowledge acquired in this study potentially facilitates the evaluation of gut morphology when specific micro-organisms are introduced into the culture system, as compared to the gnotobiotic counterparts.

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Keywords:
Gut
Gnotobiotic
Microscopy
Artemia
Morphology

A B S T R A C T

Cysts of Artemia franciscana were hatched and nauplii were reared under gnotobiotic conditions (gnotobiotic Artemia rearing system). Stereomicroscopy, computer assisted three-dimensional reconstruction, light microscopy, and transmission electron microscopy were used to study the structural and cellular morphology of their digestive tracts. The alimentary tract of gnotobiotic Artemia nauplii, fed with dead Aeromonas hydrophila and wild type strain of Saccharomyces cerevisiae, is a hooked, tubular structure which is composed of three clearly distinguishable parts, i.e. the foregut, midgut and hindgut that are freely suspended in haemolymph. The epithelium lining of the entire gut consists of a single cell layer. Enteroctyes of the foregut and hindgut are cuboidal and lined by a thin cuticle, whereas midgut enterocytes are cuboidal to columnar and possess an apical brush border. The fore- and hindgut mainly display characteristics suggestive for mechanical functions, whereas the midgut shows characteristics of absorption, storage and secretion. The gnotobiotic Artemia rearing system is most useful to investigate the effects of micro-organisms on the development of nauplii. The knowledge acquired in this study potentially facilitates the evaluation of gut morphology when specific micro-organisms are introduced into the culture system, as compared to the gnotobiotic counterparts.
the early stages of the life cycle. The knowledge gained from this study will facilitate future research which aims at comparison of the digestive tract morphology between gnotobiotic Artemia nauplii and their counterparts at same age after introduction of either one or more definite strains of micro-organisms to the culture system.

2. Materials and methods

2.1. Culturing and harvesting of yeast

Wild type (WT) strain of baker’s yeast (Saccharomyces cerevisiae) (BY4741 [genotype, Mata his3Δ 1 leu2Δ0 met15Δ0 ura3Δ0]), kindly provided by the European Saccharomyces cerevisiae Archive for Functional Analysis (University of Frankurt, Frankurt, Germany), was used as live feed for Artemia. It was cultured in minimal yeast nitrogen based (YNB), with amino acids (histidine HCl: 10 mg/l; methionine: 20 mg/l; tryptophan: 20 mg/l; inositol: 2 mg/l-Sigma, 0.67% w/v), supplemented with uracil (Sigma, 0.002% w/v), leucine (Sigma, 0.002% w/v) and D-glucose (Sigma, 0.5% w/v), agar. Harvested yeast cells were counted following the methodology described by Marques et al. (2006) and Gunasekara et al. (2010b). Yeast suspensions were stored at 4 °C and used to feed Artemia until the end of the experiment.

2.2. Culturing, harvesting, and killing of bacteria

The Aeromonas hydrophila strain (LVS3) selected for the experiment is a rod shaped, motile, gram negative, Aeromonadaceae bacterial strain. LVS3 was cultured and harvested, and the density was determined according to the methodology described by Marques et al. (2005, 2006) and Gunasekara et al. (2010b). As LVS3 cells were used for feeding, they were killed by autoclaving at 120 °C for 20 min. Bacterial suspensions were stored at 4 °C and used to feed Artemia until the end of the experiment.

2.3. Culturing and feeding of gnotobiotic Artemia

The experiment was carried out using A. franciscana originating from the Great Salt Lake, Utah. Artemia cysts were hydrated and decapsulated following the procedures described by Defoirdt et al. (2005) in order to obtain sterile cysts and subsequently sterile nauplii. Sterilization of necessary equipment, decapsulation of cysts and setting of culture tubes for hatching were done according to the procedures described by Gunasekara et al. (2010b). After about 20–24 h, 30 nauplii were transferred to fresh 50 ml sterile tubes containing 30 ml filtered autoclaved sea water made with Instant Ocean (Aquarium systems, Sarrebourg, France). All the culture tubes received living germ-free WT yeast and dead germ-free LVS3 as daily feed and were placed on a rotor turning at 4 rpm. Daily feeding was done ad libitum. As such 140.1 μg WT strain (ash free dry weight content) and 1401 μg dead bacteria were administered per culture tube during the six days of the study period as described by Marques et al. (2004a).

Sampling was done at days two, four and six for stereomicroscopy, light microscopy, and transmission electron microscopy and tested with four replicates for each.

2.4. Verification of axenity

The axenity of the decapsulated cysts, the Artemia culture water and the dead LVS3 suspension (after autoclaving) was verified using plating. Absence or presence of bacterial growth was monitored after five days of incubation at 28 °C of 100 μl of decapsulated cysts, culture medium or feed plated on Marine agar (n = 2).

2.5. Stereomicroscopy

Immediately after fixation, the specimens were viewed and photographed using a stereomicroscope (Olympus SZX 7, Olympus, Belgium) to study the gut segments in relation to external structures.

2.6. Light microscopy

Nauplii were fixed, pre-stained and further processed for histological sections following the procedures described by Gunasekara et al.
(2010b). Per day, six nauplii were cut into serial transverse sections and four nauplii were cut into longitudinal sagittal sections of 5 μm thickness with a microtome (Microm HM360, Prosan). For general histology, the sections were stained with haematoxylin (Haematoxylin (C.I. 75290), Merck KGaA, Darmstadt, Germany) and eosin (Eosin yellow (C.I. 45380), VWR International bvba/sprl, Leuven, Belgium), and for the detection of structures containing high concentrations of carbohydrate macromolecules, the sections were stained with PAS (periodic-acid-Schiff) reagent (C.I. 42500, Merck KGaA, Darmstadt, Germany). The histological sections were examined and photographed using a motorized microscope (Olympus BX 61, Olympus Belgium, Aartselaar, Belgium) linked to a digital camera (Olympus DP 50, Olympus Belgium).

2.7. Three-dimensional (3D) reconstruction

All serial histological sections were photographed using the objective lens 20× (1.0 μm × 1.0 pixel size) or 40× (0.5 μm × 0.5 μm pixel size) depending on the size of the tissue section and saved in jpeg format in two separate folders according to the magnification. The last image in each folder was obtained from the same section as the first picture of the next folder.

A plain text file for every folder was created to load the bricks of pictures in the stacked slice file format into the Amira 4.0.1 (Visage Imaging GmbH, Berlin, Germany) application, following the detailed information described by Cornillie et al. (2008).

All the slices within a brick were aligned, using an automated module, and by manual correction whenever necessary. Every pixel of each picture was assigned a grey-tone value based on the green colour channel (channel 1). Important structures on these images such as the contours of the nauplii and the fore-, mid- and hindgut of every fourth section was labeled manually with the brush tool (for internal structures) and lasso tool (for external surfaces) in the segmentation editor. The sections in-between were subsequently labeled through the interpolation command.

The voxel size of the two bricks of labeled sections was downsampled by a factor 2 in each direction (x, y and z) using the resampling module to save memory.

Constrained smoothened surface images of labeled structures were generated using SurfaceGen module. Two separate 3D images were created from two bricks of each nauplius and aligned manually using the transform editor of the Amira program.

2.8. Transmission electron microscopy (TEM)

Samples for TEM were fixed in Karnovsky’s fixative. After overnight fixation at 4 °C, specimens were washed in sodium cacodylate buffer (pH 7.4), and postfixed overnight in 1% osmium tetroxide. Subsequently, the samples were dehydrated using a graded series of alcohols (50% to 100%) and finally embedded in SPURR’s resin. After examination of semithin sections to localize the regions of interest, ultrathin sections of 60 nm were made using a Leica EM MZ 6 ultramicrotome (Leica Mircosystems GmbH), mounted on formvar coated single slot copper grids (Laborimpex N.V., Brussels, Belgium). The sections were post-stained with uranyl acetate and lead citrate (Leica EM Stain, Leica Microsystems GmbH) and viewed on a Jeol 1200 EXII TEM (JEOL Ltd., Tokyo, Japan) at 80 kV accelerating voltage.

3. Results

3.1. Stereomicroscopy

The transparent gut segments (mid- and hindgut) could be visualized clearly by stereomicroscopy. Gastric caeca and the midgut–hindgut transition zones were also clearly visible (Fig. 1a, b, c).

3.2. Three-dimensional morphology

The alimentary tract of A. franciscana nauplii is freely suspended in haemolymph and composed of three clearly distinguishable structures.
segments, i.e. the foregut, the midgut and the hindgut. The mid- and the hindgut are lined cranially with cuboidal cells (Fig. 3a, c) and where it opens into the midgut a clear transition from cuboidal to cuboidal-columnar lining cells is visible (Fig. 3a, c). The cells of the foregut contain small amounts of cytoplasmic organelles and their luminal surface is covered by a thin cuticle layer (Fig. 5a). The basement membrane separates the epithelium from the underlying muscle cells which are circularly arranged at intervals around the foregut and a longitudinal muscle layer.

3.3.2. Midgut

The mucosal lining of the entire midgut, including the two gastric caeca, is composed of a single epithelium of cuboidal to columnar cells (Fig. 3a, b, c). A brush border which stains PAS positive (Fig. 4) in the epithelial cells of the midgut are visible (Fig. 3a, b, c). The cells are bound to each other with occluding and anchoring junctions (Fig. 5b). Large, central nuclei are observed throughout the whole midgut epithelium (Fig. 5b, c, d). The cytoplasm of the midgut epithelial cells contains numerous mitochondria and a well developed endoplasmic reticulum. Golgi complexes are seen in some cells. The basal cell membrane of the midgut epithelial cells, and in particular of the cells located in the most caudal region, is folded into narrow channels, called basal infoldings, which protrude into the basal part of the cytoplasm (Fig. 5b, d). The basement membrane separates the epithelium from the underlying muscle cells which are circularly arranged at intervals around the midgut (Fig. 5d, f). A longitudinal muscle layer could not be observed in the midgut. A clear transition from a cuboidal-columnar to a cuboidal epithelium is present at the border between the midgut and the hindgut (Figs. 3b, 5e).

3.3.3. Hindgut

The hindgut is lined with a cuboidal cell layer (Figs. 3b, d, 5f). The hindgut epithelial cells do not possess apical microvilli, instead they are covered by a thin cuticle layer (Fig. 5e, f). The cells of the hindgut contain small amounts of cytoplasmic organelles. The basement membrane separates the epithelium from the underlying muscle cells which are circularly arranged at intervals around the hindgut and from a longitudinal muscle layer (Fig. 5f).

During the entire period of experiment (six days) all parts of the alimentary tract increased proportionally in the nauplii (Table 1).
Fig. 5. Transmission electron micrographs of gut segments of gnotobiotic Artemia nauplii.
Gnotobiology is a well-established science (Marques et al., 2005), which makes studying of host-microbial interactions effective. Additionally, the use of gnotobiotic organisms allows an increased control of variables, enhances the reproducibility of results, and enables the more accurate experimental designs (Pleasants, 1973). A key experimental strategy to study these interactions is to first define the morphology and functioning of the animal in the absence of all micro-organisms (i.e. under germ-free or gnotobiotic conditions) and then to evaluate the effects of adding a single or defined population of microbes (Gordon and Pesti, 1971).

Many studies have been performed in the past, using a wide range of terrestrial gnotobiotic animals such as gnotobiotic mice (Abrams et al., 1963; Banaszaz et al., 2001, 2002; Uribe et al., 1997), guinea pigs, chickens, dogs, cats, and pigs (Delluva et al., 1968). However, similar studies on aquatic animals are still scarce. Some studies have identified gnotobiotic marine and fresh water fish as a good research tool to investigate host-microbial interactions, e.g. germ-free turbot (Scopthalmus maximus) larvae by Munro et al. (1995), bacteria-free halibut (Hippoglossus hippoglossus) larvae by Verner-Jeffreys et al. (2003), gnotobiotic zebra fish (Danio rerio) by Rawls et al. (2004), or germ-free seabass (Dicentrarchus labrax) larvae by Dierckens et al. (2009) and Rebecki et al. (2009).

In the present study, A. franciscana was used as an aquatic model to describe the digestive tract morphology of the gnotobiotic nauplii. A. franciscana is an important live food for commercial production of fish and shellfish larvae (Sorgeloos et al., 1986) and a unique aquatic model to elucidate host-microbial interactions under germ-free and gnotobiotic culture conditions using various types of feed sources with simple experimental equipment (Verschueren et al., 1999, 2000).

Since it is evident that those gnotobiotic animal models are a prerequisite to study the host-microbe interactions and as morphology is used as a monitoring tool in our other studies, the study of the gnotobiotic animals and their detailed morphology before introducing any known microbiota is of high value. Hence, in the present study the digestive tract of gnotobiotic A. franciscana was studied in detail using stereomicroscopy, three-dimensional reconstruction, light microscopy, and transmission electron microscopy. Stereomicroscopy allowed studying the general appearance of the Artemia body and the digestive tract. Three-dimensional reconstructions facilitated evaluation of architectural organization and topography of the digestive tract. Histological and transmission electron microscopic examination allowed evaluating the types of cells, junctions between gut segments, the presence or absence of a cuticle or brush border. Using transmission electron microscopy, the cells could be studied for the organization of organelles, transition zones and presence of microvilli. As such, the whole study enabled evaluating the digestive tract of gnotobiotic Artemia nauplii in detail.

The alimentary tract of gnotobiotic Artemia nauplii is a hooked tube consisting of three major segments, which is in agreement with previous reports of Hootman and Conte (1974) who made a descriptive ultrastructural account on conventional second instar Artemia nauplii, Schrehardt (1987) who illustrated the morphology of adult conventional Artemia, and Criel (1991) who described conventional Artemia nauplii and adults. The mucosal cell types and the arrangement of organelles such as nuclei and mitochondria in the cells of the entire gut of conventional Artemia nauplii described by the above-mentioned authors are similar to the present findings. However, the abundance of Golgi apparatus was little less as seen in the current study. According to the description of Criel (1991), the fore- and the hindgut are surrounded by circularly arranged and a longitudinal muscle layers while the midgut is only surrounded by a circularly arranged muscle layer, as was also observed in the present study in gnotobiotic Artemia nauplii.

In the present study, microvilli of the midgut epithelial cells could be observed in gnotobiotic Artemia nauplii which are suggestive for an absorptive function as described by Kikuchi (1971) in conventional adult Artemia. The microvilli are coated with a fine fibrous material, probably mucopolysaccharides as mentioned by Criel (1991). In the current study, the brush border was positively stained with PAS reagent. This finding suggests that the microvilli are surrounded by structures containing high concentrations of carbohydrate macromolecules (e.g. glycoproteins, proteoglycan, glycogen), which is in accordance with the results of Hauber and Zabel (2005). In contrast, Hootman and Conte (1974) did not find any mucopolysaccharide glycocalyx, but they did observe numerous vesicles filling the spaces among microvilli. As no epithelial goblet cells were detected using PAS staining in nauplii at this stage, further studies are necessary to confirm the origin and biochemical nature of these compounds. The presence of infoldings of the basal cellular surfaces and associated mitochondria in the epithelial cells lining the caudal part of the midgut support the absorptive function of these cells (Hootman and Conte, 1974).

Gnotobiological techniques have been utilised to assess the ability of specific bacteria to protect live food cultures against pathogenic bacterial strains (Marques et al., 2006). Therefore in future research using such gnotobiotic systems, beneficial effects such as probiotic (single or in mixtures) or deleterious effects of micro-organisms can be screened by comparing to the gnotobiotic basic model. Similarly such effects can be compared with conventional Artemia in which the resident microbial community is more poorly defined. With stereo-microscopy, the effects of micro-organisms on individual naupliar length can be measured (Gunasekara et al., 2010a). Furthermore, histology and morphometrical analysis can facilitate comparison of the gross morphology of the various gut segments (Gunasekara et al., 2010b), while ultrastructural changes such as site specific colonization, adhesion or translocation can be verified by transmission electron microscopy. Further studies using pathogenic bacteria will eventually be of importance for investigating preventive and / or curing measures.

**Acknowledgements**

This study was supported by a Special Research Grant (Bijzonder Onderzoeksfonds, BOF, grant numbers B/07289/02 and 05B01906) of Ghent University (Belgium) awarded to the first author and by the Foundation for Scientific Research-Flanders (FWO) (Project: Probiont induced functional responses in aquatic organisms, G.0491.08).

We are indebted to Prof. Godelieve Criel for the guidance during the experiment and comments for the manuscript. Thanks are also to Prof. Annemie Decostere and Dr. Christophe Casteleyn for critically reading the manuscript. Excellent technical assistance of Lobke De Bels, Bart De Pauw, Liliana Standaert and Leen Pieters is also thankfully acknowledged.
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